UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

MESTRADO E DOUTORADO

AVALIAÇÃO DO SISTEMA ENDOTELINA NA NEFROPATIA DIABÉTICA EM PACIENTES COM DIABETE MELITO TIPO 2

CLAUDETE MARIA ZANATTA

Porto Alegre, 2009

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

AVALIAÇÃO DO SISTEMA ENDOTELINA NA NEFROPATIA DIABÉTICA EM PACIENTES COM DIABETE MELITO TIPO 2

CLAUDETE MARIA ZANATTA

ORIENTADOR: Prof. Dr. Luís Henrique Canani

Tese apresentada como requisito parcial para a obtenção de título de Doutor em Endocrinologia, à Universidade Federal do Rio Grande do Sul, Programa de Pós-graduação em Ciências Médicas: Endocrinologia.

Porto Alegre, 2009

AGRADECIMENTOS

Ao meu orientador Prof. Dr. Luís Henrique Canani, pela dedicação à pesquisa, paciência, confiança e disponibilidade. Desde que entrei no mestrado e continuando com o doutorado, contribuiu imensamente ao meu crescimento pessoal e intelectual. Sua capacidade intelectual e amplo conhecimento científico, assim como sua compreensão humana, tornaram essa caminhada mais amena.

Ao Prof. Dr. Francisco Veronese, pela orientação, conhecimento e dedicação no projeto de Imunohistoquimica. Sua valiosa contribuição enriqueceu esse projeto e contribuiu para meu crescimento científico.

À Dra. Daisy Crispim pelo seu amplo conhecimento em genética, amizade, disponibilidade e importantes contribuições no projeto de genética.

À Farmacêutica Denise Sortica pela amizade e incansável ajuda na execução de toda tese. Sempre pronta para ajudar, não medindo esforços para encontrar solução dos incontáveis e inimagináveis obstáculos que nos surpreendiam. Querida Denise, você é dessas pessoas especiais que aparecem no nosso caminho e nos enriquecem.

À Dra. Virna Carpio e à aluna da iniciação científica Melina Loretto pela contribuição e dedicação no projeto de Imunohistoquimica.

À minha família, sem eles nada teria sentido.

Ao PPG de Endocrinologia e ao GPPG do HCPA por disponibilizarem os meios para realização deste projeto. Ao CNPq, FIPE/HCPA pelo apoio financeiro.

A todas as pessoas que de alguma forma contribuíram para a execução deste trabalho.

A todos os pacientes que participaram deste estudo, pois são eles que nos instigam à pesquisa.

SUMÁRIO

Capitulo 2

Polimorfismos do Gene da Endotelina-1 estão Associados com Nefropatia Diabética em Pacientes com Diabete Melito tipo 2

Capitulo 3

Expressão da Endotelina-1 e do Receptor-A da Endotelina estão Aumentados em Pacientes com Nefropatia Diabética

LISTA DE TABELAS

Capitulo 1

Capitulo 2

Capitulo 3

LISTA DE FIGURAS

Capitulo 1

Capitulo 3

LISTA DE ABREVIATURAS

A1c	Hemoglobina glicada / glycated haemoglobin
ACEi	Angiotensin-converting enzyme inhibitors
AER	Albumin excretion rate
ARA II	Antagonistas do receptor da angiotensina II
DM	Diabete melito / diabetes mellitus
DCV	Doença cardio vascular
DN	Diabetic Nephropathy
ECA	Enzima de conversão da angiotensina
EDN1	Gene da endotelina-1 / Endothelin-1 gene
EDNRA	Gene do receptor-A da endotelina / Endothelin receptor type A gene
ET-1	Endotelina-1 / Endothelin-1
ETRA	Receptor A da endotelina / Endothelin receptor type A
ETRB	Receptor B da endotelina / Endothelin receptor type B
GFR	Glomerular filtration rate
HCPA	Hospital de Clinicas de Porto Alegre
HWE	Hardy Weinberg equilibrium
IECA	Inibidores da enzima conversora da angiotensina
MDRD	Modification of Diet in Renal Disease equation
ND	Nefropatia diabética
RAS	Sistema renina-angiotensina
SNP	*Single nucleotide polymorphism*
T2DM	Type 2 diabetes

TFG	Taxa de filtração glomerular
UKPDS	United Kingdom Prospective Diabetes Study

INTRODUÇÃO

A nefropatia diabética (ND) é uma das principais complicações crônicas do diabete melito (DM) tipo 2, sendo que dependendo da origem étnica cerca de 20 a 50% dos pacientes com DM tipo 2 desenvolvem ND ao longo da vida (1). Um estudo realizado na região metropolitana de Porto Alegre incluiu 111 pacientes com DM que iniciaram diálise e acompanhou a evolução destes pacientes por 3,6 anos (2). A sobrevida no primeiro, segundo e terceiro ano após iniciado tratamento dialítico foi de 69%, 51% e 28%, sendo que a principal causa de morte foi por doenças cardiovasculares (63%). A alta morbidade e alta mortalidade associada à DN, especialmente quando progridem para diálise, motiva a pesquisa de novas abordagens para prevenção e tratamento desta complicação. Pensando em contribuir com essa área do conhecimento, desenvolvemos o projeto de doutorado estudando o papel do sistema endotelina na ND. Vários estudos têm relacionado o sistema endotelina com a ND, devido ao seu intenso efeito hemodinâmico e na estrutura renal (3-6). As endotelinas são peptídeos vasoconstritores representados pela endotelina-1 (ET-1), endotelina-2 e endotelina-3. Estes peptídeos são produzidos em vários tecidos, onde atuam como moduladores do tônus vascular, proliferação celular e produção hormonal (7; 8). Apenas a ET-1 é produzida pelas células endoteliais, sendo, portanto, a que está relacionada à disfunção endotelial. As endotelinas agem nos tecidos alvos por meio de dois receptores. O receptor do tipo A (ETRA) e o receptor do tipo B, sendo que o ETRA apresenta maior afinidade a ET-1 e é por meio deste receptor que a ET-1 age, causando vários efeitos associados com a patogênese da DN, incluindo intensa vasoconstrição (7; 8).

Em estudo anterior, avaliamos os níveis séricos de ET-1 nos diferentes estágios da ND e observamos que os níveis séricos de ET-1 estavam maiores nos pacientes macroalbuminúricos em relação aos micro e normoalbuminúricos, sugerindo uma relação

deste sistema com a ND (9). Esta observação nos motivou a expandir o estudo deste sistema na ND. Inicialmente, o papel de variações genéticas de genes do sistema endotelina foi avaliado. A seguir, avaliamos a expressão da ET-1 e do ETRA no tecido renal. Este processo culminou na elaboração de três manuscritos. No primeiro, revisamos a contribuição dos estudos genéticos na ND. No segundo, um artigo original, aonde avaliamos o papel de dois genes do sistema endotelina na ND. O terceiro, um artigo original sobre a expressão destes genes em rins de pacientes com ND, nefropatia por IgA (controles positivos), comparando com rins normais. Desta forma, esperamos poder contribuir para o entendimento desta complicação do DM.

Referências:

1. Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA, Holman RR: Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). *Kidney Int* 63:225-232, 2003

2. Bruno RM, Gross JL: Prognostic factors in Brazilian diabetic patients starting dialysis: a 3.6-year follow-up study. *J Diabetes Complications* 14:266-271, 2000

3. Sorokin A, Kohan DE: Physiology and pathology of endothelin-1 in renal mesangium. *Am J Physiol Renal Physiol* 285:F579-589, 2003

4. Simonson MS: Endothelins: multifunctional renal peptides. *Physiol Rev* 73:375-411, 19931. Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA, Holman RR: Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). *Kidney Int* 63:225-232, 2003

5. Lee YJ, Shin SJ, Tsai JH: Increased urinary endothelin-1-like immunoreactivity excretion in NIDDM patients with albuminuria. *Diabetes Care* 17:263-266, 1994

6. De Mattia G, Cassone-Faldetta M, Bellini C, Bravi MC, Laurenti O, Baldoncini R, Santucci A, Ferri C: Role of plasma and urinary endothelin-1 in early diabetic and hypertensive nephropathy. *Am J Hypertens* 11:983-988, 1998

7. Hopfner RL, Gopalakrishnan V: Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 42:1383-1394, 1999

8. Levin ER: Endothelins. *N Engl J Med* 333:356-363, 1995

9. Zanatta CM, Gerchman F, Burttet L, Nabinger G, Jacques-Silva MC, Canani LH, Gross JL: Endothelin-1 levels and albuminuria in patients with type 2 diabetes mellitus. *Diabetes Res Clin Pract* 80:299-304, 2008

Avaliação do Sistema Endotelina na Nefropatia Diabética em Pacientes com Diabete Melito Tipo 2

RESUMO

Introdução: A nefropatia diabética (ND) é uma das principais complicações crônicas do diabete melito (DM), sendo que cerca de 25 a 40% dos pacientes com DM tipo 1 e 20 a 50% dos pacientes com DM tipo 2 desenvolvem ND ao longo da vida, dependendo da origem étnica. Estudos de agregação familiar mostram uma importante concordância para o desenvolvimento de ND em algumas famílias e reforçam a hipótese de que existem fatores genéticos envolvidos na sua patogênese. O sistema endotelina tem sido relacionado na patogênese da hipertensão arterial e desordens renais. A endotelina-1 (ET-1) regula a vasoconstrição e proliferação celular nos tecidos através da ativação do receptor tipo A (ETRA). Em tecidos de rins normais, ET-1 e ETRA estão mais expressos em vasos e em menor intensidade no glomérulo. Em modelos animais com DM, a expressão de ET-1 é cinco vezes maior, sugerindo uma potencial associação entre o sistema endotelina e ND. No presente estudo, avaliamos a associação de polimorfismos do gene da ET-1 (*EDN1*) e ETRA (*EDNRA)* com a ND em pacientes com DM tipo 2 e a expressão da ET-1 e ETRA em biópsias de rins de pacientes com ND, Nefropatia por IgA e tecido de rins normais.

Materiais e Métodos: O estudo de genética, um estudo caso controle a partir de um estudo transversal, com 548 pacientes brancos com DM tipo 2. Os casos foram considerados pacientes com proteinúria (excreção urinária de albumina (EUA) > 200 μg/min ou 174 mg/24h, em coleta de 24h ou em amostra de urina respectivamente) ou em diálise e controles pacientes com normoalbuminúria (EUA < 20μg/min ou < 17 mg/l) e DM tipo 2 por mais de 5 anos. Foram genotipados dois polimorfismos (SNP) do gene da *EDN1* (rs1800541 or T-1370G; rs57072783 or *Lys*198*Asn*) e cinco do gene do *EDNRA* (rs6842241; rs4835083;

rs4639051; rs5333 and rs5343). A análise de haplótipos foi realizada através do programa PHASE versão 2.1. A frequência dos alelos, genótipos e haplótipos foi comparada entre casos e controles. O equilíbrio de Hardy-Weinberg de cada SNP foi testado através do teste do χ^2 de Pearson. No estudo de imunohistoquimica, analisamos a expressão da ET-1 e ETRA em treze biópsias de pacientes com DM tipo 2 e ND, dez biópsias de pacientes proteinúricos por Nefropatia por IgA e treze amostras de tecido de rim normal que realizaram nefrectomia por tumor.

Resultados: Considerando um modelo dominante, a presença do alelo T do rs57072783 (TT/TG *vs.* GG) foi protetor contra DN (OR = 0.69; IC 95% 0.48-0.99, P = 0.049), enquanto a presença do alelo G do rs1800541 (GG/GT vs. TT) foi associado com OR = 0.60 (IC 95% 0.41-0.88, P=0.009). Entretanto na análise multivariada, somente o genótipo GG/GT do rs1800541 permaneceu com associação independente com a ND (P = 0.046). A expressão da ET-1 em biópsias de pacientes com ND e Nefropatia por IgA estava aumentada nas células endoteliais de capilares glomerulares e capilares peri tubulares quando comparado com controles (P = 0,001). A expressão do ETRA também foi mais intensa na ND e Nefropatia por IgA em relação aos controles (P = 0,019). Pacientes com mais altos níveis de proteinúria tiveram maior expressão do ET-1 mas não do ETRA.

Conclusão: Neste estudo mostramos que SNPs do gene da *EDN1* podem estar associados com aumentado risco para ND em pacientes brancos com DM tipo 2. Também observamos uma maior expressão da ET-1 e do ETRA em pacientes com ND e Nefropatia por IgA, sugerindo um potencial papel do sistema endotelina na ND e provavelmente em outras doenças glomerulares não relacionadas ao DM.

Palavras Chave: Endotelina-1, nefropatia diabética, diabete melito tipo 2.

Artigo 1

Determinantes Genéticos na Nefropatia Diabética: Polimorfismos do Sistema Renina-Angiotensina e do Sistema Endotelina

Autores: Zanatta CM[1], Crispim D[1], Sortica DA[1], Camargo JL[2], Gross JL[1], Canani LH[1]

[1] Serviço de Endocrinologia do Hospital de Clínicas de Porto Alegre, Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Universidade Federal do Rio Grande do Sul.

[2] Serviço de Patologia Clínica do Hospital de Clínicas de Porto Alegre.

Endereço para correspondência:

Luís H. Canani

Serviço de Endocrinologia do Hospital de Clínicas de Porto Alegre

Rua Ramiro Barcelos, 2.350, prédio 12, 4º andar

90035-003 Porto Alegre, RS

E-mail: luiscanani@terra.com.br

Resumo

A prevalência do diabete melito (DM) tem aumentado em proporções epidêmicas e, consequentemente, o risco de um maior número de pacientes desenvolver complicações crônicas também. A nefropatia diabética (ND) é uma das principais complicações crônicas, sendo que cerca de 25 a 40% dos pacientes com DM tipo 1 e 20 a 50% dos pacientes com DM tipo 2 desenvolvem ND ao longo da vida, dependendo da origem étnica. Estudos de agregação familiar mostram uma importante concordância para o desenvolvimento de ND em algumas famílias e reforçam a hipótese de que existem fatores genéticos envolvidos na sua patogênese. Existem várias maneiras de pesquisar o papel de genes na suscetibilidade para a ND e uma estratégia frequentemente utilizada é a do gene candidato. Devido ao sistema renina angiotensina ter um importante papel na regulação da pressão arterial, hemodinâmica renal, retenção de sal e inflamação, polimorfismos no gene que codifica a enzima de conversão da angiotensina (ECA) e nos demais genes envolvidos neste sistema estão entre os mais estudados. Outro sistema que deve ser considerado como um bom candidato para a predisposição à ND é o sistema endotelina. A endotelina-1 (ET-1) que age através de seu receptor A (ETRA) e receptor B (ETRB) está relacionada a distúrbios hemodinâmicos e disfunção endotelial e é um dos mais potentes vasoconstritores já identificados. Vários estudos têm relacionado o sistema endotelina no desenvolvimento da lesão renal em pacientes com DM. O entendimento de todos os fatores genéticos e de suas associações fará com que entendamos melhor essa complicação crônica do DM e com isso possamos ter uma abordagem mais efetiva em sua prevenção e tratamento.

Descritores: Diabete Melito, Nefropatia Diabética, Sistema Renina Angiotensina, Endotelina-1.

Abstract

The prevalence of diabetes mellitus (DM) is increasing in epidemic proportions, and consequently the risks of a greater number of patients develop chronic complications as well. Diabetic nephropathy (DN) is one of the main chronic complications, and about 25 to 40% of patients with type 1 DM and 20 to 50% of patients with type 2 DM develop ND lifelong, depending on ethnic origin. Familial aggregation studies show an important agreement for the development of ND in some families and strengthen the hypothesis that there are genetic factors involved in its pathogenesis. There are several ways to search the role of genes in the susceptibility to ND and a strategy commonly used is the candidate gene. Because of the renin angiotensin system plays an important role in regulating blood pressure, renal hemodynamics, salt retention and inflammation, polymorphisms in the gene encoding the converting enzyme (ACE) and other genes involved in this system are among the most studied. Another system that should be considered a good candidate for predisposition to ND is the endothelin system. Endothelin-1 (ET-1), acting through its receptor A (ETRA) and receptor B (ETRB), is related to hemodynamic disturbances and endothelial dysfunction and is one of the most potent vasoconstrictors already identified. Several studies have linked the endothelin system to the development of renal injury in patients with DM. The understanding of all genetic factors and their associations will provide us a better knowledge of this chronic diabetic complication and, thus, we may have a more effective approach in prevention and treatment.

Keywords: Diabetes Mellitus, Diabetic Nephropathy, Renin Angiotensin System, Endothelin-1.

Introdução

O diabete melito (DM) constitui um grave problema de saúde pública. O envelhecimento da população e as alterações no estilo de vida, levando à obesidade, à síndrome metabólica e à hipertensão arterial, têm aumentado a prevalência do DM em proporções epidêmicas (1). O risco de desenvolver DM tipo 2 ao longo da vida na população caucasiana americana nascida em 2000 é de 30%, enquanto em homens afro-americanos é de 40% e em mulheres afro-americanas é de 49% (2). Consequentemente espera-se um maior número de pacientes com complicações crônicas do DM. A nefropatia diabética (ND) é uma importante complicação do DM, sendo responsável por um quarto dos casos que iniciam diálise na região metropolitana de Porto Alegre (3) e a principal causa de doença renal terminal na Europa, Estados Unidos (4) e Japão (5). Estudos de agregação familiar em indivíduos com DM mostram uma forte presença de ND em algumas famílias. Esta complicação do DM também está associada à presença de doenças cardiovasculares (DCV) (6; 7).

Epidemiologia da Nefropatia Diabética

A ND é definida por um aumento muito pequeno na excreção urinária de proteínas, predominantemente da albumina. Esta anormalidade não é detectada pela medida da proteína urinária total em testes de rotina. Na fase inicial apenas testes muito sensíveis (imunoensaios ou técnicas por HPLC) conseguem detectar pequenas quantidades de albumina na urina. Esta determinação se consagrou pela terminologia de microalbuminúria. Esta alteração pode estar presente em qualquer momento do DM, mas é mais frequente após, pelo menos, 5 anos de evolução do mesmo. A evolução da microalbuminúria é muito variada. Tradicionalmente, considera-se que se nenhuma intervenção for realizada em pacientes com DM tipo 1, a microalbuminúria pode evoluir para proteinúria clínica (ou macroalbuminúria) num período

de aproximadamente 10 anos, quando os testes convencionais de urina passam a detectar essa alteração (Tabela 1). No estágio da microalbuminúria não se espera alteração na taxa de filtração glomerular (TFG), ocorrendo um declínio progressivo da TFG na fase proteinúrica que pode evoluir para doença renal terminal em aproximadamente 10 anos (8).

Em torno de 25 a 40% dos pacientes com DM tipo 1 apresentam ND após 25 anos da doença, que é a principal causa de morte nestes pacientes (9). A incidência cumulativa de microalbuminúria em pacientes com DM tipo 1 foi de 12,6% ao longo de 7,3 anos de acordo com o *The European Diabetes* (EURODIAB) *Prospectiv Complications Study Group* (10) e de 33% em um estudo de 18 anos realizado na Dinamarca (11). Proteinúria ou macroalbuminúria ocorre em 15 a 40% dos pacientes com DM tipo 1, com um pico de incidência em torno de 15 a 20 anos de DM (12; 13). Em pacientes com DM tipo 2, a prevalência de ND varia de 20 a 50%, dependendo da origem étnica (11). A incidência de microalbuminúria foi de 2,0% ao ano e a prevalência após 10 anos do diagnóstico de DM foi de 25% no *U.K. Prospective Diabetes Study* (UKPDS) (14). A prevalência de macroalbuminúria é muito variável, ficando em torno de 5% a 20% (14; 15).

Porém, a história natural da albuminúria não é bem clara. Caramori e cols. revisaram os dados das maiores séries de casos da progressão da microalbuminúria e demonstraram que somente 30 a 45% dos pacientes com microalbuminúria evoluem para a macroalbuminúria (16). Um percentual significativo regride para normoalbuminúria e outro permanece estável. Este achado é conflitante com os relatos iniciais da década de 80 quando a presença de microalbuminúria parecia predizer a evolução para a macroalbuminúria (proteinúria clínica) em aproximadamente 80% dos pacientes com DM tipo 1. Uma explicação para esta diferença observada pode ser devido que nos estudos iniciais existia uma valorização excessiva da microalbuminúria como preditor de progressão para estágios mais graves, provavelmente à seleção de casos mais graves ou representar um achado espúrio devido ao pequeno número de

pacientes acompanhados nestas séries. Outra explicação pode ser que a evolução desta condição tenha se modificado com o passar dos anos, pelas mudanças do tratamento anti-hipertensivo e da hiperglicemia. Perkins e cols. acompanharam uma coorte de 386 pacientes com DM tipo 1 microalbuminúricos por um período de 6 anos, que estavam estáveis há 2 anos, e observaram que somente 15% evoluíram para proteinúria, 40% reduziram pela metade os valores de excreção urinária de albumina e 45% permaneceram com valores de excreção urinária de albumina inalterada, na faixa de microalbuminúria (17). Neste estudo o uso de inibidores da enzima conversora da angiotensina (IECA) não se relacionou com a regressão da microalbuminúria. Entretanto, microalbuminúria de início recente, hemoglobina glicada (A1c) menor que 8,0%, pressão arterial sistólica menor que 115 mmHg, colesterol total menor que 198 mg/dL e triglicerídeo menor que 145 mg/dL foram fatores independentes associados com a regressão da microalbuminúria.

Outro aspecto não tradicional é a queda da TFG na ausência de aumento da albuminúria. Alguns pacientes com DM tipo 2 normoalbuminúricos evoluem com diminuição da TFG (18). Nestes pacientes se observou uma relação com níveis altos de triglicerídeos e com a presença da síndrome metabólica (18), sugerindo que doença aterosclerótica microvascular renal levaria à diminuição do fluxo plasmático renal e isso seria uma possível explicação para uma baixa TFG.

Genética e Nefropatia Diabética

A melhora no controle da glicemia e da pressão arterial, associado ao uso de inibidores do sistema renina-angiotensina (RAS), tem retardado o surgimento e progressão para ND terminal nos últimos anos (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm 5443a2.htm). No estudo DCCT, a terapia intensiva reduziu o risco de desenvolvimento de microalbuminúria em 34% no grupo de prevenção primária e em 43% no grupo de prevenção

secundária (19). Porém, uma grande proporção de pacientes com DM ainda desenvolve essa complicação. Estudos de agregação familiar mostram uma importante concordância para o desenvolvimento de ND em algumas famílias e reforçam a hipótese de que existem fatores genéticos envolvidos na sua patogênese (20-24). Indivíduos com DM que possuem familiares diabéticos com ND apresentam um risco significativamente maior de desenvolver doença renal em comparação com indivíduos com DM sem familiares com essa complicação (20; 23; 25; 26).

Dados epidemiológicos também indicam suscetibilidade genética para desenvolvimento da ND pelo fato de que existe um pico de incidência entre 15 a 20 anos após o diagnóstico de DM, seguido por um rápido declínio, resultando em uma incidência cumulativa de menos de 30% (27; 28). Se a ND fosse causada apenas pela hiperglicemia, sua incidência aumentaria progressivamente ao longo do tempo e a maioria dos pacientes com DM desenvolveriam doença renal, similarmente ao que acontece com a retinopatia diabética (29).

Pacientes com DM e ND apresentam mais frequentemente história familiar de hipertensão arterial e DCV do que os pacientes sem ND (30). A presença de microalbuminúria é um preditor fortemente relacionado à morte por DCV, talvez mais do que para o próprio desenvolvimento de formas mais graves de ND (11; 31; 32).

Portanto, existe a necessidade de identificar genes que predispõem ao desenvolvimento e progressão da doença renal, assim como, genes que podem causar proteção renal.

Está estimado que o genoma humano contenha em torno de 25.000 genes e mais de 10 milhões de polimorfismos genéticos (33). O desenvolvimento de uma doença complexa como a ND depende do efeito de muitas variáveis genéticas atuando sinergicamente e aditivamente uma com a outra e com fatores ambientais (34).

Existem várias maneiras de pesquisar o papel de genes na suscetibilidade para a ND (33; 35-37). Uma estratégia frequentemente utilizada é a do gene candidato (33; 38; 39). Vários estudos têm avaliado o papel genético na ND através dessa abordagem. Devido ao sistema renina angiotensina ter um importante papel na regulação da pressão arterial, hemodinâmica renal, retenção de sal e inflamação, polimorfismos no gene que codifica a enzima de conversão da angiotensina (ECA) e nos demais genes envolvidos neste sistema estão entre os mais estudados.

Polimorfismos em genes do sistema renina angiotensina

O polimorfismo de inserção/deleção (I/D; rs1799752) do gene da ECA, caracterizado pela presença (inserção, I) ou a ausência (deleção, D) de um segmento de 256 pares de bases no *intron* 16, é um dos mais estudados em relação à ND. Por isso, é um bom exemplo de alguns aspectos do estudo de genes candidatos. O alelo D deste polimorfismo tem sido descrito como associado a maior risco de desenvolver ND. Entretanto, em alguns estudos essa associação não foi encontrada. Em junho de 2008, Ng e cols. (40) publicaram uma meta-análise de 53 estudos sobre o polimorfismo I/D do gene da ECA, com um total de 17.791 pacientes. Vinte e um estudos envolviam pacientes com DM tipo 1 (n = 4.154) e 32 estudos eram com DM tipo 2 (n = 13.637). Na análise total foi observada uma razão de chances (RC) de 0,78 (IC 95% 0,70-0,87; p <0,001), indicando que o genótipo II confere uma importante proteção contra o desenvolvimento da ND quando comparado com a presença do alelo D, tanto na ND do DM tipo 1 quanto do DM tipo 2. Outro aspecto interessante, é que a frequência alélica deste polimorfismo é bastante dependente da etnia (41; 42). Por exemplo, a frequência do alelo D em Asiáticos varia de 33% a 47%, em Caucasianos varia de 46% a 66%, em Afro-Americanos é de 58%, em Africanos varia de 54% a 65% e em Franceses-Canadenses é de 62%. Como a prevalência de ND também varia de forma importante nestes

grupos étnicos (13; 43-45), este é um bom exemplo de polimorfismo que pode ter associações espúrias devido à estratificação populacional. Outro aspecto a ser ressaltado é que em doenças complexas como a ND exista uma interação entre gene e ambiente. Em teoria, uma doença com predisposição genética deverá ocorrer mais precocemente ou na presença de uma hiperglicemia não tão importante. Demonstrando este efeito, observamos que o alelo D está associado com doença renal avançada nos indivíduos com pouco tempo de exposição à hiperglicemia, enquanto nos indivíduos que desenvolvem a ND avançada após longos anos de exposição não se observa esta associação (46).

Além disso, a maioria dos estudos de genes candidatos é feita na forma de estudo de casos e de controles. Neste desenho, os casos podem ser super selecionados ou podem ser sobreviventes (viés de sobrevida), o que pode resultar em associações espúrias ou até mesmo inversas. Para analisar se o polimorfismo I/D do gene da ECA seria útil em determinar prognóstico renal e cardiovascular em pacientes com DM tipo 2, Hadjadj e cols. (47) analisaram prospectivamente os genótipos de um grande número de pacientes em 3 estudos (DIABHYCAR, DIAB2NEPHROGENE e SURDIAGENE) e não encontraram impacto na ocorrência de desfechos tais como, morte por DCV, infarto do miocárdio, acidente vascular cerebral, insuficiência cardíaca levando à hospitalização e doença renal terminal. No entanto, em outro estudo prospectivo a inibição do sistema renina angiotensina foi associada com melhor resposta de renoproteção e menor mortalidade em pacientes Chineses com DM tipo 2 que apresentavam o genótipo II ou ID (48). Outros estudos observaram melhor resposta a intervenção nos estágios de normo e microalbuminúria no DM tipo 1 e no DM tipo 2 que apresentavam o genótipo I (49; 50). No estudo RENNAL, os pacientes com DM tipo 2 e proteinúria e que apresentavam o alelo D, apresentaram melhor resposta ao tratamento com losartana em relação à doença renal quando comparado com placebo (51).

Por fim, o estudo de um polimorfismo isoladamente nem sempre é suficiente para se entender o papel do gene. A avaliação de vários polimorfismos em um gene e a predição de haplótipos pode ser útil no entendimento do papel real do gene na ND. Novamente, o gene da ECA pode ser um bom exemplo desta abordagem. A análise de três polimorfismos localizados neste gene, incluindo o de I/D, em um pequeno grupo de pacientes com DM tipo 2 (n = 458) não evidenciou associação de nenhum dos polimorfismos de forma isolada. Entretanto, a análise de haplótipos demonstrou que uma variante que incluía o alelo D estava associada com a presença de ND e apresentava um aumento de quase duas vezes nos casos em relação aos controles (52).

Indivíduos que apresentam o genótipo II mostram níveis séricos mais baixos de ECA do que indivíduos com o genótipo DD (53). Até o momento, esse polimorfismo permanece como o único *locus* associado com a patogênese e progressão da doença renal crônica e com resposta ao tratamento com drogas que afetam diretamente o sistema renina angiotensina, tais como os IECA e os antagonistas do receptor da angiotensina II (ARA II) (49). Este é um exemplo de farmacogenômica, em que o conhecimento de um genótipo tem valor na determinação do impacto de uma terapêutica medicamentosa (54).

Polimorfismos do gene do sistema endotelina

Outro sistema que deve ser considerado como um bom candidato para a predisposição à ND é o sistema endotelina. Entretanto, diferente do gene da ECA são escassos os estudos sobre variantes gênicas deste sistema. A endotelina-1 (ET-1) é a principal representante do sistema endotelina e está relacionada a distúrbios hemodinâmicos e disfunção endotelial. A ET-1 e o receptor A (ETRA) têm importante papel na manutenção do tônus vasomotor basal (55), sendo um dos mais potentes vasoconstritores já identificados (56; 57). A ET-1 tem sido implicada no desenvolvimento da lesão renal em pacientes com DM (58). Alterações

metabólicas que estão presentes no DM, como o hiperinsulinismo, a hiperglicemia e a hiperlipidemia, estimulam diretamente a liberação da ET-1 pelas células endoteliais e a expressão de seus receptores (56). ET-1 é secretada pelas células endoteliais glomerulares, células mesangiais e células epiteliais. A ativação do sistema endotelina no rim via o ETRA e o receptor B (ETRB), causa vasoconstrição, inibição da reabsorção de sal e água, aumento da proliferação celular glomerular e acúmulo de proteínas na matriz extracelular (59). Estudos em pacientes com DM tipo 2 tem relatado níveis elevados de ET-1 plasmática (60; 61). Outros estudos mostram uma correlação entre níveis plasmáticos e urinários de ET-1 e a ND (62; 63). Demonstramos um aumento progressivo da ET-1 plasmática em pacientes com DM tipo 2 de normo para micro e deste para macroalbuminúria (64) (Figura 1). Estudos em modelos animais demonstram que ocorre normalização da pressão glomerular e diminuição de deposição de proteínas na matriz extracelular, assim como diminuição da excreção urinária de albumina, com o uso do bloqueador do receptor ETRA/ETRB (bosentan) (65). Nestes animais, o uso do bosentan apresentou efeito nefroprotetor, corrigindo tanto a hiperfiltração inicial quanto a evolução para nefropatia clínica (66). Hocher e cols. (67) estudaram o uso de antagonista seletivo do ETRA (LU135252) em comparação com o antagonista combinado ETRA/ETRB (LU224332) em ratos, e mostraram que os dois antagonistas normalizaram a expressão da fibronectina e do colágeno tipo IV dentro do glomérulo, que estava aumentada após 36 semanas da indução do DM, e reduziram em 50% a proteinúria em relação aos controles. O efeito antifibrótico parece ser mediado via receptor ETRA. Um estudo em pacientes com DM tipo 2 obesos, com resistência à insulina, mostrou melhora da vasodilatação endotélio-dependente com o uso do bloqueador do ETRA (BQ123) (68).

O gene da ET-1 (*EDN1*) está localizado no cromossomo 6 (6p24.1) e contém 5 exons (http://www.ncbi.nlm.nih.gov/GenBank). Até o momento foram identificados 15 polimorfismos do tipo *single nucleotide polymorphism* (SNP) em caucasianos

(www.hapmap.org). Devido ao alto desequilíbrio de ligação entre esses polimorfismos, a genotipagem de apenas 2 polimorfismos, um localizado na região promotora (rs1800541) e outro no éxon 5 (rs57072783 ou rs5370), é suficiente para analisar toda a variabilidade do gene (69) (Figura 2). O gene que codifica o receptor ETRA (*EDNRA*) se localiza no cromossomo 4 (4q31.22) e contém 8 éxons (http://www.ncbi.nlm.nih.gv/GenBank). O consórcio *International HapMap Project* (www.hapmap.org) identificou 58 SNPs com frequência maior do que 1% nos 90 indivíduos caucasianos avaliados. Também devido aos valores de desequilíbrio de ligação entre os SNPs do gene *EDNRA*, mostra-se necessário estudar de 5 a 11 SNPs para cobrir todos os haplótipos com mais do que 10% de frequência, analisando assim 90 a 100% da variabilidade deste gene (Figura 3).

Um estudo recente avaliou a relação entre o polimorfismo rs1476046 do gene *EDN1* (G>A) e nível sérico da pró- ET-1. Observou-se uma associação com uma razão de chances estimada para ND em heterozigotos de 1,26 (0,96-1,66) e em homozigotos de 1,87 (1,13-3,12); p = 0,0072 (35).

Freedman e cols. analisaram a associação em 361 irmãos com doença renal terminal e DM e irmãos com doença renal terminal não diabética de 168 famílias de afro-americanos e não encontraram associação da doença renal com polimorfismos no gene *EDN1 (70)*. Outro estudo investigando a associação de 45 polimorfismos em 20 genes candidatos em pacientes com DM tipo 2 com e sem ND, encontraram uma associação com genes do cromossomo 6 e 7, entre eles um polimorfismo localizado no gene *EDN1* (rs1476046G/A) (71). Por outro lado, uma avaliação em uma população não diabética encontrou relação de variantes no gene *EDN1* com valores de TFG, mas nenhuma relação com excreção urinária de albumina (69).

Como esses estudos não foram conclusivos, os genes *EDN1* e *EDNRA*, por codificarem proteínas com muitos efeitos renais, continuam sendo genes candidatos para ND e futuros estudos são necessários para determinar sua relação com essa complicação.

Conclusão

A ND é uma doença multifatorial e, dessa forma, está associada tanto a fatores de risco ambientais como fatores genéticos. O entendimento de todos os fatores genéticos e de suas associações fará com que entendamos melhor essa complicação crônica do DM e com isso possamos ter uma abordagem mais efetiva em sua prevenção e tratamento.

Referências:

1. Sale MM, Woods J, Freedman BI: Genetic determinants of the metabolic syndrome. *Curr Hypertens Rep* 8:16-22, 2006

2. Narayan KM, Boyle JP, Thompson TJ, Sorensen SW, Williamson DF: Lifetime risk for diabetes mellitus in the United States. *Jama* 290:1884-1890, 2003

3. Bruno RM, Gross JL: Prognostic factors in Brazilian diabetic patients starting dialysis: a 3.6-year follow-up study. *J Diabetes Complications* 14:266-271, 2000

4. Molitch ME, DeFronzo RA, Franz MJ, Keane WF, Mogensen CE, Parving HH: Diabetic nephropathy. *Diabetes Care* 26 Suppl 1:S94-98, 2003

5. An overview of regular dialysis treatment in Japan (as of 31 December 2002). *Ther Apher Dial* 8:358-382, 2004

6. Rotimi C, Cooper R, Cao G, Sundarum C, McGee D: Familial aggregation of cardiovascular diseases in African-American pedigrees. *Genet Epidemiol* 11:397-407, 1994

7. Sale MM, Freedman BI: Genetic determinants of albuminuria and renal disease in diabetes mellitus. *Nephrol Dial Transplant* 21:13-16, 2006

8. Marshall SM: Recent advances in diabetic nephropathy. *Postgrad Med J* 80:624-633, 2004

9. Group. TDCaCTR: Effect of intensive therapy on the development and progression of diabetic nephropathy in the Diabetes Control and Complications Trial. *Kidney Int* 47:1703-1720, 1995

10. Chaturvedi N, Bandinelli S, Mangili R, Penno G, Rottiers RE, Fuller JH: Microalbuminuria in type 1 diabetes: rates, risk factors and glycemic threshold. *Kidney Int* 60:219-227, 2001

11. Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA, Holman RR: Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). *Kidney Int* 63:225-232, 2003

12. Wong TY, Shankar A, Klein R, Klein BE: Retinal vessel diameters and the incidence of gross proteinuria and renal insufficiency in people with type 1 diabetes. *Diabetes* 53:179-184, 2004

13. Young BA, Maynard C, Boyko EJ: Racial differences in diabetic nephropathy, cardiovascular disease, and mortality in a national population of veterans. *Diabetes Care* 26:2392-2399, 2003

14. Andersen AR, Christiansen JS, Andersen JK, Kreiner S, Deckert T: Diabetic nephropathy in Type 1 (insulin-dependent) diabetes: an epidemiological study. *Diabetologia* 25:496-501, 1983

15. Valmadrid CT, Klein R, Moss SE, Klein BE: The risk of cardiovascular disease mortality associated with microalbuminuria and gross proteinuria in persons with older-onset diabetes mellitus. *Arch Intern Med* 160:1093-1100, 2000

16. Caramori ML, Fioretto P, Mauer M: The need for early predictors of diabetic nephropathy risk: is albumin excretion rate sufficient? *Diabetes* 49:1399-1408, 2000

17. Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS: Regression of microalbuminuria in type 1 diabetes. *N Engl J Med* 348:2285-2293, 2003

18. Kramer CK, Leitao CB, Pinto LC, Silveiro SP, Gross JL, Canani LH: Clinical and laboratory profile of patients with type 2 diabetes with low glomerular filtration rate and normoalbuminuria. *Diabetes Care* 30:1998-2000, 2007

19. Effect of intensive therapy on the development and progression of diabetic nephropathy in the Diabetes Control and Complications Trial. The Diabetes Control and Complications (DCCT) Research Group. *Kidney Int* 47:1703-1720, 1995

20. Canani LH, Gerchman F, Gross JL: Familial clustering of diabetic nephropathy in Brazilian type 2 diabetic patients. *Diabetes* 48:909-913, 1999

21. Harjutsalo V, Katoh S, Sarti C, Tajima N, Tuomilehto J: Population-based assessment of familial clustering of diabetic nephropathy in type 1 diabetes. *Diabetes* 53:2449-2454, 2004

22. Rema M, Saravanan G, Deepa R, Mohan V: Familial clustering of diabetic retinopathy in South Indian Type 2 diabetic patients. *Diabet Med* 19:910-916, 2002

23. Seaquist ER, Goetz FC, Rich S, Barbosa J: Familial clustering of diabetic kidney disease. Evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med* 320:1161-1165, 1989

24. Yajima Y, Nakajima S: [Familial clustering and genetics of diabetic nephropathy]. *Nippon Rinsho* 55 Suppl:795-801, 1997

25. Krolewski AS: Genetics of diabetic nephropathy: evidence for major and minor gene effects. *Kidney Int* 55:1582-1596, 1999

26. Quinn M, Angelico MC, Warram JH, Krolewski AS: Familial factors determine the development of diabetic nephropathy in patients with IDDM. *Diabetologia* 39:940-945, 1996

27. Krolewski AS, Warram JH, Rand LI, Christlieb AR, Busick EJ, Kahn CR: Risk of proliferative diabetic retinopathy in juvenile-onset type I diabetes: a 40-yr follow-up study. *Diabetes Care* 9:443-452, 1986

28. Krolewski AS, Warram JH, Christlieb AR, Busick EJ, Kahn CR: The changing natural history of nephropathy in type I diabetes. *Am J Med* 78:785-794, 1985

29. Klein R, Klein BE, Moss SE, Davis MD, DeMets DL: The Wisconsin epidemiologic study of diabetic retinopathy. II. Prevalence and risk of diabetic retinopathy when age at diagnosis is less than 30 years. *Arch Ophthalmol* 102:520-526, 1984

30. Canani LH, Gerchman F, Gross JL: Increased familial history of arterial hypertension, coronary heart disease, and renal disease in Brazilian type 2 diabetic patients with diabetic nephropathy. *Diabetes Care* 21:1545-1550, 1998

31. Roglic G, Colhoun HM, Stevens LK, Lemkes HH, Manes C, Fuller JH: Parental history of hypertension and parental history of diabetes and microvascular complications in insulin-dependent diabetes mellitus: the EURODIAB IDDM Complications Study. *Diabet Med* 15:418-426, 1998

32. De Cosmo S, Bacci S, Piras GP, Cignarelli M, Placentino G, Margaglione M, Colaizzo D, Di Minno G, Giorgino R, Liuzzi A, Viberti GC: High prevalence of risk factors for cardiovascular disease in parents of IDDM patients with albuminuria. *Diabetologia* 40:1191-1196, 1997

33. Conway BR, Savage DA, Maxwell AP: Identifying genes for diabetic nephropathy--current difficulties and future directions. *Nephrol Dial Transplant* 21:3012-3017, 2006

34. Martini S, Eichinger F, Nair V, Kretzler M: Defining human diabetic nephropathy on the molecular level: integration of transcriptomic profiles with biological knowledge. *Rev Endocr Metab Disord* 9:267-274, 2008

35. Lim SC, Liu JJ, Low HQ, Morgenthaler NG, Li Y, Yeoh LY, Wu YS, Goh SK, Chionh CY, Tan SH, Kon YC, Soon PC, Bee YM, Subramaniam T, Sum CF, Chia KS: Microarray analysis of multiple candidate genes and associated plasma proteins for nephropathy secondary to type 2 diabetes among Chinese individuals. *Diabetologia* 52:1343-1351, 2009

36. Pezzolesi MG, Poznik GD, Mychaleckyj JC, Paterson AD, Barati MT, Klein JB, Ng DP, Placha G, Canani LH, Bochenski J, Waggott D, Merchant ML, Krolewski B, Mirea L, Wanic K, Katavetin P, Kure M, Wolkow P, Dunn JS, Smiles A, Walker WH, Boright AP, Bull SB, Doria A, Rogus JJ, Rich SS, Warram JH, Krolewski AS: Genome-wide association scan for diabetic nephropathy susceptibility genes in type 1 diabetes. *Diabetes* 58:1403-1410, 2009

37. He B, Osterholm AM, Hoverfalt A, Forsblom C, Hjorleifsdottir EE, Nilsson AS, Parkkonen M, Pitkaniemi J, Hreidarsson A, Sarti C, McKnight AJ, Maxwell AP, Tuomilehto J, Groop PH, Tryggvason K: Association of genetic variants at 3q22 with nephropathy in patients with type 1 diabetes mellitus. *Am J Hum Genet* 84:5-13, 2009

38. Freedman BI, Bostrom M, Daeihagh P, Bowden DW: Genetic factors in diabetic nephropathy. *Clin J Am Soc Nephrol* 2:1306-1316, 2007

39. Susztak K, Sharma K, Schiffer M, McCue P, Ciccone E, Bottinger EP: Genomic strategies for diabetic nephropathy. *J Am Soc Nephrol* 14:S271-278, 2003

40. Ng DP, Tai BC, Lim XL: Is the presence of retinopathy of practical value in defining cases of diabetic nephropathy in genetic association studies? The experience with the ACE insertion/deletion polymorphism in 53 studies comprising 17,791 subjects. *Diabetes* 57:2541-2546, 2008

41. Zintzaras E, Raman G, Kitsios G, Lau J: Angiotensin-converting enzyme insertion/deletion gene polymorphic variant as a marker of coronary artery disease: a meta-analysis. *Arch Intern Med* 168:1077-1089, 2008

42. Tien KJ, Hsiao JY, Hsu SC, Liang HT, Lin SR, Chen HC, Hsieh MC: Gender-dependent effect of ACE I/D and AGT M235T polymorphisms on the progression of urinary albumin excretion in Taiwanese with type 2 diabetes. *Am J Nephrol* 29:299-308, 2009

43. Gerchman F, Zanatta CM, Burttet LM, Picon PX, Lisboa HR, Silveiro SP, Gross JL, Canani LH: Vascular complications of black patients with type 2 diabetes mellitus in Southern Brazil. *Braz J Med Biol Res* 41:668-673, 2008

44. Young BA, Katon WJ, Von Korff M, Simon GE, Lin EH, Ciechanowski PS, Bush T, Oliver M, Ludman EJ, Boyko EJ: Racial and ethnic differences in microalbuminuria prevalence in a diabetes population: the pathways study. *J Am Soc Nephrol* 16:219-228, 2005

45. Parving HH, Lewis JB, Ravid M, Remuzzi G, Hunsicker LG: Prevalence and risk factors for microalbuminuria in a referred cohort of type II diabetic patients: a global perspective. *Kidney Int* 69:2057-2063, 2006

46. Canani LH, Costa LA, Crispim D, Goncalves Dos Santos K, Roisenberg I, Lisboa HR, Sarturi Tres G, Maia AL, Gross JL: The presence of allele D of angiotensin-converting enzyme polymorphism is associated with diabetic nephropathy in patients with less than 10 years duration of Type 2 diabetes. *Diabet Med* 22:1167-1172, 2005

47. Hadjadj S, Fumeron F, Roussel R, Saulnier PJ, Gallois Y, Ankotche A, Travert F, Abi Khalil C, Miot A, Alhenc-Gelas F, Lievre M, Marre M: Prognostic value of the insertion/deletion polymorphism of the ACE gene in type 2 diabetic subjects: results from the Non-insulin-dependent Diabetes, Hypertension, Microalbuminuria or Proteinuria, Cardiovascular Events, and Ramipril (DIABHYCAR), Diabete de type 2, Nephropathie et Genetique (DIAB2NEPHROGENE), and Survie, Diabete de type 2 et Genetique (SURDIAGENE) studies. *Diabetes Care* 31:1847-1852, 2008

48. So WY, Ma RC, Ozaki R, Tong PC, Ng MC, Ho CS, Lam CW, Chow CC, Chan WB, Kong AP, Chan JC: Angiotensin-converting enzyme (ACE) inhibition in type 2, diabetic patients-- interaction with ACE insertion/deletion polymorphism. *Kidney Int* 69:1438-1443, 2006

49. Ruggenenti P, Bettinaglio P, Pinares F, Remuzzi G: Angiotensin converting enzyme insertion/deletion polymorphism and renoprotection in diabetic and nondiabetic nephropathies. *Clin J Am Soc Nephrol* 3:1511-1525, 2008

50. Parving HH, Jacobsen P, Tarnow L, Rossing P, Lecerf L, Poirier O, Cambien F: Effect of deletion polymorphism of angiotensin converting enzyme gene on progression of diabetic nephropathy during inhibition of angiotensin converting enzyme: observational follow up study. *Bmj* 313:591-594, 1996

51. Parving HH, de Zeeuw D, Cooper ME, Remuzzi G, Liu N, Lunceford J, Shahinfar S, Wong PH, Lyle PA, Rossing P, Brenner BM: ACE gene polymorphism and losartan treatment in type 2 diabetic patients with nephropathy. *J Am Soc Nephrol* 19:771-779, 2008

52. Ng DP, Placha G, Choo S, Chia KS, Warram JH, Krolewski AS: A disease haplotype for advanced nephropathy in type 2 diabetes at the ACE locus. *Diabetes* 55:2660-2663, 2006

53. Marre M, Bernadet P, Gallois Y, Savagner F, Guyene TT, Hallab M, Cambien F, Passa P, Alhenc-Gelas F: Relationships between angiotensin I converting enzyme gene polymorphism, plasma levels, and diabetic retinal and renal complications. *Diabetes* 43:384-388, 1994

54. Penno G, Chaturvedi N, Talmud PJ, Cotroneo P, Manto A, Nannipieri M, Luong LA, Fuller JH: Effect of angiotensin-converting enzyme (ACE) gene polymorphism on progression of renal disease and the influence of ACE inhibition in IDDM patients: findings from the EUCLID Randomized Controlled Trial. EURODIAB Controlled Trial of Lisinopril in IDDM. *Diabetes* 47:1507-1511, 1998

55. Haynes WG, Webb DJ: Contribution of endogenous generation of endothelin-1 to basal vascular tone. *Lancet* 344:852-854, 1994

56. Hopfner RL, Gopalakrishnan V: Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 42:1383-1394, 1999

57. Levin ER: Endothelins. *N Engl J Med* 333:356-363, 1995

58. Sorokin A, Kohan DE: Physiology and pathology of endothelin-1 in renal mesangium. *Am J Physiol Renal Physiol* 285:F579-589, 2003

59. Simonson MS: Endothelins: multifunctional renal peptides. *Physiol Rev* 73:375-411, 1993

60. Seligman BG, Biolo A, Polanczyk CA, Gross JL, Clausell N: Increased plasma levels of endothelin 1 and von Willebrand factor in patients with type 2 diabetes and dyslipidemia. *Diabetes Care* 23:1395-1400, 2000

61. Schneider JG, Tilly N, Hierl T, Sommer U, Hamann A, Dugi K, Leidig-Bruckner G, Kasperk C: Elevated plasma endothelin-1 levels in diabetes mellitus. *Am J Hypertens* 15:967-972, 2002

62. Lee YJ, Shin SJ, Tsai JH: Increased urinary endothelin-1-like immunoreactivity excretion in NIDDM patients with albuminuria. *Diabetes Care* 17:263-266, 1994

63. De Mattia G, Cassone-Faldetta M, Bellini C, Bravi MC, Laurenti O, Baldoncini R, Santucci A, Ferri C: Role of plasma and urinary endothelin-1 in early diabetic and hypertensive nephropathy. *Am J Hypertens* 11:983-988, 1998

64. Zanatta CM, Gerchman F, Burttet L, Nabinger G, Jacques-Silva MC, Canani LH, Gross JL: Endothelin-1 levels and albuminuria in patients with type 2 diabetes mellitus. *Diabetes Res Clin Pract* 80:299-304, 2008

65. Cosenzi A, Bernobich E, Trevisan R, Milutinovic N, Borri A, Bellini G: Nephroprotective effect of bosentan in diabetic rats. *J Cardiovasc Pharmacol* 42:752-756, 2003

66. Ding SS, Qiu C, Hess P, Xi JF, Zheng N, Clozel M: Chronic endothelin receptor blockade prevents both early hyperfiltration and late overt diabetic nephropathy in the rat. *J Cardiovasc Pharmacol* 42:48-54, 2003

67. Hocher B, Schwarz A, Reinbacher D, Jacobi J, Lun A, Priem F, Bauer C, Neumayer HH, Raschack M: Effects of endothelin receptor antagonists on the progression of diabetic nephropathy. *Nephron* 87:161-169, 2001

68. Marther K, Mirzamohammadi B, Lteif A, Steinberg H, Baron A: ET-1A Receptor blockade improves endothelium-dependent vasodilation in insulin resistant obese and type 2 diabetic patients- endothelin-1A. *Diabetes* 51, 2000

69. Pinto-Sietsma SJ, Herrmann SM, Schmidt-Petersen K, Niu T, Hillege HL, Janssen WM, de Zeeuw D, de Jong P, Kreutz R: Role of the endothelin-1 gene locus for renal impairment in the general nondiabetic population. *J Am Soc Nephrol* 14:2596-2602, 2003

70. Freedman BI, Yu H, Anderson PJ, Roh BH, Rich SS, Bowden DW: Genetic analysis of nitric oxide and endothelin in end-stage renal disease. *Nephrol Dial Transplant* 15:1794-1800, 2000

71. Kankova K, Stejskalova A, Pacal L, Tschoplova S, Hertlova M, Krusova D, Izakovicova-Holla L, Beranek M, Vasku A, Barral S, Ott J: Genetic risk factors for diabetic nephropathy on chromosomes 6p and 7q identified by the set-association approach. *Diabetologia* 50:990-999, 2007

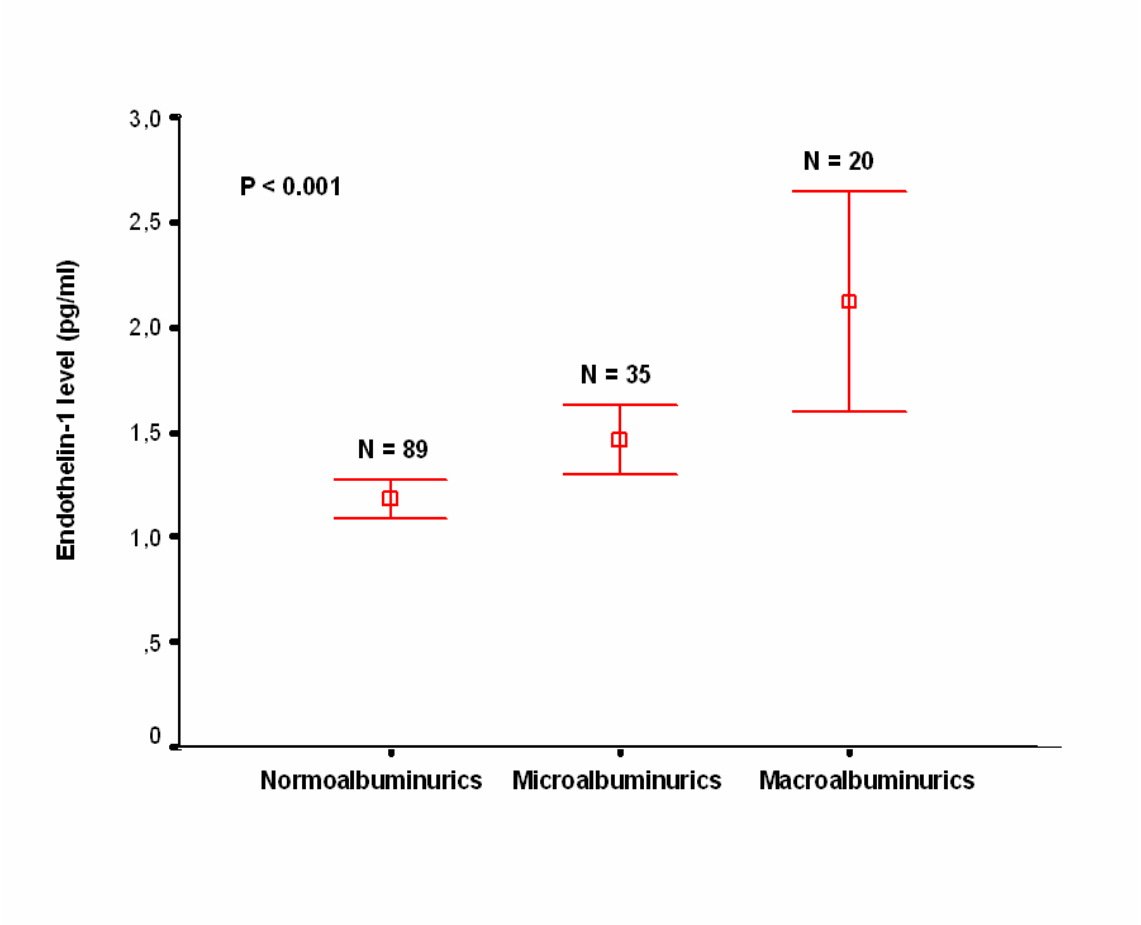
Tabela 1- Estágios da nefropatia diabética: valores da albumina urinária para diagnóstico e principais características clínicas

Estágios	Valores de albuminúria	Características clínicas
Microalbuminúria	20-199 μg/min	Aumento dos níveis da pressão arterial e ausência do descenso noturno
	30-299 mg/24h	Aumento dos triglicerídeos, colesterol total e LDL e ácidos graxos saturados
	30-299 mg/g*	Aumento dos componentes da síndrome metabólica
		Disfunção endotelial
		Associação com retinopatia diabética, amputação e doenças cardiovasculares
		Aumento da mortalidade cardivascular
		Taxa de filtração glomerular estável
Macroalbuminúria†	≥200 μg/min	Hipertensão
	≥300 mg/24h	Aumento dos triglicerídeos e colesterol total e HDL
	>300 mg/g*	Isquemia miocárdica assintomática
		Declínio progressivo da TFG

Adaptado de Diabetes Care. 2005 Jan;28(1):164-76.

*urina de amostra. †Medida da proteinúria total (≥500 mg/24h ou ≥430 mg/l em urina de amostra) também pode ser usado para definir esse estágio.

Figura 1: Níveis plasmáticos de endotelina-1 em pacientes com DM tipo 2 conforme os diferentes estágios da nefropatia diabética.

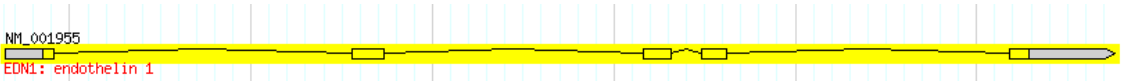


Adaptado de *Diabetes Res Clin Pract* 80:299-304, 2008

Dados apresentados como média e intervalo de confiança de 95%. Nível de ET-1 em normo: 1,18 ± 0,44; micro:1,47 ± 0,49; macro:2,12 ± 1,12 pg/ml. * P < 0.01 normo- vs. micro- vs. macroalbuminuricos.

Figura 2

A



B

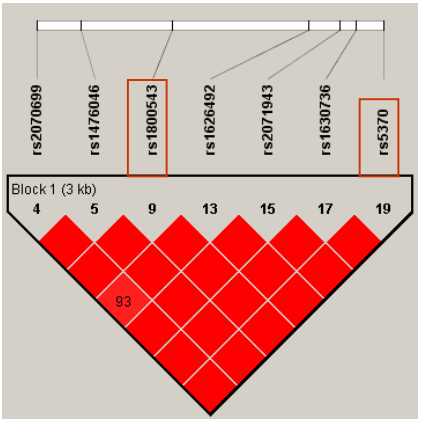


Figura 2: Gene EDN1 e esquema de desequilíbrio de ligação (DL) do *Haploview* mostrando o mapa geográfico do haplótipo EDN1 associado com DN: A) Representação esquemática do gene EDN1 no cromossomo 6 (www.hapmap.org). B) Esquema modificado do programa *Haploview* mostrando os blocos de haplótipos (D' > 0,9) estimados em 90 indivíduos caucasianos. SNPs em destaque são aqueles que foram estudados. As cores dos quadrados representam D' (valores de DL calculados por D' de Lewontin)/ LOD (*logarithm of the odds*). Quadrados sem números apresentam D' = 1 e LOD ≥ 2, significando um DL total.

Figura 3
A

B

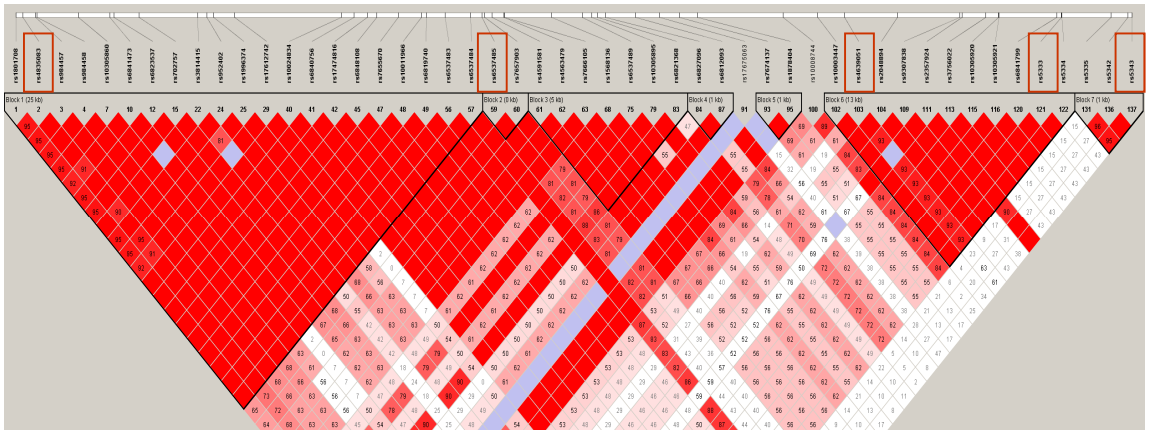


Figura 2: Gene EDNRA e esquema de desequilíbrio de ligação (DL) do *Haploview* mostrando o mapa geográfico do haplótipo EDNRA: A) Representação esquemática do gene EDNRA no cromossomo 4 (www.hapmap.org). B) Esquema modificado do programa *Haploview* mostrando os blocos de haplótipos (D' > 0,9) estimados em 90 indivíduos caucasianos. SNPs em destaque são aqueles que foram estudados. As cores dos quadrados representam D' (valores de DL calculados por D' de Lewontin)/ LOD (*logarithm of the odds*). Quadrados vermelhos sem números apresentam D' = 1 e LOD ≥ 2, significando um DL total. Quadrados azuis apresentam D' = 1 e LOD < 2. Quadrados brancos e rosas apresentam D' < 0,9 e LOD < ou ≥ 2.

Artigo 2

Endothelin-1 Gene Polymorphisms are Associated with Diabetic Nephropathy in Patients with Type 2 Diabetes Mellitus

Claudete M. Zanatta[1], Daisy Crispim[1], Denise A. Sortica[1], Jorge L. Gross[1], Luís H. Canani[1]

[1]Endocrine Division, Hospital de Clínicas de Porto Alegre, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil

Correspondence: Luís H. Canani

Serviço de Endocrinologia, Hospital de Clinicas de Porto Alegre, Rua Ramiro Barcelos 2350; prédio 12; 4º andar, Porto Alegre, RS, Brazil, 90035-003. Tel.: +55 51 21018127; Fax: +55 51 21018777;

E-mail: luiscanani@terra.com.br

Background and Aims: Diabetic nephropathy (DN) is the leading cause of end stage renal disease worldwide and is associated with increased cardiovascular mortality risk. The endothelin system has been implicated in the pathogenesis of arterial hypertension and renal disorders. Endothelin-1 (ET-1), the predominant isoform of the endothelin peptide family, regulates vasoconstriction and cell proliferation in tissues through activation of endothelin receptor type A (ETRA). In the present study, the association of DN with polymorphisms in ET-1 (*EDN1)* and ETRA (*EDNRA)* genes was evaluated in patients with type 2 diabetes (T2DM).

Materials and Methods: A case control nested to a cross-sectional study conducted in 548 white patients with T2DM. Patients with proteinuria [albumin excretion rate (AER)>200 μg/min or 174 mg/24h in 24-h timed urine or spot random sterile urine, respectively] or on dialysis were considered cases and patients with normoalbuminuria (AER <20 μg/min or <17 mg/l) and T2DM for more than 5 years were considered controls. Two polymorphisms in the *EDN1* gene (rs1800541 or T-1370G; rs57072783 or *Lys*198*Asn*) and five in *EDNRA* (rs6842241; rs4835083; rs4639051; rs5333 and rs5343) gene were genotyped. Haplotype analyses were performed in the program PHASE version 2.1. The alleles, genotypes and haplotype frequencies were compared between cases and controls. The Hardy-Weinberg equilibrium of each SNP was tested by Pearson χ^2 test.

Results: Assuming a dominant model, the presence of rs57072783 T allele (TT/TG *vs*. GG) protected against DN (OR = 0.69; 95% CI 0.48-0.99, P = 0.049), while the presence of rs1800541 G allele (GG/GT vs. TT) was associated with an OR of 0.60 (95% CI 0.41-0.88, P=0.009). However in multivariate analyses, only the GG/GT genotypes of rs1800541 polymorphism remained independently associated with DN (P = 0.046).

Conclusions: The present study shows that the polymorphisms in the ET-1 gene could be associated with an increase risk of DN in white patients with T2DM.

Key Words: Endothelin-1, diabetic nephropathy, diabetes mellitus type 2.

Introduction

Diabetic nephropathy (DN) is the leading cause of chronic kidney disease in patients starting renal replacement therapy (1) and it is associated with increased cardiovascular mortality (2). Among patients starting renal replacement therapy, the incidence of DN doubled from the years 1991–2001. Fortunately, the rate of increase has slowed down, probably because of the adoption in clinical practice of several measures that contribute to the early diagnosis and prevention of DN, which thereby decreases the progression of established renal disease (1). However, the implementation of these measures is far below the desirable goals (3).

Endothelin-1 (ET-1), the predominant isoform of the endothelin peptide family, regulates vasoconstriction and cell proliferation in tissues through activation of receptor type A (ETRA) and receptor type B (ETRB) (4). ET-1 is found in a variety of tissues and is thought to modulate vascular tone, cell proliferation and hormone production (5). Several studies documented that renal synthesis of ET-1 is increased either in animal or in human chronic nephropathies (6; 7). Endothelial dysfunction increases ET-1 production leading to vascular hypertrophy, atherogenesis and, in the kidney, glomerulosclerosis (8-10). An elevated plasma ET-1 level has been reported in patients with diabetes mellitus (11-13), and there is a progressive increase in plasma ET-1 levels according to the increased ranges of urinary albumin excretion (14).

The question remains, however, whether ET-1 is primarily involved in renal disease, or whether it is only a secondary modulator promoting progression after disease initiation. Genetic studies are very helpful in dissecting primary from secondary disease effects both in experimental models and in clinical studies. The aim of this study was to evaluate the association of genetic variation in the ET-1 gene (*EDN1)* and ETRA gene (*EDNRA)* in DN in patients with type 2 diabetes mellitus (T2DM).

Materials and Methods

Subjects

This case control nested to a cross-sectional study was carried out in 548 unrelated Caucasian-Brazilian T2DM patients who are participating in a multicentre study in the Brazilian state of Rio Grande do Sul that started recruiting patients in 2002 (15). That project originally aimed to study risk factors for chronic complications of T2DM. It includes four centers located at general hospitals in the state of Rio Grande do Sul, namely Grupo Hospitalar Nossa Senhora da Conceição, Hospital São Vicente de Paula, Hospital Universitário de Rio Grande, and Hospital de Clínicas de Porto Alegre. In this study, the ethnic groups were defined based on self-classification. All patients who described themselves as white were included. In the state of Rio Grande do Sul, white subjects are mainly from European ancestry (largely from Portugal, Spain, Italy and Germany). Patients of African ancestry or those who defined themselves as having mixed or other ancestry were not included. The ethics committees of the participating Institutions approved this study and all procedures were performed, including drawing blood samples, only after the informed consent was signed.

The patients underwent a standardized clinical and laboratory evaluation. Sitting blood pressure (BP) was measured twice after a 5-min rest using a mercury sphygmomanometer. The mean value of two measurements was used to calculate systolic antihypertensive drugs. Weight and height were used to calculate body mass index (kg/m^2). Renal status was based on the albumin excretion rate (AER) in at least two out of three collections. Patients were classified according to previous local standardization (16) as normo- (AER <20 μg/min or <17 mg/l, 24-h timed urine or spot random sterile urine, respectively), and macroalbuminurics (AER >200 μg/min or >174 mg/l or dialysis). Additionally, to be included in the control group (normoalbuminurics) the subjects had to have T2DM for five years or more. Since

many factors could affect plasma ET-1 levels, ET-1 was measured in a subgroup of subjects (n=111) after interruption of angiotensin converting enzyme (ACE) inhibitor therapy for at least 2 weeks. We excluded patients with the following conditions: renal impairment (serum creatinine ≥1.5 mg/dl); any cardiovascular event during 6 months preceding the enrollment (stroke, myocardial infarction, unstable angina, lower limb amputation, bypass surgery, or percutaneous revascularization); heart failure (New York Heart Association class II or greater); liver disease (history or elevated liver enzymes), any malignancy, or any acute inflammatory or infectious process. The mean value of plasma ET-1 was analyzed according to different genotypes of the *EDN1* and *EDNRA* polymorphisms.

Laboratory Methods

A serum sample was collected after a 12-h fast for laboratory analyses. Blood glucose was determined using the glucose oxidase method; serum creatinine by Jaffe's reaction; glycated haemoglobin (HbA1c) by ion-exchange HPLC (Merck-Hitachi L-9100 GhB Analyser, reference range: 4.7–6.0%); total plasma cholesterol and triglycerides by enzymatic methods; AER by immunoturbidimetry (Sera-Pak immuno microalbuminuria, Bayer, Tarrytown, NY, USA; mean intra- and interassay coefficients of variance of 4.5 and 7.6%, respectively). Plasma ET-1 was measured as previously described (14).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by a salting-out procedure as previously described (17). Evaluation of the polymorphism rs4639051 in the intron 3 of the *EDNRA* gene was done by digesting Polymerase Chain Reaction (PCR) products with the restriction enzyme *Hha*I (New England Biolabs, Inc., Ipswich, MA, USA). Digestion fragments were resolved on 2% agarose gels containing ethidium bromide and

viewed under ultra-violet illumination. Genotypes of the rs4639051 polymorphism were recorded using the ImageMaster System VDS (GE HealthCare, London, UK). The primers sequences used for this polymorphism are: forward 5'-GAC TAT CCC AGA CCA CAC CTT CA-3' and reverse 5'-GCT CAG GGC TGC CAA CTC C–3'.

Genotyping of the rs4835083 (intron 1), rs1568136 (intron 2), rs5333 (exon 6) and rs5343 (exon 8) polymorphisms in the *EDNRA* gene and rs1800541 (T-1370G; promoter region) and rs57072783 (*Lys*198*Asn*; exon 5) in the *EDN1* gene was performed using specific primers and probes contained in the Human Custom TaqMan Genotyping Assays 40x (Applied Biosystems, Foster City, CA; USA). One allelic probe was labeled with VIC dye and the other was labeled with FAM dye. The reactions were conducted in a 96-well plate, in a total 5 μl reaction volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Applied Biosystems), and Custom TaqMan Genotyping Assay 1x specific each polymorphism analyzed. The plates were then positioned in a real-time PCR thermal cycler (7500 Fast Real PCR System; Applied Biosystems) and heated for 10 minutes at 95ºC, followed by 40-50 cycles of 95ºC for 15 seconds and 63ºC for 1 minute. Fluorescence data files from each plate were analyzed using automated allele-calling software (SDS 2.1; Applied Biosystems).

The *EDNRA* polymorphisms were selected from the International HapMap Project (18). Due to linkage disequilibrium between some of these 58 polymorphisms, it was necessary to genotype at least five polymorphisms to estimate all haplotypes with more than a 5% frequency that would cover more than 90% of the possible haplotypes. The rs1800541 and rs57072783 polymorphisms in the *EDN1* were selected based on a previous study (19), which reported that these two polymorphisms are in almost complete linkage disequilibrium with other polymorphisms in this gene, thus covering more than 90% of the gene variability.

Statistical Analysis

Allele frequencies were determined by gene counting, and departures from the Hardy–Weinberg equilibrium (HWE) were verified using χ^2 test. Genotype and allele frequencies were compared among different groups by χ^2 test. Between all pairs of biallelic loci in the *EDNRA* or *EDN1* genes, we examined widely used measures of linkage disequilibrium (LD), Lewontin's D' |D'| and r^2 (20). The haplotypes constructed from the combination of the five *EDNRA* polymorphisms or the two *EDN1* polymorphisms and their frequencies were inferred using the Phase 2.1 program, which implements a Bayesian statistical method (21). We also used the Phase 2.1 program to compare the distributions of different *EDNRA* and *EDN1* haplotypes between case and control patients with or without ND through permutation analyses of 1,000 random replicates (21).

Clinical and laboratory characteristics were compared between groups using unpaired Student's test or χ^2 test, as appropriate. Continuous variables were expressed by mean and standard deviation. Variables with a skewed distribution (triglycerides, UAE, ET-1 and serum creatinine) were logarithmically transformed before analyses and are presented as median (interquartile range). The magnitude of the association of different genotypes and haplotypes with DN was estimated using odds ratio (OR) tests with 95% confidence interval (CI). Multivariate logistic regression analyses were performed to assess the independent association of genotypes and haplotypes with DN, as well as to control for possible confounding factors whenever a statistically significant association was found in the univariate analyses. In all cases, a two-tailed P value <0.05 was considered statistically significant. These statistical analyses were done with an SPSS package (SPSS for Windows, version 16.0).

Results

Sample description

The main clinical features of patients according to the renal status are presented in Table 1. Cases were more often males and had longer diabetes duration than controls. Cases also presented lower HDL cholesterol and higher triglycerides and blood pressure values compared to controls. By definition, serum creatinine was higher among cases compared to controls.

Genotypic and allelic distributions

The distributions of the *EDN1* and *EDNRA* genes polymorphisms in T2DM with and without DN are presented in Table 2. All genotypes were in Hardy Weinberg equilibrium (P > 0.05; data not shown). The lowest genotyping success rate among controls was obtained for the rs5333 polymorphism (95%) and among cases, for the rs5333 and rs4639051 polymorphisms (95%). The rs1800541 polymorphism in the *EDN1* gene is in a moderate LD with the rs57072783 polymorphism of the same gene (|D'| = 0.823 and r^2 = 0.788). LD coefficients (|D'| and r^2) between all biallelic combinations of the five *EDNRA* gene polymorphisms are presented in Table 3.

EDN1 polymorphisms

Cases had a lower frequency of the rs1800541 GG and GT variant genotypes compared to controls (P = 0.031). Assuming a dominant model of inheritance (GG/GT *vs.* TT), the presence of the G allele was associated with an OR of 0.60 (95% CI 0.41-0.88, P = 0.009). G allele frequency was 0.194 in controls compared to 0.134 in cases (P = 0.01). When only controls with more than five or more than ten years of T2DM were analyzed, similar results were obtained (data not shown). Variant genotypes of the rs57072783 polymorphism (TT and TG) were only statistically borderline associated with DN (P = 0.050). However, assuming a dominant model of inheritance, the presence of T allele (TT/TG *vs.* GG) showed

an OR of 0.69 (95% CI 0.48-0.99, P = 0.049). The T allele frequency was not different between cases and controls (P = 0.062). In multivariate analyses, the GG/GT genotypes of rs1800541 polymorphism remained independently associated with lower frequency of DN (P = 0.046) after controlling for gender, diabetes duration, systolic blood pressure, HbA1c and HDL cholesterol (OR 0.67 95% CI 0.42-0.89). On the other hand, the rs57072783 polymorphism (TT/TG genotypes) did not remain associated with DN after controlling for the same variables (P = 0.196).

EDNRA polymorphisms

The genotypic and allelic frequencies were similar among cases and controls for the rs4835083, rs1568136, rs5333 and rs5343 polymorphisms (all P values >0.10). Assuming recessive or dominant models of inheritance did not disclose an association of any EDNRA polymorphisms and DN. The genotypic distribution of the rs4639051 polymorphism was different between cases and controls (P = 0.04). There was a small decrease in heterozygotes AG and a small increase of the wild type genotype AA among cases. However, because the G allele is rare the genotypic distribution pattern of this polymorphism could not be characterized as additive, dominant or recessive. The allelic frequencies of the rs4639051 polymorphism were not different between cases and controls (P = 0.113). In multivariate analysis, no association of rs4639051 polymorphism was observed after controlling for gender, systolic blood pressure, diabetes duration, HbA1c and HDL cholesterol (P = 0.057).

Haplotype distributions

A Bayesian statistical method was used to estimate the frequencies of different haplotypes produced by the combination of the *EDN1* or *EDNRA* gene polymorphisms. All four expected haplotypes constructed by the combination of the two *EDN1* polymorphisms

were observed (Table 4). The five polymorphisms of the *EDNRA* gene result in 24 different haplotypes, but only those which had more than a 5% frequency are presented in Table 5. For both genes, permutation analyses showed that the haplotype distributions were not statistically different between case and control subjects.

ET-1 levels

ET-1 was measured in 111 patients who were able to stop medications that could interfere with ET-1 levels and also did not have any acute or chronic disease that could be associated with increased levels of ET-1. No difference was found regarding ET-1 levels among all polymorphisms evaluated.

Discussion

A previous study reported that patients with T2DM had elevated levels of ET-1 as compared to nondiabetic subjects (11). Furthermore, plasma ET-1 level was higher in macroalbuminuric than normoalbuminuric patients (14). ET-1 is the most powerful endogenous vasoconstrictor with profibrotic and proinflammatory effects (22). It has been found to affect three different aspects of renal physiology: vascular and mesangial tone; sodium and water excretion; and cell proliferation and matrix formation (13; 23). These studies reinforce that ET-1 is important in the pathogenesis of DN and it is a promising candidate gene for this complication.

In the present study, two variants in the *EDN1* gene were associated with DN protection in white patients with T2DM. The presence of *EDN1* rs1800541 G allele and rs57072783 T allele was associated with a decreased risk of having DN. The association was stronger for the rs1800541 polymorphism and more evident when assuming a dominant model of inheritance. Heterozygosis for rs4639051 polymorphism at *EDNRA* was also

associated with DN protection. However, none of the polymorphisms were associated with plasma ET-1 levels.

Kanková *et al*. (24), investigating potential associations between a set of 45 polymorphisms localized in 20 candidate genes and DN in T2DM patients from the Czech Republic, also reported a relationship between an *EDN1* polymorphism (8002 G/A) and this diabetic complication using a multi-locus analysis (P = 0.033). However, this association did not remained statistically significant after adjusting for diabetes duration, HbA1c, diastolic BP and the presence of other DN associated polymorphisms localized in three different genes, namely *AGER* -429T/C and 2184A/G, *LTA* 252A/G, and *NOS3* 774C/T and E298D. Another case-control study analyzing the association between 43 pathway-related candidate genes with DN in a customized microarray of 1536 polymorphisms found that the A allele of the *END1* rs1476046 G/A polymorphism was associated with an increased risk for ND with an estimated OR=1.26 (0.96-1.66) when in heterozygosis and of 1.87 (1.13-3.12) when in homozygosis (P = 0.0072)

A number of previous studies linked *EDN1* rs1800541 and rs57072783 polymorphisms with hypertension in individuals with overweight and obesity (25-27), and with HDL cholesterol metabolism (28). These are known predisposing factors for the development of DN. In the PREVEND Study cohort, Pinto-Sietsma *et al*. (19) studied the relationship between these two polymorphisms and impaired renal function in a nondiabetic population. The haplotype analysis revealed that individuals carrying both *EDN1* rs1800541 G allele and *EDN1* rs57072783 T allele showed diminished glomerular filtration and lower creatinine clearance than subjects carrying other haplotypes (19). No significant difference in ET-1 plasma levels between haplotype groups was observed. However, in this study only normo and microalbuminuric patients were analyzed, while patients with overt proteinuria were excluded (19).

Recently, the rs57072783 T allele was associated with delayed onset of T2DM and reduced risk of diabetic retinopathy in a Chinese sample (29). However, the rs57072783 T/T genotype was associate with elevated plasma ET-1 levels in pregnant women and the T allele has been associated with raised systolic BP (30). In our study, the observed protective association between the presence of the rs57072783 T allele (TT or TG, dominant inheritance model) and DN was statistically weak and did not remain independently associated with ND after adjusting for gender, diabetes duration, systolic BP, HbA1c and HDL cholesterol.

A lower ET-1 level would be expected with the protective genotypes of *EDN1* gene polymorphisms (rs1800541 and rs57072783). However, no difference was found in the present study. Since many situations could interfere with ET-1 levels, we were able to measure ET-1 in only 111 selected subjects. Probably those in whom the genetic effect would be more marked were excluded *a priori*. This would be the case for those with advanced renal disease. On the other hand, Tanaka *et al*. (31) investigated the expression of the rs57072783 polymorphism in ET-1 *in vitro*. The rs57072783 T allele cells were transfected and compared with G allele transfected cells with preproET-1 in three different cell lines. They measured the levels of ET-1 and big ET-1 in the culture supernatant and did not find a significant difference in the levels of ET-1 or big ET-1 between the T-type and G-type transfectant cells, suggesting that this polymorphism, in fact, does not have an important role in ET-1 levels. However, some effect on the processing of preproET-1 to mature ET-1 can not be ruled out. It is also possible that it is not the causal polymorphism, but only occurs in LD with a functional polymorphism in the *EDN1* gene. As far we are aware, no other study evaluated the ET-1 level and rs1800541 polymorphism.

We also used a Bayesian statistical method to estimate the frequencies of different haplotypes constructed with the combination of the *EDN1* or *EDNRA* polymorphisms. However, these haplotype analyses did not add any further information to the single

polymorphism analyses, i.e. the frequencies of all observed *EDN1* and *EDNRA* haplotypes were not statistically different between case and control subjects. The selection of the polymorphisms evaluated in our study aimed to cover the most common haplotypes, and it was based on a previous publication (19) and/or based on the HapMap Project data. Unfortunately, we can not exclude the possibility that less frequent *EDN1* or *EDNRA* gene haplotypes could have some effect on the DN development.

Among the limitations of the study we could emphasize the cross-sectional design. Therefore, we can only come to a conclusion about associations and not about causality. Still on the topic of the cross-sectional design, 116 patients were on dialysis. Among those we could expect changes in glycemic control, weight and HbA1c. This could explain not finding or decreasing the magnitude of differences for traditional risk factors between cases and controls. For that reason we decide a priori what variables to include in the multivariate analysis and not only those statistically significant in the univariate analyses. Some other factors could have interfered with the findings of the present study. For example, we can not rule out the possibility of a stratification bias in our sample, nevertheless we analyzed only self-defined white subjects, thus reducing the risk of false positive/negative associations due to this bias.

In conclusion, the present study, an independent protective association between *EDN1* gene polymorphisms and DN in white patients with T2DM was demonstrated. This seems to have a minor effect, but it is supported by an a priori hypothesis that the ET-1 system is involved in the development of DN. Larger confirmatory studies, in other populations would be needed to define the role of these polymorphisms in this diabetic complication.

References:

1. New chapter on CKD highlights changes to 15th annual USRDS report. *Nephrol News Issues* 17:25-28, 110, 2003

2. Valmadrid CT, Klein R, Moss SE, Klein BE: The risk of cardiovascular disease mortality associated with microalbuminuria and gross proteinuria in persons with older-onset diabetes mellitus. *Arch Intern Med* 160:1093-1100, 2000

3. Craig KJ, Donovan K, Munnery M, Owens DR, Williams JD, Phillips AO: Identification and management of diabetic nephropathy in the diabetes clinic. *Diabetes Care* 26:1806-1811, 2003

4. Barton M, Luscher TF: Endothelin antagonists for hypertension and renal disease. *Curr Opin Nephrol Hypertens* 8:549-556, 1999

5. Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, Eknoyan G: National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Ann Intern Med* 139:137-147, 2003

6. Ohta K, Hirata Y, Shichiri M, Kanno K, Emori T, Tomita K, Marumo F: Urinary excretion of endothelin-1 in normal subjects and patients with renal disease. *Kidney Int* 39:307-311, 1991

7. Benigni A, Perico N, Remuzzi G: Research on renal endothelin in proteinuric nephropathies dictates novel strategies to prevent progression. *Curr Opin Nephrol Hypertens* 10:1-6, 2001

8. Hocher B, Schwarz A, Reinbacher D, Jacobi J, Lun A, Priem F, Bauer C, Neumayer HH, Raschack M: Effects of endothelin receptor antagonists on the progression of diabetic nephropathy. *Nephron* 87:161-169, 2001

9. Sorokin A, Kohan DE: Physiology and pathology of endothelin-1 in renal mesangium. *Am J Physiol Renal Physiol* 285:F579-589, 2003

10. Gomez-Garre D, Ruiz-Ortega M, Ortego M, Largo R, Lopez-Armada MJ, Plaza JJ, Gonzalez E, Egido J: Effects and interactions of endothelin-1 and angiotensin II on matrix protein expression and synthesis and mesangial cell growth. *Hypertension* 27:885-892, 1996

11. Seligman BG, Biolo A, Polanczyk CA, Gross JL, Clausell N: Increased plasma levels of endothelin 1 and von Willebrand factor in patients with type 2 diabetes and dyslipidemia. *Diabetes Care* 23:1395-1400, 2000

12. Schneider JG, Tilly N, Hierl T, Sommer U, Hamann A, Dugi K, Leidig-Bruckner G, Kasperk C: Elevated plasma endothelin-1 levels in diabetes mellitus. *Am J Hypertens* 15:967-972, 2002

13. De Mattia G, Cassone-Faldetta M, Bellini C, Bravi MC, Laurenti O, Baldoncini R, Santucci A, Ferri C: Role of plasma and urinary endothelin-1 in early diabetic and hypertensive nephropathy. *Am J Hypertens* 11:983-988, 1998

14. Zanatta CM, Gerchman F, Burttet L, Nabinger G, Jacques-Silva MC, Canani LH, Gross JL: Endothelin-1 levels and albuminuria in patients with type 2 diabetes mellitus. *Diabetes Res Clin Pract* 80:299-304, 2008

15. Canani LH, Costa LA, Crispim D, Goncalves Dos Santos K, Roisenberg I, Lisboa HR, Sarturi Tres G, Maia AL, Gross JL: The presence of allele D of angiotensin-converting enzyme polymorphism is associated with diabetic nephropathy in patients with less than 10 years duration of Type 2 diabetes. *Diabet Med* 22:1167-1172, 2005

16. Zelmanovitz T, Gross JL, Oliveira JR, Paggi A, Tatsch M, Azevedo MJ: The receiver operating characteristics curve in the evaluation of a random urine specimen as a screening test for diabetic nephropathy. *Diabetes Care* 20:516-519, 1997

17. Miller L, Gray L, Beachey E, Kehoe M: Antigenic variation among group A streptococcal M proteins. Nucleotide sequence of the serotype 5 M protein gene and its relationship with genes encoding types 6 and 24 M proteins. *J Biol Chem* 263:5668-5673, 1988

18. The International HapMap Project. *Nature* 426:789-796, 2003

19. Pinto-Sietsma SJ, Herrmann SM, Schmidt-Petersen K, Niu T, Hillege HL, Janssen WM, de Zeeuw D, de Jong P, Kreutz R: Role of the endothelin-1 gene locus for renal impairment in the general nondiabetic population. *J Am Soc Nephrol* 14:2596-2602, 2003

20. Hedrick PW: Gametic disequilibrium measures: proceed with caution. *Genetics* 117:331-341, 1987

21. Stephens M, Smith NJ, Donnelly P: A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 68:978-989, 2001

22. Hopfner RL, Gopalakrishnan V: Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 42:1383-1394, 1999

23. Ding SS, Qiu C, Hess P, Xi JF, Zheng N, Clozel M: Chronic endothelin receptor blockade prevents both early hyperfiltration and late overt diabetic nephropathy in the rat. *J Cardiovasc Pharmacol* 42:48-54, 2003

24. Kankova K, Stejskalova A, Pacal L, Tschoplova S, Hertlova M, Krusova D, Izakovicova-Holla L, Beranek M, Vasku A, Barral S, Ott J: Genetic risk factors for diabetic nephropathy on chromosomes 6p and 7q identified by the set-association approach. *Diabetologia* 50:990-999, 2007

25. Asai T, Ohkubo T, Katsuya T, Higaki J, Fu Y, Fukuda M, Hozawa A, Matsubara M, Kitaoka H, Tsuji I, Araki T, Satoh H, Hisamichi S, Imai Y, Ogihara T: Endothelin-1 gene variant associates with blood pressure in obese Japanese subjects: the Ohasama Study. *Hypertension* 38:1321-1324, 2001

26. Jin JJ, Nakura J, Wu Z, Yamamoto M, Abe M, Tabara Y, Yamamoto Y, Igase M, Kohara K, Miki T: Association of endothelin-1 gene variant with hypertension. *Hypertension* 41:163-167, 2003

27. Treiber FA, Barbeau P, Harshfield G, Kang HS, Pollock DM, Pollock JS, Snieder H: Endothelin-1 gene Lys198Asn polymorphism and blood pressure reactivity. *Hypertension* 42:494-499, 2003

28. Pare G, Serre D, Brisson D, Anand SS, Montpetit A, Tremblay G, Engert JC, Hudson TJ, Gaudet D: Genetic analysis of 103 candidate genes for coronary artery disease and associated phenotypes in a founder population reveals a new association between endothelin-1 and high-density lipoprotein cholesterol. *Am J Hum Genet* 80:673-682, 2007

29. Li H, Louey JW, Choy KW, Liu DT, Chan WM, Chan YM, Fung NS, Fan BJ, Baum L, Chan JC, Lam DS, Pang CP: EDN1 Lys198Asn is associated with diabetic retinopathy in type 2 diabetes. *Mol Vis* 14:1698-1704, 2008

30. Barden AE, Herbison CE, Beilin LJ, Michael CA, Walters BN, Van Bockxmeer FM: Association between the endothelin-1 gene Lys198Asn polymorphism blood pressure and plasma endothelin-1 levels in normal and pre-eclamptic pregnancy. *J Hypertens* 19:1775-1782, 2001

31. Tanaka C, Kamide K, Takiuchi S, Kawano Y, Miyata T: Evaluation of the Lys198Asn and -134delA genetic polymorphisms of the endothelin-1 gene. *Hypertens Res* 27:367-371, 2004

Table 1. Clinical and laboratory characteristics of patients with type 2 diabetes mellitus according to renal status

	Control (n = 308)	Cases (n = 240)	P*
Sex male -n (%)	118 (38.3)	144 (60.0)	<0.001
Duration of diabetes (years)	13.4 ± 7.2	17.2 ± 9.6	<0.001
Age (years)	60.6 ± 9.7	61.2 ± 9.9	0.477
BMI (Kg/m²)	28.4 ± 4.7	28.4 ± 5.2	0.999
Systolic blood pressure (mmHg)	142 ± 23.5	149 ± 24.1	<0.001
Diastolic blood pressure (mmHg)	85 ± 13.5	86 ± 14.0	0.398
HbA1c (%)	6.88 ± 1.57	6.85 ± 2.06	0.847
Fasting plasma glucose (mg/dl)	161,8 ± 59.5	168,3 ± 75.3	0.260
Serum creatinine (mg/dl)	0.9 (0.5 – 1.4)	2.9 (0.6-13.9)	<0.001
Cholesterol total (mg/dl)	206 ± 44.2	203.1 ± 52.6	0.469
Cholesterol HDL (mg/dl)	46.7 ± 11.5	41.7 ± 11.7	<0.001
Triglycerides (mg/dl)	143 (40-659)	171 (45-1265)	<0.001

Data are expressed as mean ± SD, median (minimum-maximum) or %. *p values were computed by χ^2 or Student's t-test, as appropriate.

Table 2. Genotypic and allelic frequencies of *EDN1* and *EDNRA* polymorphisms in white type 2 diabetic patients according to renal status

	Genotype Frequencies			Allele Frequencies			
EDN1	**Controls**	**Cases**	**P***		**Controls**	**Cases**	**P***
rs1800541	**n = 304**	**n = 239**					
GG	14 (4.6)	7 (2.9)	0.031	T	0.806	0.866	0.011
GT	90 (29.6)	50 (20.9)		G	0.194	0.134	
TT	200 (65.8)	182 (76.2)					
rs57072783	**n = 289**	**n = 224**					
TT	15 (5.2)	8 (3.6)	0.050*	T	0.234	0.183	0.062
TG	105 (36.3)	66 (29.5)		G	0.766	0.817	
GG	169 (58.5)	150 (67.0)					
EDNRA							
rs4835083	**n = 300**	**n = 233**					
AA	115 (38.3)	100 (42.9)	0.513	A	0.622	0.644	0.498
AG	143 (47.7)	100 (43.9)		G	0.378	0.356	
GG	42 (14.0)	33 (14.2)					
rs1568136	**n = 308**	**n = 240**					
AA	30(10.1)	26 (10.8)	0.852	A	0.307	0.323	0.254
AT	129(42.1))	103 (42.9)		T	0.693	0.677	
TT	149(47.7)	111 (46.2)					
rs4639051	**n = 287**	**n = 230**					
AA	183 (63.4)	157 (68.3)	0.040	A	0.814	0.826	0.113
AG	103 (35.9)	66 (28.7)		G	0.186	0.174	
GG	2 (0.7)	7 (3.0)					
rs5333	**N = 284**	**n = 230**					
TT	137 (48.2)	125 (54.3)	0.373	T	0.695	0.728	0.282
TC	121 (42.6)	85 (37.0)		C	0.305	0.272	
CC	26 (9.2)	20 (8.7)					

rs5343	**n = 299**	**n = 235**					
CC	120 (40.1)	105 (43.8)	0.470	C	0.698	0.645	0.113
CT	38 (46.2)	97 (40.9)		T	0.302	0.355	
TT	41 (13.7)	36 (15.3)					

Genotype data: expressed as number (%) and data of allelic frequencies expressed as proportions. * P values were computed by χ^2 test.

Table 3. Linkage desequilibrium (|D'| and r^2) between all biallelic loci of the *EDNRA* gene.

		|D'|				
		rs4835083	**rs1568136**	**rs4639051**	**rs5333**	**rs5343**
	rs4835083	-	0.576	0.133	0.359	0.452
r^2	**rs1568136**	0.260	-	0.452	0.665	0.579
	rs4639051	0.007	0.099	-	0.802	0.635
	rs5333	0.090	0.394	0.349	-	0.657
	rs5343	0.182	0.087	0.051	0.100	-

Table 4. ***EDN1*** **and** ***EDNRA*** **haplotype frequencies constructed by PHASE 2.1 in patients with type 2 diabetes mellitus according to renal status**

Haplotype	Frequency Controls	Frequency Cases	P*
EDN1	**n = 311**	**n = 188**	
TG	**0.752**	**0.749**	
TT	**0.059**	**0.058**	
GG	**0.053**	**0.044**	
GT	**0.136**	**0.149**	**0.424**
EDNRA	**n = 242**	**n = 276**	
GAACC	**0.089**	**0.072**	
GAGCC	**0.057**	**0.050**	
GTATT	**0.100**	**0.090**	
ATATC	**0.273**	**0.286**	
ATATT	**0.178**	**0.180**	**0.278**

n = number of chromosomes. The first letter of the *END1* haplotypes refers to the rs1800541 polymorphism and the second to the rs57072783 polymorphism. The first letter of the *EDNRA* haplotypes refers to the rs4835083 polymorphism, the second to the rs1568136, the third to the rs4639051, the fourth to the 5333 and the last to the rs5343 polymorphism * P values for the comparisons of haplotypic frequencies between patients with or without diabetic nephropathy were calculated using permutations tests (1000 replications).

Artigo 3

Endothelin-1 and Endothelin A Receptor Expression are Increased in Patients with Diabetic Nephropathy

Authors: Zanatta CM[1], Veronese FV[2], Loreto MS[2], Sortica DA[1], Carpio VN[2], Eldeweiss MI[3], Silva VD[4], Lopes TG[4], Gross JL[1]; Canani LH[1].

[1]Endocrinology Division, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

[2]Nephrology Division, Hospital de Clinicas de Porto Alegre, Porto Alegre, Brazil

[3]Pathology Division, Hospital de Clinicas de Porto Alegre, Porto Alegre, Brazil

[4]Pathology Division, Hospital São Lucas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil

Short title: **Endothelin expression in diabetic nephropathy**

Key words: Endothelin-1, Endothelin A receptor, diabetic nephropathy, IgA nephropathy

Abstract

Background. Endothelin-1 (ET-1) is associated with progression of renal disease, acting *a* potent vasoconstrictor and as a growth factor of mesangial cells. In normal human kidney, ET-1 and endothelin A receptor (ETRA) are more markedly expressed in vessels, and at a lower intensity in glomerular structures. Animal models of diabetes mellitus (DM) showed that glomerular expression of ET-1 was five-fold greater, suggesting a potential association between endothelin and diabetic nephropathy (DN). The aim of this study was to determine the level of ET-1 and ETRA expression in patients with DN as compared to IgA Nephropathy (positive comparison group) and to normal kidney tissue.

Methods. Cross-sectional study comprising thirteen patients with type 2 DM and DN, ten patients with proteinuric IgA nephropathy and thirteen samples of normal kidney from tumor nephrectomies. The distribution and intensity of ET-1 and ETRA expression in renal biopsies and normal kidney tissue were determined by immunohistochemistry.

Results. Patients with DN and IgA nephropathy on biopsy had markedly increased staining for ET-1 in endothelial cells of glomerular capillaries and peritubular capillaries as compared to controls ($p < 0.001$). ETRA staining was also more intense and more diffuse in DN and IgA nephropathy than in control ($p = 0.019$). Patients with higher proteinuria levels had a greater expression of ET-1 but not of ETRA.

Conclusion. A higher expression of ET-1 and ETRA was found both in DN and IgA nephropathy, suggesting a potential role for the endothelin system in diabetic nephropathy and probably in other non diabetic glomerular diseases.

Introduction

Endothelin-1 (ET-1), a peptide of 21 amino acid residues, is the most potent vasoconstrictor known (1). There is much evidence showing that ET-1 is related to the progression of renal disease, acting as a potent growth factor of mesangial cells (2-4). The kidney is an important ET-1 production site and it is also a target for ET-1 effects. Mesangial cells are the main cells that produce and suffer the actions of ET-1 in the kidney (5), however, endothelial cells of glomerular capillaries and tubular epithelial cells also produce ET-1 (6). Endothelin A receptor (ETRA) is present in arcuate arteries and vasa recta, and endothelin B receptor (ETRB) is predominantly found in the collecting ducts, suggesting different roles in the reabsorption of salt and water. Both receptors are found in the glomerulus (2).

Animal studies showed that ET-1 is not only more markedly expressed in kidney of diabetic rats (7), but can also mediate the effect of hyperglycemia on cellular mesangial hypertrophy and synthesis of extracellular matrix proteins (8). ET-1 growth-promoting and arteriolar vasoconstricting effects in glomeruli and renal cortex, reducing renal blood flow and glomerular filtration rate (GFR), are mediated via ETRA (9; 10).

In normal human kidney, ET-1 and ETRA are expressed in greater quantity in the vascular tissue and at a lower intensity in glomerular structures (10-13). Studies in animal models of diabetes mellitus (DM) showed a five-fold increase in the expression of ET-1 in the glomerulus, suggesting a role in diabetic nephropathy (DN) (8). Additionally, experimental blockade of ET-1 receptor with antagonists corrects the initial hemodynamic changes and decreases proteinuria in rats with DN (13; 14). Angiotensin II is a potent stimulus for ET-1 secretion and its effects on mesangial matrix synthesis are attenuated by antagonists of ET-1 (15).

Studies in patients with IgA nephropathy showed significantly increased expression of ET-1 and ETRA in the kidney. In those patients, proteinuria correlated positively with ET-1

expression, and when receiving angiotensin-converting enzyme inhibitors (ACEi) a significant reduction in ET-1 expression is observed (15). In kidney transplant recipients, intragraft ET-1 expression is positively correlated with post-transplant hypertension and proteinuria, and higher levels of proteinuria are also associated with more intense expression of ET-1 and ETRA (16). Similar changes are seen in patients with lupus nephritis (17). So far, no study has evaluated ET-1 and its receptor expression in patients with DM and DN.

Patients with type 2 DM have elevated plasma levels of ET-1, independent of the presence of microvascular disease. Moreover, patients with DM and microalbuminuria have higher plasma ET-1 levels than those with DM and normoalbuminuria or hypertension and microalbuminuria without DM (18). In a previous study in type 2 DM, we reported a progressive increase in plasma ET-1 levels from normo-, micro and macroalbuminuria (19).

The aims of this study were to determine the renal expression of ET-1 and ETRA in patients with DN and to correlate this expression with renal function and proteinuria.

Patients and Methods

Subjects

Thirteen type 2 DM patients with diabetic nodular glomerulosclerosis on kidney biopsy were selected from the Nephrology Division database at Hospital de Clínicas de Porto Alegre (HCPA). Ten patients with mesangial proliferative IgA glomerulonephritis were included as a positive control group, because a high expression of endothelin was previously demonstrated in this nephropathy (15). Renal biopsies in patients with DN and IgA nephropathy were performed according to clinical indication. IgA nephropathy was defined by the presence of mesangial cell proliferation and/or matrix expansion (or focal segmental glomerular sclerosis in advanced stages) with predominant mesangial granular deposits of IgA (2+ or more) on immunofluorescence. Diabetic nephropathy was defined by diffuse capillary

basement membrane thickening with peripheral hyaline PAS positive nodules (Kimmelstiel-Wilson), with segmental or global glomerulosclerosis at advanced stages, and thickened arterioles with hyaline deposits. Histologically normal renal tissue from 13 patients who had unilateral nephrectomy for renal tumor (3 were diabetic and 10 were not diabetic) was included as control. Paraffin blocks with enough tissue for immunohistological analysis were located in the Pathology Division archive at HCPA.

The study protocol was approved by the HCPA Research Ethics Committee and registered in the Institutional Review Board (IRB) under number 00000921.

Methods

Demographic and clinical variables were collected retrospectively in patient medical records. Age, gender, ethnicity, serum creatinine, proteinuria (total protein /creatinine ratio in a morning urine sample). Proteinuria was available only for subjects with DN and IgA nephropathy. No proteinuria data were available in controls. GFR was estimated by the Modification of Diet in Renal Disease (MDRD) equation (20).

The distribution and intensity of ET-1 and ETRA expression were determined by immunohistochemistry in formalin-fixed, paraffin-embedded sections. To detect protein expression of ET-1 in kidney biopsies, a mouse monoclonal antibody (ab2786, Abcam Limited, Cambridgeshire, UK) with aorta sections as the positive control for the technique was used. This antibody shows little cross-reactivity to ET-2 and ET-3. For detection of ETRA protein expression, a rabbit polyclonal antibody (ab12977, Abcam Limited, Cambridgeshire, UK) was used. The positive control for ETRA was human large bowel. Immunohistochemistry techniques are described in detail below.

Immunohistochemistry for ET-1

Paraffin sections were cut to a 4 μm thickness and placed on pre-treated slides with Histogrip (Zymed, US), heat-fixed at 60°C for 24 hours. Deparaffinization and dehydration in alcohols were followed by washing in distilled water. Antigen retrieval was done with citrate pH 9.0 solution (Target Retrieval Solution, Dako, US) in a pressure cooker designed for use in a microwave oven (NordicWare Microwave Tender Cooker, Biogenex, US). Endogenous peroxidase was blocked with hydrogen peroxide 3% in methanol twice, for 15 min each, and washed in PBS pH 7.2. Nonspecific blocking was done with Protein Block Serum-Free solution (Dako, US) for 30 min at room temperature. Slides were then incubated overnight with primary monoclonal anti-mouse antibody to ET-1 (ab2786, Abcam Limited, Cambridgeshire, UK) at 1:1000 dilution. The reaction was amplified with the peroxidase polymer Picture Max (HRP Polymer Conjugate Broad Spectrum, Invitrogen, US) according to the manufacturer's instructions. To develop the reaction, diaminobenzidine was used for 5 min (Dako Liquid DAB Substrate Chromogen System, US). The slides were counterstaining with Harris hematoxylin for 1 min, followed by dipping in ammonia water 37 mM for 15 seconds and dehydration in alcohols. Slides were mounted and coverslipped with non permanent Entellan mounting medium (Merck, Germany).

Immunohistochemistry for ETRA

Paraffin sections were cut to a 4 μm thickness and placed on pre-treated slides with Histogrip (Zymed, US), heat-fixed at 60°C for 24 hours. Kidney tissue was deparaffinized and dehydrated in alcohols followed by washing in distilled water. Endogenous peroxidase was blocked with hydrogen peroxide 4.5% in methanol. Washing was done with TBS/Tween20 buffer between steps. Antigen retrieval was done with citrate buffer (pH 6.0) in an automated pressure cooker (Decloaking Chamber, Biocare Medical, US). Nonspecific blocking was done with normal goat serum at 1:50 and avidin D at 1:10 dilutions. Slides were then incubated

overnight with primary polyclonal anti-rabbit antibody to ETRA (ab12977, Abcam Limited, Cambridgeshire, UK) at 1:200 dilution in TBS / 1% BSA for 45 minutes, followed by incubation with secondary biotinylated anti-rabbit antibody (Biocare Medical, US) for 30 minutes. The next step was to apply the enzyme alkaline phosphatase (AK 5000, Vector Laboratories, US) for 30 minutes (1 ml of TBS / BSA 1% + l0 μl solution A + 10 μl solution B). The final step was to wash with TBS alone, and to develop the reaction with blue chromogen substrate of alkaline phosphatase (Vector Blue, Vector Laboratories, US) The slides were counterstained with Harris hematoxylin for 1 min, followed by dipping in ammonia water 37 mM for 15 seconds and dehydration in alcohols. Slides were mounted and coverslipped with non permanent Entellan mounting medium (Merck, Germany).

ET-1 and ETRA expression

Quantification of the expression of ET-1 and ETRA was performed by digital image analysis using Image Pro Plus software, version 4.5 (Media Cybernetics). Images were visualized through a Zeiss microscope (model AXIOSKOP-40, Carl Zeiss, Oberkochen, Germany), and captured using the Cool Snap-Pro CS (Media Cybernetics) camera. After selecting the hot spot areas with higher expression of ET-1 and ETRA using low power objectives, non-consecutive random fields were chosen using the greek line method to avoid field overlap (21). Thirty-five to forty fields were captured in nephrectomy tissue (mean, 37 fields) and for the entire fragment of percutaneous biopsies, 21 fields in average.

ET-1 expression

ET-1 expression was detected exclusively in endothelial cells of peritubular capillaries and glomerular capillaries, with the strongest intensity of staining in dark brown by DAB chromogen as compared to background color. Positive control was considered as the brown

staining in red blood cells, and negative control as the background. Positive events were considered when spherical or elliptical shaped stained structures, with or without the presence of red blood cells, stained by DAB at the same intensity of red blood cells. Contiguous objects were counted as a single event.

Positive event counting was performed with 200X magnification and a resolution of 1392 x 1040 pixels per 0.76 mm^2. Image analysis was performed by unbiased counting, inserting a correction grid with 1312x960 pixel amplitude, corresponding to the area of 0.66 mm^2. Positive staining was counted within the area of the grid in the captured field, and all events that touched the continuous line of the grid were also counted, those that touched the dotted line were excluded. The area (in pixels) from the field without tissue or with non-renal tissue was subtracted from the total area of the field, thereby correcting for positive events per area of renal tissue in that field. Counting was performed on an ordinal scale, and the arithmetic mean of positive events was calculated for the 35-40 selected fields in tissue nephrectomies, or for all fields in fragments of percutaneous biopsies, at each hot spot (21).

ETRA expression

Endothelin A receptor was expressed only in the cytoplasm of tubular epithelial cells, with the strongest intensity of staining in dark blue compared to background color. If one or more epithelial cells in a tubular section stained positive, that tubule was considered one event. Positive event counting and calculation of the mean to obtain a final score for ETRA (on an ordinal scale) were performed the same way as for ET-1.

Statistical analysis

Data were presented as mean and standard deviation (SD) (or median and interquartile ranges) or proportions. Comparisons between different groups were done by ANOVA.

Nominal data were analyzed using the Chi-square and Fischer exact test. For statistical analysis, ET-1 and ETRA expressions were logarithmically transformed to reduce skewness. Correlations between immunohistochemical scores of ET-1 and ETRA with renal function and proteinuria were performed using Spearman's correlation coefficient. A p value <0.05 was considered significant. Data were processed and analyzed using Statistical Package for Social Sciences for Windows, version 16.0 (SPSS Inc. Chicago, IL).

Results

Clinical and laboratory data

Clinical and laboratory data of the patients are presented in Table 1. The prevalence of arterial hypertension and the use of ACEi were similar in all three groups. Patients with IgA nephropathy were younger compared to controls and DN.

Control patients had lower serum creatinine levels and higher GFR as compared to DN and IgA nephropathy groups. Proteinuria was of a greater magnitude in patients with DN but it was also high in IgA nephropathy, as these patients had an indication for biopsy.

Immunohistochemical staining for immunoreactive ET-1

Kidney tissue from control subjects had no or only a weak expression of ET-1 in endothelial cells of glomerular and peritubular capillaries. Among patients with IgA nephropathy and DN, staining was markedly increased in endothelial cells of glomerular capillaries and was slightly increased in peritubular capillaries (Figure 1). There was a stronger staining and higher scores in DN and IgA nephropathy biopsies when compared to controls ($p <0.001$) as illustrated in Figure 2. ET-1 expression was also detected in renal vessels, mainly in endothelial cells and vascular smooth muscle cells of arteries and arterioles, and it was more prominent in biopsies of DN patients.

Immunohistochemical staining for immunoreactive ETRA

ETRA was only expressed in the cytoplasm of tubular epithelial cells, mainly in distal tubules. No staining was detected in glomeruli, peritubular capillaries and vessels. Control sections demonstrated only weak and focal expression, whereas in most of the DN and IgA nephropathy cases the staining was much more intense and diffuse (Figure 1). There were stronger staining and higher scores in DN and IgA nephropathy biopsies when compared to controls ($p = 0.019$) as illustrated in Figure 3.

ET-1 and ETRA correlation with renal function and proteinuria

Proteinuria was available for 18 subjects (11 ND and 7 IgA nephropathy). There was a positive correlation between proteinuria and ET-1 expression ($r = 0.634$, $p = 0.027$). No correlation was observed between ETRA expression and proteinuria ($r = 0.095$, $p = 0.77$). Additionally, no correlation was found between the magnitude of ET-1 or ETRA expression and GFR or serum creatinine.

Discussion

This comprehensive immunohistological analysis of the two main components of the endothelin system showed an up-regulation of ET-1 as well as ETRA expression in DN compared to normal renal tissue. The magnitude of this expression was similar to that observed in IgA nephropathy, and in both conditions ET-1 expression correlated positively with proteinuria.

Elevated plasma ET-1 levels have been reported in patients with DM (18; 22; 23). In a previous study we showed that there is a progressive increase in plasma ET-1 levels which correlated positively with increased urinary albumin excretion (19). The gene expression of ET-1 is increased in kidneys of diabetic rats, as well as in mesangial cell culture of rats

exposed to high glucose concentrations (7). Endothelial dysfunction in diabetic patients, increases ET-1 production leading to vascular hypertrophy, atherogenesis and, in the kidney, glomerulosclerosis. Endothelin receptor blockade has a nephroprotective effect, correcting both the initial hyperfiltration and its progression to clinical DN (13; 14).

In normal human kidneys, immunoreactive ET-1 was localized segmentally in endothelium of glomerular capillary loops, vessels, and peritubular capillaries, suggesting that its secretion is compartimentalized and locally regulated (24). In disease, several studies reported that renal expression of ET-1 is increased in both animal models and human chronic nephropathies (24; 25). In human subjects, increased ET-1 expression was observed in several conditions, *e.g.*, lupus nephritis, IgA nephropathy and membranous glomerulonephritis (15), acute graft rejection, chronic allograft nephropathy, and post-operative acute tubular necrosis (16; 26). This study is the first to demonstrate consistently the increased ET-1 and ETRA expression in human DN.

Endothelin-1 can be over expressed in kidney tissue in several situations. However, the pattern of ET-1 expressions seems to vary. Lehrke et al (15) showed a markedly increased ET-1 expression in proximal tubular epithelial cells and less marked but still increased expression in glomerular endothelium of subjects with IgA nephropathy. Expression of ETRA was not significantly increased in biopsy samples, but immunoreactive ETRB was greatly increased in proximal tubules but less intense in glomeruli. In lupus nephritis, mesangial staining for ET-1 was elevated suggesting that under certain conditions (i.e., inflammation) ET-1 is also present in glomerular mesangium (24). In dysfunctional kidney allografts, expression of ET-1 and ETRA was markedly increased in glomeruli and tubuli irrespective of the underlying histological diagnosis, and there was a strong correlation between proteinuria and ET-1 expressed in glomeruli and tubuli (16). In the present study, ET-1 expression was segmental and restricted to endothelial cells of glomerular and peritubular capillaries,

suggesting a local production and regulation. Unexpectedly, ETRA staining was restricted to tubular epithelial cells, mainly in distal segments, a finding to be further explored.

Previous studies in patients with IgA nephropathy demonstrated an association between proteinuria levels and intensity of ET-1 expression in glomeruli and in proximal tubular cells. Patients with high-grade proteinuria (≥2 g/24h) have a significantly higher ET-1 expression than patients with low-grade proteinuria (<2 g/24h) (15). Furthermore, a strong correlation was described between proteinuria and ET-1 expression in glomeruli and proximal tubules of recipients of kidney transplants (16). In accordance with these previous reports, in the present study there was a significant correlation between proteinuria and ET-1 expression in glomeruli and peritubular capillaries in patients with DN and IgA nephropathy ($r = 0.634$, $p = 0.027$).

Douglas et al (27) analyzed ET-1 expression in two different renal cell carcinomas (clear cells vs. papillary) showing that the endothelin axis was expressed differently in these two main subtypes. We considered normal renal tissue derived from nephrectomies due to tumors as controls and we included cases of both clear cells and papillary carcinoma subtypes. ET-1 and ETRA expression did not differ between the two subtypes (data not shown).

Therefore, due to over expression of ET-1 in many kidney diseases, increased ET-1 expression could be a non specific phenomenon secondary to kidney injury. However, studies with experimental models of DM where ETRA and ETRB were blocked by specific antagonists demonstrated a normalization of the glomerular pressure, a decrease of protein deposition in mesangial extracellular matrix and a decrease in urinary albumin excretion suggesting that the endothelin system does in fact play a role in the pathogenesis of DN (28; 29).

In conclusion, our results suggest a potential role for the endothelin system in DN and probably in other non diabetic glomerular diseases such as IgA nephropathy. These findings

are of interest and must be further explored, in view of the experimental observation that ET system blockade with specific antagonists could prevent the progression of DN.

References:

1. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332:411-415, 1988

2. Simonson MS: Endothelins: multifunctional renal peptides. *Physiol Rev* 73:375-411, 1993

3. Hopfner RL, Gopalakrishnan V: Endothelin: emerging role in diabetic vascular complications. *Diabetologia* 42:1383-1394, 1999

4. Levin ER: Endothelins. *N Engl J Med* 333:356-363, 1995

5. Sorokin A, Kohan DE: Physiology and pathology of endothelin-1 in renal mesangium. *Am J Physiol Renal Physiol* 285:F579-589, 2003

6. Herizi A, Jover B, Bouriquet N, Mimran A: Prevention of the cardiovascular and renal effects of angiotensin II by endothelin blockade. *Hypertension* 31:10-14, 1998

7. Hargrove GM, Dufresne J, Whiteside C, Muruve DA, Wong NC: Diabetes mellitus increases endothelin-1 gene transcription in rat kidney. *Kidney Int* 58:1534-1545, 2000

8. Fukui M, Nakamura T, Ebihara I, Osada S, Tomino Y, Masaki T, Goto K, Furuichi Y, Koide H: Gene expression for endothelins and their receptors in glomeruli of diabetic rats. *J Lab Clin Med* 122:149-156, 1993

9. Karet FE: Endothelin peptides and receptors in human kidney. *Clin Sci (Lond)* 91:267-273, 1996

10. Pupilli C, Brunori M, Misciglia N, Selli C, Ianni L, Yanagisawa M, Mannelli M, Serio M: Presence and distribution of endothelin-1 gene expression in human kidney. *Am J Physiol* 267:F679-687, 1994

11. Terada Y, Tomita K, Nonoguchi H, Marumo F: Different localization of two types of endothelin receptor mRNA in microdissected rat nephron segments using reverse transcription and polymerase chain reaction assay. *J Clin Invest* 90:107-112, 1992

12. Karet FE, Davenport AP: Localization of endothelin peptides in human kidney. *Kidney Int* 49:382-387, 1996

13. Ding SS, Qiu C, Hess P, Xi JF, Zheng N, Clozel M: Chronic endothelin receptor blockade prevents both early hyperfiltration and late overt diabetic nephropathy in the rat. *J Cardiovasc Pharmacol* 42:48-54, 2003

14. Cosenzi A, Bernobich E, Trevisan R, Milutinovic N, Borri A, Bellini G: Nephroprotective effect of bosentan in diabetic rats. *J Cardiovasc Pharmacol* 42:752-756, 2003

15. Lehrke I, Waldherr R, Ritz E, Wagner J: Renal endothelin-1 and endothelin receptor type B expression in glomerular diseases with proteinuria. *J Am Soc Nephrol* 12:2321-2329, 2001

16. Frank K, Zeier M, Gross ML, Waldherr R, Ritz E, Amann K: Comprehensive immunohistological analysis of the endothelin system in human kidney grafts. *Nephrol Dial Transplant* 21:1365-1372, 2006

17. Herman WH, Emancipator SN, Rhoten RL, Simonson MS: Vascular and glomerular expression of endothelin-1 in normal human kidney. *Am J Physiol* 275:F8-17, 1998

18. De Mattia G, Cassone-Faldetta M, Bellini C, Bravi MC, Laurenti O, Baldoncini R, Santucci A, Ferri C: Role of plasma and urinary endothelin-1 in early diabetic and hypertensive nephropathy. *Am J Hypertens* 11:983-988, 1998

19. Zanatta CM, Gerchman F, Burttet L, Nabinger G, Jacques-Silva MC, Canani LH, Gross JL: Endothelin-1 levels and albuminuria in patients with type 2 diabetes mellitus. *Diabetes Res Clin Pract* 80:299-304, 2008

20. Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, Eknoyan G: National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Ann Intern Med* 139:137-147, 2003

21. Gundersen HJ, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A, et al.: Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *Apmis* 96:379-394, 1988

22. Seligman BG, Biolo A, Polanczyk CA, Gross JL, Clausell N: Increased plasma levels of endothelin 1 and von Willebrand factor in patients with type 2 diabetes and dyslipidemia. *Diabetes Care* 23:1395-1400, 2000

23. Schneider JG, Tilly N, Hierl T, Sommer U, Hamann A, Dugi K, Leidig-Bruckner G, Kasperk C: Elevated plasma endothelin-1 levels in diabetes mellitus. *Am J Hypertens* 15:967-972, 2002

24. Ohta K, Hirata Y, Shichiri M, Kanno K, Emori T, Tomita K, Marumo F: Urinary excretion of endothelin-1 in normal subjects and patients with renal disease. *Kidney Int* 39:307-311, 1991

25. Benigni A, Perico N, Remuzzi G: Research on renal endothelin in proteinuric nephropathies dictates novel strategies to prevent progression. *Curr Opin Nephrol Hypertens* 10:1-6, 2001

26. Chareandee C, Herman WH, Hricik DE, Simonson MS: Elevated endothelin-1 in tubular epithelium is associated with renal allograft rejection. *Am J Kidney Dis* 36:541-549, 2000

27. Douglas ML, Richardson MM, Nicol DL: Endothelin axis expression is markedly different in the two main subtypes of renal cell carcinoma. *Cancer* 100:2118-2124, 2004

28. Hocher B, Schwarz A, Reinbacher D, Jacobi J, Lun A, Priem F, Bauer C, Neumayer HH, Raschack M: Effects of endothelin receptor antagonists on the progression of diabetic nephropathy. *Nephron* 87:161-169, 2001

29. Gomez-Garre D, Ruiz-Ortega M, Ortego M, Largo R, Lopez-Armada MJ, Plaza JJ, Gonzalez E, Egido J: Effects and interactions of endothelin-1 and angiotensin II on matrix protein expression and synthesis and mesangial cell growth. *Hypertension* 27:885-892, 1996

Table 1. Clinical and laboratory characteristics of patients with normal kidney tissue, diabetic nephropathy or IgA nephropathy

	Normal Kidney n = 13	Diabetic Nephropathy n = 13	IgA Nephropathy n = 10	P
Age (years)	62 ± 20	52 ± 15	39 ± 14	0.013*
Sex male, n (%)	8 (61.5)	8 (61.5)	7 (70.0)	1.0
White patients, n (%)	11 (84.6)	10 (76.9)	10 (100)	0.184
Serum creatinine (mg/dL)	1.0 ± 0.2	2.8 ± 2.5	2.8 ± 2.7	0.041[£]
Proteinuria (g/24h)	NA	294 (107-570)	193 (33-219)	0.056
GFR (mL/min/1.73 m^2)	78.5± 25	52.1 ± 42	50.1 ± 36	0.115
Hypertension, n (%)	7 (53.8)	11 (84.6)	3 (37.5)	0.112
ACE inhibitor use, n (%)	7 (53.8)	9 (75)	6 (75)	0.265
ET-1 score[‡]	1.52 (1.02 - 2.12)	8.86 (5.41 - 15.12)	3.16 (2.41 – 3.88)	<0.001[£]
ETRA score[‡]	2.50 (1.36 - 2.37)	4.90 (2.80 - 7.70)	4.60 (3.10 - 5.40)	0.019[£]

Data are expressed as mean ± SD or median (interquartile ranges 25 -75%); *IgA vs. normal kidney; [£]DN and IgA nephropathy vs. controls vs.; NA: not available; [‡]Data were logarithmically transformed before analysis.

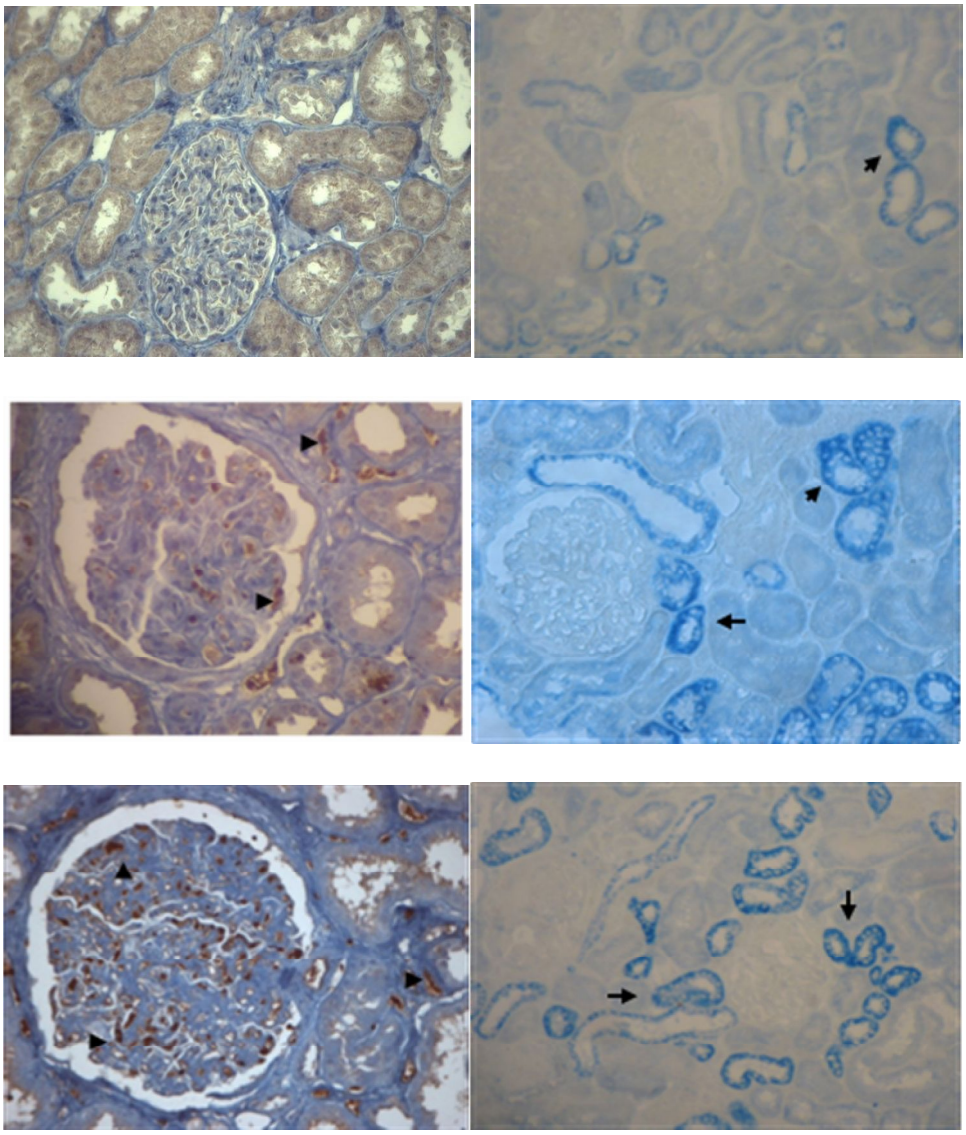


Figure 1. Representative microphotographs of the immunostaining of ET-1 in normal kidney tissue (A1), IgA nephropathy (A2) and diabetic nephropathy (A3); magnification: X200. Cells stained dark brown indicate the expression of ET-1. Representative microphotographs of the immunostaining of ETRA expression in normal tissue (B1), IgA nephropathy (B2) and diabetic nephropathy (B3); magnification: X100. Cells stained dark blue indicate the expression of ETRA. Arrowhead: increased ET-1 expression in endothelial cells of glomerular and peritubular capillaries (A2, A3) which was more intense in diabetic nephropathy (A3); no or only weak expression of ET-1 in normal renal tissue (A1). Arrow: diffuse and strong expression of ETRA in the cytoplasm of tubular epithelial cells in IgA nephropathy (B2) and diabetic nephropathy (B3); a focal and weak expression of ETRA in tubuli of normal tissue (B1).

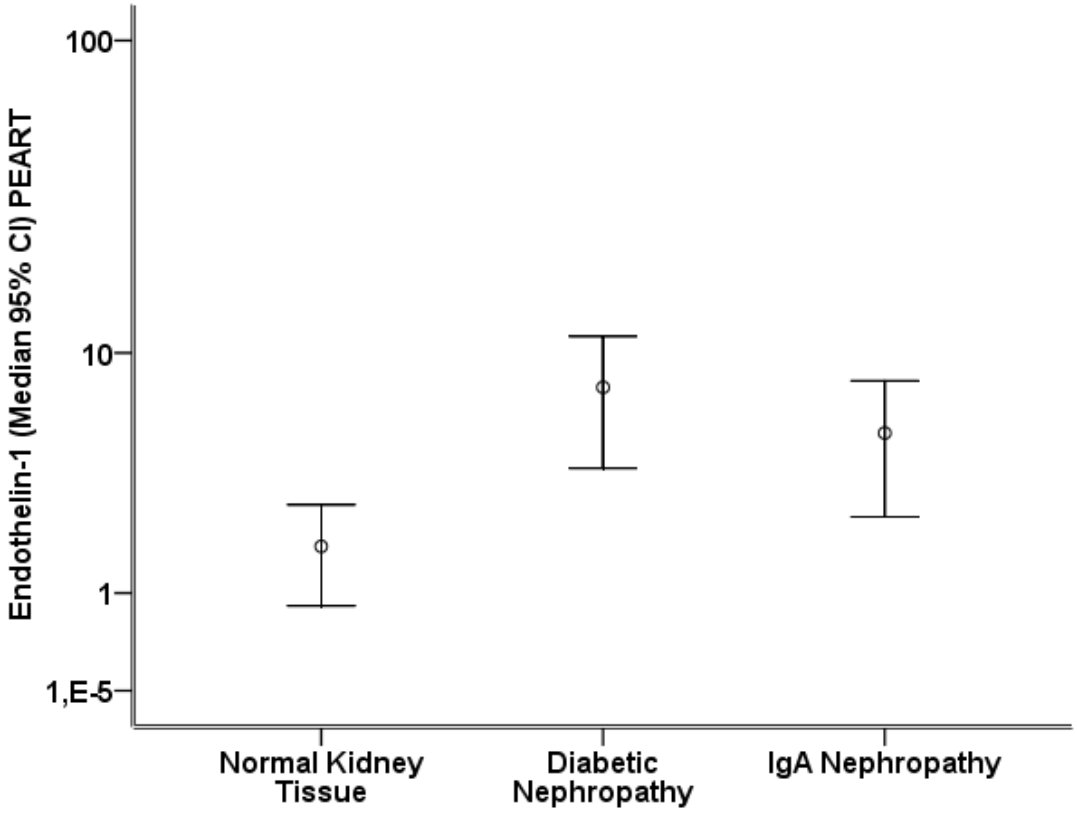


Figure 2. Score analysis of ET-1 positive events per area of renal tissue (PEART) according to renal disease.

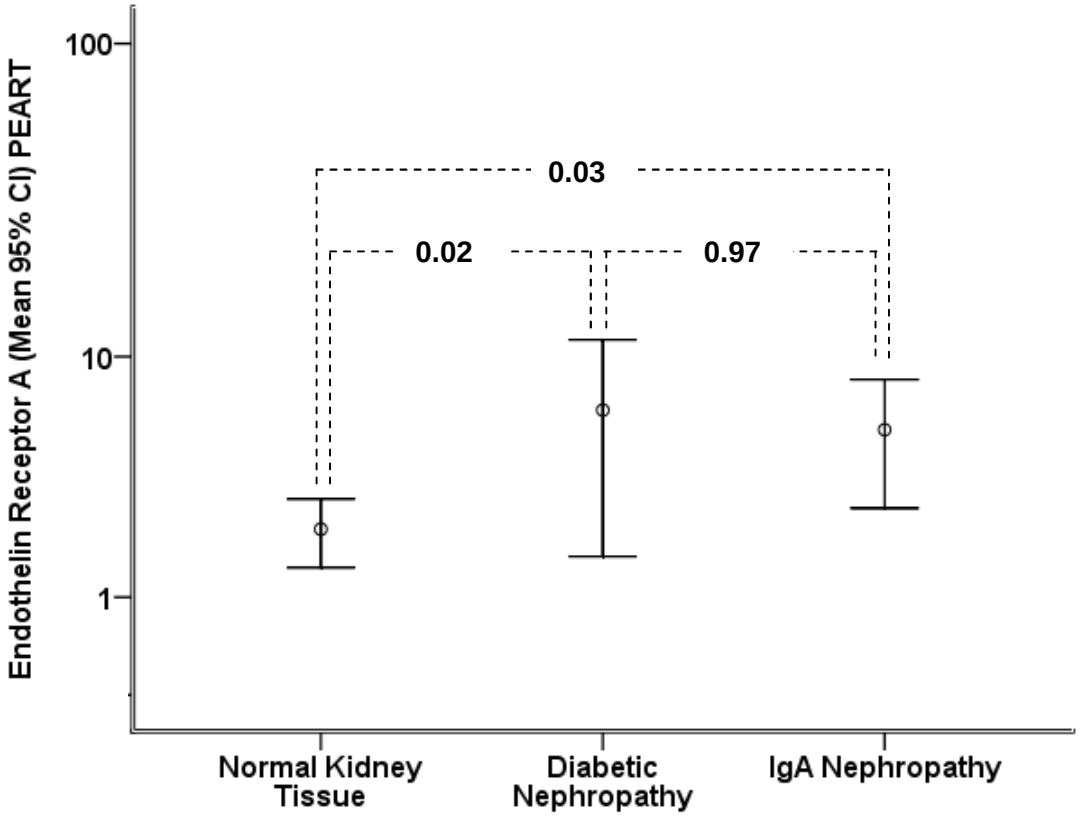


Figure 3. Score analysis of ETRA positive events per area of renal tissue (PEART) according to renal disease.

Z27a Zanatta, Claudete Maria
Avaliação do sistema endotelina na nefropatia diabética em pacientes com diabete melito tipo 2 / Claudete Maria Zanatta ; orient. Luís Henrique Canani. – 2009.
73 f.: il. color.

Tese (doutorado) – Universidade Federal Rio Grande do Sul. Faculdade de Medicina. Programa de Pós-Graduação em Ciências Médicas: Endocrinologia. Porto Alegre, BR-RS, 2009.

1. Diabetes mellitus tipo 2 2. Nefropatias diabéticas 3. Polimorfismo genético 4. Endotelina-1 5. Receptor de endotelina A I. Canani, Luis Henrique II. Título.

NLM: WK 810
Catalogação Biblioteca FAMED/HCPA