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FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

CAROLINE PEREIRA DOMINGUETI

AVALIAÇÃO DA FUNÇÃO RENAL E SUA ASSOCIAÇÃO COM FVW, ADAMTS13 E DÍMERO D EM PACIENTES DIABÉTICOS TIPO 1

Belo Horizonte - MG

Outubro – 2014

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Orientadora: Profa. Dra. Ana Paula Salles Moura Fernandes

Co-orientadora: Profa. Dra. Karina Braga Gomes Borges

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FOLHA DE APROVAÇÃO

"Avaliação da Função Renal e sua Associação com FVW, ADAMTS13 e Dímero D em Pacientes Diabéticos tipo 1"

CAROLINE PEREIRA DOMINGUETI

Tese submetida à Comissão Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS FARMACÊUTICAS, como requisito para obtenção do grau de Doutora em CIÊNCIAS FARMACÊUTICAS, área de concentração CIÊNCIAS FARMACÊUTICAS.

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Profa. Ana Paula Salles Moura Fernandes - Orientadora
UFMG

Profa. Karina Braga Gomes Borges - Coorientadora
UFMG

Profa. Joyce Maria Annichino-Bizzacchi
UNICAMP

Prof. Marcos de Bastos
IPSEMG

Profa. Ana Cristina Simões e Silva
UFMG

Profa. Maria das Graças Carvalho
UFMG

Belo Horizonte, 16 de outubro de 2014.

DEDICATÓRIA

Dedico esta tese de Doutorado ao meu pai Helder Ponciano Domingueti e à minha mãe Rozilene Fávaro Pereira Domingueti.

"Por vezes sentimos que aquilo que fazemos

não é senão uma gota de água no mar.

Mas o mar seria menor se lhe faltasse uma gota"

Madre Teresa de Caucuta

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RESUMO

A nefropatia diabética consiste na principal causa de doença renal terminal em adultos e em um fator de risco independente para doenças cardiovasculares. A disfunção endotelial juntamente com o desenvolvimento de um estado de hipercoagulabilidade têm sido associadas com o desenvolvimento das complicações vasculares diabéticas. Assim, este estudo teve como objetivo avaliar a função renal de pacientes diabéticos tipo 1, utilizando diferentes metodologias, e sua associação com os níveis plasmáticos dos biomarcadores de disfunção endotelial, FVW e ADAMTS13, e do biomarcador de hipercoagulabilidade, Dímero D. Os pacientes diabéticos foram classificados em três grupos de acordo com o ritmo de filtração glomerular (RFG): RFG ≥90mL/min/1,73m^2, n=52; RFG ≥60 e <90mL/min/1,73m^2, n=29; RFG <60mL/min/1,73m^2, n=28; e também de acordo com os níveis de albuminúria: <30mg/g, n=53; ≥30 e <300mg/g, n=26; ≥300mg/g, n=30. Os níveis plasmáticos de FVW, ADAMTS13, Dímero D e cistatina C foram determinados através da técnica de ELISA, a atividade da ADAMTS13 foi determinada por meio da técnica FRET e o RFG foi estimado através de equações baseadas na creatinina ou na cistatina C. O RFG estimado através das equações baseadas na creatinina ou na cistatina C apresentou uma boa correlação com os diferentes níveis de albuminúria. Contudo, as equações baseadas na cistatina C apresentaram uma precisão um pouco maior para detectar a presença de albuminúria acentuadamente aumentada. Níveis plasmáticos elevados de FVW, ADAMTS13 e Dímero D, uma atividade aumentada da ADAMTS13, e uma relação ADAMTS13Atividade/ADAMTS13Ag reduzida foram significativamente associados com o declínio do RFG e com o aumento da albuminúria, indicando uma associação entre a disfunção endotelial e a hipercoagulabilidade com a nefropatia no diabetes mellitus tipo 1 (DM1). Apenas a albuminúria e a cistatina C foram independentemente associadas com níveis elevados de Dímero D. Portanto, a cistatina C foi o biomarcador de função renal que apresentou uma melhor associação com a albuminúria acentuadamente aumentada e com os níveis elevados de Dímero D, o que demonstra um grande potencial deste biomarcador para avaliar simultaneamente a função renal e o risco de doenças cardiovasculares no DM1. Como todas as metodologias de avaliar a função renal apresentam vantagens e limitações, o ideal é que todas sejam utilizadas de modo

complementar. Além disso, é muito importante a busca por novos biomarcadores de função renal que possibilitem a detecção precoce da doença renal, da sua progressão e de suas complicações. Como níveis elevados de FVW e Dímero D, e uma relação ADAMTS13Atividade/ADAMTS13Ag reduzida estão associados com a nefropatia diabética, estes biomarcadores de disfunção endotelial e de hipercoagulabilidade são promissores para serem utilizados no acompanhamento da doença renal no DM1.

Palavras-chaves: Nefropatia Diabética, cistatina C, fator von Willebrand, ADAMTS13, Dímero D, albuminúria.

ABSTRACT

Diabetic nephropathy is the most important cause of end stage renal disease in adults and an idependent risk factor for cardiovascular disease. Endothelial dysfunction along with the development of a hypercoagulability state have been associated with the development of diabetic vascular complications. Thus, this study aimed to evaluate the renal function of type 1 diabetic patients using different methodologies and their association with plasma levels of biomarkers of endothelial dysfunction, VWF and ADAMTS13, and the biomarker of hypercoagulability, D-Dimer. Diabetic patients were classified into three groups according to glomerular filtration rate (GFR): GFR ≥90mL/min/1,73m^2, n=52; GFR ≥60 e <90mL/min/1,73m^2, n=29; GFR <60mL/min/1,73m^2, n=28; and also according to albuminuria: <30mg/g, n=53; ≥30 e <300mg/g, n=26; ≥300mg/g, n=30. Plasma levels of VWF, ADAMTS13, D-Dimer and cystatin C were determined by ELISA, ADAMTS13 activity was evaluated by FRET, and GFR was estimated using equations based on creatinine or cystatin C. GFR estimated by creatinine-based or cystatin-based equations presented a good correlation with different levels of albuminuria. However, equations based on cystatin C presented a slightly higher accuracy for detecting the presence of severe increased albuminuria. Increased plasma levels of VWF, ADAMTS13 and D-Dimer, increased ADAMTS13 activity, and reduced ADAMTS13Activity/ADAMTS13Ag ratio were significantly associated with the decline of GFR and increased albuminuria, indicating an association between endothelial dysfunction and hypercoagulability with nephropathy in type 1 diabetes mellitus (DM1). Only albuminuria and cystatin C were independently associated with increased levels of D-Dimer. Therefore, cystatin C was the biomarker of renal function that presented a greater association with severe increased albuminuria and with increased levels of D-Dimer, which demonstrates the great potential of this biomarker to simultaneously assess renal function and the risk of cardiovascular disease in DM1. As all the methodologies to evaluate renal function have advantages and limitations, ideally, all of them should be used complementarily. Moreover, it is very important to search for new biomarkers of renal function that enable early detection of renal disease, its progression and its complications. As increased plasma levels of VWF and D-Dimer, and reduced ADAMTS13Activity/ADAMTS13Ag ratio are associated with diabetic nephropathy,

these biomarkers of endothelial dysfunction and hypercoagulability are very promising to be used in monitoring renal disease in DM1.

Keywords: Diabetic Nephropathy, cystatin C, von Willebrand factor, ADAMTS13, D-Dimer, albuminuria.

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ADA – Associação Americana de Diabetes

ADAMTS13 – Desintegrina e Metaloproteinase com Domínio Trombospondina tipo 1

AGE – Produtos Finais de Glicação Avançada (*Advanced Glycation End-Products*)

AUC – Área Sob a Curva

CG – Cockcroft Gault

CKD-EPI – Colaboração Epidemiológica na Doença Renal (*The Chronic Kidney Disease Epidemiology Collaboration*)

DEC – Depuração da Creatinina

DM – Diabetes Mellitus

DM1 – Diabetes Mellitus tipo 1

DM2 – Diabetes Mellitus tipo 2

DMG – Diabetes Mellitus Gestacional

DRC – Doença Renal Crônica

ELISA – Ensaio Imunoadsorvente Ligado à Enzima (*Enzyme-Linked Immunosorbent Assay*)

EUA – Excreção Urinária de Albumina

FRET – Transferência de Energia de Ressonância de Fluorescência (*Fluorescence Resonance Energy Transfer*)

FVW – Fator Von Willebrand

GPIb/IX – Glicoproteína Ib/IX

GPIIb/IIIa – Glicoproteína IIb/IIIa

HbA1c – Hemoglobina Glicada

HLA – Antígeno Leucocitário Humano

ICAM-1 – Molécula de Adesão Intercelular-1

IL-1 – Interleucina-1

IL-6 – Interleucina-6

LADA – Diabetes Latente Auto-imune do Adulto (*Latent Autoimune Diabetes in Adults*)

LDL – Lipoproteína de Baixa Densidade

MDRD – Modificação Dietética na Doença Renal

NF-κB- Fator de Transcrição Nuclear kappa B

PAI-1 – Inibidor do Ativador de Plasminogênio-1

RAC – Relação Albumina-Creatinina

RFG – Ritmo de Filtração Glomerular

TNF-α – Fator de Necrose Tumoral alfa

SUMÁRIO

1 REFERENCIAL TEÓRICO

1.1 Diabetes mellitus

O diabetes mellitus (DM) consiste em um grupo de distúrbios metabólicos que possui como característica principal o desenvolvimento de hiperglicemia, a qual é resultante de uma produção deficiente de insulina pelas células beta do pâncreas e/ou uma resistência periférica à ação da insulina (ADA, 2014).

A classificação atual do DM é baseada na etiologia e não no tipo de tratamento. A classificação proposta pela Associação Americana de Diabetes (ADA) inclui quatro entidades clínicas: diabetes mellitus tipo 1 (DM1), diabetes mellitus tipo 2 (DM2), outros tipos específicos de diabetes mellitus e diabetes mellitus gestacional (DMG) (Quadro 1). Ainda há duas categorias, referidas como pré-diabetes, que se caracterizam pela glicemia de jejum alterada e tolerância à glicose diminuída. Tais categorias não são entidades clínicas, mas fatores de risco para o desenvolvimento de DM e doenças cardiovasculares (ADA, 2014).

Quadro 1 – Classificação etiológica do diabetes mellitus

I. Diabetes tipo 1
Destruição das células beta, usualmente levando à deficiência completa de insulina
A. Auto-imune
B. Idiopático
II. Diabetes tipo 2
Graus variados de diminuição de secreção e resistência à insulina
III. Outros tipos específicos
A. Defeitos genéticos da função da célula β
B. Defeitos genéticos da ação da insulina
C. Doenças do pâncreas exócrino
D. Endocrinopatias
E. Indução por drogas ou produtos químicos
F. Infecções
G. Formas incomuns de diabetes imunomediado
H. Outras síndromes genéticas algumas vezes associadas com diabetes
IV. Diabetes gestacional

Adaptado: ADA, 2014

Os critérios para o diagnóstico laboratorial do DM foram modificados pela ADA em 2010 com a finalidade de prevenir, de maneira eficaz, as complicações micro e macrovasculares. Estes critérios consistem em:

- Glicemia de jejum (durante pelo menos 8 horas) igual ou maior que 126 mg/dL (7,0 mmol/L);
- Glicemia 2 horas após sobrecarga oral de 75 g de glicose igual ou superior a 200 mg/dL (11,1 mmol/L);
- Níveis de hemoglobina glicada (HbA1c) maiores ou iguais a 6,5%;
- Glicemia casual ou aleatória maior ou igual a 200 mg/dL (11,1 mmol/L), na presença de sinais e sintomas do DM, como poliúria, polidipsia, polifagia e perda de peso inexplicada.

Na ausência de hiperglicemia inequívoca, um resultado positivo para DM de acordo com qualquer um dos três primeiros critérios deve ser confirmado em outra ocasião (ADA, 2014).

Reconhece-se ainda um grupo intermediário de indivíduos, cujos níveis de glicemia não preenchem os critérios para o diagnóstico de DM, contudo, são muito elevados para serem considerados normais. Neste grupo estão incluídas as categorias de glicemia de jejum alterada, em que a glicemia de jejum se encontra entre 100 e 125 mg/dL; tolerância à glicose diminuída, em que a glicemia 2 horas após sobrecarga oral de 75 g de glicose, se situa entre 140 e 199 mg/dL; e HbA1c alterada, em que a HbA1c está compreendida entre 5,7 e 6,4% (ADA, 2014).

1.1.1 Diabetes mellitus tipo 1

O DM1, previamente denominado diabetes insulino-dependente ou diabetes de início no jovem, resulta da destruição das células beta do pâncreas, geralmente decorrente de um processo autoimune que acarreta deficiência completa na secreção de insulina. Duas formas de DM1 podem ser identificadas: tipo 1A (forma autoimune), resultante da destruição autoimune das células beta; e tipo 1B (forma idiopática), de causa desconhecida (Canivell e Gomis, 2014).

No DM1 autoimune, há um processo de insulite e estão presentes auto-anticorpos circulantes (principalmente anticorpos anti-descarboxilase do ácido glutâmico, anti-ilhotas, anti-insulina e anti-tirosina-fosfatases). Estes anticorpos podem estar presentes meses ou anos antes do diagnóstico clínico, ou seja, na fase

pré-clínica da doença, e em até 90% dos indivíduos, quando se detecta hiperglicemia. Além do componente autoimune, há uma grande associação com determinados alelos do sistema antígeno leucocitário humano (HLA), os quais podem predispor ou proteger contra o seu desenvolvimento. O DM1 idiopático caracteriza-se pela ausência tanto de insulite como dos marcadores de autoimunidade e se observa associação com haplótipos do sistema HLA (Gross *et al.*, 2002).

O DM1 representa aproximadamente 5 a 10% de todos os casos de diabetes. Embora represente menor número, consiste na forma predominante em crianças e adolescentes (Canivell e Gomis, 2014). Estima-se que 497.100 crianças apresentem DM1 e que, a cada ano, 79.100 crianças desenvolvam a doença em todo o mundo (International Diabetes Federation, 2013). O pico de incidência do DM1 ocorre dos 10 aos 14 anos, havendo a seguir uma diminuição progressiva da incidência até os 35 anos, de modo que casos de DM1 de início após esta idade são pouco frequentes. Contudo, indivíduos de qualquer idade podem desenvolver DM1. Em geral, os pacientes apresentam índice de massa corporal normal, porém a presença de obesidade não exclui o diagnóstico (Gross *et al.*, 2002).

As principais manifestações clínicas do DM1 são decorrentes da hiperglicemia e consistem em poliúria, polidipsia, polifagia, emagrecimento, visão turva e astenia. A instalação do quadro de DM1 é relativamente abrupta, sendo que muitas vezes o indivíduo pode identificar a data de início dos sintomas. Devido à destruição das células beta do pâncreas, ocorre deficiência da secreção de insulina, o que deixa os pacientes susceptíveis à ocorrência de cetoacidose, a qual geralmente consiste na primeira manifestação da doença (Gross *et al.*, 2002). A taxa de destruição das células beta é variável, sendo geralmente mais rápida entre as crianças. A forma lentamente progressiva ocorre em adultos, sendo referida como *latent autoimune diabetes in adults* (LADA) (Canivell e Gomis, 2014).

1.1.2 Complicações crônicas do diabetes mellitus

As complicações crônicas do DM são resultantes de um estado hiperglicêmico crônico e se caracterizam por alterações vasculares e neuropáticas. As alterações vasculares podem ocorrer nos grandes vasos sanguíneos (macroangiopatia) e também nos pequenos vasos sanguíneos (microangiopatia).

Ambas resultam de um conjunto de processos que incluem glicação não-enzimática irreversível de proteínas, alteração do potencial redox celular, aumento do estresse oxidativo e do estado inflamatório, e desenvolvimento de disfunção endotelial e de um estado de hipercoagulabilidade (Oliveira *et al.*, 1998; Wautier e Guillausseau, 1998; Goldberg, 2009).

As células vasculares endoteliais apresentam um risco particular de desenvolverem hiperglicemia intracelular devido ao fato de serem livremente permeáveis à glicose. Assim, o acúmulo de glicose no meio intracelular leva à ativação de uma via metabólica secundária, a via da aldose redutase, na qual essa enzima e a sorbitol dehidrogenase catalisam o metabolismo da glicose a sorbitol e deste em frutose, respectivamente. Sendo a célula impermeável à saída destes metabólitos, estes se acumulam no meio intracelular. Estas reações são acompanhadas pela oxidação do NADPH a $NADP^+$ e redução do NAD^+ a NADH. O fluxo excessivo de glicose através desta via leva a uma alteração do potencial redox celular devido à depleção de NADPH e ao aumento da taxa citosólica $NADH/NAD^+$ (Giannini *et al.*, 2011).

Um aumento na taxa $NADH/NAD^+$ decorrente da hiperglicemia mimetiza os efeitos da hipóxia, acarretando aceleração da glicólise, com consequente aumento da síntese “de novo” do diacilglicerol proveniente de intermediários glicolíticos e subsequente ativação da via da proteína quinase C. A ativação da proteína quinase C interfere na síntese de óxido nítrico, promove um aumento da permeabilidade e da contratilidade vascular, estimula a síntese de matriz extracelular e o espessamento da membrana basal, além de promover uma ativação da resposta inflamatória através da expressão de citocinas e adesão de leucócitos (Giannini *et al.*, 2011; Kessler *et al.*, 1998).

A alteração da taxa $NADH/NAD^+$ também resulta em um aumento na produção de ânions superóxido, devido à ativação de oxidases dependentes de NADH, os quais oxidam a lipoproteína de baixa densidade (LDL), exercem efeitos citóxicos sobre as células endoteliais e promovem uma redução da disponibilidade de óxido nítrico, acarretando uma disfunção endotelial (Giannini *et al.*, 2011; Kessler *et al.*, 1998). Quando lesadas, as células endoteliais liberam moléculas pró-coagulantes, como fator von Willebrand (FVW), inibidor do ativador de plasminogênio-1 (PAI-1) e tromboxano A2, e expressam na sua superfície fator tecidual e moléculas de adesão, como P-selectina, E-selectina, molécula de adesão

vascular-1 (VCAM-1) e molécula de adesão intercelular-1 (ICAM-1), as quais promovem a interação de neutrófilos e plaquetas com o endotélio. Deste modo, a disfunção endotelial pode promover tanto um estado pró-inflamatório quanto um estado pró-coagulante (Margetic, 2012).

Quando expostas a aldoses, as proteínas são submetidas à glicação e oxidação. Inicialmente, a reação consiste em condensação da glicose com grupos amino de proteínas para formar produtos reversíveis (bases de Schiff), que podem sofrer rearranjos e formar produtos mais estáveis, porém lentamente reversíveis, denominados produtos Amadori. Após rearranjos moleculares, esses produtos Amadori podem formar os produtos finais de glicação avançada (AGE – *advanced glycation end-products*), que são irreversíveis. As moléculas ligadas aos AGEs adquirem novas propriedades e se tornam oxidantes, o que leva à produção de espécies reativas do oxigênio, as quais promovem um aumento do estresse oxidativo e um bloqueio na liberação de óxido nítrico, resultando no surgimento de lesões vasculares (Singh *et al*., 2014; Oliveira *et al*., 1998; Wautier e Guillausseau, 1998). Além disso, o acúmulo de AGEs na matriz extracelular vascular pode levar à formação de ligações cruzadas entre as proteínas da matriz, principalmente o colágeno, resultando em diminuição da elasticidade e aumento da rigidez dos vasos sanguíneos, e no espessamento da parede vascular (Singh *et al*., 2014; Schalkwijk e Miyata, 2012).

Os AGEs podem se ligar aos seus receptores (RAGE – *receptors for advanced glycation end-products*) presentes na superfície das células endoteliais, células musculares lisas, fibroblastos, linfócitos, monócitos e macrófagos, acarretando a ativação do fator de transcrição nuclear NF-κB (Giannini *et al*., 2011; Wautier e Guillausseau, 1998). Através desta ativação, é induzida a transcrição de diferentes genes, como endotelina-1, VCAM-1, ICAM-1, E-selectina, trombomodulina, fator tecidual, fator de crescimento endotelial vascular, interleucina-1 (IL-1), interleucina-6 (IL-6), fator de necrose tumoral alfa (TNF-α) e RAGE, desencadeando um estado pró-inflamatório e pró-coagulante, que contribui para a disfunção endotelial (Giannini *et al*., 2011).

O aumento da expressão das citocinas inflamatórias e das moléculas de adesão pode induzir respostas pró-inflamatórias, levando a um agravamento das complicações vasculares diabéticas. Além disso, as citocinas TNF-α, IL-1 e IL-6 são importantes mediadoras do efeito pró-coagulante das células endoteliais lesadas, já

que estas citocinas podem estimular a liberação e a expressão de moléculas pró-coagulantes, como FVW, PAI-1 e fator tecidual, e inibir a expressão de moléculas anti-coagulantes, como a trombomodulina, pelas células endoteliais (Margetic, 2012). A redução da expressão de trombomodulina associada com a indução da expressão do fator tecidual altera a superfície do endotélio de um estado anticoagulante para um estado pró-coagulante. Além disso, a produção aumentada de fatores de crescimento pode estimular o remodelamento da parede dos vasos sanguíneos, resultando em espessamento da membrana basal destes vasos, o que favorece a deposição local de proteínas e lipídeos, além de promover a esclerose e o comprometimento da função vasodilatadora. Os AGEs ainda podem reduzir a biodisponibilidade e a atividade do óxido nítrico derivado do endotélio, comprometendo ainda mais a atividade vascular (Giannini *et al.*, 2011; Oliveira *et al.*, 1998).

A lesão endotelial, o estresse oxidativo, a inflamação e as alterações crônicas no equilíbrio hemodinâmico derivadas da hiperglicemia podem iniciar um processo de aterosclerose e a formação de trombo arterial (Annichino-Bizzacchi, 2004). Durante o início do processo aterosclerótico, os proteoglicanos da matriz sequestram a LDL circulante e induzem sua oxidação. Estas lipoproteínas oxidadas consistem em moléculas altamente pró-inflamatórias que estimulam a expressão de várias moléculas de adesão pelas células endoteliais, como VCAM-1, ICAM-1 e selectinas, e a secreção de fatores de crescimento e de citocinas inflamatórias, como IL-1 e TNF-α (Giannini *et al.*, 2011; Libby, 2012).

A expressão das moléculas de adesão pelo endotélio lesado promove a ligação seletiva dos leucócitos e a sua transmigração para o interior da parede vascular. Além disso, os monócitos circulantes são recrutados e ativados, diferenciando-se em macrófagos, os quais, por fagocitarem o excesso de LDL oxidado, transformam-se em células espumosas, formando as estrias gordurosas. As células mononucleares também liberam citocinas inflamatórias, incluindo IL-1 e IL-6, as quais promovem o recrutamento de mais células inflamatórias. Devido ao efeito dos fatores pró-inflamatórios e de crescimento secretados pelos macrófagos e pelas células espumosas, as células musculares lisas se proliferam e migram da camada média para a íntima. As células musculares lisas ativadas sintetizam e secretam matriz extracelular (colágeno, elastina, proteoglicanos) acarretando a formação de fibroateroma (Giannini *et al.*, 2011).

Durante a fase inicial da aterosclerose, a trombose é infreqüente. Contudo, com a evolução do processo, a formação de fissuras ou a ulceração da placa aterosclerótica expõe substâncias altamente trombogênicas, como o fator tecidual e o FVW, o que resulta na adesão e agregação de plaquetas e no rápido crescimento do trombo. Isto ocorre em placas com fina camada fibrosa, com grande quantidade lipídica, e naquelas com grande concentração de fator tecidual (Annichino-Bizzacchi, 2004). Além de participarem da formação do trombo, as plaquetas ativadas também liberam citocinas pró-inflamatórias e fatores de crescimento, os quais promovem o recrutamento de monócitos para a placa aterosclerótica e estimulam a proliferação de fibroblastos e de células musculares lisas, o que acentua o processo aterosclerótico. As plaquetas ativadas ainda podem interagir com as células endoteliais através da P-selectina, resultando na liberação de IL-6 e na expressão de E-selectina, VCAM-1 e ICAM-1 pelas células endoteliais, acentuando a inflamação (Margetic, 2012).

A macroangiopatia diabética tem sido associada com o desenvolvimento destes processos ateroscleróticos, e tem como consequência um aumento do risco de infarto agudo do miocárdio, acidente vascular cerebral e doenças vasculares periféricas (Knudson *et al.*, 2008). Os pacientes diabéticos tipo 1 possuem um risco de mortalidade cardiovascular e de mortalidade em geral dez vezes maior do que os indivíduos sem DM, sendo que a doença cardiovascular consiste na principal causa de mortalidade entre estes pacientes (Nadeau e Reusch, 2011). Como o DM1 surge predominantemente durante a infância, os pacientes diabéticos tipo 1 apresentam um risco maior de desenvolver eventos coronarianos mais precocemente. Foi observado que a taxa de eventos cardiovasculares nos pacientes diabéticos tipo 1 excede 1% ao ano após os 45 anos e ultrapassa 3% ao ano após os 55 anos (Giannini *et al.*, 2011).

A microangiopatia diabética também consiste em importante causa de morbidade e mortalidade nos pacientes diabéticos tipo 1. Ela é representada pelo desenvolvimento da retinopatia diabética, que se manisfesta pela formação de microaneurismas nos capilares, principalmente ao redor do nervo ótico, podendo evoluir para hemorragia retiniana e cegueira; pela neuropatia diabética, que é caracterizada por alterações no sistema nervoso autônomo, as quais resultam no surgimento de diarréia, gastroparesia, hipotensão postural e impotência, além da perda de sensibilidade cutânea; e pelo desenvolvimento da nefropatia diabética, que

se manifesta por um espessamento da membrana basal glomerular, com consequente proteinúria, esclerose e fibrose renal, culminando com o desenvolvimento de insuficiência e falência renal (Wautier e Guillausseau, 1998).

1.2 Nefropatia diabética

A nefropatia diabética consiste na causa mais comum de doença renal terminal em adultos, contribuindo para aproximadamente 45% dos novos casos (Karnib and Ziyadeh, 2010). Em torno de 25 a 40% dos pacientes diabéticos tipo 1 e tipo 2 desenvolvem nefropatia 20 a 25 anos após o estabelecimento do DM (Yamagishi e Matsui, 2010). A hiperglicemia, a hipertensão arterial, a dislipidemia e a predisposição genética consistem nos principais fatores de risco para o desenvolvimento da nefropatia nestes pacientes (Gross, *et al.*, 2005).

A definição clássica da nefropatia diabética consiste em um aumento progressivo da excreção urinária de albumina (EUA), acarretando um declínio do ritmo de filtração glomerular (RFG), e eventualmente, falência renal (Marshall, 2004). Contudo, tem sido observado que a albuminúria nem sempre precede o declínio do RFG, de modo que este pode ocorrer mesmo na ausência de um aumento da EUA (Gross *et al.*, 2005). Assim, doença renal crônica (DRC) é atualmente definida como a presença de anormalidades da estrutura ou função dos rins, presentes por mais de 3 meses, com implicações para a saúde (KDIGO, 2013).

De acordo com as novas Diretrizes da National Kidney Foundation (KDIGO, 2013), deve-se classificar a DRC baseando-se na causa, na categoria do RFG e na albuminúria. Esta classificação possibilita a identificação do risco de desfechos adversos, tais como DRC progressiva, doença renal terminal, doença renal aguda, mortalidade por todas as causas e mortalidade cardiovascular (Quadro 2). Estas Diretrizes também definiram novos critérios para o diagnóstico da DRC, os quais consistem na presença de um ou mais marcadores de lesão do parênquima renal e/ou de um RFG inferior a 60 mL/min/1,73m^2 durante um período maior do que três meses (Quadro 3).

Quadro 2: Classificação da DRC de acordo com o RFG e a albuminúria

Categoria	RFG (mL/min/1,73m^2)	EUA (mg/24h)	RAC (mg/g)	Descrição
RFG				
G1	≥ 90	-	-	Normal ou aumentado
G2	60-89	-	-	Levemente diminuído*†
G3a	45-59	-	-	Levemente a moderadamente diminuído
G3b	30-44	-	-	Moderadamente a gravemente diminuído
G4	15-29	-	-	Gravemente diminuído
G5	< 15	-	-	Falência renal
Albuminúria				
A1	-	< 30	< 30	Normal ou ligeiramente aumentado
A2	-	30-299	30-299	Moderadamente aumentado*
A3	-	≥ 300	≥ 300	Acentuadamente aumentado‡

DRC = doença renal crônica; RFG = ritmo de filtração glomerular; EUA = excreção urinária de albumina; RAC = relação albumina-creatinina.
* Em relação ao nível de jovens adultos
† Na ausência de lesão renal evidente, as categorias do RFG G1 e G2 não cumprem os critérios para a DRC
‡ Incluindo a síndrome nefrótica (EUA geralmente > 2200 mg/24h ou RAC > 2220 mg/g)

Adaptado: KDIGO, 2013

Quadro 3: Critérios para o diagnóstico da DRC (qualquer um dos seguintes presentes por > 3 meses)

Marcadores de lesão renal (um ou mais)
Albuminúria (EUA ≥ 30 mg/24h ou RAC ≥ 30 mg/g)
Anormalidades no sedimento urinário
Distúrbios eletrolíticos e outros devido a lesões tubulares
Anormalidades detectadas por exame histológico
Anormalidades estruturais detectadas por exame de imagem
História do transplante renal
Ritmo de filtração glomerular diminuído
RFG < 60 mL / min por 1,73 m^2 (categorias de RFG G3a-G5)

DRC = doença renal crônica; EUA = excreção urinária de albumina; RAC = relação albumina-creatinina; RFG = ritmo de filtração glomerular.

Adaptado: KDIGO, 2013

A albuminúria e a proteinúria consistem nos principais marcadores laboratoriais de lesão do parênquima renal. A avaliação destes marcadores pode ser realizada em amostra de urina coletada durante 24 horas ou em amostra de urina isolada normalizada pela creatinina urinária. A relação albumina/creatinina (ou proteínas totais/creatinina) tem sido mais recomendada por ser um método menos sujeito a erros de coleta. A elevação da EUA deve ser confirmada em pelo menos duas de três coletas, em um período de três a seis meses (Alves, 2004).

Vários fatores podem interferir na determinação da albuminúria e, portanto, devem ser considerados durante a realização do exame. Dentre os fatores que podem elevar os níveis de albuminúria destacam-se o mau controle glicêmico e a hipertensão arterial não controlada, a presença de infecção do trato urinário, a prática de exercício físico intenso antes da coleta, a obesidade mórbida, a insuficiência cardíaca congestiva descompensada, a presença de doença infecciosa aguda ou febre, a sobrecarga protéica ou hídrica, a menstruação e a gestação. Nos pacientes com DM1, a triagem para albuminúria deve ser realizada a partir de cinco anos do diagnóstico do DM, ou antes, em pacientes persistentemente descompensados ou na adolescência. Em pacientes com DM2, a albuminúria deve ser pesquisada logo após o diagnóstico do DM (Murussi *et al*., 2008).

O aumento da EUA na nefropatia diabética ocorre principalmente devido à lesão glomerular resultante da deposição de proteínas glicadas. O aumento da pressão intraglomerular, a perda de glicosaminoglicanos carregados negativamente na membrana basal e o aumento do tamanho dos poros nesta membrana contribuem para a albuminúria. As anormalidades histológicas incluem o espessamento da membrana basal glomerular, o acúmulo de matriz mesangial e o aumento do número de células mesangiais. Com a progressão da doença renal, há uma forte associação entre expansão mesangial e declínio do RFG (Marshall, 2004; Strasinger e Lorenzo, 2009).

Alterações no interstício tubular, incluindo espessamento da membrana basal tubular, atrofia tubular, fibrose intersticial e esclerose vascular, também estão presentes no indivíduo com nefropatia. O espessamento intersticial se correlaciona com a redução do RFG, albuminúria e expansão mesangial. Além disso, a morfologia dos podócitos está anormal e pode haver perda destas células. Os podócitos fornecem um suporte estrutural para os capilares glomerulares, atenuam o aumento da pressão intraglomerular e constituem na última barreira de passagem das proteínas através do glomérulo. De modo semelhante à membrana basal, os podócitos são revestidos por moléculas carregadas negativamente, as quais auxiliam na repulsão das proteínas aniônicas, como a albumina. Assim, alterações na morfologia e no número de podócitos também podem contribuir para a albuminúria e a glomeruloesclerose na nefropatia diabética (Marshall, 2004).

Várias alterações metabólicas e hemodinâmicas induzidas pela hiperglicemia, incluindo a formação dos AGEs, a geração de espécies reativas do oxigênio e a ativação da proteína quinase C, da via poliol e do sistema renina-angiotensina, podem contribuir para o desenvolvimento e progressão da nefropatia diabética (Yamagishi e Matsui, 2010). Além disso, vários marcadores inflamatórios e pró-coagulantes, como IL-6, TNF-α, VCAM-1, ICAM-1, fibrinogênio, FVW, fator VIII, Dímero D e fator tecidual, têm sido associados com o declínio da função renal (Dubin *et al*., 2011; Sahakyan *et al*., 2010; Keller *et al*., 2008).

1.2.1 Estimativa do ritmo de filtração glomerular

O método padrão-ouro para o cálculo do RFG se baseia na determinação da depuração de substâncias radioativas, como ^{51}Cr-EDTA,^{99m}Tc-DTPA e ^{125}I-

iotalamato, ou de compostos não radioativos, como inulina, ioexol e iotalamato. A utilização destes marcadores exógenos é onerosa, pouco prática e invasiva, de modo que na prática clínica, o RFG é estimado através da utilização de um marcador endógeno, a creatinina (Kirsztajn, 2007).

A creatinina tem sido utilizada para avaliar a função renal há pelo menos 75 anos. A sua concentração no plasma depende do balanço entre a sua produção e a sua excreção. A creatinina plasmática é produzida pelas células musculares esqueléticas como um metabólito final do metabolismo energético, e também pode ser gerada, em menor extensão, pela absorção intestinal da creatinina derivada dos alimentos. A excreção da creatinina é realizada pelos rins, sendo que ela é livremente filtrada pelos glomérulos renais e, em pequena proporção, é secretada pelos túbulos renais. Portanto, a concentração plasmática de creatinina depende não apenas da função renal, mas também da dieta e da massa muscular, a qual varia de acordo com o sexo e a idade (Cirillo, 2010).

Para contornar estes interferentes da creatinina plasmática, a função renal pode ser avaliada através do cálculo da depuração da creatinina (DEC) corrigida pela superfície corporal (Quadro 4), a qual fornece uma estimativa do RFG e se correlaciona melhor com a função renal do que a creatinina plasmática. Contudo, o cálculo da DEC envolve a coleta de urina durante um período de 24 horas, a qual é pouco confiável, já que muitas vezes é realizada de modo inadequado pelo paciente (Bastos *et al.*, 2010).

Assim, foram desenvolvidas equações baseadas nos níveis séricos de creatinina, as quais incluem outras variáveis, como idade, sexo, raça e superfície corporal, para estimar o RFG. Na prática clínica, as equações de Cockcroft-Gault e do estudo Modificação Dietética na Doença Renal (MDRD) são as mais utilizadas (Cockcroft e Gault, 1976; Levey *et al.*, 1999; Levey *et al.*, 2000) (Quadro 4). Estas equações possuem a vantagem de superar as limitações da creatinina plasmática e da DEC, sem aumento de custos e tempo para avaliar a função renal. Contudo, as predições fornecidas por estas equações representam uma estimativa aproximada do RFG, não fornecendo o seu verdadeiro valor. Deste modo, todas elas possuem algumas desvantagens, não existindo uma equação ideal para estimar o RFG (Cirilo, 2010).

Quadro 4 – Equações baseadas na creatinina utilizadas para estimar o ritmo de filtração glomerular (RFG)

Depuração da creatinina (DEC)

$$DEC\ (mL/min) = \frac{\text{creatinina na urina (mg/dL)}}{\text{creatinina plasmática (mg/dL)}} \times \text{volume urinário por min (mL/min)}$$

Correção da DEC pela superfície corporal:

$$RFG\ (mL/min/1{,}73m^2) = \frac{\text{DEC (mL/min) x 1,73}}{\text{superfície corporal } (m^2)}$$

Equação de Cockcroft-Gault:

$$DEC\ (mL/min) = \frac{\text{(140 – idade em anos) x (peso em kg)}}{\text{72 x creatinina plasmática em mg/dL}} \times 0{,}85\ \text{(se mulher)}$$

Correção da DEC pela superfície corporal:

$$RFG\ (mL/min/1{,}73m^2) = \frac{\text{DEC (mL/min) x 1,73}}{\text{superfície corporal } (m^2)}$$

Equação do estudo MDRD (completa):

$$RFG\ (mL/min/1{,}73m^2) = 170 \times \text{creatinina plasmática } (mg/dL)^{-0{,}999} \times \text{idade (anos)}^{-0{,}176} \times 0{,}762\ \text{(se mulher)} \times 1{,}18\ \text{(se negro)} \times \text{uréia plasmática } (mg/dL)^{-0{,}17} \times \text{albumina plasmática } (g/dL)^{+0{,}318}$$

Equação do estudo MDRD (simplificada):

$$RFG\ (mL/min/1{,}73m^2) = 186 \times \text{creatinina plasmática } (mg/dL)^{-1{,}154} \times \text{idade (anos)}^{-0{,}203} \times 0{,}742\ \text{(se mulher)} \times 1{,}212\ \text{(se negro)}$$

Equação do estudo CKD-EPI :

Homens:

Creatinina sérica ≤ 0,9 mg/dL: $RFG\ (mL/min/1{,}73m^2) = \alpha \times [\text{creatinina no soro } (mg/dL)/0{,}9]^{-0{,}411} \times (0{,}993)^{\text{idade (anos)}}$

Creatinina sérica > 0,9 mg/dL: $RFG\ (mL/min/1{,}73m^2) = \alpha \times [\text{creatinina no soro } (mg/dL)/0{,}9]^{-1{,}209} \times (0{,}993)^{\text{idade (anos)}}$

Mulheres:

Creatinina sérica ≤ 0,7 mg/dL: $RFG\ (mL/min/1{,}73m^2) = \alpha \times [\text{creatinina no soro } (mg/dL)/0{,}7]^{-0{,}329} \times (0{,}993)^{\text{idade (anos)}}$

Creatinina sérica > 0,7mg/dL: $RFG\ (mL/min/1{,}73m^2) = \alpha \times [\text{creatinina no soro } (mg/dL)/0{,}7]^{-1{,}209} \times (0{,}993)^{\text{idade (anos)}}$

α = 141 para homens brancos, α = 144 para mulheres brancas, α = 163 para homens negros, α = 166 para mulheres negras

Adaptado: Cirilo, 2010; Levey *et al.*, 1999

A equação de Cockcroft-Gault estima a DEC, sendo necessário corrigir o resultado pela superfície corporal. Como a DEC é geralmente maior do que o RFG devido à secreção tubular da creatinina, a equação de Cockcroft-Gault tende a fornecer um valor maior do RFG do que a equação MDRD (Cirillo, 2010). Geralmente, esta secreção contribui relativamente pouco para superestimar a DEC, mas com o agravamento da doença renal e a redução da creatinina filtrada, a secreção tubular da creatinina aumenta e se torna um componente mais significativo da DEC (Massey, 2004).

A equação MDRD estima o próprio RFG, contudo, ela não é muito precisa para estimar este índice em indivíduos que apresentam a função renal normal, já que ela foi desenvolvida a partir de um estudo que incluiu apenas indivíduos com doença renal (Cirillo, 2010). Esta equação tende a subestimar o RFG de pessoas com função renal normal (MacIsaac *et al.*, 2011).

Além disso, o grupo de estudo Colaboração Epidemiológica da Doença Renal Crônica (CKD-EPI – *The Chronic Kidney Disease Epidemiology Collaboration*) desenvolveu uma nova equação para estimar o RFG baseada na creatinina sérica (Levey *et al.*, 2009) (Quadro 4). Esta equação foi elaborada a partir de um estudo que envolveu tanto pacientes com RFG reduzido quanto indivíduos com RFG dentro da faixa da normalidade, com o objetivo de superar a limitação da equação MDRD (Stevens *et al.*, 2010; Cirillo, 2010). A inclusão de indivíduos com e sem doença renal no estudo possibilitou o desenvolvimento de uma equação que apresenta uma maior precisão para estimar o RFG, um melhor valor preditivo do risco de progressão da DRC e que proporciona menos diagnósticos falso-positivos (Stevens *et al.*, 2010; Levey *et al.*, 2010). Atualmente, as novas Diretrizes da National Kidney Foundation (KDIGO, 2013) recomendam que a equação CKD-EPI seja empregada para estimar o RFG. Contudo, esta equação ainda precisa ser validada em diferentes grupos de pacientes e em diferentes populações (Stevens *et al.*, 2013).

1.2.2 Cistatina C

Determinações precisas do RFG e o reconhecimento precoce da disfunção renal são essenciais para o acompanhamento dos pacientes diabéticos, já que estes possuem um risco elevado de desenvolver DRC (Murussi *et al.*, 2008; Marshall, 2004). Deste modo, vários marcadores para a avaliação da disfunção

renal têm sido propostos. Um marcador endógeno bastante promissor para a avaliação do RFG consiste na cistatina C (Massey, 2004).

A cistatina C é uma proteína não glicosilada de baixo peso molecular (13,3 kDa) pertencente à família das cisteinoproteases. Ela é sintetizada por todas as células nucleadas a uma taxa de produção constante, podendo ser encontrada em vários fluidos biológicos, como soro, líquido seminal e líquido cefalorraquidiano (Hawkins, 2011; Murussi *et al.*, 2008). A cistatina C é livremente filtrada pelos glomérulos renais devido ao seu pequeno tamanho e carga positiva. Ao contrário da creatinina, ela não é secretada pelos túbulos renais, embora seja reabsorvida. Uma vez reabsorvida, ela é metabolizada pelas células epiteliais dos túbulos renais e não retorna à circulação sanguínea (Massey, 2004).

Fatores como processos inflamatórios e infecciosos não alteram os níveis plasmáticos da cistatina C. Além disso, não há uma variação significativa da faixa de referência para homens e mulheres, já que sua produção não depende da massa muscular (Martins *et al.*, 2003). A cistatina C também tem se mostrado melhor do que a creatinina para avaliar a função renal de populações idosas e pediátricas, pois a massa muscular reduzida presente nestes indivíduos não afeta os níveis da cistatina C, mas pode resultar em níveis plasmáticos menores de creatinina, os quais não refletem o verdadeiro RFG (Massey, 2004).

Os métodos baseados na cistatina C para estimar o RFG têm se mostrado iguais ou superiores aos métodos baseados na creatinina (MacIsaac *et al.*, 2011; Murussi *et al.*, 2008). Uma meta-análise de 49 estudos e um total de 4.492 indivíduos demonstrou que a cistatina C é um melhor preditor do RFG do que a creatinina (Dharnidharka *et al.*, 2002).

Alguns estudos ainda têm sugerido que a cistatina C é superior à DEC quando há disfunção renal subclínica, possibilitando a detecção precoce do declínio da função renal em pacientes diabéticos e não diabéticos (Massey, 2004; Perkins e Krolewski, 2009; Tan *et al.*, 2002). Pucci e cols. (2007) avaliaram a função renal de 288 pacientes diabéticos tipo 1 e tipo 2 através da determinação dos níveis plasmáticos de cistatina C e creatinina, e do cálculo do RFG através das equações de Cockcroft-Gault e MDRD, e verificaram que, em comparação com a depuração do iohexol, a cistatina C plasmática consistiu em um melhor marcador para a detecção precoce do declínio da função renal do que a creatinina plasmática e as equações baseadas na creatinina. Premaratne e cols. (2008) também observaram

que, em comparação com a depuração plasmática do ^{99m}Tc-DTPA, a estimativa do RFG baseada na cistatina C foi mais precisa do que o RFG calculado através das equações de Cockcroft-Gault e MDRD, para a detecção do declínio da função renal em pacientes diabéticos tipo 1.

Além disso, a cistatina C tem se mostrado melhor preditor da doença renal terminal e de eventos cardiovasculares nos pacientes diabéticos. Krolewski e cols. (2012) classificaram pacientes diabéticos tipo 1 e tipo 2 nos estágios 1 a 3 da DRC, através de equações baseadas na creatinina e na cistatina C para estimar o RFG, e os acompanharam durante 10 anos para verificar a ocorrência de doença renal terminal. Eles observaram que os pacientes classificados em estágio mais avançado da DRC pela equação baseada na cistatina C do que pelas equações baseadas na creatinina de fato apresentaram um risco significativamente maior de desenvolver doença renal terminal, enquanto que aqueles classificados em um estágio menos avabçado apresentaram um risco significativamente menor.

Schottker e cols. (2012) avaliaram o risco de doença cardiovascular em pacientes diabéticos com DRC, a qual foi definida através de equações que estimam o RFG com base na creatinina e com base na cistatina C. Eles verificaram que apenas a definição de DRC com base na cistatina C consistiu em preditor independente do risco de eventos cardiovasculares nos pacientes diabéticos, sugerindo que a equação baseada na cistatina C deve apresentar uma melhor utilidade clínica para predição do risco cardiovascular do que as equações baseadas na creatinina.

Várias equações têm sido desenvolvidas para estimar o RFG com base nos níveis plasmáticos de cistatina C (Quadro 5). Em geral, independente da equação utilizada, a precisão é maior do que a das equações baseadas na creatinina. Contudo, a determinação laboratorial da cistatina C é onerosa e os métodos baseados na cistatina C ainda carecem de mais estudos e de padronização antes de serem introduzidos na prática clínica (MacIsaac *et al*., 2011).

Quadro 5: Equações baseadas na cistatina C (mg/L) para estimar o ritmo de filtração glomerular

Equação	Autor
78 x (1/cistatina C) + 4	Le Bricon *et al.*, 2000
(87,1/cistatina C) – 6,87	Tan *et al.*, 2002
log (RFG) = 1,962 + [1,123 x log (1/cistatina C)]	Filler *et al.*, 2003
$77,24 \times (\text{cistatina C})^{-1,2623}$	Larsson *et al.*, 2004
-4,32 + (80,35 x 1/cistatina C)	Hoek *et al.*, 2004
$86,49 \times \text{cistatina C}^{-1,686} \times 0,948$ (se sexo feminino)	Grubb *et al.*, 2005
100/cistatina C	Perkins *et al.*, 2005
$66,8/\text{cistatina C}^{1,30}$	Rule *et al.*, 2006
(84,6/cistatina C) - 3,2	MacIsaac *et al.*, 2006
$79,901 \times \text{cistatina C}^{-1,4389}$	Flodin *et al.*, 2007
$74,835/\text{cistatina C}^{1,333}$	Beauvieux *et al.*, 2007
$127,7 \times \text{cistatina C}^{-1,17} \times \text{idade}^{-0,13} \times 0,91$ (se sexo feminino) x 1,06 (se negro)	Stevens *et al.*, 2008
$177,6 \times (\text{creatinina}/88.4)^{-0,65} \times \text{cistatina C}^{-0,57} \times \text{idade}^{-0,20} \times 0,82$ (se sexo feminino)	Stevens *et al.*, 2008
(100/cistatina C) - 14	Tidman *et al.*, 2008

Adaptado: Iliadis *et al.*, 2011; White *et al.*, 2005

1.2.3 Nefropatia diabética e aterosclerose

A associação entre a nefropatia diabética e o aumento no risco para o desenvolvimento de doenças ateroscleróticas é bem conhecida (Hahr e Molitch, 2010). No início dos anos 80, foi verificado que os pacientes diabéticos tipo 1 e tipo 2 com proteinúria apresentavam aumento de 3 a 4 vezes na mortalidade, principalmente decorrente de eventos cardiovasculares (Gross *et al.*, 2007).

Kim e cols. (2007) avaliaram a presença de aterosclerose nas artérias coronárias e aórtica de pacientes diabéticos tipo 1 com e sem nefropatia diabética através de ressonância magnética, e verificaram maior prevalência de aterosclerose nas artérias coronárias dos pacientes diabéticos com nefropatia do que daqueles sem nefropatia. O Estudo Multinacional WHO de Doença Vascular no Diabetes

confirmou a importância da proteinúria como preditor para a mortalidade decorrente de doença cardiovascular, infarto agudo do miocárdio fatal e não-fatal e acidente vascular cerebral nos pacientes diabéticos tipo 1 e tipo 2 (Fuller *et al.*, 2001).

Alguns estudos têm demonstrado que níveis moderadamente aumentados de albuminúria predizem o desenvolvimento da doença vascular aterosclerótica nos pacientes diabéticos, consistindo em um importante fator de risco cardiovascular para estes pacientes (Deckert *et al.*, 1996; Karnib e Ziyadeh, 2010). Foi verificado que pacientes com DM1 e níveis normais de albuminúria possuem um risco 2 a 4 vezes maior para o desenvolvimento de doença cardiovascular, sendo que este risco é 20 a 40 vezes maior naqueles com EUA moderadamente aumentada. Além disso, a sobrevivência média do paciente diabético após o surgimento de proteinúria é de sete anos, sendo que esta mortalidade aumentada se deve principalmente à morte por eventos coronarianos, e não propriamente à insuficiência renal (Naidoo, 2002).

Dubin e cols. (2011) observaram uma associação entre um RFG reduzido e níveis plasmáticos elevados de vários marcadores de hipercoagulabilidade, como trombomodulina, fator tecidual, Dímero D, FVW, fator VIII, complexo plasmina-α2 antiplasmina, inibidor da via do fator tissular, PAI1 e fibrinogênio, o que indica que a alteração da hemostasia pode ser um mecanismo pelo qual a função renal reduzida eleva o risco de doenças cardiovasculares.

Por outro lado, a ativação plaquetária e a hipercoagulabilidade parecem também contribuir para a patogênese das complicações microvasculares no paciente diabético e para o desenvolvimento da nefropatia diabética (Wakabayashi e Masuda, 2009; Omoto *et al.*, 1999).

1.3 Dímero D e Hipercoagulabilidade

A fibrinólise consiste na degradação da fibrina mediada pela plasmina. O sistema fibrinolítico é composto por diversas proteínas (proteases séricas e inibidores) que regulam a geração de plasmina, uma enzima ativa produzida a partir de uma pró-enzima inativa, o plasminogênio, que tem por função degradar a fibrina (Franco, 2004). O Dímero D é um produto de degradação da fibrina derivado exclusivamente da fibrina e não do fibrinogênio, sendo assim específico para

mostrar a atividade fibrinolítica secundária à formação de fibrina, consistindo em um importante marcador de hipercoagulabilidade (Lourenço, 2004).

Alguns estudos têm demonstrado uma associação entre níveis aumentados de Dímero D e o surgimento de complicações micro e macrovasculares em pacientes diabéticos. Em um estudo envolvendo crianças e adolescentes com DM1 e DM2, El Asrar e cols. (2012) observaram um aumento dos níveis plasmáticos do Dímero D nas crianças que apresentavam complicações microvasculares e uma correlação positiva entre os níveis do Dímero D e a EUA. Long e cols. (2001) ainda verificaram que pacientes diabéticos tipo 2 com proteinúria apresentaram níveis plasmáticos maiores de Dímero D do que os pacientes sem proteinúria.

Em um estudo envolvendo pacientes diabéticos tipo 2, Wakabayashi e Masuda (2009) verificaram uma associação entre o Dímero D, a presença de albuminúria moderadamente aumentada e a espessura íntimo-medial das artérias carótidas, sugerindo que o estado de hipercoagulabilidade possa estar envolvido tanto com a progressão da aterosclerose, quanto com a disfunção renal nos pacientes diabéticos.

Soares e cols. (2010) avaliaram os níveis plasmáticos de Dímero D em mulheres diabéticas tipo 2, as quais foram classificadas de acordo com a espessura íntimo-medial das artérias carótidas, e verificaram que o grupo de mulheres diabéticas com placa nas carótidas apresentou níveis plasmáticos maiores de Dímero D do que os demais grupos, sugerindo uma associação entre os níveis plasmáticos de Dímero D e a formação de placa aterosclerótica no DM. Outros estudos ainda demonstraram que há um aumento progressivo dos níveis plasmáticos de Dímero D com a progressão do DM e das complicações cardiovasculares e que os níveis plasmáticos de Dímero D podem ser úteis para o diagnóstico de pacientes diabéticos tipo 2 que possuem elevado risco de aterotrombose (Nwose *et al*., 2007; Krupinski *et al*., 2007).

1.4 Fator von Willebrand, ADAMTS13 e disfunção endotelial

O FVW é uma glicoproteína multimérica composta por subunidades idênticas de 270 kDa. Ligações dissulfeto unem as subunidades formando dímeros de aproximadamente 500 kDa, e este mesmo tipo de ligação une os dímeros formando multímeros de vários tamanhos que podem exceder 10.000 kDa (Reininger, 2008).

Os multímeros do FVW são armazenados nos corpos de Weibel-Palade das células endoteliais e nos grânulos alfa dos megacariócitos e de suas plaquetas derivadas (Bowen e Collins, 2006). O FVW pode ser secretado pelas células endoteliais através de uma via constitutiva, em que as moléculas são liberadas diretamente após a síntese, ou através de uma via regulada, em que as moléculas armazenadas são liberadas após a estimulação por secretagogos, como a histamina, a trombina e a fibrina (Reininger, 2008). Nas plaquetas circulantes somente a via regulada de secreção do FVW atua efetivamente *in vivo*. Assim, o FVW circulante no plasma é essencialmente todo derivado das células endoteliais, já que as plaquetas liberam o conteúdo dos seus grânulos alfa somente quando ativadas (Ruggeri, 2007).

O FVW participa da hemostasia primária e no processo de coagulação, onde atua como um transportador do fator VIII, impedindo a degradação deste fator pela proteína C e aumentando consideravelmente a sua meia-vida plasmática. O FVW é importante para a adesão das plaquetas aos locais de injúria vascular, onde é mediador inicial da progressão da formação do trombo no local da lesão endotelial por meio de interações específicas com o colágeno subendotelial e os receptores das plaquetas (Jenkins e O'Donnell, 2006).

Na presença de lesão vascular, a qual pode ser decorrente de um trauma ou de um processo degenerativo crônico, como a aterosclerose, o endotélio libera o FVW, o qual interage com o colágeno subendotelial, e a seguir, a GPIb/IX plaquetária liga-se ao FVW. Essa ligação possui uma rápida velocidade de associação, permitindo que a adesão plaquetária ocorra em vasos onde o sangue circula em alta velocidade. Entretanto, a interação entre a GPIb/IX e o FVW também possui uma alta taxa de dissociação, de modo que as plaquetas aderidas à parede vascular movem-se constantemente na direção do fluxo sanguíneo. Após a ativação plaquetária, a GPIIb/IIIa torna-se capaz de se ligar ao FVW, propiciando uma adesão plaquetária irreversível ao subendotélio (Morelli, 2004) (Figura 1).

A adesão das plaquetas ao subendotélio desencadeia a ativação plaquetária, o que resulta na ativação da GPIIb/IIIa plaquetária e no recrutamento de mais plaquetas para junto da lesão vascular. O FVW, e principalmente o fibrinogênio solúvel, promoverão a agregação plaquetária, formando pontes entre as plaquetas adjacentes através da ligação com a GPIIb/IIIa (Morelli, 2004) (Figura 2).

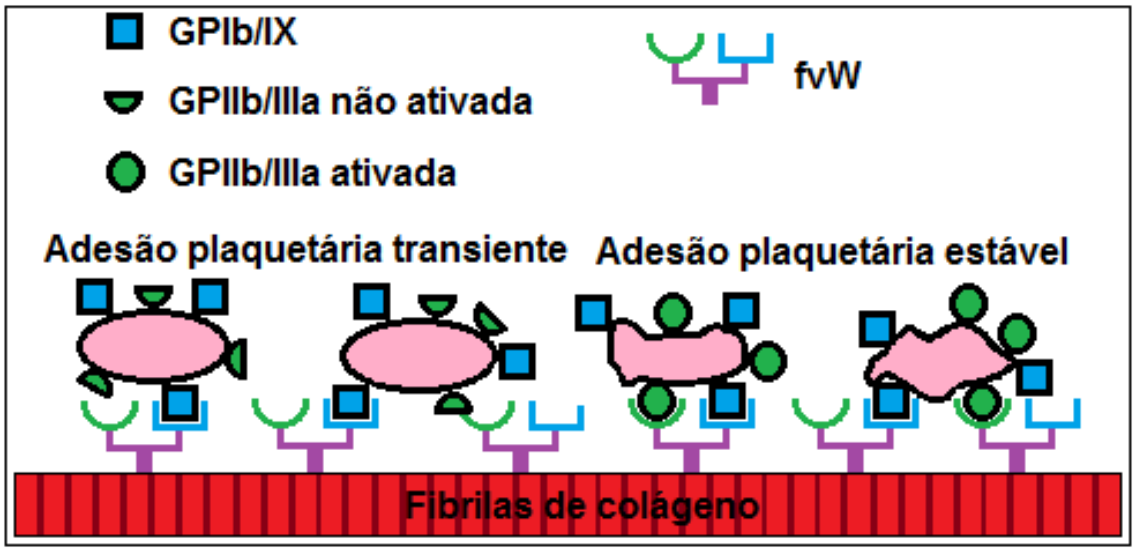

Figura 1 – Esquema do mecanismo de adesão plaquetária na circulação sanguínea. Quando há lesão vascular, as plaquetas inicialmente se aderem transientemente ao fator von Willebrand (FVW) através do receptor plaquetário GPIb/IX. Este contato reduz significativamente o movimento das plaquetas e promove uma adesão transiente destas ao subendotélio, o que resulta na ativação do receptor plaquetário GPIIb/IIIa, o qual então se liga ao seu sítio de ligação no FVW, propiciando uma adesão plaquetária irreversível ao subendotélio.

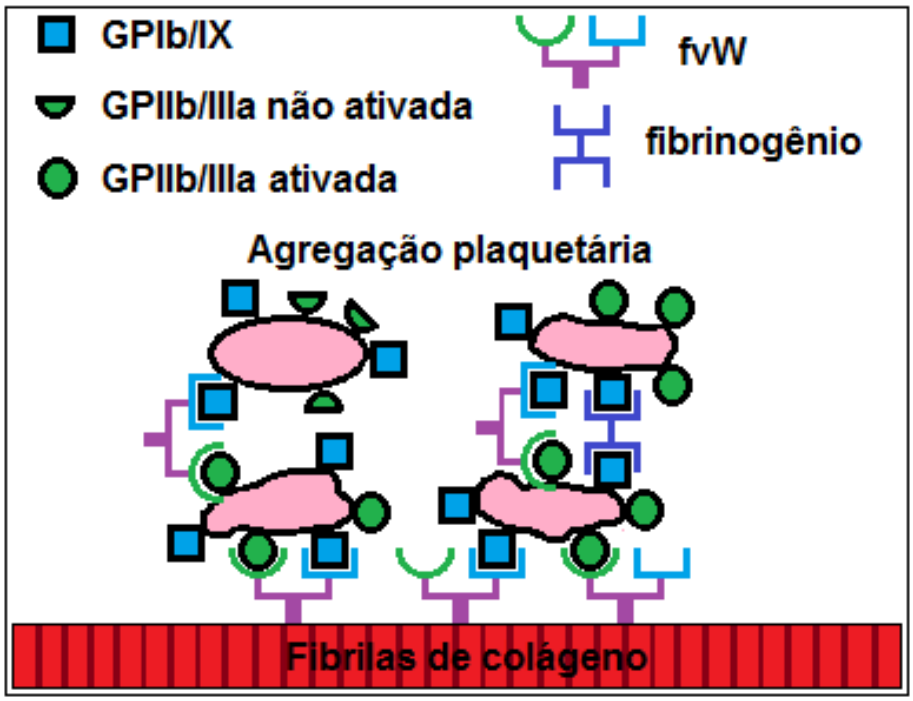

Figura 2 – Esquema do mecanismo de agregação plaquetária na circulação sanguínea. Após uma adesão estável, as plaquetas são ativadas, secretam o conteúdo dos seus grânulos e se ligam às proteínas plasmáticas, como o fibrinogênio e o fator von Willebrand (FVW), os quais formam o substrato no qual plaquetas adicionais são agregadas para formar um trombo.

A ADAMTS13, uma desintegrina e metaloproteinase com domínio trombospondina tipo 1, dependente de Zn^{2+}/Ca^{2+}, é uma protease capaz de clivar a ligação peptídica entre a tirosina na posição 1605 e a metionina na posição 1606 no domínio central A2 do FVW (Bowen e Collins, 2006). Ela é secretada constitutivamente como uma enzima ativa de 190 kDa e possui uma meia-vida plasmática de aproximadamente 2 a 3 dias (Crawley *et al.*, 2005). A ADAMTS13 é sintetizada principalmente pelo fígado, mas também é expressa nas plaquetas, nas células endoteliais e nos rins (Manea *et al.*, 2010; Turner *et al.*, 2006; Suzuki *et al.*, 2004).

Alguns estudos têm demonstrado que a ADAMTS13 pode ser clivada por proteases plasmáticas, como a trombina, a plasmina e a elastase dos granulócitos, o que resulta em uma inativação desta enzima. Por outro lado, a trombomodulina pode inibir a inativação da ADAMTS13 mediada pela trombina (Ono *et al.*, 2006; Crawley *et al.*, 2005). Estes achados podem apresentar importância fisiológica no sítio de lesão vascular, onde a inativação da ADAMTS13 pela trombina e pela plasmina poderia favorecer o recrutamento de plaquetas pelo FVW, enquanto que a trombomodulina no endotélio não lesado ao redor do sítio de lesão vascular poderia limitar a propagação da agregação plaquetária (Bowen e Collins, 2006). Por outro lado, níveis plasmáticos elevados destas proteases, o que ocorre em condições clínicas em que há um estado de hipercoagulabilidade e inflamação, podem estar associados com uma maior degradação da ADAMTS13 e com uma menor atividade desta enzima (Ono *et al.*, 2006).

Como os níveis plasmáticos do FVW aumentam quando as células endoteliais são lesadas, o FVW tem sido considerado um indicador de disfunção endotelial. Há uma associação bem estabelecida entre níveis plasmáticos elevados do FVW e o desenvolvimento de doença arterial coronariana, doença vascular periférica e eventos cerebrovasculares isquêmicos. Além disso, o FVW parece ser um marcador do risco aumentado para re-infarto e mortalidade nos pacientes com angina e nos sobreviventes após um infarto do miocárdio (Lip e Blann, 1997).

Níveis plasmáticos elevados de FVW têm sido encontrados nos pacientes diabéticos tipo 1 e tipo 2, sendo que o FVW parece ser um marcador preditivo da nefropatia diabética, sugerindo que a disfunção endotelial precede o desenvolvimento da microangiopatia diabética (Kessler *et al.*, 1998; Porta *et al.*, 1991; Targher *et al.*, 2005). Contudo, com o desenvolvimento das complicações

crônicas do diabetes, os níveis plasmáticos de FVW aumentam com a gravidade da nefropatia e parecem consistir em um fator de risco para o desenvolvimento da macroangiopatia diabética nestes pacientes (Jensen, 1989; Kessler *et al*., 1998).

Chan e cols. (2003) verificaram que a atividade do FVW está aumentada nos pacientes diabéticos tipo 1 que possuem níveis moderadamente ou acentuadamente aumentados de albuminúria em relação aos pacientes diabéticos que possuem níveis normais de albuminúria, e que estes apresentam uma atividade aumentada do FVW quando comparados com os indivíduos não diabéticos. Em um estudo longitudinal, Stehouwer e cols. (1991) observaram que houve um aumento dos níveis plasmáticos do FVW nos pacientes diabéticos tipo 1 que apresentaram um aumento da EUA durante um período de 3 anos, indicando uma associação entre a disfunção endotelial e o desenvolvimento da nefropatia diabética. Além disso, foi verificado que o aumento dos níveis plasmáticos do FVW precedeu o aumento da EUA em aproximadamente 3 anos nesses pacientes, sugerindo que a disfunção endotelial precede e pode predizer o desenvolvimento da albuminúria nos pacientes diabéticos (Stehouwer *et al*., 1995).

Stehouwer e cols. (2002) acompanharam 328 pacientes diabéticos tipo 2 durante 9 anos e verificaram que o desenvolvimento longitudinal da albuminúria foi significativamente e independentemente determinado pelos níveis iniciais de FVW e de outros marcadores de disfunção endotelial e de inflamação, sugerindo uma interrelação entre a disfunção endotelial, a inflamação crônica e o desenvolvimento da disfunção renal no DM2. Fang e cols. (2005) também encontraram níveis plasmáticos do FVW elevados em pacientes diabéticos tipo 2 com alterações renais.

Em um estudo longitudinal, Stehouwer e cols. (1992) ainda observaram que a presença de níveis moderadamente aumentados de albuminúria no início do estudo foi associada com um risco aumentado de eventos cardiovasculares somente nos pacientes que apresentavam níveis plasmáticos do FVW acima da média. Assim, é possível sugerir que a disfunção vascular deve ser o ponto de ligação entre albuminúria e doença cardiovascular aterosclerótica no DM. Outros estudos com pacientes diabéticos tipo 2 também verificaram uma associação entre níveis elevados do FVW e o desenvolvimento de doenças cardiovasculares (Soares *et al*., 2011; Verkleij *et al*., 2010; Standl *et al*., 1996).

Skeppholm e cols. (2009) verificaram um aumento significativo dos níveis plasmáticos do FVW e uma redução significativa da atividade da ADAMTS13 nos

pacientes diabéticos. Crawley e cols. (2008) ainda observaram uma associação entre níveis plasmáticos elevados do FVW e reduzidos da ADAMTS13 e um aumento do risco de desenvolvimento de infarto agudo do miocárdio. Além disso, Matsukawa e cols. (2007) demonstraram que a razão entre os níveis plasmáticos do FVW e os níveis plasmáticos da ADAMTS13 consiste em um preditor significativo do desenvolvimento de eventos trombóticos após a ocorrência do infarto agudo do miocárdio.

Lu e cols. (2008) verificaram que os pacientes com doenças renais crônicas apresentavam níveis plasmáticos significativamente elevados do FVW e uma atividade significativamente reduzida da ADAMTS13 em relação aos indivíduos saudáveis. Em um estudo com pacientes diabéticos tipo 2, Tanigushi e cols. (2010) verificaram que os pacientes com proteinúria apresentaram níveis plasmáticos de ADAMTS13 significativamente menores do que os indivíduos saudáveis. Além disso, eles observaram uma correlação positiva entre os níveis plasmáticos de ADAMTS13 e o RFG e uma correlação negativa entre a relação FVW/ADAMTS13 e o RFG. Para avaliar a macroangiopatia diabética, estes autores determinaram o espessamento médio-intimal das artérias carótidas dos pacientes e observaram que esta se correlacionou negativamente com os níveis plasmáticos de ADAMTS13 e positivamente com a relação FVW/ADAMTS13, sugerindo que níveis plasmáticos reduzidos de ADAMTS13 estão associados com um risco aumentado para o desenvolvimento da macroangiopatia diabética. Rurali e cols. (2013) também observaram que uma atividade reduzida da ADAMTS13 está associada com um risco aumentado de disfunção renal e de eventos cardiovasculares em pacientes diabéticos tipo 2.

Através da técnica de *Western-blot*, Manea e cols. (2010) verificaram a presença de ADAMTS13 na urina de indivíduos que apresentavam disfunções renais, mas não detectaram esta enzima na urina de indivíduos saudáveis, indicando que a redução dos níveis plasmáticos da ADAMTS13 em pacientes que apresentam alterações na função renal possa ser decorrente da perda desta enzima na urina.

Estudos realizados por nosso grupo de pesquisa verificaram uma elevação dos níveis do FVW associada à uma redução significativa nos níveis plasmáticos da ADAMTS13 em gestantes com pré-eclâmpsia, as quais apresentam proteinúria significativa (Alpoim *et al.*, 2011). Portanto, um dos possíveis mecanismos

responsáveis pela redução dos níveis plasmáticos de ADAMTS13 nestas pacientes pode ser a perda desta enzima na urina. De forma similar, é possível supor que as alterações nos níveis plasmáticos de ADAMTS13 e o quadro de hipercoagulabilidade observados em pacientes diabéticos com nefropatia e proteinúria estejam associados à perda de ADAMST13 na urina.

Os pacientes diabéticos com nefropatia apresentam uma resposta inflamatória maior do que os pacientes diabéticos sem alterações renais, o que pode ser demonstrado por uma maior produção de citocinas inflamatórias e uma maior ativação dos leucócitos polimorfonucleares nos pacientes com nefropatia diabética (Taslipinar *et al*., 2011; Mastei e Adamiec, 2006). Shen e cols. (2011) observaram que os pacientes com DRC apresentaram níveis maiores do FVW e TNF-α do que os indivíduos saudáveis e uma menor atividade da ADAMTS13. Além disso, eles observaram que os níveis de TNF-α se correlacionaram positivamente com os níveis do FVW e negativamente com o RFG, o que sugere que a inflamação pode ser um importante fator contribuinte para a disfunção endotelial e o comprometimento renal. Em um estudo realizado *in vitro*, Bernardo e cols. (2004) verificaram que a IL-8 e o TNF-α estimularam significativamente a liberação de FVW pelas células endoteliais e que a IL-6 inibiu a clivagem do FVW pela ADAMTS13, indicando um possível papel das citocinas inflamatórias no desequílibrio FVW/ADAMTS13.

Cao e cols. (2008) demonstraram que algumas citocinas inflamatórias podem inibir a síntese da ADAMTS13 pelas células estreladas hepáticas e pelas células endoteliais. Assim, outra possível explicação para os níveis plasmáticos reduzidos de ADAMTS13 nos pacientes diabéticos com nefropatia seria uma menor síntese hepática e/ou renal desta enzima decorrente de uma maior produção de citocinas inflamatórias nestes pacientes.

Devido à maior ativação dos leucócitos polimorfonucleares, os pacientes diabéticos possuem níveis plasmáticos mais elevados da elastase produzida por estas células do que os indivíduos não diabéticos (Piwowar *et al*., 2000). Além disso, estes pacientes apresentam um estado de hipercoagulabilidade, o que resulta em níveis plasmáticos elevados de trombina e plasmina (Carr, 2001). Alguns estudos têm demonstrado que a ADAMTS13 pode ser clivada por proteases plasmáticas, como a trombina, a plasmina e a elastase dos granulócitos (Ono *et al*., 2006; Crawley *et al*., 2005). Portanto, considerando que ocorre uma acentuação da resposta inflamatória e do estado de hipercoagulabilidade nos pacientes diabéticos

com nefropatia, é possível sugerir que estes pacientes devem apresentar níveis plasmáticos maiores de trombina, plasmina e elastase dos granulócitos, as quais poderiam promover uma degradação proteolítica da ADAMTS13 no plasma, resultando em níveis plasmáticos reduzidos desta enzima.

Assim, é possível propor três potenciais mecanismos responsáveis pelos níveis plasmáticos reduzidos da ADAMTS13 nos pacientes com nefropatia diabética (Figura 3): i) perda da enzima na urina devido ao comprometimento da função renal; ii) síntese renal e/ou hepática reduzida da enzima devido à uma maior produção de citocinas inflamatórias; iii) degradação proteolítica da enzima por proteases plasmáticas, como trombina, plasmina e elastase dos granulócitos, cujos níveis plasmáticos estão elevados devido à acentuada resposta inflamatória e estado de hipercoagulabilidade destes pacientes (Manea *et al.*, 2010; Ono *et al.*, 2006; Crawley *et al.*, 2005; Bernardo *et al.* 2004;).

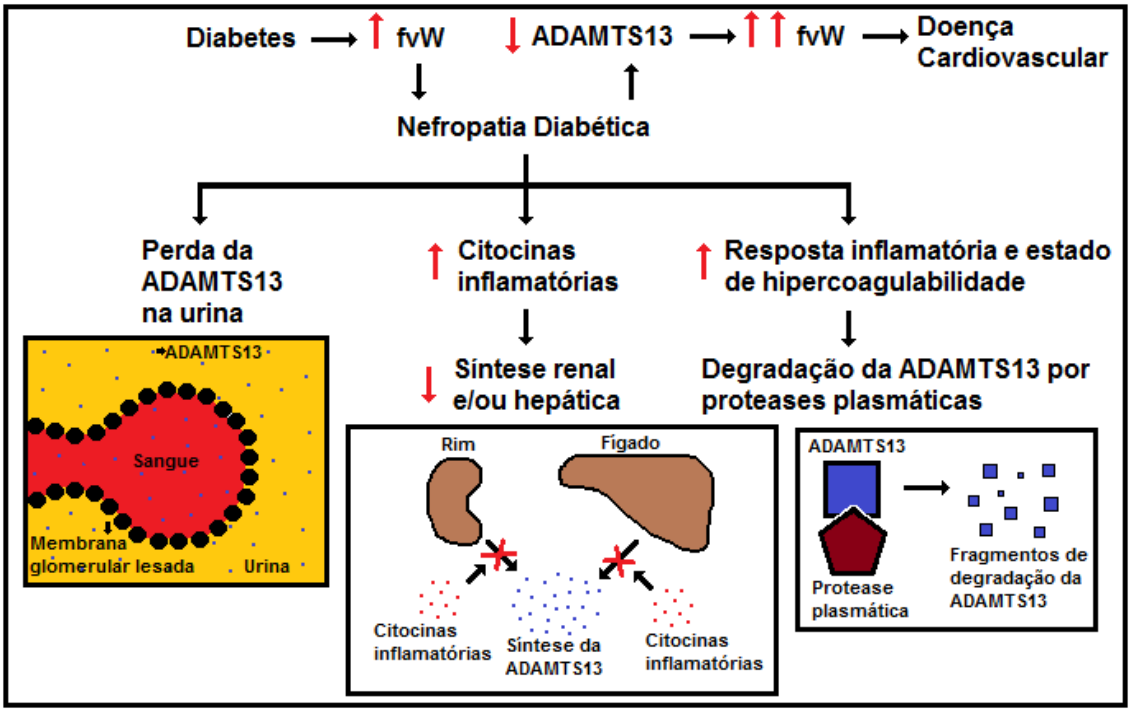

Figura 3 – Esquema dos potenciais mecanismos responsáveis pelos níveis plasmáticos reduzidos da ADAMTS13 nos pacientes com nefropatia diabética.

2 JUSTIFICATIVA

A nefropatia diabética consiste na causa mais comum de doença renal terminal em todo o mundo e em um fator de risco independente para doença cardiovascular (Marshall, 2004). O desenvolvimento de nefropatia é muito comum nos pacientes com DM1, sendo que a prevalência de proteinúria nestes pacientes pode chegar a 40% (Diretrizes SBD, 2013).

Os principais desfechos da DRC nos pacientes diabéticos são as suas complicações (anemia, acidose metabólica, desnutrição e alteração do metabolismo de cálcio e fósforo) decorrentes da perda funcional dos rins, a doença renal terminal e o óbito (principalmente por complicações cardiovasculares). Estes desfechos indesejados podem ser prevenidos ou retardados se a nefropatia for diagnosticada precocemente e as medidas nefro e cardioprotetoras implementadas o mais rápido possível (Bastos *et al.*, 2010).

Vários biomarcadores podem ser utilizados para a avaliação da função renal, como a creatinina, a ureia, a EUA, a cistatina C e o RFG, o qual pode ser estimado através de equações baseadas na creatinina e na cistatina C (Kirstajn, 2007). Contudo, todos estes biomarcadores apresentam limitações, de modo que ainda não existe um biomarcador ideal para a avaliação da função renal em diferentes grupos de pacientes (Levey *et al.*, 2014). Portanto, é importante a realização de estudos que avaliem a utilidade clínica dos biomarcadores de função renal, já estabelecidos na rotina clínica, no diagnóstico e acompanhamento dos pacientes com DM1.

Além disso, o estudo de novos analitos que estão associados com o declínio da função renal possui grande importância, já que pode levar à descoberta de biomarcadores de função renal mais eficazes ou que complementem os disponíveis. Novos biomarcadores que possibilitem o diagnóstico precoce da doença renal e do seu agravamento são muito promissores, já que podem contribuir para a adoção de medidas preventivas e terapêuticas adequadas para evitar o desenvolvimento da doença renal e retardar a sua evolução e o surgimento de complicações.

Recentemente, tem sido demonstrado que as anormalidades metabólicas decorrentes da hiperglicemia crônica, associadas com o estado inflamatório dos pacientes diabéticos, podem levar à lesão endotelial e ao desenvolvimento das complicações vasculares no DM. Desta forma, a disfunção endotelial juntamente com o estado de hipercoagulabilidade, podem indicar precocemente a evolução deste processo (Goldberg, 2009).

Assim, o estudo da associação entre biomarcadores de disfunção endotelial e de hipercoagulabilidade com diferentes níveis de função renal nos pacientes diabéticos tipo 1 possui extrema importância, já que estes biomarcadores podem ser promissores para a avaliação da função renal destes pacientes, apresentando um grande potencial para serem utilizados no diagnóstico e no acompanhamento da doença renal.

O presente trabalho será apresentado na forma de artigos, sendo os dois primeiros trabalhos de revisão e os três seguintes artigos originais com os resultados obtidos.

3 OBJETIVOS

3.1 Objetivo geral

Avaliar a relação entre biomarcadores de disfunção endotelial, FVW e ADAMTS13, e do biomarcador de hipercoagulabilidade, Dímero D, com diferentes níveis de função renal em pacientes com DM1.

3.2 Objetivos específicos

- Avaliar a função renal de pacientes com DM1 por meio da determinação dos níveis plasmáticos de creatinina, ureia e cistatina C, da estimativa do RFG através de equações baseadas na creatinina e na cistatina C, e da avaliação da EUA;
- Comparar o RFG estimado através de equações baseadas na creatinina e na cistatina C, com base na EUA;
- Determinar a atividade da ADAMTS13 e os níveis plasmáticos de FVW, ADAMTS13 e Dímero D e calcular as relações FVW/ADAMTS13 e ADAMTS13atividade/ADAMTS13Ag dos pacientes com DM1, comparando-os com com os níveis de função renal;
- Avaliar a associação entre os diferentes biomarcadores de função renal com os níveis plasmáticos de Dímero D;
- Avaliar quais biomarcadores hemostáticos estão independentemente associados com a presença de nefropatia no DM1.

4 DELINEAMENTO EXPERIMENTAL

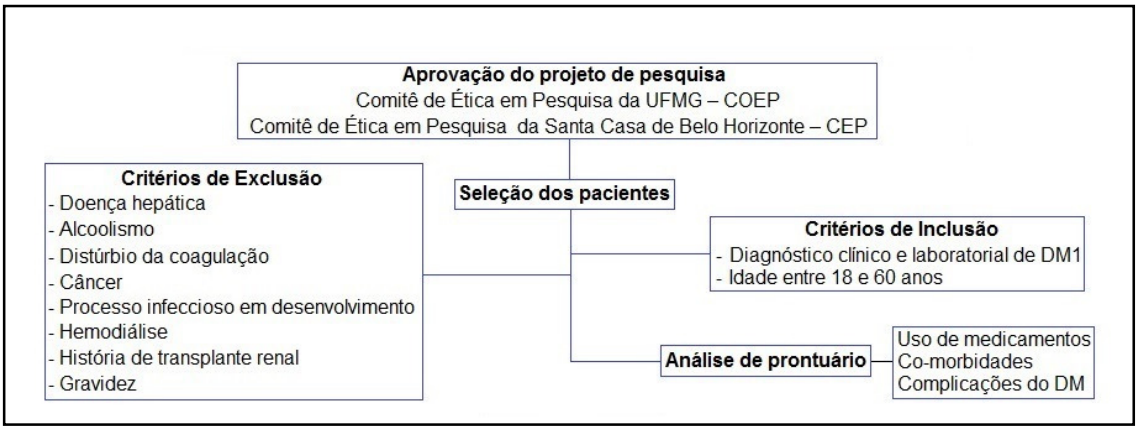

Figura 4 – Esquema da seleção dos pacientes para o estudo.

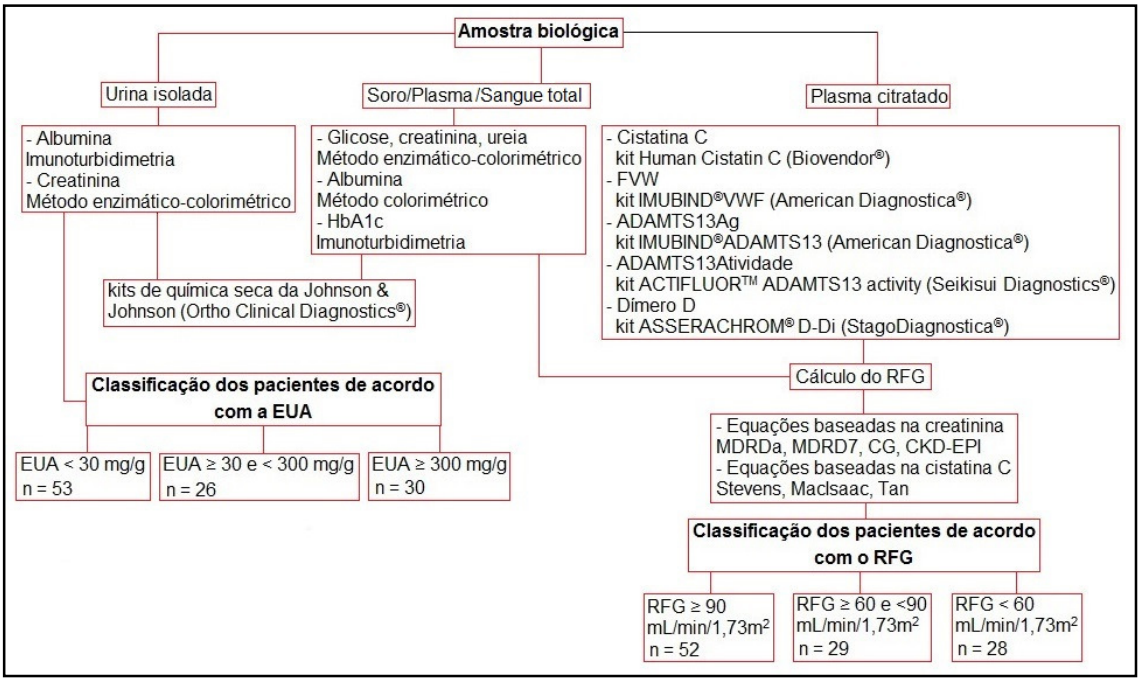

Figura 5 – Esquema das análises laboratoriais realizadas no estudo e da classificação dos pacientes.

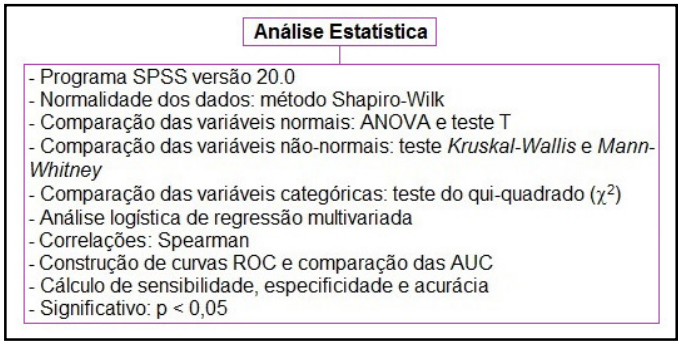

Figura 6 – Esquema da análise estatística realizada no estudo.

5 RESULTADOS

5.1 Artigo publicado

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Hypercoagulability and cardiovascular disease in diabetic nephropathy

- Caroline Pereira Domingueti,
- Luci Maria Sant'Ana Dusse,
- Maria das Graças Carvalho,
- Karina Braga Gomes,
- Ana Paula Fernandes

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Abstract

Diabetic nephropathy is the leading cause of end stage renal disease (ESRD) and an important risk factor for cardiovascular disease. Recent studies have shown that increased plasma levels of Von Willebrand factor (VWF) and reduced plasma levels of enzyme ADAMTS13 are associated with diabetic nephropathy and an increased risk of developing cardiovascular disease, suggesting that these markers of hypercoagulability may contribute to an increased risk of cardiovascular disease in diabetic patients with impaired renal function. However, it is still not clear whether VWF and ADAMTS13 are only markers of cardiovascular events or whether they play an active role in the development of these events. It is also unclear how renal injury may affect ADAMTS13 levels, leading consequently to hypercoagulability. The association of diabetic nephropathy, atherosclerotic cardiovascular disease and these hypercoagulability markers is discussed in this review. Insights on the role that renal dysfunction and other possible mechanisms may have in ADAMTS13 metabolism, leading to reduced levels of this enzyme and increased hypercoagulability are also presented.

Highlights

► Von Willebrand factor (VWF) is associated with diabetic nephropathy ► ADAMTS13 may contribute to risk of cardiovascular disease in diabetic nephropathy ► Renal injury may affect ADAMTS13 levels leading to hypercoagulability

Keywords

- Diabetic nephropathy;
- Von Willebrand factor;
- ADAMTS13;

- Cardiovascular disease;
- Hypercoagulability

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1. Introduction

Diabetes mellitus is one of the main risk factors for the development of chronic arterial obstructive disease, acute myocardial infarction, stroke and gangrene of the lower limbs **[1]**. Diabetic patients have a 2- to 4-fold increased risk of heart disease and stroke than people without diabetes. Cardiovascular complications are the leading cause of morbidity and mortality among diabetic patients, accounting for some 50% of all diabetes fatalities **[2]** and **[3]**.

Diabetic nephropathy is the most common cause of end stage renal disease (ESRD), contributing to approximately 45% of new cases, and is an independent risk factor for cardiovascular disease **[3]**. The classic definition of diabetic nephropathy is a progressive increase in urinary albumin excretion, leading to a decline in glomerular filtration, and eventually, an end stage renal failure. The earliest clinical manifestation of diabetic nephropathy is the presence of microalbuminuria, whose risk can be reduced through a strict control of blood glucose and blood pressure **[4]**.

Around 25% to 40% of type 1 and type 2 diabetic patients develop diabetic nephropathy within 20 to 25 years of the onset of diabetes **[5]**. Hyperglycemia, high blood pressure and genetic predisposition are the main risk factors for the development of nephropathy in these patients **[6]**.

Several metabolic and hemodynamic alterations induced by hyperglycemia, including the formation of advanced glycation end products (AGEs), the generation of reactive oxygen species and the activation of protein kinase C, polyol pathway and renin–angiotensin system, are considered to contribute to the development and progression of diabetic nephropathy **[5]**.

The association between diabetic nephropathy and heart disease is already well established. In the early 1980s, it was found that patients with type 1 and type 2 diabetes with proteinuria showed a 3- to 4-fold increased mortality, mainly due to cardiac events **[7]**. The WHO Multinational Study of Vascular Disease in Diabetes confirmed the importance of proteinuria, as predictor for cardiovascular disease mortality, fatal and non-fatal myocardial infarction and stroke in type 1 and type 2 diabetic patients **[8]**.Currently, the presence of microalbuminuria has been considered an important risk factor for developing atherosclerotic cardiovascular disease in diabetic patients **[9]** and **[3]**.

Some studies have shown that the high risk of cardiovascular disease in diabetic patients with nephropathy is associated to increased plasma levels of Von Willebrand factor (VWF) and reduced of ADAMTS13 **[10]**,**[11]** and **[12]**.

VWF is a glycoprotein that plays an important role in platelet thrombus formation, whereas ADAMTS13 is a proteolytic enzyme that is responsible for degradation of large multimers of VWF released in the plasma by endothelial cells and platelets **[13]**. Increased plasma levels of VWF, which reflect damage to endothelial cells and a hypercoagulability state, have been reported in atherosclerosis and diabetes **[11]**. Thus, VWF and ADAMTS13 seem to be important players on the interface between diabetic nephropathy, hypercoagulability and atherosclerotic cardiovascular

disease. The association of these hypercoagulability markers with the development of diabetic nephropathy and atherosclerotic cardiovascular disease is discussed in this review.

2. Von Willebrand factor

VWF is a multimeric glycoprotein composed by identical subunits of 270 kDa. Disulphide bonds link the subunits into dimers of approximately 500 kDa, and then, the dimers are linked into multimers of various sizes that may exceed 10.000 kDa. The VWF gene is located on the short arm of chromosome 12 and comprises 180 kb and 52 exons. VWF is synthesized only by endothelial cells and megakaryocytes **[14]**.

Newly synthesized VWF multimers are stored in the Weibel–Palade bodies of endothelial cells and in the alpha-granules of megakaryocytes and their platelet derivatives. VWF can be secreted by endothelial cells through a constitutive pathway, in which the molecules are released directly after synthesis, or through a regulated pathway, in which the stored molecules are released after stimulation by secretagogues, such as histamine, thrombin and fibrin **[13]**. In circulating platelets, only the regulated pathway of VWF secretion is effectively operative *in vivo*. Therefore, VWF in plasma is essentially from endothelial cell origin, since platelets release their alpha-granules content only when activated **[15]**.

VWF is involved in primary hemostasis and in coagulation process, in which it acts as a carrier of factor VIII, preventing its degradation by protein C and greatly increasing its plasma half-life. VWF is important for platelet adhesion at sites of vascular damage, where it mediates the initial progression of thrombus formation at the site of endothelial injury through specific interactions with the subendothelial collagen and platelet receptors **[16]**. VWF-mediated platelet adhesion is especially important in regions where there is a high shearing stress, as in the arterioles and stenosed arteries **[17]**.

Under vascular injury, VWF interacts with the subendothelial collagen, and then, the glycoprotein (GP)Ib/IX on platelets surface binds transiently to VWF. After platelet activation, GPIIb/IIIa is able to bind to VWF, providing an irreversible platelet adhesion to the subendothelium **[17]**.

Platelets adhesion to sub-endothelium initiates their activation, granules contend release and in recruitment of more platelets to the place of vascular injury. VWF, and especially, soluble fibrinogen, will promote platelet aggregation by forming bridges between adjacent platelets by binding to GPIIb/IIIa **[17]**. In pathological conditions with high shearing stress, as in stenosis, platelets can form aggregates by binding to VWF through GPIb/IX, even in the absence of platelet activation. The lack of necessity for platelet activation may be the main determinant for the quick platelets accumulation in the stenosed arteries, leading to acute thrombotic occlusion **[15]**.

The interaction between VWF and platelets is essential for an effective hemostasis. When this interaction is compromised due to VWF deficiency or dysfunction or GPIb/IX alteration bleeding disorders occur **[13]**.

VWF stored in Weibel–Palade bodies and alpha-granules is composed by largest multimeric species, the ultra large VWF (UL-VWF), which is the most thrombogenic form of the VWF. UL-VWF are not

usually found in the blood, so that its controlled secretion at sites of injury allow the release of the most thrombogenic VWF forms directly where they are needed **[15]**.

UL-VWF pro-thrombotic activity results from the increased number of binding sites for platelets, which promote increased binding strength to GPIb/IX. Besides, in UL-VWF the A1 domain is exposed and is much more accessible for binding to platelets. The accessibility of the binding site of the A1 domain to GPIb/IX is also dependent on the fluid dynamic forces exerted on the molecule and on its immobilization on the surface. VWF molecule is exposed to a high shearing stress when it is adhered to the endothelial surface, after secretion. The high shearing stress promotes a change from the globular conformation of UL-VWF to an extended conformation, which exposes the A1 domain, favoring the promotion of platelet adhesion by VWF**[14]**. However, even the VWF molecules present in the fluid phase without immobilization may be extended when the shearing stress is high enough. In this extended conformation, there is also a greater exposure of the A2 domain, which owns the site of cleavage by ADAMTS13. Therefore, the shearing stress also promotes the proteolysis of UL-VWF **[18]**.

3. ADAMTS13

In 1998, Furlan et al. **[19]** and Tsai and Lian **[20]** isolated a protease enable to cleave the peptide bond between tyrosine at position 1605 and methionine at position 1606 in the central A2 domain of VWF. In 2001, Zheng et al. **[21]** identified this protease as ADAMTS13, a disintegrin and metalloproteinase with thrombospondin type 1 domain, dependent on Zn^{2+}/Ca^{2+}. Later, it was found that this protease is synthesized primarily by the liver but is also expressed on platelets, endothelial cells and kidney and is normally present in plasma **[22]**, **[23]** and **[24]**.

ADAMTS13 is constitutively secreted as an active enzyme, with a plasma concentration of approximately 1 µg/mL. It is a stable enzyme that has a plasma half-life of approximately 2 to 3 days **[25]**. The ADAMTS13 gene is located on the long arm of chromosome 9, has 29 exons and spans 37 kb of genomic sequence. ADAMTS13 has multiple domains and a predicted molecular weight of 145 kDa, which differs from the molecular weight of approximately 190 kDa observed for ADAMTS13 purified from human plasma. This difference between the calculated and the observed molecular weights is probably due to the glycosylation of the protein **[26]**.

Under physiological conditions, ADAMTS13 cleaves UL-VWF and removes them from circulation, which is the only known substrate for this enzyme. Cleavage occurs at the peptide bond between Tyr1605 and Met1606, located in the VWF A2 domain and generates shorter multimers **[13]**. UL-VWF is not uniformly cleaved into minimal fragments of identical size by ADAMTS13. Instead, they suffer a partial and regulated proteolysis by this enzyme **[14]**.

ADAMTS13 can bind to VWF under static conditions and under conditions of shearing stress. However, this interaction may be ineffective with respect to proteolysis unless the shearing stress is high enough to stretch the VWF and expose the A2 domain that owns the site of cleavage by ADAMTS13, which is usually hidden among the A1 and A3 domains much larger **[13]**. VWF cleavage in their extended conformation induced by the shearing stress results in the release of VWF from the

endothelial surface and allows the molecule to adopt a globular conformation, which reduces its accessibility to platelets [18]. Therefore, in addition to promoting platelet adhesion mediated by UL-VWF, by exposing the A1 domain binding to GPIb/IX, which is necessary for thrombus formation, the shearing stress also favors the cleavage of these large multimers by ADAMTS13, which may represent an intensely regulated control mechanism of thrombogenesis [13]. On the other hand, amino acid substitutions in VWF can significantly increase its proteolysis by ADAMTS13, resulting in the loss of high molecular weight and intermediate size multimers from circulation, predisposing individuals to bleeding, in von Willebrand disease [16].

Deficiency or dysfunction of ADAMTS13 results in the absence of cleavage or partial cleavage of UL-VWF and predisposes to spontaneous formation of intravascular platelet aggregates, resulting in thrombotic thrombocytopenic purpura (TTP). TTP can be congenital, caused by a mutation in ADAMTS13 gene, or acquired, caused by the presence of auto-antibodies against ADAMTS13. In both of them occur a serious deficiency of ADAMTS13 activity (< 5%), which results in a severe pathological state. ADAMTS13 deficiency in these patients results in the formation of VWF and platelet-rich thrombi in the microcirculation of the heart, brain, kidneys, liver, spleen and adrenals. These patients often develop stroke, neurological and renal complications [27].

Some studies have shown that ADAMTS13 can be cleaved by plasma proteases, such as thrombin, plasmin and granulocyte-elastase, resulting in the inactivation of this enzyme. On the other hand, thrombomodulin may inhibit the thrombin-mediated inactivation of ADAMTS13 [25] and [28]. These findings may have a physiological importance at the site of vascular injury, where the inactivation of ADAMTS13 by thrombin and plasmin could promote the recruitment of platelets by VWF. In addition, thrombomodulin could limit the propagation of platelet aggregation in the not injured endothelium around the site of vascular injury [13]. On the other hand, in clinical conditions associated to hypercoagulability state and inflammation, thrombin and plasmin plasma levels and granulocyte-elastase are higher, which may be associated with an increased degradation of ADAMTS13 and, consequently, a lower activity of this enzyme [28].

4. Von Willebrand factor, ADAMTS13 and hypercoagulability

As plasma levels of VWF increase when endothelial cells are injured, increased VWF levels have been proposed as a possible indicator of endothelial dysfunction [29]. The inflammatory response involved in the genesis and progression of atherosclerotic plaques may also promote an increased secretion of VWF, resulting in local recruitment of platelets. This could contribute to the association between increased plasma levels of VWF and the development of acute arterial thrombosis and myocardial infarction [14] and [30].

There is a well-established association between plasma levels of VWF and the development of coronary artery disease, peripheral vascular disease and ischemic cerebrovascular events. In addition, VWF appears to be a marker of increased risk for re-infarction and mortality in patients with angina and in survivors after a myocardial infarction [29]. Some studies also have shown an

association between elevated plasma levels of VWF and factor VIII and the development of ischemic heart disease and venous thromboembolism**[31]** and **[32]**.

Several clinical conditions, such as diabetes mellitus, sepsis, inflammatory vascular disease and chronic kidney disease have also been associated with increased plasma levels of VWF and factor VIII, and consequently, with a greater propensity to the development of thrombotic events **[33]** and **[34]**. The increased plasma levels of VWF found in diabetic patients are linked to an increased production of inflammatory cytokines, a chronic state of oxidative stress, an impairment of NO production and an accelerated production of AGEs **[35]**.

Hyperglycemia induces an overproduction of superoxide by the mitochondrial electron transport chain, blocks endothelial nitric oxide synthase (eNOS) activation, activates protein kinase C and factor kB (NF-kB), resulting in an increased production of reactive oxygen specimens and reduced of nitric oxide (NO). Transcription factors, such as NF-kB induce inflammatory gene expression, resulting in an increased production of inflammatory cytokines and a higher expression of cell adhesion molecules **[36]**. Production of AGEs is also accelerated by hyperglycemia. AGEs can bind to plasma proteins, changing their properties and making them oxidants, and can activate endothelial cells through binding to specific receptors, stimulating the synthesis of tissue factor and IL-6. The inflammatory state associated with increased production of peroxides and oxygen free radicals cause endothelial dysfunction, which leads to an increased production of tissue factor and VWF **[11]**.

Feys et al. **[37]** found that plasma levels and activity of ADAMTS13 are reduced in different physiological and pathological conditions associated with an increased risk for thrombosis, such as pregnancy, cardiac surgery, liver cirrhosis and inflammatory disease. Crawley et al. **[38]** observed an association between increased plasma levels of VWF and reduced of ADAMTS13 with an increased risk for myocardial infarction. Furthermore, Matsukawa et al. **[39]** demonstrated that the ratio of plasma levels of VWF and ADAMTS13 is a significant predictor for the development of thrombotic events after the occurrence of acute myocardial infarction.

Through studies involving deficient mice for ADAMTS13 gene, Chauhan et al. **[40]** showed that the deficiency of this enzyme results in an increased recruitment and adhesion of leukocyte in inflamed veins, and that these processes are dependent on VWF and platelet adhesion. Therefore, the balance between reduced activity of ADAMTS13 and increased VWF plasma levels observed during inflammatory conditions may contribute to accelerate this process, leading to pro-thrombotic effect.

Imbalance between ADAMST13 activity (deficiency of ADAMTS13 activity < 5%) and VWF plasma levels, as mentioned before, is an underlying mechanism, resulting in a severe pathological state in TTP patients. However, there are some reports of TTP secondary to collagen vascular diseases in which ADAMTS13 levels are normal, which is also a severe pathological state. The mechanism of TTP underlying with collagen vascular diseases, in contrast to classical TTP, might be associated to increased release of VWF in plasma due to an intense endothelial damage and inflammation caused by auto-antibodies **[41]**. It has been demonstrated *in vitro* that IL-6 interrupts or inhibits cleavage of VWF by ADAMTS13 and IL-8 and TNF-α precipitate a release of VWF by activating endothelial cells **[42]**. Because VWF are produced excessively in the presence of increased cytokine with primary disease, ADAMTS13 cannot cleave excessive multimers. Therefore, the imbalance between VWF

and ADAMTS13 caused by inflammatory cytokines could trigger TTP in patients with collagen vascular diseases **[42]** and **[43]**. Diabetes is another condition where inflammatory responses may also disrupt the balance between ADAMST13 activity and VWF plasma levels resulting in pro-thrombotic state.

5. Diabetic nephropathy and hypercoagulability

Several markers of hypercoagulability are increased in diabetic patients, such as prothrombin activation fragment 1 + 2, fibrinopeptide A, D-dimer, thrombin–antithrombin complex (TAT), plasmin-α 2 antiplasmin complex (PAP), fibrinogen, factor VII, factor VIII, factor XI, factor XII, tissue factor, kallikrein, VWF and plasminogen activator inhibitor-1 (PAI-1). Platelet aggregation is also exacerbated in diabetic patients, and plasma levels of platelet release products, such as β-thromboglobulin, platelet factor 4 and thromboxane β 2 are increased in diabetes, indicating platelet hyperactivity **[44]**, **[45]** and **[46]**.

Platelet activation and hypercoagulability may contribute to the high risk for cardiovascular events in diabetic patients. They also seem to contribute to the pathogenesis of microvascular complications in these patients, such as the development of diabetic nephropathy **[47]** and **[48]**. Increased VWF plasma levels, which reflect the occurrence of endothelial injury have been found in type 1 and type 2 diabetic patients. VWF may be a predictive marker of diabetic nephropathy, suggesting that endothelial dysfunction precedes the development of diabetic microangiopathy **[11]**, **[49]**, **[50]** and **[51]**. Nonetheless, along with the development of chronic complications of diabetes, plasma levels of VWF increases with the severity of nephropathy and seems to be a risk factor for the development of diabetic macroangiopathy in these patients **[10]** and **[11]**. Therefore, it is not completely clear how the kinetics of these two events is connected and which one precedes each other.

The association between diabetic nephropathy and increased risk for the development of atherosclerotic disease is well known **[52]**. Kim et al. **[53]** evaluated the presence of atherosclerosis in coronary and aorta arteries in type 1 diabetic patients with and without diabetic nephropathy using cardiovascular magnetic resonance imaging, and found a higher prevalence of atherosclerosis in coronary arteries of diabetic patients with nephropathy than in those without nephropathy.

Go et al. **[54]** evaluated the relationship between the decline of renal function and cardiovascular events in a low-risk population of more than one million of patients followed for a period of 2.84 years. Compared with subjects with eGFR > 60 mL/min/1.73 m^2, those with eGFR of 45–59 mL/min/1.73 m^2; 30–44 mL/min/1.73 m^2; 15–29 mL/min/1.73 m^2; and < 15 mL/min/1.73 m^2 showed an increased risk of cardiovascular mortality of 1.4; 2.0; 2.8; and 3.4, respectively. This study clearly showed an independent correlation between the levels and gradual decline of renal function with the increased rates of cardiovascular mortality.

Some studies have shown that microalbuminuria predicts the development of atherosclerosis vascular disease in diabetic patients, consisting in an important cardiovascular risk factor for these patients**[3]** and **[9]**. It was found that patients with type 1 diabetes and normoalbuminuria have a 2- to

4-fold increased risk for the development of cardiovascular disease, and this risk is 20- to 40-fold higher in those with microalbuminuria. In addition, the median survival of diabetic patients after the onset of proteinuria is seven years, and this increased mortality is mainly due to coronary death, rather than to renal failure **[55]**.

Valmadrid et al. **[56]** evaluated the association of microalbuminuria and proteinuria with the risk of cardiovascular mortality in 840 patients with type 2 diabetes followed for a period of 12 years, and found that patients with microalbuminuria and proteinuria had a 1.84 and 2.61-fold increased risk of death from cardiovascular disease than patients with normoalbuminuria, respectively. Mattock et al. **[57]** followed 146 type 2 diabetic patients over a period of seven years and found that the presence of microalbuminuria was a significant risk factor for all-cause mortality (relative risk 3.94) and cardiovascular mortality (relative risk 7.40).

The United Kingdom Prospective Diabetes Study showed that there is a trend towards an increased risk of cardiovascular death with worsening nephropathy in patients with type 2 diabetes, with an annual rate of cardiovascular death of 0.7% for patients without nephropathy, 2.0% for those with microalbuminuria, 3.5% for those with macroalbuminuria and 12.1% for those with ESRD. Besides, patients with macroalbuminuria were more likely to die from cardiovascular disease than to develop renal failure **[58]**.

Increased risk of atherosclerotic disease in patients with diabetic nephropathy has been attributed to several mechanisms including hypertension, dyslipidemia, and abnormalities in coagulation and fibrinolysis. Diabetic nephropathy is associated with an atherogenic lipoprotein profile, including high levels of LDL cholesterol, VLDL cholesterol and lipoprotein(a), and reduced levels of HDL cholesterol. Moreover, a hypercoagulability state characterized by increased plasma levels of VWF, PAI-1, factor VII and fibrinogen has been reported. Reduced renal function can still lead to an accumulation of AGEs in the circulation and tissues, which can accelerate atherosclerosis **[53]**.

Therefore, there is a gradual and independent association between renal function decline and risk of cardiovascular death in patients with diabetic nephropathy, with the risk of cardiovascular death outweighing the risk of developing renal failure in these patients **[54]** and **[55]**.

Dubin et al. **[59]** observed an association between reduced estimated glomerular filtration rate (eGFR) and increased plasma levels of several markers of hypercoagulability, such as thrombomodulin, tissue factor, D-dimer, VWF, factor VIII, PAP, tissue factor pathway inhibitor (TFPI), PAI-1 and fibrinogen, which indicates that the alteration of hemostasis may be one mechanism by which reduced renal function promotes an increased risk of cardiovascular disease.

Lu et al. **[60]** found that patients with chronic kidney disease had significantly increased plasma levels of VWF and significantly reduced ADAMTS13 activity as compared to healthy subjects. Tanigushi et al. **[12]**determined the plasma levels of VWF and ADAMTS13 and calculated the VWF/ADAMTS13 rate in 86 patients with type 2 diabetes mellitus and 26 healthy subjects in order to evaluate the relationship between these levels and renal function. They observed a positive correlation between plasma levels of ADAMTS13 and the eGFR and a negative correlation between the VWF/ADAMTS13 rate and the eGFR. In addition, diabetic patients were divided into three groups: with normoalbuminuria, microalbuminuria and nephropathy. It was found that only the group with

nephropathy had plasma levels of ADAMTS13 significantly lower compared to healthy subjects. To evaluate the diabetic macroangiopathy, these authors also determined the carotid intima-media thickness (IMT) of 68 patients with diabetes mellitus and observed a negative correlation between the levels of ADAMTS13 and the carotid IMT and a positive correlation between the VWF/ADAMTS13 rate and the carotid IMT. Based on this study, it is possible to suggest that reduced plasma levels of ADAMTS13 are associated with diabetic nephropathy and an increased risk for the development of diabetic macroangiopathy.

By employing Western blot analysis, Manea et al. **[22]** verified the presence of ADAMTS13 in the urine of individuals with renal dysfunction, but did not detect this enzyme in the urine of healthy subjects, indicating that the reduced plasma levels of ADAMTS13 in patients with impaired renal function might be caused by the loss of this enzyme in the urine. Studies conducted by our research group found increased VWF levels and factor VIII activity associated with a significant reduction in plasma levels of ADAMTS13 in pregnant women with preeclampsia and in patients undergoing hemodialysis **[61]** and **[62]**. It is noteworthy that there is the presence of significant proteinuria in pre-eclampsia. These findings led us to hypothesize a possible role of kidney on ADAMTS13 synthesis or metabolism. Similarly, it can be assumed that the alterations in plasma levels of ADAMST13 and the hypercoagulability state observed in diabetic patients with nephropathy and proteinuria could be associated with the loss of ADAMST13 in the urine.

Inflammatory responses are more intense in diabetic patients with nephropathy than in diabetic patients without renal impairment, which can be demonstrated by an increased production of inflammatory cytokines and activation of polymorphonuclear leukocytes in patients with diabetic nephropathy **[63]** and **[64]**. Increased plasma levels of inflammatory cytokines cause endothelial damage, which results in increased plasma levels of VWF **[65]** and **[66]**. Therefore, inflammatory states and renal dysfunction, or even the interaction between these two factors are potential triggers of the imbalance between ADAMST13 activity and VWF plasma levels.

Inflammatory responses may directly affect ADAMST13 levels. Cao et al. **[67]** demonstrated that some inflammatory cytokines can inhibit the synthesis of ADAMTS13 by hepatic stellate cells and endothelial cells. In addition, during inflammatory responses, due to the increased activation of polymorphonuclear leukocytes there are increased plasma levels of elastase produced by these cells in diabetic patients than in subjects without diabetes **[68]**. Furthermore, these patients have a hypercoagulability state which results in increased plasma levels of thrombin and plasmin and evidences suggest that ADAMTS13 can be cleaved by plasma proteases, like thrombin, plasmin and granulocyte elastase **[25]**, **[28]** and **[44]**.

Altogether, these findings may lead to several different proposals on the potential mechanisms leading to decreased ADAMTS13 levels in diabetic patients with renal injury (**Fig. 1**): i) lower hepatic and/or renal synthesis of this enzyme in result of the increased production of inflammatory cytokines in these patients; ii) increased plasma levels of thrombin, plasmin and elastase of granulocytes, due to intense inflammatory response and hypercoagulability state in patients with diabetic nephropathy, could promote the proteolytic degradation of ADAMTS13 in the plasma; iii) impaired renal function

might result in the loss of this enzyme in the urine. However, all these potential mechanisms still wait for further investigations and more conclusive evidences.

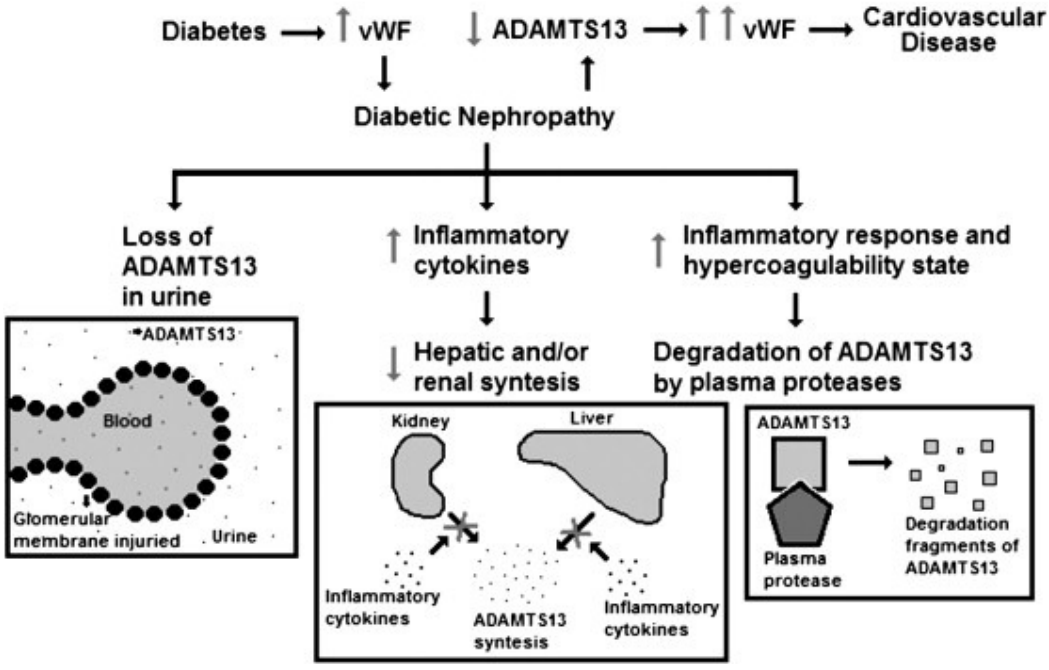

Fig. 1.

Schematic representation of the potential mechanisms leading decreased ADAMST13 levels in diabetic patients with renal injury: i) loss of ADAMTS13 in urine; ii) lower hepatic and/or renal synthesis of ADAMTS13 in result of the increased production of inflammatory cytokines; iii) proteolytic degradation of ADAMTS13 by plasma proteases, whose levels are increased due to intense inflammatory response and hypercoagulability state.

Another aspect that remains unclear is whether VWF and ADAMTS13 are only markers of endothelial dysfunction, diabetic nephropathy and cardiovascular events or whether they play an active role in the development of the microangiopathy and macroangiopathy in diabetic patients. It is possible to propose that the endothelial dysfunction present in diabetic patients as a result of the hypercoagulability state and higher inflammatory response could lead to increased levels of VWF. The endothelial dysfunction and increased levels of VWF, in turn, might contribute to the development of renal injury. Moreover, the onset of nephropathy could enhance the hypercoagulability state and inflammatory response in these patients, resulting in a lowering of ADAMTS13 levels and a stronger increase of VWF levels, which might contribute to the development of atherosclerotic cardiovascular disease (**Fig. 1**). However, this hypothesis still waits for further investigation.

6. Conclusion

The mechanisms that are responsible for the association between nephropathy and atherosclerosis in diabetic patients have not been fully elucidated. Several evidences indicate that VWF and ADAMTS13 imbalance may contribute to the increased hypercoagulability and consequent risk of atherosclerotic cardiovascular disease in patients with diabetic nephropathy. However, it is still

unclear whether VWF and ADAMTS13 are only markers of diabetic nephropathy and cardiovascular events or whether they play an active role in the development of these events. Furthermore, it is not clear what triggers the imbalance between VWF and ADAMTS13 in these patients.

The potential triggers of this imbalance are renal dysfunction and inflammatory state, or even the interaction between these two factors. It is possible to suggest that the hypercoagulability state and increased inflammatory response present in diabetic patients could lead to endothelial dysfunction and increased levels of VWF, which might contribute to the development of renal injury. Then, the onset of nephropathy could enhance the hypercoagulability state and inflammatory response in these patients, resulting in low ADAMTS13 levels and increased VWF levels. As ADAMTS13 is required for cleavage and plasma clearance of VWF, reduced levels of this enzyme also contribute to increase plasma levels of VWF in patients with diabetic nephropathy, leading to an imbalance between VWF and ADAMTS13, which might contribute to the development of atherosclerotic cardiovascular disease. Thus, according to this hypothesis, VWF and ADAMTS13 imbalance would have an effective role on the development of renal injury and cardiovascular events in diabetic patients, presenting a great importance in the development of these complications in diabetes.

Nonetheless, it is also not clear how renal injury may affect ADAMTS13 levels, leading consequently to an intensified hypercoagulability state. Some potential mechanisms may be raised: i) lower hepatic and/or renal synthesis of ADAMTS13 as result of the increased production of inflammatory cytokines in patients with diabetic nephropathy; ii) proteolytic degradation of ADAMTS13 by plasma proteases, whose levels are increased due to intense inflammatory responses and hypercoagulability state; iii) loss of this enzyme in the urine due to impaired renal function. Therefore, additional studies are needed to elucidate which of these potential mechanisms are responsible for the relationship between renal injury and reduced levels of ADAMTS13 in patients with diabetes and other clinical conditions.

Finally, a better understanding of the association between diabetic nephropathy, hypercoagulability and atherosclerotic cardiovascular disease may possibly enable the discovery of new markers, applicable to clinical and laboratory monitoring of patients with diabetes and other diseases in which renal dysfunction and hypercoagulability are progressive conditions, contributing to the introduction of more effective prevention measures for these clinical conditions.

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5.2 Artigos submetidos

DIABETES MELLITUS: THE LINKAGE BETWEEN INFLAMMATION, HYPERCOAGULABILITY AND VASCULAR COMPLICATIONS

Caroline Pereira Domingueti[1], Luci Maria Sant'Ana Dusse[1], Maria das Graças Carvalho[1], Lirlândia Pires de Sousa[1],Karina Braga Gomes[1], Ana Paula Fernandes[1]

1- Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte – Minas Gerais, Brazil

Address correspondence to

Caroline Pereira Domingueti

Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais Av. Antônio Carlos, 6627 - Pampulha - Belo Horizonte – MG – Brazil, CEP 31270-901.

Telephone: (55) (31) 3409-6902

e-mail: caroldomingueti@yahoo.com.br

Abstract

Vascular complications are the leading cause of morbidity and mortality among patients with type 1 and type 2 diabetes mellitus. These vascular abnormalities are a result of a chronic hyperglycemic state, which leads to an increase in oxidative stress and inflammatory state, and the development of endothelial dysfunction and a hypercoagulability state. It has been demonstrated that there is an interrelationship between endothelial dysfunction, inflammation and hypercoagulability in type 1 and type 2 diabetes and that these processes play an important role in the development of vascular complications in diabetic patients. Moreover, it has been observed that several endothelial, inflammatory and pro-coagulant biomarkers, such as VWF, IL-6, TNF-α, D-dimer and PAI-1, are increased in diabetic patients who have microvascular complications, such as nephropathy, and macrovascular complications, such as cardiovascular disease. This review addresses the interrelationship between endothelial dysfunction, hypercoagulability and inflammation in the development of vascular complications in type 1 and type 2 diabetes and the association between endothelial (VWF), inflammatory (IL-6 and TNF-α) and pro-coagulant (D-dimer and PAI-1) biomarkers and the development of these complications.

Keywords: Diabetes mellitus, vascular complications, endothelial dysfunction, hypercoagulability, inflammation

Introduction

Diabetes mellitus (DM) is a metabolic disorder which affect about 383 million adults, accounting for 8.3% of adult population worldwide, projected to reach 592 million in 2035, or 10.1% of adults, which is equivalent to the appearance of about more than three people with diabetes once every 10 seconds. Approximately 50% of affected individuals are unaware of the diagnosis [1].

DM is the fifth leading cause of death worldwide, accounting for 5.2% of all deaths. Its chronic nature, severity of complications and the means necessary to control them become diabetes a disease very costly not only for affected individuals and their families, but also for the health system. Costs directly related to diabetes range from 2.5% to 15% of the annual health budget, depending on their prevalence and the sophistication of the treatment available [2].

This disorder is one of the main risk factors for the development of myocardial infarction, stroke and peripheral vascular disease [3, 4]. Diabetic patients are four to five times more likely to develop heart disease and stroke than individuals without DM, and cardiovascular complications are the leading cause of morbidity and mortality among patients with type 1 (DM1) and type 2 diabetes (DM2) [3, 5, 6, 7]. As DM1 arises predominantly during childhood, DM1 patients have a higher risk of developing coronary events earlier. It was observed that the rate of cardiovascular events in DM1 patients exceeds 1% per year after 45 years old and more than 3% per year after 55 years old [6].

DM is also one of the main risk factors for developing chronic kidney disease (CKD). The risk of developing nephropathy is about 30% and 20% in DM1 and DM2, respectively [8]. Diabetic nephropathy is the most common cause of end stage renal desease (ESRD), contributing to approximately 45% of new cases, and is also an independent risk factor for cardiovascular disease [5, 9].

Recent studies have shown that an interrelation between inflammation and metabolic abnormalities in diabetes leads to endothelial injury and the development of vascular complications. It has been suggested that the early indicator of these effects is endothelial dysfunction together with the development of a pro-coagulant state [10].

Therefore, the aim of this review was to explore the relationship between endothelial dysfunction, inflammation and hypercoagulability in the development of vascular complications in DM1 and DM2 through the association between

endothelial (VWF – Von Willebrand Factor), inflammatory (IL-6 – Interleukin-6 and TNF-α – Tumor Necrosis Factor-alfa) and pro-coagulant (D-Dimer and PAI-1 – Plasminogen Activator Inhibitor-1) factors and the development of these complications.

Vascular Complications in Diabetes Mellitus

Vascular complications of DM are derived from a chronic hyperglycemic state and can occur both in large blood vessels, characterizing diabetic macroangiopathy, as in small blood vessels, consisting of diabetic microangiopathy. These vascular abnormalities are the result of irreversible non-enzymatic glycation of proteins, alteration in cellular redox potential, increase of oxidative stress and inflammatory state, and the development of endothelial dysfunction and a hypercoagulability state (Figure 1) [10, 11, 12].

Vascular endothelial cells exhibit a particular risk of developing intracellular hyperglycemia, since glucose can penetrates these cells by passive diffusion, not being necessary the action of insulin. Thus, the accumulation of intracellular glucose leads to the activation of a secondary metabolic pathway, the aldose reductase, wherein the aldose reductase and sorbitol dehydrogenase catalyzes the metabolism of glucose to sorbitol and sorbitol to fructose respectively. These reactions go together with oxidation of NADPH to $NADP^+$ and reduction of NAD^+ to NADH. The excessive flow of glucose through this pathway leads to a change in redox potential due to depletion of cellular NADPH and an increase in $NADH/NAD^+$ cytosolic rate (Figure 2) [6].

This increase in $NADH/NAD^+$ rate caused by hyperglycemia imitates the effects of hypoxia, causing an acceleration of glycolysis, leading to increased "de novo" synthesis of diacylglycerol from glycolytic intermediates and subsequent activation of protein kinase C (PKC). Activation of PKC interferes with the synthesis of nitric oxide, promotes increased vascular permeability and contractility, stimulates synthesis of extracellular matrix and thickening of basement membrane, and promotes an inflammatory response through activation of cytokines and adhesion molecules. The alteration in $NADH/NAD^+$ rate also results in an increased production of superoxide due to activation of oxidases NADH dependent, which oxidize low-density-lipoprotein (LDL), have cytotoxic effects on endothelial cells and promotes a reduction in the availability of nitric oxide, leading to endothelial dysfunction [6, 13]. When damaged, endothelial cells release pro-coagulant molecules, such as VWF,

PAI-1 and tromboxan A2, and express on their surfaces tissue factor (TF) and adhesion molecules, such as P-selectin, E-selectin, vascular adhesion molecular-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), which mediate the interaction between neutrophils and platelets with endothelium. Therefore, endothelial dysfunction can promote both a pro-inflammatory and a pro-coagulant state [14].

When exposed to aldoses, proteins are submitted to glycation and oxidation. Initially, the reaction consists of condensation of glucose with amino groups of proteins to form an instable and reversible Schiff bases product, which may undergo rearrangements to form more stable products called Amadori products (such as glycated hemoglobin). After molecular rearrangements, such products may form the advanced glycation end-products (AGEs), which are irreversible. The molecules linked to AGEs acquire new properties and become oxidants. This process leads to the production of reactive oxygen species, which promote an increase in oxidative stress and prevents the release of nitric oxide, resulting in appearance of vascular lesions. AGEs may also reduce the bioavailability and activity of endothelium-derived nitric oxide, further compromising vascular activity (Figure 3) [11, 12, 15].

AGEs can bind to its receptor (RAGE - receptors for advanced glycation end-products) present on the surface of endothelial cells, smooth muscle cells, fibroblasts, lymphocytes, monocytes and macrophages, resulting in the activation of nuclear transcription factor NF-κB (Nuclear Factor-KappaB) [6, 12, 15]. After this activation, NF-κB induces the transcription of various genes, such as endothelin-1, VCAM-1, ICAM-1, E-selectin, thrombomodulin, TF, vascular endothelial growth factor (VEGF), IL-1, IL-6, TNF-α and RAGE, triggering an inflammatory and pro-coagulant state, which causes endothelial activation [6, 16].

The increased expression of inflammatory cytokines and adhesion molecules can amplify the inflammatory responses, leading to an aggravation of diabetic vascular complications. Also, pro-inflammatory cytokines TNF-α, IL-1 and IL-6 are important to mediate the pro-coagulant effect of damaged endothelial cells, since these cytokines can stimulate the release and expression of pro-coagulant molecules, such as VWF, PAI-1 and TF, and inhibit the expression of anti-coagulant molecules, such as trombomodulin, by endothelial cells [14]. Reduced expression of thrombomodulin associated with induction of TF expression alters the surface of the endothelium of an anti-coagulant state to a pro-coagulant state. Besides, increased

production of growth factors, such as VEGF, fibroblast growth factor (FGF), can stimulate the remodeling of blood vessel wall, resulting in a thickening of the basement membrane, which favors local deposition of proteins and lipids, and promotes sclerosis and an impaired vasodilation [6, 11].

Vascular injury, oxidative stress, inflammation and chronic alterations in the hemodynamic balance deriving from alterations underlying hyperglycemia may initiate a process of atherosclerosis and the formation of arterial thrombus [17]. During the early atherosclerotic process, matrix proteoglycans sequester circulating LDL and induce its oxidation. These oxidized lipoproteins consists of highly pro-inflammatory molecules that stimulate the expression of several adhesion molecules by endothelial cells, such as VCAM-1, ICAM-1 and selectins, and the secretion of growth factors, such as VEGF, FGF, insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF), inflammatory cytokines, such as IL-1 and TNF-α, and chemokines, such as monocyte chemoattractant protein-1 (MCP-1) [6, 18].

The expression of adhesion molecules by injured endothelium promotes the selective binding of leukocytes and their transmigration into vascular wall. Furthermore, circulating monocytes are recruited and activated, differentiating into macrophages, which phagocyte the excess of oxidized LDL and transform into foam cells, forming fatty streaks. Mononuclear cells also release inflammatory cytokines, including IL-1 and IL-6, which promote the recruitment of additional inflammatory cells. Because of the effect of pro-inflammatory and growth factors secreted by macrophages and foam cells, smooth muscle cells proliferate and migrate from tunica media to intima. Activated smooth muscle cells synthesize and secrete extracellular matrix (collagen, elastin, proteoglycans) leading to formation of a fibroatheroma [6].

During the initial phase of atherosclerosis, thrombosis is uncommon. However, with the progress of the process, the formation of fissures or ulceration of the plaque exposes highly thrombogenic substances, such as TF and VWF, which results in adhesion and aggregation of platelets and a rapid growth of the thrombus. This occurs in plaques with a thin fibrous layer, with a large amount of lipid, and in those with a high concentration of TF [17]. Besides participating in the formation of thrombus, activated platelets also release pro-inflammatory cytokines and growth factors, which promote the recruitment of monocytes to atherosclerotic plaque and stimulate proliferation of fibroblasts and smooth muscle cells, accentuating

atherosclerotic process. Activated platelets can also interact with endothelial cells through P-selectin, resulting in release of IL-6 and MCP-1 and in expression of E-selectin, VCAM-1 and ICAM-1 by endothelial cells, exacerbating the inflammation [14].

Therefore, it is incontestable the role that endothelial dysfunction, hypercoagulability and inflammation plays in the development of vascular complications in diabetes. Several inflammatory, endothelial and pro-coagulant biomarkers, such as IL-6, TNF-α, D-dimer, PAI-1 and VWF, among others, are increased in patients with DM1 and DM2, and it is observed an increase even more intense in the levels of these biomarkers in patients with vascular complications [19, 20].

Von Willebrand Factor and Endothelial Dysfunction

Multimeric VWF is a glycoprotein composed of identical subunits of 270 kDa. Disulfide bonds link the subunits to form dimers of approximately 500 kDa, and then, this same type of connection links the dimers to form multimers of various sizes that may exceed 10,000 kDa. VWF is synthesized only by endothelial cells and megakaryocytes, and is stored in Weibel-Palade bodies of endothelial cells and alpha granules of platelets [5, 21].

VWF is engaged in primary hemostasis and coagulation process, in which it acts as a carrier of factor VIII, preventing the degradation of this factor by protein C, and considerably increasing its plasma half-life. VWF is important in platelet adhesion to sites of vascular injury, where it mediates the initial progression of thrombus formation at the site of endothelial injury through specific interactions with subendothelial collagen and platelet receptors [5, 22]. The largest multimeric species of VWF, called ultra large VWF, are the most active and thrombogenic species [21].

As plasma levels of VWF increase when endothelial cells are damaged, VWF has been considered a biomarker of endothelial dysfunction. There is a well-established association between plasma levels of VWF and the development of coronary artery disease, peripheral vascular disease and ischemic cerebrovascular events. Furthermore, VWF seems to be a biomarker of increased risk for mortality and reinfarction in patients with angina and in survivors after a myocardial infarction [23]. Some studies have also shown an association between increased plasma levels of VWF and the development of ischemic heart disease and venous thrombosis [24, 25].

Increased plasma levels of VWF have been found in patients with DM1 and DM2, and VWF seems to be a predictive biomarker of diabetic nephropathy in these patients [13, 26, 27]. Domingueti *et al.* [28] observed an increased VWF levels in DM1 patients with microalbuminuria and proteinuria compared to those with normoalbuminuria. They also verified that DM1 patients with mild (≥60 and <90 mL/min/1.73m^2) and severe (<60 mL/min/1.73m^2) GFR decline presented a higher VWF activity in comparison to those with normal GFR (≥90 and < 130 mL/min/1.73m^2). Chan *et al.* [29] also found that DM1 patients with increased UAE presented a higher activity of VWF in comparison to diabetic patients with normoalbuminuria, and that normoalbuminuric patients have increased activity of VWF when compared to healthy subjects.

In a study of DM1 patients, who were followed for 3 years, it was observed that there was an increase in VWF levels in patients who developed microalbuminuria during this time, indicating an association between endothelial dysfunction and the development of diabetic nephropathy [30]. In another study, Stehouwer *et al.* [31] found that increased plasma levels of VWF preceded the development of microalbuminuria in DM1 patients in approximately three years, suggesting that endothelial dysfunction precedes and may predict the development of microalbuminuria in diabetic patients.

Fang *et al.* [32] evaluated plasma levels of VWF in DM2 patients with and without nephropathy and observed increased levels of this biomarker in patients with renal dysfunction. Stehouwer *et al.* [33] followed 328 patients with DM2 for 9 years and observed that the longitudinal development of urinary albumin excretion (UAE) in these patients was significantly and independently determined by the baseline levels of VWF and other biomarkers of endothelial dysfunction and inflammation, such as E-selectin, t-PA, protein C reactive (PCR) and fibrinogen, indicating an interrelationship between endothelial dysfunction, chronic inflammation and the development of renal dysfunction in DM2.

In a study involving patients with DM2 who were followed for 53 months, it was found an increase in plasma levels of VWF in patients who developed microalbuminuria or macroalbuminuria. Furthermore, it was observed that the presence of microalbuminuria at baseline was associated with an increased risk of cardiovascular events only in patients who had plasma levels of VWF above the mean. Thus, it is possible to suggest that vascular dysfunction must be the link

between albuminuria and atherosclerotic cardiovascular disease in diabetes [34]. In another study involving 290 patients with DM2 who were followed for 10 years, Standl *et al.* [35] observed that VWF was an important risk factor for cardiovascular mortality.

Domingueti *et al.* [28] determined ADAMTS13 activity and plasma levels of ADAMTS13, an enzyme responsible for cleavage and inactivation of VWF, and calculated ADAMTS13activity/ADAMTS13antigen ratio in DM1 patients. They observed a reduction in ADAMTS13activity/ADAMTS13antigen ratio with the GFR decline and with UAE increase in these patients. Tanigushi *et al.* [36] determined plasma levels of VWF and ADAMTS13 and calculated FVW/ADAMTS13 ratio in DM2 patients with and without nephropathy and found a negative correlation between this ratio and GFR. To assess diabetic macroangiopathy, the authors determined carotid intima-media thickness (CIMT) of diabetic patients and observed a positive correlation between FVW/ADAMTS13 ratio and CIMT. Rurali *et al*. [37] also have demonstrated that decreased ADAMTS13 activity is associated with an increased risk of renal and cardiovascular events in DM2 patients.

Soares *et al.* [38] evaluated plasma levels of VWF in DM2 women, which were classified according to CIMT and observed increased plasma levels of VWF in patients with greater CIMT. In a study involving diabetic patients with peripheral arterial occlusive disease, Skeppholm *et al.* [39] found a significant increase in plasma levels of VWF in these patients. Verkleij *et al.* [40] also observed that DM2 patients with cardiovascular disease had increased levels of VWF than patients without cardiovascular disease.

Zareba *et al.* [41] evaluated plasma levels of VWF in 846 non-diabetic subjects, 125 DM2 patients and 74 DM1 patients two months after the occurrence of a myocardial infarction. They found that patients with DM2 and DM1 had increased VWF levels than individuals without diabetes, suggesting that endothelial damage is an important mechanism contributing to the occurrence of cardiac and vascular events in diabetic patients.

Plasminogen Activator Inhibitor-1, D-Dimer, and Hypercoagulability

Fibrinolysis is the degradation of fibrin mediated by plasmin. Fibrinolytic system is composed of several proteins (inhibitors and serine proteases) which regulate generation of plasmin, an active enzyme produced from an inactive pro-enzyme, plasminogen, whose primary function is to degrade fibrin. Physiological

plasminogen activators are tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) [42].

PAI-1 is a 50 kDa glycoprotein which, in active conformation, is an efficient inhibitor of plasmin formation by inhibiting t-PA, and thus, it is a potent inhibitor of the fibrinolytic system. Alterations in function and/or concentration of PAI-1may lead to a hypercoagulability state. PAI-1 has been classified as an acute phase protein, and its production can be induced by IL-1 and TNF-α, which are associated with vascular inflammation and atherothrombosis [43].

D-dimer is a fibrin degradation product (FDP) that derives only from fibrin and not from fibrinogen, thus being specific to show fibrinolytic activity secondary to the formation of fibrin, consisting of an important biomarker of hypercoagulability [44].

Long *et al.* [45] found that DM2 patients with albuminuria have higher D-dimer plasma levels than diabetic patients without albuminuria. In a study involving children and adolescents with DM1 and DM2, El Asrar *et al.* [46] observed increased serum levels of D-dimer in children with diabetic microvascular complications and a correlation between plasma levels of D-dimer and UAE. Domingueti *et al.* [28] verified that D-dimer levels is increased in microalbuminuric DM1 patients compared to normoalbuminuric ones, and is even higher in proteinuric patients. They also found an increase in D-dimer levels with the decline of GFR. Therefore, it is possible to suggest that there is a hypercoagulability state in diabetes, which may contribute to the development of microvascular complications.

In a study involving patients with DM2, Wakabayashi and Masuda [47] found that D-dimer is associated with microalbuminuria in these patients and that UAE rate is significantly correlated with CIMT, suggesting that the hypercoagulability state may be involved both with the progression of atherosclerosis as well as glomerular dysfunction in diabetic patients.

Soares *et al.* [48] evaluated plasma levels of D-dimer in women with DM2, and classified these patients into three groups according to CIMT. They observed that the group of diabetic women with carotid plaque showed increased plasma levels of D-dimer than the other groups, suggesting that plasma levels of D-dimer are associated with the formation of atherosclerotic plaque in diabetes.

Nwose *et al.* [49] analyzed plasma levels of D-dimer in 343 individuals, which were divided into 7 groups: family history of diabetes, pre-diabetes with/without cardiovascular disease, diabetes with/without cardiovascular disease and

cardiovascular disease only. They found that there was a progressive increase in plasma levels of D-dimer with the progression of diabetes and cardiovascular complications, indicating that plasma levels of D-dimer can be useful to indicate the progression of diabetes and macrovascular complications. In a study of DM2 patients with carotid atherosclerotic plaques, Krupinski *et al.* [50] demonstrated that plasma levels of D-dimer can be helpful in diagnosing diabetic patients who have a high risk of atherothrombosis.

Targher *et al.* [27] evaluated plasma levels of PAI-1 in DM1 patients without clinical macrovascular disease, and found that these patients had increased levels of this biomarker than healthy individuals. Moreover, they observed that patients with advanced microvascular complications displayed increased levels of PAI-1 than those with early stage microvascular complications or without these complications. Other studies have shown that DM1 and DM2 patients with nephropathy showed increased levels of PAI-1 than patients without renal dysfunction [51, 52].

Hagiwara *et al.* [53] analyzed renal expression of PAI-1 by real-time PCR in a DM1 rat model and in a DM2 rat model and found increased renal expression of PAI-1 in both models, suggesting that increased renal expression of PAI-1 may play an important role in the development of pathogenesis of diabetic nephropathy.

Verkleij *et al.* [40]) evaluated plasma levels of PAI-1 in DM2 patients with and without cardiovascular disease and found that patients with cardiovascular disease had increased levels of PAI-1. In a study of DM1 patients who suffered a myocardial infarction, Zareba *et al.* [41] found that these patients had increased levels of PAI-1 than non-diabetic individuals who have suffered a myocardial infarction. Pratte *et al.* [54] also demonstrated a correlation between plasma levels of PAI-1 and calcification of coronary arteries in patients with DM1.

Interleukin-6, Tumor Necrosis Factor Alpha and Inflammation

IL-6 is a pro-inflammatory cytokine produced by endothelial cells, leukocytes, adipocytes and mesangial cells. Its expression is associated with renal mesangial proliferation and tubular atrophy, indicating that it plays a role in the progression of renal disease [55].

TNF-α is a pleiotropic cytokine produced mainly by macrophages and monocytes, which is involved in systemic inflammation. TNF-α induces a local inflammatory response, in which it initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to the site of

infection. It also activates NF-κB signaling, mediating the transcription of many cytokines involved in the survival and proliferation, inflammatory response and cell adhesion, and apoptotic factors. TNF-α is a cytotoxic cytokine for glomerular mesangial cells and epithelial cells, and may induce renal injury. It has been shown that an increase in urinary levels of TNF-α precedes the increase in albuminuria in diabetes and that urinary levels of TNF-α increases with the progression of diabetic nephropathy, suggesting that TNF-α may contribute to the development and progression of renal injury in diabetes [55].

Schram *et al.* [56] studied the association of inflammatory biomarkers IL-6 and TNF-α with vascular complications in 543 DM1 patients, and found that plasma levels of these biomarkers were associated with albuminuria, retinopathy and cardiovascular disease. Several studies have shown that DM1 and DM2 patients with nephropathy have increased plasma levels of pro-inflammatory cytokines TNF-α and IL-6 than patients without renal dysfunction, indicating an important role of inflammation in the development of nephropathy in diabetes [20, 51, 57, 58].

Shikano *et al.* [59] evaluated plasma and urinary levels of IL-6 in 72 DM2 patients and found a significant increase of these levels with the progression of nephropathy. They also noted that IL-6 plasma levels correlated with plasma levels of fibrinogen and other biomarkers of atherosclerosis, suggesting an association between IL-6 and the development of atherosclerosis in diabetes. Heo *et al.* [60] also observed that diabetic patients who suffered a myocardial infarction showed increased levels of IL-6 than diabetic patients with stable angina.

In a study involving patients with DM2, Kajitani *et al.* [61] found that TNF-α plasma levels correlated with UAE and that IL-6 plasma levels and TNF-α correlated with CIMT, suggesting that inflammation must be a risk factor common between nephropathy and atherosclerosis in diabetes. Saremi *et al.* [62] also found an association between IL-6 plasma levels and coronary artery calcification in DM2 patients.

Wu *et al.* [63] analyzed plasma levels of TNF-α and IL-6 in the progression of macrovascular complications in DM2 patients and non-diabetic individuals to assess the predictive value of these biomarkers in the incidence of macrovascular complications. They found that the incidence of atherosclerosis was greater in diabetic patients than in non-diabetic subjects, and that levels of TNF-α and IL-6

increased annually in both groups. Moreover, they observed that diabetic patients who developed atherosclerosis showed increased levels of TNF-α and IL-6 than non-diabetic patients who developed atherosclerosis, and that levels of TNF-α and IL-6 had a stronger predictive value for the development of atherosclerosis in diabetic patients than in those without diabetes.

Resolution of inflammation is impaired in diabetic patients, which contributes to the increased levels of TNF-α, IL-6 and other pro-inflammatory cytokines in these patients, and to the development and progression of nephropathy and atherosclerosis. Recent studies have shown that pro-resolving lipid mediators, such as lipoxins, resolvins and protectins play an important role in the resolution of inflammation by inhibiting polymorphonuclear and monocyte recruitment and changing the cytokine milieu from pro-inflammatory to pro-resolving. Therefore, these pro-resolving lipid mediators have a great potential as therapeutic targets in renal and cardiovascular diseases in diabetes [64, 64].

Conclusion

Microvascular and macrovascular complications are responsible for a great morbidity and mortality among patients with DM1 and DM2. These vascular abnormalities result of a chronic hyperglycemic state, which leads to increased oxidative stress and inflammatory state, and the development of endothelial dysfunction and a hypercoagulability state.

Recently, it has been demonstrated that there is an interrelationship between endothelial dysfunction, inflammation and hypercoagulability in DM1 and DM2 and that these processes play a very important role in the development of vascular complications. Several studies have shown that plasma levels of endothelial biomarkers, such as VWF, inflammatory biomarkers, such as IL-6 and TNF-α, and pro-coagulant biomarkers, such as D-dimer and PAI-1, are increased in diabetic patients who have microvascular complications, such as nephropathy, and macrovascular complications, such as cardiovascular disease. Furthermore, it has been suggested that some of these biomarkers can predict the development of these complications in diabetic patients.

Therefore, the study of endothelial, inflammatory and pro-coagulant biomarkers are very important for a better understanding of the pathophysiologic mechanisms involved in the onset of vascular complications in D. Moreover, it is promising the clinical and laboratory use of these biomarkers for predicting the risk of

cardiovascular and renal complications in diabetic patients and for monitoring these patients, which may contribute to the introduction of more effective preventive measures.

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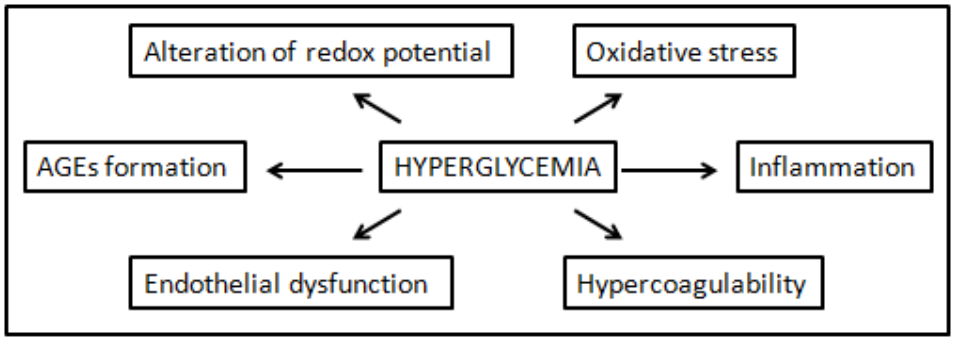

Figure 1: Mechanisms involved in the development of vascular complications in diabetes mellitus.

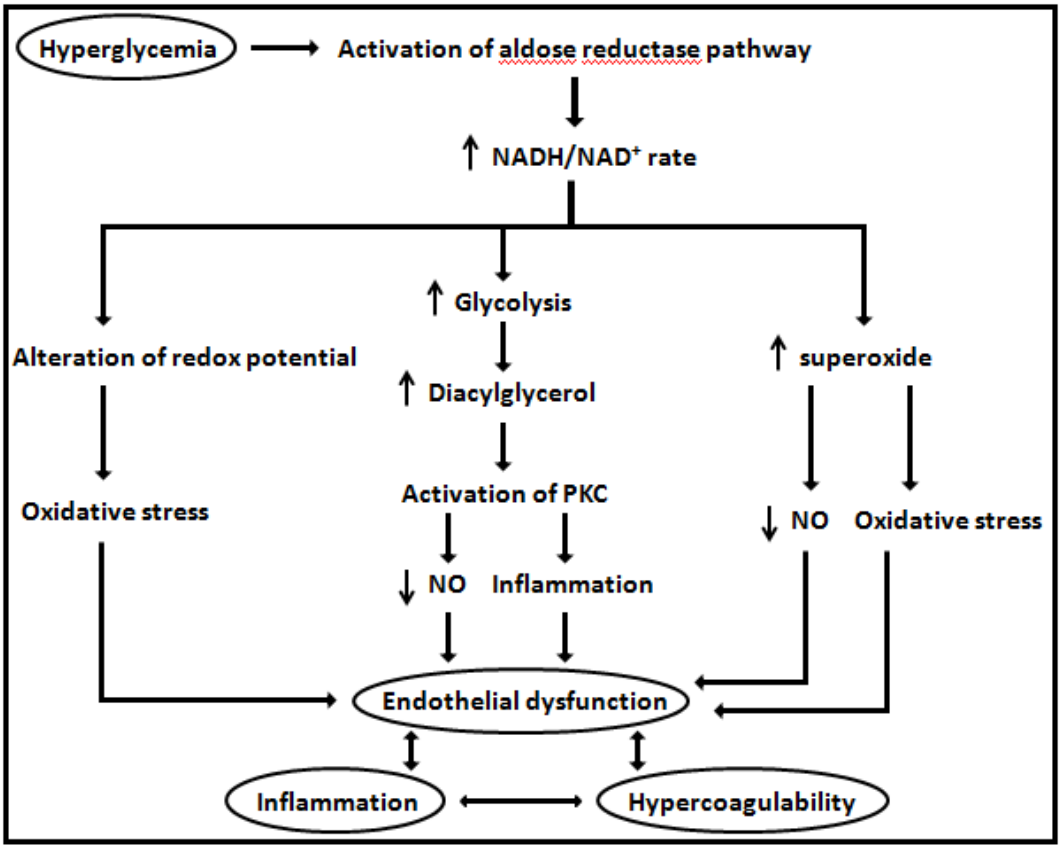

Figure 2: Mechanism by which hyperglycemia can promote vascular complications through activation of aldose reductase pathway.

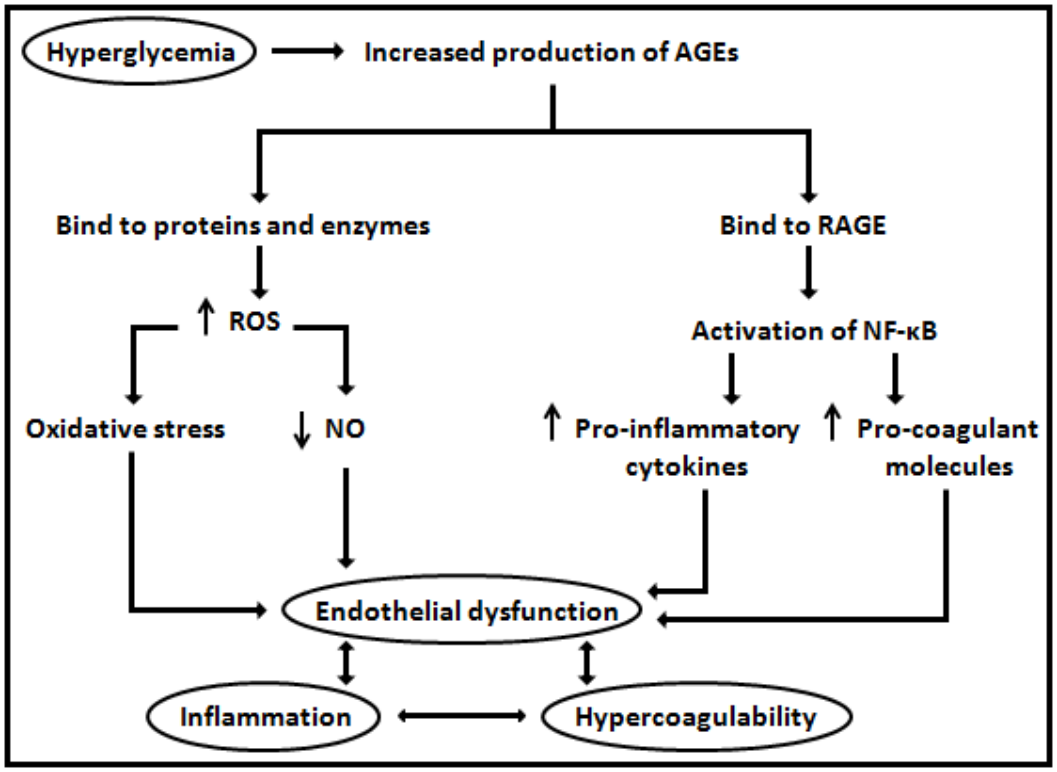

Figure 3: Mechanism by which hyperglycemia can promote vascular complications through increased production of advanced glycation end-products (AGEs).

EVALUATION OF CREATININE-BASED AND CYSTATIN C-BASED EQUATIONS FOR ESTIMATION OF GLOMERULAR FILTRATION RATE IN TYPE 1 DIABETIC PATIENTS ACCORDING TO ALBUMINURIA

Caroline Pereira Domingueti[1], Rodrigo Bastos Fóscolo[2], Ana Cristina Simões e Silva[3], Luci Maria S. Dusse[1], Janice Sepúlveda Reis[4], Maria das Graças Carvalho[1], Ana Paula Fernandes[1] and Karina Braga Gomes[1]

1- Departament of Clinical and Toxicological Analysis, Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte – Minas Gerais, Brazil
2- Departament of Medical Clinic, Faculty of Medicine, Federal University of Minas Gerais, Belo Horizonte – Minas Gerais, Brazil
3- Departament of Pediatrics, Faculty of Medicine, Federal University of Minas Gerais, Belo Horizonte – Minas Gerais, Brazil
4- Departament of Endocrinology and Metabolism, Institute of Education and Research of Santa Casa of Belo Horizonte, Belo Horizonte – Minas Gerais, Brazil

Address correspondence to

Karina Braga Gomes

Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais Av. Antônio Carlos, 6627 - Pampulha - Belo Horizonte – MG – Brazil, CEP 31270-901.

Telephone: (55) (31) 3409-6902

e-mail: karinabgb@gmail.com

Abstract

Aim: We compared glomerular filtration rate (GFR), as estimated by creatinine-based and cystatin C-based equations, according to albuminuria, in type 1 diabetes (DM1), in an observational case-control study.

Methods: DM1 patients were classified according to albuminuria: normoalbuminuric (n=63), microalbuminuric (n=30), macroalbuminuric (n=32). GFR was calculated using creatinine-based (aMDRD and CKD-EPI) and cystatin C-based (Stevens, MacIsaac and Tan) equations. Correlation of GFR estimated by the formulas with albuminuria was evaluated by Spearman Correlation. ROC curves were constructed to compare AUCs of GFR estimated by equations, in reference to macroalbuminuria. Sensibility, specificity and accuracy were calculated for a cut-off<60 mL/min/1.73m^2.

Results: GFR estimated by creatinine-based and cystatin C-based equations significantly differed among normoalbuminuric, microalbuminuric and macroalbuminuric patients. Spearman correlation and AUCs of GFR estimated by creatinine-based and cystatin C-based formulas were very similar to each other. However, cystatin C-based equations presented better correlation with albuminuria and higher AUCs than creatinine-based ones, and also presented the best accuracy to detect macroalbuminuric patients.

Conclusion: GFR estimated by all creatinine-based and cystatin C-based equations permitted the differentiation between DM1 patients according to albuminuria. However, cystatin C-based equations may better correlate with macroalbuminuria.

Keywords: Albuminuria; Cystatin C; Glomerular Filtration Rate; Diabetic Nephropathy.

1. Introduction

Diabetic nephropathy is characterized by a progressive increase in urinary albumin excretion (UAE), resulting in glomerular filtration declining and, eventually, renal failure [1]. It is the most important cause of end stage renal disease (ESRD), and an independent risk factor for cardiovascular disease [2]. It is estimated that 25 to 40% of type 1 (DM1) and type 2 (DM2) diabetic patients develop renal disease 20 to 25 years after the diagnosis of diabetes [3].

Diabetic nephropathy can be divided into stages, according to UAE. The initial stage or incipient nephropathy is characterized by microalbuminuria, while the advanced stage or clinical nephropathy is characterized by macroalbuminuria [4]. UAE is the most important biomarker of renal parenchyma injury, and is used to diagnose and to establish the prognosis of nephropathy [5]. The evaluation of UAE must be conducted along with glomerular filtration rate (GFR) estimation to assess renal function in diabetic patients, since some patients with normoalbuminuria present a decline in GFR [4, 6].

The most used creatinine-based equations to estimate GFR in clinical practice are Cockcroft-Gault (CG) and abbreviated Modification of Diet in Renal Disease (aMDRD) [7, 8]. However, the CG formula tends to overestimate GFR, especially in patients with severe renal disease, and aMDRD tends to subestimate GFR of persons with normal renal function [9, 10, 11]. Besides, the CG formula was developed before standardization of creatinine assays and cannot be re-expressed for use with these assays [12]. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) research group developed a new creatinine-based equation to estimate GFR as an attempt to overcome the limitations of the other formulas [13]. However, this new equation also has some limitations and there is still no ideal equation to estimate GFR [14,15].

Cystatin C, a new endogenous biomarker of renal function, was also proposed to assess GFR [16, 17, 18]. Some authors have suggested that cystatin C-based equations are equal or superior to creatinine-based ones, although they emphasize that further studies are necessary to evaluate its efficacy in different groups of patients [16, 17].

In this study, we compared the estimation of GFR by different creatinine-based and cystatin C-based equations in DM1 patients. In addition, we evaluated the correlation of estimated GFR by these equations according to levels of albuminuria.

2. Materials and Methods

The clinical records of 240 consecutive DM1 patients being assisted at Endocrinology Ambulatories of the University Hospital (*Hospital das Clínicas*) and Santa Casa de Misericórdia/Belo Horizonte, Brazil, from November 2011 to September 2012 were analysed. After application of exclusion criteria, 125 patients with clinical and laboratorial diagnosis of DM1 [19], 18 to 60 years of age, were selected for this study.

DM1 patients with hepatic disease, alcoholism, coagulation or haemostatic abnormalities, malignant diseases, acute infectious, history of kidney transplantation, pregnancy and undergoing hemodialysis were excluded from the study.

All procedures performed in this study were in accordance with the 2000 Declaration of Helsinki as well as the Declaration of Istanbul 2008. This study was approved by the Research Ethics Committee of Federal University of Minas Gerais (CAAE – 0392.0.203.000-11) and informed consent was obtained from all individual participants included in the study. The research protocol did not interfere with any medical recommendations or prescriptions.

Data regarding age, sex, weight, height, time of diagnosis of diabetes, presence of retinopathy and neuropathy, use of antihypertensive, statin and AAS were obtained from medical records. Fasting glucose, creatinine and urea were determined by enzymatic method; albumin was assessed by colorimetric method and HbA1c was determined by immunoturbidimetric method; using Johnson & Johnson dry chemistry technology kits (Ortho Clinical Diagnostics®) and VITROS 4600 analyzer. Cystatin C was measured by ELISA, using Human Cistatin C kit (Biovendor®). UAE was determined in urine samples collected after at least 4 hours of urinary retention, and urinary albumin was normalized by urinary creatinine. Urinary albumin was evaluated by immunoturbidimetric method and urinary creatinine was assessed by enzymatic

method, using Johnson & Johnson dry chemistry technology kits (Ortho Clinical Diagnostics®) and VITROS 4600 analyzer.

Normoalbuminuria was defined as < 30 mg of albumin/g of creatinine, microalbuminuria as ≥ 30 e < 300 mg of albumin/g of creatinine and macroalbuminuria as ≥ 300 mg of albumin/g of creatinine. The presence of microalbuminuria or macroalbuminuria was confirmed in two out of three occasions, over a period between three and six months [6].

The estimated glomerular filtration rate was calculated using two creatinine-based equations: eGFR-aMDRD expressed for standardized serum creatine [20] and eGFR-CKD-EPI [13]. Three cystatin C-based equations were also used: eGFR-Stevens [16], eGFR-MacIsaac [17], eGFR-Tan [18] (Table 1). CG formula was not used because it cannot be re-expressed for standardized serum creatinine [12].

Patients were also classified according to eGFR assessed by creatinine-based and cystatin C-based equations into three groups: eGFR ≥ 90 mL/min/1.73m^2; eGFR ≥ 60 and < 90 mL/min/1.73m^2; eGFR< 60 mL/min/1.73m^2.

Statistical comparisons were performed using SPSS software (version 20.0, SPSS). The Shapiro-Wilk test was used to test the normality of the variables. Data normally distributed were expressed as mean ± SD and were compared by ANOVA and T test. Data not normally distributed were expressed as median (percentiles 25% - 75%) and were compared by Kruskal-Wallis H test and Mann-Whitney U test, followed by Bonferroni correction. Categorical variables were expressed as frequencies and compared using chi-square test (χ^2). The correlation of the GFR estimated by the formulas with UAE was evaluated by Spearman Correlation. eGFR was categorized into three groups (≥ 90, ≥ 60 and < 90 and < 60 mL/min/1.73m^2) and was compared to normoalbuminuria, microalbuminuria and macroalbuminuria using chi-square test (χ^2) and kappa index was calculated. eGFR was also categorized into two groups (≥ 60 and < 60 mL/min/1.73m^2) and was compared to normoalbuminuria or microalbuminuria and macroalbuminuria using chi-square test (χ^2) and odds ratio was calculated. ROC curves were constructed to compare the

area under curve (AUC) of GFR estimated by different equations, in reference to macroalbuminuria. Sensibility, specificity and accuracy were calculated for a cut-off < 60 mL/min/1.73m^2. Differences were considered significant when $p \leq 0.05$.

3. Results

The clinical records of all consecutive 240 DM1 patients being assisted at the Endocrinology Ambulatories of University Hospital (Hospital das Clínicas) and Santa Casa de Misericórdia/Belo Horizonte, Brazil, from November 2011 to September 2012 were analysed and 15 were excluded from the study due to presence of at least one of the following exclusion criteria: hepatic disease, alcoholism, haemostatic abnormalities, malignant diseases, acute infectious, history of kidney transplantation, pregnancy or undergoing hemodialysis. Therefore, 125 DM1 patients were included in this observational case-control study and their characteristics and clinical variables are presented in Table 2.

Patients with macroalbuminuria presented lower BMI than patients with micro and normoalbuminuria ($p = 0.001$ and $p = 0.006$, respectively) and an increased frequency of retinopathy, more frequent use of antihypertensive, statin and AAS than patients with normoalbuminuria ($p < 0.001$, $p < 0.001$, $p = 0.001$, $p = 0.001$, respectively). There were no significant differences among the groups regarding to age, sex, time of diagnosis and frequency of neuropathy. Fasting glucose was lower in patients with microalbuminuria as compared to patients with normoalbuminuria ($p < 0.001$). Higher levels of HbA1c and reduced serum albumin were observed in patients with macroalbuminuria, when compared to those with normoalbuminuria ($p = 0.009$ and $p = 0.007$, respectively). Patients with microalbuminuria presented increased levels of urea and UAE than patients with normoalbuminuria ($p = 0.001$ and $p < 0.001$, respectively) and patients with macroalbuminuria had higher levels of urea, creatinine, cystatin C and UAE than the other groups ($p < 0.001$). eGFR was reduced in patients with microalbuminuria compared to patients with normoalbuminuria, independent of the equation used to estimate GFR (eGFR-aMDRD, $p = 0.004$; eGFR-CKD-EPI, $p = 0.001$; eGFR-Tan, $p = 0.003$; eGFR-MacIsaac, $p = 0.003$; eGFR-Stevens, $p = 0.002$). eGFR was also reduced in patients

with macroalbuminuria as compared to patients with micro and normoalbuminuria, independent of the equation used to estimate GFR ($p < 0.001$).

The classification of patients according to GFR as estimated by the formulas (eGFR ≥ 90 mL/min/1.73m^2; eGFR ≥ 60 and < 90 mL/min/1.73m^2; eGFR< 60 mL/min/1.73m^2) was correlated to the classification of patients according to UAE (Table 3). The kappa index and Spearman correlation were used to assess the quality of the patients classification. The classification of patients according to GFR estimated by aMDRD equation had a regular correlation with the classification of patients according UAE ($0.20 \leq k \leq 0.39$), while GFR estimated by CKD-EPI and cystatin C-based equations presented a moderate correlation ($0.40 \leq k \leq 0.59$). Spearman correlation has shown that GFR estimated by cystatin C-based equations presented a better correlation with UAE than GFR estimated by creatinine-based equations.

Patients were also classified into two groups according to GFR estimated by the formulas (eGFR ≥ 60 and < 60 mL/min/1.73m^2) and GFR was associated to presence or absence of macroalbuminuria (Table 4). The reduced GFR estimated by all the formulas presented a significant association with macroalbuminuria, as observed by odds ratio analysis [OR of 14.8 (5.7 – 38.9), 21.2 (7.1 – 62.8) and 31.9 (10.5 – 97.3) for aMDRD, CKD-EPI and cystatin C-based equations, respectively]. It was also observed that the GFR estimated by cystatin C-based equations presented a better association with macroalbuminuria than the GFR estimated by creatinine-based equations.

ROC curves were constructed to compare AUCs of GFR estimated by the formulas in reference to presence of macroalbuminuria and very similar AUCs were observed. However, it was verified that cystatin C-based equations presented higher AUCs than creatinine-based equations. We also calculated sensitivity, specificity and accuracy for a cut-off < 60 mL/min/1.73m^2 and observed that aMDRD and cystatin C-based equations presented the same sensitivity to detect patients with macroalbuminuria, while CKD-EPI and cystatin C-based equations presented the same specificity. However, aMDRD formula presented a worse specificity than cystatin C-based equation, while CKD-EPI formula presented a worse sensitivity.

Accuracy was very similar among the formulas, however, cystatin C-based equations presented higher accuracy than creatinine-based equations (Table 5).

4. Discussion

Nephropathy is a very common complication in DM1 patients. Early detection of this complication is a relevant issue, since the majority of patients with nephropathy have hypertension, dyslipidemia and an increased risk of cardiovascular disease and renal failure [4]. Several biomarkers and formulas based in these biomarkers may be used to estimate GFR and to evaluate renal function. However, all of them have limitations and there is still no ideal biomarker. Moreover, very few studies have compared the performance of them in DM1 patients. Here, we have compared two important biomarkers, creatinine and cystatin C, and their most used derived formulas to estimate GFR. Furthermore, we evaluated the correlation of these formulas with the levels of UAE, a recognized biomarker of renal injury, in DM1 patients.

As expected, clinical findings differed in diabetic patients according to UAE. Increased frequencies of use of antihypertensive, statin and AAS were observed in patients with macroalbuminuria, as expected [4, 21]. An increased frequency of retinopathy was also detected, which can be explained by the fact that retinopathy and nephropathy are both microvascular complications of diabetes and may have similar pathological mechanisms [21]. Patients with macroalbuminuria also presented increased levels of HbA1c, indicating a poor glycemic control, which is indeed a risk factor for diabetic nephropathy, and decreased BMI, since anemia and malnutrition are common conditions in patients with renal disease [4, 21].

In this study, patients with normoalbuminuria, microalbuminuria and macroalbuminuria could be distinguished, regardless the creatinine-based equation used to estimate GFR, although serum creatinine was not discriminatory enough to differentiate patients with normoalbuminuria and microalbuminuria. This result is due to the fact that serum creatinine is affected by several other factors besides GFR and it is less sensitive to detect mild renal dysfunction. It has been demonstrated that GFR declines to approximately half the normal level before serum creatinine rises above the upper limit of normality [22]. GFR as estimated by all cystatin C-based equations also differed according to the levels of UAE, while plasma cystatin C did

not distinguish patients with normoalbuminuria and microalbuminuria. Therefore, estimated GFR was better to assess renal dysfunction than a single measurement of plasma cystatin C levels. In contrast, other studies have shown that serial measurements of plasma cystatin C can be useful to detect early renal dysfunction in DM1 patients [23, 24].

Some patients with normoalbuminuria presented reduced GFR, independent of the equation used to estimate GFR. Decline in GFR has been detected in DM1 and DM2 patients with normoalbuminuria [25, 26]. While this finding may suggest the presence of renal dysfunction in the absence of albuminuria, the decline of GFR might also be due to underestimation of GFR by the equations used. We have observed that when aMDRD equation was used to estimate GFR, an increased number of patients with normoalbuminuria presented reduced GFR. This is in agreement with other studies which have demonstrated that this formula tends to underestimate GFR in patients with normal renal function [9, 24].

Regardless of the equation used to estimate GFR, some patients with macroalbuminuria presented normal GFR or just a mild decline in GFR. This result suggests that simultaneous assessment of UAE and GFR are complementary for diagnosis of DM1 patients with nephropathy. Many patients with microalbuminuria also presented normal GFR, regardless of the equation used to estimate GFR, which can be explained by the fact that microalbuminuria is an earlier biomarker of renal injury and a risk factor for progression of nephropathy [6, 27].

The classification of patients according to GFR as estimated by aMDRD equation presented the worst correlation with the UAE classification of patients, while CKD-EPI formula presented the best correlation, according to Kappa index evaluation. Among creatinine-based equations, aMDRD formula also presented a worse accuracy to detect patients with macroalbuminuria, as compared to CKD-EPI formula. Besides, CKD-EPI formula was better associated with UAE than aMDRD equation, as shown by Spearman Correlation. This result is in agreement with other studies, which have demonstrated that CKD-EPI equation is more accurate than aMDRD formula to evaluate renal function [12, 28, 29, 30]. Indeed, National Kidney

Foundation has recently recommended the use of CKD-EPI formula to evaluate GFR [30].

The odds ratio analysis has demonstrated that the GFR < 60 mL/min/1.73m^2 estimated by cystatin C-based and creatinine-based equations presented similar associations with macroalbuminuria, indicating that a reduced GFR estimated by any the formulas is significantly associated with renal injury. However, cystatin C-based equations presented a slightly increased association with macroalbuminuria than creatine-based equations. Spearman Correlation and ROC curves comparing AUCs for creatinine-based and cystatin C-based formulas were also very similar to each other. However, cystatin C-based equations presented a slightly better correlation with UAE and higher AUCs to detect patients with macroalbuminuria than creatinine-based equations. Although cystatin C-based equations have presented the same sensitivity of aMDRD formula and the same specificity of CKD-EPI formula to detect patients with macroalbuminuria, they have showed a better specificity than aMDRD formula, a better sensitivity than CKD-EPI formula and a higher accuracy than both formulas. These data suggest a better association between cystatin C-based formulas with the presence of renal injury. In agreement, Yoo *et al.* [31] have demonstrated that the annual change in GFR estimated by cystatin C-based formula reflected the progression of albuminuria more accurately than GFR estimated by aMDRD formula in DM2 patients.

Nonetheless, the superiority of cystatin C-based equations to evaluate the presence of renal dysfunction in diabetic patients is still controversial. Thus, some authors have suggested that cystatin C-based formulas and serum cystatin C improve the assessment of renal function of these patients compared to creatinine-based formulas [18, 23, 24, 32, 33] while others suggest that they have the same efficacy to evaluate renal function of diabetic patients [34, 35]. Therefore, further studies are still necessary to elucidate this issue and to assess whether cystatin C-based equations really improve the assessment of GFR or not.

The transversal design of this study is a limitation, since longitudinal determinations of the GFR are more relevant for monitoring renal function than a unique measure. Indeed, it has been demonstrated that serial measurements of cystatin C may be

more useful for evaluation of early renal function decline than GFR estimated by creatinine-based equations [24] Therefore, further longitudinal studies aiming to evaluate the association between GFR estimated by different cystatin C-based and creatinine-based equations with albuminuria are relevant and necessary.

In conclusion, all creatinine-based and cystatin C-based equations were able to estimate GFR in DM1 patients and correlated with different levels of UAE. However, cystatin C-based equations showed a slight increase in accuracy. On the other hand, as cystatin C is not always available in clinical practice and there is no evident superiority of cystatin C-based formulas to assess GFR, we suggest that creatinine-based formulas, particularly CKD-EPI formula, might be used to evaluate renal function in DM1 patients. We emphasize that further studies aiming to discover new renal biomarkers are extremely important, since there is still no ideal biomarker to evaluate GFR and to detect early decline of renal function.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Table 1 – Creatinine-based and cystatin C-based equations used to estimate GFR

aMDRD
eGFR (mL/min/1.73m^2) = 175 x serum creatinine (mg/dL)$^{-1.154}$ x age (years)$^{-0.203}$ x 0.742 (if female) x 1.212 (if black)
CKD-EPI
Male:
Serum creatinine ≤ 0.9 mg/dL: eGFR (mL/min/1,73m^2) = α x (serum creatinine in mg/dL/0.9)$^{-0.411}$ x (0,993)^{age}
Serum creatinine > 0.9 mg/dL: eGFR (mL/min/1.73m^2) = α x (serum creatinine in mg/dL/0.9)$^{-1.209}$ x (0.993)^{age}
Female:
Serum creatinine ≤ 0.7 mg/dL: eGFR (mL/min/1.73m^2) = α x (serum creatinine in mg/dL/0.7)$^{-0.329}$ x (0,993)^{age}
Serum creatinine > 0.7 mg/dL: eGFR (mL/min/1.73m^2) = α x (serum creatinine in mg/dL/0.7)$^{-1.209}$ x (0,993)^{age}
α = 141 for non-black male, 144 for non-black female, 163 for black male, 166 for black female
Stevens
127.7 x serum cystatin C (mg/L)$^{-1.17}$ x age (years)$^{-0.13}$ x 0.91 (if female) x 1.06 (if black)
MacIsaac
(84.6/serum cystatin C in mg/L) – 3.2
Tan
(87.1/serum cystatin C in mg/L) – 6.87

Table 2 – Characteristics of diabetic patients according to albuminuria

	Patients with normoalbuminuria	Patients with microalbuminuria	Patients with macroalbuminuria	p
Number of Individuals (n)	63	30	32	
Age (years)	30 ± 8	37 ± 11	34 ± 10	NA
Sex/male (n, %)	25 (55.6)	6 (13.3)	14 (31.1)	NA
BMI (kg/m^2)	24.5 ± 3.3	26.1 ± 3.4	21.8 ± 2.6**†	0.001** 0.006†
Time of Diagnosis (years)	18 ± 8	17 ± 6	20 ± 5	NA
Retinopathy (n,%)	18 (32.7)	14 (25.5)	23 (41.8)**	<0.001**
Neuropathy (n,%)	11 (55.0)	3 (15.0)	6 (30.0)	NA
Use of Antihypertensive (n, %)	31 (38.8)	21 (26.2)	28 (35.0)**	<0.001**
Use of Statin (n, %)	12 (30.0)	12 (30.0)	16 (40.0)**	0.001**
Use of AAS (n, %)	5 (23.8)	4 (19.0)	12 (57.1)**	0.001**
Fasting Glucose (mg/dL)	176 ± 76	148 ± 69*	149 ± 68	< 0.001*
HbA1c (%)	8.1 ± 1.3	8.2 ± 1.2	9.8 ± 2.2**	0.009**
Creatinine (mg/dL)	0.79 (0.66 – 0.89)	0.91 (0.68 – 1.10)	1.53 (1.03 – 2.16)**†	<0.001** <0.001†
Urea (mg/dL)	30 ± 7	35 ± 9*	48 ± 15**†	0.001* <0.001** <0.001†
Albumin (g/dL)	4.1 ± 0.4	3.9 ± 0.4	3.9 ± 0.4**	0.007**
Cystatin C (ng/mL)	734 (651 – 842)	831 (672 – 941)	1834 (1074 – 2558)**†	< 0.001** < 0.001†
eGFR-aMDRD (mL/min/1.73m^2)	101 (89 – 112)	85 (57 – 105)*	45 (26 – 72)**†	0.004* < 0.001** < 0.001†
eGFR-CKD-EPI (mL/min/1.73m^2)	114 (104 – 123)	98 (74 – 118)*	49 (28 – 83)**†	0.001* < 0.001** < 0.001†
eGFR-Tan (mL/min/1.73m^2)	109 (95 – 119)	90 (71 – 105)*	41 (27 – 74)**†	0.003* < 0.001** < 0.001†
eGFR-MacIsaac (mL/min/1.73m^2)	112 (97 – 121)	92 (73 – 107)*	43 (30 – 77)**†	0.003* < 0.001** < 0.001†
eGFR-Stevens (mL/min/1.73m^2)	112 (89 – 129)	86 (67 – 106)*	37 (25 – 72)**†	0.002* < 0.001** < 0.001†
UAE (mg/g de	7 (4 – 14)	68 (49 – 150)*	926	< 0.001*

creatinine)	(475 – 1535)**†	< 0.001**
		< 0.001†

Normally-distributed data were expressed as mean ± SD and compared by ANOVA and T test. Not normally distributed data were expressed as median (percentiles 25% – 75%) and compared by the Kruskal-Wallis H test and Mann-Whitney U test, followed by Bonferroni correction. Categorical variables were expressed as frequencies n (%) and compared using the chi-square test (χ^2).

* P < 0.05 for patients with microalbuminuria compared to patients with normoalbuminuria.

** P < 0.05 for patients with macroalbuminuria compared to patients with normoalbuminuria.

†P < 0.05 for patients with macroalbuminuria compared to patients with microalbuminuria.

Table 3 – Comparison of classification of patients according to level of GFR decline estimated by the different formulas and urinary albumin excretion and correlation of the GFR estimated by the formulas with urinary albumin excretion

Classification of patients	Patients with normoalbuminuria	Patients with microalbuminuria	Patients with macroalbuminuria	Kappa index	Spearman correlation
Total (n)	63	30	32		
eGFR-aMDRD (mL/min/1.73m^2)				0.326	-0.508
≥ 90	42 (66.7)	14 (46.7)	4 (12.5)		
≥ 60 and < 90	17 (27.0)	8 (26.7)	6 (18.8)		
< 60	4 (6.3)	8 (26.7)	22 (68.8)		
eGFR-CKD-EPI (mL/min/1.73m^2)				0.450	-0.529
≥ 90	54 (85.7)	16 (53.3)	6 (18.8)		
≥ 60 and < 90	6 (9.5)	11 (36.7)	7 (21.9)		
< 60	3 (4.8)	3 (10.0)	19 (59.4)		
eGFR-Tan (mL/min/1.73m^2)				0.433	-0.609
≥ 90	51 (81.0)	16 (53.3)	4 (12.5)		
≥ 60 and < 90	11 (17.5)	9 (30.0)	6 (18.8)		
< 60	1 (1.6)	5 (16.7)	22 (68.8)		
eGFR-MacIsaac (mL/min/1.73m^2)				0.429	-0.608
≥ 90	52 (82.5)	17 (56.7)	4 (12.5)		
≥ 60 and < 90	10 (15.9)	8 (26.7)	6 (18.8)		
< 60	1 (1.6)	5 (16.7)	22 (68.8)		
eGFR-Stevens (mL/min/1.73m^2)				0.428	-0.575
≥ 90	48 (76.2)	14 (46.7)	5 (15.6)		
≥ 60 and < 90	14 (22.2)	11 (36.7)	5 (15.6)		
< 60	1 (1.6)	5 (16.7)	22 (68.8)		

Variables were compared using the chi-square test (χ^2) and Kappa index was calculated. Spearman correlation was calculated for variables non-categorized.

Table 4 – Association between GFR estimated by the formulas < 60 mL/min/1.73m^2 and macroalbuminuria

Classification of patients	Patients with normoalbuminuria or microalbuminuria	Patients with macroalbuminuria	Odds Ratio (95% Confidence Interval)	p
Total (n)	93	32		
eGFR-aMDRD (mL/min/1.73m^2)				
≥ 60	81 (87.1)	10 (31.2)	14.8 (5.7 – 38.9)	< 0.001
< 60	12 (12.9)	22 (68.8)*		
eGFR-CKD-EPI (mL/min/1.73m^2)				
≥ 60	87 (93.5)	13 (40.6)	21.2 (7.1 – 62.8)	< 0.001
< 60	6 (6.5)	19 (59.4)*		
eGFR-Tan (mL/min/1.73m^2)				
≥ 60	87 (93.5)	10 (31.2)	31.9 (10.5 – 97.3)	< 0.001
< 60	6 (6.5)	22 (68.8)*		
eGFR-MacIsaac (mL/min/1.73m^2)				
≥ 60	87 (93.5)	10 (31.2)	31.9 (10.5 – 97.3)	< 0.001
< 60	6 (6.5)	22 (68.8)*		
eGFR-Stevens (mL/min/1.73m^2)				
≥ 60	87 (93.5)	10 (31.2)	31.9 (10.5 – 97.3)	< 0.001
< 60	6 (6.5)	22 (68.8)*		

Variables were compared using the chi-square test (χ^2) and odds ratio (95% confidence interval) was calculated.
* P < 0.05 for patients with macroalbuminuria compared to those with normoalbuminuria or microalbuminuria.

Table 5 – AUC of the GFR estimated by the formulas in reference to macroalbuminuria, sensitivity, specificity and accuracy for GFR < 60 mL/min/1.73m^2

Formula	AUC	Sensitivity (%)	Specificity (%)	Accuracy (%)
eGFR-aMDRD	0.819 (0.730 – 0.908)	68.8	87.1	82.4
eGFR-CKD-EPI	0.830 (0.738 – 0.922)	59.4	93.5	84.8
eGFR-Tan	0.866 (0.784 – 0.947)	68.8	93.5	87.2
eGFR-MacIsaac	0.869 (0.788 – 0.950)	68.8	93.5	87.2
eGFR-Stevens	0.843 (0.752 – 0.933)	68.8	93.5	87.2

Area under curves (AUCs) are presented as median (95% confidence interval). Sensitivity was defined as the percentage of patients with eGFR < 60 mL/min/1.73m^2 among patients with macroalbuminuria. Specificity was defined as the percentage of patients with eGFR ≥ 60 mL/min/1.73m^2 among patients with normoalbuminuria or microalbuminuria. Accuracy was defined as the percentage of patients with eGFR < 60 mL/min/1.73m^2 and macroalbuminuria and with eGFR ≥ 60 mL/min/1.73m^2 and normoalbuminuria or microalbuminuria among all patients.

ASSOCIATION OF DIFFERENT BIOMARKERS OF RENAL FUNCTION WITH D-DIMER LEVELS IN TYPE 1 DIABETIC PATIENTS

Caroline Pereira Domingueti[1], Rodrigo Bastos Fóscolo[2], Luci Maria Sant'Ana Dusse[1], Janice Sepúlveda Reis[3], Maria das Graças Carvalho[1], Karina Braga Gomes[1], Ana Paula Fernandes[1]

1- Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte – Minas Gerais, Brasil
2- Departamento de Clínica Médica, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte – Minas Gerais, Brasil
3- Departamento de Endocrinologia e Metabologia, Instituto de Ensino e Pesquisa da Santa Casa de Belo Horizonte, Belo Horizonte – Minas Gerais, Brasil

Address correspondence to

Caroline Pereira Domingueti

Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais Av. Antônio Carlos, 6627 - Pampulha - Belo Horizonte – MG – Brazil, CEP 31270-901.

Telephone: (55) (31) 3409-6902

e-mail: caroldomingueti@yahoo.com.br

Abstract

Evaluate the association between different renal biomarkers with D-Dimer levels, an important biomarker of hypercoagulability, in type 1 diabetic (DM1) patients. DM1 patients were divided into two groups: low D-Dimer levels (<318ng/mL), which included first and second D-Dimer tertiles, and high D-Dimer levels (≥318ng/mL), which included third D-Dimer tertile. D-Dimer and cystatin C were measured by ELISA. Creatinine and urea were determined by enzymatic method. Albuminuria was assessed by immunoturbidimetry. GFR was calculated using creatinine-based and cystatin C-based equations. Frequency of patients with renal disease, as defined by different renal biomarkers, related to high D-Dimer levels was evaluated using chi-square test, and odds ratio was calculated. Patients of high D-Dimer group presented an increased frequency of renal disease, evaluated by different renal biomarkers. Albuminuria, cystatin C and cystatin C-based eGFR presented a better association [OR of 7.5(3.1–18.4), 9.0(3.8–21.1), 8.0(3.1–20.7), respectively] with high D-Dimer levels, than creatinine, urea, and creatinine-based eGFR. Only increased albuminuria and cystatin C were independently associated with high D-Dimer levels, even after adjusting for sex and age. Albuminuria, cystatin C and cystatin C-based eGFR present a better association with D-Dimer levels, and consequently, with hypercoagulability status than urea, creatinine and eGFR based on creatinine.

Keywords: D-Dimer, Type 1 Diabetes Mellitus, Glomerular Filtration Rate, Cystatin C, Creatinine, Albuminuria.

1. Introduction

Diabetic nephropathy is defined as a progressive rise in urinary albumin excretion (UAE), leading to glomerular filtration declining and, eventually, renal failure [1]. It is the most important cause of end stage renal disease (ESRD), and an independent risk factor for cardiovascular disease, responsible for increased mortality. It is estimated that nearly 30% of diabetic patients develop renal disease [2, 3].

Several biomarkers can be used to evaluate renal function in diabetic patients, as creatinine and urea. However, various factors can influence their levels besides renal disease, therefore the estimative of glomerular filtration rate (GFR) is more used in clinical practice [4, 5]. Another important biomarker of renal injury is UAE, which is very used to diagnosis and prognosis of nephropathy [4, 6]. Cystatin C, a new endogenous biomarker of renal function, was also proposed to assess GFR and has been shown very promising to evaluate renal function [6, 7].

D-Dimer is a specific degradation product of cross-linked fibrin clots. It is a classic hypercoagulability biomarker, useful in the diagnosis of thromboembolic events [8]. There is an association between D-Dimer levels with the development of atherothrombosis and cardiovascular complications in diabetic patients, indicating that D-Dimer can be useful to evaluate the risk of cardiovascular disease in these patients [8, 9, 10]. D-Dimer levels also increase with the progression of renal disease in diabetic patients, indicating that hypercoagulability could be a link between diabetic nephropathy and the increased risk of cardiovascular outcomes [11, 12, 13].

A biomarker that is capable to detect renal function decline and that is simultaneously associated with a higher hypercoagulability status could be of great value, since it could be useful to detect diabetic patients with renal disease that present an increased risk of cardiovascular disease, contributing to an early adoption of reno and cardioprotective therapies and, consequently, to a reduction of mortality.

Therefore, this study aimed to evaluate the association between different biomarkers of renal function with D-Dimer levels to assess which biomarkers are better associated with the hypercoagulability status in type 1 diabetic (DM1) patients.

2. Materials and methods

All procedures performed in this study were in accordance with the 2000 Declaration of Helsinki as well as the Declaration of Istanbul 2008. This study was approved by the Research Ethics Committee of Federal University of Minas Gerais (CAAE – 0392.0.203.000-11) and an informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations or prescriptions.

The clinical records of 240 consecutive DM1 patients being assisted at Endocrinology Ambulatories of the University Hospital (*Hospital das Clínicas*) and Santa Casa de Misericórdia/Belo Horizonte, Brazil, from November 2011 to September 2012 were analysed. After application of exclusion criteria, 125 patients with clinical and laboratorial diagnosis of DM1 [14], 18 to 60 years of age, were selected for this study. Data regarding age, sex, weight, height, time of diagnosis of DM1, use of antihypertensive, statin and AAS were obtained from medical records.

DM1 patients with hepatic disease, alcoholism, coagulation or haemostatic abnormalities, malignant diseases, acute infectious, history of kidney transplantation, pregnancy and undergoing hemodialysis were excluded from the study.

Creatinine and urea were determined by enzymatic method; albumin was assessed by colorimetric method and HbA1c was determined by immunoturbidimetric method, using Johnson & Johnson dry chemistry technology kits (Ortho Clinical Diagnostics®) and VITROS 4600 analyser. Cystatin C and D-Dimer were measured by ELISA, using Human Cistatin C kit (Biovendor®) and ASSERACHROM® D-Di kit (StagoDiagnostica®), respectively. UAE was determined in urine samples collected after at least 4 hours of urinary retention, and urinary albumin was normalized by urinary creatinine. Urinary albumin was evaluated by immunoturbidimetric method and urinary creatinine was assessed by enzymatic method, using Johnson & Johnson dry chemistry technology kits (Ortho Clinical Diagnostics®) and VITROS 4600 analyser. UAE ≥ 30 mg/g of creatinine was confirmed in two out of three occasions, over a period between three and six months, and median was calculated [6].

The estimated glomerular filtration rate was calculated using four creatinine-based equations: eGFR-CG [15], eGFR-MDRD7 [16], eGFR-MDRDa [17] and eGFR-CKD-EPI [18]. Three cystatin C-based equations were also used: eGFR-Stevens [19], eGFR-MacIsaac [20], eGFR-Tan [21] (Table 1).

Statistical comparisons were performed using SPSS software (version 20.0, SPSS). Diabetic patients were divided into tertiles based on D-Dimer levels and were classified into two groups: low D-Dimer levels (< 318 ng/mL), which included first and second D-Dimer tertiles, and high D-Dimer levels (≥ 318 ng/mL), which included third D-Dimer tertile [22]. The Shapiro-Wilk test was used to test the normality of the variables. Data normally distributed were expressed as mean ± SD and were compared by ANOVA and T test. Data not normally distributed were expressed as median (percentiles 25% - 75%) and were compared by Kruskal-Wallis H test and Mann-Whitney U test. Categorical variables were expressed as frequencies and compared using chi-square test (χ^2). Presence of renal disease was evaluated using each renal biomarker, which were dichotomized using as cut-off ≥ 1.3 mg/dL, ≥ 40 mg/dL, ≥ 0.92 mg/L, ≥ 300 mg/g, and < 60 mL/min/1,73m^2, for creatinine, urea, cystatin C, UAE, and eGFR, respectively [23, 24, 25]. Frequency of patients with renal disease, as defined by different renal biomarkers, related to high D-Dimer levels was evaluated using chi-square test (χ^2) and odds ratio was calculated. Correlation between non-categorized renal biomarkers and D-Dimer levels were evaluated by Spearman Correlation. Multivariate logistic regression analysis was performed to assess which dichotomized renal biomarkers are independently associated with high D-Dimer levels. Variables included in this analysis were previously associated with high D-Dimer levels in bivariate logistic regression analysis ($p < 0.2$), and consisted on creatinine, urea, cystatin C, UAE, GFR estimated by cystatin C-based formulas and GFR estimated by creatinine-based formulas. Odds ratio unadjusted and adjusted for age and sex were calculated. Differences were considered significant when $p \leq 0.05$.

4. Results

The characteristics and clinical variables of the DM1 patients included in this cross-sectional study are presented in Table 2.

Patients with high D-Dimer levels were older ($p = 0.003$) and presented an increased frequency of use of antihypertensive than those with low D-Dimer levels ($p = 0.001$). Frequency of males was decreased in high D-Dimer group compared to low D-Dimer one ($p = 0.003$). There were no significant differences among groups regarding to BMI, time of diagnosis, HbA1c levels, use of statin and use of AAS. Reduced serum albumin was observed in patients of high D-Dimer group, when compared to low D-Dimer one ($p = 0.006$). Patients with high D-Dimer levels presented increased levels of creatinine, urea, cystatin C and UAE than patients with low D-Dimer levels ($p = 0.001$, $p < 0.001$, $p < 0.001$ and $p = 0.004$, respectively). eGFR was reduced in patients with high D-Dimer levels compared to those with low D-Dimer levels ($p < 0.001$ for all formulas), independent of the formula used to estimate GFR.

Patients with high D-Dimer levels presented a greater impairment of renal disease, evaluated by different renal biomarkers (creatinine, urea, cystatin C, UAE and GFR estimated by formulas based on creatinine and on cystatin C) when compared to patients with low D-Dimer levels (Table 3). Odds ratio analysis demonstrated that increased UAE and cystatin C levels, as well as decreased cystatin C-based eGFR formulas presented a stronger association with higher D-Dimer levels when compared to other renal parameters.

Patients with UAE ≥ 300 mg/g, cystatin C ≥ 0.92 mg/L and cystatin C-based eGFR < 60 mL/min/1.73m^2 presented a better association with high D-Dimer levels [OR of 7.5 (3.1–18.4), 9.0 (3.8–21.1) and 8.0 (3.1–20.7), respectively], than patients with creatinine ≥ 1.3 mg/dL, urea ≥ 40 mg/dL and creatinine-based eGFR < 60 mL/min/1.73m^2 [OR of 5.3 (2.1 – 13.3) for creatinine, 3.3 (1.5 – 7.3) for urea, 6.4 (2.6 – 16.1) for MDRDa equation, 5.1 (2.3 – 11.7) for MDRD7 equation, 6.8 (2.4 – 19.2) for CG equation and 6.0 (2.3 – 15.7) for CKD-EPI equation]. Cystatin C levels and cystatin C-based eGFR formulas also correlated better with D-Dimer levels (Spearman correlation was 0.476 for cystatin C, -0.465 for eGFR-Tan, -0.464 for eGFR-MacIsaac, -0.516 for eGFR-Steven) than other renal biomarkers (Spearman correlation was 0.174 for creatinine, 0.238 for urea, 0.314 for UAE, -0.386 for eGFR-MDRDa, -0.391 for eGFR-MDRD7, -0.332 for eGFR-CG, -0.416 for eGFR-CKD-EPI) (Table 4). Multivariate analysis logistic regression has shown that only increased

UAE and cystatin C levels are independently associated with high D-Dimer levels, even after adjusting for sex and age (Table 5). Odds ratio was 4.2 (1.4 – 12.8) and 9.5 (2.4 – 36.6) for cystatin C ≥ 0.92 mg/L and UAE ≥ 300 mg/g in adjusted analysis, respectively.

5. Discussion

Diabetic nephropathy is associated with an increased mortality, mainly due to cardiovascular outcomes [6]. It has been demonstrated that the risk of cardiovascular death gradually increases with progressing stages of nephropathy [26, 27]. Therefore, simultaneously evaluation of renal function and hypercoagulability status, using a unique biomarker, would be of great value. Here, we have evaluated the association between different biomarkers of renal function with hypercoagulability status, as assessed by D-Dimer levels, in DM1 patients.

Some studies have found an association between increased D-Dimer levels and presence of increased UAE levels in diabetic patients [28, 29, 30]. In this study, increased levels of different renal biomarkers, such as creatinine, urea, cystatin C and UAE, and decreased eGFR were observed in patients with high D-Dimer levels. Accordingly, these patients also presented an increased frequency of renal disease, regardless the renal biomarker used to evaluate renal function, corroborating the association between renal function decline and increased hypercoagulability status.

Haase *et al.* [31] has demonstrated that D-Dimer plasma levels are higher in older people and in females, which was also found in this study. Increased frequency of use of antihypertensive was observed in patients with high D-Dimer levels, which was expected since these patients also presented an increased frequency of renal disease. Antihypertensive is commonly prescribed to diabetic patients with nephropathy to protect renal function [32]. These patients also presented reduced serum albumin, which is in accordance to the increased UAE.

The relationship between increased UAE levels with higher risk of cardiovascular disease in DM1 patients has been demonstrated by several authors [26, 33, 34]. After the onset of proteinuria, median survival is about only seven years and this increased mortality is mainly due to cardiovascular death, rather than to renal failure

[35]. Increased levels of cystatin C have also been associated with the development of cardiovascular events [36, 37, 38, 39]. Some authors have even shown that cystatin C and eGFR based on cystatin C are stronger predictors of cardiovascular outcomes in diabetic and elderly patients than creatinine and eGFR based on creatinine [38, 39].

In agreement, in this study, UAE, cystatin C and eGFR based on cystatin C presented a better association, as assessed by odds ratio analysis, with higher D-Dimer levels than urea, creatinine and eGFR based on creatinine. Cystatin C levels and cystatin C-based eGFR also presented a better correlation with D-Dimer levels than other renal biomarkers. These results suggest that UAE, cystatin C and eGFR based on cystatin C present a better association with hypercoagulability status than other renal biomarkers, and might be able to detect haemostatic changes that is not completely captured by measures of urea, creatinine and eGFR based on creatinine. However, further longitudinal studies that directly assess the development of cardiovascular disease are still necessary to confirm the superiority of UAE and cystatin C to predict this risk in comparison to other renal biomarkers.

Regression logistic multivariate analysis has demonstrated that only increased UAE and cystatin C levels are independently associated with high D-Dimer levels, and that this association remained even after adjusting for sex and age, which are variables that can influence D-Dimer levels [31]. This result indicates that these biomarkers are independently associated with hypercoagulability status. Patients with increased UAE may lose important natural anticoagulant molecules in urine, such as antithrombin, protein C and protein S, which could intensify the hypercoagulability status [40].

6. Conclusions

In conclusion, UAE, cystatin C and eGFR based on cystatin C present a better association with high D-Dimer levels than urea, creatinine and eGFR based on creatinine. UAE and cystatin C are also independently associated with high D-Dimer levels. These findings suggest that UAE and cystatin C might present an important utility to evaluate simultaneously renal function decline and the hypercoagulability status in DM1 patients.

7. Acknowlegments

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Table 1 – Creatinine-based and cystatin C-based equations used to estimate glomerular filtration rate

Cockcroft-Gault
eGFR (mL/min1.73m^2) = $\frac{(140 - \text{age in years}) \times (\text{weight in kg})}{72 \times \text{serum creatinine in mg/dL}}$ x 0.85 (if woman) x $\frac{1.73}{\text{body surface (m}^2\text{)}}$
MDRD7
eGFR (mL/min/1.73m^2) = 170 x serum creatinine (mg/dL)$^{-0.999}$ x age (years)$^{-0.176}$ x 0.762 (if female) x 1.18 (if black) x serum urea (mg/dL)$^{-0.17}$ x serum albumin (g/dL)$^{+0.318}$
MDRDa
eGFR (mL/min/1.73m^2) = 186 x serum creatinine (mg/dL)$^{-1.154}$ x age (years)$^{-0.203}$ x 0.742 (if female) x 1.212 (if black)
CKD-EPI
Male:
Serum creatinine ≤ 0.9 mg/dL: eGFR (mL/min/1.73m^2) = α x (serum creatinine in mg/dL/0.9)$^{-0.411}$ x (0,993)^{age}
Serum creatinine > 0.9 mg/dL: eGFR (mL/min/1.73m^2) = α x (serum creatinine in mg/dL/0.9)$^{-1.209}$ x (0,993)^{age}
Female:
Serum creatinine ≤ 0.7 mg/dL: eGFR (mL/min/1.73m^2) = α x (serum creatinine in mg/dL/0.7)$^{-0.329}$ x (0,993) ^{age}
Serum creatinine > 0.7 mg/dL: eGFR (mL/min/1.73m^2) = α x (serum creatinine in mg/dL/0.7)$^{-1.209}$ x (0,993)^{age}
α = 141 for non-black male, 144 for non-black female, 163 for black male, 166 for black female
Stevens
127.7 x serum cystatin C (mg/L)$^{-1.17}$ x age (years)$^{-0.13}$ x 0.91 (if female) x 1.06 (if black)
MacIsaac
(84.6/serum cystatin C in mg/L) – 3.2
Tan
(87.1/serum cystatin C in mg/L) – 6.87

Table 2 – Characteristics of diabetic patients classified according to D-Dimer levels.

	Low D-Dimer Group	High D-Dimer Group	p
Number of Individuals (n)	82	43	
Age (years)	32 (24 – 37)	35 (30 – 45)*	0.003
Sex/male (n, %)	37 (45.1)	8 (18.6)*	0.003
BMI (kg/m^2)	24 ± 3	23 ± 3	NS
Time of Diagnosis (years)	18 ± 8	20 ± 6	NS
Use of Antihypertensive (n, %)	44 (53.7)	36 (83.7)*	0.001
Use of Statin (n, %)	22 (26.8)	18 (41.9)	NS
Use of AAS (n, %)	10 (12.2)	11 (25.6)	NS
HbA1c (%)	8.5 (7.5 – 9.8)	8.4 (7.6 – 8.4)	NS
Creatinine (mg/dL)	0.81 (0.66 – 0.92)	1.02 (0.71 – 1.45)*	0.001
Urea (mg/dL)	31 ± 7	42 ± 17*	< 0.001
Albumin (g/dL)	4.1 ± 0.4	3.8 ± 0.4*	0.006
Cystatin C (mg/L)	0.74 (0.64 – 0.85)	1.11 (0.86 – 1.97)*	< 0.001
eGFR-MDRDa (mL/min/1.73m^2)	105 (83 – 119)	69 (42 – 96)*	< 0.001
eGFR-MDRD7 (mL/min/1.73m^2)	88 (66 – 103)	58 (34 – 87)*	< 0.001
eGFR-CG (mL/min/1.73m^2)	110 ± 34	78 ± 38*	< 0.001
eGFR-CKD-EPI (mL/min/1.73m^2)	112 (91 – 123)	76 (43 – 104)*	< 0.001
eGFR-Tan (mL/min/1.73m^2)	107 ± 23	66 ± 36*	< 0.001
eGFR-MacIsaac (mL/min/1.73m^2)	109 ± 23	68 ± 36*	< 0.001
eGFR-Stevens (mL/min/1.73m^2)	106 ± 35	59 ± 31*	< 0.001
UAE (mg/g of creatinine)	8 (4 – 18)	44 (6 – 157)*	0.004
D-Dimer (ng/mL)	191 (134 – 233)	484 (381 – 639)*	< 0.001

Normally-distributed data were expressed as mean ± SD and compared by ANOVA and T test. Not normally distributed data were expressed as median (percentiles 25% – 75%) and compared by the Kruskal-Wallis H test and Mann-Whitney U test, followed by Bonferroni correction. Categorical variables were expressed as frequencies n (%) and compared using the chi-square test (χ^2).

* $p < 0.05$ for high D-Dimer group compared to low D-Dimer group.

NS: not significant. BMI: body mass index. UAE: urinary albumin excretion. AAS: acetylsalicylic acid.

Table 3 – Frequency of patients with and without renal dysfunction, evaluated by different biomarkers, according to D-Dimer levels.

Classification of patients	Low D-Dimer Group	High D-Dimer Group	p	Odds Ratio (95% confidence interval)
Total (n)	82	43		
Creatinine			< 0.001	5.3 (2.1 – 13.3)
< 1.3 mg/dL	73 (89.0)	26 (60.5)*		
≥ 1.3 mg/dL	9 (11.0)	17 (39.5)*		
Urea			0.002	3.3 (1.5 – 7.3)
< 40 mg/dL	61 (75.3)	20 (46.5)*		
≥ 40 mg/dL	21 (25.6)	23 (53.5)*		
Cystatin C			< 0.001	9.0 (3.8 – 21.1)
< 0.92 mg/L	62 (75.6)	11 (25,6)*		
≥ 0.92 mg/L	20 (24.4)	32 (74.4)*		
UAE			< 0.001	7.5 (3.1 – 18.4)
< 300 mg/g	72 (87.8)	21 (48.8)*		
≥ 300 mg/g	10 (12.2)	22 (51.2)*		
eGFR-MDRDa			< 0.001	6.4 (2.6 – 16.1)
≥ 60 mL/min/1.73m^2	73 (89.0)	24 (55.8)*		
< 60 mL/min/1.73m^2	9 (11.0)	19 (44.2)*		
eGFR-MDRD7			< 0.001	5.1 (2.3 – 11.7)
≥ 60 mL/min/1.73m^2	67 (81.7)	20 (46.5)*		
< 60 mL/min/1.73m^2	15 (18.3)	23 (53.5)*		
eGFR-CG			< 0.001	6.8 (2.4 – 19.2)
≥ 60 mL/min/1.73m^2	76 (92.7)	28 (65.1)*		
< 60 mL/min/1.73m^2	6 (7.3)	15 (34.9)*		
eGFR-CKD-EPI			< 0.001	6.0 (2.3 – 15.7)
≥ 60 mL/min/1.73m^2	74 (90.2)	26 (60.5)*		
< 60 mL/min/1.73m^2	8 (9.8)	17 (39.5)*		
eGFR-Tan			< 0.001	8.0 (3.1 – 20.7)
≥ 60 mL/min/1.73m^2	74 (90.2)	23 (53.5)*		
< 60 mL/min/1.73m^2	8 (9.8)	20 (46.5)*		
eGFR-MacIsaac			< 0.001	8.0 (3.1 – 20.7)
≥ 60 mL/min/1.73m^2	74 (90.2)	23 (53.5)*		
< 60 mL/min/1.73m^2	8 (9.8)	20 (46.5)*		
eGFR-Stevens			< 0.001	8.0 (3.1 – 20.7)
≥ 60 mL/min/1.73m^2	74 (90.2)	23 (53.5)*		
< 60 mL/min/1.73m^2	8 (9.8)	20 (46.5)*		

Variables were compared using the chi-square test (χ^2) and odds ratio (95% confidence interval) was calculated.
* $p < 0.05$ for high D-Dimer group compared to low D-Dimer group.
UAE: urinary albumin excretion.

Table 4 – Correlation of different renal biomarkers with D-Dimer levels.

Biomarker	Spearman Correlation	p
Creatinine	0.174	0.070
Urea	0.238*	0.012
Cystatin C	0.476**	< 0.001
UAE	0.314**	0.005
eGFR-MDRDa	-0.386**	< 0.001
eGFR-MDRD7	-0.391**	< 0.001
eGFR-CG	-0.332**	< 0.001
eGFR-CKD-EPI	-0.416**	< 0.001
eGFR-Tan	-0.465**	< 0.001
eGFR-MacIsaac	-0.464**	< 0.001
eGFR-Stevens	-0.516**	<0.001

**Correlation is significant at the 0.01 level.
*Correlation is significant at the 0.05 level.
UAE: urinary albumin excretion.

Table 5 – Renal biomarkers that correlated independently with high D-Dimer levels compared to low D-Dimer levels.

Variable	Odds Ratio (95% confidence interval) Unadjusted	p*	Odds Ratio (95% confidence interval) Adjusted for Sex and Age	p*
Cystatin C ≥ 0.92 mg/L	5.5 (2.1 – 14.3)	< 0.001	4.230 (1.4 – 12.8)	0.010
UAE ≥ 300 mg/g	3.1 (1.1 – 8.7)	0.029	9.464 (2.4 – 36.6)	0.001

Data was evaluated by multivariate logistic regression analysis and are presented as odds ratio (95% Confidence Interval). NS = not significant.
* $p < 0.05$ for high D-Dimer group compared to low D-Dimer group.

Von Willebrand factor, ADAMT13 and D-Dimer are associated with different levels of nephropathy in type 1 diabetes mellitus

[VWF, ADAMTS13, D-Dimer and diabetic nephropathy]

Caroline Pereira Domingueti[1], Luci Maria S. Dusse[1], Rodrigo Bastos Fóscolo[2], Janice Sepúlveda Reis[3], Joyce Maria Annichino-Bizzacchi[4], Fernanda Loureiro de Andrade Orsi[4], Bruna de Moraes Mazetto[4], Maria das Graças Carvalho[1], Karina Braga Gomes[1], Ana Paula Fernandes[1]*

1- Departament of Clinical and Toxicological Analysis, Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte – Minas Gerais, Brazil
2- Departament of Medical Clinic, Faculty of Medicine, Federal University of Minas Gerais, Belo Horizonte – Minas Gerais, Brazil
3- Departament of Endocrinology and Metabolism, Institute of Education and Research of Santa Casa of Belo Horizonte, Belo Horizonte – Minas Gerais, Brazil
4- Laboratory of Hemostasis, Hemocenter of Campinas, University of Campinas – São Paulo, Brazil

***Corresponding author**

e-mail: apfernandes.ufmg@gmail.com (APF)

Abstract

We have investigated whether VWF, ADAMTS13, and D-Dimer were associated with different levels of renal function in type 1 diabetic (DM1) patients in an observational case-control study. DM1 patients were classified according to level of renal function through eGFR-MDRDa: ≥90 and <130mL/min/1,73m^2, n=52 (control group), ≥60 and <90mL/min/1,73m^2, n=29 (mild eGFR decline group), <60mL/min/1,73m^2, n=28 (severe eGFR decline group), and urinary albumin excretion (UAE). VWF, ADAMTS13, and D-Dimer plasma levels were determined by ELISA. ADAMTS13 activity was determined by FRET. VWF levels were increased in patients with mild (P=0.001) and severe (P<0.001) eGFR decline as compared to the control group. ADAMTS13 levels were also increased in mild (P=0.029) and severe (P=0.002) eGFR decline groups in comparison to the control group, while ADAMTS13 activity was increased only in the severe eGFR decline group as compared to the control group (P=0.006). No significant differences were observed among the groups regarding VWF/ADAMTS13 ratio. ADAMTS13 activity/ADAMTS13 Ag ratio was reduced in patients with mild (P=0.013) and severe (P=0.015) eGFR decline as compared to the control group. D-Dimer levels were increased in patients with mild (P=0.006) and severe (P<0.001) eGFR decline as compared to the control group; it was also higher in patients with severe eGFR decline as compared to the mild eGFR decline group (P=0.019). Similar results were found for UAE classification. Increased VWF, ADAMTS13, and D-Dimer levels and decreased ADAMTS13 activity/ADAMTS13 Ag ratio are associated with renal dysfunction in DM1 patients, suggesting that endothelial dysfunction and hypercoagulability are associated with nephropathy in DM1.

Keywords: ADAMTS13 human protein; D-Dimer; Diabetic nephropathies; Diabetes mellitus, Type 1; Von Willebrand factor.

Introduction

Diabetic nephropathy is the most common cause of end stage renal disease (ESRD) worldwide, contributing to approximately 45% of new cases [1, 2]. Early detection of nephropathy in diabetic patients is, therefore, essential to preventing or delaying the progression of renal disease and the development of cardiovascular complications [3].

Metabolic abnormalities resulting from chronic hyperglycemia and the associated inflammatory state in diabetic patients may lead to endothelial injury and consequent vascular complications. It has been suggested that endothelial dysfunction and hypercoagulability state are the earliest indicators of this process [4].

The Von Willebrand factor (VWF) is a multimeric glycoprotein involved in primary hemostasis and in the coagulation process, acting as a carrier of factor VIII, which prevents its degradation by activated protein C. As VWF is released when endothelial cells are injured, it is an important biomarker of endothelial dysfunction [5]. VWF promotes platelet adhesion at vascular damage sites, where it mediates the progression of thrombus formation through specific interactions with subendothelial collagen and platelet receptors [6]. Elevated VWF has been found in patients with type 1 diabetes mellitus (DM1) and has been identified as a predictive biomarker of micro and macroangiopathy in these patients [7].

ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13) is an enzyme responsible for the cleavage of large multimers of VWF, released into plasma by endothelial cells and platelets, and an imbalance between VWF and ADAMTS13 plasma levels may also contribute to the development of micro and macrovascular complications in diabetic patients[7, 8]. VWF/ADAMTS13

and ADAMTS13 activity/ADAMTS13 Ag ratio have also been described as markers of endothelial dysfunction [9].

Moreover, elevated plasma levels of D-Dimer have been found in DM1 patients with microvascular complications, and it seems that hypercoagulability might be involved with the progression of atherosclerosis as well as with renal dysfunction in diabetic patients [10, 11]. D-Dimer consists of an important biomarker of hypercoagulability, since it is a fibrin degradation product that derives only from fibrin, but not from fibrinogen, and therefore is specific to fibrinolytic activity secondary to fibrin formation [12].

Based on the evidence that these biomarkers are associated with endothelial dysfunction and hypercoagulability, this study aimed to investigate the relationship among VWF, ADAMTS13, and D-Dimer with different levels of renal dysfunction in DM1 patients. To the best of our knowledge, our study was the first one to evaluate ADAMTS13 Ag levels and ADAMTS13 activity in DM1 with different degrees of renal dysfunction.

Materials and Methods

Ethical aspects

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study was approved by the Research Ethics Committee of Federal University of Minas Gerais (CAAE – 0392.0.203.000-11) and written informed consent was obtained from all individual participants included in the study. The research protocol did not interfere with any medical recommendations or prescriptions.

Studied population

The clinical records of 240 consecutive DM1 patients being assisted at Endocrinology Ambulatories of the University Hospital (*Hospital das Clínicas*) and Santa Casa de Misericórdia/Belo Horizonte, Brazil, from November 2011 to September 2012 were analysed. After application of exclusion criteria, 125 patients with clinical and laboratorial diagnosis of DM1 [13], 18 to 60 years of age, were selected for these study. Blood samples were drawn from these selected patients and biochemistry and haemostatic parameters were assessed. 14 patients presented hyperfiltration (eGFR ≥ 130 mL/min/1.73m^2) and were also excluded from the study. Finally, 109 DM1 patients were included in this observational case-control study.

DM1 patients with hepatic disease, alcoholism, hemostatic abnormalities, malignant diseases, acute infectious, pregnancy, renal hyperfiltration, undergoing hemodialysis and history of kidney transplantation or cardiovascular diseases were excluded from the study.

Study protocol

A detailed history and clinical variables of each patient were obtained from medical records: age, sex, BMI, time of diabetes diagnosis, presence of diabetes complications such as retinopathy and neuropathy, and use of medication such as antihypertensive, statin, and AAS.

DM1 patients were placed into three groups, according to level of renal function: patients with eGFR-MDRDa ≥ 90 and < 130 mL/min/1.73m^2, n = 52 (control group); patients with eGFR-MDRDa ≥ 60 and < 90 mL/min/1.73m^2, n = 29 (mild eGFR decline group); and patients with eGFR-MDRDa < 60 mL/min/1.73m^2, but not undergoing hemodialysis, n = 28 (severe eGFR decline group) [14]. They were also

placed into three groups according to urinary albumin excretion (UAE): normoalbuminuria, n = 53, microalbuminuria, n = 26, and proteinuria, n = 30 [14].

Determination of biochemistry parameters

Fasting glucose and creatinine levels were determined by enzymatic methods in serum samples, and HbA1c was determined by the immunoturbidimetric method in EDTA whole blood samples, using Johnson & Johnson dry chemistry technology kits (Ortho Clinical Diagnostics®) and VITROS 4600 analyzer. UAE was determined in urine samples collected after at least 4 hours of urinary retention in the morning, and urinary albumin was normalized by urinary creatinine. Urinary albumin was evaluated by the immunoturbidimetric method and urinary creatinine was assessed by the enzymatic method, using Johnson & Johnson dry chemistry technology kits (Ortho Clinical Diagnostics®) and VITROS 4600 analyzer.

Normoalbuminuria was defined as < 30 mg/g of creatinine, microalbuminuria as ≥ 30 and < 300 mg/g of creatinine, and proteinuria as ≥ 300 mg/g of creatinine. The presence of microalbuminuria or proteinuria was confirmed in two out of three occasions over a period between three and six months [14].

Estimation of glomerular filtration rate

The estimated glomerular filtration rate was calculated using the abbreviated Modification of Diet in Renal Disease formula [eGFR-MDRDa: 186 x plasma creatinine (mg/dL)$^{-1,154}$ x age (years)$^{-0,203}$ x 0.742 (if female) x 1.212 (if black)] [15].

Hemostatic parameters measurement

VWF, ADAMTS13 antigen, and D-Dimer plasma levels were determined by ELISA, using IMUBIND®VWF kit (American Diagnostica®), IMUBIND®ADAMTS13 kit (American Diagnostica®), and ASSERACHROM® D-Di kit (StagoDiagnostica®),

respectively. ADAMTS13 activity was assessed by fluorescence resonance energy transfer (FRET) assay, using ACTIFLUOR™ ADAMTS13 activity kit (Seikisui Diagnostics®). Intra- and inter-assay coefficients of variations were, respectively, 9% and 13% for VWF, 4.0% and 7.3% for ADAMYS13 antigen, <6% and <10% for D-Dimer, and 4.1% and 4.4% for ADAMTS13 activity.

Statistical analysis

Statistical comparisons were performed using SPSS software (version 13.0, SPSS). The Shapiro-Wilk test was used to test whether continuous variables were normally distributed. Normally-distributed data were expressed as mean ± SD and compared by ANOVA and T test. Not normally distributed data were expressed as median (percentiles 25% – 75%) and compared by the Kruskal-Wallis H test and Mann-Whitney U test, followed by Bonferroni correction. Categorical variables were expressed as frequencies and compared using the chi-square test (χ^2). A bivariate logistic regression analysis was performed to assess which hemostatic variables were associated with eGFR-MDRDa < 90 mL/min/1.73m^2, eGFR-MDRDa < 60 mL/min/1.73m^2, UAE ≥ 30 mg/g of creatinine, and UAE ≥ 300 mg/g of creatinine. A multivariate logistic regression analysis was also performed to assess which variables were independently associated with the same dependent variables described above. Variables included in this analysis were previously associated with eGFR decline and renal injury in bivariate logistic regression analysis ($p < 0.2$) and consisted on VWF, ADAMTS13 activity/ADAMTS13 Ag ratio, D-Dimer, age, BMI, time of diabetes diagnosis, HbA1c, use of antihypertensive, use of statin and AAS. Some of these variables were classified into different categories, using the following values as a cut off: < 7% and ≥ 7%, for HbA1c; ≥ 18 and < 30 years old, ≥ 30 and < 45 years old, ≥ 45 years old, for age; < 25 kg/m^2, ≥ 25 and < 30 kg/m^2, ≥ 30 kg/m^2,

for BMI; and ≤ 10 years, > 10 and ≤ 20 years, > 20 years, for time of diabetes diagnosis. Differences were considered significant when $P \leq 0.05$.

Results

Characteristics of the study group

Characteristics and clinical variables of the 109 DM1 patients included in this cross-sectional study are shown in Table 1.

Patients with severe eGFR decline were older than those with mild eGFR decline and the control group (P = 0.001). Patients with severe eGFR decline had lower BMI than that found in the control group (P = 0.007). There were no significant differences among the groups regarding sex, time of diabetes diagnosis, fasting glucose, HbA1c, and presence of neuropathy. However, a higher frequency of retinopathy was observed in the group of patients with severe eGFR decline as compared to the other groups (P<0.001). There was also a higher frequency of use of antihypertensive and AAS in the severe eGFR decline group of patients as compared to the other groups (P < 0.001) and the use of statin in patients with mild and severe eGFR decline as compared to the control group (P < 0.001).

VWF, ADAMTS13, and D-Dimer

Plasma levels of VWF, ADAMTS13 Ag, ADAMTS13 activity, and D-Dimer were measured in diabetic patients, after which VWF/ADAMTS13Ag, VWF/ADAMTS13 activity and ADAMTS13 activity/ADAMTS13 Ag ratios were calculated (Table 2).

VWF plasma levels were elevated in patients with mild and severe eGFR decline as compared to the control group (P = 0.001 and P < 0.001, respectively). ADAMTS13 Ag levels were also elevated in mild and severe eGFR decline groups of patients as compared to the control group (P = 0.029 and P = 0.002, respectively), while

ADAMTS13 activity was elevated only in severe eGFR decline group as compared to the control group (P = 0.006).

No significant differences were observed among the groups regarding the VWF/ADAMTS13 Ag and VWF/ADAMTS13 activity ratio. Conversely, the ADAMTS13 activity/ADAMTS13 Ag ratio was reduced in patients with mild and severe eGFR decline as compared to the control group (P = 0.013 and P = 0.015, respectively).

D-Dimer plasma levels were elevated in patients with mild and severe eGFR decline as compared to the control group (P = 0.006 and P < 0.001, respectively) and was also higher in patients with severe eGFR decline as compared to the mild eGFR decline group of patients (P = 0.019).

Differences among VWF, ADAMTS13 Ag, ADAMTS13 activity, and D-Dimer were also evaluated in patients classified according to UAE to evaluate the association of these hemostatic parameters with the development of renal injury (Table 3). The association of these parameters with UAE was similar to that observed in eGFR decline.

Bivariate and Multivariate logistic regression analysis

The bivariate logistic regression analysis demonstrated that VWF, ADAMTS13 Ag, ADAMTS13 activity, ADAMTS13 actvity/ADAMTS13 Ag and D-Dimer were associated with eGFR-MDRDa < 90 mL/min/1.73m^2, eGFR-MDRDa < 60 mL/min/1.73m^2, UAE ≥ 30 mg/g of creatinine and UAE ≥ 300 mg/g of creatinine.

The multivariate logistic regression analysis showed that FVW, ADAMTS13 activity/ADAMTS13 Ag, D-Dimer, and use of statin were independently correlated with eGFR-MDRDa < 90 mL/min/1.73m^2 when compared to eGFR-MDRDa ≥ 90 mL/min/1.73m^2. Moreover, only D-Dimer and use of antihypertensive or AAS were

independently correlated with eGFR-MDRDa < 60 mL/min/1.73m^2 as compared to eGFR-MDRDa ≥ 60 mL/min/1.73m^2. VWF, ADAMTS13 activity/ADAMTS13 Ag, and D-Dimer were also independently correlated with UAE ≥ 30 mg/g of creatinine when compared to UAE < 30 mg/g of creatinine. Furthermore, only D-Dimer and the use of AAS were independently correlated with UAE ≥ 300 mg/g of creatinine as compared to UAE < 300 mg/g of creatinine (Table 5).

Discussion

There are scant data in the literature correlating the progression of nephropathy, endothelial dysfunction, and hypercoagulability in DM1 patients. In this study, we used VWF, ADAMST13, and D-Dimer to assess endothelial dysfunction and hypercoagulability in DM1 patients with different and progressive levels of renal dysfunction.

Increased frequencies of use of antihypertensive and AAS were observed in patients with severe eGFR decline, while an increased frequency of use of statin was verified in patients with mild and severe renal dysfunction. According to the multivariate regression analysis, antihypertensive use was independently associated with eGFR < 60 mL/min/1.73m^2, which may be expected, given that renal dysfunction contributes to the development of hypertension and angiotensin converting enzyme inhibitors may protect renal function and prevent the progression of nephropathy [16]. The use of AAS was also independently associated with eGFR < 60 mL/min/1.73m^2 and with UAE ≥ 300 mg/g. Indeed, the use of an antiplatelet agent is usually recommended to patients presenting this condition [16], considering that nephropathy is associated with an increased risk of cardiovascular disease [17, 18]. Moreover, statin use was independently associated with eGFR < 90 mL/min/1.73m^2, which may be expected, since GFR decline is a risk factor for dyslipidemia [16].

A high frequency of retinopathy has been also detected in DM1 patients, which shares common pathological mechanisms with nephropathy, since both are microvascular complications related to diabetes [16]. A more advanced age and reduced BMI were also seen in patients with severe renal dysfunction, which are very common and expected clinical characteristics in DM1 patients [3, 19].

Elevated levels of VWF were observed in DM1 patients with mild and severe renal dysfunction, in relation to the DM1 control group, as assessed either by an increase in UAE or by GFR declining. High VWF levels were also associated with eGFR-MDRDa < 90 and < 60 mL/min/1.73m^2, and with UAE ≥ 30 and ≥ 300 mg/g, as demonstrated by bivariate logistic regression analysis. Accordingly, elevated VWF levels have correlated with microalbuminuria and proteinuria in a transversal study [20], and with the development of microalbumuria in a longitudinal study [21], in DM1 patients. Besides DM1 patients, similar findings were also reported in type 2 diabetic (DM2) patients with low GFR [22]. Moreover, there is evidence suggesting that a rise in VWF levels preceded the development of microalbuminuria in DM1 patients [23]. Altogether, these findings support the proposal that elevated VWF levels and endothelial dysfunction are associated with nephropathy in DM1, and might be useful to predict the development of renal disease in these patients.

We have also observed elevated ADAMTS13 Ag levels in DM1 patients with mild and severe renal dysfunction, and increased ADAMTS13 activity in patients with severe renal dysfunction, as compared to the control group. High ADAMTS13 levels and activity were also associated with eGFR-MDRDa < 90 and < 60 mL/min/1.73m^2, and with UAE ≥ 30 and ≥ 300 mg/g, as verified by bivariate logistic regression analysis. Increased ADAMTS13 activity has been also observed in DM2 patients with micro and macroangiopathy [24]. One possible explanation for this result is the

presence of a compensatory mechanism, by which ADAMTS13 synthesis is increased due to the marked elevation in VWF plasma levels, as nephropathy progresses. This compensatory elevation in ADAMTS13 Ag levels and increase in ADAMTS13 activity could also be responsible for keeping VWF/ADAMTS13 Ag and VWF/ADAMTS13 activity ratio unchanged in patients with renal dysfunction. Conversely, the ADAMTS13 activity/ADAMTS13 Ag ratio was reduced in patients with mild and severe renal dysfunction, as compared to the control group, and a low ADAMTS13 activity/ADAMTS13 Ag ratio was associated with eGFR-MDRDa < 90 and < 60 mL/min/1.73m^2, and with UAE ≥ 30 and ≥ 300 mg/g, as observed by bivariate logistic regression analysis, indicating that the rise in ADAMTS13 Ag levels is not accompanied by a proportional increase in ADAMTS13 activity in these patients. Therefore, the activity of each ADAMTS13 molecule may be inhibited in DM1 patients with mild and severe renal dysfunction, reducing VWF cleavage. The imbalance between ADAMTS13 activity and ADAMTS13 Ag levels in these patients may well be associated with the progressive inflammatory process, frequently seen in DM1 [4]. This hypothesis is supported by an *in vitro* study [25], which has demonstrated that IL-6 inhibited VWF cleavage by ADAMTS13, suggesting that inflammatory status is associated with reduced ADAMTS13 activity. However, as DM1 is an autoimmune disease, autoantibodies against ADAMTS13 may be present and could also partially explain the imbalance between ADAMTS13 activity and ADAMTS13 Ag levels, considering that they can directly inhibit ADAMTS13 activity [26]. A recent report [27] has provided evidence that ADAMTS13 deficiency exacerbates the progress of atherosclerotic lesions via a VWF-dependent inflammatory mechanism. As the inflammatory status may reduce ADAMTS13 activity, preventing VWF cleavage [23], it is plausible that the elevation of VWF

levels in DM1 patients with renal dysfunction is a consequence of both the endothelial dysfunction and the chronic inflammation provoked by hyperglycemia.

To our knowledge, a single study[9] has found elevated levels of D-Dimer in children and adolescents with DM1 and DM2 and nephropathy. High D-Dimer plasma levels were also verified in DM2 patients with increased UAE [28]. In the present study, increased D-Dimer levels were detected in DM1 adult patients with mild renal dysfunction as compared to the DM1 control group, and even higher levels were observed in patients with severe renal dysfunction, as assessed by an increase in UAE or by GFR declining. High D-Dimer levels were also associated with eGFR-MDRDa < 90 and < 60 mL/min/1.73m^2, and with UAE ≥ 30 and ≥ 300 mg/g, as demonstrated by bivariate logistic regression analysis. These findings suggest that hypercoagulability is associated with renal disease since its early stages in DM1 patients.

Statin and aspirin use were also independently associated with GFR < 90 mL/min/1.73m^2 and GFR < 60 mL/min/1.73m^2, respectively. Interestingly, statin has pleiotrophic effects, and in addition of reducing cholesterol levels, it can also reduce inflammation, oxidative stress and platelet aggregation [29]. Moreover, a direct impact of statin use on D-Dimer has been demonstrated in healthy subjects [30]. Therefore, it could be expected that D-Dimer levels would be even higher in patients with mild and severe GFR decline, if most of them were not taken statin. In fact, D-Dimer levels were significantly lower (287 ± 112 ng/mL) in patients with GFR < 90 mL/min/1.73m^2 that were taking statin (n = 30) as compared with those not using statin (n= 27) (383 ± 209 ng/mL) (P = 0.043) (data not shown). Aspirin may also affect coagulation, by inhibiting platelet aggregation and consequently microthrombi formation [31] and it may have also lowered D-Dimer levels in this group of patients.

In agreement, D-Dimer levels were significantly lower (283 ± 108 ng/mL) in patients with GFR < 60 mL/min/1.73m^2 that were taking aspirin (n = 13) as compared with those not using aspirin (n= 15) (434 ± 175 ng/mL) (P = 0.014) (data not shown). Therefore, these data suggest that the hypercoagulability status of patients with severe renal dysfunction could be even higher than the levels detected in this study, since these medications may have impacted their D-Dimer levels. However, the more frequent use of these medications among patients with severe renal dysfunction did not prevent the disclosure of significant independent associations between different stages of renal dysfunction and hypercoagulability status.

Increased D-Dimer levels in patients with renal dysfunction could be a consequence of endothelial dysfunction, since it can lead to VWF release, which promotes platelet adhesion and aggregation and, thus, microthrombi formation [6]. Endothelial vascular damage still impairs the conversion of protein C into its activated form, since this activation depends on the endothelial protein C receptor and thrombomodulin, which are expressed at high levels in undamaged microvasculature [32]. Protein C is a potent anticoagulant and antiinflammatory molecule, responsible for cleavage and inhibition of coagulation factors FVIIIa and FVa [33]. Therefore, impairment of its activation may increase inflammation and hypercoagulability status. The even more marked elevation in D-Dimer levels in patients with severe renal dysfunction could be partially explained by proteinuria, which involves the loss of important natural anticoagulant proteins, such as antithrombin, protein C and protein S, which may intensify the hypercoagulability status [34]. At this point, it is clear that the etiology of the underlying hypercoagulabity in DM1 that is originally triggered by hyperglycemia, is complex and multifactorial, resulting from the interaction of multiple

factors. Nonetheless, the understanding of these factors may provide valuable information on the pathogenesis of renal disease in patients with DM1.

There is a known relationship between the progression of nephropathy and an increased risk of cardiovascular disease in both DM1 and DM2 [35, 36]. In addition, some authors have argued that elevated levels of VWF and D-Dimer are associated with the development of cardiovascular disease in DM1 and DM2, since endothelial dysfunction and hypercoagulability could be important risk factors for the development of diabetic macroangiopathy [37, 38]. Since this is not a prospective study, patients were not followed up for cardiovascular disease events. Considering the increased risk of cardiovascular disease in DM1 with renal impairment, the results reported herein may guide and be further considered in prospective studies.

Interestingly, the multivariate logistic regression analysis demonstrated that VWF and ADAMTS13 activity/ADAMTS13 Ag ratio are independently associated with eGFR < 90 mL/min/1.73m^2 and UAE ≥ 30 mg/g. It is noteworthy that alterations in VWF and ADAMTS13 activity/ADAMTS13 Ag ratio are associated with both mild and severe renal dysfunction. Conversely, D-Dimer plasma levels are independently associated not only with eGFR < 90 mL/min/1.73m^2 and UAE ≥ 30 mg/g, but also with eGFR < 60 mL/min/1.73m^2 and UAE ≥ 300 mg/g. These findings suggest that endothelial dysfunction and hypercoagulability are evident at early stages of renal disease, since VWF and D-Dimer plasma levels are already elevated and ADAMTS13 activity/ADAMTS13 Ag ratio is reduced in patients with mild eGFR decline or microalbuminuria, whilst hypercoagulability is also associated with later stages of renal dysfunction. Therefore, these hemostatic parameters may complement each other on the follow-up of renal dysfunction in DM1 patients as well as their risk for cardiovascular disease. However, as previously mentioned,

longitudinal studies are needed to confirm that the coagulation factors measured are markers of diabetic nephropathy. It is noteworthy that the patients included in this study did not have history of thrombosis, cardiovascular or malignant diseases and other conditions that are frequently associated with hypercoagulability. Moreover, the single clinical characteristics significantly different among groups, BMI and age, were not independently associated with the measures coagulation markers, as indicated by the multivariate regression analysis.

In conclusion, elevated VWF, ADAMTS13 Ag, and D-Dimer plasma levels and increased ADAMTS13 activity, as well as decreased ADAMTS13 activity/ADAMTS13 Ag ratio, are associated with eGFR declining and UAE increasing in DM1 patients. The small sample size and transversal design of this study limited our analysis on the relationship between endothelial dysfunction and hypercoagulability with the progression of nephropathy.

Despite the limitations of a transversal study, our data suggest that levels of endothelial dysfunction and hypercoagulability biomarkers are altered in DM1 patients with nephropathy, as evaluated by GFR decline or by increased UAE. These findings may be useful for guiding future studies aimed at acquiring a better understanding of the mechanisms linking these aspects and nephropathy progression in DM1 patients. They may also provide new instruments for follow up of DM1 patients with nephropathy and the risk of cardiovascular disease.

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Table 1 – Characteristics of DM1 patients according to estimated glomerular filtration rate (eGFR)

	Patients with eGFR-MDRDa ≥90 and <130 mL/min/1.73m^2	Patients with eGFR-MDRDa ≥60 and <90mL/min/1.73m^2	Patients with eGFR-MDRDa <60mL/min/1.73m^2	p
Number of Individuals (n)	52	29	28	
Age (years)	32 (25 – 37)	32 (28 – 35)	41 (32 – 48)**†	0.001** 0.001†
Sex/male (n, %)	22 (56.4)	5 (12.8)	12 (30.8)	NS
BMI (kg/m^2)	24.6 ± 3.7	23.7 ± 2.7	22.2 ± 2.4**	0.007**
Time of Diagnosis (years)	19 ± 8	19 ± 7	22 ± 5	NS
Retinopathy (n,%)	15 (29.4)	11 (21.6)	25 (49.0)**†	< 0.001** < 0.001†
Neuropathy (n,%)	6 (31.6)	7 (36.8)	6 (31.6)	NS
Use of Antihypertensive (n, %)	26 (36.1)	19 (26.4)	27 (37.5)**†	< 0.001** < 0.001†
Use of Statin (n, %)	8 (21.1)	12 (31.6)*	18 (47.4)**	< 0.001* < 0.001**
Use of AAS (n, %)	4 (20.0)	3 (15.0)	13 (65.0)**†	< 0.001** < 0.001†
Fasting Glucose (mg/dL)	145 (92 – 253)	159 (91 – 217)	128 (81 – 280)	NS
HbA1c (%)	8.3 ± 1.2	8.0 ± 1.1	8.5 ± 1.3	NS
Creatinine (mg/dL)	0.74 (0,67 – 0.85)*	1.00 (0.88 – 1.10)*	1.66 (1.41 – 2.11)**†	< 0.001* < 0.001** < 0.001†
eGFR-MDRDa (mL/min/1.73m^2)	109 ± 11	74 ± 10*	38 ± 13**†	< 0.001* < 0.001** < 0.001†
UAE (mg/g of creatinine)	5 (3 – 13)	28 (10 – 154)*	496 (64 – 1417)** †	< 0.001* < 0.001** < 0.001†

Normally-distributed data were expressed as mean ± SD and compared by ANOVA and T test. Not normally distributed data were expressed as median (percentiles 25% – 75%) and compared by the Kruskal-Wallis H test and Mann-Whitney U test, followed by Bonferroni correction. Categorical variables were expressed as frequencies n (%) and compared using the chi-square test (χ^2).

* P < 0.05 for patients with eGFR-MDRDa ≥ 60 and < 90 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 90 and < 130 mL/min/1.73m^2

** P < 0.05 for patients with eGFR-MDRDa < 60 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 90 and < 130 mL/min/1.73m^2

† P < 0.05 for patients with eGFR-MDRDa < 60 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 60 and < 90 mL/min/1.73m^2.

NS = not significant.

Table 2 –VWF, ADAMTS13Ag and D-Dimer plasma levels, ADAMTS13 activity, and ratios in DM1 patients classified according to eGFR-MDRDa.

	Patients with eGFR-MDRDa ≥90 and < 130 mL/min/1.73m^2	Patients with eGFR-MDRDa ≥60 and <90mL/min/1.73m^2	Patients with eGFR-MDRDa <60mL/min/1.73m^2	p
Number of Individuals (n)	52	29	28	
VWF (mU/mL)	1031 ± 264	1290 ± 377*	1396 ± 408**	0.001* < 0.001**
ADAMTS13Ag (ng/mL)	309 (250 – 528)	503 (286 – 603)*	549 (351 – 635)**	0.029* 0.002**
ADAMTS13 activity (%)	95 ± 16	104 ± 20	108 ± 19**	0.006**
VWF/ ADAMTS13Ag	2.9 ± 1.0	2.7 ± 1.0	2.7 ± 1.1	NS
VWF/ ADAMTS13 Activity	11.7 ± 4.0	12.8 ± 4.2	13.2 ± 4.6	NS
ADAMTS13 Activity/ ADAMTS13Ag	0.30 (0.19 – 0.39)	0.20 (0.16 – 0.30)*	0.19 (0.18 – 0.28)**	0.013* 0.015**
D-Dimer (ng/mL)	178 (128 – 264)	239 (195 – 385)*	361 (232 – 536)** †	0.006* < 0.001** 0.019†

Normally-distributed data were expressed as mean ± SD and compared by ANOVA and T test. Not normally distributed data were expressed as median (percentiles 25% – 75%) and compared by the Kruskal-Wallis H test and Mann-Whitney U test, followed by Bonferroni correction.

* P < 0.05 for patients with eGFR-MDRDa ≥ 60 and < 90 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 90 and < 130 mL/min/1.73m

** P < 0.05 for patients with eGFR-MDRDa < 60 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 90 and < 130 mL/min/1.73m^2

† P < 0.05 for patients with eGFR-MDRDa < 60 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 60 and < 90 mL/min/1.73m^2.

NS = not significant.

Table 3 – VWF, ADAMTS13Ag and D-Dimer plasma levels, ADAMTS13 activity and ratios in the DM1 patients classified according to urinary albumin excretion.

	Patients with normoalbuminuria	Patients with microalbuminuria	Patients with macroalbuminuria	p
Number of Individuals (n)	53	26	30	
VWF (mU/mL)	1050 ± 280	1319 ± 377*	1428 ± 431**	0.003* < 0.001**
ADAMTS13Ag (ng/mL)	297 (235 – 507)	504 (384 – 609)*	571 (338 – 661)**	0.002* < 0.001**
ADAMTS13 activity (%)	95 ± 17	100 ± 15	113 ± 21**†	< 0.001** 0.007†
VWF/ ADAMTS13Ag	3.3 (1.9 – 4.1)	2.9 (2.1 – 3.2)	2.4 (1.9 – 3.5)	NS
VWF/ ADAMTS13 Activity	11.3 ± 3.7	13.5 ± 4.0	11.6 ± 3.2	NS
ADAMTS13 Activity/ ADAMTS13Ag	0.32 (0.20 – 0.40)	0.18 (0.18 – 0.19)*	0.18 (0.18 – 0.20)**	0.002* < 0.001**
D-Dimer (ng/mL)	200 ± 73	292 ± 153*	441 ± 253**†	0.001* < 0.001** 0.013†

Normally-distributed data were expressed as mean ± SD and compared by ANOVA and T test. Not normally distributed data were expressed as median (percentiles 25% – 75%) and compared by the Kruskal-Wallis H test and Mann-Whitney U test, followed by Bonferroni correction.
* P < 0.05 for patients with microalbuminuria compared to patients with normoalbuminuria
** P < 0.05 for patients with proteinuria compared to patients with microalbuminuria
† P < 0.05 for patients with proteinuria compared to patients with microalbuminuria.
NS = not significant.

Table 4 – Association between hemostatic parameters and eGFR-MDRDa < 90 mL/min/1.73m^2, eGFR-MDRDa < 60 mL/min/1.73m^2, UAE ≥ 30 mg/g of creatinine and UAE ≥ 300 mg/g of creatinine.

Variable	eGFR-MDRDa < 90 mL/min/1.73m^{2*}	eGFR-MDRDa < 60 mL/min/1.73m^{2**}	UAE ≥ 30 mg/g of creatinine†	UAE ≥ 300 mg/g of creatinine††
VWF	1.003	1.002	1.003	1.002
	(1.001 – 1.004)	(1.001 – 1.003)	(1.001 – 1.004)	(1.001 – 1.003)
	P < 0.001	P = 0.002	P < 0.001	P = 0.001
ADAMTS13Ag	1.003	1.003	1.005	1.004
	(1.001 – 1.004)	(1.001 – 1.005)	(1.003 – 1.007)	(1.001 – 1.006)
	P = 0.001	P = 0.016	P < 0.001	P = 0.002
ADAMTS13 activity	1.033	1.029	1.036	1.053
	(1.010 – 1.057)	(1.004 – 1.025)	(1.013 – 1.060)	(1.024 – 1.082)
	P = 0.005	P = 0.025	P = 0.002	P < 0.001
ADAMTS13 Activity/ ADAMTS13Ag	0.001	0.006	1×10^{-4}	1×10^{-4}
	(1×10^{-4} – 0.057)	(1×10^{-4} – 0.942)	(1×10^{-4} - 2×10^{-4})	(1×10^{-4} – 0.014)
	P = 0.001	P = 0.047	P < 0.001	P = 0.008
D-Dimer	1.009	1.007	1.009	1.007
	(1.004 – 1.013)	(1.004 – 1.011)	(1.004 – 1.013)	(1.003 – 1.010)
	P < 0.001	P < 0.001	P < 0.001	P < 0.001

Data was evaluated by bivariate logistic regression analysis and are presented as odds ratio (95% Confidence Interval).
* P < 0.05 for patients with eGFR-MDRDa < 90 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 90 mL/min/1.73m^2 (mild + severe x control group)
** P < 0.05 for patients with eGFR-MDRDa < 60 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 60 mL/min/1.73m^2 (severe x mild + control group)
† P < 0.05 for patients with UAE ≥ 30 mg/g of creatinine compared to patients with UAE < 30 mg/g of creatinine (mild + severe x control group)
†† P < 0.05 for patients with UAE ≥ 300 mg/g of creatinine compared to patients with UAE < 300 mg/g of creatinine (severe x mild + control group)

Table 5 – Variables that correlated independently with eGFR-MDRDa < 90 mL/min/1.73m^2 , eGFR-MDRDa < 60 mL/min/1.73m^2, UAE ≥ 30 mg/g of creatinine and UAE ≥ 300 mg/g of creatinine.

Variable	eGFR-MDRDa < 90 mL/min/1.73m^{2*}	eGFR-MDRDa < 60 mL/min/1.73m^{2**}	UAE ≥ 30 mg/g of creatinine†	UAE ≥ 300 mg/g of creatinine††
VWF	1.003 (1.001 – 1.005) P = 0.008	NS	1.003 (1.001 – 1.005) P = 0.015	NS
ADAMTS13 Activity/ ADAMTS13Ag	1 x 10^{-4} (3 x 10^{-7} – 0.109) P = 0.008	NS	1 x 10^{-10} (1 x 10^{-16} – 1 x 10^{-4}) P = 0.001	NS
D-Dimer	1.010 (1.004 – 1.016) P = 0.002	1.008 (1.004 – 1.012) P < 0.001	1.006 (1.000 – 1.013) P = 0.047	1.007 (1.004 – 1.010) P < 0.001
Use of Antihypertensive	NS	12.249 (1.371 – 109.467) P = 0.025	NS	NS
Use of Statin	13.962 (3.191 – 61.083) P < 0.001	NS	NS	NS
Use of AAS	NS	6.636 (1.711 – 25.735) P = 0.006	NS	4.794 (1.449 – 15.863) P = 0.010

Data was evaluated by multivariate logistic regression analysis and are presented as odds ratio (95% Confidence Interval). NS = not significant.

* P < 0.05 for patients with eGFR-MDRDa < 90 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 90 mL/min/1.73m^2 (mild + severe x control group)

** P < 0.05 for patients with eGFR-MDRDa < 60 mL/min/1.73m^2 compared to patients with eGFR-MDRDa ≥ 60 mL/min/1.73m^2 (severe x mild + control group)

† P < 0.05 for patients with UAE ≥ 30 mg/g of creatinine compared to patients with UAE < 30 mg/g of creatinine (mild + severe x control group)

†† P < 0.05 for patients with UAE ≥ 300 mg/g of creatinine compared to patients with UAE < 300 mg/g of creatinine (severe x mild + control group)

6 CONSIDERAÇÕES FINAIS

6.1 Avaliação de equações baseadas na creatinina ou na cistatina C para a estimativa do RFG nos pacientes diabéticos tipo 1 de acordo com a EUA

Até o momento, poucos estudos foram realizados para comparar a performance do RFG estimado por meio de equações baseadas na creatinina e na cistatina C no DM1. Portanto, no presente estudo, nós comparamos o RFG estimado através de diferentes equações baseadas na creatinina ou na cistatina C, correlacionando este com os níveis de EUA, um importante biomarcador de lesão renal, em pacientes diabéticos tipo 1.

O RFG estimado através das diferentes equações baseadas na creatinina ou na cistatina C foi capaz de distinguir os pacientes com níveis de EUA normais, moderadamente aumentados ou acentuadamente aumentados, enquanto que os níveis plasmáticos de creatinina e de cistatina C não foram capazes de fazer esta distinção. Este resultado indica que o RFG estimado consiste em um melhor marcador para acompanhar a evolução da doença renal no DM1 do que a avaliação apenas dos níveis plasmáticos de creatinina ou de cistatina C. Contudo, alguns estudos demonstraram que determinações seriadas dos níveis plasmáticos de cistatina C podem ser úteis para a detecção precoce da disfunção renal no DM1 (Premaratne *et al*., 2008; Caramori *et a*l., 2003).

Nós observamos que alguns pacientes com níveis normais de albuminúria apresentaram um declínio do RFG, enquanto que alguns pacientes com níveis de EUA acentuadamente aumentados apresentaram um RFG normal, indicando que a determinação da EUA e do RFG são complementares para o diagnóstico e o acompanhamento da doença renal no DM1, e que ambas devem ser realizadas.

A classificação dos pacientes de acordo com o RFG estimado pela equação MDRDa apresentou a pior correlação com a classificação dos pacientes de acordo com a EUA, enquanto que a fórmula CKD-EPI apresentou a melhor correlação, a qual foi avaliada pelo índice kappa. Dentre as equações baseadas na creatinina, a fórmula MDRDa também apresentou uma pior precisão do que a fórmula CKD-EPI para detectar os pacientes com níveis de EUA acentuadamente aumentados. Além disso, a equação CKD-EPI apresentou uma melhor associação com a EUA do que a equação MDRDa, o que foi demonstrado pela correlação de Spearman. Este resultado está de acordo com outros estudos, os quais demonstraram que a

equação CKD-EPI é mais indicada para avaliar a função renal do que a fórmula MDRDa (Vucic Lovrencic *et al*., 2012; Murata *et al*., 2011; Cirillo *et al*., 2010).

A análise de odds ratio demonstrou associações semelhantes entre um RFG < 60 mL/min/1,73m^2 e a EUA acentuadamente aumentada, quando este foi estimado pelas equações baseadas na cistatina C ou na creatinina, indicando que um RFG reduzido estimado por quaisquer das fórmulas está significativamente associado com a presença de lesão renal. Contudo, as equações baseadas na cistatina C apresentaram uma associação um pouco melhor com a EUA acentudamente aumentada do que as equações baseadas na creatinina. A correlação de Spearman e as curvas ROC comparando as áreas sob a curva (AUCs) para as equações baseadas na creatinina e na cistatina C foram muito semelhantes entre si. Contudo, as equações baseadas na cistatina C apresentaram uma correlação um pouco melhor com a EUA, AUCs maiores e uma melhor acurácia para detectar os pacientes com níveis de EUA acentuadamente aumentados do que as equações baseadas na creatinina, sugerindo uma melhor associação das fórmulas baseadas na cistatina C com a presença de lesão renal. Em concordância com nosso estudo, Yoo e cols. (2011) demonstraram que alterações anuais no RFG estimado pela equação baseada na cistatina C refletiu melhor a progressão da albuminúria do que o RFG estimado pela fórmula MDRDa em pacientes diabéticos tipo 2.

Portanto, de acordo com os nossos resultados, é possível concluir que todas as equações baseadas na creatinina e na cistatina C utilizadas para estimar o RFG apresentaram uma boa correlação com os diferentes níveis de EUA. Contudo, as equações baseadas na cistatina C apresentaram uma precisão um pouco maior para detectar os pacientes com níveis de EUA acentuadamente aumentados. Como a cistatina C nem sempre está disponível nos laboratórios clínicos e como não há uma superioridade evidente das equações baseadas na cistatina C para estimar o RFG, sugerimos que as equações baseadas na creatinina, principalmente a fórmula CKD-EPI, sejam utilizadas para avaliar a função renal dos pacientes diabéticos tipo 1. É importante enfatizar a extrema importância de estudos futuros que visem a descoberta de novos biomarcadores de função renal, já que ainda não existe um biomarcador ideal para avaliar o RFG e detectar precocemente o declínio da função renal.

6.2 Associação entre diferentes biomarcadores da função renal com os níveis de Dímero D em pacientes diabéticos tipo 1

No presente estudo, nós avaliamos a associação entre diferentes biomarcadores da função renal, incluindo creatinina, ureia, albuminúria, cistatina C, e RFG estimado através de equações baseadas na creatinina e na cistatina C, com o estado de hipercoagulabilidade avaliado pelos níveis plasmáticos de Dímero D, nos pacientes diabéticos tipo 1.

No nosso estudo, os pacientes com níveis plasmáticos elevados de Dímero D apresentaram níveis aumentados de creatinina, ureia, cistatina C e albuminúria e um RFG estimado por equações baseadas na creatinina ou na cistatina C reduzido. Estes pacientes também apresentaram uma maior frequência de doença renal, independente do biomarcador utilizado para avaliar a função renal, confirmando a associação entre o declínio da função renal e o aumento do estado de hipercoagulabilidade. De modo semelhante, outros estudos também encontraram uma associação entre níveis elevados de Dímero D e uma EUA aumentada ou um RFG reduzido em pacientes diabéticos (El Asrar *et al.*, 2012; Wakabayashi e Masuda, 2009; Long *et al.*, 2001).

Nós também observamos que a EUA, a cistatina C e o RFG estimado através de equações baseadas na cistatina C apresentaram uma melhor associação com os níveis plasmáticos elevados de Dímero D do que a ureia, a creatinina e o RFG estimado através de equações baseadas na creatinina. Os níveis plasmáticos de cistatina C e o RFG estimado através de equações baseadas na cistatina C ainda se correlacionaram melhor com os níveis de Dímero D do que os outros biomarcadores da função renal. Estes resultados sugerem que a EUA, os níveis plasmáticos de cistatina C e o RFG estimado através de equações baseadas na cistatina C possuem uma melhor associação com o estado de hipercoagulabilidade do que os outros biomarcadores da função renal, o que é importante, já um já que um maior estado de hipercoagulabilidade está associado com um maior risco de DVC. Contudo, estudos longitudinais que avaliam diretamente o desenvolvimento de doenças cardiovasculares são necessários para confirmar esta hipótese.

A análise de regressão logística multivariada ainda demonstrou que apenas níveis elevados de EUA e de cistatina C foram independentemente associados com os níveis elevados de Dímero D, e que esta associação permaneceu mesmo após

ajuste para sexo e idade, as quais consistem em variáveis que podem influenciar os níves plasmáticos de Dímero D (Haase *et al*., 2013). Este achado indica que estes biomarcadores da função renal estão independentemente associados com o estado de hipercoagulabilidade, podendo ser úteis para a avaliação simultânea da presença de doença renal e do estado de hipercoagulabilidade nos pacientes diabéticos tipo 1.

6.3 Fator Von Willebrand, ADAMTS13 e Dímero D e sua associação com diferentes níveis de nefropatia no DM1

Na literatura, há uma escassez de estudos que correlacionam diferentes níveis de nefropatia com a disfunção endotelial e a hipercoagulabilidade nos pacientes diabéticos tipo 1. Portanto, no presente estudo, nós avaliamos os níveis plasmáticos de FVW, ADAMTS13 e Dímero D, e a atividade da ADAMTS13, em pacientes diabéticos tipo 1 com diferentes níveis de função renal com o objetivo de analisar a associação entre a disfunção endotelial e a hipercoagulabilidade com a nefropatia nestes pacientes.

Nós verificamos que os pacientes diabéticos tipo 1 com disfunção renal leve ou grave, avaliada tanto por um aumento da EUA quanto por um declínio do RFG, apresentaram níveis plasmáticos maiores do FVW do que os pacientes diabéticos tipo 1 sem disfunção renal. Na análise de regressão logística bivariada, níveis elevados de FVW também foram associados com RFG < 90 e < 60 mL/min/1,73m^2, e com EUA ≥ 30 e ≥ 300 mg/g, sugerindo que a disfunção endotelial está associada com a progressão da nefropatia no DM1. Outros autores também encontraram níveis plasmáticos elevados do FVW em pacientes diabéticos tipo 1 que apresentavam um aumento da EUA (Chan *et al*., 2003; Stehouwer *et al*., 1991) ou uma redução do RFG (Almquist *et al*., 2012). Além disso, há evidências de que o aumento dos níveis plasmáticos do FVW deve preceder o aumento da EUA no DM1, indicando que a disfunção endotelial possa predizer o desenvolvimento da doença renal nestes pacientes (Stehouwer *et al*., 1995).

Nós também observamos níveis plasmáticos elevados da ADAMTS13 nos pacientes diabéticos tipo 1 com disfunção renal leve ou grave, e uma atividade aumentada da ADAMTS13 nos pacientes com disfunção renal grave, em comparação com o grupo controle. Na análise de regressão logística bivariada,

níveis elevados de ADAMTS13 e uma atividade aumentada da ADAMTS13 foram associados com RFG < 90 e < 60 mL/min/1,73m^2, e com EUA ≥ 30 e ≥ 300 mg/g. De modo semelhante, outro estudo encontrou uma atividade aumentada da ADAMTS13 em pacientes diabéticos tipo 2 com micro ou macroangiopatia (Oggianu *et al*., 2013). Uma possível explicação para este resultado seria a presença de um mecanismo compensatório, em que a síntese da ADAMTS13 estaria aumentada devido ao aumento intenso dos níveis plasmáticos do FVW que ocorre com a progressão da nefropatia. Este aumento compensatório nos níveis e na atividade da ADAMTS13 ainda poderia ser responsável pela manutenção das relações FVW/ADAMTS13Antígeno e FVW/ADAMTS13Atividade inalteradas nos pacientes com disfunção renal.

Por outro lado, a relação ADAMTS13Atividade/ADAMTS13Antígeno estava reduzida nos pacientes com disfunção renal leve ou grave, quando comparados ao grupo controle, e uma relação ADAMTS13Atividade/ADAMTS13Antígeno reduzida foi associada com RFG < 90 e < 60 mL/min/1,73m^2, e com EUA ≥ 30 e ≥ 300 mg/g na análise de regressão logística bivariada. Este resultado demonstra que o aumento dos níveis plasmáticos da ADAMTS13 não foi acompanhado por um aumento proporcional da sua atividade nestes pacientes. Assim, é possível inferir que a atividade de ADAMTS13 possa estar reduzida nos pacientes diabéticos tipo 1 com disfunção renal leve ou grave, o que poderia reduzir a clivagem do FVW. Uma possível explicação para o desequilíbrio entre a atividade da ADAMTS13 e os níveis plasmáticos da ADAMTS13 nestes pacientes poderia ser a presença de um processo inflamatório progressivo, o qual resultaria em níveis elevados de IL-6, a qual poderia reduzir a atividade da ADAMTS13, comprometendo a clivagem do FVW. Esta hipótese é sustentada por um estudo *in vitro* conduzido por Bernardo e cols. (2004), os quais demonstraram que a IL-6 inibiu a clivagem do FVW pela ADAMTS13, sugerindo que o estado inflamatório pode contribuir para reduzir a atividade desta enzima. Por outro lado, como o DM1 é uma doença autoimune (Szablewski, 2014), auto-anticorpos contra a ADAMTS13 podem estar presentes, o que poderia explicar parcialmente o desequilíbrio entre a atividade da ADAMTS13 e os níveis plasmáticos da ADAMTS13, uma vez que estes podem inibir diretamente a atividade da enzima (Scheiflinger *et al*., 2003). Um estudo recente (Gandhi *et al*., 2012) demonstrou que a deficiência da ADAMTS13 pode intensificar a progressão da lesão aterosclerótica por meio de um mecanismo inflamatório dependente do

FVW. Como o estado inflamatório pode reduzir a atividade da ADAMTS13, inibindo a clivagem do FVW pela ADAMTS13 (Bernardo *et al*., 2004), é possível especular que os níveis aumentados do FVW nos pacientes diabéticos tipo 1 com disfunção renal podem ser decorrentes tanto da disfunção endotelial quanto da inflamação crônica provocada pela hiperglicemia.

No nosso estudo ainda foram encontrados níveis plasmáticos aumentados de Dímero D nos pacientes diabéticos tipo 1 com disfunção renal leve em comparação com o grupo controle, e níveis ainda maiores nos pacientes com disfunção renal grave, a qual foi avaliada tanto por um aumento da EUA quanto por um declínio do RFG. Na análise de regressão logística bivariada, níveis elevados de Dímero D foram associados com RFG < 90 e < 60 mL/min/1,73m^2, e com EUA ≥ 30 e ≥ 300 mg/g. Estes achados sugerem uma associação entre um estado de hipercoagulabilidade exacerbado com a nefropatia no DM1. Resultados semelhantes foram encontrados em estudos que avaliaram os níveis plasmáticos de Dímero D em crianças e adolescentes com DM1 e DM2 e nefropatia (El Asrar *et al*., 2012) e em pacientes adultos com DM2 e albuminúria aumentada (Long *et al*., 2001).

O aumento dos níveis plasmáticos do Dímero D nos pacientes com disfunção renal pode ser uma consequência da disfunção endotelial, já que esta pode promover o desenvolvimento de uma superfície pró-coagulante, e resultar na liberação de FVW, o qual promove a adesão e a ativação plaquetária, acarretando a formação de microtrombos, contribuindo para o desenvolvimento de um estado de hipercoagulabilidade. A lesão do endotélio vascular ainda pode comprometer a conversão da proteína C na sua forma ativada, já que esta ativação depende da presença do receptor endotelial da proteína C e da trombomodulina, os quais estão expressos em níveis elevados na microvasculatura intacta (Scaldaferri *et al*., 2007). A proteína C ativada possui importantes funções antiinflamatória e anticoagulante, sendo responsável pela clivagem e inibição dos fatores da coagulação FVIIIa e FVa, de modo que o comprometimento da sua ativação pode resultar em um aumento do estado inflamatório e de hipercoagulabilidade (Dahlbach e Villoutreix, 2005). O aumento ainda mais acentuado dos níveis plasmáticos do Dímero D nos pacientes com disfunção renal grave pode ser parcialmente explicado pela presença de proteinúria, a qual pode resultar na perda de proteínas anticoagulantes naturais

importantes na urina, como a antitrombina, a proteína C e a proteína S, intensificando o estado de hipercoagulabilidade (Kato *et al*., 2010).

A análise de regressão logística multivariada demonstrou que os níveis plasmáticos do FVW e a relação ADAMTS13Atividade/ADAMTS13Antígeno estão independentemente associados com um RFG < 90 mL/min/1,73m^2 e com uma EUA ≥ 30 mg/g, enquanto que os níveis plasmáticos de Dímero D estão independentemente associados não somente com um um RFG < 90 mL/min/1,73m^2 e com uma EUA ≥ 30 mg/g, mas também com um RFG < 60 mL/min/1,73m^2 e com uma EUA ≥ 300 mg/g. Estes achados sugerem que a disfunção endotelial e a hipercoagulabilidade estão associadas com os estágios mais precoces da doença renal e que a hipercoagulabilidade ainda está associada com os estágios mais tardios da nefropatia no DM1.

Portanto, é possível inferir que os biomarcadores de disfunção endotelial, FVW e relação ADAMTS13Atividade/ADAMTS13Antígeno, e o biomarcador de hipercoagulabilidade, Dímero D, possam ser complementares uns aos outros no acompanhamento da doença renal nos pacientes diabéticos tipo 1. Além disso, o Dímero D consiste em um biomarcador interessante para a avaliação do estado de hipercoagulabilidade e do risco de doenças cardiovasculares no DM1. Contudo, estudos longitudinais são necessários para confirmar estas hipóteses.

O pequeno tamanho amostral e o desenho transversal consistiram nas principais limitações do nosso estudo, já que impossibilitaram a avaliação do poder preditivo dos biomarcadores de disfunção endotelial e de hipercoagulabilidade para o desenvolvimento e a progressão da nefropatia diabética. Portanto, a realização de estudos longitudinais seria bastante interessante para verificar se o aumento dos níveis plasmáticos do FVW e do Dímero D, e a redução da relação ADAMTS13Atividade/ADAMTS13Antígeno, precede o desenvolvimento e o agravamento da doença renal no DM1, ou se eles consistem apenas em biomarcadores, cujos níveis se alteram em decorrência da progressão da nefropatia diabética.

7 CONCLUSÕES

Os resultados obtidos neste estudo nos permitem concluir que:

- Todas as equações baseadas na creatinina ou na cistatina C para estimar o RFG apresentaram uma boa correlação com os diferentes níveis de EUA nos pacientes diabéticos tipo 1 e maior sensibilidade para detectar níveis de EUA acentuadamente aumentados;
- Níveis plasmáticos elevados de FVW, ADAMTS13 e Dímero D, uma atividade aumentada da ADAMTS13, e uma relação ADAMTS13Atividade/ADAMTS13Antígeno reduzida, estão associados com o declínio do RFG e com o aumento da EUA nos pacientes diabéticos tipo 1;
- O FVW, a relação ADAMTS13Atividade/ADAMTS13Antígeno e o Dímero D estão independentemente associados com níveis de EUA moderadamente aumentados;
- A relação ADAMTS13Atividade/ADAMTS13Antígeno e o Dímero D estão independentemente associados com um leve declínio no RFG;
- O Dímero D está independentemente associado com níveis de EUA acentuadamente aumentados e com um grave declínio no RFG;
- A EUA, a cistatina C e o RFG estimado através de equações baseadas na cistatina C apresentaram uma melhor associação com níveis plasmáticos elevados de Dímero D do que a ureia, a creatinina e o RFG estimado através de equações baseadas na creatinina;
- A EUA e a cistatina C estão independentemente associadas com o estado de hipercoagulabilidade nos pacientes diabéticos tipo 1.

8 PERPECTIVAS

Os resultados obtidos neste estudo abrem novas perpectivas, tais como:

- Realizar estudos semelhantes em outras populações com o objetivo de avaliar a validade externa dos achados e possibilitar a generalização das conclusões;
- Avaliar a associação do estado inflamatório com a progressão da nefropatia nos pacientes diabéticos tipo 1, através da dosagem da PCR ultra-sensível e de citocinas e quimiocinas inflamatórias no plasma e na urina;
- Compreender melhor os mecanismos responsáveis pelo aumento dos níveis plasmáticos e da atividade da ADAMTS13 nos pacientes diabéticos tipo 1 com nefropatia, por meio da avaliação da síntese da hepática e renal da enzima em camundongos diabéticos com disfunção renal;
- Avaliar o poder preditivo do FVW, do Dímero D e da relação ADAMTS13Atividade/ADAMTS13Antígeno para o desenvolvimento e a progressão da nefropatia em pacientes diabéticos tipo 1 por meio de estudos longitudinais;
- Avaliar a associação entre níveis elevados de cistatina C e o risco de desenvolvimento de doenças cardiovasculares nos pacientes diabéticos tipo 1 por meio de estudos longitudinais.

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ANEXO 1

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

Projeto: CAAE - 0392.0.203.000-11

Interessado(a): Profa. Ana Paula Salles Moura Fernandes
Departamento de Análises Clínicas e Toxicológicas
Faculdade de Farmácia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 21 de setembro de 2011, o projeto de pesquisa intitulado **"Estudo da enzima ADAMTS13 e de outros marcadores de hipercoagulabilidade em pacientes diabéticos e a relação com o desenvolvimento de nefropatia e aterosclerose"** 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 2

GRUPO
SANTA CASA
DE BELO HORIZONTE

Registro CEP: 006/2012 (Este número deve ser citado nas correspondências referentes a este projeto).

Belo Horizonte, 30 de março de 2012.

Ilma. Sra.
Dra. Ana Paula Salles Moura Fernandes
Pesquisadora Responsável

Informamos-lhe que o Comitê de Ética em Pesquisa (CEP) da Santa Casa de Misericórdia de Belo Horizonte, em reunião do dia 30 de março de 2012, analisou e **aprovou** o projeto de pesquisa intitulado: "Estudo de enzima ADAMTS13 e de outros marcadores de hipercoagulabilidade em pacientes diabéticos e a relação com o desenvolvimento de nefropatia e aterosclerose.", registrado neste CEP sob número 006/2012, no qual V. Sa. figura como pesquisadora responsável.

OBS.:
Após o início da pesquisa, o pesquisador responsável deverá enviar ao CEP relatórios semestrais e final (para o primeiro semestre o prazo é 30 de junho: para o segundo semestre é 31 de dezembro).

Atenciosamente,

Dr. Francisco das Chagas Lima e Silva
Coordenador do CEP

ANEXO 3

FICHA CLÍNICA

Projeto: **"Estudo da enzima ADAMTS13 e de outros marcadores de hipercoagulabilidade em pacientes diabéticos e a relação com o desenvolvimento de nefropatia e aterosclerose."**

Número de identificação:_____ Número do prontuário:_____
Data da coleta:_____

1. Identificação

Nome:_____
Nascimento:_____ Sexo: M__ F__ Naturalidade:_____
Endereço:_____ Bairro:_____
Cidade:_____ Estado:____ CEP:_____
Tel:_____ Cel:_____

2. Dados clínicos

Peso (kg):_____ Altura (m):_____ IMC (kg/m^2):_____
Circunferência abdominal (cm):_____
Pressão sanguínea (mmHg):_____
Espessura íntimo-medial das artérias carótidas (mm):_____
Data do diagnóstico do diabetes: _____
História familiar: ( ) sim ( ) não
Patologias associadas:_____
_____
_____
Medicamentos em uso:_____
_____
_____
Complicações do diabetes:_____
_____
_____

ANEXO 4

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

PROJETO DE PESQUISA: "Estudo da enzima ADAMTS13 e de outros marcadores de hipercoagulabilidade em pacientes diabéticos e a relação com o desenvolvimento de nefropatia e aterosclerose"

Prezado (a) Sr.(a),

Este projeto tem por objetivo estudar a coagulação do sangue, os processos inflamatórios e as doenças renais que podem ocorrer nos pacientes diabéticos. Para obter a conclusão da pesquisa, será necessário comparar os resultados dos exames dos pacientes com diabetes que possuem problemas renais com os resultados de pacientes com diabetes que não possuem problemas renais e de indivíduos não diabéticos (grupos controles).

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 10 mL de seu sangue e uma amostra da sua urina 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. As amostras coletadas serão utilizadas para a realização de alguns exames de laboratório com o objetivo de estudar os problemas circulatórios e renais que podem ocorrer nos pacientes diabéticos.

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. Ana Paula Salles Moura Fernandes da Faculdade de Farmácia / UFMG no telefone 3409-6884 ou com a farmacêutica Caroline Pereira Domingueti no telefone 3409-6902.

Desde já agradecemos sua valiosa participação.

_____

Profa. Ana Paula Salles Moura Fernandes

_____

Caroline Pereira Domingueti

Data:___/___/_____

**Santa Casa de Misericórdia – Av. Francisco Sales, 1111,
Bairro Santa Efigênia, Belo Horizonte, MG – Brasil – Cep 30.150.221 – Telefone 31 3238-8100**

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