UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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MARCUS SILVA MICHELS

ESTUDO DA ASSOCIAÇÃO DOS POLIMORFISMOS -866G/A, ALA55VAL E INS/DEL DO GENE DA PROTEÍNA DESACOPLADORA 2 (*UCP2*) NA EXPRESSÃO DESSE GENE EM RIM HUMANO

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Bacharel em Biomedicina.

Orientador: Prof. Dr. Luís Henrique Canani
Co-orientadora: Profª. Drª. Daisy Crispim

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SUMÁRIO

LISTA DE ABREVIATURAS E SÍMBOLOS

°C	Graus Celsius
AER	*Albumin Excretion Rate*
AGE	*Advanced Glycation End-Products*
AH	*Arterial Hypertension*
Ala	Alanina
ATP	*Adenosine Triphosphate* (Trifosfato de Adenosina)
BMI	*Body Mass Index*
BP	*Blood Pressure*
bp	*base pairs*
cDNA	DNA complementar
CKD	*Chronic Kidney Disease*
Del	Deleção
DKD	*Diabetic Kidney Disease*
DM	Diabetes mellitus
DM1	Diabetes mellitus tipo 1
DM2	Diabetes mellitus tipo 2
DNA	Ácido Desoxirribonucleico
DR	*Diabetic Retinopathy*
DRC	Doença Renal Crônica
DRD	Doença Renal do Diabetes
eGFR	*Estimated Glomerular Filtration Rate*
ESRD	*End-Stage Renal Disease*

GAPDH	Gliceraldeído 3-Fosfato Desidrogenase
HDL	*High Density Lipoprotein*
IC	Intervalo de Confiança
Ins	Inserção
PCR	Reação em Cadeia da DNA Polimerase
RC	Razão de Chance
RNA	Ácido Ribonucleico
ROS	*Reactive Oxygen Species* - Espécies Reativas de Oxigênio
RT-qPCR	*Real Time Quantitative Polymerase Chain Reaction*
T1DM	*Type 1 Diabetes mellitus*
T2DM	*Type 2 Diabetes mellitus*
TFG	Taxa de Filtração Glomerular
TFGe	Taxa de Filtração Glomerular Estimada
UCP1	Proteína Desacopladora 1
UCP2	Proteína Desacopladora 2
UCP3	Proteína Desacopladora 3
UTR	*Untranslated Region*
Val	Valina

RESUMO

Introdução e Objetivos. A proteína desacopladora 2 (UCP2) diminui a produção de espécies reativas de oxigênio (ROS) pela mitocôndria. A superprodução de ROS é um dos maiores contribuintes para a patogênese de complicações crônicas do diabetes, como a doença renal do diabetes (DRD). Assim, polimorfismos deletérios no gene *UCP2* são fatores de risco candidatos para a DRD. Neste estudo, nós investigamos se os polimorfismos -866G/A, Ala55Val e Ins/Del do gene *UCP2* estavam associados com DRD em pacientes com diabetes mellitus do tipo 2 (DM2), e se eles tinham um efeito na expressão gênica de *UCP2* em biópsias de tecido renal humano.

Pacientes e Métodos. Neste estudo de caso-controle, as frequências dos polimorfismos -866G/A, Ala55Val e Ins/Del do gene *UCP2*, assim como as frequências dos haplótipos constituídos por estes, foram analisadas em 287 pacientes com DM2 e DRD, e em 281 pacientes com DM2 sem esta complicação e com mais de 10 anos de duração de DM2. No estudo transversal envolvendo pacientes que se submeteram à nefrectomia terapêutica, a expressão do gene *UCP2* foi avaliada em 42 amostras de biópsia renal estratificada de acordo com a presença do haplótipo mutado -866A/55Val/Ins do gene *UCP2*.

Resultados. Análise por regressão logística multivariada mostrou que o haplótipo 866A/55Val/Ins foi um fator de risco independente para a DRD (RC = 2,136, IC 95% 1,036-4,404), embora nem o genótipo nem as frequências alélicas dos polimorfismos -866G/A, Ala55Val e Ins/Del tenham diferido estatisticamente entre os grupos caso e controle. Interessantemente, pacientes com T2DM portadores do haplótipo mutado

mostraram taxa de filtração glomerular estimada (TFGe) diminuída quando comparados a indivíduos portadores do haplótipo de referência (*P* ajustado = 0.035). Nas amostras de biópsia renal, a expressão do gene *UCP2* foi significativamente diminuída em portadores do haplótipo mutado do gene *UCP2* quando comparada a amostras de rim de pacientes portadores do haplótipo de referência (0,32 ± 1,20 *vs*. 1,85 ± 1,16 *n fold change*; *P* ajustado < 0,000001).

Conclusões. Os dados apresentados sugerem que o haplótipo -866A/55Val/Ins do gene *UCP2* está associado com um risco aumentado para DRD e com uma menor TFGe em pacientes com DM2. Além disso, esse haplótipo mutado foi associado à diminuição da expressão do gene *UCP2* no rim humano.

1. INTRODUÇÃO

1.1. Diabetes Mellitus

Diabetes mellitus (DM) representa um grupo de doenças metabólicas caracterizadas por hiperglicemia crônica, seja ela pela deficiência na secreção de insulina ou por falha na ação desta, ou ambos. Diferentes processos patogênicos estão envolvidos no desenvolvimento do diabetes, desde destruição auto-imune de células beta-pancreáticas (DM1) a uma série de anormalidades que culminam em resistência insulínica nas células dos tecidos alvo (DM2). A hiperglicemia crônica que ocorre no DM está associada ao dano, à disfunção ou à falha de diversos órgãos, em especial o coração, os vasos sanguíneos, os nervos, os olhos e os rins [1].

Sob uma perspectiva epidemiológica, segundo a Federação Internacional de Diabetes – IDF, do inglês, *International Diabetes Federation* –, a maioria dos 382 milhões de pacientes com DM tem entre 40 e 59 anos e 80% deles vivem em países de baixa ou média renda. Segundo o mesmo órgão, o DM foi a causa de 5,1 milhões de mortes somente no ano de 2013. Ainda no mesmo ano, o gasto em saúde gerado pelo DM foi de US$548 bilhões de dólares, o que representa 11% dos gastos mundiais em saúde. Previsões indicam que no ano de 2035 o gasto em saúde gerado pelo DM será de US$627 bilhões, bem como o número de pacientes com DM aumentará em 55% [2].

O DM é uma doença com alta prevalência, afetando principalmente pessoas em idade produtiva e idosos, como é possível observar na figura 1, à seguir. Essa doença apresenta ainda elevada taxa de morbidade, sendo isso um reflexo das complicações crônicas que ocorre em pacientes com DM [2, 3].

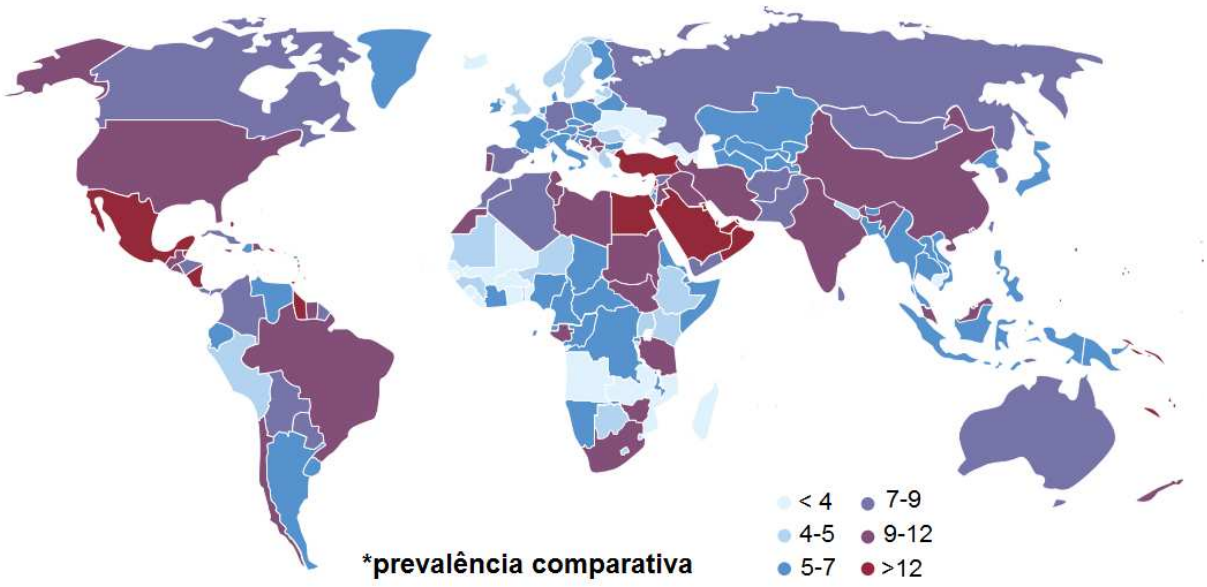

Figura 1. Prevalência* (%) de diabetes em adultos (20-79 anos), no ano de 2013. Adaptado de: IDF: *IDF Diabetes Atlas,* 6ª edição.

Do ponto de vista clínico, os sintomas mais marcantes da hiperglicemia incluem poliúria, polidipsia, perda de peso, e às vezes, polifagia e visão borrada. A falta de monitoramento glicêmico pode levar alguns pacientes hiperglicêmicos, sobre tudo os que apresentam DM1, ao quadro de cetoacidose [1]. Como este trabalho de conclusão estudou pacientes com DM2, o próximo tópico traz mais detalhes sobre tal forma de DM.

1.1.1. Diabetes Mellitus Tipo 2

O DM2 corresponde a 90-95% dos casos de DM em todo o mundo. Previamente conhecido como DM não-insulino dependente, o DM2 inclui indivíduos que apresentam resistência a tal hormônio e geralmente algum grau de deficiência insulínica. Pacientes com esse tipo de DM são em geral obesos, e a obesidade por si só causa certo grau de resistência à insulina [1].

Devido ao desenvolvimento gradual de hiperglicemia, pacientes com DM2 podem viver anos com níveis aumentados de glicose sanguínea antes que haja desenvolvimento dos sintomas clássicos que ajudem no diagnóstico da doença. Considerando que tais pacientes têm níveis normais de secreção de insulina, níveis de glicose elevados gerariam níveis ainda maiores de insulinemia, dado o fato de as células beta-pancreáticas funcionarem normalmente. Assim, a secreção de insulina em tais pacientes tornar-se-ia defeituosa e insuficiente para compensar a resistência a esse hormônio [1].

Fatores de risco para o desenvolvimento de DM2 incluem idade, obesidade e falta de atividade física. As pessoas mais frequentemente afetadas pelo DM2 são mulheres que apresentaram DM gestacional prévia e também indivíduos com hipertensão e dislipidemia. A frequência desta doença pode ainda variar de acordo com a etnia. Por ser uma doença multifatorial o DM2 também está associado a uma forte predisposição genética. Entretanto, o componente genético dessa doença é complexo e não está claramente definido. O que se sabe, porém, é que pessoas com DM2 têm risco aumentado de desenvolver complicações crônicas macro e microvasculares [1], assunto tratado no próximo tópico.

1.1.2. Complicações crônicas do DM

Pacientes com DM tem risco aumentado de desenvolver uma série de complicações que podem incapacitá-los e até levá-los a óbito [2]. Tais complicações estão ilustradas na figura 2.

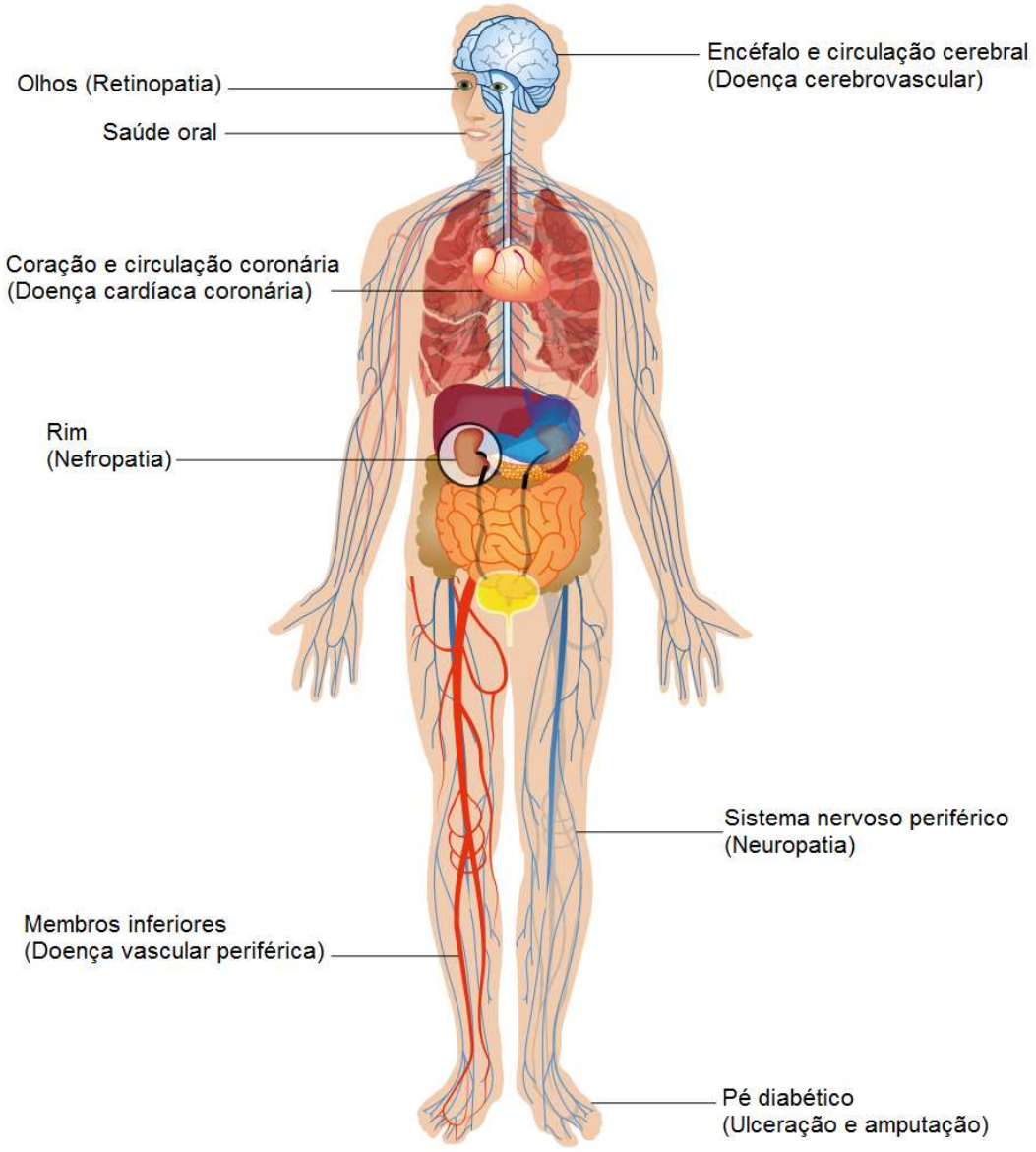

Figura 2. As principais complicações do Diabetes. Adaptado de: IDF: *IDF Diabetes Atlas,* 6ª edição.

Dentre as complicações microvasculares do diabetes, a retinopatia diabética é a principal causa de cegueira em adultos [4]; a doença renal diabética é a causa mais comum de doença renal de estágio final e transplante de rim em vários países bem como a maior causa de diálise [5]; e a neuropatia periférica é responsável por 50-75% das amputações de membro inferior não-traumáticas [6]. Como a prevalência do DM2 tem crescido em países em desenvolvimento, o impacto social e econômico dessas complicações crônicas tem acompanhado essa tendência [2].

Como este trabalho tem como alvo de estudo a doença renal do diabetes, essa complicação crônica do DM é tratada com mais detalhes à seguir.

1.1.2.1. Doença renal do diabetes

A Doença Renal do Diabetes (DRD) é a maior causa de doença renal crônica (do inglês, *chronic kidney disease* - CKD) e doença renal de estágio final (do inglês, *end-stage-renal disease* - ESRD) em todo o mundo, sendo um grande preditor de mortalidade em pacientes com DM2 e acometendo 20% a 30% destes [5, 7, 8]. Ainda, essa doença é a maior responsável por transplantes renais em vários países [5]. Nos Estados Unidos, cerca de 200.000 pessoas são tratadas para ESRD por apresentarem DRD, sendo que a cada ano, 50.000 novos indivíduos iniciam diálise [7, 8]. A taxa de incidência de diálise poderia ser ainda maior se fossem considerados os casos de pacientes com DRD que morrem devido a doenças cardiovasculares antes de atingirem a ESRD. Pacientes com DRD que apresentam

ESRD se deparam com uma taxa de mortalidade anual de 20%, o que é maior que a taxa de mortalidade de muitos cânceres sólidos, como câncer de próstata, de mama e até mesmo câncer de células renais [7].

O primeiro sinal de DRD é a albuminúria (índice albumina/creatinina > 30 mg/g). A proteinúria persistente é no início moderadamente aumentada (30-300 mg/g), e progride para albuminúria severamente aumentada (>300 mg/g), culminando na doença renal de estágio final [7, 9]. Todavia, estudos têm demonstrado que a microalbuminúria pode regredir, além de haver pacientes com DRD progressiva sem proteinúria significativa [7, 10-12].

Junto da albuminúria persistência, ocorre também o declínio da taxa de filtração glomerular (TFG) [7, 9]. A TFG estimada (TFGe) é amplamente usada para indicar a função renal [13]. Uma forma de se obter a TFGe é a partir do cálculo de MDRD (*Modification of Diet for Renal Disease*), que utiliza os níveis de creatinina sérica e também leva em consideração o sexo, a idade e a etnia. Entretanto, a TFGe indica apenas função, mas não dano renal, haja vista a TFG pode aumentar em até 40% acima do valor normal em pacientes com DRD que já apresentam aumento da pressão de filtração e hipertrofia glomerular [14].

A realização de diagnóstico histopatológico de DRD, considerado o "padrão-ouro", não é rotina na clínica, já que a maioria dos médicos é relutante em submeter seus pacientes à biopsia renal, visto que tal procedimento envolve um risco muito alto [7]. Com relação às alterações fisiopatológicas da DRD, os principais danos presentes nesses pacientes podem ser encontrados na figura 3, à seguir. Inicia-se com espessamento da membrana basal glomerular e aumento da deposição de colágeno por células mesangiais na matriz extracelular. Ainda, ocorre obliteração de pedicelos secundários e diminuição da quantidade de podócitos.

Esses fatores contribuem para que ocorra excreção de proteínas, incluindo a albumina, como citado anteriormente. Além disso, outras regiões do néfron são afetadas. No túbulo contorcido proximal, por exemplo, ocorre atrofia do epitélio tubular, com perda de microvilosidades, diminuição da quantidade de capilares e também infiltrado inflamatório.

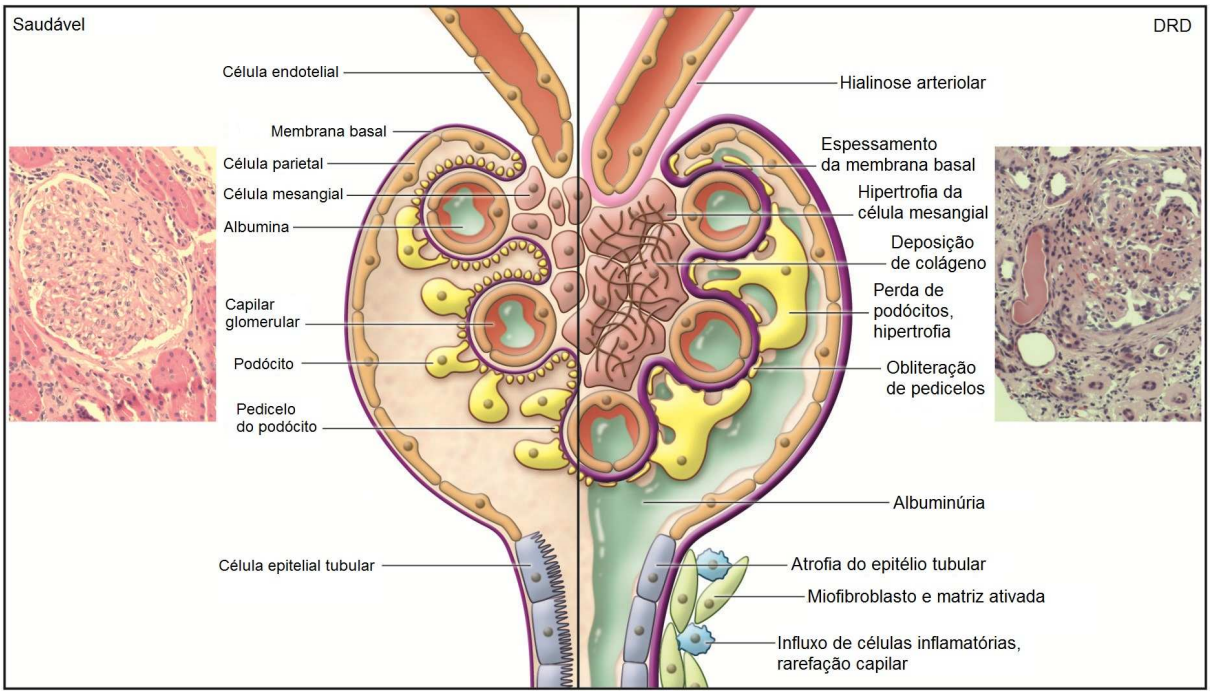

Figura 3. Lesões patológicas na DRD. O glomérulo de um paciente saudável inclui arteríola aferente, capilares glomerulares, células endoteliais, membrana basal, podócitos células epiteliais parietais, células túbulo-epiteliais, e é impermeável à albumina. Em contraste, o glomérulo de um paciente diabético apresenta hialinose arteriolar, expansão mesangial, deposição de colágeno, espessamento da membrana basal, perda e hipertrofia de podócitos, albuminúria, atrofia do epitélio tubular, acúmulo de matriz e miofibroblastos ativados, influxo de células inflamatórias e rarefação de capilares. Também é mostrado tecido renal de

glomérulo saudável e de paciente com DRD (corado com Ácido Periódico de Schiff). Magnificação original, 400x. Adaptado de Reidy K et. al. 2014.

A DRD e demais complicações crônicas tem sido relacionadas com a superprodução de espécies reativas de oxigênio, sendo consequência desta [15, 16]. Assim, o próximo tema desta introdução trata da cadeia transportadora de elétrons e sua relação com o DM e suas complicações.

1.2. Cadeia Respiratória Mitocondrial

As mitocôndrias são organelas essenciais às células eucarióticas, regulando vários processos vitais para a sobrevivência e a função celular, como produção de energia, controle redox, homeostase de cálcio, controle de vias metabólicas e até de morte celular programada [17].

No contexto da produção de energia, a cadeira transportadora de elétrons é composta pelos complexos I, II, III e IV, pela coenzima Q, pelo citocromo C e pela ATP sintase. Carreadores de prótons na forma reduzida, como NADH e $FADH_2$, doam elétrons para esses complexos, iniciando o processo que culminará na produção de moléculas de ATP e redução de moléculas de O_2 em água. NADH doa elétrons para o complexo I (NADH:ubiquinona oxidorredutase). Esse complexo transfere elétrons para a coenzima Q (ubiquinona). Essa, por sua vez, recebe também elétrons do complexo II (succinato:ubiquinona oxidorredutase), provenientes de moléculas de $FADH_2$. Os elétrons são transferidos da coenzima Q para o

complexo III (ubiquinol:citocromo C oxidorredutase). À seguir, esses elétrons fluem pelo citocromo C, pelo complexo IV (citocromo C oxidase) até chegarem às molécula de O_2, reduzindo-as à água. Os elétrons que fluem pelos complexos I, III e IV geram um gradiente de prótons que é necessário para a atividade da ATP sintase (Figura 4) [16, 17].

1.2.1. Espécies Reativas de Oxigênio (ROS) e Complicações Crônicas do DM

Fisiologicamente, espécies reativas de oxigênio são geradas durante o processo de fosforilação oxidativa. Todavia, a hiperglicemia característica do DM aumenta a produção de doadores de elétrons provenientes do ciclo do ácido tricarbolíxico. Essas moléculas irão doar seus elétrons para os complexos da cadeia respiratória, o que irá aumentar ainda mais o potencial de membrana mitocondrial. Porém, tal processo faz que o complexo III pare de transferir elétrons para o citocromo C. Isso aumenta a meia vida de intermediários de radicais livres da ubiquinona. Com isso, O_2 molecular é reduzido a ânion superóxido [16]. Resumindo, há uma superprodução de espécies reativas de oxigênio. A figura 4 ilustra o processo.

No ano 2000, Du *et al.* propuseram a hipótese de que a hiperglicemia intracelular levaria a uma superprodução de espécies reativas de oxigênio (ROS). Tal hipótese unifica quatro vias principais de como a hiperglicemia leva às complicações macro e microvasculares do diabetes. São elas: o aumento do fluxo de glicose pela via da aldose redutase; o aumento da formação de produtos finais de

glicação avançada; a ativação de isoformas da proteína cinase C (PKC); e o aumento do fluxo de glicose para a via da hexaminase [16]. As alterações nessas vias estimulam fatores de crescimento como o TFG-β (do inglês, *transforming growth factor beta*), que no glomérulo renal, promove um acúmulo de matriz extracelular, levando a DRD.

Neste contexto, mecanismos fisiológicos que diminuam a superprodução de ROS, como as proteínas desacopladoras (UCPs), são alvos de estudo interessantes para o DM.

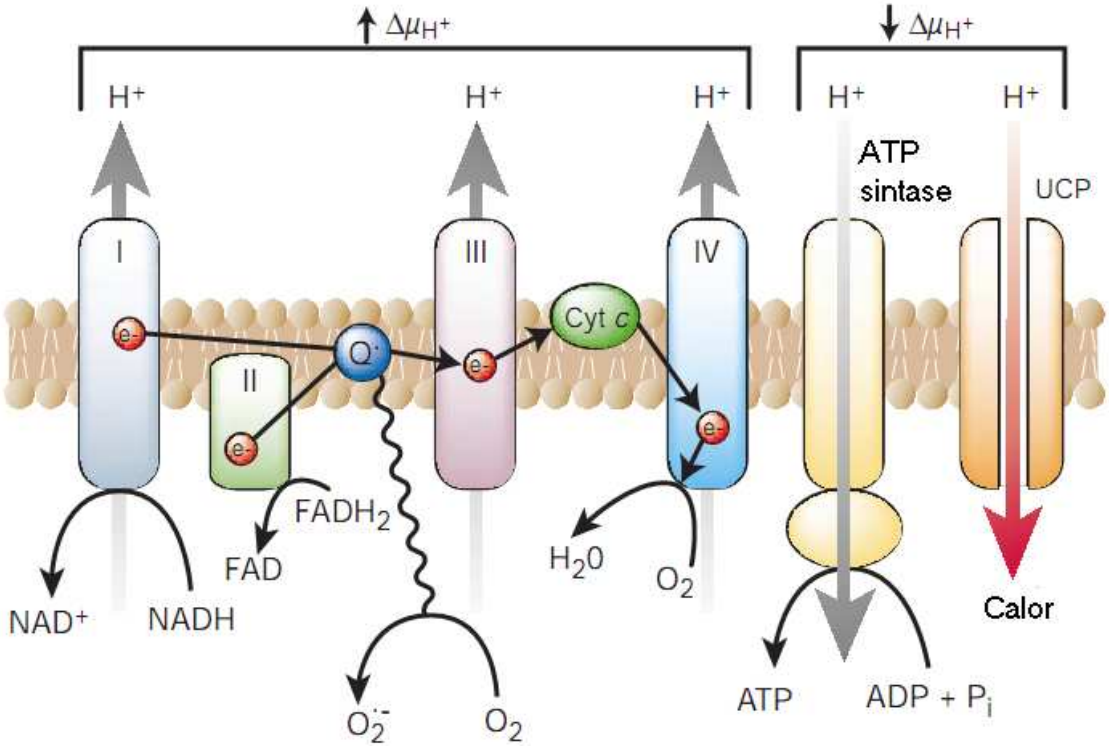

Figure 4 Produção de superóxido pela cadeia transportadora de elétrons. Com a hiperglicemia, há maior disponibilidade de doadores de elétrons provenientes do ciclo de Krebs (NADH e FADH2), os quais geram um aumento no potencial de membrana mitocondrial pelo maior bombeamento de prótons através da membrana mitocondrial interna. Isso inibe o transporte de elétrons pelo complexo III, elevando a

meia vida de intermediários de radicais livres da coenzima Q, reduzindo oxigênio molecular a superóxido. Adaptado de Brownlee *et al*. 2001.

1.3. Proteínas Desacopladoras

As proteínas desacopladoras (UCPs) são membros de uma família de proteínas carreadoras de ânions localizadas na membrana mitocondrial interna [18]. As isoformas apresentam estrutura similar, mas em mamíferos, são diferentemente expressas nos tecidos. A UCP1 é principalmente encontrada em tecido adiposo marrom [19, 20], enquanto a UCP2 está distribuída por vários tecidos e células, e a UCP3 é quase restrita ao músculo esquelético [17, 20].

Elas transportam prótons do espaço intermembranas para a matriz mitocondrial. Dessa forma, elas diminuem a energia próton-motiva disponível para a atividade da ATP sintase. Esse desacoplamento permite a ocorrência de funções tecido-específicas como a termogênese (UCP1), regulação do metabolismo de ácidos graxos livres e transporte (UCP2 e UCP3), atenuação da produção de espécies reativas de oxigênio (ROS) pela mitocôndria (UCP1-3), e regulação da secreção de insulina pelas células beta-pancreáticas (UCP2), todos eles mecanismos associados a patogênese do DM2 e suas complicações crônicas [17, 18, 20, 21].

1.3.1. Polimorfismos nos Genes *UCP*

A relação entre os *loci* dos genes *UCP 1-5* e a susceptibilidade ao DM2 e suas complicações foi investigada em vários estudos genéticos e grande atenção tem sido dada ao polimorfismo -3826A/G (rs1800592) na região promotora do gene *UCP1*, aos polimorfismos -866G/A (rs659366), Ala55Val (C/T; rs660339) e Ins/Del (Inserção/deleção de 45 pares de base), respectivamente, na região promotora, no éxon 4 e na região 3' UTR (éxon 8) do gene *UCP2* (Figura 5), e ao polimorfismo -55C/T (rs1800849) na região promotora do gene *UCP3* [17, 19, 20, 22, 23].

Um dos principais mecanismos que relacionam hiperglicemia às complicações microvasculares do DM é a superprodução mitocondrial de ROS [5, 24]. Entretanto, apesar do reconhecido papel da UCP2 na proteção contra o estresse oxidativo, há poucos estudos que avaliam a associação entre polimorfismos no gene *UCP2* e a ocorrência de complicações crônicas do T2DM [17, 25-27], como a DRD.

Portanto, a proposta deste trabalho de conclusão de curso foi estudar a associação de polimorfismos no gene *UCP2* com susceptibilidade à DRD e também avaliar se esses polimorfismos estão associados a alterações na expressão do gene *UCP2* em amostras de rim humano.

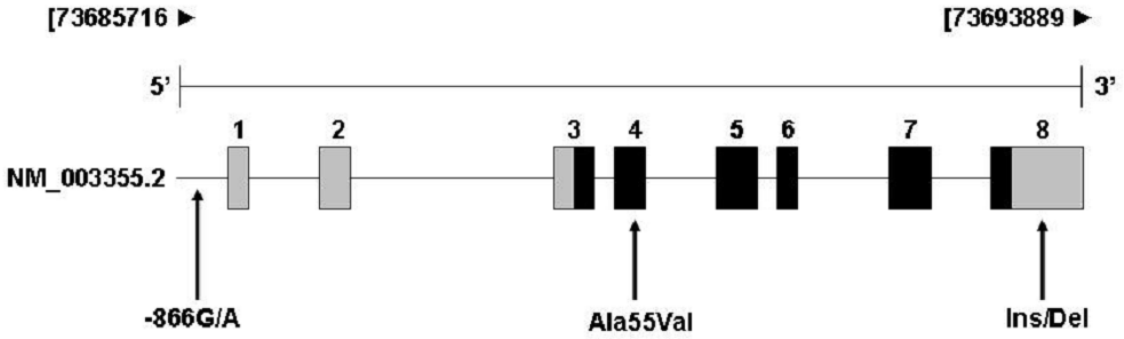

Figura 5. Mapa do *locus* do gene *UCP2* no cromossomo 11 (região 11q13). O oito éxons (retângulos) estão numerados da esquerda para a direita de acordo com a região transcricional. Os retângulos pretos representam as regiões codificantes, e os retângulos cinza representam a região não codificante, incluindo a região 3'UTR do éxon 8. As setas verticais os polimorfismos mais comumente associados com T2DM ou suas complicações crônicas microvasculares. Adaptado de Souza et. al., 2011.

2. OBJETIVOS

- Avaliar se os polimorfismos -866G/A, Ala55Val e Ins/Del estão associados à doença renal diabética em pacientes com DM2.

- Avaliar se os alelos -866A, 55Val e Ins no gene *UCP2* têm um efeito diferencial na expressão deste gene em células de rim humano em comparação ao genótipo selvagem -866G / 55Ala / Del.

3. ARTIGO CIENTÍFICO

O artigo intitulado "*Polymorphisms of the UCP2 gene are associated with glomerular filtration rate in type 2 diabetic patients*" foi formatado conforme normas para publicação junto ao periódico Arquivos Brasileiros de Endocrinologia e Metabologia.

Polymorphisms of the *UCP2* gene are associated with glomerular filtration rate in type 2 diabetic patients

Marcus Michels[1], Bianca M. de Souza[1,2], Denise A. Sortica[1,2], Ana Paula Bouças[1,2], Jakeline Rheinheimer[1,2], Marjoriê P. Buffon[1,2], Luís H. Canani[1,2] and Daisy Crispim[1,2]

1. Endocrine Division, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil

2. Postgraduate Program in Medical Sciences: Endocrinology, Universidade Federal do Rio Grande do Sul. Porto Alegre, Rio Grande do Sul, Brazil

Corresponding author:

Dr. Daisy Crispim, Rua Ramiro Barcelos 2350, Prédio 12, 4° andar. CEP 90035-003, Porto Alegre, RS, Brazil. E-mail: dcmoreira@hcpa.ufrgs.br.

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Resumo

Introdução e Objetivos. A proteína desacopladora 2 (UCP2) diminui a produção de espécies reativas de oxigênio (ROS) pela mitocôndria. A superprodução de ROS é um dos maiores contribuintes para a patogênese de complicações crônicas do diabetes, como a doença renal do diabetes (DRD). Assim, polimorfismos deletérios no gene UCP2 são fatores de risco candidatos para a DRD. Neste estudo, nós investigamos se os polimorfismos -866G/A, Ala55Val e Ins/Del do gene UCP2 estavam associados com DRD em pacientes com diabetes mellitus do tipo 2 (DM2), e se eles tinham um efeito na expressão gênica de UCP2 em biópsias de tecido renal humano.

Pacientes e Métodos. Neste estudo de caso-controle, as frequências dos polimorfismos -866G/A, Ala55Val e Ins/Del do gene UCP2, assim como as frequências dos haplótipos constituídos por estes, foram analisadas em 287 pacientes com DM2 e DRD, e em 281 pacientes com DM2 sem esta complicação e com mais de 10 anos de duração de DM2. No estudo transversal envolvendo pacientes que se submeteram à nefrectomia terapêutica, a expressão do gene UCP2 foi avaliada em 42 amostras de biópsia renal estratificada de acordo com a presença do haplótipo mutado -866A/55Val/Ins do gene UCP2.

Resultados. Análise por regressão logística multivariada mostrou que o haplótipo 866A/55Val/Ins foi um fator de risco independente para a DRD (RC = 2,136, IC 95% 1,036-4,404), embora nem o genótipo nem as frequências alélicas dos polimorfismos -866G/A, Ala55Val e Ins/Del tenham diferido estatisticamente entre os grupos caso e controle. Interessantemente, pacientes com T2DM portadores do haplótipo mutado mostraram taxa de filtração glomerular estimada (TFGe) diminuída quando comparados a indivíduos portadores do haplótipo de referência (P ajustado = 0.035). Nas amostras de biópsia renal, a expressão do gene UCP2 foi significativamente diminuída em portadores do haplótipo mutado do gene UCP2 quando comparada a amostras de rim de pacientes portadores do haplótipo de referência (0,32 $\pm$ 1,20 vs. 1,85 $\pm$ 1,16 n fold change; P ajustado < 0,000001).

Conclusões. Os dados apresentados sugerem que o haplótipo -866A/55Val/Ins do gene UCP2 está associado com um risco aumentado para DRD e com uma menor TFGe em pacientes com

DM2. Além disso, esse haplótipo mutado foi associado à diminuição da expressão do gene UCP2 no rim humano.

Abstract

Background and Aims. Uncoupling protein 2 (UCP2) reduces production of reactive oxygen species (ROS) by mitochondrial. ROS overproduction is one of the major contributors to the pathogenesis of chronic diabetic complications, such as diabetic kidney disease (DKD). Thus, deleterious polymorphisms in the *UCP2* gene are candidate risk factors for DKD. In this study, we investigated whether *UCP2* -866G/A, Ala55Val and Ins/Del polymorphisms were associated with DKD in patients with type 2 diabetes mellitus (T2DM), and whether they had an effect on *UCP2* gene expression in human kidney tissue biopsies.

Subjects and Methods. In a case-control study, frequencies of the *UCP2* -866G/A, Ala55Val and Ins/Del polymorphisms, as well as frequencies of the haplotypes constituted by them, were analyzed in 287 T2DM patients with DKD and 281 T2DM patients without this complication and with more than 10 years of T2DM duration. In a cross-sectional study comprising patients who undergone therapeutic nephrectomy, *UCP2* gene expression was evaluated in 42 kidney biopsies samples stratified according to the presence of the *UCP2* mutated -866A/55Val/Ins haplotype.

Results. Multivariate logistic regression analysis showed that the -866A/55Val/Ins haplotype was an independent risk factor for DKD (OR = 2.136, 95% CI 1.036-4.404), although neither genotype nor allele frequencies of the individual -866G/A, Ala55Val and Ins/Del polymorphisms differed statistically between case and control groups. Interestingly, T2DM patients carrying the mutated haplotype showed decreased estimated glomerular filtration rate (eGFR) when compared to subjects with the reference haplotype (adjusted P = 0.035). In kidney biopsy samples, *UCP2* gene expression was significantly decreased in *UCP2* mutated haplotype carriers when compared to kidneys from patients with the reference haplotype (0.32 $\pm$ 1.20 *vs*. 1.85 $\pm$ 1.16 n fold change; adjusted P < 0.000001).

Conclusions. Data reported here suggest that the *UCP2* -866A/55Val/Ins haplotype is associated with an increased risk for DKD and with a lower eGFR in T2DM patients. Furthermore, this mutated haplotype was associated with decreased *UCP2* gene expression in human kidneys.

Introduction

Diabetic kidney disease (DKD), also known as diabetic nephropathy, is a major chronic complication of diabetes mellitus (DM) and the leading cause of end-stage renal disease (ESRD) that requires dialysis treatment or kidney transplantation (1, 2). This complication affects approximately 40% of type 2 DM (T2DM) patients, and is an important cause of morbidity and mortality among these subjects (2-4). Usually, DKD is a progressive disorder characterized by pathophysiological alterations resulting from the diabetic state, which begin with glomerular hyperfiltration and renal hypertrophy, and might progress to proteinuria and a gradual decrease in glomerular filtration rate (GFR) (1, 4).

Although hyperglycemia, arterial hypertension (AH) and dyslipidemia are known risk factors for DKD, a subset of subjects with poorly controlled DM do not develop this complication, indicating that genetic factors might have a key role in its pathogenesis (5). In fact, several studies have shown that genetic susceptibility contributes to the development of DKD in both type 1 and type 2 DM (6-9). Therefore, great efforts have been made to identify genetic variants associated with DKD; however, results are still inconclusive with different variants associated with small effects in different populations (1).

It is well known that hyperglycemia causes an important increase in the production of reactive oxygen species (ROS) by mitochondria (6, 10). In this context, Du *et al*. (11) proposed a unifying hypothesis linking important pathways involved in the pathogenesis of DKD. Accordingly to this hypothesis, hyperglycemia-induced mitochondrial superoxide overproduction results in an increased activation of protein kinase C isoforms, increased formation of advanced glycation end-products (AGE), acceleration of glucose flux through the aldose reductase pathway, and an increased glucose flux into the hexosamine pathway. These alterations stimulate growth factors that result in extracellular matrix accumulation, leading to DKD.

Uncoupling protein 2 (UCP2) belongs to an anion-carrier protein family located in the mitochondrial inner membrane (12, 13), and it is expressed in many tissues, including white adipose tissue, pancreatic islets, retinal cells and kidneys (14-17). UCP2 mildly uncouples

substrate oxidation from ATP synthesis, thereby dissipating the membrane potential energy and, consequently, decreasing ATP production by mitochondrial respiratory chain (15, 18). The uncoupling thus leads to tissue-specific functions such as regulation of free fatty acid metabolism, inhibition of insulin secretion from pancreatic beta-cells and, importantly, decreasing ROS formation by mitochondria (12, 17). Thus, polymorphisms in the *UCP2* gene might be involved in the development of DKD or other diabetic complications.

Taking into consideration the role of UCP2 in the protection against oxidative stress, our group previously investigated whether three common *UCP2* gene polymorphisms (-866G/A, Ala55Val and Ins/Del), also described an association with T2DM (19), could be also associated with diabetic retinopathy (DR) in a Brazilian population of diabetic patients (20). Our data showed that the -866A/55Val/Ins haplotype was associated with increased risk for proliferative DR in both type 1 and type 2 diabetic patients. More recently, we evaluated if the -866A/55Val/Ins haplotype was associated with changes in *UCP2* gene expression in retina from cadaveric cornea donors. Interestingly, carriers of the mutated haplotype showed a lower *UCP2* gene expression in retina than homozygous for the reference haplotype (-886G/55Ala/Del) (21).

Therefore, in this study, we investigated whether the *UCP2* -866G/A, Ala55Val and Ins/Del polymorphisms were associated with DKD in T2DM patients, and whether they had an effect on *UCP2* gene expression in human kidney tissue biopsies.

Subjects and Methods

Type 2 DM patients, nondiabetic controls, and phenotype measurements

A total of 568 unrelated T2DM patients were enrolled in the study. The sample population comprised 287 T2DM patients with DKD (cases) and 281 T2DM patients without this complication and with known DM duration of at least 10 years (controls). T2DM patients were participating in a multicenter study that started recruiting patients in Southern Brazil in 2002. That project was designed to study genetic risk factors associated with T2DM and its chronic complications, such as DKD and DR. It initially had four participating centers located in teaching hospitals in the Brazilian State of Rio Grande do Sul, specifically Grupo Hospitalar Conceição, Hospital São Vicente de Paula, Hospital Universitário de Rio Grande, and Hospital de Clínicas de Porto Alegre. A detailed description of the study can be found elsewhere (22). T2DM was defined as a diagnosis of DM after the age of 35 years, with no insulin therapy during the first year after diagnosis and no previous episodes of ketoacidosis (23). The ethnic group was defined based on self-classification, and the ethnic proportion between case and controls was as follows: 21.9% of black patients in the case group and 21.3% of black patients in the control group (P=0.860).

A standard questionnaire was used to collect information about age, age at T2DM diagnosis, and drug treatment. All T2DM patients underwent physical and laboratory evaluations, as previously described (20, 24). Briefly, they were weighed bare feet, wearing light outdoor clothes and their height was measured. Body mass index (BMI) was calculated as weight (kg)/height (meters)^2. Office blood pressure (BP) was measured in sitting position, on the left arm, after a 5-min rest by a trained research, with a mercury sphygmomanometer. The mean of two measurements taken 1 min apart was used to calculate systolic and diastolic BP. AH was defined as BP levels ≥140/90 mmHg at the initial visit and at two follow-up visits within 1 month of the initial visit, or if the presence of AH was previously register on medical records.

The diagnosis of DKD was primarily based on the albumin excretion rate (AER) in at least two out of three consecutive 24-h timed urine samples in a 6-month period. Patients were classified as having normal to mildly increased AER (AER < 30 mg/24h, **control group**),

moderately increased AER (AER 30–299 mg/24h) or severely increased AER (AER ≥ 300 mg/24h) (25). Therefore, the **case group** with DKD was constituted by patients having moderately to severely increased AER. Patients with other causes of albuminuria or renal diseases other than DKD were excluded. Moreover, independently of AER, patients were also evaluated regarding their estimated GFR (eGFR) (25). eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) equation (http://nephron.org/mdrd_gfr_si), which takes into account the following parameters: age, gender, ethnicity and creatinine value. According to the MDRD equation, eGFR values above 60 ml/min/1.73m^2 should be interpreted as "above 60 ml/min/1.72m^2", not an exact number. An experienced ophthalmologist assessed all patients for DR using fundoscopy through dilated pupils. DR was classified as absent, non-proliferative DR or proliferative DR (26). DR classification was based on the most severe degree of retinopathy in the worst affected eye.

Serum and plasma samples were taken after 12 hours of fasting for laboratory analyses. Glucose levels were determined using the glucose oxidase method. HbA1c measurements were performed by different methods and the results were traceable to the Diabetes Control and Complications Trial (DCCT) method by off-line calibration or through conversion formulae (27). Creatinine was measured by the Jaffé reaction; total plasma cholesterol, HDL cholesterol and triglycerides by enzymatic methods, and albuminuria by immunoturbidimetry (Sera-Pak immuno microalbuminuria, Bayer, Tarrytown, NY, USA; mean intra and interassay coefficients of variance of 4.5% and 11% respectively) (28). Patients interrupted the use of angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists for at least one week before having their albuminuria measured.

The protocol was approved by the Hospital ethical committees, and all patients gave their written informed consent.

Kidney samples and phenotype measurements

To investigate *UCP2* gene expression in the presence of different *UCP2* haplotypes, kidney biopsies were obtained from 118 patients who undergone therapeutic nephrectomy suggested by an urologist in Hospital de Clínicas de Porto Alegre. A standardized form was used to collect

information from medical records about age, sex, presence of AH and DM, smoking habits, and occurrence of other diseases. Peripheral blood samples were collected from each subject for DNA extraction and genotyping of the *UCP2* polymorphisms of interest. Following genotyping, subjects were divided into groups according to the presence of the *UCP2* mutated haplotype (-866A/55Val/Ins).

Most of subjects had their kidney removed due to malignant disease. After nephrectomy, an excised normal kidney biopsy was snap-frozen in liquid nitrogen and stored at -80°C until *UCP2* mRNA expression analysis. Only kidney samples from non-diabetic subjects and containing normal tissue without visible tumors at optical microscopy were eligible for inclusion in the study.

The protocol was approved by the Hospital's ethical committee, and all patients gave their written informed consent.

Genotyping

DNA was extracted from peripheral blood leucocytes by a standardized salting-out procedure. The -866G/A polymorphism (rs659366) in the promoter region of the *UCP2* gene was determined by digesting polymerase chain reaction (PCR) products with the restriction enzyme *MluI* (Invitrogen Life Technologies, Inc., CA, USA), as previously described (21). Digestion fragments were resolved on 2% agarose gels containing GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA) and visualized under ultraviolet illumination. A sample of DNA (whose genotype was identified by sequencing) was used as a positive control to evaluate the completeness of PCR product digestion. Evaluation of the *UCP2* 45 bp Ins/Del polymorphism in the 3' untranslated region (UTR) of exon 8 was done by PCR, as previously described (21). Briefly, primers amplified products of 457 bp (insertion allele) or 412 bp (deletion allele), which were resolved on 2.5% agarose gels stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc.) and visualized under ultraviolet light. Genotypes of the -866G/A and Ins/Del polymorphisms were recorded using the ImageMaster System VDS (GE HealthCare, London, UK).

Genotyping of the Ala55Val (C/T) polymorphism (rs660339) in exon 4 of the *UCP2* gene was determined using primers and probes contained in the Human Custom TaqMan Genotyping

188 Assay 40x (Life Technologies, Foster City, CA, USA). Primer and probe sequences can be
189 found elsewhere (21). The reactions were conducted in 96-well plates, in a total 5 µL reaction
190 volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1 x (Life Technologies), and
191 Custom TaqMan Genotyping Assay 1x. Plates were then positioned in a real-time PCR thermal
192 cycler (7500 Fast Real PCR System; Life Technologies) and heated for 10 min at 95°C, followed
193 by 45 cycles of 95°C for 5s and 62°C for 1min. Fluorescence data files from each plate were
194 analyzed using automated allele-calling software (SDS 2.1; Life Technologies).
195
196 *RNA isolation*
197 Kidney tissue biopsies (250 mg) were homogenized in phenol-guanidine isothiocyanate
198 (Invitrogen - Life Technologies). RNA was extracted with chloroform and precipitated with
199 isopropanol by centrifugation (12,000 x g) at 4°C. RNA pellet was washed twice with 75% ethanol
200 and ressuspended in 10-50 µL of diethylpyrocarbonate treated water.
201 Concentration and quality of total RNA samples were assessed using a NANODROP 2000
202 spectrophotometer (Thermo Scientific Inc., DE, USA). Only RNA samples which achieved
203 adequate purity ratios (A260/A280 = 1.9–2.1) were used for subsequent analyses (29). In addition,
204 RNA integrity and purity were also checked on agarose gel containing GelRed™ Nucleic Acid Gel
205 Stain (Biotium, Inc.). The mean RNA concentration (± SD) isolated was 16.8 ± 31.4 µg / 250 mg
206 kidney tissue biopsy.
207
208 *Quantification of UCP2 gene expression by Real-Time qPCR*
209 Real-time reverse transcription-PCR was performed in two separate reactions: first, RNA
210 was reverse transcribed into cDNA, then cDNA was amplified by quantitative real-time PCR (RT-
211 qPCR). Reverse transcription of 5 µg of RNA into cDNA was carried out using the SuperScript™
212 VILO Master Mix for RT-PCR (Invitrogen - Life Technologies), following the manufacturer's
213 protocol for the random primer method.
214 RT-qPCR experiments were performed in a 7500 Fast Real-Time PCR System Thermal
215 Cycler (Life Technologies). Experiments were performed by monitoring in real-time the increase in

fluorescence of the SYBER® Green dye (30). Primers for *UCP2* and *GAPDH* genes were designed using published human gene sequences and the Primer Express 3.0 Software (Life Technologies), and they were projected to target two consecutive exons of a gene in order to prevent the amplification of any contaminating genomic DNA. Primer sequences were as follows: *UCP2* F 5'-TTGGGTTCAAGGCCACAGAT-3', *UCP2* R 5'-CCAGCCCCAAGAAACTTCAC-3', *GAPDH* F 5'-ACCCACTCCTCCACCTTTG-3', and *GAPDH* R 5'-CTCTTGTGCTCTTGCTGGG-3'.

PCR reactions were performed using 10 µL of 2x Fast SYBER® Green Master Mix (Life Technologies), 1 µL (1 ng/µL) of forward and reverse primers for *UCP2* or *GAPDH* and 1 µL of cDNA template (1.25 µg/µL), in a total volume of 20 µL. Each sample was assayed in triplicate and a negative control was included in each experiment. The thermocycling conditions for these genes were as follows: an initial cycle of 95°C for 20 seconds, followed by 50 cycles of 95°C for 3 seconds and 60°C for 1 minute. RT-qPCR specificity was determined using melting curve analyses and all primers generated amplicons that produced a single sharp peak during the analyses.

Quantification of *UCP2* mRNA was performed by relative quantification using the comparative ΔΔCq method (29, 31), and expressed relative to the reference gene (*GAPDH*). Validation assays were done by amplification of the target (*UCP2*) and reference (*GAPDH*) genes, separately, using serial dilutions of a cDNA sample. As a requirement of this method, both target and reference genes exhibited equal amplification efficiencies (*E* = 95-105%) in all experiments. The ΔΔCq method calculates changes in gene expression as relative fold differences (n-fold change) between an experimental and an external calibrator sample (29, 31).

UCP2 gene expression was analyzed in 42 kidney samples: 15 carrying the reference haplotype (-866G/55Ala/Del) in homozygosis, 15 heterozygous, and 12 carrying the mutated haplotype (-866A/55Val/Ins) in homozygosis. These numbers were sufficient to detect a 0.5 n fold difference between groups (beta = 80%, α = 0.05).

Statistical analyses

Allele frequencies were determined by gene counting and departures from the Hardy-Weinberg equilibrium (HWE) were verified using the χ^2 test. Allele and genotype frequencies were compared between groups using the χ^2 test. The haplotypes constructed from the combination of the three *UCP2* polymorphisms and their frequencies were inferred using the PHASE 2.1 program, which implements a Bayesian statistical method (32).

Clinical and laboratory characteristics and *UCP2* mRNA concentrations were compared between groups by using unpaired Student's t-test, one-way ANOVA or χ^2 test, as appropriate. Variables with normal distribution are presented as mean ± SD or percentage. Variables with a skewed distribution were logarithmically transformed before analyses and are presented as median (minimum – maximum values) or mean (95% CI).

The magnitude of the association of different *UCP2* polymorphisms or haplotypes with DKD was estimated using odds ratio (OR) tests with 95% CI. Multivariate logistic regression analyses were performed to assess the independent association of individual *UCP2* polymorphisms or haplotypes with DKD, as well as to control for possible confounding factors whenever a statistically significant association was found in univariate analyses. DM duration was not included as an independent variable in these analyses because the control group (without DKD) was selected based on this feature. Multiple linear regression analysis was performed with eGFR (logarithmic) as a dependent variable and age, sex, HAS, T2DM duration, and the presence of the mutated *UCP2* haplotype as independent variables. Moreover, linear regression analysis was performed with *UCP2* gene expression (logarithmic) as dependent variable and age, sex, diagnosis of DM and presence of the *UCP2* mutated haplotype as independent variables. Pearson's correlation test was used to assess correlations between different quantitative variables. A *P* value of <0.05 was considered statistically significant. These statistical analyses were done with SPSS version 18.0 (SPSS, Chicago, IL, USA).

Results

Study of the association between UCP2 polymorphisms and DKD

As expected, T2DM patients with DKD differed significantly from control patients for gender, T2DM duration, HDL cholesterol, triglycerides and creatinine levels, and occurrence of DR (**Table 1**). Frequencies of the *UCP2* -866G/A, Ala55Val and Ins/Del genotypes did not differ between white and black T2DM patients (all P values > 0.300). Neither genotype nor allele frequencies of the -866G/A, Ala55Val and Ins/Del polymorphisms differed statistically between cases with DKD and controls without this complication (**Table 2**), and all genotypes were in agreement with those predicted by the HWE in all groups (P > 0.05). Frequencies of mutated genotype carriers (dominant model) were also similar between groups, and the adjustment for covariables did not change these results (**Table 2**). It is worth mentioning that these polymorphisms remained not associated with DKD when taking into account recessive or additive inheritance models (data not shown). Moreover, frequencies of the analyzed *UCP2* polymorphisms were not significantly different between patients with moderately or severely increased AER (P ≥ 0.20, data not shown).

As already described, the -866G/A polymorphism is in almost complete LD with the Ala55Val polymorphism (|D'| = 0.991, r^2 = 0.905), but only in moderate LD with the Ins/Del polymorphism (|D'| = 0.855, r^2 = 0.485) in our population (20). The Ala55Val polymorphism is also in partial LD with the Ins/Del polymorphism (|D'| = 0.878, r^2 = 0.471). Seven haplotypes produced by the combination of the -866G/A, Ala55Val and Ins/Del polymorphisms were inferred in the total sample of T2DM patients. Haplotypes -866G/55Val/Del (reference; 52.5%), -866A/55Val/Del (13.0%) and -866A/55Val/Ins (mutated; 25.7%) were inferred in frequencies higher than 5% and altogether accounted for 91.2% of the observed haplotypes, with the remaining 8.8% being shared among haplotypes -866G/55Ala/Ins, -866G/55Val/Del, -866G/55Val/Ins, and -866A/55Ala/Del. Taking into consideration the results of our previous study showing that the mutated -866G/55Val/Ins haplotype was associated with increased risk for proliferative DR (20), only subjects carrying the mutated -866A/55Val/Ins haplotype (homozygosis/heterozygosis) or the reference -866G/55Ala/Del haplotype were selected for subsequent analyses. Of note, frequencies

of the mutated haplotype were similar between white and black T2DM patients: frequencies of the mutated haplotype in a recessive model: 9.6% in white patients *vs*. 8.4 in black patients (P = 0.702); frequencies in a dominant model: 50.3% in white vs. 48.9% in black patients (P= 0.762).

The frequency of the mutated haplotype (recessive model) was higher in patients with DKD (11.5%) as compared to control patients (6.5%); however, this difference did not reach formal statistical significance (P = 0.071). Interestingly, after adjusting for age, gender, treatment with ACE-inhibitors, triglycerides levels and eGFR, homozygosis for the mutated haplotype was statistically associated with risk for DKD (OR = 2.136, 95% CI 1.036-4.404; **Table 2**).

Interestingly, T2DM patients carrying the minor alleles of the analyzed *UCP2* polymorphisms showed decreased eGFR when compared to subjects homozygous for the reference genotypes (**Figure 1**). Accordingly, patients carrying the mutated haplotype (dominant model) showed a decreased eGFR when compared to subjects with the reference haplotype (P = 0.018; Figure 1), and this difference remained statistically significant after adjusting for age, gender, HAS and T2DM duration (β = -2.231, P = 0.035).

UCP2 gene expression in human kidney biopsies according to the presence of the -866A/55Val/Ins haplotype

UCP2 gene expression was analyzed in 42 human kidney biopsies collected from 15 patients homozygous for the *UCP2* reference haplotype (-866G/55Ala/Del), 15 heterozygous, and 12 homozygous for the *UCP2* mutated haplotype (-866A/55Val/Ins). The main clinical characteristics of this group were as follows: mean age was 58.3 $\pm$ 1.2 years, men comprised 45.2 % (n = 19) of the sample, 57.1% (n = 24) of all patients had AH, and 19.0% (n = 8) had DM.

The mean $\pm$ SD *UCP2* mRNA concentration in the whole kidney tissue group was 0.88 $\pm$ 1.39 n fold change (logarithmic scale). No significant difference was observed when *UCP2* gene expression was analyzed by gender (men: 0.84 $\pm$ 1.59 *vs*. women: 0.91 $\pm$ 1.21 n fold change; P = 0.879), AH status (normotensive: 0.59 $\pm$ 1.57 *vs*. hypertensive: 0.97 $\pm$ 1.31 n fold change; P = 0.442), or presence of DM (non-diabetic patients: 0.76 $\pm$ 1.44 *vs*. DM patients: 0.93 $\pm$ 1.30; P = 0.762 n fold change). *UCP2* gene expression did not correlated with age (r^2 = -0.054, P = 0.739).

323 *UCP2* gene expression in kidney samples stratified by the presence of the selected
324 *UCP2* haplotypes is depicted in **Figure 2**. *UCP*2 gene expression was decreased in kidneys from
325 *UCP2* mutated haplotype carriers when compared to kidneys from patients with the reference
326 haplotype (0.32 ± 1.20 *vs*. 1.85 ± 1.16 n fold change, respectively; $P < 0.0000001$). *UCP2* gene
327 expression was similar between patients heterozygous or homozygous for the mutated haplotype
328 ($P = 0.750$ from Tukey's post hoc test; **Figure 2**). After linear regression analysis, the presence of
329 the mutated haplotype remained significantly associated with decreased UCP2 gene expression
330 after controlling for age, sex and presence of DM ($\beta = -1.913$, $P < 0.00001$).

Discussion

In the present study, we investigated the frequencies of the *UCP2* -866G/A, Ala55Val and Ins/Del polymorphisms in a sample of T2DM patients subdivided according to presence/absence of DKD. Homozygosis for the -866A/55Val/Ins haplotype was associated with risk for DKD after adjustment for covariables. Furthermore, the minor alleles of the analyzed *UCP2* polymorphisms as well as presence of the mutated haplotype were associated with lower eGFR when compared to subjects homozygous for reference genotypes or -866G/55Ala/Del haplotype.

Although UCP2 plays an acknowledged role in protection against oxidative stress (33), and although oxidative stress is one of the major contributors to the pathogenesis of chronic diabetic complications (10), only a few studies have evaluated the association between *UCP2* polymorphisms and DKD or related phenotypes. Rudofsky *et al.* (34) reported that German type 1 DM patients carrying the -866A allele had reduced prevalence of diabetic peripheral neuropathy when compared with patients with the G/G genotype; however, they did not find any association between the -866G/A polymorphism and DKD or DR, which could be explained by the small sample number analyzed (n = 227). Rudofsky *et al*. (35) studying T2DM patients from German also did not observe any association between -866G/A polymorphism and DKD, DR or diabetic peripheral neuropathy. In addition, Lindholm *et al*. (36) reported that Ins/Del polymorphism was not associated with DKD in 434 T2DM patients from Scandinavia. Tripathi *et al*. (37) reported a significant association between the Ins/Del polymorphism and risk for ESRD (OR = 8.856; 95% CI 3.458-22.667) in subjects from North India; nevertheless, this result should be interpreted with caution since genotype distributions of this polymorphism were not in HWE in the control group. None of these studies evaluated the association between *UCP2* polymorphisms and eGFR. Further studies are urgently needed to evaluate the association between *UCP2* polymorphisms and DKD and related features in other populations.

Functional polymorphisms can influence gene expression and regulate the final quantity of protein in a given tissue. Therefore, in this study, we also demonstrated that human kidney biopsy samples from patients carrying the mutated *UCP2* -866A/55Val/Ins haplotype, in heterozygosis or

359 homozygosis, showed a 5-fold decrease in *UCP2* gene expression when compared to kidneys
360 from patients with the reference haplotype. This finding is biologically plausible since both -866G/A
361 and Ins/Del polymorphisms have been reported as functional polymorphisms (21, 38-42).

362 In humans, the *UCP2* -866A allele has been reported as being associated with either
363 increased (38, 39) or decreased (21, 41, 43) *UCP2* mRNA levels. A possible explanation for these
364 conflicting results is that this polymorphism seems to be involved in putative binding sites for
365 specific transcription factors (39). Thus, preferential binding of some transcriptional factor to the G
366 or A allele in the *UCP2* promoter could confer tissue-specific advantages to either allele (39). The
367 Ins/Del polymorphism is located in the 3´UTR region of the *UCP2* gene, and it seems to be
368 functional because mRNA transcribed from the *UCP2* sequence containing the Ins allele displayed
369 a shorter half-life in a fetal myoblast cell line than mRNA transcribed from the sequence carrying
370 the Del allele (36). The Ala55Val polymorphism causes a conservative amino acid change and,
371 until this date, there has been no indication that it causes a functional change in the protein.
372 Therefore, taking into account that the Ala55Val polymorphism is in tight LD with the -866G/A
373 polymorphism and in moderate LD with the Ins/Del polymorphism, it is probable that this
374 polymorphism is only reflecting the -866G/A or Ins/Del polymorphism effects on *UCP2* gene
375 expression.

376 Further studies are necessary to better define if the -866G/A and Ins/Del polymorphisms
377 have a synergistically effect on *UCP2* gene expression or if one of them has a major effect on it.
378 Alternatively, there is a possibility that the three analyzed *UCP2* polymorphisms are not
379 themselves responsible for the observed association with DKD, only being in LD with a still
380 unknown functional polymorphism. Nevertheless, previous functional studies indicate that the -
381 866G/A and Ins/Del polymorphisms could be directly leading to changes in *UCP2* gene expression
382 (43, 44). Moreover, the -866A allele was reported as being associated with lower plasma total
383 antioxidant status (increase oxidative stress) in DM patients with coronary heart disease (45),
384 which could explain the association of the mutated haplotype containing this allele with risk for
385 diabetic complications, including DKD.

Considering the data presented here, we therefore hypothesized that the decreased *UCP2* gene expression in kidney from carriers of the -866A/55Val/Ins haplotype might be associated with increased ROS in this tissue. Thereby, T2DM patients carrying the mutated *UCP2* haplotype could have an increased risk for DKD development since UCP2 concentration in their kidneys might not be enough to compensate the oxidative stress produced by chronic hyperglycemia. In agreement with our hypothesis, a recent study showed that genipin, an UCP2 inhibitor, dramatically boosted oxidative stress in rat renal proximal tubular cells incubated with high glucose concentrations, and this exacerbated cellular apoptosis due to an increase in caspase-3 activation (46). In addition, He *et al*. (47) demonstrated that HUVECs (human umbilical vein endothelial cells) treated with high glucose showed an upregulation of caspase-3 and cytochrome c and the downregulation of Bcl-2 when compared to cells incubated with normal glucose concentrations. *UCP2* overexpression was able to inhibit the apoptosis of HUVECs induced by hyperglycemia. Based on these results, the authors suggested the application of UCP2 as a new protective factor for chronic diabetic complications. In contrast, Qiu *et al*. (48), reported that oral administration of genipin to diabetic mice postponed the progression of DKD, attenuating glomerular basement membrane thickness, and restoring the expression of podocin and WT1 in podocytes. They concluded that the improvement in podocyte injury was probably through the suppression of UCP2 in diabetic kidneys, which attenuated glucose-induced albumin leakage through podocytes monolayer. Thus, the role of UCP2 in kidneys still needs to be clarified.

Some factors could have interfered with the results of our case-control study. First, we cannot rule out the possibility of population stratification bias when analyzing our samples, although the number of black patients was similar in case and control groups, and frequencies of the analyzed *UCP2* polymorphisms were also similar between white and black patients. Moreover, both case and control groups were recruited from the same hospital, thus reducing the risk of false positive/negative associations due to this bias. Second, we cannot exclude the possibility of a type II error when investigating the association between the analyzed polymorphisms and DKD. We had more than an 80% power (α = 0.05) to detect an OR ≥ 1.7 for the association with the -866G/A and Ala55Val polymorphisms, and we had an 80% power to detect an OR ≥ 2.0 for the Ins/Del

414 polymorphism. Thus, we cannot rule out the possibility that these polymorphisms would be
415 associated with DKD with lower ORs. The results of our *UCP2* gene expression study in kidney
416 biopsies also should be interpreted with caution as most of our sample was constituted by patients
417 who undergone nephrectomy, and for whom we did not have information about DKD diagnosis.
418 Thus, further studies will be necessary to confirm whether the -866A/55Val/Ins haplotype is also
419 associated with changes in *UCP2* gene expression in kidneys from DM patients with different
420 degrees of DKD and eGFR.

421 In conclusion, data reported here suggest that the *UCP2* -866A/55Val/Ins haplotype is
422 associated with an increased risk of DKD and with a lower eGFR in T2DM patients. Furthermore,
423 this mutated haplotype was associated with decreased *UCP2* gene expression in human kidneys.
424 Further additional studies will be necessary to confirm the association between the *UCP2* -
425 866A/55Val/Ins haplotype and DKD as well as to elucidate how this haplotype increases the risk of
426 this diabetic complication. Moreover, therapeutic strategies to counteract ROS through reinforcing
427 the action of UCP2 should be explored.

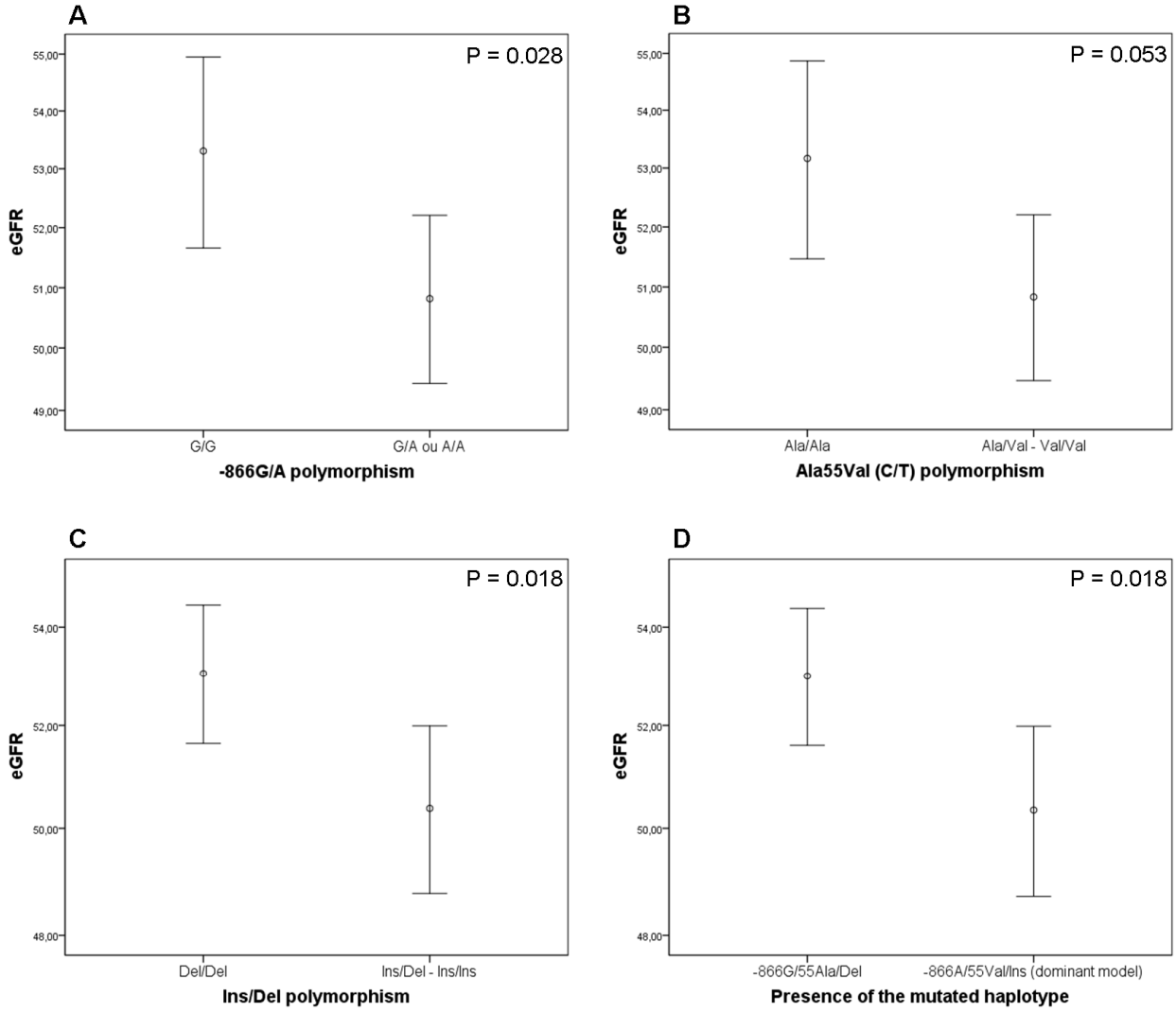
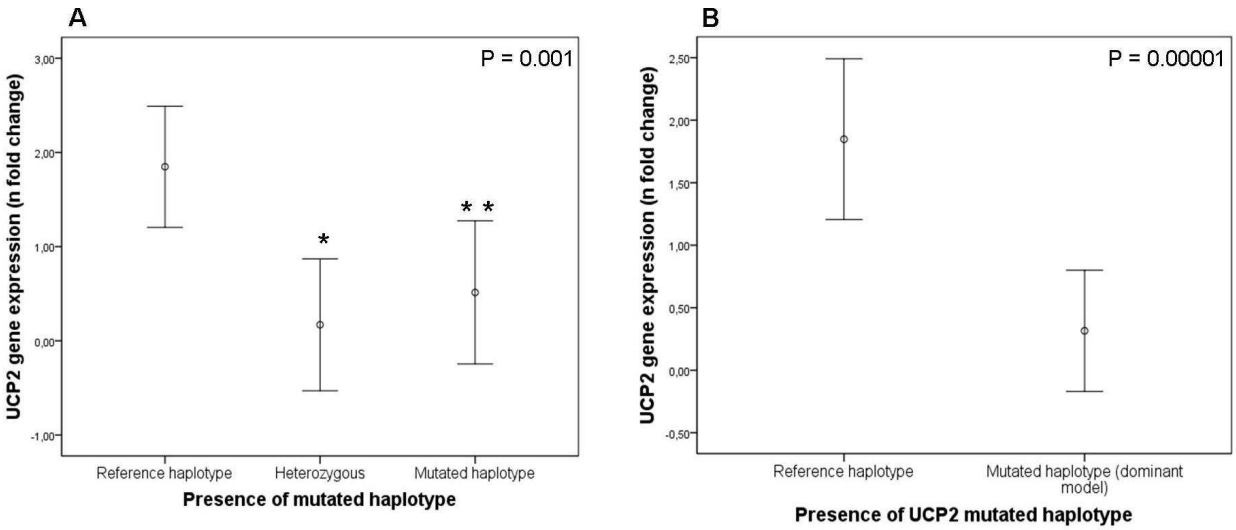


Figure 1: Estimated glomerular filtration rate (eGFR) in T2DM patients according to different *UCP2* polymorphisms and presence of the mutated *UCP2* haplotype. **A)** eGFR in patients stratified according to the presence of the A allele of the -866G/A polymorphism (dominant model). **B)** eGFR in patients stratified according to the presence of the Val allele of the Ala55Val polymorphism (dominant model). **C)** eGFR in patients according to the presence of the Ins allele of the Ins/Del polymorphism (dominant model). **D)** eGFR in patients according to the presence of the *UCP2* mutated haplotype (-866A/55Val/Ins; dominant model). P values were obtained using Student's t-tests. Data are presented as mean (95% CI).



437

438 **Figure 2**: *UCP2* gene expression in human kidney biopsies according to the presence of the *UCP2*
439 mutated haplotype. **A)** *UCP2* gene expression in subjects homozygous for the reference haplotype
440 (-866G/55Ala/Del), heterozygous (reference/mutated haplotypes) or homozygous for the mutated
441 haplotype (-866A/55Val/Ins). P value was obtained using One-Way ANOVA test. * P = 0.001 (post-
442 hoc Tukey's test). ** P = 0.02 (post-hoc Tukey's test). **B)** *UCP2* gene expression in patients
443 stratified according to the presence of the *UCP2* mutated haplotype (dominant model). P value was
444 obtained using Studant's t-test. Data are presented as mean (95% CI) of *UCP2* gene expression in
445 logarithmic scale.

446 **Table 1.** Clinical and laboratory characteristics of T2DM patients broken down by the presence
447 of diabetic kidney disease.

	Control group (n = 281)	**Case group** (n = 287)	**P* value**
Age (years)	61.4 ± 9.6	60.2 ± 10.2	0.281
Gender (% males)	34.2	56.4	< 0.000001
Ethnicity (% black)	21.9	21.3	0.860
T2DM duration (years)	16.5 ± 6.5	14.7 ± 9.1	0.006
BMI (kg/m²)	28.4 ± 4.7	29.0 ± 5.1	0.153
HbA1c (%)	6.99 ± 1.98	6.88 ± 2.12	0.537
Systolic BP (mmHg)	142.5 ± 22.3	144.8 ± 22.6	0.226
Diastolic BP (mmHg)	85.8 ± 13.1	86.3 ± 13.4	0.669
Total cholesterol (mg/dL)	208.9 ± 45.0	213.8 ± 49.1	0.275
HDL cholesterol (mg/dL)	46.1 ± 12.1	42.9 ± 12.6	0.002
Triglycerides (mg/dL)	145 (35 – 892)	164 (44 – 1470)	0.005
Diabetic retinopathy (%)	42.8	66.2	< 0.000001
Creatinine (µg/dL)	0.9 (0.5 – 2.99)	1.1 (0.4 – 13.6)	< 0.000001

448 Data are mean ± SD, median (minimum-maximum values) or %. BMI, body mass index; BP, blood
449 pressure; HbA1c, glycated hemoglobin; T2DM, type 2 diabetes mellitus. *P values are according to
450 χ^2 test or t-test as appropriate.

451 **Table 2.** Genotype and allele distributions of *UCP2* polymorphisms in T2DM patients with and without DKD.

***UCP2* polymorphisms**	**Control group**	**Case group**	**Unadjusted P value***	**Adjusted OR (95% CI) / P value§**
-866 (G/A)	n = 278	n = 287		
G/G	99 (35.6)	101 (35.2)	0.965	1
G/A	131 (47.1)	134 (46.7)		0.763 (0.446-1.304) / 0.322
A/A	48 (17.3)	52 (18.1)		0.825 (0.424-1.607) / 0.572
G	0.592	0.585	0.875	-
A	0.408	0.415		
Dominant Model				
G/G	99 (35.6)	101 (35.2)	0.987	1
G/A + A/A	179 (64.4)	186 (64.8)		0.780 (0.471-1.293) / 0.336
Ala55Val (C/T)	n = 281	n = 287		
C/C	93 (33.1)	102 (35.5)	0.828	1
C/T	135 (48.0)	133 (46.3)		0.700 (0.407-1.202) / 0.196

T/T	53 (18.9)	52 (18.2)		0.793 (0.408-1.542) / 0.494
C	0.571	0.587	0.629	-
T	0.429	0.413		
Dominant Model				
C/C	93 (33.1)	102 (35.5)	0.600	1
C/T + T/T	188 (66.9)	185 (64.5)		0.726 (0.436-1.209) / 0.219
45 bp Ins/Del	n = 278	n = 287		
D/D	132 (47.5)	144 (50.5)	0.181	1
I/D	124 (44.6)	110 (38.3)		0.753 (0.459-1.235) / 0.261
I/I	22 (7.9)	33 (11.5)		1.218 (0.539-2.752) / 0.636
D	0.698	0.700	0.978	-
I	0.302	0.300		
Dominant model				
D/D	132 (47.5)	144 (50.2)	0.578	1

I/D + I/I	146 (52.5)	143 (49.8)		0.822 (0.513-1.317) / 0.415
Presence of *UCP2* mutated haplotype	n = 278	n = 287		
Dominant model				
Other haplotypes	141 (50.7)	145 (50.5)	0.963	1
_A/_Val/_Ins[a]	137 (49.3)	142 (49.5)		0.816 (0.498-1.336) / 0.418
Recessive model				
Other haplotypes	260 (93.5)	255 (88.9)	0.071	1
A Val Ins / A Val Ins[b]	18 (6.5)	32 (11.1)		2.136 (1.036-4.404) / 0.040

Data are presented as number of carriers (%) or proportion. *P values were computed using χ^2 tests to compare control (T2DM patients without DKD and with more than 10 years of DM duration) and case (T2DM patients with DKD) groups. § Adjusted OR (95% CI) / P values adjusted for age, gender, treatment with ACE-inhibitors, triglycerides levels, and eGFR (logarithmic scale) in logistic regression analyses. [a] Presence of the mutated A Val Ins haplotype (homozygosis + heterozygosis; dominant model); [b] Mutated haplotype in homozygosis *vs*. other haplotypes (recessive model).

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Conflict of interest

Nothing to declare.

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4. CONCLUSÕES E PERSPECTIVAS

Os dados apresentados neste trabalho de conclusão de curso sugerem que o haplótipo mutado 866A/55Val/Ins está associado com um risco aumentado para DRD e com uma menor TFGe em pacientes com T2DM. Ademais, esse haplótipo mutado foi associado com uma diminuição na expressão do gene *UCP2* em amostras de rim humano. Estudos adicionais serão necessários para confirmar esta associação entre o haplótipo -866A/55Val/Ins do gene *UCP2* e a DRD assim como elucidar de que forma este haplótipo aumenta o risco dessa complicação crônicas do diabetes. Além disso, estratégias terapêuticas para contrabalançar as espécies reativas de oxigênio através do reforço da ação da UCP2 deveriam ser exploradas.

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