UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOLOGIA GERAL
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

TESE DE DOUTORADO

DIABETES MELLITUS TIPO 2 E SUAS COMPLICAÇÕES - ASSOCIAÇÃO COM NOVOS MARCADORES INFLAMATÓRIOS E MOLECULARES

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BELO HORIZONTE - MG

Abril / 2017

KATHRYNA FONTANA RODRIGUES

DIABETES MELLITUS TIPO 2 E SUAS COMPLICAÇÕES - ASSOCIAÇÃO COM NOVOS MARCADORES INFLAMATÓRIOS E MOLECULARES

Tese apresentada ao programa de Pós-Graduação em Genética do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do título de doutora em Genética.
Orientadora: Profa. Dra. Karina Braga Gomes Borges

BELO HORIZONTE - MG
Abril / 2017

043 Rodrigues, Kathryna Fontana.
Diabetes Mellitus tipo 2 e suas complicações: associação com novos marcadores inflamatórios e moleculares [manuscrito] / Kathryna Fontana Rodrigues. – 2017.

156 f. : il. ; 29,5 cm.

Orientadora: Profa. Dra. Karina Braga Gomes Borges.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas.

1. Genética - Teses. 2. Diabetes Mellitus tipo 2. 3. Inflamação - Teses. 4. Obesidade - Teses. 5. Marcadores biológicos. 6. Polimorfismo (Genética) - Teses. I. Borges, Karina Braga Gomes. II. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. III. Título.

CDU: 575

Ficha elaborada pela Biblioteca do Instituto de Ciências Biológias da UFMG

Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Genética

ATA DA DEFESA DE TESE

93/2017
entrada
2°/2013
CPF:
089.221.336-19

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Às oito horas e trinta minutos do dia **06 de abril de 2017**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"DIABETES MELLITUS TIPO 2 E SUAS COMPLICAÇÕES - ASSOCIAÇÃO COM NOVOS MARCADORES INFLAMATÓRIOS E MOLECULARES"**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, a Presidente da Comissão, **Karina Braga Gomes Borges**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

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Pelas indicações, a candidata foi considerada: APROVADA
O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.
Belo Horizonte, 06 de abril de 2017.

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"DIABETES MELLITUS TIPO 2 E SUAS COMPLICAÇÕES - ASSOCIAÇÃO COM NOVOS MARCADORES INFLAMATÓRIOS E MOLECULARES"

Kathryna Fontana Rodrigues

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Genética Molecular, de Microrganismos e Biotecnologia

Linha de Pesquisa:

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“É exatamente disso que a vida é feita: de momentos. Momentos que temos que passar, sendo bons ou ruins, para o nosso próprio aprendizado. Nunca esquecendo do mais importante: nada nessa vida é por acaso. Absolutamente nada”.

(Chico Xavier)

Dedico este trabalho aos meus pais, fonte inesgotável de compreensão, apoio e amor. Sem eles a realização deste projeto não seria possível. Mais uma vez, esta vitória é de vocês.

AGRADECIMENTOS

À Deus, presença constante em minha vida, dando-me força e paciência para enfrentar os momentos mais críticos desta caminhada. Agradeço por ter colocado no meu caminho tantas “pessoas-anjos” que foram inspiradoras e fundamentais para a realização deste trabalho.

Aos meus pais, fonte inesgotável de amor, carinho e apoio incondicional. Sei que vocês abdicaram de muitos sonhos em prol da realização dos meus. Nunca mediram esforços para me ajudar a superar as dificuldades e sempre acreditaram em mim. Minha eterna gratidão a Deus por ter me dado esses pais maravilhosos. Amo vocês!

À minha orientadora, professora Dra. Karina Braga Gomes Borges, pela paciência, compreensão, dedicação e amizade. Só você poderia ter sido minha orientadora por tantos anos. Obrigada por ter me dado os melhores conselhos, os mais necessários “puxões de orelha”, os mais bonitos exemplos de mulher e profissional.

À minha família, em especial a minha vovó Celina, por sempre dedicarem a mim suas preces e orações. Obrigada pelo carinho.

À minha Nina que na simplicidade e pureza de um animalzinho, enche minha vida de amor e alegria. Minha pequena guerreira, por vezes você foi mais forte do que seus donos. Você nos ensina todos os dias como ser simples para a vida e para o amor.

Ao programa de Pós-graduação em Genética por ter me acolhido novamente como aluna de pós-graduação. Agradeço aos professores, aos funcionários e colegas pelo convívio sempre amigável e troca de conhecimentos.

À CAPES, CNPq e Fapemig que concederam apoio financeiro durante todo o doutorado, permitindo a execução deste projeto.

Aos professores da Faculdade de Farmácia da UFMG que me ensinaram muito mais do que o necessário para ser uma Farmacêutica. Foram muitas as lições de ética, humanidade e respeito. Especialmente agradeço aos professores do Departamento de Análises Clínicas e Toxicológicas que me acolheram desde a iniciação científica

até hoje permitindo que o quarto andar da FAFAR fosse uma continuação da minha casa.

Aos funcionários e alunos dos Laboratórios de Bioquímica Clínica, Biologia Molecular e Hematologia Clínica da Faculdade de Farmácia da UFMG. Obrigada pelo convívio, amizade e apoio profissional em todas as etapas do projeto. Mais que colegas, somos amigos e nossas “loucuras” de cada dia não me deixaram perder o bom humor. Aprendi muito com cada um de vocês!

À “Caverna do dragão” mais épica da história: Fred, Angel, Poldo, Dani, Anderson, Leandro, Camila, Michelle, Vanêssa... *Best friends forever*!

À Daniela Diniz, minha “pequena”, que de tão pequena foi por vezes muito maior do que eu e me ajudou a superar problemas pessoais e profissionais. Obrigada pela amizade, pelas conversas longas (que você não gosta) pelo celular, pelos conselhos e por estar sempre disposta a ajudar. Valeu demais amiga!

A todos os amigos que minha vida profissional de farmacêutica-bioquímica me deu de presente. Nunca imaginei que ao escolher esta profissão encontraria tanta gente linda, doida e apaixonante pelo caminho. Aos colegas do IPSEMG, UNA, Estudo Clínico da Vacina da Dengue e Hemominas, meu sincero “muito obrigada, adoro vocês coleguinhas!”.

Aos amigos que a vida me deu e foram (e ainda são) responsáveis por aqueles momentos descontraídos e leves que recarregam as baterias da gente.

Às colaboradoras deste projeto (Dra. Adriana, Valéria, Cláudia e Fernanda) que agregaram muito conhecimento e contribuíram imensamente para a execução deste projeto.

Aos pacientes do Ambulatório de Endocrinologia da Santa Casa e demais voluntários que gentilmente aceitaram ser incluídos neste trabalho e literalmente “doaram o sangue” para a pesquisa.

Enfim, a todos que direta ou indiretamente me ajudaram a tornar este sonho realidade.

Muito obrigada!

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LISTA DE ABREVIATURAS E SIGLAS

1αOHD3	*1,α-hydroxyvitamin D3*
25(OH)D	25-hidroxivitamina D
AAS	Ácido acetilsalicílico
ADA	*American Diabetes Association* (Associação Americana de Diabetes)
AG	Ácido graxo
AGEs	*Advanced glycation end-products* (Produtos finais de glicação avançada)
AGL	Ácido graxo livre
Akt	*Protein kinase B* (Proteína quinase B)
AMPK	*AMP-activated protein kinase* (Proteína quinase ativada por AMP)
AP-1	*Activator protein 1* (Proteína ativadora 1)
ASC	*Apoptosis associated speck-like protein*
AVC	Acidente vascular cerebral
BMI	B*ody mass index* (Índice de massa corporal)
CKD	*Chronic kidney disease* (Doença rena crônica)
CRP	*C-reactive protein* (Proteína C reativa)
DAG	Diacilglicerol
DCV	Doença cardiovascular
DM	Diabetes mellitus
DM1	Diabetes mellitus tipo 1
DM2	Diabetes mellitus tipo 2
DMG	Diabetes mellitus gestacional
DN	*Diabetic nephropathy* (Nefropatia diabética)
DRC	Doença renal crônica
DRD	Doença renal do diabetes
EM	Edema macular
EMPs	*Endothelial cell-derived microparticles* (Micropartículas derivadas de células endoteliais)
eNOS	*Endothelial nitric oxide synthase* (Óxido nítrico sintase endotelial)
EROs	Espécies reativas de oxigênio

ESM-1	*Endothelial cell-specific molecule 1*
EUA	Excreção urinária de albumina
FNDC5	*Fibronectin type III domain-containing protein 5*
GAD65	Anticorpo antidescarboxilase do ácido glutâmico
GFR	*Glomerular filtration ratio* (Taxa de filtração glomerular)
GJ	Glicemia de jejum
GLUT	*Glucose transporter* (Transportador de glicose)
Hb	*Hemoglobin* (Hemoglobina)
HbA1c	*Glycated hemoglobin* (Hemoglobina glicada)
HLA	Antígeno leucocitário humano
HOMA-IR	*Homeostasis model assessement of insulin resistance*
Hp	*Haptoglobin* (Haptoglobina)
hs-CRP	*High-sensitivity C-reactive protein* (Proteína C reativa de alta sensibilidade)
HWE	*Hardy-weinberg equilibrium* (Equilíbrio de Hardy-Weinberg)
IA	Anticorpo antitirosina-fosfatase
ICAM-1	*Intercellular Adhesion Molecule 1*
IDF	*International Diabetes Federation* (Federação Internacional do Diabetes)
IFN	Interferon
IKKβ	*I kappa B kinase beta* (I kapa B quinase beta)
IL	*Interleukin* (Interleucina)
IL-1R	*Interleukin 1 receptor* (Receptor de interleucina 1)
IMC	Índice de massa corporal
IQR	*Interquartile range* (Intervalo interquartílico)
IRS	*Insulin receptor substrate* (Substrato do receptor de insulina)
JNK	*c-Jun N-terminal kinase* (Quinase c-Jun N-terminal)
LDL	Lipoproteína de baixa densidade
LFA-1	*Leukocyte function-associated antigen 1*
LMPs	L*eukocyte-derived microparticles* (Micropartículas derivadas de leucócitos)
MAPK	*Mitogen-activated protein kinase* (Proteína quinase ativada por mitógeno)
MCP-1	*Monocyte chemoattractant protein 1* (Proteína quimiotática de monócitos 1)

MPs	*Microparticles* (Micropartículas)
mRNA	RNA mensageiro
NADPH	*Nicotinamide adenine dinucleotide phosphate hydrogen*
NALP	*Neutrophilic alkaline phosphatases*
ND	Nefropatia diabética
NFK	*National Kidney Foundation* (Fundação Nacional do Rim)
NF-κB	*Nuclear factor kappa B* (Fator nuclear kapa B)
NLR	*NOD-like receptors*
NLRP3	*NLR family pyrin domain containing 3*
NO	*Nitric oxide* (Óxido nítrico)
NR	Neuropatia diabética
OMS	Organização Mundial da Saúde
PAI-1	*Plasminogen activator inhibitor 1* (Inibidor do ativador de plasminogênio 1)
PCG1-α	*Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha*
PCR	Proteína C reativa
PCR	*Polymerase chain reaction* (Reação em cadeia da polimerase)
PI3q	Fosfatidilinositol 3-quinase
PKC	*Protein kinase C* (Proteína quinase C)
PMPs	*Platelet-derived microparticles* (Micropartículas derivadas de plaquetas)
RAGE	*Receptor advanced glycation end-products* (Receptor de produtos finais de glicação avançada)
RD	Retinopatia diabética
ROS	*Reactive oxygen species* (Espécies reativas de oxigênio)
SBD	Sociedade Brasileira de Diabetes
SD	*Standard deviation* (Desvio padrão)
SNP	*Single-nucleotide polymorphism* (Polimorfismo de nucleotídeo único)
SPSS	*Statistical Package of the Social Sciences*
T2D	*Type 2 diabetes* (Diabetes tipo 2)
T2DM	*Type 2 diabetes mellitus* (Diabetes mellitus tipo 2)
TA	Temperatura ambiente

TF	*Tissue factor* (Fator tissular)
TFG	Taxa de filtração glomerular
TFMPs	*Microparticles that express tissue factor* (Micropartículas que expressam fator tissular)
TGF-β	*Transforming growth factor beta* (Fator de crescimento transformante beta)
TLR	*Toll-like receptor* (Receptor tipo Toll)
TNFR	*Tumor necrosis factor receptor* (Receptor do fator de necrose tumoral)
TNF-α	*Tumor necrosis factor alpha* (Fator de necrose tumoral alfa)
TOTG	Teste oral de tolerância à glicose
VCAM-1	*Vascular cell adhesion molecule 1*
VEGF	*Vascular endothelial growth factor* (Fator de crescimento endotelial vascular)
Znt	Anticorpo antitransportador de zinco

RESUMO

O diabetes mellitus tipo 2 (DM2) é o tipo mais prevalente de diabetes mellitus e afeta milhões de pessoas em todo o mundo, alcançando números que caracterizam uma epidemia da doença. A patogênese do DM2 e de suas complicações microvasculares está associada a um estado inflamatório crônico subclínico. Observa-se, portanto, ativação do sistema imune e de vias de sinalização inflamatórias intracelulares, alteração do padrão de expressão e secreção de mediadores inflamatórios e adipocinas, disfunção endotelial e estresse oxidativo. O acúmulo de gordura visceral tem papel fundamental nos eventos que culminam com a resistência à insulina e o DM2. Este trabalho teve por objetivo avaliar a associação de marcadores inflamatórios, bioquímicos e moleculares com o DM2, suas complicações microvasculares e comorbidades. Foram selecionados 102 pacientes com diagnóstico clínico e laboratorial de DM2 e 62 controles não-diabéticos. Os resultados indicaram que pacientes com DM2 apresentam maiores níveis de IL-6, irisina, haptoglobina, micropartículas circulantes (micropartículas derivadas de plaquetas, células endoteliais, leucócitos e carreadoras de fator tissular) e menores níveis de endocan, quando comparados a controles não-diabéticos. Além disso, os níveis de visfatina mostraram-se diminuídos nos estágios mais avançados da doença renal do diabetes. O índice de massa corporal, utilizado como marcador do *status* de obesidade, mostrou influência sobre os níveis de IL-6, irisina e haptoglobina. O polimorfismo IL-10 -819 T/C revelou efeito sobre os níveis de IL-10 no DM2. Além disso, o genótipo Hp1-Hp1 do polimorfismo no gene da haptoglobina foi mais frequente entre os pacientes com DM2 e parece seguir um modelo de herança dominante. Estes resultados reforçam que marcadores bioquímicos e de ativação celular estão alterados no DM2 e podem ser influenciados pela obesidade e também por polimorfismos genéticos. A melhor compreensão do comportamento destes marcadores no DM2 poderá auxiliar na elucidação da patogênese da doença, bem como na adoção de estratégias complementares no acompanhamento dos indivíduos com este diagnóstico.

Palavras-chave: diabetes mellitus tipo 2, inflamação, obesidade, complicações microvasculares, marcadores bioquímicos, polimorfismos.

ABSTRACT

Type 2 diabetes mellitus (T2DM) is the most prevalent form of diabetes mellitus, a disease of epidemic proportions that affects millions of people worldwide. The pathogenesis of T2DM and its microvascular complications are associated with a chronic subclinical inflammation, characterized by activation of the immune system and intracellular inflammatory signaling pathways, altered expression/secretion pattern of inflammatory mediators and adipokines, endothelial dysfunction and oxidative stress. Visceral obesity plays an important role in the events that culminate in insulin resistance and T2DM. The objective of this research was to evaluate the association of biochemical and molecular inflammatory markers with T2DM, its microvascular complications and comorbidities. 102 patients with clinical and laboratory diagnosis of T2DM and 62 non-diabetic controls where selected for participation. The results indicated that T2DM patients had higher IL-6, irisin, haptoglobin, and circulating microparticles levels (platelet-, endothelial cells- and leukocytes-derived microparticles as well as tissue factor carriers); and lower endocan levels when compared to non-diabetic controls. Furthermore, visfatin levels are decreased in advanced stages of diabetic nephropathy. Body mass index, a marker of obesity status, was shown to influence in IL-6, irisin, and haptoglobin levels. IL-10 -819T/C polymorphism affected IL-10 levels in T2DM. Hp1-Hp1 genotype of the haptoglobin gene polymorphism was more frequent in the T2DM group and presented a dominant inheritance pattern. These results indicate that biochemical and cellular activation markers levels are altered in T2DM and they can be influenced by obesity status and genetic polymorphisms. Understanding how these markers influence the onset and development of T2DM may be useful to elucidate the pathogenesis mechanisms and may as well provide insights to ameliorate the clinical follow-up.

Keywords: type 2 diabetes mellitus, inflammation, obesity, microvascular complications, biochemical markers, polymorphisms.

1. INTRODUÇÃO

O diabetes mellitus (DM) é um grupo heterogêneo de distúrbios metabólicos que têm em comum a hiperglicemia (SBD, 2016). A elevação dos níveis glicêmicos ocorre principalmente pela produção deficiente de insulina pelo pâncreas ou devido a uma ação ineficiente do hormônio nos tecidos periféricos, como tecido adiposo, hepático e muscular esquelético, caracterizando um quadro de resistência à insulina (KHARDORI, 2013). Dados epidemiológicos indicam que o número de pacientes com DM tem aumentado em todo o mundo, alcançando proporções epidêmicas. Segundo o *International Diabetes Federation* (IDF), estima-se que em 2015 havia 415 milhões de pessoas com DM no mundo. Em 2040 o número de afetados poderá alcançar a marca de 642 milhões de indivíduos, o que representará um crescimento de 54,6% no número de casos de DM. Ainda segundo o IDF, o Brasil ocupa atualmente a quarta posição no *ranking* dos países com maior número de casos de DM (IDF, 2015).

Entre as diferentes formas de DM, o diabetes mellitus tipo 2 (DM2) é sem dúvida a mais prevalente delas, sendo responsável por cerca de 90-95% dos casos (ADA, 2017).

Os principais fatores de risco associados ao desenvolvimento do DM2, além do envolvimento genético, dizem respeito a fatores ambientais relacionados ao estilo de vida moderno, caracterizado pelo sedentarismo, alto consumo de gorduras e açúcares, tabagismo e obesidade (SBD, 2016).

As complicações do DM são as responsáveis pelo alto índice de morbi-mortalidade da doença gerando um grande impacto econômico no sistema de saúde, além de perdas significativas na qualidade de vida dos pacientes e familiares. As complicações macrovasculares dizem respeito à doença aterosclerótica, elevando o DM à condição de fator de risco independente para as doenças cardiovasculares (DCVs). Por outro lado, as complicações microvasculares são importantes causas de doença renal crônica (DRC) e diálise (doença renal do diabetes), cegueira (retinopatia) e neuropatias debilitantes (SBD, 2016; ADA, 2017).

Há pelo menos duas décadas a patogênese do DM2 e de suas complicações têm sido associadas a um estado inflamatório crônico de baixo grau e à ativação do sistema imune inato (MEDZHITOV, 2008; MANCUSO, 2016). A obesidade, principalmente o acúmulo de gordura visceral, tem papel importante neste contexto, visto que a hipertrofia e hiperplasia do tecido adiposo estão associadas à ativação de vias de sinalização intracelulares responsáveis pela produção e secreção de

mediadores inflamatórios, bem como eventos moleculares que culminam com a resistência à insulina (RODRÍGUEZ et al., 2015).

Estudos atuais sugerem que os níveis de citocinas pró e anti-inflamatórias, adipocinas, marcadores bioquímicos e de disfunção endotelial, devem estar alterados no DM2. Por outro lado, a expressão destes marcadores está sob forte controle genético, envolvendo desde polimorfismos de nucleotídeo único (SNPs) até mecanismos mais robustos de controle pré e pós-transcricional envolvidos na regulação da expressão destas proteínas e de seus receptores. Adicionalmente, a ativação celular observada no DM2 em decorrência do processo inflamatório altera os níveis de micropartículas (MPs) circulantes, e estas, por sua vez, atuam amplificando os sinais inflamatórios e a disfunção endotelial.

Diversos estudos têm sido conduzidos em diferentes populações buscando caracterizar perfis moleculares e bioquímicos de inflamação associados ao DM2, sua progressão e desenvolvimento de complicações macro e microvasculares. Entretanto, os resultados ainda são conflitantes e os mecanismos que associam a inflamação e a ativação do sistema imune ao desenvolvimento da doença precisam ser melhor elucidados. Além disso, ainda não há a descrição de um marcador sanguíneo que consiga estabelecer, com precocidade, o risco ou o desenvolvimento destas complicações.

Desta forma, este trabalho objetivou investigar a associação de marcadores inflamatórios, bioquímicos e moleculares, com o DM2, suas complicações microvasculares e comorbidades. A melhor compreensão sobre a fisiopatologia da doença poderá contribuir para a adoção de novas estratégias para o acompanhamento dos pacientes e melhorar seu prognóstico.

2. REVISÃO DA LITERATURA

2.1 Diabetes mellitus: conceito, classificação e critérios laboratoriais de diagnóstico

Segundo a Sociedade Brasileira de Diabetes (SBD), o DM é um grupo heterogêneo de distúrbios metabólicos que têm em comum a hiperglicemia, resultante de defeitos na ação da insulina, na secreção desta ou em ambos (SBD, 2016).

A homeostase da glicose garante a manutenção de sua concentração no sangue em um estreito intervalo, independente dos períodos de alimentação e jejum. Esse balanço é regulado pela absorção de glicose a partir do intestino, a produção desta pelo fígado e seu metabolismo nos tecidos periféricos; envolvendo, portanto, várias vias metabólicas e a ação de diversos hormônios com características hipo e hiperglicemiantes (SALTIEL e KAHN, 2001).

A insulina é o principal hormônio envolvido no metabolismo da glicose. Consiste em um polipeptídeo anabólico (peso molecular 5,8 KDa) produzido pelas células beta das ilhotas de Langerhans na porção endócrina do pâncreas (SAAD et al., 2007). Ela atua como o principal regulador da concentração de glicose no sangue por promover a captação deste açúcar, principalmente no tecido muscular esquelético e adiposo; estimular a síntese de proteínas, ácidos graxos (AG) e glicogênio (glicogênese); bem como bloqueia a produção hepática de glicose (gliconeogênese), a lipólise e a proteólise (CINTRA et al., 2011).

A insulina atua por meio da interação com seu receptor, uma proteína heterotetramérica com atividade quinase intrínseca. Este receptor é composto por duas subunidades alfa e duas subunidades beta, ligadas por pontes dissulfeto, que atuam como uma enzima alostérica na qual a subunidade alfa inibe a atividade tirosina quinase da subunidade beta. A ligação da insulina à subunidade alfa, totalmente extracelular, permite que a subunidade beta, transmembrana, adquira atividade de tirosina quinase levando a uma alteração conformacional, autofosforilação de múltiplos resíduos de tirosina e também fosforilação de outras proteínas citoplasmáticas, conhecidas como substratos do receptor de insulina (IRS). Os IRS-1 e IRS-2 fosforilados são responsáveis pela ativação da via PI3q/Akt que participa na promoção da exocitose das vesículas contendo transportadores de glicose (GLUT) para a membrana celular, permitindo a captação de glicose nos

tecidos dependentes de insulina (SALTIEL e KAHN, 2001; SAAD et al., 2007; CINTRA et al., 2011).

Conforme definido anteriormente, a hiperglicemia no DM pode ocorrer devido à produção deficiente de insulina pelo pâncreas ou devido a uma ação ineficiente do hormônio nos tecidos periféricos, caracterizando um quadro de resistência à insulina. Esta resistência pode apresentar-se em decorrência de uma menor atividade dos receptores de insulina, por uma diminuição no número destes, por alterações estruturais que levam a uma deficiência na ligação insulina-receptor, ou ainda por uma diminuição da proteína transportadora no tecido muscular esquelético e adiposo (GLUT4) (KHARDORI, 2013).

Clinicamente, os principais sintomas associados ao DM são poliúria, polidipsia e perda não explicada de peso (SBD, 2016), podendo ser acompanhados de polifagia, prurido, visão turva, cansaço e infecções recorrentes, principalmente na pele e sistemas urinário e genital (SBD, 2015).

A *American Diabetes Association* (ADA) recomenda a classificação do DM em: diabetes mellitus tipo 1 (DM1), DM2, diabetes mellitus gestacional (DMG) e outros tipos específicos de diabetes (ADA, 2017). Também podem ser citados dois estádios intermediários, referidos como “pré-diabetes”: a glicemia de jejum alterada e a tolerância à glicose diminuída. Ambos não são entidades clínicas, mas representam risco aumentado para o desenvolvimento de DM e DCVs (SBD, 2016; ADA, 2017).

O DM1 é caracterizado pela destruição das células beta do pâncreas com consequente perda da capacidade secretora de insulina, podendo chegar até a deficiência absoluta deste hormônio. O DM1 pode ser subdividido em tipos 1A (autoimune) e 1B (idiopático) (SBD, 2016; ADA, 2017).

O DM 1A corresponde a cerca de 5-10% dos casos de DM e apresenta marcadores de autoimunidade responsáveis pela destruição das células beta. Os principais autoanticorpos encontrados são os anti-ilhota ou antígenos específicos da ilhota e incluem os anticorpos anti-insulina, antidescarboxilase do ácido glutâmico (GAD 65), antitirosina-fosfatases (IA2 e IA2B) e antitransportador de zinco (Znt). Estes anticorpos podem ser determinados meses ou anos antes do diagnóstico clínico da doença. A taxa de destruição das células beta é bastante variável, normalmente mais rápida em crianças e adolescentes e mais lenta em adultos, ambos os casos, entretanto, com forte predisposição a cetoacidose. A patogênese

do DM 1A está associada a fatores genéticos (condição poligênica) e ambientais. Os genes *DR* e *DQ* do sistema do antígeno leucocitário humano (HLA) classe II estão em forte desequilíbrio de ligação e são responsáveis por mais de 50% do risco de desenvolver DM 1A. Sugere-se que cerca de 90% dos pacientes possuem um ou ambos os haplótipos: DR4/DQ8 e DR3/DQ2. Os principais fatores ambientais que podem desencadear a autoimunidade em indivíduos geneticamente predispostos são: infecções virais (citomegalovírus B4, por exemplo), fatores nutricionais (introdução precoce na dieta da proteína do leite bovino, glúten, deficiência de vitaminas A e D), dentre outros (ERLICH et al., 2008; SIMMONS e MICHELS, 2015; SBD, 2016).

O DM 1B, por sua vez, não possui uma etiologia conhecida, nem marcadores de autoimunidade e tampouco associa-se a haplótipos do sistema HLA classe II. Corresponde à minoria dos casos de DM1 e os afetados apresentam graus variáveis de deficiência de insulina e predisposição a cetoacidose (SIMMONS e MICHELS, 2015; SBD, 2016).

O DM2 corresponde a cerca de 90-95% dos casos de DM e é causado por uma combinação de resistência periférica à ação da insulina e resposta compensatória inadequada da secreção desta; além de defeitos na regulação da produção hepática de glicose. Normalmente, a hiperglicemia se desenvolve de forma silenciosa por vários anos, e o diagnóstico clínico e laboratorial da doença ocorre por volta dos 40 anos de idade quando os sintomas desta hiperglicemia se tornam evidentes. Além da contribuição dos fatores genéticos, fatores ambientais também estão fortemente associados a este tipo de DM, como obesidade (principalmente a obesidade visceral), dieta rica em gorduras, envelhecimento, sedentarismo e tabagismo (SAAD et al., 2007; SBD, 2016; ADA, 2017).

O DMG refere-se a qualquer quadro de intolerância à glicose, de magnitude variável, diagnosticado durante a gravidez (normalmente entre a 24ª-28ª semana de gestação). Está associado tanto a resistência à insulina quanto a diminuição da função das células beta. Estima-se que o DMG pode ocorrer em 1-14% das gestações, elevando os riscos de complicações no parto e morbimortalidade perinatal. As mulheres que tiveram DMG têm maior risco de desenvolver DM2 (cerca de 10 a 63%) dentro de 5 a 16 anos após o parto (SAAD et al., 2007; SBD, 2016; ADA, 2017).

Finalmente, os outros tipos específicos de diabetes são aqueles de etiologias diversas como defeitos genéticos na função das células beta ou na ação da insulina, doenças do pâncreas exócrino, endocrinopatias, induzido por medicamentos ou agentes químicos, infecções e síndromes genéticas associadas ao DM, como as Síndromes de Down, Turner e Klinefelter (SAAD et al., 2007; SBD, 2016; ADA, 2017).

Os critérios laboratoriais para o diagnóstico do DM são preconizados pela ADA e adotados em quase todo o mundo: valor de glicemia plasmática após oito horas de jejum ≥ 126 mg/dL (7,0 mmol/L); valor de glicemia plasmática após 120 minutos da ingestão de 75 g de glicose anidra dissolvida em água (Teste Oral de Tolerância a Glicose - TOTG) ≥ 200 mg/dL (11,1 mmol/L); valor de hemoglobina glicada (HbA1c) ≥ 6,5% (48 mmol/mol - teste realizado por metodologia certificada pelo *National Glycohemoglobin Standartization Program* com padrão fornecido pelo *Diabetes Control and Complication Trial*); ou em pacientes com sintomas clássicos de hiperglicemia (poliúria, polidipsia e perda ponderal) e/ou em crise hiperglicêmica um valor de glicemia plasmática aleatória ≥ 200 mg/dL (11,1 mmol/L - dosagem realizada a qualquer hora do dia independente do horário das refeições). Quaisquer destes achados devem ser confirmados realizando um novo teste em outro momento (SBD, 2016; ADA, 2017).

Os estádios intermediários entre a homeostase normal da glicose e o DM também seguem critérios laboratoriais. A glicemia de jejum alterada refere-se a valor de glicemia de jejum inferior ao *cut-off* para o diagnóstico do DM, contudo mais elevada do que o valor de referência normal (≥ 100 mg/dL e < 126 mg/dL). Já a tolerância à glicose diminuída representa uma anormalidade na regulação da glicose no estado pós-sobrecarga, diagnosticada por meio do TOTG, com valor de glicemia plasmática após 2 horas entre 140 - 199 mg/dL (SBD, 2016; ADA, 2017).

2.2 Epidemiologia do diabetes mellitus

O número de indivíduos diabéticos vem aumentado em todos os países, alcançando proporções epidêmicas. De acordo com o *IDF Diabetes Atlas*, estima-se que em 2015 havia 415 milhões (prevalência 8,8%) de diabéticos em todo o mundo, sendo 75% deles residentes em países em desenvolvimento e cerca de 193 milhões

sem o diagnóstico adequado da doença. Calcula-se que em 2040 o número de diabéticos no mundo pode chegar a 642 milhões (prevalência 10,4%), evidenciando que o DM é uma das maiores emergências em saúde pública do século XXI (IDF, 2015).

O aumento no número de indivíduos com DM pode estar associado ao crescimento e envelhecimento populacional, ao estilo de vida urbano caracterizado pelo sedentarismo, maus hábitos alimentares e obesidade, bem como aos avanços no tratamento da doença, proporcionando maior sobrevida aos pacientes (WHO, 2002). Considerando-se o gênero, não há diferença significativa entre o número de homens e mulheres com DM, mas dados atuais indicam que há aproximadamente 15,6 milhões de mulheres diabéticas a mais do que homens (IDF, 2015).

No *ranking* dos países com maior número de diabéticos entre 20-79 anos, o Brasil ocupa a quarta posição com 14,3 milhões (prevalência de 10,2%) de indivíduos portadores da doença, ficando atrás somente da China (109,6 milhões), Índia (69,2 milhões) e Estados Unidos (29,3 milhões). Estimativas apontam que em 2040 o Brasil terá 23,3 milhões de indivíduos com DM (IDF, 2015).

A Organização Mundial de Saúde (OMS) prevê que o DM será a sétima principal causa de morte em 2030 (WHO, 2013). Dados do IDF apontam que em 2015, 5 milhões de pessoas entre 20-79 anos morreram em decorrência do DM e suas complicações, sendo aproximadamente 130,7 mil mortes somente no Brasil. Assim, o DM e suas complicações representam atualmente 14,5% do total de causas de morte no mundo; estimando-se uma morte a cada seis segundos em consequência dos danos hiperglicêmicos (IDF, 2015).

Em sua última edição, o *IDF Diabetes Atlas* destaca o aumento do número de casos de DM em populações indígenas no mundo, muitas vezes alcançando prevalência superior àquela observada na população geral do país (IDF, 2015). Estudo conduzido por Dal Fabbro e colaboradores (2014) com 948 índios Xavante no Mato Grosso/Brasil indica que a prevalência de DM2 entre eles é de 28,2%, sendo 18,4% em homens e 40,6% em mulheres. Ainda neste trabalho, observou-se que 17,5% da população avaliada era hipertensa e 50,8% obesa. Esses dados sinalizam a importância dos fatores ambientais e do estilo de vida no desenvolvimento do DM2, a vulnerabilidade desse grupo para doenças crônicas e suas complicações, além da necessidade de implantação de políticas públicas de saúde com vistas à prevenção do DM nestas populações.

Considerando a epidemia global de diabetes, sua natureza crônica e complicações, observa-se que o DM é uma doença muito onerosa para as famílias dos indivíduos afetados e para os sistemas de saúde (público ou privado). Estima-se que em 2015 foram gastos 673 bilhões de dólares no mundo em cuidados de saúde com o DM; e em 2040 esse gasto poderá alcançar a marca dos 802 bilhões de dólares. Neste quesito, novamente o Brasil está numa posição de destaque, pois ocupa o quinto lugar no *ranking* dos países que mais gastam com o DM: em 2015 foram 22 bilhões de dólares, podendo chegar a 36 bilhões em 2040 (IDF, 2015).

Os gastos com o DM podem ser divididos em gastos diretos, relacionados a medicamentos, testes diagnósticos, procedimentos ambulatoriais e hospitalares, dietas, consultas médicas; e gastos indiretos relacionados ao absenteísmo, licença médica, aposentadoria e morte precoce (IDF, 2015). O *Brazilian Study on Diabetes Costs* (BAHIA et al., 2011) estimou que o custo anual para o atendimento ambulatorial do paciente com DM2 é de US$2108/*per capita*, dos quais US$1335 para custos diretos e US$773 para custos indiretos. Foi observado um aumento nos custos *per capita* de acordo com a duração da doença, nível de cuidado de saúde (primário, secundário e terciário) bem como a presença de complicações micro e macrovasculares. Sugere-se, portanto a necessidade de se direcionar os esforços de saúde para o âmbito da prevenção e controle do DM e suas complicações.

2.3 Complicações microvasculares do diabetes mellitus

A hiperglicemia é um fator que predispõe ao desenvolvimento de complicações crônicas micro e macrovasculares características de todas as formas de DM. As complicações microvasculares normalmente são a retinopatia diabética (RD), doença renal do diabetes (DRD) e neuropatia diabética (NR), tornando o DM a principal causa de cegueira adquirida, DRC com ingresso em programas de diálise e transplante, além do desenvolvimento de neuropatias debilitantes em indivíduos em idade produtiva. As complicações macrovasculares são representadas pela própria doença aterosclerótica que compreende a doença arterial coronariana, acidente vascular encefálico (AVE) e doença arterial periférica (SAAD et al., 2007; SBD, 2016; ADA, 2017). Sabe-se que o DM é um fator de risco independente para DCVs, visto que em pacientes diabéticos a doença aterosclerótica tem evolução mais

rápida, com alta gravidade e acomete maior número de vasos (FOX et al., 2007). Um estudo longitudinal que acompanhou pacientes com DM2 por cerca de 10 anos, mostrou uma redução de 17% do risco de desenvolver as principais complicações cardiovasculares nos indivíduos cujo controle glicêmico foi realizado de forma intensiva quando comparado a um grupo que seguiu a terapia padrão (HAYWARD et al., 2015).

O desenvolvimento e a progressão das complicações microvasculares correlacionam-se com a duração do DM e o controle glicêmico. Embora todas as células de um paciente diabético estejam expostas a níveis elevados de glicose, o dano hiperglicêmico é limitado a alguns tipos celulares, seja pela formação de produtos finais de glicação avançada (*advanced glycation end-products* - AGEs) ou por aqueles cujo transporte de glicose não é modulado pela ação da insulina e assim apresentam hiperglicemia intracelular (SAAD et al., 2007). Estudos clínicos randomizados de larga-escala - *UKPDS*, *ADVANCE* e *VADT* - revelam de forma contundente os benefícios que o rígido controle glicêmico apresenta no que diz respeito à prevenção e prognóstico das complicações microvasculares do DM (UKPDS GROUP et al., 1998; ADVANCE GROUP et al., 2008; DUCKWORTH et al., 2009).

A patogênese da RD, DRD e NR ainda não está completamente elucidada. Estudos sumarizam que a ativação de vias inflamatórias intracelulares em tecidos específicos, o estresse oxidativo, a produção de fatores de crescimento, citocinas, quimiocinas e outros mediadores inflamatórios podem estar associados ao desenvolvimento destas complicações (BROWNLEE, 2001; KING, 2008; MARCOVECCHIO et al., 2011; FORBES e COOPER, 2013; REIDY et al., 2014; SANDIREDDY et al., 2014; WAN et al., 2015).

Desta forma, os principais mecanismos descritos que fazem a conexão entre a hiperglicemia e a patogênese das complicações microvasculares são (BROWNLEE, 2001; KING, 2008; MARCOVECCHIO et al., 2011; FORBES e COOPER, 2013; REIDY et al., 2014; SANDIREDDY et al., 2014; WAN et al., 2015):

1) *Aumento do fluxo de glicose pela via dos polióis*. Em condições hiperglicêmicas, nas células que independem da ação da insulina para a entrada de glicose, ocorre um aumento do consumo de NADPH pela aldose redutase para conversão intracelular da glicose em sorbitol. Como consequência, o acúmulo de sorbitol causa

estresse osmótico e lesão vascular. Além disso, a menor disponibilidade de NADPH diminui a regeneração da glutationa reduzida, exacerbando o estresse oxidativo.

2) *Aumento do fluxo de glicose pela via da hexosamina*. O excesso de glicose intracelular faz com que uma parte da frutose-6-fosfato seja desviada da via glicolítica produzindo UDP-N-acetilglicosamina. A glicosilação do fator de transcrição Sp1, pela adição de N-acetilglicosamina, está associada ao aumento da expressão do inibidor do ativador de plasminogênio 1 (PAI-1) e do fator de crescimento transformante beta (TGF-β), proteínas associadas ao processo hemostático e inflamatório. Além disso, outras proteínas nucleares e citoplasmáticas podem ser modificadas por glicosilação, interferindo na função destas.

3) *Glicação não-enzimática de proteínas intra e extracelulares levando a formação de AGEs*. A modificação de proteínas e componentes da matriz extracelular pelos AGEs implica em alteração do trânsito destas moléculas, de suas funções e formas de interação. A ligação dos AGEs com os receptores de produtos finais de glicação avançada (RAGEs), principalmente em células endoteliais, mesangiais e macrófagos, induz a produção de espécies reativas de oxigênio (EROs) e ativa o fator de transcrição nuclear kapa B (NF-κB), aumentando a expressão de citocinas/quimiocinas pró-inflamatórias, fatores de crescimento, bem como de substâncias pró-coagulantes.

4) *Ativação de isoformas da proteína quinase C (PKC)*. A hiperglicemia intracelular aumenta a síntese *de novo* do segundo mensageiro lipídico, o diacilglicerol (DAG), que por sua vez ativa isoformas de PKC (principalmente as isoformas β e δ). Por consequência, há aumento da expressão da enzima óxido nítrico sintase endotelial (eNOS), endotelina-1, fator de crescimento do endotélio vascular (VEGF), TGF-β1, PAI-1, fibronectina e colágeno tipo IV. Além disso, a ativação dessas isoformas de PKC promove a ativação de NF-κB e de oxidases de membrana NAD(P)H dependentes.

Todos os mecanismos discutidos acima causam anormalidades do fluxo sanguíneo, aumento da permeabilidade vascular e da síntese de matriz extracelular, oclusão de vasos e isquemia, angiogênese e ativação das vias de sinalização pró-inflamatórias, os quais contribuem para a fisiopatologia da RD, DRD e NR.

A RD é a principal causa de cegueira em pessoas com idade entre 20 e 74 anos. Estima-se que após 20 anos de diagnóstico de DM, mais de 90% dos pacientes com DM1 e 60% daqueles com DM2 apresentarão algum grau de RD. Os

principais fatores de risco associados ao desenvolvimento e progressão desta complicação são: tempo de diagnóstico do DM, mau controle glicêmico, hipertensão, dislipidemia e doença renal do diabetes (ADA, 2017). Clinicamente, a RD pode ser classificada em não-proliferativa, proliferativa e edema macular (principal causa de perda visual), cujos critérios de diagnóstico atualmente aceitos foram definidos por Wilkinson e colaboradores (2003) tendo como base o *Diabetic Retinopathy Study* (DRS - 1976 a 1979) e *Early Treatment Diabetic Retinopathy* (ETDRS - 1985 a 1997) (**Quadro 1**).

Quadro 1: Classificação da retinopatia diabética e do edema macular.

Gravidade da retinopatia	**Achados à oftalmoscopia sob dilatação pupilar**
Sem RD aparente	Sem alterações
RD não proliferativa leve	Microaneurismas apenas
RD não proliferativa moderada	Achados mais abundantes que na RD não proliferativa leve e menos abundantes que na RD não proliferativa grave
RD não proliferativa grave	Presença de um dos seguintes achados: mais de 20 hemorragias retinianas em cada um dos quatro quadrantes retinianos, ensalsichamento venoso em dois quadrantes ou microanormalidades vasculares intrarretinianas em um quadrante
RD proliferativa	Presença de neovasos e/ou hemorragia vítrea ou pré-retiniana
Gravidade do edema macular	**Achados à oftalmoscopia sob dilatação pupilar**
EM aparentemente ausente	Ausência de espessamento retiniano ou exsudatos duros no polo posterior
EM aparentemente presente	Presença de espessamento retiniano ou exsudatos duros no polo posterior
Se EM presente classifica-se em:	
EM leve	Algum grau de espessamento de retina ou exsudatos duros no polo posterior, porém distantes do centro foveal
EM moderado	Espessamento de retina próximo ao centro da mácula, mas ainda não atingindo seu centro
EM grave	Espessamento de retina ou exsudatos duros atingindo o centro da mácula

RD: retinopatia diabética; EM: edema macular. Adaptado de: SBD, 2016.

As fases iniciais da RD são caracterizadas pela perda de células endoteliais e pericitos nos capilares retinianos por apoptose, microaneurismas e hemorragias intrarretinianas de ponto. Com a evolução da doença, essas hemorragias aumentam em número e tamanho, acompanhado pelos *cotton-wool*, indicando falência da microcirculação da retina e isquemia. Um exame oftalmoscópico revela veias dilatadas, tortuosas e de calibre irregular, enquanto as artérias são pouco perfundidas. Nos estágios mais avançados, pode-se evoluir para a fase proliferativa com aumento da expressão de fatores de crescimento, neovascularização da retina e do disco óptico, risco de descolamento tracional de retina e aumento da probabilidade de perda da visão (FRANK, 2004; SAAD et al., 2007; WAN et al., 2015).

A quebra da barreira hemato-retiniana é outra alteração importante na RD culminando no edema macular. Este edema é caracterizado pelo aumento da permeabilidade dos pequenos vasos sanguíneos da mácula, formação de depósitos extravasculares de lípides e lipoproteínas, recrutamento de macrófagos, acúmulo de material fibrinóide e formação de exsudato duro. Embora o edema macular não cause cegueira total, ele está associado a perdas severas da visão central, visto que a mácula é a porção central da retina e a responsável pela maior parte da visão funcional. O edema macular pode estar presente desde as fases iniciais da RD até nos casos de doença proliferativa grave, acometendo cerca de 30% dos pacientes com DM2 com mais de 20 anos de diagnóstico (FRANK, 2004; WAN et al., 2015; SBD, 2016).

O risco de cegueira em decorrência da RD pode ser reduzido a menos de 5% quando o diagnóstico desta complicação for realizado de forma precoce e o tratamento feito corretamente (principalmente o acompanhamento/controle dos fatores de risco e das comorbidades, laserterapia) (SBD, 2016). Portanto, os pacientes diabéticos devem dispor de rigoroso e periódico acompanhamento oftalmológico, a fim de evitar a instalação de lesões irreversíveis.

A DRD refere-se a doença renal causada pelo DM na qual, frequentemente, observa-se alteração na taxa de filtração glomerular (TFG) e na excreção urinária de albumina (EUA). A DRD ocorre em 20-40% dos pacientes com DM e é a principal causa de DRC e ingresso em programas de diálise. Além disso, a DRD é fortemente associada ao aumento da mortalidade por DCVs (SBD, 2016; ADA, 2017).

Desde 2015, a ADA se posiciona a favor do desuso dos termos micro e macroalbuminúria, uma vez que a albuminúria ocorre *on a continuum* e a progressão destes estágios não é inexorável (ADA, 2015; ADA, 2017). Portanto, a ADA recomenda o uso dos termos albuminúria normal e albuminúria elevada, sendo que este último engloba as categorias anteriormente denominadas de micro e macroalbuminúria (ADA, 2017). Seguindo esta linha, a *Kidney Disease Improving Global Outcomes* (KDIGO) concorda com o posicionamento da ADA, porém sugere a adoção da seguinte divisão: albuminúria normal (< 30 mg/g = mg albumina/g de creatinina), albuminúria elevada (30 a 300 mg/g) e albuminúria muito elevada (> 300 mg/g) (LEVEY et al., 2011; ADA, 2017).

A SBD recomenda, portanto, que o termo nefropatia diabética (ND) deve ser utilizado apenas para pacientes com proteinúria detectável e persistente, em geral associada à elevação da pressão arterial (SBD, 2016).

O rastreamento e acompanhamento da DRD devem ser realizados de forma periódica baseando-se na medida da albuminúria e na estimativa da TFG (SBD, 2016; ADA, 2017) (**Quadros 2** e **3**).

Quadro 2: Valores de albuminúria utilizados para o diagnóstico de doença renal do diabetes.

Amostra casual de urina	Valores
Concentração de albumina	≥ 14 mg/L
Índice albumina:creatinina	≥ 30 mg/g
Amostra de urina de 24h	≥ 30 mg/24 horas

Adaptado de: SBD, 2016.

O desenvolvimento e a progressão da DRD envolvem processos complexos, dadas a diversidade de populações celulares presentes nos rins e as várias funções exercidas por este órgão. Portanto, a DRD pode ser caracterizada por lesões glomerulares, tubulares, intersticiais e vasculares (FORBES e COOPER, 2013). A fase inicial da DRD é caracterizada por alterações hemodinâmicas que culminam na hiperfiltração glomerular, seguida por um longo período de declínio da função e do número de glomérulos com consequente instalação da DRC. As principais alterações morfológicas e estruturais observadas ao longo da evolução da DRD são:

expansão mesangial, espessamento da membrana basal glomerular, depósito de colágeno, hialinose arterial, perda e hipertrofia de podócitos, atrofia do epitélio tubular, acúmulo de miofibroblastos ativados, influxo de células inflamatórias e perda da arquitetura capilar (DRONAVALLI et al., 2008; REIDY et al., 2014)

Quadro 3: Doença renal do diabetes - estágios de classificação da doença renal crônica de acordo com a taxa de filtração glomerular e a excreção urinária de albumina.

Estágio	Descrição	Taxa de filtração glomerular ($mL/min/1,73m^2$)
1	TFG normal ou elevada *	≥ 90
2	TFG levemente reduzida *	60-89
3A	Moderada redução da TFG	45-59
3B	Redução marcada da TFG	30-44
4	Redução grave da TFG	15-29
5	Insuficiência renal	< 15

TFG: taxa de filtração glomerular. *Excreção urinária de albumina elevada, segundo Levey et al., 2011. Adaptado de: SBD, 2016.

O rastreamento da DRD deve ser iniciado imediatamente após o diagnóstico clínico/laboratorial de DM2. A detecção precoce e diferencial com outras doenças renais, bem como o tratamento adequado, permitem bom controle da DRD e prevenção para os desfechos cardiovasculares (ADA, 2017).

A NR é um grupo heterogêneo de distúrbios neurológicos que afeta cerca de 50% dos pacientes com DM. Dado o amplo acometimento patológico do sistema nervoso, a NR é responsável pela piora significativa da qualidade de vida dos indivíduos por incapacitação e diminuição da sobrevida. As alterações neuropáticas afetam o sistema nervoso periférico (somático e autônomo), sendo que suas principais formas são representadas pela polineuropatia sensorimotora simétrica e neuropatia autonômica (SBD, 2016).

O diagnóstico das diversas formas de NR baseia-se na caracterização do quadro clínico, avaliando sinais e sintomas típicos, além da realização de testes neurológicos. O comprometimento somático normalmente se caracteriza por dor, sensação de queimação e formigamento, pontadas, choques, desconforto ou dor ao

toque (inclusive em lençóis e cobertores), diminuição ou perda da sensibilidade tátil, térmica ou dolorosa. Ainda que a predominância de sintomas se localize nos membros inferiores, os membros superiores também podem ser afetados. Além disso, a ausência de sinais e sintomas de parestesia não exclui a neuropatia, pois alguns pacientes evoluem direto para a perda total de sensibilidade. Por sua vez, o comprometimento autonômico se caracteriza principalmente por alterações cardiovasculares (taquicardia de repouso, intolerância ao exercício, hipotensão ortostática, arritmias cardíacas), gastrintestinais (gastroparesia, constipação, diarréia, náusea, vômito) e genitourinárias (disfunção erétil, ejaculação retrógrada, incontinência urinária, infecções recorrentes do trato urinário) (BOULTON et al., 2005; SBD, 2016, ADA, 2017).

Assim como nas demais complicações microvasculares, a NR também tem sua patogênese ligada a causas metabólicas e vasculares que resultam em vasoconstrição, isquemia e hipóxia com consequente deterioração das fibras mielinizadas e diminuição da velocidade de condução dos estímulos nervosos (FORBES e COOPER, 2013).

Conforme exposto, a ocorrência de complicações microvasculares é bastante frequente no curso do DM, não sendo raro observar a “triopatia diabética” - RD, DRD e NR - em pacientes com pouco tempo de diagnóstico do DM2. Assim, além da doença de base, essas complicações também são um importante problema de saúde pública pela redução significativa na qualidade de vida e na sobrevida dos pacientes. Torna-se evidente, portanto, que o diagnóstico precoce do DM, o adequado controle glicêmico e o reconhecimento prematuro das lesões são estratégias básicas de prevenção e controle da progressão destas complicações.

2.4 Inflamação, obesidade e diabetes mellitus

A inflamação é uma resposta adaptativa desencadeada por estímulos nocivos, tais como infecções, estresse e dano tecidual, que visa restaurar a homeostase dos sistemas fisiológicos (MEDZHITOV, 2008). As doenças humanas modernas, incluindo o DM2 e a obesidade, têm em comum duas características: perturbação da homeostase e inflamação crônica. Segundo esse conceito, as variáveis homeostáticas não se mantêm constantes, mas sim dentro de um intervalo

aceitável capaz de se ajustar de acordo com as necessidades fisiológicas, condições ambientais e infecções (KOTAS e MEDZHITOV, 2015).

A inflamação observada no DM2 e na obesidade é conhecida como meta-inflamação ou inflamação metabólica. Ela está associada ao mau funcionamento tecidual no qual há um desequilíbrio homeostático em um ou em vários sistemas fisiológicos que não estão diretamente e funcionalmente relacionados à defesa do hospedeiro ou ao reparo tecidual (MEDZHITOV, 2008). Além disso, observa-se que a perda do controle homeostático impede a resolução do processo inflamatório que se torna crônico e contribui para a patogênese desta doença (NATHAN e DING, 2010).

Desde a década de 90, tornou-se evidente que a inflamação tem um papel central na patogênese da obesidade e do DM2. Esta conexão foi primeiro reportada por Hotamisligil e colaboradores (1993), os quais demonstraram que: adipócitos expressam constitutivamente citocinas pró-inflamatórias como o fator de necrose tumoral alfa (TNF-α); a expressão de TNF-α é mais elevada em roedores obesos; e por fim, a neutralização de TNF-α por anticorpos leva à diminuição da resistência insulínica nesses animais. De fato, trabalhos contemporâneos a este evidenciaram que o TNF-α interfere na via de sinalização da insulina, inibindo a atividade de tirosina quinase do seu receptor no tecido adiposo e muscular esquelético de ratos (HOTAMISLIGIL et al.,1994a; HOTAMISLIGIL et al.,1994b). Além disso, foi observado que a expressão do mRNA do TNF-α e os níveis da proteína são maiores no tecido adiposo de indivíduos obesos quando comparado a indivíduos magros e que há uma correlação positiva entre a expressão de TNF-α no tecido adiposo e o grau de hiperinsulinemia (HOTAMISLIGIL et al.,1995). Em suma, obesidade e DM2 são alterações metabólicas caracterizadas por um estado inflamatório crônico de baixo grau com níveis circulantes de citocinas e quimiocinas pró-inflamatórias, proteínas de fase aguda, adipocinas, mediadores lipídicos e ácidos graxos livres (AGL) mais elevados (WELLEN e HOTAMISLIGIL, 2005; MANCUSO, 2016).

O tecido adiposo pode ser basicamente dividido em tecido adiposo marrom e branco. O papel do primeiro é reconhecidamente a produção de calor (isolante térmico) sendo encontrado em grande quantidade nos recém-nascidos e sofrendo involução significativa nos adultos. Por sua vez, o tecido adiposo branco, presente em diferentes partes do corpo humano, é responsável, principalmente, pelo estoque de energia na forma de triglicérides. O tecido adiposo visceral se refere

coletivamente aos depósitos intra-abdominais de tecido adiposo branco que se comportam como um sensor do estado metabólico do organismo (WENSVEEN et al., 2015).

A obesidade foi reconhecida pela OMS em 1948 como uma doença (JAMES, 2008). De fato, o acúmulo de gordura visceral é um fator de risco independente para o desenvolvimento de alterações metabólicas, como resistência à insulina e DM2, além de aumentar do risco de DCVs e de morte prematura (KOPELMAN, 2000). Observa-se que nas últimas décadas a obesidade alcançou o *status* de pandemia e se destaca como um grave problema de saúde pública, tanto em países desenvolvidos quanto em desenvolvimento (NG et al., 2014).

Atualmente, o tecido adiposo é considerado um órgão endócrino capaz de produzir e secretar uma série de produtos bioativos denominados de adipocinas. As adipocinas são hormônios (adiponectina, leptina, resistina), citocinas (TNF-α, IL-6, IL-1β), fatores de crescimento (TGF-β, VEFG), proteínas de fase aguda (proteína C reativa - PCR, haptoglobina - Hp), substâncias vasoativas (PAI-1, fator tissular, angiotensina II), além de irisina, visfatina, entre outras. Elas exercem efeito sobre a biologia dos adipócitos (funções autócrina e parácrina), mas também são secretadas para a corrente sanguínea e participam de vários processos biológicos (função endócrina) (MANCUSO, 2016). O tecido adiposo visceral é ainda um sítio importante de células do sistema imune (neutrófilos, macrófagos, linfócitos T e células dendríticas) que têm papel importante no controle da estrutura e homeostase tecidual (WANG et al., 2008; RODRÍGUEZ et al., 2015; WENSVEEN et al., 2015). Pode-se concluir, portanto, que o tecido adiposo é um componente fundamental na regulação metabólica e nas respostas imune e inflamatória.

O aumento da massa adiposa na obesidade se dá pela hiperplasia (aumento do número) e pela hipertrofia (aumento do tamanho) dos adipócitos com alterações no padrão de secreção das adipocinas e mudança nos tipos de células imunes que predominam no tecido (**Figura 1**). O tecido adiposo magro é caracterizado pela presença de macrófagos do tipo M2, linfócitos T *helper* 2 (Th2) $CD4^+$, células T reg e eosinófilos. Esses tipos celulares estão relacionados principalmente a produção de citocinas anti-inflamatórias (IL-4, IL-5, IL-10, IL-13), manutenção da polarização M2 dos macrófagos do tecido adiposo, inibição da ativação de células imunes e da citotoxicidade das células *Natural Killer*. Na obesidade, entretanto, observa-se o predomínio de tipos celulares associados a um perfil pró-inflamatório (macrófagos

M1, linfócitos T - Th1 $CD4^+$, T $CD8^+$, Th17, *Natural Killer* - e B, neutrófilos, mastócitos e células dendríticas) com aumento da secreção de TNF-α, IL-1β, IL-6, IFN-γ. A redução da relação de macrófagos M2/M1 no tecido adiposo obeso ocorre devido à polarização dos macrófagos residentes M2 para o perfil M1, induzido principalmente por citocinas pró-inflamatórias; ou pelo recrutamento de monócitos pró-inflamatórios que, ao alcançarem o tecido, se diferenciam em macrófagos M1 (XU, 2013; RODRÍGUEZ et al., 2015; WENSVEEN et al., 2015; KHODABANDELOO et al., 2016).

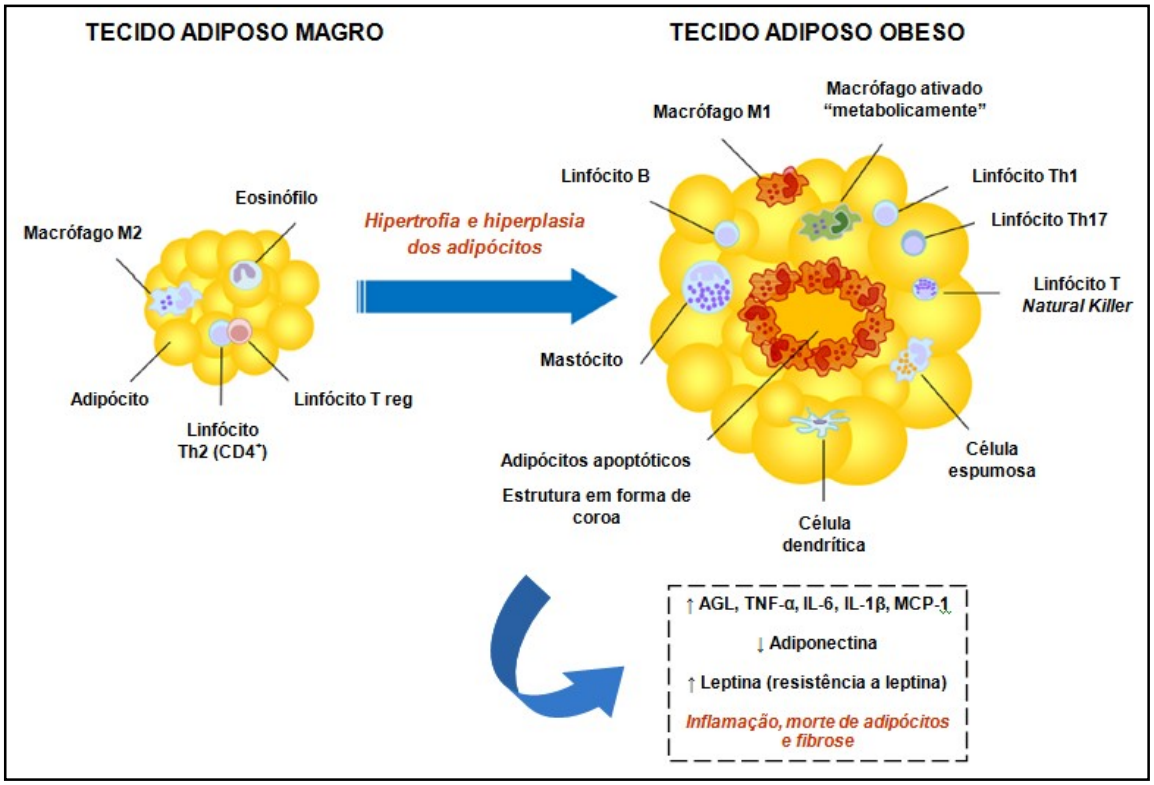


Figura 1: Inflamação crônica na obesidade e mudanças nas populações celulares do tecido adiposo. O tecido adiposo magro possui um perfil celular que secreta citocinas anti-inflamatórias como IL-4, IL-10 e IL-13. A hiperplasia e hipertrofia deste tecido na obesidade é acompanhada pela mudança dos tipos celulares com predomínio de células pró-inflamatórias como macrófagos M1 e linfócitos T e B. Como consequência, tem-se o aumento da produção de mediadores pró-inflamatórios (TNF-α, IL-6, IL-1β, MCP-1), alteração da secreção de adipocinas, inflamação, morte celular e fibrose. Adipócitos apoptóticos são circundados por macrófagos M1 formando uma estrutura em forma de coroa. AGL: ácidos graxos livres, IL: interleucina, MCP-1: proteína quimiotática de monócitos 1, TNF-α: fator de necrose tumoral alfa. Adaptado de: RODRÍGUEZ et al., 2015.

Esse ambiente pró-inflamatório, característico da obesidade, promove a disfunção do tecido adiposo e consequente diminuição sistêmica da sensibilidade à insulina. A hipóxia observada é responsável pela alteração na expressão de genes,

aumento da secreção de adipocinas pró-inflamatórias e indução da glicólise anaeróbica com aumento das concentrações de lactato, que por sua vez ativam vias de sinalização inflamatórias em macrófagos. À disfunção dos adipócitos segue-se o aumento da morte celular devido à ativação das vias intrínseca e extrínseca de apoptose e fibrose do tecido (TRAYHURN, 2013).

Vários mecanismos moleculares têm sido propostos para explicar como a obesidade e a inflamação contribuem para a resistência à insulina e consequentemente para o desenvolvimento do DM2. Trabalhos recentes destacam o envolvimento das vias IKKβ/NF-κB e JNK/AP1 no desenvolvimento da resistência à insulina (CHEN et al., 2015; KHODABANDELOO et al., 2016; LACKEY e OLEFSKY, 2016; SOLINAS e BECATTINI, 2016).

- *Via IKKβ/NF-κB:* a IκB quinase β (IKKβ) é uma serina quinase capaz de fosforilar resíduos de serina do IRS-1, com consequente menor ativação da via PI3q/Akt e comprometimento das ações metabólicas da insulina. Adicionalmente, IKKβ catalisa a fosforilação de dois resíduos conservados de serina (ser32 e ser36) da IκBα, provocando a degradação do complexo proteico citoplasmático e a liberação de NF-κB, que se transloca para o núcleo ligando-se a sequências específicas dos promotores de genes que codificam mediadores pró-inflamatórios (**Figura 2**).

- *Via JNK/AP-1:* as JNKs (c-Jun-N-terminal) são proteínas quinases serina/treonina que pertencem à família das proteínas quinases ativadas por mitógenos (MAPK). São ativadas pela fosforilação em resíduos específicos de treonina e tirosina em resposta a uma variedade de sinais celulares. De forma semelhante à IKKβ, JNK também fosforila resíduos de serina do IRS-1 inibindo a via de sinalização da insulina. Além disso, JNK ativa o fator de transcrição c-Jun que pode formar homodímeros ou heterodímeros com c-Fos dando origem ao complexo de transcrição AP-1 (proteína ativadora 1). Este complexo se liga a sequências específicas dos promotores de genes metabólicos e pró-inflamatórios, ativando-os. Além disso, sabe-se que JNK pode inibir a secreção de insulina a partir das células beta via mediadores pró-inflamatórios como IL-1β (**Figura 2**).

Muitos estímulos podem ativar as vias IKKβ/NF-κB e JNK/AP-1 (**Figura 2**). As citocinas pró-inflamatórias TNF-α e IL-1β ativam essas vias através de mecanismos mediados pela interação com seus receptores clássicos. AGL, presentes em excesso na obesidade, podem se ligar aos receptores do tipo Toll (TLR), principalmente TLR4, ativando essas vias de sinalização. Além disso, a

hiperglicemia favorece a formação dos AGEs que são reconhecidos por seus receptores (RAGEs). O aumento da concentração de AGL, estimulada pela lipólise nos adipócitos, também é responsável pelo estresse do retículo endoplasmático, disfunção mitocondrial com geração de EROs, aumento da produção de ceramidas e ativação de isoformas de PKC em virtude do acúmulo intracelular de DAG. Uma vez ativadas, as vias produzem mediadores inflamatórios que retroalimentam os processos, favorecendo a inflamação crônica sistêmica (SHOELSON et al., 2006; CHEN et al., 2015; KHODABANDELOO et al., 2016).

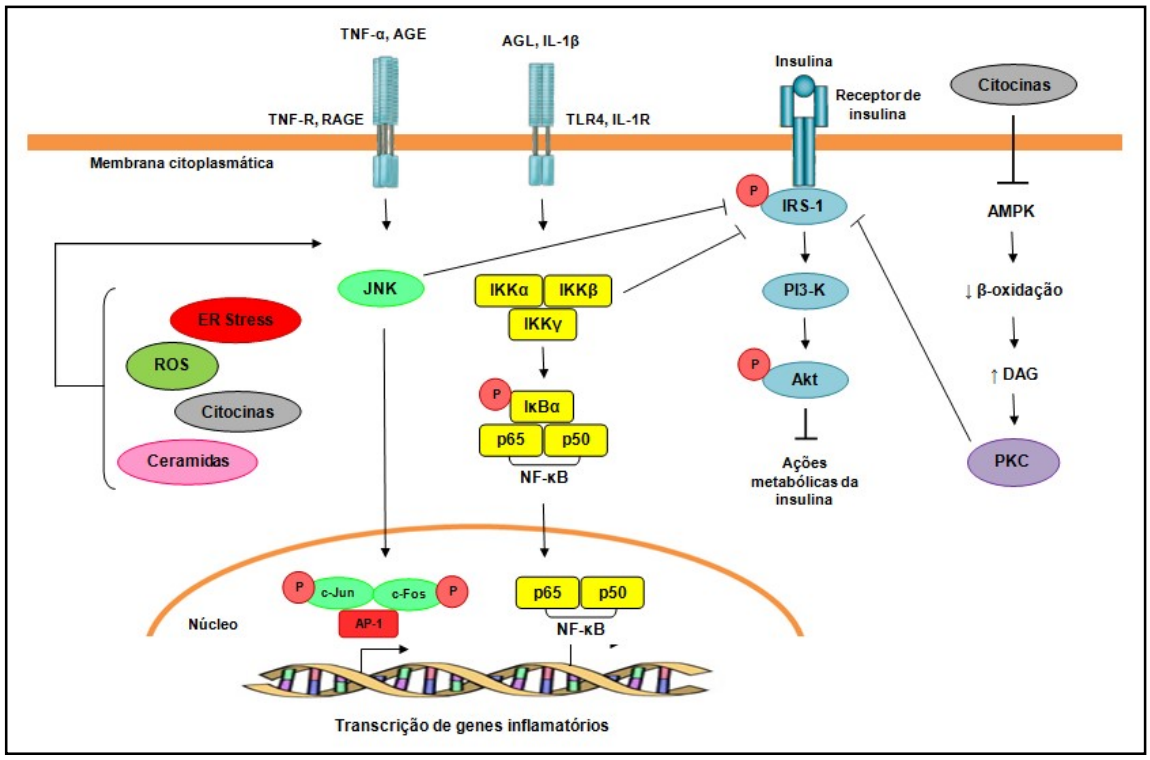


Figura 2: Mecanismos moleculares que associam obesidade, inflamação e resistência à insulina. Na obesidade, as vias IKKβ/NF-κB e JNK/AP-1 são ativadas em adipócitos, hepatócitos e macrófagos associados. Os estímulos que ativam essas vias incluem os ligantes para os receptores TNFR, IL-1R, TLR4 ou RAGE, estresse do retículo endoplasmático, disfunção mitocondrial com geração de ROS, ceramidas, citocinas pró-inflamatórias e isoformas ativadas de PKC. A ativação de IKKβ com consequente translocação de NF-κB para o núcleo aumenta a expressão de genes relacionados à inflamação. De forma semelhante, a ativação de JNK e a formação do complexo de transcrição AP-1 também aumenta a expressão de genes inflamatórios. IKKβ, PKC e as MAPK (JNK e ERC1/2) promovem a fosforilação de resíduos de serina, principalmente do IRS-1, regulando negativamente a sinalização da insulina. Citocinas pró-inflamatórias como TNF-α inibem a atividade de AMPK provocando redução da oxidação de ácidos graxos, acúmulo de DAG e ativação de isoformas de PKC. A disfunção mitocondrial também aumenta as concentrações intracelulares de DAG. AGE: produtos finais de glicação avançada, AGL: ácidos graxos livres, Akt: proteína quinase B, AMPK: proteína quinase ativada por AMP, AP-1: proteína ativadora 1, DAG: diacilglicerol, ER: retículo endoplasmático, IKK: I kapa B quinase, IL: interleucina, IRS-1: substrato do receptor de insulina 1, JNK: quinase c-Jun N-terminal, NF-κB: fator nuclear kapa B, PI3-K: fosfatidilinositol 3-quinase, PKC: proteína quinase C, RAGE: receptor de produtos finais de glicação avançada, ROS: espécies reativas de oxigênio, TLR: receptor do tipo Toll, TNF-R: receptor do fator de necrose tumoral alfa, TNF-α: fator de necrose tumoral alfa. Fonte: Elaborada pelo autor.

O inflamassoma é um complexo proteico de alto peso molecular presente no citoplasma de células imunes ativadas que promove a ativação de caspases inflamatórias (MARTINON et al., 2002). É constituído basicamente por proteínas da família dos receptores NLR (*NOD-like receptors*), proteínas adaptadoras como NALP (*neutrophilic alkaline phosphatases*) e ASC (*apoptosis associated speck-like protein*), além da caspase-1 (BROZ e DIXIT, 2016). A formação do inflamassoma é deflagrada em resposta a sinais de infecção, dano tecidual ou alterações metabólicas. Tem por função promover a maturação e liberação das citocinas pró-inflamatórias IL-1β e IL-18, amplificar os sinais inflamatórios, além de causar morte celular por piroptose (LATZ et al., 2013; CHOI e RYTER, 2014; BROZ e DIXIT, 2016).

O inflamassoma NLRP3 pode ser considerado um sensor da homeostase metabólica, visto que alguns metabólitos cujas concentrações estão elevadas na obesidade e no DM2 podem atuar como ativadores desta via (ABDERRAZAK et al., 2015; HANEKLAUS e O'NEILL, 2015). Tem sido descrito que a ativação do inflamassoma NLRP3 requer dois sinais. O primeiro pode ser desencadeado pela glicose, LDL oxidada e AGL, promovendo a ativação do fator de transcrição NF-κB para expressão de NLRP3 e pró-IL-1β. Pró-IL-18 é constitutivamente expressa e tem sua concentração aumentada mediante a ativação celular. O segundo sinal pode ser proveniente de outros metabólitos e EROs, deflagrando a formação do inflamassoma. O recrutamento de ASC pela NLRP3 é pré-requisito absoluto para a formação do inflamassoma. O complexo NLRP3-ASC sofre oligomerização, a pró-caspase-1 é convertida em caspase-1 e esta última catalisa a clivagem das proformas de IL-1β e IL-18 promovendo a secreção não-clássica destas citocinas (LATZ et al., 2013; CHOI e RYTER, 2014; HANEKLAUS e O'NEILL, 2015; BROZ e DIXIT, 2016) (**Figura 3**).

Estudos indicam que o inflamassoma NLRP3 exerce efeito sobre a inflamação associada à obesidade, resistência à insulina e DM2. Schroder et al. (2010) propuseram que o inflamassoma NLRP3 pode contribuir para a falência de células beta e manutenção do estado hiperglicêmico. Vandanmagsar et al. (2011) observaram redução da expressão de NLRP3 e da inflamação em indivíduos obesos e diabéticos que perderam peso devido à restrição calórica ou prática de exercício físico. Stienstra et al. (2011) também observaram que camundongos deficientes em

NLRP3, ASC e caspase-1 são resistentes ao desenvolvimento de obesidade induzida por dieta rica em gorduras.

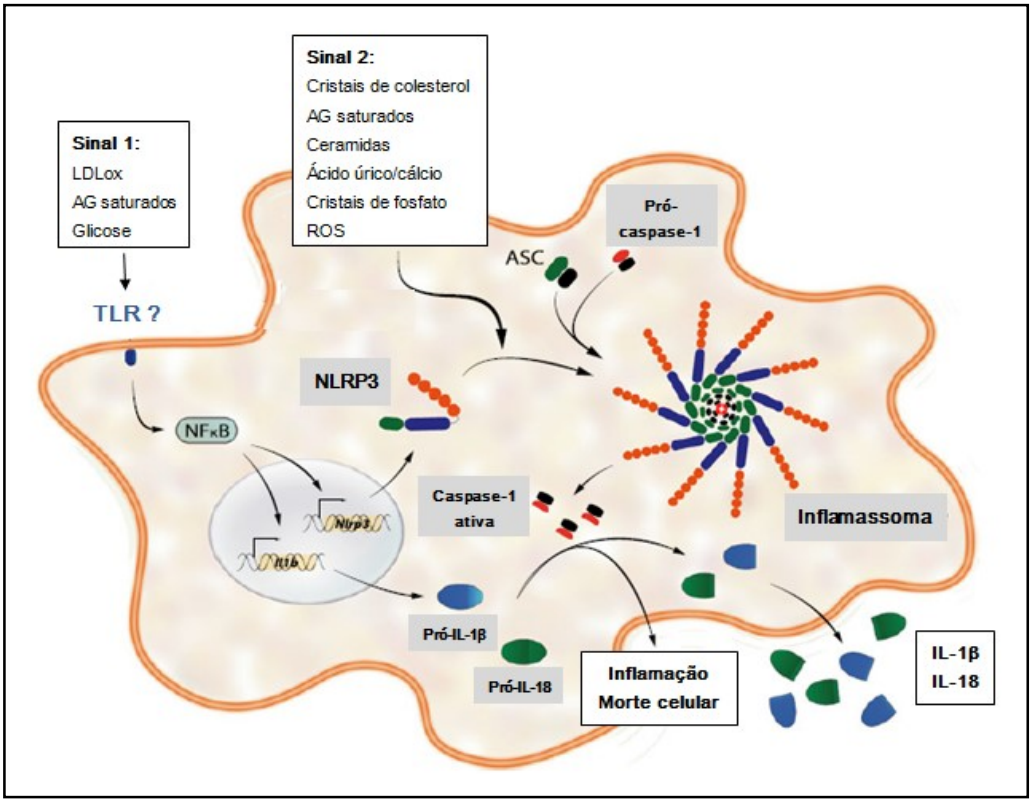


Figura 3: Ativação do inflamassoma NLRP3 por metabólitos. Metabólitos cujas concentrações estão elevadas no DM2 e na obesidade podem induzir a ativação do inflamassoma NLRP3 que promove a secreção de IL-1β e IL-18 a partir da clivagem proteolítica de suas proformas pela caspase-1 ativada. Estas potentes citocinas pró-inflamatórias podem interferir na via de sinalização da insulina no fígado e tecidos muscular e adiposo, além de amplificar os sinais inflamatórios e causar morte celular. AG: ácidos graxos, ASC: *apoptosis associated speck-like protein*, IL: interleucina, LDLox: lipoproteína de baixa densidade oxidada, NF-κB: fator nuclear kapa B, ROS: espécies reativas de oxigênio. Adaptado de: HANEKLAUS e O'NEILL, 2015.

O aumento dos níveis circulantes de IL-1β contribui para a resistência à insulina por promover diretamente a fosforilação de resíduos de serina do IRS-1 e por induzir a produção de TNF-α, que também é, sabidamente, um regulador negativo da via de sinalização da insulina. Além disso IL-1β e IL-18 promovem o recrutamento de linfócitos Th1 $CD4^+$ para o tecido adiposo amplificando a resposta inflamatória (ABDERRAZAK et al., 2015).

Está cada vez mais evidente que a ativação do sistema imune inato e a resposta inflamatória têm papel importante na patogênese da obesidade, resistência à insulina e DM2. O conhecimento detalhado destes mecanismos poderá elucidar as

principais características desses distúrbios metabólicos, bem como ampliar as pesquisas para novas abordagens terapêuticas. Além disso, a descrição e validação de marcadores sanguíneos associados a estes mecanismos auxiliará no diagnóstico e prevenção das complicações do DM2.

3. JUSTIFICATIVA

É inequívoca a evidência de que o processo inflamatório associado a ativação do sistema imune e obesidade contribuem para a patogênese do DM2 e suas complicações microvasculares, embora os mecanismos ainda não tenham sido completamente elucidados.

Trabalhos anteriores do nosso grupo de pesquisa demonstraram que existe uma associação entre polimorfismos nos genes do TGF-β1 e IL-10 com complicações microvasculares e comorbidades do DM2 (RODRIGUES et al., 2015), além da relação entre a ativação de vias inflamatórias intracelulares periféricas em indivíduos com DM2 e obesos (PIETRANI et al., 2015).

Considerando nossos resultados prévios, as proporções epidêmicas alcançadas pelo DM2 e obesidade, bem como o conhecimento de que o padrão de secreção das adipocinas é alterado pelos processos imune-inflamatórios, este trabalho justifica-se na medida em que se pretende avaliar a associação de marcadores anti e pró-inflamatórios (irisina, citocinas, visfatina e haptoglobina), marcadores de ativação celular (micropartículas) e disfunção endotelial (endocan) com o DM2, suas complicações microvasculares e comorbidades comumente observadas nestes pacientes.

É importante ressaltar que poucos são os trabalhos na população brasileira que buscaram caracterizar um extenso perfil de marcadores moleculares e inflamatórios em um mesmo grupo amostral.

Assim, acredita-se que a melhor compreensão sobre o comportamento destes marcadores no DM2, da resposta inflamatória crônica e da fisiopatologia da doença, será importante para a adoção de novas estratégias complementares no acompanhamento dos pacientes e prognóstico.

4. OBJETIVOS

4.1 Objetivo geral

Avaliar a associação de marcadores inflamatórios, bioquímicos e moleculares, com o diabetes mellitus tipo 2, suas complicações microvasculares e comorbidades.

4.2 Objetivos específicos

Comparar, entre um grupo de pacientes com DM2 (grupo caso) e controles não-diabéticos, os seguintes parâmetros:

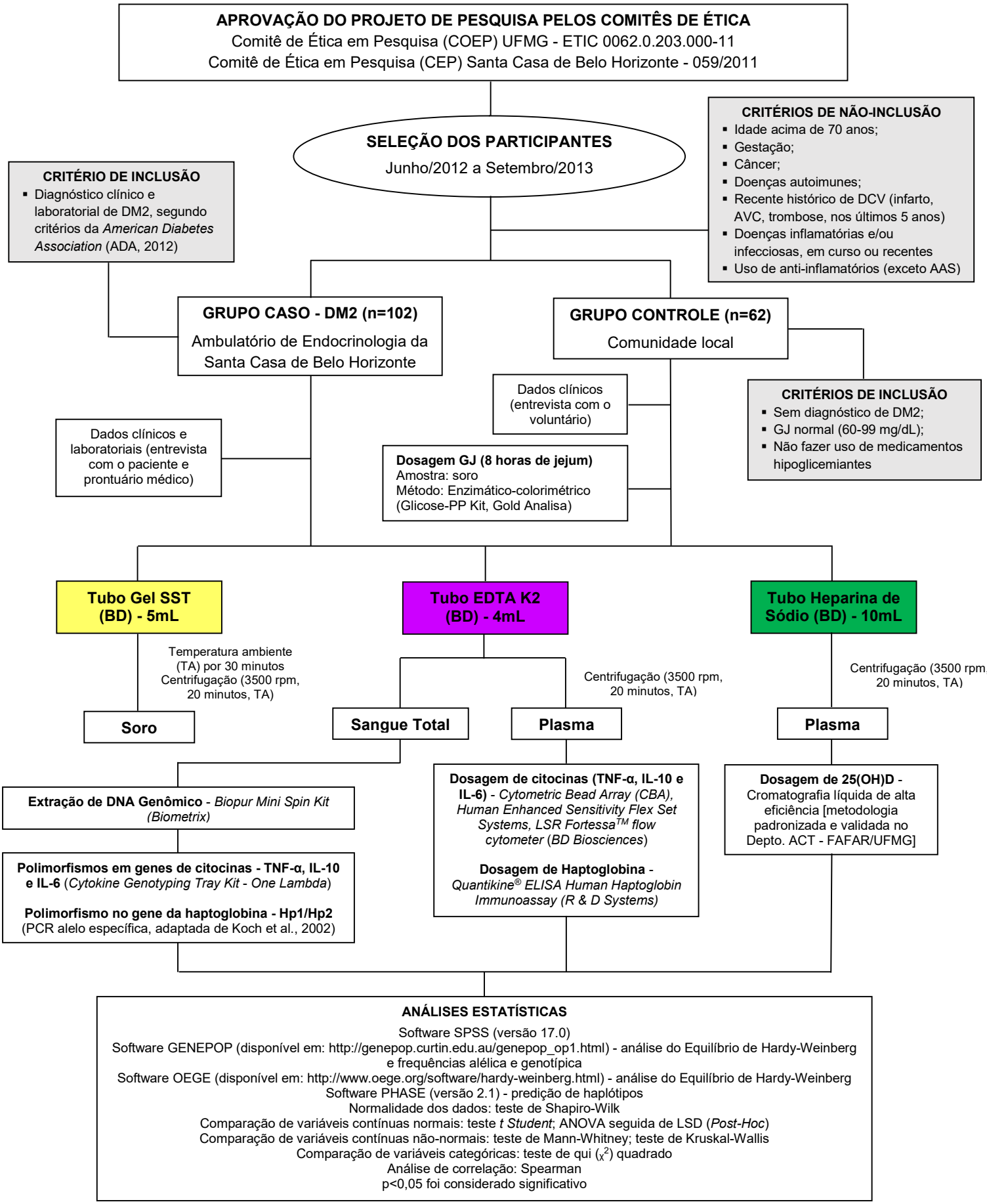
- Genótipos dos polimorfismos TNF-α -308G/A (rs1800629), IL-6 -174G/C (rs1800795), IL-10 -1082G/A (rs1800896), IL-10 -819T/C (rs1800871) e IL-10 -592C/A (rs1800872) e os níveis plasmáticos das respectivas citocinas.

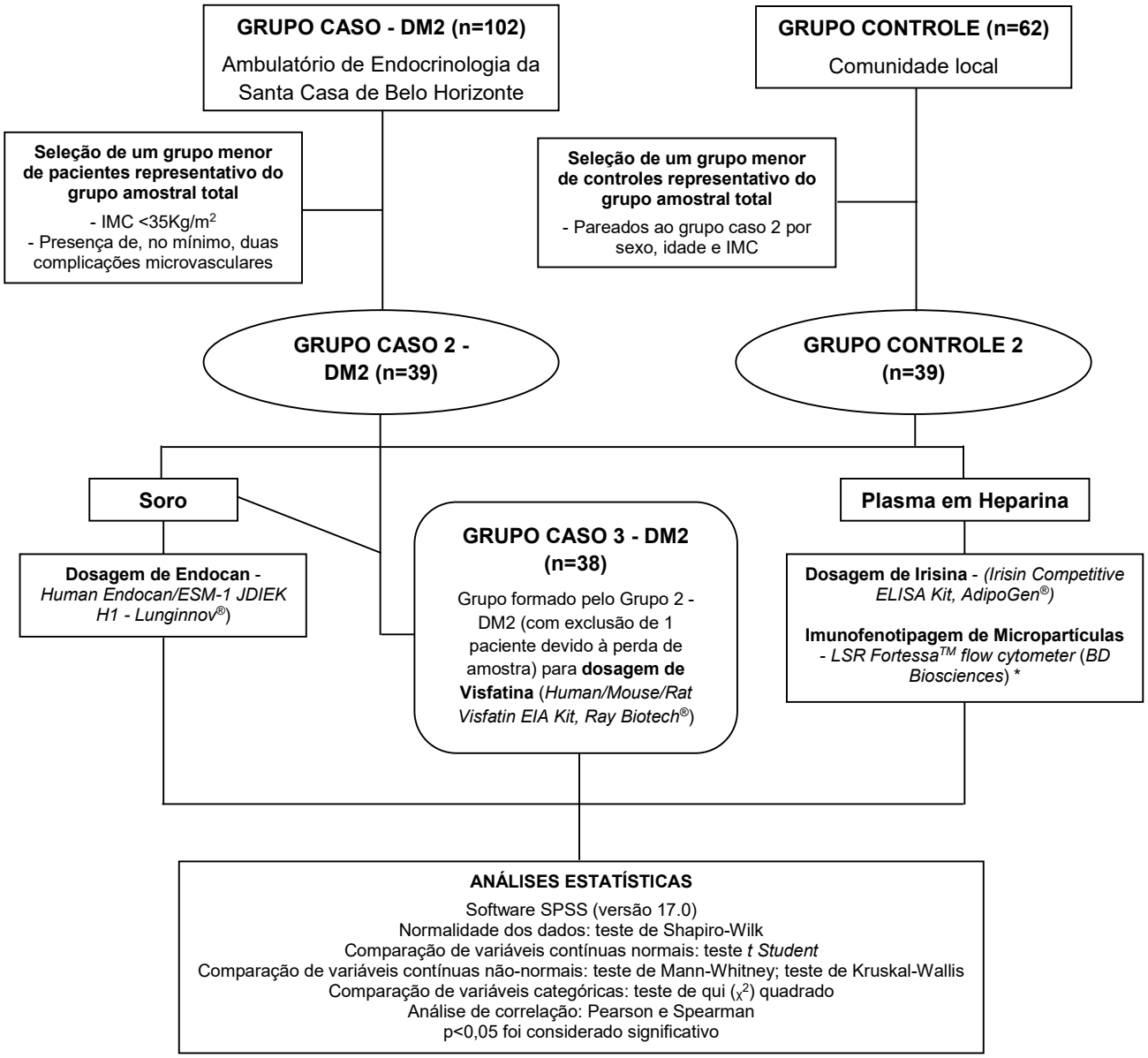
- Níveis dos marcadores inflamatórios, a saber: endocan, irisina, visfatina e haptoglobina; associando-os às complicações microvasculares e comorbidades do diabetes, e correlacionando-os à parâmetros clínicos e laboratoriais.

- Genótipos do polimorfismo Hp1/Hp2 no gene da haptoglobina.

- Níveis de micropartículas circulantes originadas de plaquetas, células endoteliais, leucócitos e carreadoras de fator tissular, correlacionando-os a parâmetros clínicos e laboratoriais.

5. DELINEAMENTO EXPERIMENTAL


APROVAÇÃO DO PROJETO DE PESQUISA PELOS COMITÊS DE ÉTICA
Comitê de Ética em Pesquisa (COEP) UFMG - ETIC 0062.0.203.000-11
Comitê de Ética em Pesquisa (CEP) Santa Casa de Belo Horizonte - 059/2011
SELEÇÃO DOS PARTICIPANTES
Junho/2012 a Setembro/2013
CRITÉRIO DE INCLUSÃO
Diagnóstico clínico e laboratorial de DM2, segundo critérios da American Diabetes Association (ADA, 2012)
CRITÉRIOS DE NÃO-INCLUSÃO
Idade acima de 70 anos;
Gestação;
Câncer;
Doenças autoimunes;
Recente histórico de DCV (infarto, AVC, trombose, nos últimos 5 anos)
Doenças inflamatórias e/ou infecciosas, em curso ou recentes
Uso de anti-inflamatórios (exceto AAS)
GRUPO CASO - DM2 (n=102)
Ambulatório de Endocrinologia da Santa Casa de Belo Horizonte
GRUPO CONTROLE (n=62)
Comunidade local
Dados clínicos e laboratoriais (entrevista com o paciente e prontuário médico)
Dados clínicos (entrevista com o voluntário)
CRITÉRIOS DE INCLUSÃO
Sem diagnóstico de DM2;
GJ normal (60-99 mg/dL);
Não fazer uso de medicamentos hipoglicemiantes
Dosagem GJ (8 horas de jejum)
Amostra: soro
Método: Enzimático-colorimétrico (Glicose-PP Kit, Gold Analisa)
Tubo Gel SST (BD) - 5mL
Tubo EDTA K2 (BD) - 4mL
Tubo Heparina de Sódio (BD) - 10mL
Temperatura ambiente (TA) por 30 minutos
Centrifugação (3500 rpm, 20 minutos, TA)
Centrifugação (3500 rpm, 20 minutos, TA)
Centrifugação (3500 rpm, 20 minutos, TA)
Soro
Sangue Total
Plasma
Plasma
Extração de DNA Genômico - Biopur Mini Spin Kit (Biometrix)
Polimorfismos em genes de citocinas - TNF-α, IL-10 e IL-6 (Cytokine Genotyping Tray Kit - One Lambda)
Polimorfismo no gene da haptoglobina - Hp1/Hp2 (PCR alelo específica, adaptada de Koch et al., 2002)
Dosagem de citocinas (TNF-α, IL-10 e IL-6) - Cytometric Bead Array (CBA), Human Enhanced Sensitivity Flex Set Systems, LSR Fortessa™ flow cytometer (BD Biosciences)
Dosagem de Haptoglobina - Quantikine® ELISA Human Haptoglobin Immunoassay (R & D Systems)
Dosagem de 25(OH)D - Cromatografia líquida de alta eficiência [metodologia padronizada e validada no Depto. ACT - FAFAR/UFMG]
ANÁLISES ESTATÍSTICAS
Software SPSS (versão 17.0)
Software GENEPOP (disponível em: http://genepop.curtin.edu.au/genepop_op1.html) - análise do Equilíbrio de Hardy-Weinberg e frequências alélica e genotípica
Software OEGE (disponível em: http://www.oege.org/software/hardy-weinberg.html) - análise do Equilíbrio de Hardy-Weinberg
Software PHASE (versão 2.1) - predição de haplótipos
Normalidade dos dados: teste de Shapiro-Wilk
Comparação de variáveis contínuas normais: teste t Student; ANOVA seguida de LSD (Post-Hoc)
Comparação de variáveis contínuas não-normais: teste de Mann-Whitney; teste de Kruskal-Wallis
Comparação de variáveis categóricas: teste de qui (χ^2) quadrado
Análise de correlação: Spearman
p<0,05 foi considerado significativo



*O grupo controle utilizado na imunofenotipagem de MPs foi composto por apenas 30 indivíduos devido à ausência de amostras de plasma em heparina para alguns participantes.

6. ARTIGOS PUBLICADOS

Capítulo 1:

IL-6, TNF-α, and IL-10 levels/polymorphisms and their association with type 2 diabetes mellitus and obesity in Brazilian individuals

IL-6, TNF-α, and IL-10 levels/ polymorphisms and their association with type 2 diabetes mellitus and obesity in Brazilian individuals

Kathryna Fontana Rodrigues[1], Nathalia Teixeira Pietrani[1], Adriana Aparecida Bosco[2], Fernanda Magalhães Freire Campos[3], Valéria Cristina Sandrim[4], Karina Braga Gomes[3]

ABSTRACT

Objective: This study aimed to investigate the association of plasma TNF-α, IL-6, and IL-10 levels and cytokine gene polymorphisms [TNF-α (-308 G→A), IL-6 (-174 C→G) and IL-10 (-1082 A→G, -819 T→C and -592 A→C)] in type 2 diabetes mellitus (T2DM) and obese patients. **Subjects and methods:** One hundred and two T2DM patients and 62 controls were included in this study. Cytokine plasma levels were measured by the Cytometric Bead Array method. Genotyping was carried out by the polymerase chain reaction. **Results:** IL-6 levels were significantly different between T2DM patients and controls. Interestingly, IL-6 levels were higher in T2DM patients with BMI > 30 kg/m² compared with other patients and obese controls. The genotype and allele frequencies were similar between patients and controls. In the T2DM group, the SNP IL-10 -819 T/C showed a difference between the cytokine level and genotypes: IL-10 level in the TT genotype was significantly higher when compared to CC genotype. **Conclusions:** These results suggest an association between IL-6 levels and obesity, and IL-10 levels and the SNP -819 T/C in T2DM. Knowledge of these variants in T2DM might contribute to a better understanding of the role of inflammation in the etiology and progression of this disease.

Keywords
Type 2 diabetes mellitus; polymorphisms; interleukin-6; interleukin-10; tumor necrosis factor-alpha

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Received on May/3/2016
Accepted on Nov/25/2016

DOI: 10.1590/2359-3997000000254

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia and represents a significant global health problem (1). According to the International Diabetes Federation (IDF), diabetes mellitus is a major metabolic disease affecting approximately 415 million people worldwide and it is expected to reach 642 million in 2040 (2).

The pathogenesis of insulin resistance and T2DM has been associated with a subclinical chronic inflammation and activation of the immune system; however, what triggers this inflammation is still unclear (3,4). Some studies have shown that T2DM patients have higher levels of inflammatory markers such as interleukin-6 (IL-6), C reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-α (TNF-α), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) (5-10).

Furthermore, it is known that obesity, especially the visceral type, is an independent risk factor for T2DM development (11). In fact, adipose tissue is an endocrine organ that co-regulates whole-body metabolism. It is able to produce a variety of cytokines (TNF-α, IL-6, IL-1β) and other bioactive products, such as leptin, resistin, and monocyte chemoattractant protein-1 (MCP-1/CCL2) (12,13). Adipose tissue in an obese individual is characterized by the presence of pro-inflammatory immune cells ($CD8^+$ T lymphocytes, IFN-γ^+ Th1 cells, B cells, mast

IL-6, TNF-α, and IL-10 levels/polymorphisms and their association with type 2 diabetes mellitus and obesity in Brazilian individuals

Abbreviated title: Cytokines, type 2 diabetes, and obesity

Kathryna Fontana Rodrigues[1], Nathalia Teixeira Pietrani[1], Adriana Aparecida Bosco[2], Fernanda Magalhães Freire Campos[3], Valéria Cristina Sandrim[4], Karina Braga Gomes[3]

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ABSTRACT

Purpose: This study aimed to investigate the association of plasma TNF-α, IL-6, and IL-10 levels and cytokine gene polymorphisms [TNF-α (-308 G→A), IL-6 (-174 C→G) and IL-10 (-1082 A→G, -819 T→C and -592 A→C)] in type 2 diabetes mellitus (T2DM) and obese patients. **Materials and Methods:** 102 T2DM patients and 62 controls were included in this study. Cytokine plasma levels were measured by the Cytometric Bead Array method. Genotyping was carried out by the polymerase chain reaction. **Results:** IL-6 levels were significantly different between T2DM patients and controls. Interestingly, IL-6 levels were higher in T2DM patients with BMI > 30 Kg/m^2 compared with other patients and obese controls. The genotype and allele frequencies were similar between patients and controls. In the T2DM group, the SNP IL-10 -819 T/C showed a difference between the cytokine level and genotypes: IL-10 level in the TT genotype was significantly higher when compared to CC genotype. **Conclusions:** These results suggest an association between IL-6 levels and obesity, and IL-10 levels and the SNP -819 T/C in T2DM. Knowledge of these variants in T2DM might contribute to a better understanding of the role of inflammation in the etiology and progression of this disease.

Keywords: Type 2 diabetes mellitus, Polymorphisms, Interleukin-6, Interleukin-10, Tumor Necrosis Factor-Alpha.

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia and represents a significant global health problem [1]. According to the International Diabetes Federation (IDF), diabetes mellitus is a major metabolic disease affecting approximately 415 million people worldwide and it is expected to reach 642 million in 2040 [2].

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Furthermore, it is known that obesity, especially the visceral type, is an independent risk factor for T2DM development [11]. In fact, adipose tissue is an endocrine organ that co-regulates whole-body metabolism. It is able to produce a variety of cytokines (TNF-α, IL-6, IL-1β) and other bioactive products, such as leptin, resistin, and monocyte chemoattractant protein-1 (MCP-1/CCL2) [12, 13]. Adipose tissue in an obese individual is characterized by the presence of pro-inflammatory immune cells ($CD8^+$ T lymphocytes, IFN-γ^+ Th1 cells, B cells, mast cells, neutrophils, and M1 macrophages) attracted by chemokines secreted from stressed adipocytes in response to lipid overload [14].

The expression of pro- and anti-inflammatory cytokines may be modulated by single nucleotide polymorphisms (SNPs) located in the regulatory regions of genes [15, 16]. Some studies have investigated the association among TNF-α, IL-6, and IL-10 gene polymorphisms with metabolic diseases [17-25]. Despite these reports examining the association of inflammation markers and SNPs in cytokine genes, much controversy remains as to their role in diabetes occurrence [26-34].

In this study, we evaluated the role of cytokines in T2DM and obesity by measuring plasma TNF-α, IL-6, and IL-10 levels. We also investigated whether these levels are modulated by polymorphisms located in the regulatory regions of genes [TNF-α (-308 G/A, rs1800629), IL-6 (-174 C/G, rs1800795), and IL-10 (-1082 G/A, rs1800896; -819 T/C, rs1800871; and -592 A/C, rs1800872)]. Higher IL-6 levels were

found in T2DM patients and our results suggest that obesity acts synergistically with T2DM by modulating the increase of this cytokine. Although this study failed to demonstrate that these polymorphisms could modulate TNF-α, IL-6, and IL-10 plasma levels, the IL-10 -819 T/C polymorphism seems to influence IL-10 levels in T2DM.

2. SUBJECTS AND METHODS

2.1 Ethical aspects

This study was approved by the Ethics Committee of the Federal University of Minas Gerais (Minas Gerais, Brazil)-ETIC 0062.0.203.000-11-and Santa Casa Hospital (Minas Gerais, Brazil)-059/2011; in accordance with the Helsinki Declaration. Informed consent was obtained from all subjects.

2.2 Study design

This cross-sectional study was conducted with 102 patients with clinical and laboratory diagnosis of T2DM (19 men and 83 women) and 62 non-diabetic controls (12 men and 50 women); both groups were aged from 32 to 70 years and matched by gender, age, and body mass index (BMI) in a 2:1 case/control proportion, according to the sample calculation based on the mean values for each cytokines level obtained from a small sample of the groups (power = 0.95; significance level = 0.05). The patients were selected from the Clinic of Endocrinology (Santa Casa Hospital, Minas Gerais, Brazil), and the controls were selected from the local community between June 2012 and September 2013. T2DM diagnosis was based on the American Diabetes Association (ADA) criteria [1]. The controls showed normal levels of fasting glucose (60-99 mg/dL) and no use of hypoglycemic drugs. Were excluded subjects older than 70 years, pregnant, with cancer, autoimmune disease, recent history of cardiovascular disease (heart attack, stroke, thrombosis in the last five years), and current or recent infections and/or inflammatory processes.

2.3 Clinical and laboratorial data

Clinical (gender, age, BMI, waist circumference, waist-hip ratio, T2DM onset, and hypertension), and laboratorial data (fasting glucose, HbA1c, and post-prandial

glucose) were obtained for all of the T2DM patients through interviews and medical records. The criteria used for determining hypertension were: systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 80 mmHg, or use of antihypertensive drugs, and comply with the criteria adopted by the ADA [1]. Clinical data (gender, age, BMI, waist circumference, and waist-hip ratio) for the controls were obtained through interviews.

The fasting glucose in the control group was measured in serum samples after eight hours fasting. The serum samples were centrifuged at 1,100 x g for 20 min at 25ºC and the assays performed immediately. The tests were performed using the enzyme-colorimetric method, BTR 811 spectrophotometer (Biotron, Minas Gerais, Brazil), and Glicose-PP kit (Gold Analisa, Minas Gerais, Brazil), following the manufacturer's instructions. The concentrations of fasting glucose were expressed in mg/dL.

Serum samples were used for quantification high sensitivity C reactive protein (hs-CRP). They were centrifuged at 1,100 x g for 20 min at 25ºC and stored at -80ºC until analysis. The tests were performed using the immunoturbidimetric method, System Vitros Chemistry 5.1 FS (Ortho Clinical Diagnostics, New York, USA), and hsCRP VITROS Chemistry Products (Ortho Clinical Diagnostics, New York, USA), following the manufacturer's instructions. All samples were assayed at the same time. The concentrations of hs-CRP were expressed in mg/L.

2.4 Determination of cytokine plasma levels

Samples collected in EDTA were centrifuged at 1,100 x g for 20 min at 25ºC to obtain plasma, which was stored at -80ºC until analysis. Data acquisition and analysis were performed in an LSR Fortessa™ flow cytometer (BD Biosciences Pharmingen, California, USA) using FCAP Array Software version 1.0.1. TNF-α, IL-6, and IL-10 levels were determined by the Cytometric Bead Array (CBA) method using Human Enhanced Sensitivity Flex Set Systems (BD Biosciences Pharmingen, California, USA), following the manufacturer's instructions. All samples were assayed at the same time. The concentrations of each cytokine were expressed in fg/mL.

2.5 Cytokine gene polymorphism analysis

Genomic DNA was extracted from whole blood collected in EDTA using Biopur Mini Spin Kit (Biometrix, São Paulo, Brazil). The polymorphisms were determined

using the Cytokine Genotyping Tray Kit (One Lambda Inc., California, USA), which employs Polymerase Chain Reaction-Sequence Specific Primers (PCR-SSP), followed by electrophoresis in 2.5% agarose gel stained with GelGreen Stain (Biotium Inc., California, USA). In order to evaluate the reproducibility rate of genotyping, 10% of the samples in both groups were randomly selected to be re-genotyped. The results showed 100% agreement. The polymorphisms analyzed in the present study were: TNF-α (-308 G/A, rs1800629), IL-6 (-174 C/G, rs1800795), IL-10 (-1082 G/A, rs1800896; -819 T/C, rs1800871; and -592 A/C, rs1800872).

2.6 Statistical analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were tested using an exact test (available at: http://genepop.curtin.edu.au/genepop_op1.html). All of the statistical analyses were performed with Statistical Package of the Social Sciences (SPSS) version 17.0. An analysis of normality was performed by Shapiro-Wilk test. Data are presented as “mean ± (standard deviation-SD)” (parametric variables), “median (interquartile range-IQR)” (non-parametric variables), or “percentage of total (categorical variables)”.

Comparisons between the two groups were made with Student’s *t*-test for parametric variables and the Mann-Whitney test for non-parametric variables. Comparisons of non-parametric variables between three groups were performed with the Kruskal-Wallis test. When differences were detected, they were compared in pairs by the Mann-Whitney method, followed by Bonferroni’s Correction. The comparison of categorical variables was performed using the chi-square test (χ^2).

Differences in genotype and allele frequencies between the groups (T2DM patient and control) were tested by Pearson’s χ^2-test or Fisher’s Test. IL-10 haplotype estimation was conducted by PHASE software version 2.1. We excluded haplotypes whose frequencies were less than 5%. The differences in the haplotype frequencies between the groups were tested by χ^2-test.

Linear regression analysis was performed for evaluating the confounding influence of variables in cytokines plasma levels. Gender, age, BMI, waist circumference, waist-hip ratio, and fasting glucose were considered as independent variables.

Spearman’s correlations were computed only in the T2DM patient group to

assess correlations between cytokine levels, anthropometric, and laboratorial data. A *p*-value < 0.05 was considered statistically significant.

3. RESULTS

Clinical and laboratorial characteristics of T2DM patients and controls are summarized in **Table 1**. Groups were matched by gender, age, and BMI (p>0.05 for all). Waist circumference and waist-hip ratio were higher in T2DM patients than in controls (p<0.0001 for both). When analyzing cytokine levels in two groups, IL-6 levels were higher in the diabetic patients (p=0.001), and no significant differences were observed for TNF-α (p=0.332) and IL-10 (p=0.317). Diabetic patients on treatment with insulin (n=18, 17.65%), oral antidiabetic drugs (n=17, 16.67%) or insulin plus oral antidiabetic drugs (n=67, 65.68%) show similar levels of cytokines compared with each other (p>0.05 for all; **Supplementary Material-SM1**).

We have evaluated cytokines levels according to BMI categories (BMI < 25 – lean, 25 ≤ BMI < 30 – overweight, and BMI ≥ 30 Kg/m^2 – obese) within each group (T2DM patient or control) and between these groups (**Table 2**). In T2DM group, only IL-6 levels were different between patients (p=0.001) when compared to those with BMI < 25 Kg/m^2 *versus* BMI ≥ 30 Kg/m^2 (p=0.001). Comparison among 25 ≤ BMI < 30 Kg/m^2 *versus* BMI ≥ 30 Kg/m^2 was not significant after Bonferroni's correction (p>0.02). No differences were found in the cytokine levels considering only control group. Finally, when comparing T2DM patients versus controls, IL-6 levels were higher in obese patients than in obese controls (p=0.019). No other associations were found for TNF-α and IL-10 levels and BMI categories.

We performed an analysis of genotype and allele frequencies of polymorphisms for cytokine genes TNF-α, IL-6, and IL-10. All polymorphisms were under Hardy-Weinberg equilibrium (p>0.025). We found no differences between genotype and allele frequencies when compared T2DM and control groups (p>0.05 for all; **Supplementary Material-SM2**). Additionally, no difference regarding the IL-10 haplotype analyses was observed between these groups (data not shown).

Aiming to evaluate whether polymorphisms in TNF-α, IL-6, and IL-10 genes modulate cytokine plasma levels, we compared their levels and genotypes considering all the individuals (T2DM patients and controls) and performed an

analysis to predict their inheritance pattern. However, no differences were found ($p>0.05$ for all; **Table 3**). Interestingly, when these comparisons were applied only in the T2DM patient group (**Table 4**), IL-10 -819 T/C polymorphism showed a difference between cytokine levels and genotypes ($p=0.021$): IL-10 levels in the TT genotype [256 (207) fg/mL] were significantly higher than in the CC genotype [182 (57) fg/mL] ($p=0.006$). No differences were found for IL-6 levels and -174 C/G polymorphism after Bonferroni's correction ($p>0.02$) and TNF-α levels and -308 G/A polymorphism ($p>0.05$). Regarding the inheritance pattern, only IL-10 -592 A/C polymorphism showed significant differences in IL-10 levels ($p=0.039$) when compared to the CC genotype [182 (57) fg/mL] and AC+AA genotypes [192 (115) fg/mL], suggesting a recessive model.

We investigated the correlation between cytokine plasma levels and anthropometric and laboratorial data in the T2D patients group. IL-6 levels showed a significant positive correlation with BMI ($r=0.314$, $p=0.002$), waist circumference ($r=0.318$, $p=0.002$), hs-CRP ($r=0.452$, $p<0.0001$), and IL-10 levels ($r=0.336$, $p=0.001$). No other significant correlation was observed between cytokines levels (TNF-α and IL-10) and these parameters. Furthermore, fasting glucose and hs-CRP showed a significant negative and positive correlations, respectively, with BMI ($r=-0.365$, $p<0.0001$; $r=0.476$, $p<0.0001$) and waist circumference ($r=-0.278$, $p=0.005$; $r=0.442$, $p<0.0001$).

Finally, the linear regression analysis did not show an independent association between gender, age, BMI, waist circumference, waist-hip ratio, and fasting glucose with TNF-α, IL-6, and IL-10 levels ($p>0.05$ for all).

4. DISCUSSION

This study evaluated the importance of TNF-α, IL-6, and IL-10 levels and their association with gene polymorphisms in T2DM disease and the obesity.

Clinical and laboratorial characteristics of T2DM patients and controls showed that T2DM patients have higher waist circumferences and waist-hip ratios when compared to the controls. These results are consistent with the knowledge that not only obesity, but mainly the distribution of body fat (mostly upper body obesity), influence glucose metabolism and are independent risk factors for developing T2D

[11].

Among the cytokines levels measured, only IL-6 levels were higher in the T2DM group. IL-6 is a multifunctional cytokine and is secreted by many types of cells, mainly T cells, macrophages, endothelial cells, smooth muscle cells, adipocytes, and hepatocytes [35]. Furthermore, IL-6 regulates/stimulates production of cell adhesion molecules, chemotactic mediators, and acute phase protein, and mediates the release of other cytokines that amplify the inflammatory response [35, 36, 37]. Similar to our result, other studies have shown that T2DM individuals have higher circulating IL-6 levels when compared to non-diabetic controls [38-43]. The increased levels of IL-6 and other inflammatory markers (IL-1β, CRP) emerge as early predictors of T2DM, preceding its clinical onset [44].

Obesity is also associated with a state of low-grade inflammation [11, 13] and elevated levels of IL-6, which has been commonly described in obese diabetic patients or only in obese individuals [38, 45, 46]. We performed an analysis in order to investigate the influence of BMI in cytokine levels. For the control group, no differences were found. However, IL-6 levels in obese T2DM patients (BMI ≥ 30 Kg/m^2) were higher than lean (BMI < 25 Kg/m^2) and overweight (25 ≤ BMI < 30 Kg/m^2) patients (tendency), although the linear regression analysis has not showed an independent association between these parameters. Moreover, obese T2DM patients presented higher IL-6 levels when compared to obese controls. These results suggest that higher BMI values in T2DM are associated with increased IL-6 levels, but some other variable appears to act synergistically, since this association was not independent. When compared to the control group, T2DM seems to act synergistically with obesity to promote an increase in IL-6 levels.

Although no difference was found in TNF-α and IL-10 levels between the groups (T2DM patients and controls) and the BMI categories, these cytokines have been associated with T2DM. TNF-α is a pro-inflammatory cytokine produced by a variety of cell-types, mainly macrophages, lymphocytes, and adipocytes [13]. Some studies found higher TNF-α levels in T2DM patients when compared with non-diabetic controls [39, 40, 43].

IL-10 is an anti-inflammatory cytokine that plays an important role in the regulation of the immune system leading to decreased cytokine production, reducing tissue factor expression, inhibiting matrix-degrading metalloproteinase, and promoting the phenotypic switching of lymphocytes to the Th2 phenotype [47]. This

cytokine is produced by T-cells, B-cells, monocytes, and macrophages, and it is estimated that 75% of the variation in IL-10 production is genetically determined [47]. An important study showed that IL-10 level was lower in subjects with impaired glucose tolerance or T2DM when compared with subjects with normal glucose tolerance and showed an inverse correlation with BMI [48]. Conversely, Al-Shukailli et al. [49] found higher IL-10 levels in T2DM patients when compared with healthy controls. Taken together, it is not clear whether higher IL-10 levels confer protection against T2DM development by decreased pro-inflammatory cytokines production, or increased IL-10 levels in T2DM result in a compensatory response against the elevation of pro-inflammatory mediators, primarily TNF-α and IL-6.

No polymorphism showed a difference in allele and genotype frequencies when compared to T2DM patients and the control group. Therefore, no polymorphism in this study was associated with T2DM. However, the association of these polymorphisms with T2DM remains unclear. In 2011, Fen et al. [26] in a meta-analysis did not find a significant association between TNF-α -308 G/A polymorphism and T2DM risk when considering Caucasian and Asian populations. In contrast, in 2014, a meta-analysis conducted by Zao et al. [27] indicated that TNF-α -308A allele could be a risk factor for the development for T2DM in Asian subjects. Similarly, Golshani et al. [28] found that TNF-α -308 GA+AA genotypes are associated with higher risk for T2DM development in an Iranian population. According to Qi et al. [29], IL-6 -174 C/G polymorphism is not associated with the risk of T2DM development; however, a recent study [23] shows a significant association between T2DM and IL-6 -174G allele. Finally, four recent meta-analyses evaluated the association between IL-10 gene polymorphisms (-1082 G/A, -819 T/C, and -592 A/C) and the risk of T2DM development. The -819 T/C and -592 A/C polymorphisms did not show an association with the disease in these studies [30-33]. However, Li et al. [31] and Hua et al. [30] found an association between the -1082GA genotype and -1082G allele, respectively, with T2DM. Additionally, a case-control study conducted by Bai et al [34] found higher risk for T2DM development associated with -1082 GA+GG and -592 AC+AA genotypes. These conflicting results are probably related to the sample size and different genetic background of the populations. Larger scale genome studies are required to further evaluate these associations.

The plasma levels of IL-6 were significantly different between -174 C/G polymorphism genotypes, but this difference was not maintained after Bonferroni's

correction. The patients with IL-10 -819TT genotype showed higher IL-10 levels than patients with -819CC genotype. Previous studies [50, 51] showed that, not only the -819T allele, but also the ATA haplotype (-1082A, -819T, -592A) are related to lower transcriptional activity and, consequently, lower IL-10 levels. However, no further study was carried out on diabetic patients to prove the association between transcriptional activity of IL-10 gene and IL-10 serum/plasma levels and -819 T/C polymorphism.

IL-6 levels were positively correlated with BMI, waist circumference, hs-CRP, and IL-10 levels. Similarly, hs-CRP also showed a significant positive correlation with BMI and waist circumference. Obesity, especially visceral obesity, is characterized by the increased size of adipocytes and recruitment of immune cells (mainly macrophages), which display a pro-inflammatory profile. These cells are responsible for the increased production of inflammatory mediators and acute phase proteins, such as IL-6 and CRP [12]. Considering these events, it is expected that IL-6 and hs-CRP levels are positively correlated with each other and with anthropometric parameters that express weight gain (BMI) and the increase in upper body fat (waist circumference) in the T2DM group. Finally, the positive correlation between IL-6 and IL-10 levels showed that, in T2DM, the increase of pro-inflammatory mediators may cause a compensatory increase in anti-inflammatory cytokines to control subclinical inflammation.

The small sample size, owing to the strict selection criteria for patients and controls, and absence of functional analysis of polymorphisms can be considered as the main limitations of this study, since the effect of the polymorphisms on protein activity was not evaluated. Indeed, the statistical power became lower when the total number of the participants in each group was classified according to BMI categories, but as cytokine levels are associated with adiposity, this selection was necessary in order to avoid a bias in the results.

Therefore, further studies with a much larger sample exploring other populations (different genetic background) and others clinical characteristics as a practice of physical activities, are needed to better understand the role of these polymorphisms in the subclinical inflammation in T2DM.

5. CONCLUSION

Few studies have evaluated inflammatory markers and cytokines genes polymorphisms in T2DM Brazilian patients. Considering the increased number of diabetic patients in Brazil and the population's genetic background, improved knowledge on the markers that contribute to the etiology and progression of T2DM are important for prevention, diagnosis, and follow-up of this disease.

Taken together, our results show that IL-6 and IL-10 levels and the SNP -819 T/C in IL-10 gene are associated with the subclinical inflammation in the T2DM. Moreover, the association between IL-6 levels and obesity in T2DM indicates that weight control may be an action adopted for preventing inflammatory status in T2DM.

DECLARATION OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGMENTS

The authors thank FAPEMIG, CAPES, CNPq/Brazil, and PRPq/UFMG for financial support. VCS and KBG are grateful to CNPq Research Fellowship.

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Table 1: Clinical and laboratorial characteristics in T2DM patients and controls.

Parameters	T2DM (n= 102)	Control (n=62)	p
Gender (Male/Female) %	18.6 / 81.4	19.3 / 81.7	0.908
Age (years)	56 (12)	53 (18)	0.358
BMI (Kg/m^2)			
BMI < 25	22.6 (3.7)	23.4 (2.8)	0.503
25 ≤ BMI < 30	28.3 (3.0)	27.6 (1.9)	0.215
BMI ≥ 30	37.8 (9.2)	39.3 (11.0)	0.850
Waist circumference (cm)	108.1 ± 16.8	96.9 ± 16.2	<0.0001*
Waist-hip ratio	1.0 ± 0.1	0.9 ± 0.1	<0.0001*
T2DM onset (%)[a]			
≤ 10 years	40.2	Not applicable	-
> 10 years	56.9		-
Hypertension (Yes/No) %	92.2 / 7.8	-	-
Fasting glucose (mg/dL)	126.5 (79.0)	85.3 (10.3)	<0.0001*
Post-prandial glucose (mg/dL)	203.0 (118.0)	-	-
HbA1c (%)	8.9 ± 1.9	-	-
hs-CRP (mg/L)	3.5 (6.6)	2.7 (2.6)	0.094
TNF-α (fg/mL)	91 (42)	95 (64)	0.332
IL-6 (fg/mL)	805 (993)	476 (516)	0.001*
IL-10 (fg/mL)	194 (67)	185 (57)	0.317

Abbreviations: BMI (Body Mass Index), HbA1c (Glycated Hemoglobin), hs-CRP (high sensitivity C Reactive Protein), TNF-α (Tumor Necrosis Factor - Alpha), IL-6 (Interleukin-6), and IL-10 (Interleukin-10).

Normal variables (Waist circumference, Waist-hip ratio, and HbA1c): the data are shown as “mean ± SD”. No normal variables (Age, BMI, Fasting glucose, Post-prandial glucose, hs-CRP, TNF-α, IL-6, and IL-10): the data are shown as “median (IQR)”. Categorical variables (Gender, T2DM onset, and Hypertension): the data are shown as “percentage of total”.

[a]Missing data for three patients.

*p<0.05 was considered statistically significant.

Table 2: Cytokines levels in T2DM patients and controls according to BMI categories.

Cytokine (fg/mL)	BMI (Kg/m^2)		T2DM (n= 102)	Control (n= 62)	p'
TNF-α	BMI < 25		86 (48)	90 (75)	0.786
	25 ≤ BMI < 30		92 (48)	86 (68)	0.748
	BMI ≥ 30		92 (42)	112 (58)	0.061
		p	0.922	0.294	
IL-6	BMI < 25		452 (412)	412 (492)	0.422
	25 ≤ BMI < 30		731 (917)	532 (439)	0.458
	BMI ≥ 30		1118 (1506)	663 (534)	0.019*
		p	0.001*	0.356	
IL-10	BMI < 25		232 (102)	192 (48)	0.172
	25 ≤ BMI < 30		188 (71)	169 (64)	0.947
	BMI ≥ 30		185 (55)	190 (37)	0.473
		p	0.085	0.353	

Abbreviations: BMI (Body Mass Index), TNF-α (Tumor Necrosis Factor - Alpha), IL-6 (Interleukin-6), and IL-10 (Interleukin-10).
Cytokine levels: no normal variable; the data are shown as “median (IQR)”.
*p<0.05 was considered statistically significant (comparison within the groups: T2DM or Control).
*p’<0.05 was considered statistically significant (comparison between the groups: T2DM *versus* Control).

Mann-Whitney test with Bonferroni’s Correction for IL-6 levels (T2DM group):
*p^1 – p^3: were considered statistically significant if p< 0.02.

p^1: [BMI < 25 (T2DM) *versus* 25 ≤ BMI < 30 (T2DM)] = 0.334
p^2: [BMI < 25 (T2DM) *versus* BMI ≥ 30 (T2DM)] = 0.001*
p^3: [25 ≤ BMI < 30 (T2DM) *versus* BMI ≥ 30 (T2DM)] = 0.024

Table 3: Cytokines levels according to genotypes considering all the individuals (T2DM patients and controls).

Polymorphism		Cytokine (fg/mL)	p
TNF-α (-308 G/A) (rs1800629)	GG (n= 125)	95 (55)	0.705
	GA (n= 38)	90 (63)	
	AA (n= 1)	47.5 ǂ	
IL-6 (-174 C/G) (rs1800795)	CC (n=10)	1381 (2892)	0.361
	CG (n= 50)	565 (754)	
	GG (n= 104)	674 (770)	
IL-10 (-1082 G/A) (rs1800896)	GG (n= 20)	204 (55)	0.278
	GA (n= 73)	182 (63)	
	AA (n= 71)	185 (65)	
IL-10 (-819 T/C) (rs1800871)	TT (n= 20)	214 (136)	0.215
	TC (n= 69)	182 (69)	
	CC (n= 75)	193 (55)	
IL-10 (-592 A/C) (rs1800872)	AA (n= 21)	208 (135)	0.409
	AC (n= 68)	182 (68)	
	CC (n= 75)	193 (55)	

Abbreviations: TNF-α (Tumor Necrosis Factor - Alpha), IL-6 (Interleukin-6), and IL-10 (Interleukin-10).
Cytokine levels: no normal variable; the data are shown as “median (IQR)”.
$p<0.05$ was considered statistically significant.
ǂ data only one individual.

Table 4: Cytokines levels according to genotypes in T2DM patients group.

Polymorphism		Cytokine (fg/mL)	p	p'
TNF-α (-308 G/A) (rs1800629)	GG (n= 78)	95 (45)	0.137	-
	GA (n= 23)	85 (44)		
	AA (n= 1)	47.5 ǂ		
IL-6 (-174 C/G) (rs1800795)	CC[1] (n= 7)	1901 (3167)	0.026*	p^a= 0.047
	CG[2] (n= 26)	496 (746)		p^b= 0.210
	GG[3] (n= 69)	878 (945)		p^c= 0.023
IL-10 (-1082 G/A) (rs1800896)	GG (n= 12)	202 (62)	0.181	-
	GA (n= 47)	178 (68)		
	AA (n= 43)	199 (87)		
IL-10 (-819 T/C) (rs1800871)	TT[1] (n= 13)	256 (207)	0.021*	p^a= 0.066
	TC[2] (n= 40)	184 (90)		p^b= 0.006*
	CC[3] (n= 49)	182 (57)		p^c= 0.207
IL-10 (-592 A/C) (rs1800872)	AA (n= 14)	249 (212)	0.055	-
	AC (n= 39)	185 (91)		
	CC (n= 49)	182 (57)		

Abbreviations: TNF-α (Tumor Necrosis Factor - Alpha), IL-6 (Interleukin-6), and IL-10 (Interleukin-10).
Cytokine levels: no normal variable; the data are shown as “median (IQR)”.
*p<0.05 was considered statistically significant.
*p’<0.02 was considered statistically significant (Mann-Whitney test with Bonferroni’s Correction).
p^a: genotype 1 *versus* genotype 2
p^b: genotype 1 *versus* genotype 3
p^c: genotype 2 *versus* genotype 3
ǂ data only one individual.

SUPPLEMENTARY MATERIAL

Table SM1: Cytokines levels according to types of pharmacological treatment in T2DM group.

Cytokine (fg/mL)	Types of treatment			p
	Insulin (n= 18)	Oral antidiabetic drugs (n= 17)	Insulin plus oral antidiabetic drugs (n= 67)	
TNF-α	94 (51)	74 (35)	92(51)	0.440
IL-6	911 (1546)	729 (877)	783 (1518)	0.530
IL-10	187 (63)	180 (42)	194 (78)	0.511

Abbreviations: TNF-α (Tumor Necrosis Factor - Alpha), IL-6 (Interleukin-6), and IL-10 (Interleukin-10).
Cytokine levels: no normal variable; the data are shown as “median (IQR)”.
$p<0.05$ was considered statistically significant.

Table SM2: Distributions of genotypes and alleles frequencies in T2DM patients and controls.

Polymorphisms		T2DM (n=102) (%)	Control (n=62) (%)	p
TNF-α (-308 G/A) (rs1800629)	**Genotypes**			0.965
	GG	78 (76.5)	47 (75.8)	
	GA	23 (22.6)	15 (24.2)	
	AA	1 (0.9)	0	
	Alleles			0.966
	G	179 (87.8)	109 (87.9)	
	A	25 (12.2)	15 (12.1)	
IL-6 (-174 C/G) (rs1800795)	**Genotypes**			0.348
	CC	7 (6.8)	3 (4.8)	
	CG	26 (25.5)	24 (38.7)	
	GG	69 (67.7)	35 (56.5)	
	Alleles			0.326
	C	40 (19.6)	30 (24.2)	
	G	164 (80.4)	94 (75.8)	
IL-10 (-1082 G/A) (rs1800896)	**Genotypes**			0.864
	GG	12 (11.8)	8 (12.9)	
	GA	47 (46.1)	26 (41.9)	
	AA	43 (42.1)	28 (45.2)	
	Alleles			0.863
	G	71 (34.8)	42 (33.9)	
	A	133 (65.2)	82 (66.1)	
IL-10 (-819 T/C) (rs1800871)	**Genotypes**			0.674
	TT	13 (12.8)	7 (11.3)	
	TC	40 (39.2)	29 (46.8)	
	CC	49 (48.0)	26 (41.9)	
	Alleles			0.665
	T	66 (32.4)	43 (34.7)	
	C	138 (67.6)	81 (65.3)	
IL-10 (-592 A/C) (rs1800872)	**Genotypes**			0.742
	AA	14 (13.7)	7 (11.3)	
	AC	39 (38.3)	29 (46.8)	
	CC	49 (48.0)	26 (41.9)	
	Alleles			0.733
	A	67 (32.8)	43 (34.7)	
	C	137 (67.2)	81 (65.3)	

Abbreviations: TNF-α (Tumor Necrosis Factor - Alpha), IL-6 (Interleukin-6), and IL-10 (Interleukin-10). $p<0.05$ was considered statistically significant.

Capítulo 2:

Endocan: a new biomarker associated with inflammation in type 2 diabetes mellitus?

DIABETES/METABOLISM RESEARCH AND REVIEWS
Diabetes Metab Res Rev 2015; **31**: 479–480.
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/dmrr.2639

LETTER TO THE EDITOR

Endocan: a new biomarker associated with inflammation in type 2 diabetes mellitus?

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Received: 9 November 2014
Revised: 23 January 2015
Accepted: 27 January 2015

Dear editor,

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes whose pathogenesis is associated with a chronic state of low-grade inflammation. Although the mechanisms are not completely known, the subclinical inflammation is a part of the insulin resistance and is closely related to complications of this metabolic disorder and obesity [1]. Endocan or endothelial cell-specific molecule 1 (ESM-1) is a dermatan sulfate proteoglycan, whose expression occurs in endothelial cells [2]. *In vitro*, ESM-1 interferes with molecular mechanisms of immune cell migration by blocking the interaction between leukocyte function-associated antigen-1 and intercellular adhesion molecule-1 [3]. We evaluated the ESM-1 levels in T2DM patients compared with a control group. T2DM patients ($n = 39$, 33 women; 59.74 ± 6.47 years) and controls ($n = 39$, 28 women; 53.03 ± 8.78 years) matched by gender, age and body mass index (T2DM 27.52 ± 4.39 kg/m^2; control 26.77 ± 4.24 kg/m^2) were included. The subjects were recruited from Santa Casa Hospital (Belo Horizonte, Minas Gerais, Brazil) and from local community in the period of June 2012 to September 2013. T2DM diagnosis was based on the American Diabetes Association [4]. Subjects older than 70 years with cancer or autoimmune disease, with current or recent infectious process, with history of cardiovascular disease, and with treatment of anti-inflammatory drugs were excluded. The concentrations of ESM-1 (human endocan/ESM-1 JDIEK H1 – Lunginnov®) were measured in serum by ELISA, according to the manufacturer's instructions. The study was approved by the Ethics Committee of the Federal University of Minas Gerais and Santa Casa Hospital. Informed consent was obtained from all the patients. ESM-1 concentrations in T2DM patients (1.75 ± 0.78 ng/mL) were significantly lower than controls (2.20 ± 0.54 ng/mL; $p = 0.005$ – Student's t-test) (Figure 1). In addition, we observed a negative correlation between waist circumference and ESM-1 levels ($r^2 = -0.155$, $p = 0.013$ – Pearson correlation coefficient), but no significant correlations were found between these levels and body mass index in T2DM patients. The results indicate that lower serum ESM-1 levels can be associated with the chronic state of inflammation in T2DM, because decreased ESM-1 levels enable leukocyte adhesion and migration through the endothelium [3,5]. The significant negative correlation with waist circumference also suggests that ESM-1 may be linked with visceral obesity, an independent risk factor for T2DM development. Therefore, hyperinsulinaemia, frequently observed in T2DM patients, can inhibit ESM-1 gene expression in adipocytes, increasing the inflammatory process [6]. As inflammation and obesity are hallmark of T2DM, other clinical

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes whose pathogenesis is associated with a chronic state of low-grade inflammation. Although the mechanisms are not completely known, the subclinical inflammation is a part of the insulin resistance and is closely related to complications of this metabolic disorder and obesity [1]. Endocan or endothelial cell-specific molecule 1 (ESM-1), is a dermatan sulfate proteoglycan, whose expression occurs in endothelial cells [2]. *In vitro*, ESM-1 interferes with molecular mechanisms of immune cell migration by blocking the interaction between leukocyte function-associated antigen-1 and intercellular adhesion molecule-1 [3]. We evaluated the ESM-1 levels in T2DM patients compared with a control group. T2DM patients (n=39, 33 women; 59.74 ± 6.47 years) and controls (n=39, 28 women; 53.03 ± 8.78 years) matched by gender, age and body mass index (T2DM 27.52 ± 4.39 kg/m^2; control 26.77 ± 4.24 kg/m^2) were included. The subjects were recruited from Santa Casa Hospital (Belo Horizonte, Minas Gerais, Brazil) and from local community in the period of June 2012 to September 2013. T2DM diagnosis was based on the American Diabetes Association [4]. Subjects older than 70 years with cancer or autoimmune disease, with current or recent infectious process, with history of cardiovascular disease, and with treatment of anti-inflammatory drugs were excluded. The concentrations of ESM-1 (human endocan/ESM-1 JDIEK H1 - Lunginnov®) were measured in serum by ELISA, according to the manufacturer's instructions. The study was approved by the Ethics Committee of the Federal University of Minas Gerais and Santa Casa Hospital. Informed consent was obtained from all the patients. ESM-1 concentrations in T2DM patients (1.75 ± 0.78 ng/mL) were significantly lower than controls (2.20 ± 0.54 ng/mL; p=0.005 - Student's *t*-test) (**Figure 1**). In addition, we observed a negative correlation between waist circumference and ESM-1 levels (r= -0.155, p=0.013 - Pearson correlation coefficient), but no significant correlations were found between these levels and body mass index in T2DM patients. The results indicate that lower serum ESM-1 levels can be associated with the chronic state of inflammation in T2DM, because decreased ESM-1levels enable leukocyte adhesion and migration through the endothelium [3, 5]. The significant negative correlation with waist circumference also suggests that ESM-1 may be linked with visceral obesity, an independent risk factor for T2DM development. Therefore, hiperinsulinemia, frequently observed in T2DM patients, can inhibit ESM-1 gene expression in adipocytes, increasing the inflammatory process [6]. As inflammation and obesity are

hallmark of T2DM, other clinical disorders that present any of these characteristics have also shown changes in ESM-1 levels, such as coronary artery disease, hypertension, chronic kidney disease, sepsis and cancer [7-11]. Therefore, as Endocan does not seem to be specific to T2DM, others studies are necessary in order to evaluate the role of ESM-1 in the regulation of inflammatory processes and its potentiality as a therapy for delaying the complications in T2DM.

ACKNOWLEDGMENTS: The authors thank FAPEMIG, CAPES, CNPq/Brazil, and PRPq/UFMG for financial support. L.P.S., V.C.S. and K.B.G. are grateful to CNPq Research Fellowship.

CONFLICTS OF INTEREST

There is no conflict of interest.

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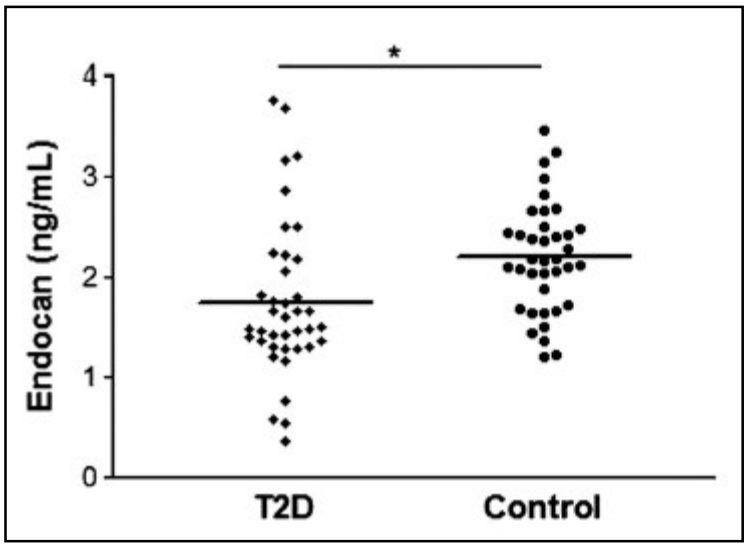

Figure 1. Endocan (endothelial cell-specific molecule 1) levels for type 2 diabetes mellitus patient and control groups (Student's *t*-test; *p<0.05).

Capítulo 3:

Circulating irisin is increased in type 2 diabetes mellitus and correlates with fasting glucose levels

APOLLO MEDICINE 13 (2016) 152–155

Available online at www.sciencedirect.com

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Original Article

Circulating irisin is increased in type 2 diabetes mellitus and correlates with fasting glucose levels

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ARTICLE INFO

Artide history:
Received 16 January 2016
Accepted 29 February 2016
Available online 20 March 2016

Keywords:
Irisin
Type 2 diabetes mellitus
Obesity
Glucose metabolism
Physical activity

ABSTRACT

Background/aims: Irisin is a hormone expressed in muscle, which is an exercise-induced myokine triggerin gbrowning of white adipose tissue and increases metabolic genes expression.
Methods: We investigated the irisin levels in 39 type 2 diabetes mellitus patients (T2D) compared to 39 control individuals.
Results: Irisin plasma levels were higher in T2D patients when compared with the control group ($p < 0.0001$). Non-obese T2D individuals presented higher irisin levels when compared with non-obese and obese controls ($p < 0.0001$ and $p = 0.011$, respectively). Obese T2D individuals showed higher irisin levels when compared with non-obese control ($p = 0.003$), but no difference was observed when compared with obese controls ($p > 0.05$). A positive correlation was observed between irisin and fasting glucose levels in the T2D group ($r = 0.341$, $p = 0.033$). No difference regarding irisin levels was found when T2D individuals who practice physical activity and sedentary individuals were compared ($p = 0.632$).
Conclusion: This result indicates that T2D is associated with an irisin release in order to compensate the insulin resistance observed in the skeletal muscle on diabetes, and that the poor glycaemic control contributes for enhancement of this state.

1. Introduction

Irisin is a glycosylated polypeptide hormone expressed in muscle as the type 1 membrane precursor protein FNDC5 (fibronectin type III domain containing 5), which is proteolytically cleaved and secreted.[1] This protein has been described as an exercise-induced myokine triggering browning of white adipose tissue and increasing metabolic genes expression.[1,2] Moreover, it is produced in response to activation of peroxisome proliferator activated receptor-c coactivator-1 α (PGC-1 α).[3]

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http://dx.doi.org/10.1016/j.apme.2016.02.010

Circulating irisin is increased in type 2 diabetes mellitus and correlates with fasting glucose levels

Short title: Irisin in type 2 diabetes mellitus

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ABSTRACT

Background/aims: Irisin is a hormone expressed in muscle, which is an exercise-induced myokine triggering browning of white adipose tissue and increases metabolic genes expression. **Methods:** We investigated the irisin levels in 39 type 2 diabetes mellitus patients (T2D) compared to 39 control individuals. **Results:** Irisin plasma levels were higher in T2D patients when compared with the control group ($p<0.0001$). Non-obese T2D individuals presented higher irisin levels when compared with non-obese and obese controls ($p<0.0001$ and $p=0.011$, respectively). Obese T2D individuals showed higher irisin levels when compared with non-obese control ($p=0.003$), but no difference was observed when compared with obese controls ($p>0.05$). A positive correlation was observed between irisin and fasting glucose levels in the T2D group ($r=0.341$, $p=0.033$). No difference regarding irisin levels was found when compared T2D individuals who practice physical activity and sedentary individuals ($p=0.632$). **Conclusion:** This result indicates that T2D is associated with an irisin release in order to compensate the insulin resistance observed in the skeletal muscle on diabetes; and that the poor glycemic control contributes for enhance of this state.

Keywords: irisin; type 2 diabetes mellitus; obesity; glucose metabolism, physical activity.

1. INTRODUCTION

Irisin is a glycosylated polypeptide hormone expressed in muscle as the type 1 membrane precursor protein FNDC5 (fibronectin type III domain containing 5), which is proteolytically cleaved and secreted [1]. This protein has been described as an exercise-induced myokine triggering browning of white adipose tissue and increasing metabolic genes expression [1, 2]. Moreover, it is produced in response to activation of peroxisome proliferator activated receptor-c coactivator-1 α (PGC-1 α) [3].

Some studies have demonstrated that irisin causes increase in total body energy expenditure, enhanced thermogenesis, increased oxidative metabolism, weight loss, besides improvement glucose tolerance and insulin resistance [1, 4-6]. Circulating irisin levels have been evaluated in cross-sectional studies involving type 2 diabetes (T2D) patients [7-12], but its association with the disease is still conflicting.

In this study, the irisin plasma levels in T2D patients were evaluated comparing with a control group, as well as the association with anthropometric and laboratory parameters.

2. MATERIAL AND METHODS

2.1 Subjects

This cross-sectional study was conducted with 39 patients with clinical and laboratory diagnosis of T2D [33 women and 6 men; 61 (9) years; BMI 27.5 ± 4.4 Kg/m^2] and 39 non-diabetic controls [28 women and 11 men; 53 (11) years; BMI 26.7 ± 4.2 Kg/m^2]. T2D diagnosis was based on the American Diabetes Association criteria [13]. The patient group was recruited from Santa Casa Hospital (Belo Horizonte, Minas Gerais, Brazil) and control group from local community; in the period of June/2012 – September/2013.

This study was approved by the Ethics Committee of the Federal University of Minas Gerais and Santa Casa Hospital. Informed consent was obtained from all the patients.

Anthropometric and laboratory data were obtained from medical records. The controls showed normal levels of fasting glucose (60 - 99 mg/dL) and no use of hypoglycemic drugs. The exclusion criteria adopted were: individuals older than 70

years, with history of cardiovascular diseases, cancer, autoimmune disease, with current or recent infectious process, and with treatment of anti-inflammatory drugs.

2.2 Clinical and laboratorial data

Clinical data (gender, age, BMI) were obtained for all the subjects through interviews or medical records. The fasting glucose was measured in serum samples after eight hours fasting. The serum samples were centrifuged at 3,500 rpm for 20 min at 25ºC and the assays performed immediately. The tests were performed using enzyme-colorimetric method, BTR 811 spectrophotometer (Biotron, Minas Gerais, Brazil), and Glicose-PP kit (Gold Analisa, Minas Gerais, Brazil), following the manufacturer's instructions. The concentrations of fasting glucose were expressed in mg/dL. The irisin levels (Irisin Competitive ELISA Kit, AdipoGen®, Switzerland) were measured in heparin plasma samples (centrifuged at 1.100 x g for 20 min at 25ºC and stored at -70ºC until analysis) by ELISA, according to the manufacturer's instructions.

2.3 Statistical analysis

All of the statistical analyses were performed with Statistical Package of the Social Sciences (SPSS) version 17.0. The analysis of normality was performed by Shapiro-Wilk test. Data are presented as "mean ± (standard deviation – SD)" (parametric variables) or "median (interquartile range – IQR)" (non-parametric variables). Comparisons between two groups were made by Student's t-test for parametric variables and Mann-Whitney test for non-parametric variables. Comparisons of non-parametric variables were performed by Kruskal-Wallis test between three groups. When differences were detected, they were compared in pairs by the Mann-Whitney method, followed by Bonferroni's Correction. Spearman's correlations were computed in the T2D patient group to assess correlations between irisin plasma levels and fasting glucose. A p-value < 0.05 was considered statistically significant.

3. RESULTS

The groups were matched by gender (Chi-square test, p=0.329) and BMI (Student t-test, p=0.391).

Irisin plasma levels were higher in T2D patients [3.54 (3.01) µg/mL] when compared with the control group [2.01 (1.44) µg/mL] - Mann-Whitney test: $p<0.0001$ (**Figure 1**).

It was also observed that irisin levels are different between T2D and control groups (Kruskal Wallis test, $p<0.0001$) when considered weight criteria: BMI <30 Kg/m^2 as non-obese and BMI ≥30 Kg/m^2 as obese. Non-obese T2D individuals presented higher irisin levels [4.08 (3.76) µg/mL] when compared with non-obese [2.08 (1.33) µg/mL] and obese controls [1.70 (2.52) µg/mL] - Mann Whitney test with Bonferroni correction: $p<0.0001$ and $p=0.011$, respectively. Furthermore, obese T2D individuals showed higher irisin levels [2.89 (1.06) µg/mL] when compared with non-obese control [2.08 (1.33) µg/mL] - Mann Whitney test with Bonferroni correction: $p=0.003$ (**Figure 2**), but no difference when compared with obese controls ($p>0.013$ after correction). No statistical difference was observed when compared irisin levels in both non-obese and obese individuals considering the same group (patients or control) were compared.

A positive correlation was observed between irisin and fasting glucose levels in the T2D group (Spearman's correlation, $r=0.341$, $p=0.033$ - **Figure 3**).

No difference regarding irisin levels was found when compared individuals who practice physical activity - at least three times a week, during 30 minutes [48.72% total group, irisin = 2.81 (2.35) µg/mL or 46.15% for T2D patients, irisin = 3.38 (3.71) µg/mL] and sedentary individuals [51.28% total group, irisin = 2.74 (2.14) µg/mL or 53.85% for T2D patients, irisin = 4.00 (2.87) µg/mL] - Mann-Whitney test, $p=0.676$ and $p=0.632$, respectively.

4. DISCUSSION

Our results showed that irisin levels are elevated in T2D when compared to control group and correlate with fasting glucose levels. These results indicate that (1) T2D is associated with an irisin resistance state similar to insulin resistance observed in the skeletal muscle on diabetes, and that the poor glycemic control contributes for enhance of this state; or (2) irisin release is increased in order to compensate the insulin resistance observed in T2D patients.

Similar results regarding irisin levels and its correlation with fasting glucose were found by García-Fontana et al. (2015) and Park et al. (2013) [9, 11]. Higher irisin levels in other metabolic diseases are shown on previous papers as a compensatory mechanism of insulin resistance. Recently, Park et al. [11] reported significantly higher irisin levels in subjects with metabolic syndrome (MetS) compared to controls. High levels of irisin in T2D were also correlated with glycated haemoglobin (HbA1c) [3].

Duan et al. (2016) observed that oral administration of irisin lowered the blood glucose in STZ-induced diabetic mice. Moreover, the oral glucose tolerance was improved, and serum insulin was significantly elevated in diabetic mice after irisin-injection treatment [14]. Xin et al. (2015) also showed that irisin improved glucose tolerance and glucose uptake as evidenced by GLUT4 translocation in diabetic skeletal muscle of a mouse model of diabetes, established by feeding with high-fat diet. Irisin also increased glucose uptake in myocytes cultured in high glucose/high fatty acid medium [15]. Other study demonstrated that irisin alleviates endothelial dysfunction in T2D mice model via reducing oxidative/nitrative stresses, suggesting that irisin may be a promising molecule for the treatment of vascular complications of diabetes [16].

Although the irisin levels have been increased in T2D patients when compared to controls, obesity is associated with decrease of irisin in the present study. The results from other studies have been controversial regarding obesity and irisin levels [17]. Moreno-Navarrete et al. (2013) observed that circulating irisin decreased in association with obesity, but diabetics patients were no included in that analysis [18]. It is generally proposed an anti-obesity effect of irisin. A hypothesis would be that irisin performs a protective factor against obesity mediated by the browning of white adipose tissue and the increase of the body mass. By considering pathological states of obesity, physiological irisin could not keep the balance of energy storage and expenditure, resulting in an adipose tissue hypertrophy and substantial increase of fat storage [19].

Al-Daghri et al. (2015) observed a positive correlation between frequency and intensity of physical activities and serum irisin levels in healthy individuals, but not in T2D subjects [3]. In fact, no such association was noted in T2D patients in our study. The possible reason for this difference may be related to the metabolic dysfunction in T2D. It has been observed that altered metabolic states as observed in T2D could

lead to abnormalities in the exercise-dependent pathway through the expression of PGC-1α and consequently, FNDC5 function [3].

The main limitation of this study is a small sample size, however, our study showed an important role of irisin in T2D and obesity. Although it is not possible to consider the irisin as diagnostic test for T2D, the recombinant irisin protein could be used for treatment of T2D complications, since it promotes the increase of total energy expenditure and attenuates the insulin resistance [17].

Although further studies are needed to better elucidate how irisin circulating levels are modulated in the context of T2D and its comorbidities as obesity, the use of irisin may prove beneficial treatment for these metabolic diseases.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS: Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). KBG is grateful to CNPq Research Fellowship (PQ).

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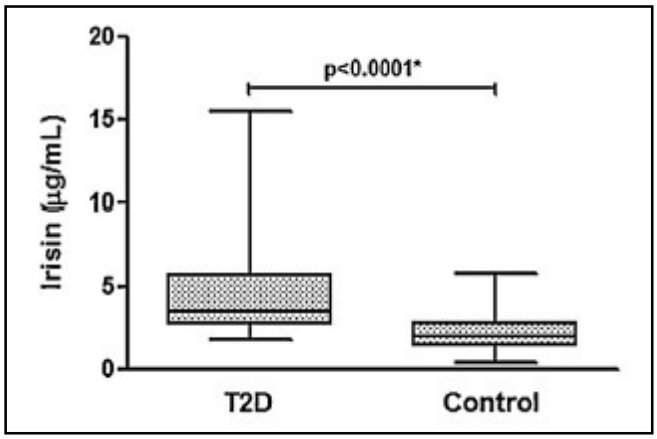

Figure 1: Irisin plasma levels in type 2 diabetes (T2D) patients and controls. *Mann-Whitney test, p<0.05 was considered significant.

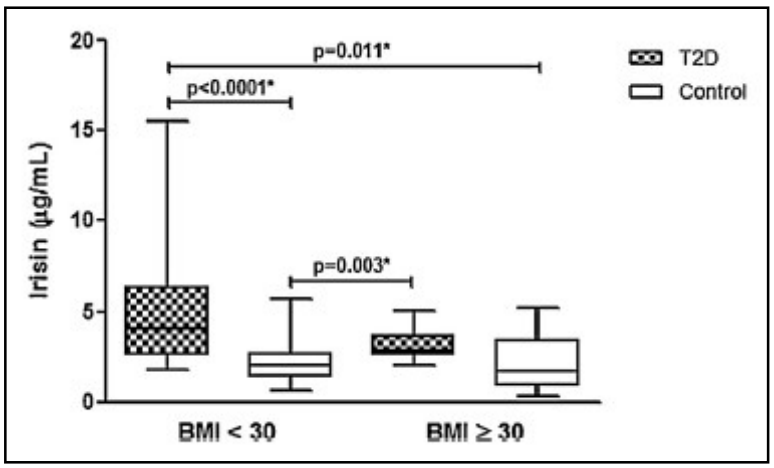

Figure 2: Irisin plasma levels in type 2 diabetes (T2D) and controls groups according to body mass index (BMI) - *Mann-Whitney test with Bonferroni correction, p<0.013 was considered significant.

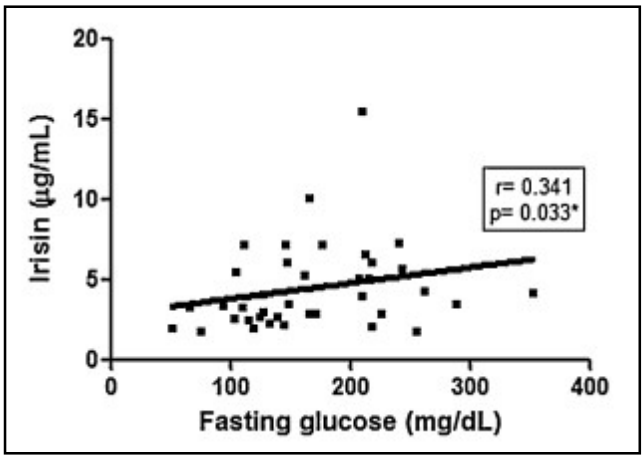

Figure 3: Correlation coefficient between irisin plasma levels and fasting glucose in type 2 diabetes (T2D) group - *Spearman's correlation, $p<0.05$ was considered significant.

Capítulo 4:

Visfatin levels are decreased in advanced stages of diabetic nephropathy

RENAL FAILURE

http://informahealthcare.com/rnf
ISSN: 0886-022X (print), 1525-6049 (electronic)

Ren Fail, 2015; 37(9): 1529–1530
 DOI: 10.3109/0886022X.2015.1074520

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LETTER TO THE EDITOR

Visfatin levels are decreased in advanced stages of diabetic nephropathy

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Visfatin, also known as nicotinamide phosphoribosyltransferase or pre-B-cell colony-enhancing factor 1, is a ubiquitous adipokine firstly described in 2005 by Fukuhara et al.[1] The visfatin is upregulated in visceral fat of both humans and mice and have insulin-mimetic effects by direct binding to insulin receptor, leading to glucose uptake. In addition to adipocytes, skeletal muscle, bone marrow, hepatocytes, lymphocytes, and mesangial cells can produce visfatin.[2] Higher visfatin levels have been associated with obesity, type 2 diabetes mellitus (T2DM) and insulin resistance.[3-5] Furthermore, visfatin acts as an inductor of the production of proinflammatory cytokines (IL-6, IL-1β, TNF-α) and reactive oxygen species generation.[6] Diabetic nephropathy (DN) is a common cause of chronic kidney disease (CKD) and is associated with the increased morbidity and premature mortality in diabetic patients.[7] CKD is associated with an abnormal high adipokines level that may be caused by increased fat tissue production and/or reduced renal clearance of them.[8] Recent studies showed that visfatin levels are changed in CKD (DN or not) in parallel to the glomerular dysfunction, severity of proteinuria and endothelial dysfunction.[9-12] We investigated the association between visfatin levels and stages of CKD in T2DM patients with DN and the correlations with anthropometric and laboratory parameters. Thirty-eight patients [33 women and 5 men; age 59.7 ± 6.5 years; body mass index (BMI) 27.5 ± 4.4 kg/m^2; waist circumference 97.9 ± 12.3 cm; serum creatinine 0.9 (0.6) mg/dL; blood urea 37,5 (19.0) mg/dL] were recruited from Santa Casa Hospital (Belo Horizonte, Minas Gerais, Brazil) in the period of June 2012 - September 2013. Subjects older than 70 years, with cancer or autoimmune disease, with current or recent infectious process, with history of cardiovascular disease, and with treatment of anti-inflammatory drugs were excluded. T2DM diagnosis was based on the American Diabetes Association criteria.[7] DN was defined as albumin excretion rate >30mg/24h at least 2 of 3 specimens collections within a 3- to 6-month, and without coexisting renal diseases from causes other than diabetes.[7] According to National Kidney Foundation (NKF), CKD was classified based on glomerular filtration ratio (GFR), estimated by Cockcroft-Gault equation:[13,14] stage 1 (GFR ≥90 mL/min/1.73^2), stage 2 (GFR 60-89 mL/min/1.73^2), stage 3 (GFR 30-59 mL/min/1.73^2), stage 4 (GFR 15-29 mL/min/1.73^2) and stage 5 (GFR <15 mL/min/1.73^2 or dialysis). The concentrations of visfatin (Human/Mouse/Rat Visfatin EIA Kit, Ray Biotech Inc., GA) were measured in serum samples by ELISA, according to the manufacturer's instructions. All samples were assayed at the same time. The concentrations of

visfatin were expressed in ng/L. Anthropometric and laboratory data were obtained from medical records. The study was approved by the Ethics Committee of the Federal University of Minas Gerais and Santa Casa Hospital. Informed consent was obtained from all the patients. The T2DM patients were classified in stage 2 [n=11; GFR 71.94 (15.94) mL/min/1.73^2], stage 3 [n=21; GFR 49.01 (15.69) mL/min/1.73^2], and stage 4 [n=6; GFR 18.39 (8.86) mL/min/1.73^2]. Visfatin levels were different between the CKD stages [stage 2 - 34.06 (56.59) ng/L; stage 3 - 21.25 (27.74) ng/L; stage 4 - 19.57 (14.51) ng/L; p= 0.042 - Kruskal Wallis' test); but this difference was significant only when compared the stage 2 *versus* stage 4 (p= 0.015 - Mann Whitney's test) (**Figure 1**). These findings were independent of fasting glucose and HbA1c levels, as well as comorbidities - retinopathy, arterial hypertension and dyslipidemia (p>0.05 for all). Contrary to expected, visfatin levels were not associated with BMI categories (BMI < 25, 25 ≤ BMI < 30 and BMI ≥ 30 Kg/m^2) (p= 0.247 – Kruskal Wallis' test). Furthermore, visfatin levels showed significant correlations with GFR (r= 0.389, p= 0.016) and serum creatinine (r= -0.402, p= 0.012). Taken together, our data suggest that CKD progression is associated with lower visfatin levels, independently of adiposity. This finding is contradictory to some previous studies,[9,11,12] which suggested an increased visfatin levels due to a decline in GFR. It is important to note that these studies evaluated different groups of patients with CKD, such as glomerulonephritis, polycystic kidney disease, vasculitis and malignant hypertension; while others just compared diabetic and control group without to consider nephropathy stages, which may affect the results. Since it is known that visfatin can be produced in glomerular mesangial cells,[2] but not whether visfatin levels can itself influence the renal damage progression, our results are preliminary and suggest two hypotheses: 1) the CKD progression in T2DM increases the urinary excretion of visfatin, reducing its plasma concentration; or 2) advanced stages of DN reduce local synthesis of visfatin by mesangial cells, decreasing its plasma levels. The main limitations of this study are absence a control group and a small sample size. However, as the role of visfatin levels in DN is still not clear, further studies are needed to better elucidate this association and to evaluate the potential of visfatin as a laboratory marker of nephropathy progression.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ACKNOWLEDGMENTS

The authors thank FAPEMIG, CAPES, CNPq/Brazil, and PRPq/UFMG for financial support. V.C.S. and K.B.G. are grateful to CNPq Research Fellowship (PQ).

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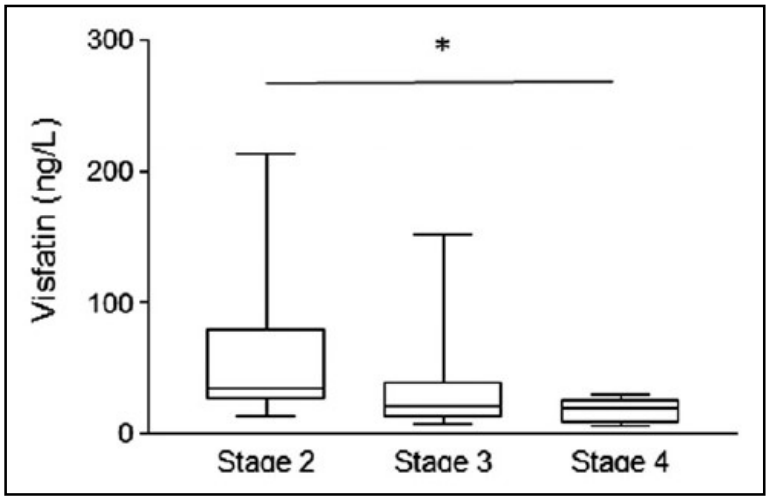


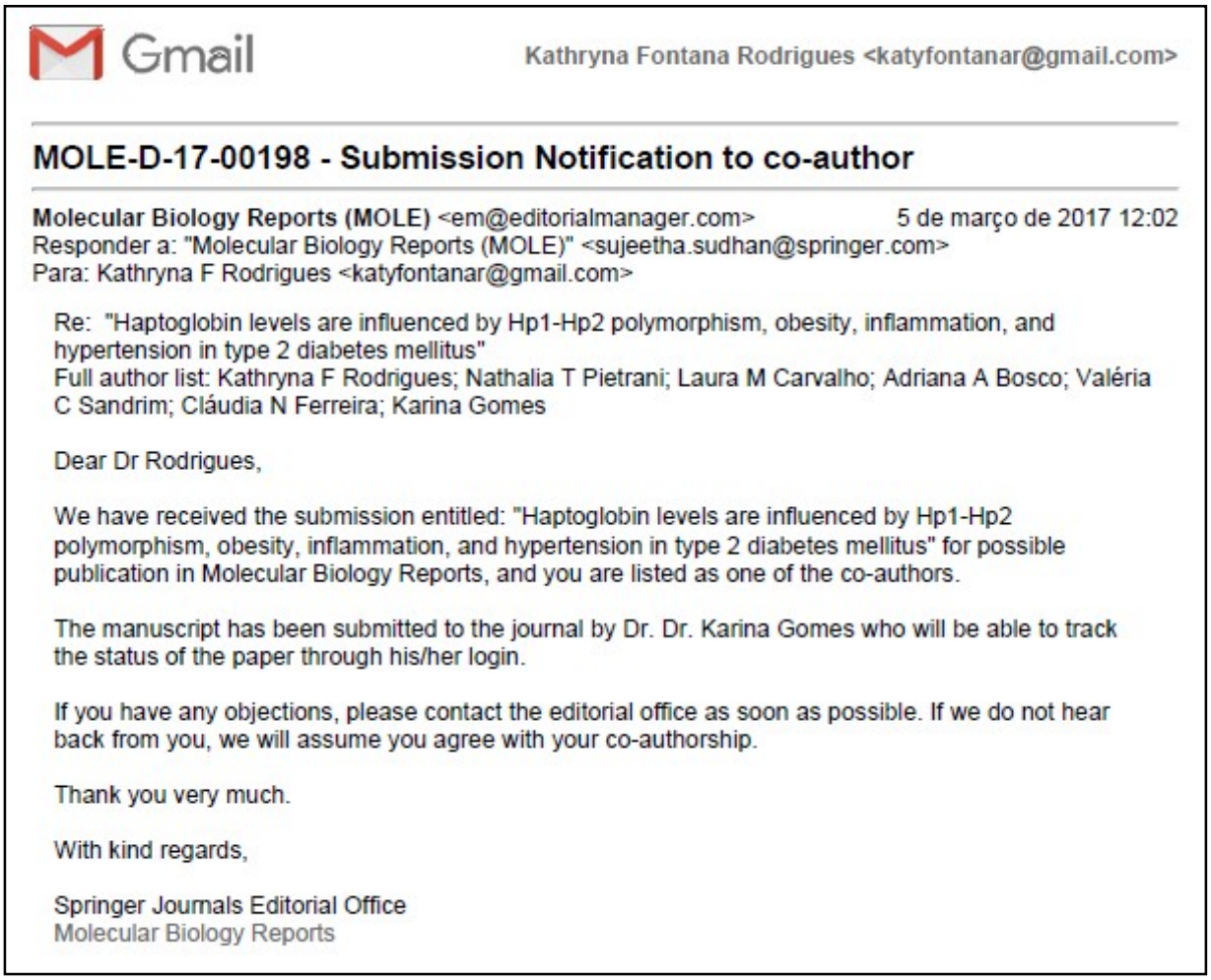
Figure 1. Visfatin levels for type 2 diabetes mellitus patients and different stages of CKD (*p<0.05 was considered significant - Kruskal-Wallis test).

7. ARTIGOS SUBMETIDOS PARA PUBLICAÇÃO

Capítulo 5:

Haptoglobin levels are influenced by Hp1-Hp2 polymorphism, obesity, inflammation, and hypertension in type 2 diabetes mellitus

Comprovante de submissão do artigo original intitulado "*Haptoglobin levels are influenced by Hp1-Hp2 polymorphism, obesity, inflammation, and hypertension in type 2 diabetes mellitus*" para a revista *Molecular Biology Reports*.



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MOLE-D-17-00198 - Submission Notification to co-author

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Responder a: "Molecular Biology Reports (MOLE)" <sujeetha.sudhan@springer.com>
Para: Kathryna F Rodrigues <katyfontanar@gmail.com>

Re: "Haptoglobin levels are influenced by Hp1-Hp2 polymorphism, obesity, inflammation, and hypertension in type 2 diabetes mellitus"
Full author list: Kathryna F Rodrigues; Nathalia T Pietrani; Laura M Carvalho; Adriana A Bosco; Valéria C Sandrim; Cláudia N Ferreira; Karina Gomes

Dear Dr Rodrigues,

We have received the submission entitled: "Haptoglobin levels are influenced by Hp1-Hp2 polymorphism, obesity, inflammation, and hypertension in type 2 diabetes mellitus" for possible publication in Molecular Biology Reports, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dr. Karina Gomes who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

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Molecular Biology Reports

Haptoglobin levels are influenced by Hp1-Hp2 polymorphism, obesity, inflammation, and hypertension in type 2 diabetes mellitus

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is an inflammatory condition associated with obesity and increased oxidative stress. Haptoglobin (Hp) is an acute reactant that scavenges extracorpuscular hemoglobin from circulation preventing heme-iron oxidative damage. We aimed to evaluate the association between Hp levels with Hp1-Hp2 gene polymorphism, clinical and laboratorial parameters in T2DM individuals. 102 T2DM patients and 62 controls were included in this study. Hp plasma levels were quantified using ELISA assay and Hp genotyping was carried out by specific two-step allelic polymerase chain reaction. Hp levels were higher in T2DM patients when compared with controls (p=0.005). Hypertensive T2DM patients showed higher Hp levels when compared with normotensive patients (p=0.021). Obese T2DM patients had higher Hp levels when compared with obese controls (p=0.009) and with non-obese T2DM patients (p=0.003). Hp1-Hp1 genotype showed association with T2DM according to an additive (OR=3.038, 95% CI 1.127 - 8.192; p=0.036) and dominant model (OR=0.320, 95% CI 0.118 - 0.839; p=0.010), but Hp2 allele carriers contributes with higher Hp levels in T2DM than controls. Waist circumference (p=0.002), BMI (p=0.001), IL-6 (p=0.012), and hs-CRP (p=0.001) were positively correlated with Hp levels in T2DM group. These results suggest that Hp levels are influenced by Hp1-Hp2 polymorphism, obesity, inflammatory status and hypertension in T2DM.

Keywords: haptoglobin; type 2 diabetes mellitus; polymorphism; inflammation; obesity; hypertension

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic disease and the most prevalent form of diabetes. It is estimated that, in developed countries, about 87-91% of diabetic patients present T2DM [1]. The number of people with diabetes is rapidly growing worldwide and reaching epidemic proportions.

The pathogenesis of T2DM has been associated with a subclinical chronic inflammation and activation of the immune system [2]. Some studies have shown that T2DM patients have higher levels of acute-phase response markers, including sialic acid, α-1 acid glycoprotein, C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1) and proinflammatory cytokines, mainly interleukin (IL) 6, IL-1β, and tumor necrosis factor alpha (TNF-α) [2, 3, 4].

Haptoglobin (Hp) is an acute-phase reactant α2-glycoprotein (54 kDa) that primarily scavenges the hemoglobin (Hb) released into circulation either by hemolysis or by normal red blood cell turnover, preventing Hb-related oxidative damage [5]. Hp is mainly synthesized by hepatocytes and to some extent by non-hepatic cells and organs, including adipocytes, lung, spleen, kidney and heart [6, 7]. The Hp-Hb soluble complex, characterized by high affinity and stability, is not filtered through the glomeruli but is transported to the liver and other tissues to be degraded by Hp-Hb scavenger receptors, such as the CD163 receptor found on the surface of monocytes, macrophages, and Kupffer cells [8, 9]. The Hp-Hb-CD163 complex induces the production of several anti-inflammatory and antioxidative mediators [10].

Synthesis of Hp is substantially lower in fetal than in adult human liver [9]. Hp levels increase several fold (3- to 8-fold) in response to local or systemic inflammatory stimuli [11]. The major inducers are proinflammatory cytokines such as IL-1, IL-6, and TNF-α [12], since there are binding sites for cytokines on the regulatory regions of the Hp gene promoter, with control of transcription rates and subsequent protein synthesis [13]. One study demonstrated in an 8-week follow-up streptozotocin-induced diabetes model that changes in Hp expression are strongly associated with TNF-α and IL-6 serum levels and TNF-α/IL-6 ratio [14].

The Hp gene is located on the long arm of chromosome 16 (16q22.2) and presents polymorphism characterized by two alleles: Hp1 and Hp2. The Hp1 allele has five exons and is conserved among species. The Hp2 allele is human-specific and has seven exons that likely arose from a duplication (non-homologous crossing

over) involving exons 3 and 4 of the Hp1 allele. The allelic combinations result in three genotypes and structurally/functionally distinct phenotypes: Hp1-Hp1, Hp2-Hp1, and Hp2-Hp2 [9].

Hp is synthesized as a single chain, which is cleaved in an amino-terminal α-chain and a carboxy-terminal β-chain, linked by disulfide bonds. The α-chain encoded by Hp1 allele (α1 - 9kDa) is smaller than that produced by Hp2 allele (α2 - 16 to 20 kDa). The β-chain (40kDa) is identical for both Hp alleles and contains the main domains involved in Hp-Hb binding. Therefore, the stoichiometry of the mature Hp protein is genotype-dependent: Hp1-Hp1 individuals produce a single linear homodimer, Hp2-Hp2 produce cyclic polymers, and in heterozygous subjects, the Hp protein can be found as linear homodimer and/or multimers [15, 16, 17]. Besides the structural differences, Hp1 and Hp2 protein products presents other functional differences: the ability to bind the Hb, the capacity to bind the Hp-Hb complex to the CD163 receptor, the clearance rates of the Hp-Hb complex from circulation, and the inhibitory effect on prostaglandin synthesis (Hp anti-inflammatory action), which are markedly greater in Hp1-Hp1 than Hp2 carrier [18].

The frequencies of Hp alleles and genotypes vary worldwide and depend on racial background. Previous studies have indicated that Hp1 allele is most frequent in Africa and South America and less frequent in Southeast Asia. In western countries, the average distribution of Hp genotypes is 16% Hp1-Hp1, 48% Hp2-Hp1, and 36% Hp2-Hp2, which correspond to allele frequencies about 40% Hp1 and 60% Hp2 [9, 19].

Since T2DM and its complications have been associated with increased oxidative stress induced primarily by hyperglycaemia [20], Hp can present an important role in this context due to its antioxidant activity by scavenging extracorpuscular Hb, which can initiate a free radical reaction by releasing heme iron [17, 20].

In spite of the existence of some reports examining the association between T2DM and Hp [20-30] in different populations, the results are still controversial. Therefore, in this study we intended to evaluate the association between Hp levels and Hp1-Hp2 gene polymorphism, as well as clinical and laboratorial parameters in a group of T2DM Brazilian patients.

2. MATERIAL AND METHODS

2.1Ethical aspects

This study was approved by the ethics committees of Federal University of Minas Gerais (Minas Gerais, Brazil) - ETIC 0062.0.203.000-11 - and of Santa Casa Hospital (Minas Gerais, Brazil) - 059/2011 - conforms to the ethical guidelines of the Declaration of Helsinki. All participants provided written informed consent prior to entering the study.

2.2 Experimental design

This cross-sectional, case-control study was conducted with 164 subjects aged 32-70 years. The case group included 102 patients with clinical and laboratorial diagnosis of T2DM, according to the criteria established by the American Diabetes Association (ADA) [31], while 62 gender-, age- and body mass index (BMI)-matched non-diabetic individuals were classified as the control group. A 2:1 case/control proportion was adopted, according to a sample calculation based on the mean values of Hp levels from a sample of the groups (power=0.95; significance level=0.05). T2DM patients were recruited at the Clinic of Endocrinology (Santa Casa Hospital, Minas Gerais, Brazil) from June 2012 to September 2013. Control group subjects were recruited from the local community during the same period. The exclusion criteria included the following parameters: age over 70 years**,** pregnancy, cancer, autoimmune diseases, recent history of cardiovascular disease (e.g. heart attack, stroke, thrombosis in the last five years), and current or recent infections and/or inflammatory processes. Interviews and medical records were utilized to obtain clinical and laboratorial data for all T2DM patients. Presence or absence of hypertension was defined by the clinical staff at the Clinic of Endocrinology (Santa Casa Hospital, Minas Gerais, Brazil), conforming to the criteria adopted by ADA [31]: systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 80 mmHg, or use of antihypertensive drugs. Controls presented normal fasting blood glucose levels (60 - 99 mg/dL) and they didn’t use hypoglycemic drugs.

2.3 Blood sampling

Venous blood samples were collected from each participant in EDTA and anticoagulant-free tubes, allowed to clot for 30 min, and centrifuged at 1,100 x g for

20 min at 25ºC. The supernatant fractions obtained (plasma and serum) were aliquoted in microtubes and stored at -80ºC until biochemical analysis. An aliquot of whole blood collected in EDTA was also stored for later genomic DNA extraction and Hp genotyping analysis.

2.4 Biochemical measurements

Subjects in the control group had their fasting glucose blood levels measured immediately after centrifugation of serum samples obtained after eight hours of fasting. The tests were performed with an enzyme-colorimetric method, using Glicose-PP kit (Gold Analisa, Minas Gerais, Brazil) following the manufacturer's instructions, and BTR 811 spectrophotometer (Biotron, Brazil).

The high-sensitivity C reactive protein levels (hs-CRP) were measured in serum samples using the immunoturbidimetric method, hsCRP VITROS Chemistry Products (Ortho Clinical Diagnostics, New York, USA) following the manufacturer's instructions, and System Vitros Chemistry 5.1 FS (Ortho Clinical Diagnostics, New York, USA).

The plasma levels of cytokines TNF-α, IL-10, and IL-6 were determined by the Cytometric Bead Array (CBA) method using Human Enhanced Sensitivity Flex Set Systems (BD Biosciences Pharmingen, California, USA), following the manufacturers' instructions. Data acquisition and analysis were performed in LSRFortessa™ flow cytometer (BD Biosciences Pharmingen, California, USA) using FCAP Array Software version 1.0.1.

Hp plasma levels were quantified using an enzyme linked immunosorbent assay (ELISA) - Quantikine® ELISA Human Haptoglobin Immunoassay (R & D Systems, Minneapolis, USA), which equally detect both human forms Hp1-Hp1 and Hp2-Hp2, following the manufacturer's protocol.

hs-CRP, cytokines, and Hp levels were measured at the same time for all samples.

2.5 Haptoglobin genotyping

Genomic DNA was obtained using Biopur Mini Spin Kit (Biometrix Biotecnologia®, Curitiba, Brazil). Hp genotyping was performed with a specific two-step allelic polymerase chain reaction (PCR) technique as described by Koch et al. [32]. In the first reaction, the 1757 bp allele 1 specific fragment and the 3481 bp allele

2 specific fragment were amplified with primer A (5'-GAGGGGAGCTTGCCTTTCCATTG-3') and primer B (5'-GAGATTTTTGAGCCCTGGCTGGT-3'). In the second reaction, only the 349 bp allele 2 specific sequence was amplified, using primer C (5'-CCTGCCTCGTATTAACTGCACCAT-3') and primer D (5'-CCGAGTGCTCCACATAGCCATGT-3'). PCR conditions were as follow: initial denaturing at 95ºC for 2 min, followed by 35 cycles of 95ºC for 1 min, 69ºC for 2 min (1 min for reaction 2), 69ºC for 2 min (1 min for reaction 2) and final extension of 7 min at 72ºC. PCR products were visualized in 1% agarose gel electrophoresis later stained with ethidium bromide solution (10 mg/mL - Sigma-Aldrich, Missouri, USA).

2.6 Statistical analysis

The data were tested for normality by Shapiro-Wilk test. Parametric variables were presented as mean ± standard deviation (SD), non-parametric variables as median (interquartile range - IQR), and percentage of total for categorical variables.

Comparisons for parametric variables were performed with Student's t-test or Analysis of Variance (ANOVA) followed by LSD Post-Hoc test; and for non-parametric variables was used Mann-Whitney test or Kruskal-Wallis and Bonferroni's correction when necessary.

Hardy-Weinberg equilibrium (HWE) was evaluated using exact tests by GENEPOP and OEGE on-line softwares (available at: http://genepop.curtin.edu.au/genepop_op1.html and http://www.oege.org/software/hardy-weinberg.html, respectively). Differences in genotypic and allelic frequencies between the groups were tested with chi-square (χ^2) test with residual analysis.

Correlation analyses between Hp levels, clinical and biochemical parameters in T2DM group were performed with Spearman's correlation test.

All statistical analyses were performed with the Statistical Package of the Social Sciences (SPSS) version 17.0, and a p-value $\leq$0.05 was considered statistically significant.

3. RESULTS

Table 1 presents the clinical and laboratorial characteristics of case (T2DM) and control groups. The groups were matched by gender, age, and BMI ($p>0.05$ for all). T2DM patients displayed higher waist circumference ($p<0.0001$), waist-hip ratio ($p<0.0001$), fasting glucose ($p<0.0001$), and IL-6 levels ($p=0.001$) when compared to non-diabetic controls.

Hp plasma levels were higher in the T2DM group [1.15 (0.52) g/L] than controls [0.88 (0.58) g/L] ($p=0.005$ - **Figure 1**). Interestingly, hypertensives T2DM patients (n= 94) showed higher Hp levels [1.19 ± 0.46 g/L] when compared to those normotensives patients (n= 8) [0.80 ± 0.26 g/L] ($p=0.021$ - **Figure 1**).

Analyses of Hp levels were performed considering obesity status: BMI < 30Kg/m^2 (non-obese) and BMI ≥ 30Kg/m^2 (obese). Obese T2DM patients had higher Hp levels when compared with obese controls ($p=0.009$) and non-obese T2DM patients ($p=0.003$ - **Table 2**). Hp levels no exhibited differences when compared case and control groups considering BMI < 30Kg/m^2 ($p=0.634$). The control group also showed no difference among Hp levels and BMI categories ($p=0.959$).

We performed the association analysis of the Hp gene polymorphism with T2DM comparing to control group (**Table 3**). The polymorphism is in Hardy-Weinberg equilibrium ($p>0.025$) for either group. The genotypic frequencies were different between cases and controls subjects ($p=0.036$), and residual analyses revealed that the Hp1-Hp1 genotype was more frequent in T2DM patients when compared to non-diabetic controls. The frequencies of Hp1 allele (T2DM: 0.49; Control: 0.38) and Hp2 allele (T2DM: 0.51; Control: 0.62) were different between the groups (OR=1.606; 95% CI 0.994-2.599; $p=0.041$). The dominant model for inheritance (Hp2-Hp2 + Hp2-Hp1 vs Hp1-Hp1) showed a significant association (OR=0.320; 95% CI 0.118 - 0.839; $p=0.010$) with T2DM, which was not observed in the recessive model ($p=0.418$).

In order to evaluate if the Hp gene polymorphism could modulate the circulating levels of the protein, the Hp levels were compared in accordance to their genotypes for both groups and separately (T2DM or control) (**Table 4**). The analysis revealed that in the additive and dominant models, the Hp levels are lower in Hp2 allele carrier, considering all the participants ($p=0.001$; $p=0.020$, respectively) and only control subjects ($p=0.008$; $p=0.035$, respectively). T2DM patients did not show differences in Hp levels between the genotypes (all $p>0.05$).

Finally, the correlation between Hp plasma levels and clinical/laboratorial parameters in the T2DM group was investigated (**Figure 2**). Hp levels showed a

significant positive correlation with waist circumference (r=0.298, p=0.002), BMI (r=0.317, p=0.001), IL-6 (r=0.255, p=0.012), and hs-CRP (r=0.382, p=0.001).

4. DISCUSSION

This study evaluated the association between Hp levels, Hp1-Hp2 polymorphism, and clinical/laboratorial parameters in T2DM patients. The data demonstrated that T2DM is associated with higher Hp levels, which are modulated by Hp gene polymorphism, obesity, inflammation, and hypertension.

Clinical and laboratorial characteristics of T2DM patients and controls showed that T2DM patients have higher waist circumference and waist-hip ratio. These findings reinforce the knowledge that not only obesity, but mainly upper body obesity, influence negatively glucose metabolism and are independent risk factors to T2DM development [33]. In addition, T2DM patients also exhibited higher IL-6 levels when compared with controls. Higher levels of proinflammatory cytokines (e.g. IL-6, IL-1β, TNF-α) have been also found in T2DM patients in other studies [34, 35, 36] showing that the etiopathogenesis of this disease is closely related with activation of inflammatory mechanisms.

The diabetic group exhibited higher Hp levels than controls. Analysis of Hp levels considering obesity status indicated that obese T2DM patients have higher Hp levels than non-obese T2DM patients and obese controls. Additionally, Hp levels in T2DM group were positively correlated with waist circumference, BMI, IL-6, and hs-CRP. Taken together these results emphasize that increased Hp levels are associated with obesity and inflammation in T2DM group. In agreement, Van Campenhout et al [20] in a case-control study with T2DM patients from Belgium found higher Hp levels in patients than controls, and positive correlation between these levels and CRP. This study indicated that higher Hp levels in diabetes mellitus are a condition associated with disturbances in iron metabolism and inflammatory status contributing for increase in oxidative stress parameters. More recently, Mohieldein et al [21] also found higher Hp levels in T2DM patients from Saudi Arabia compared to control subjects. Moreover, the increase in Hp levels was dependent to BMI: obese (BMI ≥ 30 Kg/m^2) and overweight (BMI 25-29.9 Kg/m^2) T2DM patients showed higher Hp levels than lean patients (BMI <25 Kg/m^2).

IL-6 is widely produced by subcutaneous and intra-abdominal adipose tissue. In turn, it stimulates the liver synthesis of Hp [37], and further Hp is produced by adipocytes [10]. Chiellini et al [38] demonstrated that Hp gene is upregulated in white adipose tissue of the obese db/db mice compared to their lean littermates and that pro-inflammatory cytokines are important signal for this regulation. Moreover, serum Hp levels were considered a marker of obesity in humans, and BMI and CRP independent determinants of this serum levels in females [39].

The diabetic state is associated with decreased antioxidant defenses and disturbances in iron metabolism [20]. Hp antioxidant function is impaired by the glycosylated fraction of Hb (GlyHb), because even after GlyHb-Hp complex formation, the GlyHb can continue to oxidatively modify proteins within the vessel wall increasing endothelial injury [40]. Besides, GlyHb seems to release free iron (redox-active form - Fe^{2+}) more readily than nonglycosylated Hb [41]. Iron in oxidation state 2+ is one of the most reactive pro-oxidants because it catalyses the generation of the highly reactive hydroxyl radicals that are able to initiate and propagate the lipid peroxidation, glycoxidation, and DNA damage [42].

Hypertensives T2DM patients showed higher Hp levels when compared with normotensives patients. It is known that diabetes mellitus is associated with cell-free Hb release due to endothelial injury. The cell-free Hb acts as a potent scavenger of NO (nitric oxide), through a high-speed deoxygenation reaction, inducing vasoconstriction and oxidative tissue damages [43]. NO is produced by endothelium and is a central modulator of vascular tone, inhibits platelet aggregation and leukocytes adhesion, and exerts antioxidant and anti-inflammatory effects [18]. The increase of Hp levels observed in the hypertension could be related to compensatory mechanism in order to bind the cell-free Hb, reducing the NO scavenging and maintaining the normal blood pressure. Boretti et al [44] showed in animal models (dogs and guinea) as compartmentalization of Hb and its interaction with Hp (Hp-Hb complex) could help to regulate the blood pressure. Some studies have indicated that Hp1-Hp2 polymorphism can modulate NO bioavailability, since individuals with Hp2-Hp2 genotype and preeclampsia [45] or T2DM [46] exhibited lower plasma nitrite concentrations when compared with individuals with others genotypes.

We found that Hp1-Hp1 genotype was more frequent in T2DM than controls and followed a dominant inheritance model, although Hp2 allele was more frequent in both groups. Stern et al [22] found that Hp1-Hp1 phenotype is associated with T2DM

patients (Mexicans-americans). However, some studies that have found association between Hp2-Hp2 genotype and T2DM [23-28]. Hp1-Hp1 protein has ability to bind more Hb than other phenotypes, with more efficiency in promoting the uptake of the Hp–Hb complex by the CD163 receptor, which is cleared from circulation faster than Hp2-Hp2 protein [47]. Moreover, Hp2-Hp2 phenotype is associated with increased cardiovascular risk in T2DM patients [40, 48]. Consequently, this result was not expected, but shows the genetic characteristic of Brazilian population, which is the result of European, African, and Amerindian miscegenation.

The more elevated Hp levels in controls were observed in Hp1-Hp1 when compared to Hp2 carriers, in accordance with Langlois and Delanghe [9] and Kasvosve et al [49]. However, in T2DM it seems that Hp2-Hp2 genotype contributes for elevated levels of the Hp protein, similarly to Hp1-Hp1 carriers. Since the Hp expressed by Hp2 allele presents lower ability to bind the Hb and the Hp-Hb complex to the CD163 receptor, this condition could compromise the Hp functions in T2DM. Therefore, these results suggest that, although the T2DM presents higher Hp levels (possibly due to inflammatory status), most of their function is ineffective.

Some limitations of this work and contradictory results compared to other studies are known, as small sample size, which is justified by the strict selection criteria for patients and controls, different techniques used for phenotype/genotype Hp individuals, or different genetic background of the populations studied. However, its data present new and clinically important information about the Hp profile in T2DM and other studies should be conducted in order to expand the comprehension about Hp role and T2DM in other populations.

5. CONCLUSION

Considering the epidemic number of T2DM patients in the word, including Brazil, improved knowledge about inflammatory marks associated with diabetes mellitus can contribute to ameliorate the clinical follow-up.

Our results suggest that higher Hp levels are associated with T2DM and are influenced by BMI, inflammatory status and hypertension. Higher frequency of Hp1 homozygous was observed in T2DM patients, however Hp2 allele contributes with higher protein levels, which could compromise the function of Hp in this group.

DECLARATION OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGMENTS

The authors thank FAPEMIG, CAPES, and CNPq/Brazil for financial support. VCS and KBG are grateful to CNPq Research Fellowship.

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Table 1: Clinical and laboratorial characteristics of T2DM patients and controls.

Parameters	T2DM (n=102)	Control (n=62)	p
Gender			
Male (%)	18.6	19.4	0.908
Female (%)	81.4	80.6	
Age (years)	56 (12)	53 (18)	0.358
BMI			
< 30 Kg/m^2	25.5 (5.2)	24.8 (4.5)	0.204
≥ 30 Kg/m^2	37.8 (9.2)	39.3 (11.0)	0.850
Waist circumference (cm)	108.1 ± 16.8	96.9 ± 16.2	<0.0001*
Waist-hip ratio	1.0 (0.1)	0.9 (0.1)	<0.0001*
T2DM onset [a]			
< 10 years (%)	33.3	Not applicable	-
≥ 10 years (%)	63.7		-
Fasting glucose (mg/dL)	126.5 (79.0)	85.3 (10.3)	<0.0001*
Post-prandial glucose (mg/dL)	203.0 (118.0)	-	-
HbA1c (%)	8.9 ± 2.0	-	-
hs-CRP (mg/L)	2.8 (4.4)	2.7 (2.6)	0.512
TNF-α (fg/mL)	88.4 (42.3)	94.8 (63.4)	0.287
IL-6 (fg/mL)	805.1 (992.7)	506.5 (510.1)	0.001*
IL-10 (fg/mL)	187.2 (63.8)	184.6 (57.1)	0.520

T2DM (Type 2 diabetes mellitus), BMI (Body mass index), HbA1c (Glycated hemoglobin), hs-CRP (High sensitivity C reactive protein), TNF-α (Tumor necrosis factor alpha), IL (Interleukin)
[a] Missing data for three patients
- Not evaluated for control group
*p<0.05 was considered statistically significant - Student's t-test (parametric variables) or Mann-Whitney test (non-parametric variables)

Table 2: Haptoglobin levels in T2DM and control groups according to obesity status.

Obesity status		Haptoglobin (g/L)		
		T2DM	Control	p
BMI <30 Kg/m^2		1.00 ± 0.41	0.96 ± 0.41	0.634
BMI ≥30 Kg/m^2		1.27 ± 0.47	0.95 ± 0.40	0.009*
	p'	0.003*	0.959	

T2DM (Type 2 diabetes mellitus), BMI (Body mass index)
*p<0.05 was considered statistically significant (comparison between case and control groups)
*p'<0.05 was considered statistically significant (comparison inside case or control groups)
Student's t-test

Table 3: Analysis of haptoglobin gene polymorphism in different inheritance models in T2DM and control individuals.

Additive model	T2DM	Control	OR	95% CI	p
Hp1-Hp1	29 (28.4%)[a]	7 (11.3%)[b]	3.038	1.127 - 8.192	
Hp2-Hp1	43 (42.2%)	33 (53.2%)	0.956	0.468 - 1.949	0.036*
Hp2-Hp2	30 (29.4%)	22 (35.5%)	Reference		
Recessive model					
Hp2-Hp2	30 (29.4%)	22 (35.5%)	0.758	0.366 – 1.567	0.418
Hp1-Hp1 + Hp2-Hp1	72 (70.6%)	40 (64.5%)			
Dominant model					
Hp2-Hp2 + Hp2-Hp1	73 (71.6%)	55 (88.7%)	0.320	0.118 - 0.839	0.010*
Hp1-Hp1	29 (28.4%)	7 (11.3%)			

T2DM (Type 2 diabetes mellitus)
*p<0.05 was considered statistically significant
[a] more frequent; [b] less frequent - Qui-square (x^2) test with residual analysis

Table 4: Haptoglobin plasma levels according to their genotypes.

Additive model	All individuals	p	T2DM	p	Control	p
Hp1-Hp1[A]	1.22 (0.54)		1.20 ± 0.35		1.24 ± 0.35	
Hp2-Hp1[B]	0.99 (0.69)	0.001*	1.24 ± 0.54	0.076	1.03 ± 0.42	0.008*
Hp2-Hp2[C]	0.80 (0.67)		1.00 ± 0.40		0.77 ± 0.30	
Dominant model						
Hp1-Hp1	1.22 (0.54)	0.020*	1.20 ± 0.35	0.541	1.24 ± 0.35	0.050*
Hp2-Hp1 + Hp2-Hp2	0.96 (0.67)		1.14 ± 0.50		0.92 ± 0.40	

T2DM (Type 2 diabetes mellitus)
*p<0.05 was considered statistically significant

Additive model (All individuals) - Mann-Whitney test with Bonferroni's Correction (p<0.017 was considered statistically significant):
A x B: p=0.288
A x C: p<0.0001*
B x C: p=0.007*

Additive model (Control) - ANOVA with LSD Post-Hoc test
A x B: p=0.182
A x C: p=0.006*
B x C: p=0.016*

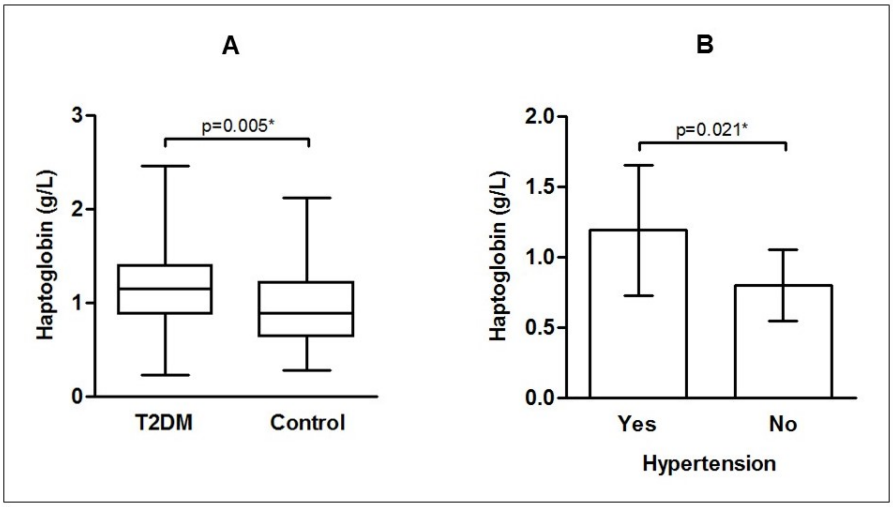

Figure 1: Haptoglobin plasma levels in case and control groups (A), and only in T2DM group considering presence/absence of hypertension (B). (A) Mann-Whitney test; (B) Student's t test; *p<0.05 was considered statistically significant.

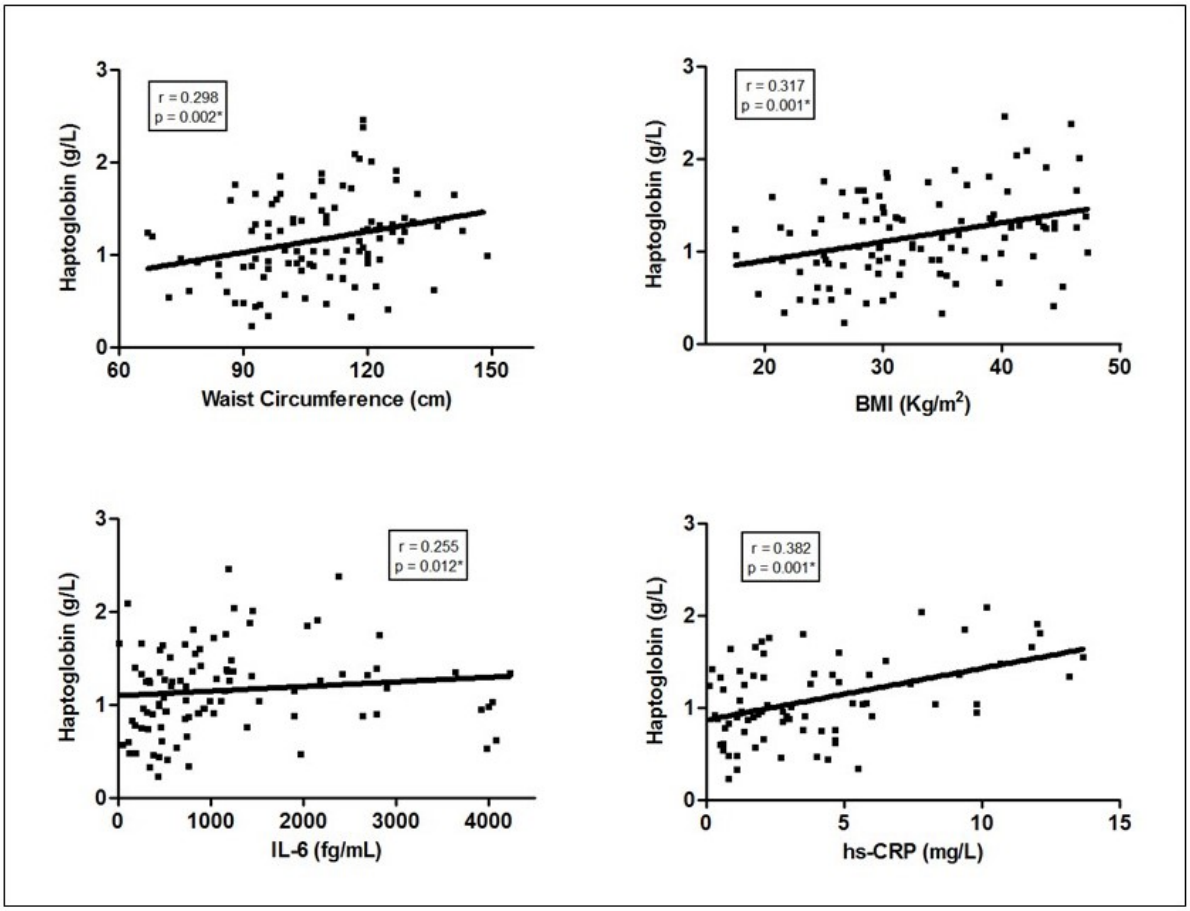

Figure 2: Correlation coefficients between haptoglobin levels, clinical, and laboratorial data in T2DM patients. BMI (body mass index), IL (interleukin), hs-CRP (high-sensitivity C-reactive protein). Spearman's correlation test; *p<0.05 was considered statistically significant.

Capítulo 6:

Microparticles in patients with type 2 diabetes mellitus and nephropathy are associated with gender and vitamin D levels

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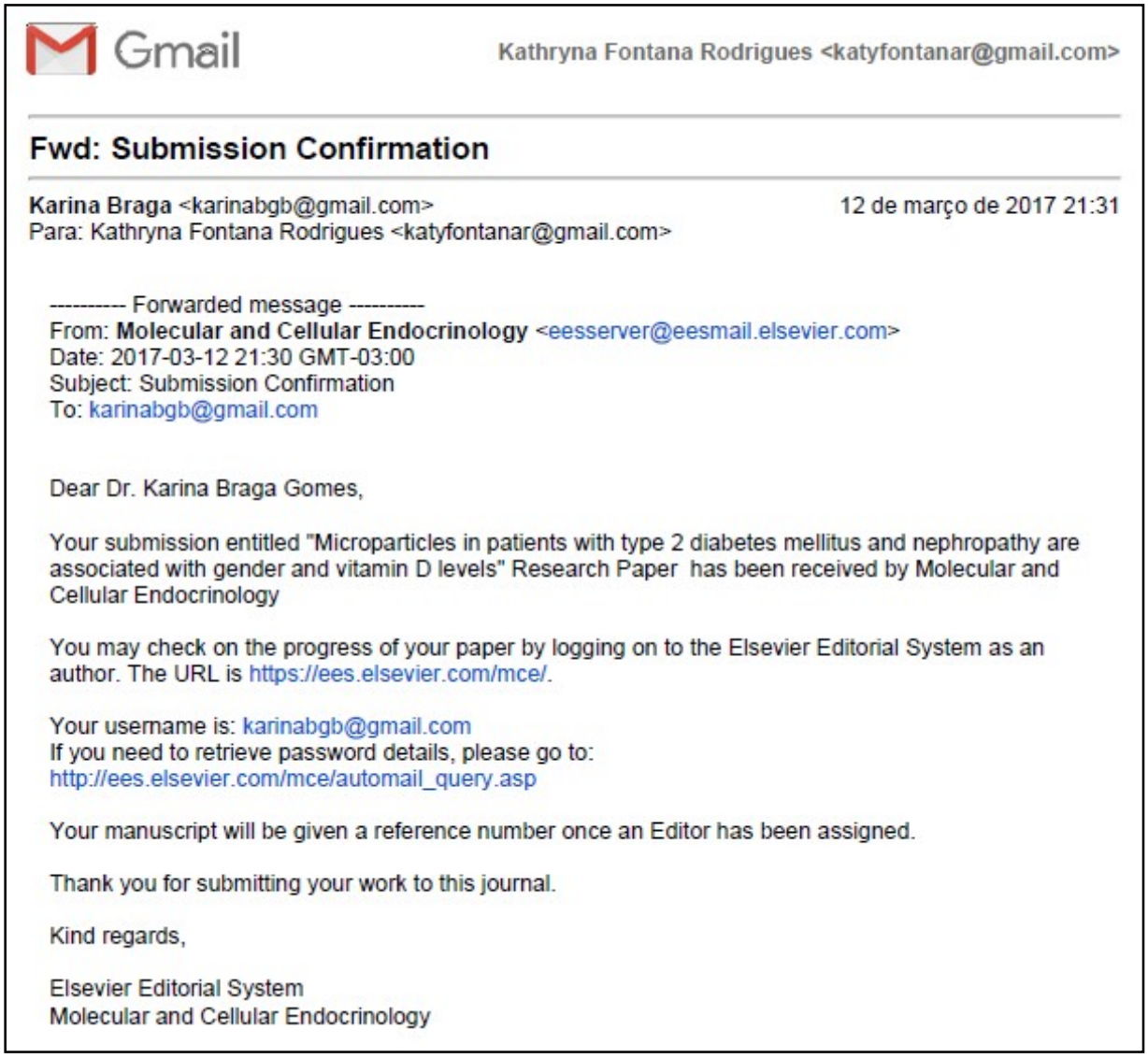

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Microparticles in patients with type 2 diabetes mellitus and nephropathy are associated with gender and vitamin D levels

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is associated with chronic low-grade inflammation. Microparticles (MPs) are extracellular microvesicles released during the apoptosis and cellular activation, with pro-coagulant and -inflammatory activities involved in endothelial dysfunction in T2DM patients with nephropathy (DN). This study aimed to evaluate MPs profile in T2DM patients and correlate it with biochemical parameters. 39 T2DM with DN and 30 non-diabetic control were included. MPs from platelets (PMPs), leukocytes (LMPs), endothelial cells (EMPs), and that express tissue factor (TFMPs) were measured in plasma by flow cytometry. We observed higher PMPs, LMPs, EMPs, and TFMPs (all $p<0.0001$) levels in T2DM than controls. For T2DM group, MPs levels were influenced by gender, but not by obesity nor T2DM onset. Fasting glucose and 25-hydroxyvitamin D levels showed correlation with circulating MPs levels. These results suggest that T2DM is associated with higher MPs levels, which exhibited correlation with metabolic and inflammatory alterations in T2DM.

Keywords: type 2 diabetes mellitus, microparticles, inflammation, obesity, nephropathy.

1. INTRODUCTION

Diabetes mellitus is a complex and chronic illness whose prevalence has reached epidemic proportions in worldwide, becoming the largest global health emergencie of the 21st century [International Diabetes Federation, 2015]. According to the International Diabetes Federation (IDF), there were 415 million of diabetic patients worldwide in 2015 (a prevalence of 8.8%) and this number will reach 642 million (a prevalence of 10.4%) in 2040. About 87-91% of the patients have type 2 diabetes mellitus (T2DM) [International Diabetes Federation, 2015].

The pathogenesis of T2DM and its macro- and microvascular complications have been associated with a subclinical chronic inflammation, metabolic stress, and activation of the immune system [Shoelson et al., 2006; Kotas and Medzhitov, 2015; Wensveen et al., 2015]. Adipose tissue homeostasis plays an important role in the development of insulin resistance and T2DM. The increase of visceral adipose tissue mass (hypertrophy and hyperplasia) is associated with a shift in adipokines/cytokines production, immune cell activation (M1-phenotype polarization in the adipose tissue macrophages and cell Th1-type response), and activation of intracellular inflammatory signaling pathways such as IKKβ/NF-κB and JNK [Cruz et al., 2013; Wensveen et al., 2015].

Microparticles (MPs) are extracellular microvesicles of 0.1 to 1 μm diameter that are released during the apoptosis and activation of many cell types, such as platelets, leukocytes, erythrocytes, endothelial cells, vascular smooth muscle cells, under influence of cytokines, thrombin, endotoxins or physical stimuli, as well as shear stress or hypoxia [Normura et al., 2008]. Once released, MPs carries cell surface proteins (receptors, transporters, antigens) and cell components (cyto/nuclear proteins - annexins, cytokines, growth factors, angiogenic and heat shock proteins; DNA; mRNA; and miRNA) of their cells of origin [Müller, 2012; Burger et al., 2013]. MPs represent a potentially important method of cell-cell communication and they have been associated with cellular activation and dysfunction [Burger et al., 2013; Normura, 2016; Santilli et al., 2016].

Although the precise molecular mechanism of the MPs formation is unknown, cytoskeletal reorganization and alterations in phospholipid symmetry are essential to their formation [Burger et al., 2013]. Briefly, after cellular activation or apoptotic stimulus occurs, the membrane remodeling with the formation of blebs within it. This

step requires an increase in intracellular calcium concentration, changes in membrane lipid asymmetry, and cytoskeleton protein reorganization. The loss of plasma membrane asymmetry leads to exposure of phosphatidylserine on the outer leaflet and the release of MPs from the cells [Boulanger et al., 2006; Morel et al., 2011; Normura, 2016].

The knowledge about MPs clearance is still unclear. They circulate in the plasma of healthy individuals, but are found in others body fluids, including saliva, urine, bile, tears, synovial and cerebral spinal fluids. It has been proposed that clearance of MPs may occur via direct phagocytosis, after opsonization, by endocytosis or by phospholipase-mediated degradation [Foley and Conway, 2016].

Higher levels of specific MPs subsets have been associated with several disorders, such as inflammatory and prothrombotic diseases [Mooberry et al., 2016], thromboembolic events [Campello et al., 2016], preeclampsia [Marques et al., 2013], polycystic ovary syndrome [Carvalho et al., 2017], autoimmune diseases [Burbano et al., 2015], cancer [Goubran et al., 2015], infectious disease [Campos et al., 2010] and T2DM [Li et al., 2016].

Some studies have demonstrated that MPs are independent predictors for diabetes-related vascular complications [Diamant et al., 2002; Normura et al., 2004; Berezin et al., 2015]. MPs present pro-coagulant activity by binding to cells via specific adhesion receptors, thereby stimulating both tissue factor (TF) and cytokines production, which promote coagulation, inflammation and endothelial dysfunction [Santilli et al., 2016].

In spite of the existence of some reports examining the association of MP levels and T2DM [Li et al., 2016], the results are still controversial and the relationship with clinical variables and diabetic nephropathy (DN) was poorly explored. Therefore, in this study we investigated the associations between MPs levels (platelet-, leukocyte-, endothelial cell-derived MPs and MPs that express TF) with T2DM and clinical and biochemical parameters in a group of Brazilian patients.

2. MATERIAL AND METHODS

2.1 Subjects

Sixty-nine subjects were selected for this cross-sectional case-control study. The case group included 39 patients with clinical and laboratorial diagnosis of T2DM, according to the criteria established by the American Diabetes Association (ADA) [American Diabetes Association, 2012], while 30 gender- and body mass index (BMI)-matched non-diabetic individuals were classified as the control group. T2DM patients were recruited at the Clinic of Endocrinology (Santa Casa Hospital, Minas Gerais, Brazil) from June 2012 to September 2013. Control group subjects were recruited from the local community during the same period. The exclusion criteria included the following parameters: age over 70 years**,** pregnancy, cancer, autoimmune diseases, and recent history of cardiovascular disease (e.g. heart attack, stroke, and thrombosis in the last five years), and current or recent infections and/or inflammatory processes. Interviews and medical records were utilized to obtain clinical and laboratorial data (e.g. fasting glucose, post-prandial glucose, serum creatinine, and blood urea levels) for all T2DM patients. Controls presented normal fasting glucose levels (60 - 99 mg/dL) and they didn't use hypoglycemic drugs.

All the T2DM patients exhibited diabetic kidney disease or DN that was defined, according to ADA criteria, as albumin excretion rate >30mg/24h at least 2 of 3 specimens collections within a 3- to 6-month, and without coexisting renal diseases from causes other than diabetes [American Diabetes Association, 2012]. In agreement with the National Kidney Foundation, the chronic kidney disease (CKD) classification was based on glomerular filtration ratio estimated (eGFR) by Cockcroft-Gault equation: stage 1 (eGFR ≥90 mL/min/1.73^2), stage 2 (eGFR 60-89 mL/min/1.73^2), stage 3 (eGFR 30-59 mL/min/1.73^2), stage 4 (eGFR 15-29 mL/min/1.73^2), and stage 5 (eGFR <15 mL/min/1.73^2 or dialysis) [Cockcroft and Gault, 1976; Levey et al., 2003].

This study was approved by the ethics committees of Federal University of Minas Gerais (Minas Gerais, Brazil) - ETIC 0062.0.203.000-11 - and of Santa Casa Hospital (Minas Gerais, Brazil) - 059/2011 - conforms to the ethical guidelines of the Declaration of Helsinki. All participants provided written informed consent prior to entering the study.

2.2 Blood sampling

Venous blood samples were collected from each participant in heparin sodium and anticoagulant-free tubes, allowed to clot for 30 min, and centrifuged at 1,100 x g for 20 min at 25ºC. The supernatant fractions obtained (plasma and serum) were aliquoted in microtubes and stored at -80ºC until biochemical analysis.

2.3 Biochemical analysis

The fasting glucose levels in the control group were measured in serum samples after eight hours fasting. The tests were performed using the enzyme-colorimetric method, BTR 811 spectrophotometer (Biotron, Brazil), and Glicose-PP kit (Gold Analisa, Brazil), following the manufacturer's instructions.

The high-sensitivity C reactive protein levels (hs-CRP) were measured in serum samples using the immunoturbidimetric method, hsCRP VITROS Chemistry Products (Ortho Clinical Diagnostics, US) following the manufacturer's instructions, and System Vitros Chemistry 5.1 FS (Ortho Clinical Diagnostics, US). All samples were assayed at the same time.

The 25-hydroxyvitamin D [25(OH)D] levels were measured in heparin sodium plasma samples using reversed-phase high performance liquid chromatography (HPLC) with UV detection (Thermo Finnigan Surveyor, US), according to Hymoller and Jensen (2011) modified. The 25(OH)D2 (≥98% pure) and 25(OH)D3 (≥99% pure) were used as standards and 1,α-hydroxyvitamin D3 (1αOHD3 - ≥97% pure) as an internal standard (all standards were Sigma Aldrich®). The samples were protected from the light until assay and were assayed at the same time.

2.4 Microparticles flow cytometry assay

The purification of the MPs was performed according to Campos et al. (2010). The plasma samples (platelet-poor plasma in heparin sodium) were centrifuged at 14,000 x g for 5 minutes at room temperature in order to obtain platelet-free plasma. The supernatant was aspirated and diluted 1:3 in citrated phosphate buffered saline (PBS) containing heparin. Then, the samples were centrifuged at 14,000 x g for 90 minutes at 15ºC, the supernatant was removed and the resultant MPs pellet was resuspended in 1X annexin V binding buffer (BD Pharmigen™, US).

MPs isolated were gated based on their forward (FSC) and side (SSC) scatter distribution when compared to the distribution of synthetic 0.7-0.9 µm SPHERO™ Amino Fluorescent Particles (Spherotech Inc., US) (**Figure 1A**). The

presence of phosphatidylserine residues on the MPs surface was assessed for their positive staining with monoclonal antibodies against annexin V labeled with fluorescein isothiocyanate (FITC) (BD Pharmingen™, US). Cell-specific monoclonal antibodies were used to identify the source of the MPs: CD41-PerCP-Cy5.5 (eBioscience, US) for platelet-derived MPs (PMPs), CD45-APC (BD Pharmingen™, US) for leukocyte-derived MPs (LMPs), CD51/61-PE (BD Pharmingen™, US) for endothelial cell-derived MPs (EMPs), and CD142-PE (eBioscience, US) for MPs that express TF (TFMPs) (**Figure 1C - T2DM, and 1D - control groups**). The specific monoclonal antibody was corrected by isotype-matched control antibodies mouse - FITC, PerCP, APC, and PE (**Figure 1B**) to eliminate nonspecific labeling.

The manufacturer's instructions about antibodies concentrations were strictly followed. The samples were analyzed in a LSR Fortessa Cytometer (BD Biosciences, US) using FACSDiva™ 6.2 software (BD Biosciences, US) for data acquisition, following by analysis performed on the FlowJo® software (Tree Star).

To determine the absolute number of MPs per microliter (MPs/μL), the cytometer was set to operate with a high flow rate for 60 seconds for each sample. The MPs/μL was calculated as described by Campos et al. (2010): MPs/μL = (N x 400) / (60 x 100), in which N is equal to the number of events, 400 is the total volume of sample into the tube prior to analysis, 60 is the volume of the analyzed sample, and 100 is the original volume of MPs suspension. This formula was validated using Trucount tubes (BD Pharmingen™, US) in some samples randomly selected, according to the manufacturer's instructions.

2.5 Statistical analysis

Statistical analysis was performed with the Statistical Package of the Social Sciences (SPSS) version 17.0. The data were tested for normality by Shapiro-Wilk test. Normal data were presented as “mean ± standard deviation (SD)”, non-normal data as “median (interquartile range - IQR)”, and categorical data as “frequency”.

We performed Student’s t test for normal variables, Kruskal-Wallis or Mann-Whitney test for non-normal, and qui-square (x^2) test for categorical variable. Correlation analysis were assessed using Spearman’s correlation test. For all analyses a p-value <0.05 was considered statistically significant.

3. RESULTS

Clinical and laboratorial data of both case (T2DM) and control groups are presenting on **Table 1**. The groups were matched by gender and obesity status [body mass index (BMI) <30Kg/m^2 - non-obese, BMI ≥30Kg/m^2 - obese] ($p>0.05$ for all). T2DM patients displayed higher waist circumference ($p=0.012$), waist-hip ratio ($p<0.0001$), fasting glucose ($p<0.0001$), and lower 25(OH)D levels ($p<0.0001$) when compared to non-diabetic controls.

We observed higher total levels of MPs in T2DM [153.40 (142.15) MPs/μL] than in control group [85.33 (76.08) MPs/μL; $p<0.0001$] **(Figure 2A)**. Moreover, higher levels of circulating PMPs [T2DM: 167.53 (206.73) MPs/μL; control 113.83 (97.05) MPs/μL; $p<0.0001$], LMPs [T2DM: 119.13 (86.27) MPs/μL; control 53.80 (44.42) MPs/μL; $p<0.0001$], EMPs [T2DM: 157.53 (131.53) MPs/μL; control 86.50 (71.65) MPs/μL; $p<0.0001$], and TFMPs [T2DM: 169.93 (213.47) MPs/μL; control 99.60 (87.53) MPs/μL; $p<0.0001$] were observed in T2DM patients when compared with control subjects **(Figure 2B, 2C, 2D, and 2E)**.

Aiming to evaluate whether obesity status could influence MPs levels, we compared the PMPs, LMPs, EMPs, and TFMPs levels between obese and non-obese T2DM patients. Although all the MPs levels were higher in obese group, no differences were found ($p>0.05$ for all; **Table 2**). Similarly, T2DM onset (<10 years and ≥10 years) also showed no influence of these MPs levels ($p>0.05$ for all, data not shown).

We performed an analysis considering MPs levels and the DN stage. Interestingly, all the MPs levels showed an increase according the progression of diabetic kidney disease, although no significant difference was observed ($p>0.05$ for all; **Table 3**).

In order to evaluate whether gender influenced circulating MPs levels, we compared these levels between male and female individuals with T2DM and controls. In the T2DM group, LMPs ($p=0.028$), EMPs ($p=0.005$), and TFMPs ($p=0.039$) showed higher levels in women [122.67 (109.43) MPs/μL; 174.67 (131.63) MPs/μL; 198.20 (249.03) MPs/μL, respectively] when compared with men [79.67 (57.50) MPs/μL; 102.20 (68.42) MPs/μL; 132.90 (75.13) MPs/μL, respectively] (**Figure 3A**). No differences were found in the control group ($p>0.05$ for all; **Figure 3B**).

Finally, we investigated the correlation between MPs levels and laboratorial data (**Figure 4**). Considering all the subjects, fasting glucose showed a significant positive correlation with PMPs (r=0.286, p=0.017), LMPs (r=0.549, p<0.0001), EMPs (r=0.462, p<0.0001), and TFMPs (r=0.343, p=0.004) levels. Besides, 25(OH)D levels exhibited a significant negative correlation with LMPs (r=-0.423, p=0.001) and EMPs (r=-0.415, p=0.001). No other significant correlation was observed between MPs levels and laboratorial data.

4. DISCUSSION

This study evaluated the association between MPs levels (PMPs, LMPs, EMPs, and TFMPs) and clinical and biochemical parameters in T2DM patients. The data demonstrated that T2DM is associated with higher MPs levels, which are influenced by gender, but not by obesity status nor T2DM onset. Also, fasting glucose and 25(OH)D levels showed correlation with circulating MPs levels.

Clinical and laboratorial characteristics of the groups showed that T2DM patients have higher waist circumference and waist-hip ratio than control subjects. These findings are in agreement with the knowledge that not only obesity, but mainly upper body obesity, influences negatively glucose metabolism and is an independent risk factor to T2DM development [American Diabetes Association, 2017]. In addition, T2DM patients exhibited lower 25(OH)D levels when compared with non-diabetic controls. Vitamin D exhibits anti-inflammatory effects and lower 25(OH)D levels have been also found in T2DM patients in others studies [Bajaj et al., 2014; Reddy et al., 2015; Zoppini et al., 2015]. Clemente-Postigo et al. (2015) in a cross-sectional study reported a negative correlation between 25(OH)D levels and insulin resistance (acessed by HOMA-IR) and glucose levels. Conversely, a clinical trial controlled by placebo demonstrated that vitamin D3 replacement improves insulin sensitivity (based on HOMA-IR) and HbA1c levels in T2DM patients (Jehle et al., 2014).

T2DM group showed higher total MPs levels when compared with controls. A recent systematic review and meta-analysis conducted by Li et al. (2016) indicated that circulating total MPs, PMPs, EMPs, and monocyte-derived MPs (MMPs), but not LMPs, present higher levels in T2DM patients when compared with non-diabetic controls. In agreement with our data, Diamant et al. (2002) and Feng et al. (2010)

also found higher TFMPs and LMPs levels, respectively, in T2DM patients than healthy subjects.

MPs are heterogeneous population of small vesicles with variety biological activities, providing a bridge between coagulation and inflammation [Foley and Conway, 2016]. MPs can be associated with endothelial dysfunction and vasculopathies observed in T2DM, since MPs influence cellular processes involved in the pathogenesis of these complications.

MPs have ability to promote coagulation mainly by externalization of anionic phospholipids (phosphatidylserine) and the subsequent assembly of coagulation complexes (TF:FVIIa; FVIIIa:FIXa; FVa:FXa) and thrombin formation [Owens and Mackman, 2011]. Besides, certain populations of MPs, mainly PMPs, have the capacity to capture and incorporate TF in order to promote coagulation [Foley and Conway, 2016]. Some studies have suggested that EMPs, MMPs, and lymphocyte-derived MPs are capable of promoting oxidative stress in the endothelium by increasing production of superoxide anion (O_2^-), inhibiting endothelial nitric oxide synthase (eNOS) function, thus decreasing nitric oxide (NO) production [Mostefai et al., 2008; Burger et al., 2012, Burger et al., 2013; Santilli et al., 2016]. Furthermore, MPs appear to represent both a consequence of, and contributor to, inflammation. Pro-inflammatory stimuli increase MPs release, which may directly promote an inflammatory response, including the production of cytokines and chemokines, and the recruitment of inflammatory cells [Chen and Nuñez, 2010; Burger et al., 2013]. PMPs and EMPs increase expression of cell adhesion molecules, and allow the cellular interactions with endothelium and the recruitment of inflammatory cells [Buesing et al., 2011; Burger et al., 2013].

We observed that obesity status did not influence MPs levels. Similarly, Zhang et al. (2014) found higher PMPs levels in T2DM patients when compared non-diabetic individuals, but independently of obesity status. In other words, although obesity is associated with chronic systemic inflammation [Wensveen et al., 2015], in diabetic patients there are indications that obesity per se is not enough to change the circulating MPs levels.

T2DM onset (over or less 10 years) showed no influence in MPs levels. We hypothesized that, although T2DM patients with a longer disease onset are exposed for more time to the deleterious chronic inflammation effects, the MPs levels are not

influenced by this time, since cellular activation and MPs release occur independently of the clinical disease onset.

Diabetic kidney disease is the leading cause of end-stage renal disease increasing the risk of cardiovascular diseases [American Diabetes Association, 2017]. Although the MPs levels showed no significant difference according to the DN progression, the results show clearly the increase in the median levels for all MPs subtypes in the later stage of DN. Probably, the limited sample size compromised the significance between the groups. Considering that CKD patients exhibit significant platelet activation and endothelial dysfunction (early signal for renal function deterioration), Almquist et al. (2016) found that T2DM patients with CKD 3-4 stages had elevated PMPs and EMPs levels when compared with diabetic patients with normal eGFR. Contrary, Lu et al. (2015) found higher PMPs and EMPs levels in stage 1-2 CKD patients than those in stage 3-4. Hence, the variation in the MPs levels according to DN stage is controversial. However, taken together, the studies show an increase of MPs associated to inflammation and hemostasis, once initiated the DN.

LMPs, EMPs, and TFMPs showed higher levels in T2DM women than T2DM men, and no differences were found in control group. These results suggest that diabetic women have an increase in pro-coagulant and -inflammatory MPs release, caused by both T2DM and decline in endogenous estrogen levels, since the median age of T2DM group showed several women around menopause period. It is known that estrogen modulates antithrombotic characteristics on the vascular endothelium and, during the menopause, it is observed estrogen decline, low-grade inflammation, and impaired endothelial function, contributing to atherosclerotic risk [Jayachandran et al., 2009; Figueroa-Veja et al., 2015].

Positive correlation between fasting glucose and PMPs, LMPs, EMPs, and TFMPs levels shows the importance of glycemic control as a strategy for decrease inflammatory and thrombotic risk in diabetes. Cheng et al. (2013) demonstrated that T2DM patients after bariatric surgery and dramatic improvement of glycemic control, exhibited decrease in EMPs, PMPs, and TFMPs levels.

As discuss above, lower 25(OH)D levels have been associated with T2DM. Vitamin D seems to play an important role in the insulin secretion/action [Maestro et al., 2002], which inhibits the NF-κB pathway activation and stimulates regulatory T cells proliferation [Giulietti et al., 2007]. Thus, negative correlations between LMPs,

EMPs, and 25(OH)D levels are in accordance with anti-inflammatory role of vitamin D.

The limitation of our study includes the small sample size, but it was supplanted by the homogeneity of the group, within all patients presented DN. Therefore, the results should be validated in other populations in order to include the MPs as potential biomarkers for T2DM diagnostic and DN status.

5. CONCLUSION

MPs were originally believed to act as inert cellular debris, but now they are emerging as important diseases biomarkers associated with endothelial injury, inflammation, and thrombotic states. Considering the T2DM epidemiological importance and its pathogenesis complex, studies are needed to improve the knowledge about inflammatory markers associated and how they can contribute to ameliorate the clinical follow-up.

Our results suggest that T2DM is associated with higher PMPs, EMPs, LMPs, and TFMPs levels, which are influenced by gender in this group. Also, glycemic control and 25(OH)D levels showed correlation with circulating MPs levels. In conclusion, circulating MPs levels should be evaluated in large-scale studies in order to confirm the potential role as T2DM biomarker.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank FAPEMIG, CAPES and CNPq/Brazil for financial support. KBG is grateful to CNPq Research Fellowship.

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Table 1: Characterization of type 2 diabetes mellitus and control groups.

Variables	T2DM (n=39)	Control (n=30)	p
Gender			
Male (%)	6 (15.4)	9 (30.0)	0.145
Female (%)	33 (84.6)	21 (70.0)	
Age (years)	61 (9)	52 (10)	<0.0001*
Obesity status			
BMI < 30 Kg/m^2	25.6 ± 3.2	24.7 ± 2.7	0.198
BMI ≥ 30 Kg/m^2	32.9 ± 2.2	31.6 ± 0.4	0.398
Waist circumference (cm)	97.9 ± 12.3	90.7 ± 10.7	0.012*
Waist-hip ratio	0.96 ± 0.08	0.88 ± 0.08	<0.0001*
T2DM onset			
< 10 years (%)	20.5	NA	-
≥ 10 years (%)	79.5	NA	-
Fasting glucose (mg/dL)	165.4 ± 66.2	85.9 ± 7.1	<0.0001*
Post-prandial glucose (mg/dL)	228.7 ± 82.4	-	-
HbA1c (%)	8.9 (2.8)	-	-
Serum creatinine (mg/dL)	0.9 (0.6)	-	-
Blood urea (mg/dL)	38 (19)	-	-
eGFR (mL/min/1.73^2)			
Stage 1 (n= 0)	0	-	-
Stage 2 (n= 11)	71.9 (15.9)	-	-
Stage 3 (n= 22)	49.0 (15.7)	-	-
Stage 4 (n= 6)	18.4 (8.9)	-	-
hs-CRP (mg/L)	2.1 (4.0)	2.2 (2.9)	0.828
25(OH)D (ng/mL)	16.9 (15.6)	30.7 (15.5)	<0.0001*

T2DM (Type 2 diabetes mellitus), BMI (Body mass index), HbA1c (Glycated hemoglobin), eGFR (Estimated glomerular filtration rate), hs-CRP (High sensitivity C reactive protein), 25(OH)D (25-hydroxyvitamin D), NA (Not Applicable)
(-) Variable not evaluated in control group
Categorical variable: Qui square (x^2) test; Normal variables: Student's t-test; Non-normal variables: Mann-Whitney test
*p<0.05 was considered statistically significant

Table 2: Microparticles levels according obesity status in type 2 diabetes mellitus.

Microparticles (MPs/µL)	Obesity status		p
	BMI < 30 Kg/m^2 (n=29)	BMI ≥ 30 Kg/m^2 (n=10)	
PMPs	158.47 (184.13)	292.67 (277.35)	0.222
LMPs	116.20 (97.47)	130.50 (112.12)	0.479
EMPs	151.67 (129.73)	174.30 (160.48)	0.359
TFMPs	165.53 (181.50)	291.60 (289.03)	0.198

MPs (Microparticles), BMI (Body mass index), PMPs (Platelets-derived MPs), LMPs (Leukocytes-derived MPs), EMPs (Endothelial cell-derived MPs), TFMPs (MPs that express tissue factor)
*p<0.05 was considered statistically significant; Mann-Whitney test

Table 3: Microparticles levels according diabetic nephropathy stage.

Microparticles (MPs/µL)	Diabetic nephropathy			p
	Stage 2 (n=11)	Stage 3 (n=22)	Stage 4 (n=6)	
PMPs	135.80 (236.07)	193.27 (189.59)	199.60 (303.67)	0.503
LMPs	97.87 (37.20)	120.90 (121.12)	130.47 (146.82)	0.626
EMPs	141.73 (47.53)	162.80 (148.32)	222.07 (243.07)	0.475
TFMPs	135.27 (218.93)	193.67 (202.63)	205.13 (312.57)	0.427

MPs (Microparticles), PMPs (Platelets-derived MPs), LMPs (Leukocytes-derived MPs), EMPs (Endothelial cell-derived MPs), TFMPs (MPs that express tissue factor)
*p<0.05 was considered statistically significant; Kruskal-Wallis test

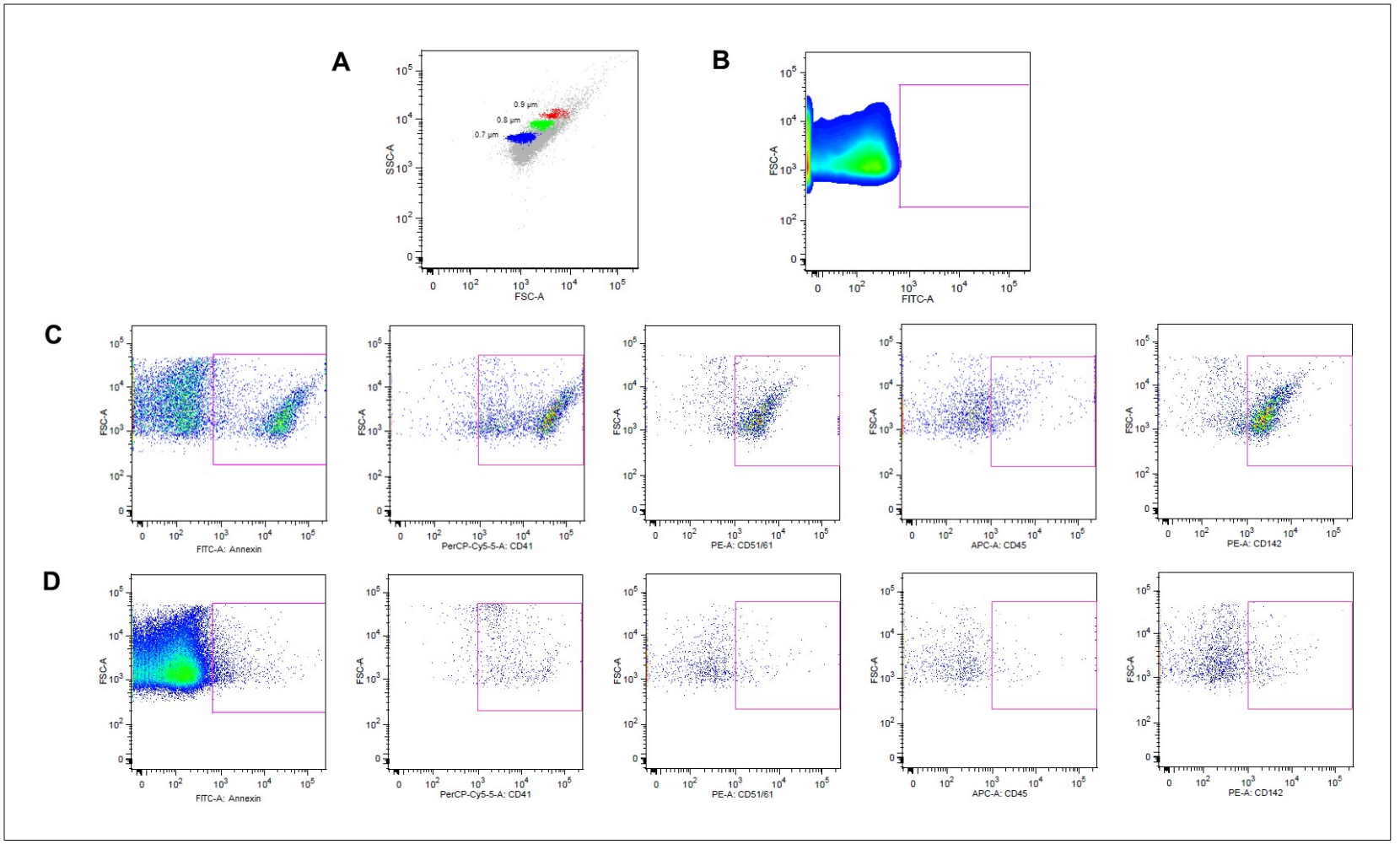

Figure 1: Microparticles measurements by flow cytometry. Microparticles (MPs) isolated were gated based on their forward (FSC) and side (SSC) scatter distribution as compared to 0.7-0.9 µm synthetic MPs (A). Mouse IgG FITC-conjugated isotype control (B). Events present on the gate were accessed for their annexin V positive staining using FITC-conjugated monoclonal antibodies and subsequent identification of the source of MPs, using cell-specific monoclonal antibodies (CD41-PerCP-Cy5.5 for platelets-, CD51/61-PE for endothelial-, CD45-APC for leukocytes-derived MPs, and CD142-PE for MPs expressing tissue factor, respectively) in type 2 diabetes mellitus (C) and control groups (D).

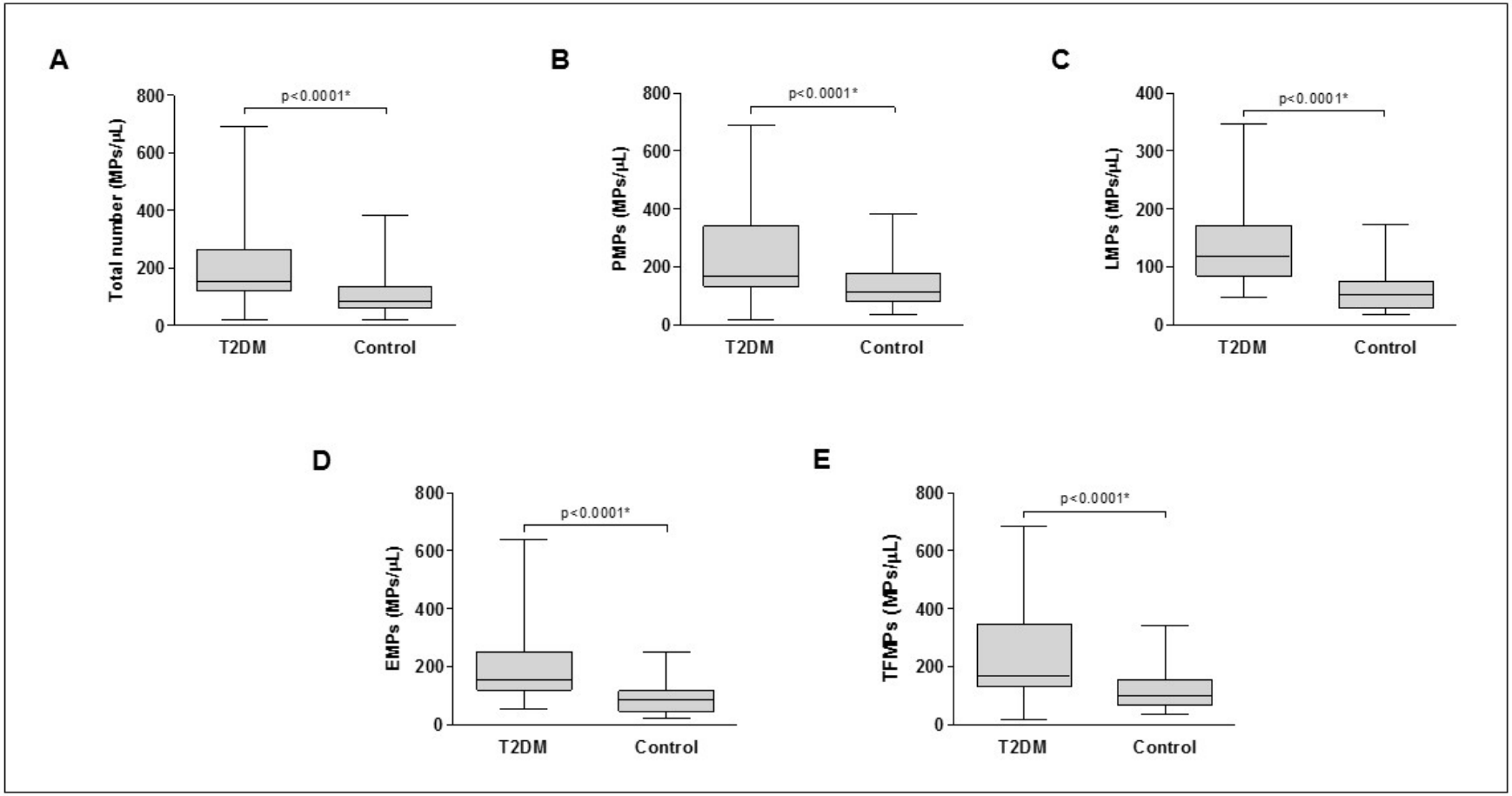

Figure 2: Distribution of microparticles levels (MPs/µL) between type 2 diabetes mellitus and control groups. Total number of MPs (represents the sum of: PMPs, LMPs, EMPs, and TFMPs) (A). Platelets-derived MPs (PMPs - annexin V and CD41 positive) (B). Leukocytes-derived MPs (LMPs – annexin V and CD45 positive) (C). Endothelial cell-derived MPs (EMPs – annexin V and CD51/61 positive) (D). MPs that express tissue factor (TFMPs – annexin V and CD142 positive) (E). MPs (microparticles), T2DM (type 2 diabetes mellitus). *p<0.05 was considered statistically significant; Mann-Whitney test.

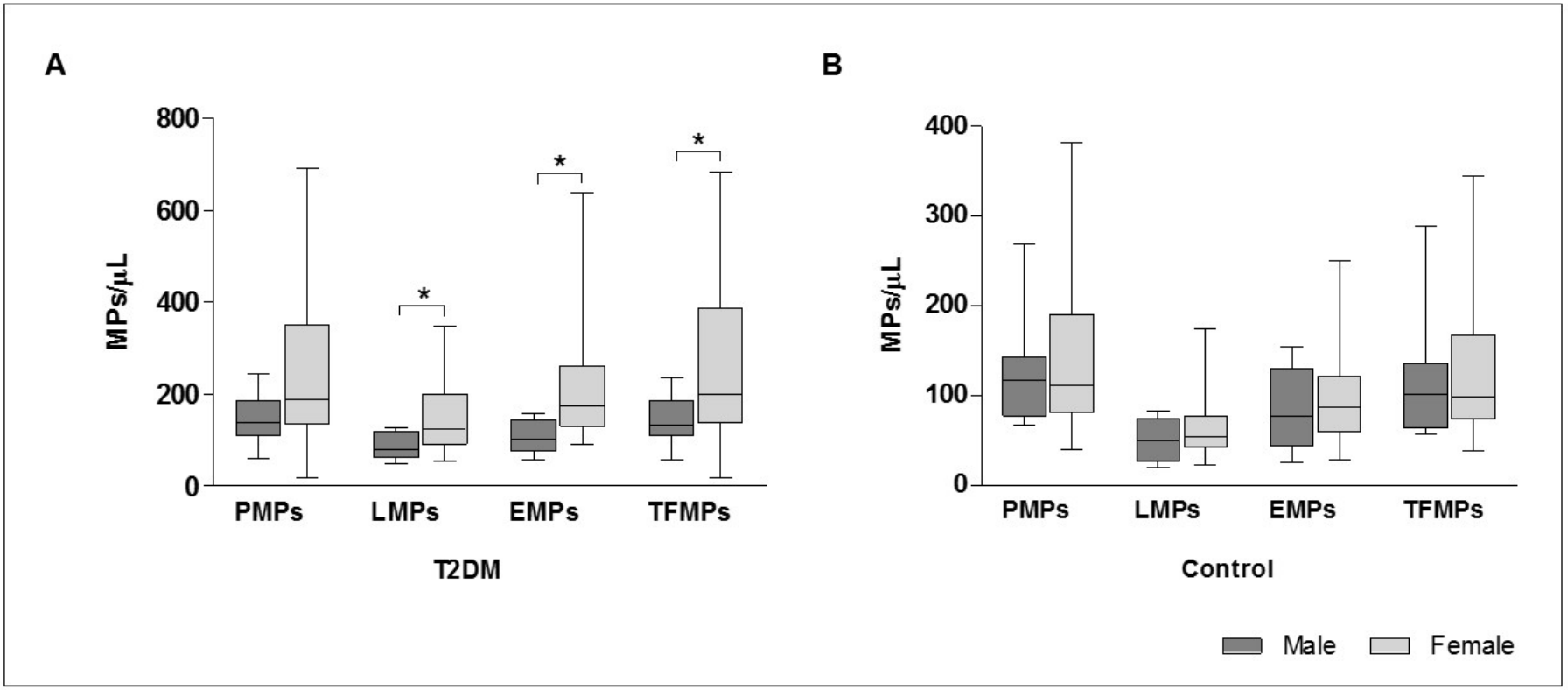

Figure 3: Distribution of microparticles levels (MPs/µL) considering gender in type 2 diabetes mellitus and control groups. MPs (microparticles), T2DM (type 2 diabetes mellitus), PMPs (platelets-derived MPs), LMPs (leukocytes-derived MPs), EMPs (endothelial cell-derived MPs), TFMPs (MPs that express tissue factor). *$p<0.05$ was considered statistically significant; Mann-Whitney test.

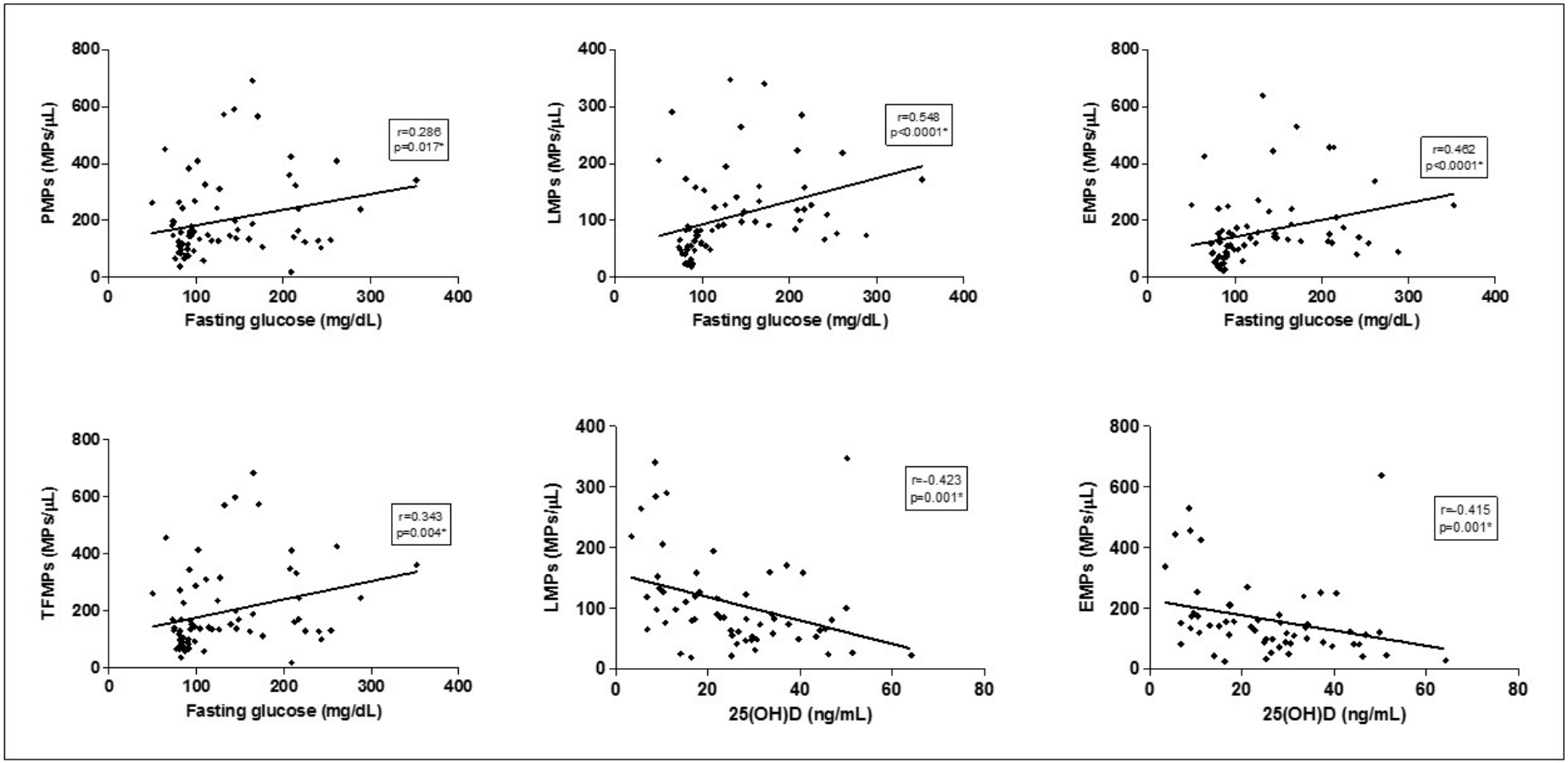

Figure 4: Correlation coefficient between microparticles levels and laboratorial data. MPs (microparticles), PMPs (platelets-derived MPs), LMPs (leukocytes-derived MPs), EMPs (endothelial cell-derived MPs), TFMPs (MPs that express tissue factor), 25(OH)D (25-hydroxyvitamin D). *p<0.05 was considered statistically significant; Spearman's correlation test.

8. DISCUSSÃO

O presente estudo investigou a associação de marcadores inflamatórios, bioquímicos e moleculares, com o DM2, suas complicações microvasculares e comorbidades.

A avaliação dos níveis de citocinas nos grupos DM2 e controle revelou que os níveis de IL-6 foram mais altos para os pacientes com DM2. Além disso, os níveis de IL-6 mostraram ser influenciados pelo IMC, exibindo correlação direta com este parâmetro. Este achado está em acordo com o conhecimento de que o DM2 é uma condição associada a um perfil pró-inflamatório (SHOELSON et al., 2006) e que a obesidade, especialmente a visceral, tem papel fundamental na patogênese da doença, uma vez que adipócitos obesos produzem e secretam citocinas pró-inflamatórias (WENSVEEN et al., 2015).

Os polimorfismos de citocinas não exibiram associação com o DM2 e apenas o polimorfismo IL-10 -819T/C mostrou afetar os níveis de IL-10 neste grupo. De fato, a associação destes polimorfismos com o DM2 ainda permanece conflitante (QI et al., 2006; HUA et al., 2013; LI et al., 2013; ZHAO et al., 2014) e estudos funcionais que avaliem o efeito das variantes moleculares sobre a expressão/secreção de citocinas são necessários.

O endocan é um proteoglicano solúvel secretado pelas células endoteliais que atua nos processos de migração e proliferação celular e angiogênese. É, portanto, um marcador da ativação e disfunção endotelial (BALTA et al., 2015). Os níveis de endocan foram mais baixos nos pacientes com DM2 quando comparados aos controles. Sabendo-se que o endocan se liga a integrina LFA-1 (*leukocyte function-associated antigen 1*) na superfície de leucócitos, inibindo diretamente a ligação destes ao ICAM-1 (BÉCHARD et al., 2001), sugere-se que mais baixos níveis de endocan no DM2 favoreçam a adesão e migração de leucócitos através do endotélio vascular, contribuindo para o agravamento da disfunção endotelial e inflamação.

A irisina é uma adipomiocina que atua sobre o metabolismo, aumentando o gasto energético, melhorando a tolerância à glicose e a resistência à insulina, além de promover perda de peso (PERAKAKIS et al., 2017). Os mecanismos pelos quais a irisina atua sobre o metabolismo, bem como a sua estrutura e as vias de sinalização do seu receptor, ainda não foram totalmente elucidados. Nós encontramos maiores níveis de irisina no grupo com DM2 quando comparado ao controle. Este achado é corroborado pelos trabalhos de Park et al. (2013) e Gárcia-Fontana et al. (2016) que também encontraram maiores níveis de irisina em

pacientes com síndrome metabólica e DM2, respectivamente. Além disso os níveis de irisina foram maiores em pacientes não obesos e positivamente correlacionados com a glicemia de jejum. Estes resultados permitem propor que o DM2 é associado a um estado de resistência a irisina, que pode ser agravado pelo descontrole glicêmico, ou ainda que a secreção de irisina pode ser aumentada para compensar a resistência à insulina observada no DM2.

A visfatina é uma adipocina que apresenta marcantes efeitos pró-inflamatórios, pois induz a ativação do fator NF-κB e a consequente produção de citocinas pró-inflamatórias (GARTEN et al., 2015). Altos níveis de visfatina têm sido associados ao DM2 e obesidade (CHEN et al., 2006; SANDEEP et al., 2007). Avaliando os níveis de visfatina, segundo o grau da doença renal do diabetes, observamos que o declínio da função renal é acompanhado da diminuição dos níveis de visfatina de forma independente da glicemia de jejum, HbA1c, RD, hipertensão e dislipidemia. Pode-se sugerir, portanto, que a doença renal do diabetes aumenta a taxa de excreção urinária da visfatina, reduzindo seus níveis, ou que a produção renal local de visfatina é afetada devido a deterioração da função do órgão.

A Hp é uma proteína de fase aguda responsável por se ligar a Hb livre conduzindo-a para ser degradada no fígado e prevenindo, desta forma, os efeitos pró-oxidantes desta (COSTACOU e LEVY, 2012). A estrutura da proteína e suas funções são altamente dependentes do polimorfismo Hp1-Hp2 (ASLEH e LEVY, 2005). Níveis mais altos de Hp foram encontrados no grupo com DM2 quando comparado aos controles. Ainda observou-se que estes níveis são influenciados pela obesidade e pelo polimorfismo Hp1-Hp2. Tomados em conjunto, estes resultados indicam que, embora o DM2 esteja associado a maiores níveis de Hp, a maior frequência do alelo Hp2 no grupo caso demonstra que esta proteína, embora em maior quantidade, não é eficiente em proteger o organismo dos danos oxidativos da Hb livre.

Por fim, foi realizada uma análise dos níveis de MPs circulantes em um grupo de pacientes com DM2 e nefropatia. As MPs são microvesículas liberadas durante a ativação e apoptose celular que carregam, seja na superfície da membrana plasmática ou no citoplasma, moléculas e marcadores das células de origem (SANTILLI et al., 2016). Além de atuarem na comunicação intercelular, as MPs atuam amplificando o processo inflamatório e a disfunção endotelial, pois ativam a

produção de citocinas/quimiocinas pró-inflamatórias e têm propriedades pró-coagulantes (FOLEY e CONWAY, 2016). Mais altos níveis de PMPs, EMPs, LMPs e TFMPs foram encontrados no grupo caso quando comparado aos controles; e estes níveis foram dependentes do gênero e correlacionados aos níveis de vitamina D e glicemia de jejum. Os níveis de MPs circulantes não foram associados ao IMC, ao tempo de diagnóstico do DM2, nem tampouco ao estadiamento da doença renal do diabetes. Estes dados evidenciam que a ativação celular que culmina na liberação de MPs está associada aos mecanismos de controle glicêmico, hemostático e pró-inflamatório no DM2.

9. CONCLUSÃO

Os resultados obtidos neste trabalho indicam que o DM2 está associado a um perfil alterado de marcadores bioquímicos (citocinas, irisina, visfatina e haptoglobina), de ativação celular (micropartículas circulantes) e disfunção endotelial (endocan), evidenciando o caráter pró-inflamatório da sua patogênese. Além disso, alguns destes marcadores, como citocinas e haptoblogina, têm seus níveis influenciados por polimorfismos genéticos. Os marcadores avaliados neste trabalho, uma vez validados em estudos de maior escala e em outras populações, poderão ser incorporados na clínica para o acompanhamento do paciente com DM2.

10. PERSPECTIVA DE TRABALHO

Residência pós-doutoral no Programa de Pós-graduação em Genética (ICB/UFMG) sob supervisão da Profa. Dra. Karina Braga Gomes Borges com projeto intitulado: “O papel dos miRNAs circulantes no diabetes mellitus tipo 2 e suas complicações”. Este projeto tem o objetivo de avaliar a expressão de miRNAs circulantes em pacientes com DM2 e identificar MPs como carreadoras destes miRNAs, associando-os às complicações microvasculares da doença. Cumpre ressaltar que este projeto dará continuidade à linha de pesquisa que vêm sendo desenvolvida sobre o papel de marcadores bioquímicos e moleculares associados à inflamação no DM2. A aluna Kathryna Fontana Rodrigues foi classificada no processo seletivo de pós-doutorado do referido programa de pós-graduação, regido pelo edital “seleção de bolsista de pós-doutorado CAPES/PNPD - Outubro 2016” e a implementação da bolsa tem previsão para ocorrer em Julho/2017.

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ANEXOS

ANEXO A - Aprovação do Comitê de Ética em pesquisa da UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Parecer nº. ETIC 0062.0.203.000-11

Interessado(a): Profa. Karina Braga Gomes Borges
Depto. de Análises Clínicas e Toxicológicas
Faculdade de Farmácia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 06 de maio de 2011, após atendidas as solicitações de diligência, o projeto de pesquisa intitulado **"O papel das citocinas no desenvolvimento do diabetes mellitus tipo 2"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

ANEXO B - Aprovação do Comitê de Ética e Pesquisa da Santa Casa de Belo Horizonte

GRUPO SANTA CASA DE BELO HORIZONTE

Comitê de Ética em Pesquisa

Registro CEP: 059/2011 (Este número deve ser citado nas correspondências referentes a este projeto).

Belo Horizonte, 18 de agosto de 2011.

Ilma. Sra.
Drª. Karina Braga Gomes Borges
Pesquisadora Responsável

Parecer:

O Comitê de Ética em Pesquisa (CEP) da Santa Casa de Misericórdia de Belo Horizonte, em reunião do dia 29 de julho de 2011, analisou e **aprovou** o projeto de pesquisa intitulado "**O papel das citocinas no desenvolvimento do diabetes mellitus tipo 2"**, registrado neste CEP sob número 059/2011 no qual V. Sa. figura como pesquisadora responsável.

Atenciosamente,

Francisco Chagas L. Silva

Dr. Francisco das Chagas Lima e Silva
Coordenador do CEP

ANEXO C - Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

PROJETO DE PESQUISA: "O papel de biomarcadores inflamatórios no desenvolvimento do diabetes mellitus tipo 2 e suas complicações"

Prezado (a) Sr.(a),

Este projeto tem por objetivo estudar como a inflamação pode afetar o desenvolvimento do diabetes, bem como as complicações da doença que o paciente pode apresentar. Para obter a conclusão da pesquisa, será necessário comparar os resultados dos exames dos pacientes com diabetes com os resultados de indivíduos sem a doença (grupo controle).

A coleta de amostras de sangue venoso inclui um pequeno risco de acidente de punção, representado, principalmente por extravasamento sanguíneo de pequena gravidade, que pode resultar em leve dor localizada e formação de um pequeno hematoma. Para minimizar este risco, a coleta de sangue será realizada por um profissional com capacidade técnica e experiência. Será utilizado material descartável de boa qualidade (agulhas e tubos a vácuo), visando o êxito da coleta.

Você está sendo convidado para participar desta pesquisa como voluntário, sem custo algum pelos exames realizados. Se você quiser participar poderá fazê-lo doando 20 mL de seu sangue para o uso nesta pesquisa, sendo este material armazenado em condições adequadas para pesquisas. Se você não quiser participar, não haverá qualquer problema e se você fizer parte do grupo de pacientes, não haverá alteração no seu tratamento e assistência recebida pelo seu médico caso você não aceite participar do estudo.

Seu nome será mantido em segredo, não sendo divulgado em nenhuma hipótese.

Se você estiver de acordo, por favor, assine esta folha.

De acordo:_____

(Assinatura)

Nome:
Data:___/___/_____

Qualquer dúvida sobre a sua participação neste estudo, por favor, entre em contato com a Profa. Karina Braga Gomes Borges no telefone 3409-6895- Faculdade de Farmácia/UFMG.

Desde já agradecemos sua valiosa participação.

Nome:_____

Assinatura:_____

Pesquisador responsável

Data:

COEP - Comitê de Ética em Pesquisa - Av. Antônio Carlos, 6627, Unidade Administrativa II - 2º andar - Sala 2005, Campus Pampulha, Belo Horizonte, MG – Brasil. telefax 31 3409-4592. coep@prpq.ufmg.br

ANEXO D - Ficha Clínica

FICHA DE AVALIAÇÃO DE PACIENTE

Data da entrevista: ____/____/____ **Data da coleta:** ____/____/____

Nome do paciente: _____

Idade: _____ anos **Sexo:** _____

Endereço e telefone: _____

_____

Peso: _____ Kg **Altura:** _____ m **IMC:** _____

Circunferência abdominal: _____ cm **Circunferência do quadril:** _____ cm

Tempo de diagnóstico: _____

Co-morbidades: _____

_____

Medicamentos em uso: _____

_____

_____

Tabagista: ___ Sim (Cigarros por dia: _____)
___ Não
___ Ex (Há quanto tempo parou? _____)

Etilista: ___ Sim (Consumo diário:_____)
___ Não
___ Ex (Há quanto tempo parou? _____)

Atividade física: ___ Sim (Atividade: _____ / Frequência: _____)
___ Não

Há alguma doença presente em mais de um membro da família? Qual? Grau de parentesco?

_____

_____

Outras informações:

Exames laboratoriais:

Glicemia jejum:	_____	Uréia:	_____
Glicemia pós-prandial:	_____	PCR:	_____
HB A1C:	_____	Leptina:	_____
CT:	_____	Adiponectina:	_____
LDL:	_____	Insulina:	_____
HDL:	_____	Proteinúria:	_____
VLDL:	_____	Microalbuminúria:	_____
TG:	_____	Peptídeo C:	_____
Creatinina sérica:	_____	Outros exames:	_____
			_____
			_____
			_____

ANEXO E - Artigos publicados como co-autora

Inflammation & Cell Signaling 2015; 2: e926. doi: 10.14800/ics.926; © 2015 by Nathalla T. Pietrani, *et al.*
http://www.smartscitech.com/index.php/ics

RESEARCH ARTICLE

Peripheral activation of inflammatory intracellular signaling pathways and their correlation with IL6, IL10 and TNFα in obesity and type 2 diabetes mellitus

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Received: July 22, 2015
Published online: August 11, 2015

***Background*: We investigated the activation of intracellular signaling pathways in individuals with and without T2D/obesity and correlated them with clinical characteristics and IL6, IL10 and TNFα serum levels. *Methods*: This study included 42 patients with T2D and 42 healthy controls matched by BMI. Intracellular signaling pathways Akt, p38 and ERK in peripheral blood mononuclear cells (PBMCs); IL6, IL10 and TNFα serum levels were evaluated. *Results:* Western blot analysis revealed that phosphorylation values of Akt, p38 and ERK were not significant different between the groups; but, once individuals were stratified according to BMI, activation of Akt and ERK was associated with obesity and T2D while the activation of p38 with obesity. The phosphorylation values of these proteins were also positively correlated with BMI and waist circumference. IL6 levels correlated with P-p38 in control group, while IL10 and TNFα did not correlated with these pathways. *Conclusions*: These results suggest that there is a link among obesity, T2D and intracellular signaling pathways in peripheral circulating immune cells, which may be associated with systemic low grade inflammation observed in T2D and obese patients.**

Keywords: type 2 diabetes; obesity; intracellular pathways; cytokines; inflammation

To cite this article: Nathalla T. Pietrani, *et al.* Peripheral activation of inflammatory intracellular signaling pathways and their correlation with IL6, IL10 and TNFα in obesity and type 2 diabetes mellitus. Inflamm Cell Signal 2015; 2: e926. doi: 10.14800/ics.926.

THE JOURNAL OF
Obstetrics and Gynaecology Research
doi:10.1111/jog.13250 J. Obstet. Gynaecol. Res. 2017

Vitamin D receptor polymorphisms and the polycystic ovary syndrome: A systematic review

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Abstract

Polycystic ovary syndrome (PCOS) is the most frequent endocrinological disorder that affects women of reproductive age, leading to metabolic alterations, such as hyperandrogenism, obesity, menstrual irregularities, insulin resistance, and polycystic ovaries. The etiology remains unclear, but several genetic and environmental factors have been correlated with manifestations of this syndrome. Vitamin D plays important roles in metabolic pathways affected by PCOS, including calcium homeostasis, the insulin pathway, and sex hormone synthesis. Vitamin D concentration has been related with the severity of this disorder, and vitamin D receptor polymorphisms have been shown in some studies to have an association with some of the patterns presented by PCOS. The objective of this study is to provide an up-to-date review about vitamin D receptor polymorphisms and their association with PCOS.

Key words: hyperandrogenism, insulin resistance, obesity, polycystic ovary syndrome, polymorphisms, vitamin D receptor.

Molecular and Cellular Endocrinology 443 (2017) 155–162

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Microparticles: Inflammatory and haemostatic biomarkers in Polycystic Ovary Syndrome

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ARTICLE INFO

Article history:
Received 17 November 2016
Received in revised form
10 January 2017
Accepted 10 January 2017
Available online 11 January 2017

Keywords:
Polycystic Ovary Syndrome
Microparticles
Platelets
Leukocytes
Endothelial cells
Tissue factor

ABSTRACT

Polycystic Ovary Syndrome (PCOS) is associated with a chronic low-grade inflammation and predisposition to hemostatic and atherosclerotic complications. This case-control study evaluated the microparticles (MPs) profile in patients with the PCOS and related these MPs to clinical and biochemical parameters. MPs derived from platelets (PMPs), leuckocytes (LMPs) and endothelial cells (EMPs) were evaluated, as well as MPs expressing tissue factor (TFMPs), by flow cytometry, comparing women with PCOS ($n = 50$) and a healthy control group ($n = 50$). PCOS women presented increased total MPs, PMPs, LMPs and EMPs levels when compared to control group (all $p < 0.05$). TFMPs was similar between the groups ($p = 0.379$). In conclusion, these MPs populations could be useful biomarkers for association with thrombosis and cardiovascular disease in PCOS women.