UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS:

ENDOCRINOLOGIA

ESTUDO DA ASSOCIAÇÃO DE POLIMORFISMOS NOS GENES DAS UCPS COM SUSCETIBILIDADE AO DIABETES MELLITUS TIPO 2 E DOENÇA RENAL DO DIABETES

TESE DE DOUTORADO

BIANCA MARMONTEL DE SOUZA

Porto Alegre, novembro de 2014

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“A verdadeira viagem do descobrimento

não consiste em procurar novas paisagens,

mas em ter novos olhos”

Marcel Proust

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- Artigo original: "Associations between *UCP1* -3826A/G, *UCP2* -866G/A, Ala55Val and Ins/Del, and *UCP3* -55C/T Polymorphisms and Susceptibility to Type 2 Diabetes Mellitus: Case-Control Study and Meta-Analysis" (artigo publicado na revista PLOS One em 2013).

- Artigo original: "Polymorphisms of the *UCP2* gene are associated with diabetic kidney disease and glomerular filtration rate in type 2 diabetic patients and with decreased *UCP2* gene expression in human kidney" (a ser submetido à revista European Journal of Endocrinology).

LISTA DE ABREVIATURAS PARA A INTRODUÇÂO

CRM	Cadeia respiratória mitocondrial
DM	*Diabetes Mellitus*
DM1	*Diabetes Mellitus* Tipo 1
DM2	*Diabetes Mellitus* Tipo 2
DRC	Doença renal crônica
DRD	Doença renal do diabético
EROs	Espécies reativas de oxigênio
FOXA1	*Forkhead Box A1*
IMC	Índice de massa corporal
NP	Neuropatia periférica
PGC-1α	*PPARγ coativator-1α*
PPAR	*Peroxisome proliferator-activated receptor*
RI	Resistência à insulina
RD	Retinopatia diabética
SIRT-1	*Sirtuin-1*
SREBP-1c	S*terol regulatory element binding-protein-1c*
TFG	Taxa de filtração glomerular
TFGe	Taxa de filtração glomerular estimada
TAB	Tecido adiposo branco
TAM	Tecido adiposo marrom
UCPs	Proteínas desacopladoras
UCP1	Proteína desacopladora 1
UCP2	Proteína desacopladora 2

UCP3	Proteína desacopladora 3
β3-AR	3-adrenérgico

LISTA DE ABREVIATURAS PARA OS ARTIGOS ORIGINAIS

3`UTR	3' untranslated region
AER	Albumin excretion rate
AGE	Glycation end-products
AH	Arterial hypertension
BCL-2	Linfoma de células B
BMI	Body mass index
BP	Blood pressure
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
T2DM	Type 2 diabetes mellitus
DKD	Diabetic kidney disease
DPN	Diabetic peripheral neuropathy
DR	Diabetic retinopathy
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
FFA	Free fatty acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GHb	Glycated haemoglobin
GFR	Glomerular filtration rate
HWE	Hardy-Weinberg equilibrium
LD	Linkage disequilibrium
MeSH	Medical subject headings
MDRD	Modification of Diet in Renal Disease

NOS	Newcastle-Ottawa Scale
OR	Odds ratio
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
REM	Random effect model
RT-qPCR	Quantitative real-time PCR
FEM	Fixed effect model
PCR	Polymerase chain reaction
UCPs	Uncoupling proteins
UCP1	Uncoupling protein 1
UCP2	Uncoupling protein 2
UCP3	Uncoupling protein 3

SUMÁRIO

RESUMO

Está bem estabelecido que fatores genéticos têm um papel importante no desenvolvimento do *diabetes mellitus* tipo 2 (DM2) bem como de suas complicações crônicas e que indivíduos geneticamente suscetíveis podem desenvolver essa doença após exposição a fatores de risco ambientais. Sendo assim, grandes esforços têm sido feitos para se identificar os genes associados com DM2 e suas complicações crônicas.

As proteínas desacopladoras 1 a 3 (UCP1-3) são membros de uma superfamília de proteínas localizadas na membrana mitocondrial interna. A UCP1 é expressa principalmente no tecido adiposo marrom, a UCP2 é amplamente distribuída em diversos tecidos, enquanto a UCP3 é basicamente restrita ao músculo esquelético. Essas proteínas desacoplam a oxidação dos substratos da síntese de ATP pela ATP-sintase, dissipando o potencial de membrana e, consequentemente, diminuindo a produção de ATP pela cadeia respiratória mitocondrial. A dissipação de energia pelas UCPs está associada a diversas funções: produção de calor (UCP1), regulação do metabolismo e transporte de ácidos graxos livres (UCP2 e UCP3), diminuição da formação de espécies reativas de oxigênio (EROs) (UCP1-3) e regulação negativa da secreção de insulina (UCP2), mecanismos envolvidos na patogênese do DM2 e/ou de suas complicações crônicas. Dessa forma, polimorfismos nos genes *UCP1-3* podem estar envolvidos no desenvolvimento destas doenças.

A relação entre polimorfismos nos genes *UCP1-3* e suscetibilidade ao DM2 tem sido investigada em diversas populações. No entanto, o impacto desses polimorfismos no DM2 ainda está em debate, com resultados contraditórios sendo relatados. Sendo assim, realizou-se um estudo de caso-controle na nossa população, seguido de uma revisão sistemática e metanálise dos estudos disponíveis na literatura para se avaliar se os seguintes polimorfismos estavam associados com suscetibilidade ao DM2: -3826A/G

(*UCP1*); -866G/A, Ala55Val e Ins/Del (*UCP2*) e -55C/T (*UCP3*). Cabe ressaltar que os resultados do nosso estudo caso-controle foram incluídos na metanálise.

No estudo de caso-controle, em indivíduos brancos, não encontramos nenhuma associação dos polimorfismos analisados com suscetibilidade ao DM2. Nossos resultados da meta-análise demonstraram que os polimorfismos -3826A/G (*UCP1*; rs1800592), -866G/A (*UCP2*; rs659366) e Ins/Del (*UCP2*), não estão associados com DM2. Por outro lado, o alelo Ala do polimorfismo Ala55Val (rs660339) no gene *UCP2* e o alelo C do polimorfismo -55C/T (rs1800849) no gene *UCP3* foram associados com risco para o DM2 em asiáticos, mas não em europeus.

Está bem definido que as UCP1-3 diminuem a formação de EROs pela mitocôndria e que a superprodução de EROs é um dos principais fatores envolvidos na patogênese das complicações crônicas diabéticas. Entre as UCPs, a UCP2 é a mais expressa na retina e rins; portanto, polimorfismos neste gene podem estar envolvidos na patogênese dessas complicações. Recentemente, nós relatamos que o haplótipo mutado -866A/55Val/Ins no gene *UCP2* foi associado com risco aumentado para retinopatia diabética proliferativa em pacientes diabéticos tipo 1 e 2. Posteriormente, também demonstramos que portadores deste haplótipo mutado tinham uma expressão diminuída de *UCP2* na retina humana de doadores de córnea em comparação aos homozigotos para o haplótipo de referência (-886G/55Ala/Del).

No presente estudo, nós investigamos se os polimorfismos -866G/A, Ala55Val e Ins/Del (*UCP2*) estavam associados com doença renal do diabético (DRD) em pacientes com DM2 (delineamento caso-controle) e também se existia algum efeito destes polimorfismos sobre a expressão de *UCP2* em biópsias de tecido renal humano (delineamento transversal). No estudo de caso-controle foram analisados 287 pacientes com DM2 e DRD (casos) e 281 pacientes com DM2 sem esta complicação e com mais

de 10 anos de duração do DM2 (controle). No estudo transversal foram incluídas 42 amostras de biópsia de rim obtidas a partir de indivíduos que sofreram nefrectomia terapêutica.

No grupo de pacientes com DM2, a homozigose para o haplótipo -866A/55Val/Ins foi um fator de risco independente para DRD (RC = 2,136, IC 95% 1,036-4,404). Interessantemente, pacientes com DM2 portadores do haplótipo mutado apresentaram diminuição da taxa de filtração glomerular estimada (TFGe) quando comparados com indivíduos com o haplótipo de referência. Em amostras de biópsias renais, a expressão do gene *UCP2* foi significativamente menor em portadores do haplótipo mutado quando comparados com o haplótipo de referência (0,32 ± 1,20 *vs*. 1,85 ± 1,16 *n fold change*; p ajustado < 0,000001).

Em conclusão, os polimorfismos -3826A/G (*UCP1*), -866G/A, A55Val e Ins/Del (*UCP2*) e -55C/T (*UCP3*) não estão associados com DM2 em indivíduos brancos da nossa população. Entretanto, nossa meta-análise mostrou uma associação significativa entre os polimorfismos A55Val e -55C/T e risco para DM2 em populações asiáticas. Já a presença do haplótipo *UCP2* -866A/55Val/Ins foi associada com aumento do risco para DRD e com diminuição da TFGe em pacientes com DM2. Além disso, este haplótipo foi associado com a diminuição da expressão do gene *UCP2* em rins humanos.

ABSTRACT

It is well established that genetic factors play an important role in the development of type 2 *diabetes mellitus* (T2DM) and its chronic complications, and that genetically susceptible subjects might develop the disease after being exposed to environmental risk factors. Therefore, great efforts have been made to identify genes associated with T2DM and its chronic complications.

Uncoupling proteins 1, 2 and 3 (UCP1-3) are members of an anion-carrier protein family located in the mitochondrial inner membrane. UCP1 is mainly expressed in brown adipose tissue, UCP2 is widely distributed in several tissues, whereas UCP3 is mainly restricted to the skeletal muscle. These proteins act by uncoupling the oxidation of substrates from ATP synthesis by ATP-synthase, thereby dissipating the membrane potential and, consequently, decreasing the ATP production by the mitochondrial respiratory chain. This uncoupling effect then leads to different functions, such as thermogenesis (UCP1), regulation of free fatty acids metabolism and transport (UCP2 and UCP3), reduction in reactive oxygen species (ROS) formation (UCP1-3), and negative regulation of insulin secretion (UCP2), mechanisms involved in the pathogenesis of T2DM and/or its chronic complications. Therefore, polymorphisms in *UCP1-3* genes might be associated with the development of these diseases.

The relationship between *UCP1-3* polymorphisms and susceptibility to T2DM has been investigated in several populations. Nevertheless, the impact of these polymorphisms on T2DM is still under debate, with contradictory results being reported. Thus, we performed a case-control study in our population followed by a systematic review and meta-analysis of published studies in order to evaluate whether the following polymorphisms were associated with T2DM susceptibility: -3826A/G

(*UCP1*), -866G/A, Ala55Val and Ins/Del (*UCP2*) and -55C/T (*UCP3*). It is worth noting that results obtained in our case-control study were also included in the meta-analysis.

In the case-control study, analyzing white subjects, we did not found any association between the analyzed polymorphisms and susceptibility for T2DM. Meta-analysis results showed that the -3826A/G (*UCP1*; rs1800592), -866G/A (*UCP2*; rs659366) and Ins/Del (*UCP2*) polymorphisms were not associated with T2DM. In contrast, the A allele of the Ala55Val polymorphism (*UCP2*; rs660339) and the C allele of the -55C/T polymorphism (*UCP3*; rs1800849) were significantly associated with risk to T2DM in Asians but not in Europeans.

It is well stablished that UCP1-3 decrease ROS formation by mitochondria, and that ROS overproduction is one of the major contributors to the pathogenesis of chronic diabetic complications. Among UCPs, UCP2 has the higher expression in human retina and kidneys; therefore, polymorphisms in this gene could be involved in the pathogenesis of these complications. Recently, we reported that the -866A/55Val/Ins haplotype in the *UCP2* gene was associated with increased risk for proliferative diabetic retinopathy in both type 1 and type 2 diabetic patients. Afterwards, we also demonstrated that mutated haplotype carriers had a decreased *UCP2* gene expression in retina from cornea donors when compared to retina from subjects homozygous for the reference haplotype (-886G/55Ala/Del).

In the present study, we investigated whether the -866G/A, Ala55Val and Ins/Del polymorphisms (*UCP2*) were associated with diabetic kidney disease (DKD) in T2DM patients (case-control design), and also if they had any effect on *UCP2* gene expression in human kidney tissue biopsies (cross-sectional design). In the case-control study, we analyzed 287 T2DM patients with DKD (cases) and 281 T2DM patients

without this complication and with more than 10 years of T2DM duration (controls). In the cross-sectional study, we included 42 kidney biopsy samples obtained from patients who undergone therapeutic nephrectomy.

In the T2DM group, homozygosis for the mutated haplotype (-866A/55Val/Ins) was an independent risk factor for DKD (OR = 2.136, 95% CI 1.036-4.404). Interestingly, T2DM patients carrying the mutated haplotype showed decreased estimated glomerular filtration rate (eGFR) when compared to subjects with the reference haplotype. In kidney biopsy samples, *UCP2* gene expression was significantly decreased in *UCP2* mutated haplotype carriers when compared to reference haplotype (0.32 ± 1.20 vs. 1.85 ± 1.16 n fold; adjusted $P < 0.000001$).

In conclusion, -3826A/G (*UCP1*), -866G/A, A55Val and Ins/Del (*UCP2*) and -55C/T (*UCP3*) polymorphisms are not associated with T2DM in white subjects from our population. However, our meta-analysis showed a significant association between Ala55Val and -55C/T polymorphisms and risk for T2DM in Asians but not in Europeans. The presence of the *UCP2* -866A/55Val/Ins haplotype seems to be associated with risk for DKD and with a decreased eGFR in T2DM patients. Moreover, this mutated haplotype was associated with decreased *UCP2* gene expression in human kidneys.

INTRODUÇÃO

O *Diabetes Mellitus* e suas complicações crônicas

Estima-se que 7,6% da população brasileira tenha *diabetes mellitus* [1] [2]. Esta doença constitui um sério problema de saúde pública devido a sua alta prevalência, taxas aumentadas de morbidade e mortalidade e a altas repercussões econômicas e sociais relacionadas ao impacto de suas complicações crônicas, as quais comprometem a qualidade de vida e produtividade dos indivíduos afetados [3].

O Diabetes Mellitus Tipo 2 (DM2) corresponde a 90-95% dos casos de DM, geralmente ocorre em indivíduos com mais de 40 anos de idade e obesos e é caracterizado por uma hiperglicemia crônica causada por um desequilíbrio entre a ação e a secreção de insulina [3]. Na maioria dos casos, a anormalidade inicial detectável é uma diminuição na sensibilidade das células-alvo (principalmente músculos, tecido adiposo e fígado) à ação da insulina (resistência à insulina - RI). Para compensar a RI, as células-beta pancreáticas aumentam a secreção desse hormônio (hiperinsulinemia), mantendo uma glicemia normal. Entretanto, com o passar do tempo, ocorre um esgotamento na capacidade secretória dessas células, fazendo com que a homeostase glicêmica no jejum não possa mais ser mantida e a forma clínica dessa doença seja detectada [4, 5].

O DM2 é uma desordem multifatorial cuja suscetibilidade é determinada pela interação de múltiplos fatores genéticos e ambientais [6, 7]. O dramático aumento na prevalência de DM2 observada nas últimas décadas pode ser explicado por mudanças nos padrões alimentares e de atividade física. Entretanto, reconhece-se que estas mudanças ambientais provavelmente só levam ao DM2 na presença de fatores genéticos

de risco. Dessa forma, tem-se buscado a identificação de genes associados ao DM2 e diversos estudos têm se focado em genes relacionados ao gasto energético, uma vez que esta doença está frequentemente associada com obesidade [8, 9].

Como o DM2 é frequentemente assintomático em seus estágios iniciais, os pacientes com essa forma de DM podem permanecer sem diagnóstico por vários anos. No entanto, mesmo uma hiperglicemia crônica discreta pode provocar lesões estruturais no endotélio vascular e no tecido nervoso que causam danos, disfunções e falhas de diversos órgãos e tecidos, levando ao aparecimento das complicações crônicas do DM [3]. Essas complicações podem ser categorizadas em microvasculares [retinopatia diabética (RD), doença renal do diabetes (DRD) e neuropatia periférica (NP)] ou macrovasculares (infarto agudo do miocárdio, acidente vascular cerebral e gangrena) [3]. Dentre as complicações crônicas microvasculares do DM, a RD é a principal causa de cegueira adquirida em adultos [10], a DRD é a causa mais frequente de falência e necessidade de transplante de rins em vários países [11] e a NP é responsável por 50-75% das amputações não-traumáticas de membros inferiores [12].

De uma forma geral, a presença dessas complicações depende do tempo de DM, idade do paciente, hipertensão arterial, dislipidemia, suscetibilidade genética do paciente ao tipo de complicação e a intensidade e persistência da hiperglicemia [13]. Um dos principais mecanismos pelo qual a hiperglicemia leva ao aparecimento das complicações microvasculares do DM é através do aumento da produção de espécies reativas de oxigênio (EROs) pela mitocôndria [10, 14, 15]. Neste contexto, Du *et al*. [16] propuseram uma teoria unificadora unindo importantes rotas envolvidas na patogênese das complicações microvasculares do DM. De acordo com esta hipótese, a produção aumentada de EROs, induzida pela hiperglicemia, resulta no aumento da ativação de isoformas da proteína quinase C, na formação aumentada de produtos de

glicação avançada, na aceleração do fluxo de glicose através da via da aldose redutase e no aumento do fluxo de glicose na via da hexosamina. Estas alterações, com variação determinada em parte por fatores genéticos interagindo com fatores ambientais, culminarão no desenvolvimento das complicações microvasculares do DM [15].

A DRD, até pouco tempo atrás conhecida como nefropatia diabética (ND), é uma importante complicação crônica do DM, sendo a principal causa de doença renal crônica (DRC) terminal, que requer tratamento dialítico ou transplante renal [11, 17]. Esta complicação afeta aproximadamente 40% de pacientes com DM2, sendo uma importante causa de morbidade e mortalidade entre esses pacientes [1, 17, 18]. No Rio Grande do Sul, cerca de 37% dos pacientes com DM2 apresentam doença renal [19]. Normalmente, a DRD é uma doença progressiva, caracterizada por alterações fisiopatológicas resultantes do ambiente diabético, que começam com a hiperfiltração renal e hipertrofia glomerular, podendo progredir para proteinúria e uma diminuição gradual da taxa de filtração glomerular (TFG) [11, 18]. A albuminúria reflete a gravidade da disfunção renal, podendo ser atualmente classificada em: 1) albuminúria normal ou levemente aumentada, 2) albuminúria moderadamente aumentada ou, 3) albuminúria severamente aumentada [20]. Além disso, recomenda-se a estimativa da TFG para o rastreamento apropriado da DRD, uma vez que, alguns pacientes com valores normais de albumina já podem apresentar uma diminuição na TFG [21]. Sendo assim, atualmente, as medidas de albuminúria e TFG estimada constituem os principais critérios diagnósticos da DRD, guiando o rastreamento, classificação e o tratamento dessa complicação [22].

Diversos estudos demonstram um alto grau de agregação familiar na ocorrência de DRD em pacientes com DM2. Parece haver um subgrupo de pacientes diabéticos que jamais desenvolve essa complicação mesmo tendo um controle metabólico deficiente.

Por outro lado, há indivíduos que desenvolvem DRD apesar de terem suas glicemias rigidamente controladas. Isso sugere que, além dos fatores de risco tradicionais para o desenvolvimento dessas complicações, existe também um forte componente genético que predispõe o seu desenvolvimento [15, 23-25]. Sendo assim, grandes esforços têm sido feitos tentando-se identificar variantes genéticas associadas com DRD; entretanto, os resultados ainda são inconclusivos com diferentes variantes estando associadas com pequenos efeitos em diferentes populações [11].

As proteínas desacopladoras mitocondriais

Proteínas desacopladoras 1, 2 e 3 (UCPs) fazem parte de uma superfamília de proteínas transportadoras e estão localizadas na membrana mitocondrial interna. Estas proteínas têm similaridades nas suas estruturas, mas possuem uma expressão tecidual diferente [26, 27]. Nos últimos anos, houve uma intensificação na pesquisa das propriedades e da regulação das UCPs, além da busca de suas funções. Diversos estudos demonstraram que através do transporte de prótons do espaço intermembranas para a matriz mitocondrial, as UCPs desacoplam a oxidação dos substratos da síntese de ATP, dissipando a energia do potencial de membrana e, consequentemente, diminuindo a produção de ATP pela cadeia respiratória mitocondrial (CRM) [28-30] (**Figura 1**). A dissipação de energia pelas UCPs pode ter diversas funções: produção de calor (UCP1), regulação do metabolismo e transporte de ácidos graxos livres (UCP2 e 3), diminuição da formação de EROs (UCP1 a 3) e regulação da secreção de insulina (UCP2), mecanismos associados à patogênese do DM2 e/ou suas complicações crônicas [26, 28, 30, 31].

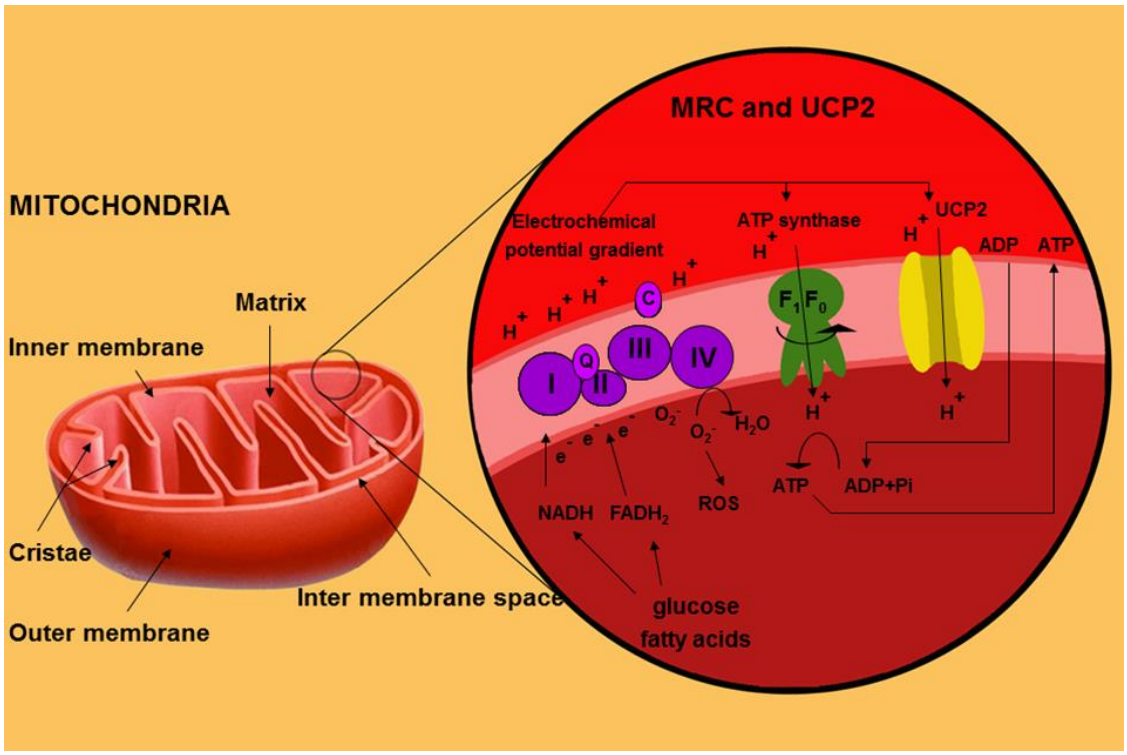

Figura 1. Figura representativa da dissipação de energia mediada pelas UCPs - representada pela UCP2 (Souza *et al.*, Arq Bras Endocrinol Metabol, 2011).

A UCP1 é expressa principalmente no tecido adiposo marrom (TAM) e atua na termogênese induzida pela dieta ou frio, regulação e metabolismo do gasto energético e proteção contra estresse oxidativo [27, 28]. Recentemente, foi demonstrado que em certas condições fisiológicas, a UCP1 também é expressa no músculo esquelético, tecido adiposo branco (TAB), células da retina, timo e ilhotas pancreáticas [27, 32-35]. Embora as funções da UCP1 nestes outros tecidos não estejam tão bem estabelecidas como no TAM, sua principal função parece ser a proteção contra o estresse oxidativo [36, 37].

O gene *UCP1* está localizado no cromossomo 4 em humanos e contém seis éxons e 5 íntrons. Sua expressão é regulada principalmente pelo sistema nervoso simpático via o receptor β3-adrenérgico (β3-AR). Além disso, a expressão de *UCP1* é aumentada por frio, hormônios retinóide e tireoidanos, AMPc e ácidos graxos não-

esterificados e inibida por nucleotídeos purina [27, 30]. Camundongos *knockouts* para o gene *Ucp1* não ficaram obesos e apenas mostraram um aumento na sensibilidade à exposição ao frio. Por outro lado, camundongos transgênicos com a expressão da *Ucp1* aumentada no TAB foram resistentes à obesidade depois de serem alimentados com uma dieta rica em gordura saturada [38]. Em humanos, a expressão de *UCP1* na gordura intraperitoneal de indivíduos obesos é 50% menor do que na gordura de indivíduos com peso normal, apesar do fato de que, em indivíduos adultos, a quantidade de TAM intercalado no meio do TAB é relativamente baixa (aproximadamente 1 adipócito marrom/200 adipócitos brancos) [27, 39, 40].

A UCP2 tem uma distribuição tecidual bastante ampla e, em humanos, é expressa no TAM, TAB, músculo esquelético, coração, rins, fígado, pulmões, baço, timo, medula óssea, cérebro, trato gastrointestinal, ilhotas pancreáticas e células da retina [28, 35, 41-43]. Embora o RNAm da UCP2 seja encontrado em diversos tecidos, parece que os níveis desse RNAm não são proporcionais a quantidade de proteína expressa, o que pode ser explicado por diferentes mecanismos de regulação pós-transcricionais e pós-traducionais [43, 44]. O gene *UCP3* é principalmente expresso no músculo esquelético [28, 39], mas recentemente foi relatado que este gene também é expresso em ilhotas pancreáticas humanas ou tecido adiposo sob certas condições fisiológicas ou patogênicas [34, 43, 45].

Em humanos, os genes *UCP2* e *UCP3* estão localizados no cromossomo 11 (região 11q13) com a região promotora do gene *UCP2* distante apenas 7kb à direita do *stop codon* do gene *UCP3* [9]. Esta região está ligada a taxa metabólica basal e porcentagem de gordura corporal em humanos e, devido à localizão próxima dos dois genes, estudos de ligação não conseguem distinguir a contribuição individual de cada um [46]. O gene *UCP2* tem oito éxons e 7 íntrons, com os dois primeiros éxons sendo

não-codificantes [47]. Já o gene *UCP3* contem 7 éxons, sendo um não-codificante, e apresenta múltiplos sítios de iniciação da transcrição [38]. A sequência do gene *UCP2* humano é 57% similar ao do gene *UCP1* e 71% similar ao gene *UCP3* [47].

Tanto a UCP2 como a UCP3 apenas causam um "desacoplamento leve" da CRM, dissipando levemente a energia do potencial de membrana mitocondrial e, consequentemente, diminuindo levemente a produção de ATP pela CRM [37, 39, 43, 48]. Entretanto, ao contrário da UCP1, a UCP2 e UCP3 apenas desacoplam a CRM após indução por frio, EROs, coenzima-Q, altos níveis de glicose ou ácidos graxos não-esterificados, exercício de alto impacto, sepse ou hipertireoidismo. Suas expressões são inibidas por purina e IL-1β [49, 50]. De uma forma geral, as expressões gênicas destas UCPs são reguladas positivamente por PPAR (*peroxisome proliferator-activated receptor*)-α (no fígado e músculo esquelético), PPAR-γ (no TAB), PPAR-δ (no músculo esquelético e coração), PGC-1α (*PPARγ coativator-1α*) e SREBP-1c (*sterol regulatory element binding-protein-1c*) e negativamente por SIRT-1 (sirtuin-1) e FOXA1 (*forkhead Box A1*) [43, 51, 52].

O "desacoplamento leve" da CRM devido à atividade da UCP2 ou UCP3 resulta na diminuição da produção de EROs pela CRM [52-54]. De acordo com isso, macrófagos [55] e células-beta pancreáticas [56] de camundongos *knockout Ucp2*$^{-/-}$ apresentam uma produção elevada de EROs. Da mesma forma, mitocôndrias isoladas de músculo de camundongos *knockout Ucp3*$^{-/-}$ também produzem mais EROs [57] e apresentam um aumento de marcadores de estresse oxidativo em proteínas [58]. Já, células INS-1E derivadas de células-beta murinas modificadas geneticamente para superexpressar o gene *Ucp2* apresentam uma sobrevivência aumentada após tratamento com o radical livre H_2O_2 [59]. Interessantemente, Cui *et al*. [60] demonstraram que células endoteliais da retina de bovinos incubadas com glicose elevada apresentaram

expressão aumentada de *Ucp2*, o que as protegeu do dano causado pelo aumento de EROs induzido por glicotoxicidade, sugerindo um papel da UCP2 na patogênese das complicações microvasculares do DM.

A atividade desacopladora da UCP2 é um importante regulador negativo da secreção de insulina pelas células-beta pancreáticas. Isso acontece porque o aumento na taxa de ATP/ADP na mitocôndria causa o fechamento dos canais de potássio sensíveis a ATP na cadeia mitocondrial interna, levando a despolarização da membrana e abertura dos canais de cálcio. O consequente influxo de Ca^{2+} para o citoplasma das células-beta engatilha a secreção dos grânulos contendo insulina. A UCP2 diminui a geração de ATP, consequentemente, diminuindo a secreção dos grânulos contendo insulina [26, 47, 61]. Por exemplo, a superexpressão de *UCP2* (obtida através de um vetor adenoviral contendo o cDNA completo do gene *UCP2* humano) suprime completamente a secreção de insulina estimulada pela glicose [62]. Adicionalmente, camundongos *knockout* Ucp$2^{-/-}$ apresentam uma secreção aumentada de insulina e níveis menores de glicose plasmática em comparação a camundongos expressando o gene normal [63].

Como já comentado, ácidos graxos são reguladores fisiológicos tanto da UCP2 como da UCP3 em uma maneira tecido-específica, através da via do PPAR-α, PPAR-γ e SRBP-1c [50]. Em uma linhagem de adipócitos, ácidos graxos insaturados marcadamente induzem a expressão de *UCP2* [64]. Outras linhagens celulares derivadas do fígado e células-beta pancreáticas também aumentam a expressão de *UCP2* após a adição de ácidos graxos livres ao meio de cultura [50].

Neste contexto, a função primária da UCP3 parece ser o transporte de ânions de ácidos graxos para fora da matriz mitocondrial, protegendo a mitocôndria dos danos causados pela peroxidação de lipídeos e permitindo taxas contínuas de oxidação dos ácidos graxos em condições de abundância exagerada dessas macromoléculas. Pacientes

com DM2 possuem um decréscimo característico nas taxas de fosforilação oxidativa mitocondrial, o que é acompanhado por uma redução de cerca de 50% nos níveis de UCP3 no músculo esquelético [65]. Sabe-se que concentrações aumentadas de metabólitos de ácidos graxos nas células musculares estão associadas à diminuição na sensibilidade à insulina [66]. Em geral, camundongos normais que se alimentam de uma dieta rica em gorduras exibem RI. Em oposição, camundongos transgênicos que expressam níveis altos de *UCP3* no músculo estão completamente protegidos da RI induzida por dieta; provavelmente porque a UCP3 esteja aumentando a taxa de oxidação da gordura na mitocôndria [67].

Além disso, estudos realizados com proteoliposomas, mitocôndrias isoladas e células intactas sugerem que as UCP2 e UCP3 têm um papel importante no transporte direto de ácidos graxos ou hidroperóxidos de ácidos graxos [30, 49]. Estas proteínas parecem exportar ânions de ácidos graxos para fora da matriz mitocondrial quando há um excesso de ácidos graxos dentro da mitocôndria, protegendo as células do dano oxidativo causado pelo excesso da peroxidação de ácidos graxos poli-insaturados (lipotoxicidade) e, consequentemente, também reduzindo a produção de EROs [37].

A disfunção das células-beta é um evento secundário a exposição prolongada destas células a níveis elevados de glicose (glicotoxicidade) e lipídeos (lipotoxicidade), condições frequentemente associadas à obesidade e ao DM2. O mecanismo pelo qual a glicolipotoxicidade desencadeia a disfunção das células-beta não é bem conhecido; entretanto, evidências indicam que a produção de EROs faz parte deste mecanismo [68]. Está bem estabelecido que células-beta expostas por um longo período a glicolipotoxicidade aumentam a expressão de *UCP2* em uma forma de se protegerem contra o estresse oxidativo [51]. Entretanto, a produção aumentada de UCP2 leva a uma diminuição na produção de insulina, podendo predispor ao DM2 [26, 30, 37].

Recentemente, foi relatado que níveis elevados de glicose nas células-beta, além de dobrar a taxa de expressão de *UCP2* também diminuir em cerca de 40% a expressão de *UCP3*, demonstrando que essas proteínas podem ter papéis distintos na regulação da insulina, embora pouco se saiba sobre o papel da UCP3 nestas células [34, 45].

Portanto, as UCPs estão envolvidas no gasto energético, regulação do metabolismo e transporte de ácidos graxos livres, diminuição do estresse oxidativo e regulação da secreção de insulina, mecanismos relacionados à patogênese do DM2, bem como de suas complicações crônicas. Dessa forma, polimorfismos nos genes *UCP1-3* podem estar associados ao desenvolvimento do DM2 e/ou de suas complicações crônicas, como a RD e DRD.

Polimorfismos nos genes *UCP1-3* e risco para DM2 e suas complicações crônicas

Diversos polimorfismos têm sido estudados nos genes das *UCP1-3* sobre suas possíveis associações com obesidade e DM2; entretanto, apenas um pequeno número destes polimorfismos tem mostrado associação consistente com essas características em mais de uma população [8, 9].

O principal polimorfismo no gene *UCP1* que tem sido estudado sobre sua associação com obesidade, DM2 e características associadas em algumas populações é o polimorfismo -3826A/G (rs1800592) localizado na região promotora do gene. Vários estudos suportam a associação deste polimorfismo com obesidade, índice de massa corporal (IMC), níveis reduzidos de HDL-colesterol, níveis aumentados de triglicerídeos ou LDL- colesterol e pressão sistólica ou diastólica aumentadas (revisado em [8, 27]). De fato, o polimorfismo -3826A/G foi associado com a redução do RNAm *UCP1* no tecido adiposo intraperitoneal de indivíduos obesos, indicando que ele tem

importância funcional [69]. Entretanto, apenas uns poucos estudos indicam a associação desse polimorfismo -3826A/G com DM2 ou RI [70, 71], enquanto outros indicam que este polimorfismo não está associado com estas características (revisado em [8, 27]).

Os principais polimorfismos investigados no gene *UCP2* são o -866G/A (rs659366) na região promotora, Ala55Val (rs660339) (C/T) no éxon 4 e o Ins/Del, uma inserção/deleção de 45bp na região 3´UTR do éxon 8 [26]. Resultados dos estudos de associação destes polimorfismos com DM2 e características associadas são bastante variáveis, enquanto alguns estudos mostraram associação de um ou mais destes polimorfismos com DM2, obesidade, secreção de insulina e RI, outros estudos não encontraram nenhuma associação positiva com essas características (revisado em [9, 26, 34, 72, 73]). Em 2011, Xu *et al*. [74] realizaram uma metanálise de 17 artigos que investigaram a associação de 2 destes 3 polimorfismos no gene *UCP2*. Os dados da metanálise não mostraram nenhuma associação entre o polimorfismo -866G/A e DM2 nas populações investigadas, mas indicaram uma associação do polimorfismo Ala55Val com risco para DM2 em asiáticos, mas não em europeus. Esta metanálise não investigou o polimorfismo Ins/Del. Mais recentemente, a metanálise realizada por Qin *et al*. [75] na população chinesa demonstrou que os polimorfismos -866G/A e Ala55Val não estão associados com DM2 nesta população. Cabe ressaltar que estudos funcionais indicam que os polimorfismos -866G/A e Ins/Del parecem ter um efeito funcional direto na expressão do gene *UCP2* (revisado em [26]).

O polimorfismo no gene *UCP3* mais frequentemente relatado como estando associado à obesidade e/ou DM2 é o polimorfismo -55C/T (rs1800849) na região promotora desse gene [9, 34, 76-78]; entretanto, os resultados são ainda contraditórios e a associação com estas características deve ser mais bem definida em diferentes populações. O alelo T deste polimorfismo foi associado com expressão aumentada do

RNAm *UCP3* no músculo esquelético [79] e também com uma alta taxa de gasto energético basal comparado ao alelo selvagem [80]. Gable *et al*. [81] relataram que homens saudáveis homozigotos para o alelo T do polimorfismo -55C/T e também para o alelo A do polimorfismo -866 G/A no gene *UCP2* têm um risco de desenvolver DM2 após 10 anos de 4,20 (IC 95% = 1,7-10,37), o que não foi observado quando o efeito de cada polimorfismo foi analisado separadamente. A metanálise realizada por Xu *et al*. [74] demonstrou que o polimorfismo -55C/T é um fator de risco para o DM2 nas populações de asiáticos analisadas, mas não em europeus.

Apesar do fato que as UCP1-3 têm um papel conhecido na proteção contra o estresse oxidativo [82] e que o estresse oxidativo é um dos principais mecanismos patogênicos das complicações crônicas do DM [14], apenas alguns estudos investigaram a associação entre polimorfismos nos genes *UCP1-3* e risco para as complicações microvasculares do DM, como a RD e DRD. Rudofsky *et al*. [83] relataram que pacientes com DM1 portadores do alelo -866A do gene *UCP2* ou do alelo -55T no gene *UCP3* tinham uma prevalência reduzida de NP quando comparados com pacientes com o genótipo G/G. Entretanto, estes autores não encontraram nenhuma associação entre este polimorfismo e DRD ou RD. O polimorfismo -3826A/G no gene *UCP1* não foi associado com nenhuma destas complicações [83]. Rudofsky *et al*. [84] mostraram que os polimorfismos -3826A/G (*UCP1*), -866G/A (*UCP2*) e -55C/T (*UCP3*) não estavam associados com risco para DRD, RD ou NP em alemães com DM2. Os polimorfismos -3826A/G (*UCP1*), Ins/Del (*UCP2*) e -55C/T (*UCP3*) também não foram associados com DRD em pacientes com DM2 da Escandinávia [85].

Por outro lado, dados recentes do nosso grupo [86] mostraram que o haplótipo -866A/55Val/Ins do gene *UCP2* foi um fator de risco independente para RD proliferativa, a forma mais grave de RD, quando presente em uma (RC = 2,12; p =

0,006) ou duas cópias (RC = 5,30; p = 0,00001) em pacientes com DM2 do Rio Grande do Sul. Esta associação com risco para RD proliferativa também foi confirmada em uma amostra de pacientes com DM1 (RC = 6,02; p = 0,005, para o modelo recessivo). Mais recentemente, nós avaliamos se este haplótipo mutado (-866A/55Val/Ins) estava associado com mudanças na expressão do gene *UCP2* na retina de doadores de córnea. De acordo com os nossos dados anteriores, indivíduos portadores do haplótipo mutado tiveram uma menor expressão de *UCP2* na retina quando comparados com o haplótipo de referência (-886G/55Ala/Del) [41]. Interessantemente, outro estudo do nosso grupo relatou a associação do genótipo G/G do polimorfismo -3826A/G no gene *UCP1* com risco aumentado para RD em pacientes com DM1 (RC = 3,503; p = 0,043) e que portadores do alelo G deste polimorfismo apresentavam níveis maiores de RNAm e proteína na retina humana quando comparados com indivíduos com o genótipo G/G [35].

Sendo assim, a elucidação das bases moleculares das complicações crônicas microvasculares do DM pode levar à identificação de pacientes que apresentam maior predisposição para o seu desenvolvimento ou um pior prognóstico. Além disso, a identificação de haplótipos de alto risco pode auxiliar no diagnóstico mais precoce e/ou na prevenção de suas complicações crônicas por meio de uma intervenção mais intensa nos fatores de risco ambientais associados a essas patologias.

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PARTE I

Associations between *UCP1* -3826A/G, *UCP2* -866G/A, Ala55Val and Ins/Del, and *UCP3* -55C/T Polymorphisms and Susceptibility to Type 2 Diabetes Mellitus: Case-Control Study and Meta-Analysis

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Erratum

Article: "Associations between *UCP1* -3826A/G, *UCP2* -866G/A, Ala55Val and ns/Del, and *UCP3* -55C/T Polymorphisms and Susceptibility to Type 2 Diabetes Mellitus: Case-Control Study and Meta-Analysis"

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Page:	**Paragraph:**	**Line:**	**It reads:**	**Read as:**
1 (Abstract)	3	6	55Val	Ala55
1 (Abstract)	3	6	-55C/T	-55C
5 (Results)	5	5	55Val	Ala55
5 (Results)	5	8	55Val	Ala55
5 (Results)	6	9	-55T	-55C
7 (Discussion)	2	13	55Val	Ala55
7 (Discussion)	2	15	-55T	-55C

Associations between *UCP1* -3826A/G, *UCP2* -866G/A, Ala55Val and Ins/Del, and *UCP3* -55C/T Polymorphisms and Susceptibility to Type 2 Diabetes Mellitus: Case-Control Study and Meta-Analysis

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Abstract

Background: Some studies have reported associations between five uncoupling protein (*UCP*) *1–3* polymorphisms and type 2 diabetes mellitus (T2DM). However, other studies have failed to confirm the associations. This paper describes a case-control study and a meta-analysis conducted to attempt to determine whether the following polymorphisms are associated with T2DM: -3826A/G (*UCP1*); -866G/A, Ala55Val and Ins/Del (*UCP2*) and -55C/T (*UCP3*).

Methods: The case-control study enrolled 981 T2DM patients and 534 nondiabetic subjects, all of European ancestry. A literature search was run to identify all studies that investigated associations between *UCP1–3* polymorphisms and T2DM. Pooled odds ratios (OR) were calculated for allele contrast, additive, recessive, dominant and co-dominant inheritance models. Sensitivity analyses were performed after stratification by ethnicity.

Results: In the case-control study the frequencies of the *UCP* polymorphisms did not differ significantly between T2DM and nondiabetic groups ($P>0.05$). Twenty-three studies were eligible for the meta-analysis. Meta-analysis results showed that the Ala55Val polymorphism was associated with T2DM under a dominant model (OR = 1.27, 95% CI 1.03–1.57); while the -55C/T polymorphism was associated with this disease in almost all genetic models: allele contrast (OR = 1.17, 95% CI 1.02–1.34), additive (OR = 1.32, 95% CI 1.01–1.72) and dominant (OR = 1.18, 95% CI 1.02–1.37). However, after stratification by ethnicity, the *UCP2* 55Val and *UCP3* -55C/T alleles remained associated with T2DM only in Asians (OR = 1.25, 95% CI 1.02–1.51 and OR = 1.22, 95% CI 1.04–1.44, respectively; allele contrast model). No significant association of the -3826A/G, -866G/A and Ins/Del polymorphisms with T2DM was observed.

Conclusions: In our case-control study of people with European ancestry we were not able to demonstrate any association between the *UCP* polymorphisms and T2DM; however, our meta-analysis detected a significant association between the *UCP2* Ala55Val and *UCP3* -55C/T polymorphisms and increased susceptibility for T2DM in Asians.

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Introduction

Diabetes mellitus (DM) has elevated prevalence, morbidity and mortality rates and the social and economic repercussions of its chronic complications compromise both the quality of life and productivity of those affected, making it a serious public health problem. Type 2 DM (T2DM) accounts for 90–95% of DM cases worldwide and usually occurs in obese subjects over 40 years of age. It is characterized by chronic hyperglycaemia caused by a combination of insulin resistance and inadequate compensatory insulin secretion [1].

Type 2 DM is a multifactorial condition and susceptibility to it is determined by the combined effects of multiple genetic and environmental factors [2]. The most likely explanation for the dramatic increase in T2DM prevalence observed over the past two decades is changing patterns of diet and physical activity. However, it is believed that these environmental changes may only lead to T2DM in the presence of a permissive genetic background [2]. Great effort has therefore been exerted in attempts to identify genes associated with T2DM and a number of studies have focused on genes related to energy expenditure,

such as those encoding the mitochondrial uncoupling proteins (UCPs) [3,4,5,6].

Uncoupling proteins 1, 2 and 3 are members of an anion-carrier protein family located in the mitochondrial inner membrane [7]. These proteins share structural similarities, but have different tissue expression in mammals [8]. The original UCP, UCP1, is mainly expressed in brown adipose tissue [6,9]. It was recently shown that under certain pathological conditions, such as hyperglycaemia, UCP1 is also expressed in skeletal muscle, white adipose tissue, retinal cells and pancreatic beta cells [8,10,11]. Uncoupling protein 2 is distributed across a wide range of tissue and cell types, whereas UCP3 is mainly restricted to skeletal muscle [5,9].

Over the last few years, several studies have shown that UCPs reduce metabolic efficiency by dissociating substrate oxidation in mitochondria from ATP synthesis by the mitochondrial respiratory chain. This is thought to be accomplished by promoting net translocation of protons from intermembrane space, across inner mitochondrial membrane, to mitochondrial matrix, thereby dissipating the potential energy available for ATP synthesis and consequently reducing ATP production [12]. This uncoupling effect enables homologue-specific and tissue-specific functions such as thermogenesis (UCP1), regulation of free fatty acid (FFA) metabolism and transport (UCP2 and UCP3), attenuation of production of reactive oxygen species (ROS) by mitochondria (UCP1–3), and regulation of insulin secretion by pancreatic beta cells (UCP2), all of which are mechanisms associated with T2DM pathogenesis [5,7,9,12].

Therefore, the relationship between *UCP loci* and susceptibility to T2DM has been investigated in a number of genetic studies and particular attention has been focused on the -3826A/G (rs1800592) polymorphism in the promoter region of the *UCP1* gene, the -866G/A polymorphism (rs659366) in the promoter region, the Ala55Val (C/T; rs660339) polymorphism in exon 4 and the Ins/Del polymorphism, which is an insertion/deletion of 45 bp in the 3′ untranslated region (3′UTR) of exon 8 of the *UCP2* gene, and the -55C/T (rs1800849) polymorphism in the promoter region of the *UCP3* gene. The results of these studies are not uniform. While some of them have demonstrated associations between one or more of these polymorphisms and T2DM or related characteristics such as obesity, others were unable to detect any associations between the polymorphisms and such characteristics (reviewed in [3,4,5,6,9]).

Considering that T2DM is caused by interactions between a large number of environmental and genetic factors, it is not unexpected that some genetic association studies will fail to confirm associations with the disease even if they actually exist. Indeed, T2DM has been described as a "geneticist's nightmare" and it may be the case that it will be necessary to study huge numbers of patients in order to elucidate the associations between even a single polymorphism and the disease [13]. Therefore, as part of the ongoing effort to examine the hypothesis that *UCP* polymorphisms are associated with T2DM risk, we performed a case-control study of Brazilian Caucasian subjects followed by a meta-analysis of the literature on the subject.

Materials and Methods

Case-control study

Ethical approval of the research protocol. The information obtained from the study did not influence patients' diagnosis or treatment. The study protocol was approved by Ethic Committee in Research from Hospital de Clínicas de Porto Alegre and all patients and nondiabetic subjects provided informed consent in writing. All clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki.

Type 2 Diabetes Mellitus population. The sample population comprised 981 unrelated T2DM patients participating in a multicenter study that began recruiting patients in Southern Brazil in 2002. That project was designed to study risk factors for DM and its chronic complications. It initially included four centres in teaching hospitals located in the Brazilian state of Rio Grande do Sul, specifically the Grupo Hospitalar Conceição, the Hospital São Vicente de Paula, the Hospital Universitário de Rio Grande, and the Hospital de Clínicas de Porto Alegre. A detailed description of that study can be found elsewhere [14]. Type 2 DM was diagnosed according to the American Diabetes Association criteria [1].

All patients had European ancestry (mostly of Portuguese, Spanish, Italian and German descent). Ethnicity was defined by self-report. A standard questionnaire was used to collect information on age, age at DM diagnosis and drug treatment and all patients underwent physical examination and laboratory tests. They were weighed barefoot, wearing light outdoor clothing and their height was measured. Body mass index (BMI) was calculated as weight (kg)/height (meters^2). Blood pressure (BP) was measured twice, in the sitting position, after a 5-min rest, and with a 2-min interval between measurements, using a mercury sphygmomanometer (Korotkoff phases I and V). The means of both measurements were used to calculate systolic and diastolic BP. Arterial hypertension was defined as BP $\geq$140/90 mm Hg, but patients on antihypertensive drugs were defined as hypertense irrespective of BP at the time of assessment.

The characteristics of the T2DM patients analyzed in this study were as follows: mean age was 59.52$\pm$10.63 years, mean T2DM duration was 13.33$\pm$9.08 years, mean age at T2DM diagnosis was 46.7$\pm$11.6 years, mean glycated haemoglobin (GHb) was 7.18$\pm$2.05%, and mean BMI was 28.84$\pm$5.39 kg/m^2. Males comprised 47.4% of the sample, and 72.2% of all patients had arterial hypertension.

Nondiabetic sample. The control group contained 534 nondiabetic volunteers attending the blood donation facility at the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) (mean age = 44.0$\pm$7.8 years; males = 55.0%). None of these people had DM or a family history of the disease. All of them had European ancestry.

Laboratory analyses. Serum samples were collected for laboratory testing after 12 hours fasting. Glucose levels were determined using the glucose oxidase method; GHb was assayed using an ion-exchange HPLC procedure (Merck-Hitachi L-9100 analyzer, Merck, Darmstadt, Germany; reference range: 4.7–6.0%); and total plasma cholesterol, HDL-C, and triglycerides were all tested using enzymatic methods. The fraction of LDL cholesterol was calculated using the Friedewald equation. The nondiabetic subjects did not undergo any of these laboratory tests.

Genotyping. DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. The -866G/A (rs659366) polymorphism in the promoter region of the *UCP2* gene was detected by digesting polymerase chain reaction (PCR) products with the restriction enzyme *Mlu*I (Invitrogen Life Technologies, Inc., San Diego, CA, USA) as previously described [15]. Digestion fragments were resolved on 2% agarose gels containing GelRed^{TM} Nucleic Acid Gel Stain (Biotium Inc., CA, USA) and viewed under ultraviolet light. A DNA sample with a known genotype (identified by sequencing) was used as a positive control to evaluate the completeness of PCR product digestion. The 45 bp Ins/Del polymorphism in the 3′UTR region of exon 8 of the *UCP2* gene was detected by PCR using primers that have

been described elsewhere [16]. The primers amplified products of 457 bp (insertion allele) or 412 bp (deletion allele), which were then resolved on 2% agarose gels stained with GelRed™ Nucleic Acid Gel Stain and viewed under ultraviolet light [17]. Genotypes of the -866G/A and Ins/Del polymorphisms were recorded using the ImageMaster System VDS (GE HealthCare, London, UK).

Primers and probes contained in the 40× Human Custom TaqMan Genotyping Assay (Assays-By-Design Service; Life Technologies, Foster City, CA; USA) were used to genotype the Ala55Val (C/T) polymorphism (rs660339) in exon 4 of the *UCP2* gene, the -3826A/G (rs1800592) polymorphism in the promoter region of the *UCP1* gene, and the -55C/T (*rs1800849*) polymorphism in the promoter region of the *UCP3* gene. Reactions were conducted in 96-well plates, in a 5 µl total reaction volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1× (Life Technologies), and Custom TaqMan Genotyping Assay 1× specific for each polymorphism (Life Technologies). The plates were then placed in a real-time PCR thermal cycler (7500 Fast Real Time PCR System; Life Technologies) and heated for 10 minutes at 95°C, followed by 50 cycles of 95°C for 15 seconds and 63°C for 1 minute. Fluorescence data files from each plate were analyzed using automated allele-calling software (System Sequence Detection v.1.4; Life Technologies).

Genotyping success rates were more than 95% for all polymorphisms and the calculated error rate based on PCR duplicates was less than 3%.

Statistical analyses for the case-control study. Allele frequencies were determined by gene counting and departures from the Hardy-Weinberg equilibrium (HWE) were verified using goodness-of-fitness χ^2 tests. Allele and genotype frequencies were compared between groups using the χ^2 test. Logistic regression analyses were performed to assess independent associations between the *UCP* polymorphisms and T2DM, after adjustment for age and gender. Two-tailed P values$<$0.05 were considered statistically significant. Statistical analyses were conducted using SPSS version 18.0 (SPSS, Chicago, IL, USA).

Meta-analysis

Search strategy and eligibility criteria. This study was designed and described in accordance with current guidelines [18,19]. PubMed and Embase were searched systemically to identify all available genetic studies of associations between T2DM and the most-often studied polymorphisms of *UCP* genes (*UCP1* -3826A/G, *UCP2* -866G/A, *UCP2* Ala55Val, *UCP2* Ins/Del and *UCP3* -55C/T) using the following medical subject headings (MeSH): "Diabetes mellitus, type 2" AND ("mitochondrial uncoupling protein" OR "SLC25A27 protein, human" OR "mitochondrial uncoupling protein 2" OR "mitochondrial uncoupling protein 3") AND ("mutation" OR "frameshift mutation" OR "germ-line mutation" OR "INDEL mutation" OR "mutation, missense" OR "point mutation" OR "codon, nonsense" OR "sequence deletion" OR "polymorphism, genetic" OR "polymorphism, single nucleotide" OR "polymorphism, restriction fragment length"). The search was limited to human and English or Spanish language papers and was completed on August 24, 2012. All of the articles identified were also searched manually to identify any other relevant citations.

Two investigators (D.A.S and A.P.B.) independently reviewed the titles and abstracts of all articles selected in order to evaluate whether the studies were eligible for inclusion in the meta-analysis. Disagreements were resolved by discussion between them and when necessary a third reviewer (D.C.) was consulted. Where abstracts did not provide enough information regarding the inclusion and exclusion criteria, the full text of the article was retrieved for evaluation. We included observational studies (case-control or cross-sectional designs) that compared one or more of the *UCP* polymorphisms in question between a known number of T2DM patients and nondiabetic subjects. Studies were excluded from the analysis if the genotype distributions in control group deviated from those predicted by the HWE, if they did not have sufficient data to estimate an OR with 95% CI or if they did not employ validated genotyping methods. If data were duplicated and had been published more than once, the most complete study was chosen.

Data extraction and quality control assessment. Data were independently extracted by two investigators (B.M.S. and L.A.B.) using a standardized abstraction form, and consensus was sought in all extracted items. When consensus could not be reached, differences in data extraction were resolved by a third reviewer (D.C.) and by referencing the original publication. The information extracted from each individual study was as follows: name of first author, publication year, ethnicity and number of subjects in case and control groups, age, gender, BMI, genotype and allele frequencies in case and control subjects and OR (95% CI).

Two investigators (B.M.S and L.A.B.) independently assessed the quality of each eligible study using the Newcastle-Ottawa Scale (NOS) for assessing quality of case-control studies in meta-analysis [20]. The NOS contains eight items, categorized into three dimensions including selection, comparability, and exposure. For each item a series of response options is provided. A star system is used to allow a semi-quantitative assessment of study quality, such that the highest quality studies are awarded a maximum of one star for each item, with the exception of the item related to comparability, which allows two stars to be assigned. The total NOS score therefore ranges from zero to nine stars [21].

Statistical analysis for meta-analyses. Control subjects' genotype distributions were tested for conformity with HWE using a goodness-of-fitness χ^2 test. Gene-disease associations were measured using OR (95% CI) estimation based on the following genetic inheritance models: (1) allele contrast; (2) additive model; (3) recessive model; (4) dominant model and (4) co-dominant model [22,23].

Heterogeneity was tested using a χ^2-based Cochran's Q statistic and inconsistency was assessed with the I^2 metric. Heterogeneity was considered statistically significant at P$<$0.10 for the Q statistic and $I^2>50\%$ for the I^2 metric statistic. Where significant heterogeneity was detected, the DerSimonian and Laird random effect model (REM) was used to calculate OR (95% CI) for each individual study and for the pooled effect; where heterogeneity was not significant, the fixed effect model (FEM) was used for this calculation [24,25].

Meta-regression and sensitivity analyses were carried out to identify key studies with a substantial impact on inter-study heterogeneity. The factors investigated by meta-regression were age, gender, BMI and ethnicity. Sensitivity analyses were performed after stratifying the studies by ethnicity given that the *UCP* polymorphisms show different frequencies in different ethnic groups.

Risk of publication bias was assessed using funnel plot graphics, analyzed both visually and with the Begg and Egger test [26]. The significance of the intercept was determined by the *t* test, as proposed by Egger, with P$<$0.10 considered indicative of statistically significant publication bias. All statistical analyses were performed using Stata 11.0 software (StataCorp, College Station, TX, USA).

Results

Case-control study

Table 1 lists the genotype and allele frequencies of the *UCP1* -3826A/G, *UCP2* -866G/A, *UCP2* Ala55Val, *UCP2* Ins/Del and *UCP3* -55C/T polymorphisms. The genotype frequencies of all polymorphisms were in agreement with those predicted by the HWE in non-diabetic subjects (P>0.05) and did not differ significantly between T2DM and nondiabetic groups (**Table 1**). These results did not change after adjustment for age and gender (**Table 1**). Furthermore, the allele frequencies of these polymorphisms were similar in T2DM patients and nondiabetic subjects (**Table 1**). It is worth mentioning that the frequencies of these five *UCP* polymorphisms also did not differ statistically when assuming dominant, recessive, additive or co-dominant models of inheritance (P>0.05).

Meta-analysis

Literature search and characteristics of eligible studies. **Figure 1** is a flow diagram illustrating the strategy used to identify and select studies for inclusion in the meta-analysis. A total of 582 potentially relevant citations were retrieved by searching the electronic databases and 539 of them were excluded during the review of titles and abstracts. Forty-three articles therefore appeared to be eligible at this point and their full texts were evaluated. However, after reading the full text, another 21 studies were excluded because of missing information or ineligible study designs or because they genotyped other *UCP* polymorphisms, but not the ones of interest here (**Figure 1**). A total of 23 articles fulfilled the eligibility criteria and were included in the meta-analyses: 22 that had been identified through the database searches [15,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46] in addition to the case-control study we describe above, which was also included in the analysis.

Table 1. Genotype and allele distributions of *UCP* polymorphisms in type 2 diabetes patients and non-diabetic subjects.

***UCP* Polymorphisms**	**Cases**	**Controls**	**Unadjusted P***	**Adjusted OR, 95% CI/P†**
UCP1 -3826A/G	*n* = 981	*n* = 534		
A/A	489 (49.9)	263 (49.3)	0.694	1
A/G	370 (37.7)	211 (39.5)		1.018 (0.696–1.489)/0.926
G/G	122 (12.4)	60 (11.2)		0.984 (0.554–1.748)/0.956
A	0.687	0.690	0.510	-
G	0.313	0.310		
UCP2 -866G/A	*n* = 778	*n* = 435		
G/G	272 (35.0)	152 (34.9)	0.950	1
G/A	372 (47.8)	211 (48.5)		1.136 (0.760–1.697)/0.534
A/A	134 (17.2)	72 (16.6)		1.405 (0.807–2.444)/0.229
G	0.589	0.592	0.909	-
A	0.411	0.408		
UCP2 Ala55Val	*n* = 784	*n* = 453		
Ala/Ala	265 (33.8)	142 (31.3)	0.539	1
Ala/Val	371 (47.3)	229 (50.6)		0.871 (0.578–1.313)/0.510
Val/Val	148 (18.9)	82 (18.1)		1.116 (0.650–1.917)/0.691
Ala	0.575	0.566	0.716	-
Val	0.425	0.434		
***UCP2* Ins/Del**	*n* = 779	*n* = 461		
Del/Del	379 (48.7)	226 (49.0)	0.699	1
Ins/Del	314 (40.3)	191 (41.4)		0.880 (0.598–1.295)/0.516
Ins/Ins	86 (11.0)	44 (9.6)		1.387 (0.734–2.621)/0.313
Del	0.688	0.697	0.642	-
Ins	0.312	0.303		
***UCP3* -55C/T**	*n* = 822	*n* = 351		
C/C	559 (68.0)	239 (68.1)	0.988	1
C/T	231 (28.1)	99 (28.2)		0.841 (0.561–1.260)/0.400
T/T	32 (3.9)	13 (3.7)		0.678 (0.256–1.796)/0.434
C	0.821	0.822	0.983	-
T	0.179	0.178		

Data are presented as number of carriers (%) or proportion of sample. The control group contained non-diabetic subjects and cases were type 2 diabetic patients.
*P values were computed using χ^2 tests to compare case and control groups.
†P values were computed using logistic regression analysis and are adjusted for age and gender.
doi:10.1371/journal.pone.0054259.t001

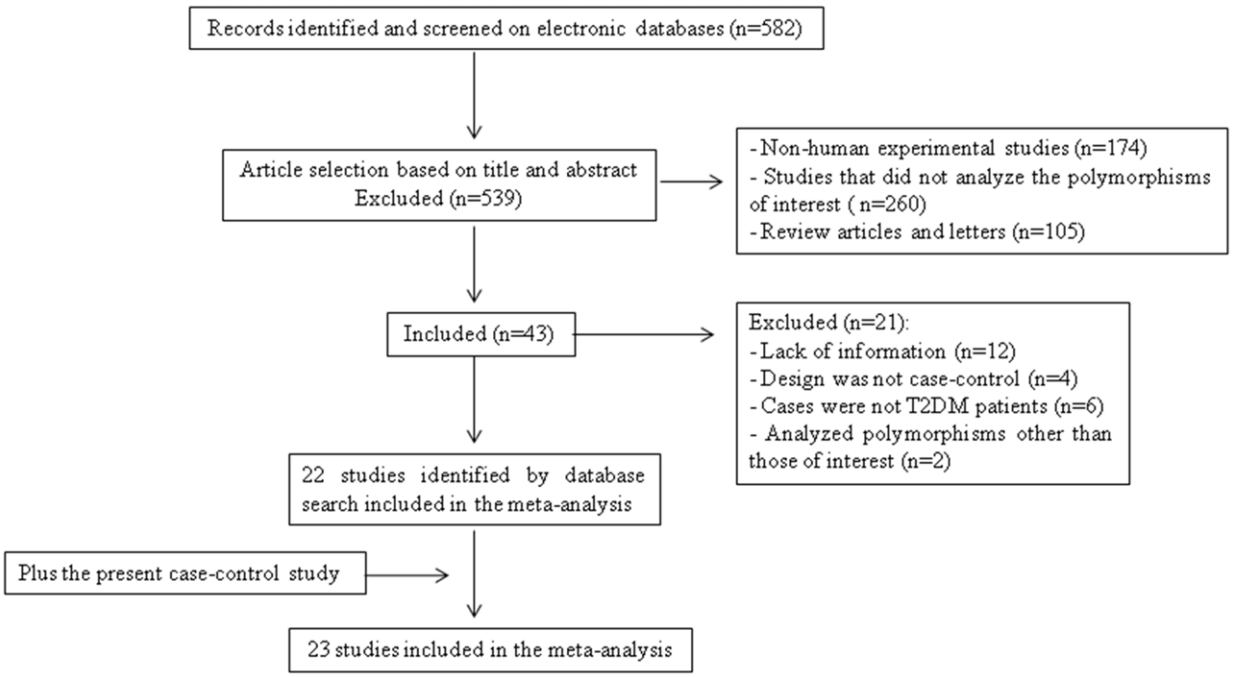

Figure 1. Flowchart illustrating the search strategy used to identify association studies of *UCP1–3* polymorphisms and type 2 diabetes mellitus for the meta-analysis.
doi:10.1371/journal.pone.0054259.g001

Table S1 summarizes the characteristics of the 23 studies included in the meta-analysis. Seven of these studies analyzed the *UCP1* -3826A/G polymorphism (3071 cases/2561 controls), 12 analyzed the *UCP2* -866G/A polymorphism (4487/4229), 5 analyzed the *UCP2* Ala55Val polymorphism (2112/1841), 3 analyzed the *UCP2* Ins/Del polymorphism (1010/699), and 8 investigated the *UCP3* -55C/T polymorphism (3370/3690). **Table S2** lists the genotype and allele distributions and OR (95% CI) for the *UCP* polymorphisms in case and control samples from the different studies reviewed.

Table S3 shows the quality of each individual study, assessed using the NOS scale. The highest quality studies were awarded nine stars. In general, most studies were considered as having good quality selection, comparability and exposure. None of the studies scored less than six stars and 47.8% of the studies had eight or nine stars.

Quantitative synthesis. **Table 2** summarizes the results of the pooled analyses for associations between *UCP* polymorphisms and susceptibility to T2DM. Gene-disease associations were measured for the following genetic inheritance models: allele contrast, additive, recessive, dominant and co-dominant. **Figure 2** illustrates the pooled OR for the associations between T2DM and *UCP1* -3826A/G and *UCP3* -55C/T polymorphisms and **Figure 3** illustrates the pooled OR for the associations between the three *UCP2* polymorphisms and T2DM, both assuming the allele contrast model.

Our results revealed no significant associations between T2DM and *UCP1* -3826A/G, *UCP2* -866G/A or *UCP2* Ins/del polymorphisms, irrespective of whether allele contrast, additive, recessive, dominant or co-dominant models of inheritance were used (**Table 2**). Furthermore, no significant associations were found between these polymorphisms and T2DM when allele contrast models were used after stratification for ethnicity (**Table 2**).

The *UCP2* Ala55Val polymorphism was significantly associated with T2DM risk, but only when assuming a dominant inheritance model (REM OR 1.27, 95% CI 1.03–1.57) (**Table 2**). Furthermore, stratification by ethnicity revealed that, assuming an allele contrast model, the 55Val allele was significantly associated with risk of T2DM in Asians (REM OR 1.25, 95% CI 1.02–1.51), but not in Europeans (REM OR 0.90, 95% CI 0.63–1.27). When assuming the dominant model, the 55Val allele was also associated with risk of T2DM in Asians (REM OR 1.34, 95% CI 1.02–1.76).

The *UCP3* -55C/T polymorphism was significantly associated with T2DM when assuming allele contrast (REM OR = 1.17, 95% CI 1.02–1.34), additive (REM OR = 1.32, 95% CI 1.01–1.72) or dominant (REM OR = 1.18, 95% CI 1.02–1.37) models of inheritance. Additionally, a marginally significant association with T2DM was observed for the recessive (FEM OR = 1.11, 95% CI 1.00–1.24) and co-dominant (FEM OR = 1.05, 95% CI 1.00–1.10) models. Stratification by ethnicity showed that, when assuming an allele contrast model, the *UCP3* -55T allele was associated with T2DM in Asians (REM OR = 1.22, 95% CI 1.04–1.44) but not in Europeans (REM OR = 1.12, 95% CI 0.91–1.38). In the same way, when assuming a dominant model, this polymorphism was associated with T2DM only Asians (REM OR = 1.28, 95% CI 1.06–1.55).

As can be observed in **Table 2**, there was significant heterogeneity between studies investigating the *UCP* polymorphisms when assuming the allele contrast model for the whole sample. Furthermore, significant heterogeneity was also observed between studies when assuming some of the other models of inheritance for these polymorphisms (I^2>50%; **Table 2**). To investigate this finding in greater depth, sex, age, BMI and ethnicity were used as covariates in the meta-regression analyses performed for the five *UCP* polymorphisms under different inheritance models. None of the covariates used in univariate meta-regression analyses could individually explain the heterogeneity observed (data not shown). Similarly, multivariate meta-

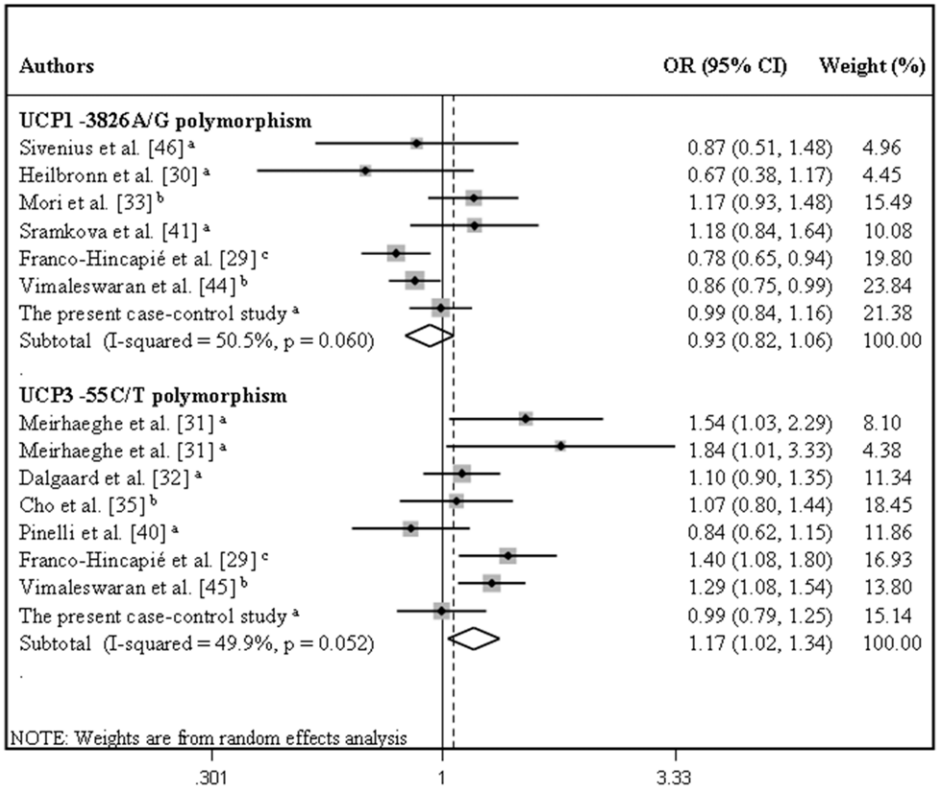

Figure 2. Forest plots showing individual and pooled ORs (95% CI) for the associations between the *UCP1* -3826A/G and *UCP3* -55C/T polymorphisms and type 2 diabetes mellitus under an allele contrast inheritance model. The areas of the squares reflect the weight of each individual study and the diamonds illustrate the random-effects summary ORs (95% CI). [a] European population; [b] Asian population; [c] Mixed population.
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regression analyses performed using these covariates also failed to explain the inter-study heterogeneity (data not shown).

Sensitivity analyses were carried out in order to estimate the influence of each individual study on the meta-analysis results obtained when assuming allele contrast inheritance models by repeating the meta-analysis omitting a different study each time. Our results showed that no individual study significantly influenced the inter-study heterogeneity or the pooled ORs for the *UCP2* Ala55Val, *UCP2* Ins/Del and *UCP3* -55C/T polymorphisms (data not shown). In contrast, two studies [34,39] were largely responsible for the heterogeneity observed in the meta-analysis of *UCP2* -866G/A polymorphism. Heterogeneity was eliminated by exclusion of these two studies ($I^2 = 0\%$ for the allele contrast model), but this did not significantly change the pooled OR (OR = 1.01, 95% CI 0.98–1.05). Similarly, heterogeneity among studies of the *UCP1* -3826A/G polymorphism was effectively reduced ($I^2 = 32.7\%$ for the allele contrast model) by exclusion of one study [33] and the recalculated pooled OR almost reached statistical significance (OR = 0.89, 95% CI 0.79–1.00).

No significant publication bias was detected in any of the inheritance models assessed for any of the *UCP* polymorphisms analyzed (**Figures S1 and S2**), which suggests that our data are statistically robust.

Discussion

Uncoupling protein 1, UCP2 and UCP3 are regarded as candidate genes for obesity and T2DM because they have been found to decrease mitochondrial membrane potential and mediate proton leak [9,47]. Mutations reducing the activity or expression of these proteins could theoretically reduce energy expenditure by increasing coupling of oxidative phosphorylation, thereby contributing to the development of obesity and, consequently, T2DM. Moreover, mutations in *UCP2* regulatory regions leading to increased expression could directly cause or exacerbate decreased glucose-stimulated insulin secretion, because of a reduced ATP/ADP ratio in pancreatic beta-cells and could therefore possibly contribute to the development of T2DM [47]. These are the reasons why the roles played by the *UCP1* -3826A/G, *UCP2* -866G/A, *UCP2* Ala55Val, *UCP2* Ins/Del and *UCP3* -55C/T polymorphisms in T2DM risk have been studied extensively, but the results of these association studies remain inconclusive (reviewed in [3,4,5,6,9,47]). In an attempt to arrive at a more definitive conclusion about the associations between *UCP* polymorphisms and T2DM, we performed a case-control study of a Brazilian Caucasian population and a meta-analysis of genetic association studies on the subject.

Our case-control study showed that the genotype and allele frequencies of *UCP1* -3826A/G, *UCP2* -866G/A, *UCP2* Ala55Val, *UCP2* Ins/Del and *UCP3* -55C/T polymorphisms were similar in T2DM patients and non-diabetic subjects, suggesting that these polymorphisms are not important risk factors for T2DM in our population. It is also worth mentioning that the frequencies of these polymorphisms in our samples were similar to frequencies observed in other Caucasian populations [27,28,30,31,34,35,40,41,42,45].

Certain factors unrelated to the *UCP* polymorphisms could have interfered with the findings of our case-control study. First, even

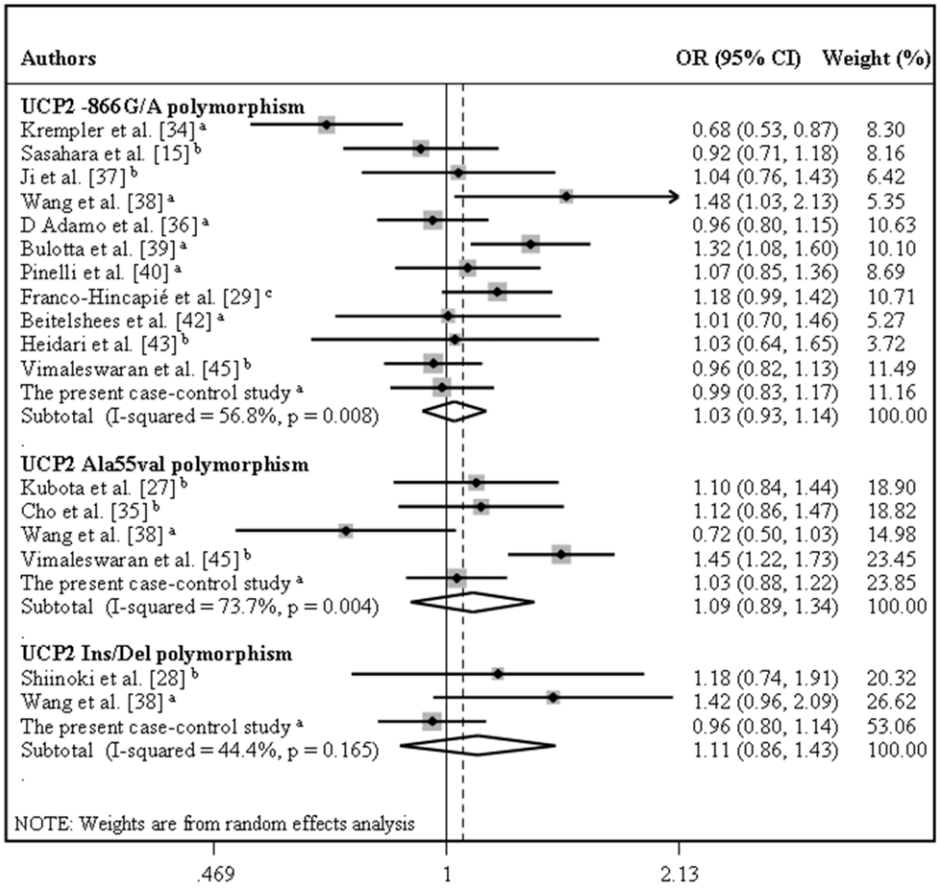

Figure 3. Forest plots showing individual and pooled ORs (95% CI) for the associations between the *UCP2* -866G/A, Ala55Val and Ins/Del polymorphisms and type 2 diabetes mellitus under an allele contrast inheritance model. The areas of the squares reflect the weight of each individual study, and the diamonds illustrate the random-effects summary ORs (95% CI). [a] European population; [b] Asian population; [c] Mixed population.
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though only Brazilian Caucasian subjects were studied, both T2DM patients and nondiabetic subjects were recruited from the same hospitals, and the OR obtained was adjusted for age and gender, we cannot rule out the possibility of population stratification bias when analysing our samples. Thus, our results should be interpreted with caution since we did not estimate the ancestral genetic background of our samples using genetic markers, which would be the best way to exclude population stratification bias due to ethnic admixture. Second, we cannot fully exclude the possibility of type II error when analyzing associations between the *UCP* polymorphisms and T2DM. Our statistical power to detect an OR $\geq$1.4 was >80% for the *UCP1* and *UCP2* polymorphism, but was less than 80% for the *UCP3* -55C/T polymorphism due to the low frequency of the -55T allele. On the other hand, our power was less than 80% for all *UCP* polymorphisms when considering an OR of 1.14 (95% CI 1.12–1.16), which is the mean OR obtained for the $\cong$20 genetic variants so far consistently associated with risk of T2DM [48]. Moreover, we did not take multiple testing corrections into account when estimating the statistical power. Consequently, our power to detect an association between the analyzed polymorphisms and T2DM could be even smaller. Third, our non-diabetic subjects are approximately 15 years younger than diabetic patients. Although, we tried to minimize this problem adjusting OR by age and sex and selecting a control group with a mean age similar to the mean age at T2DM diagnosis of the diabetic sample, we cannot exclude the possibility that this difference could have influenced our results given that increasing age is an important risk factor for developing T2DM. Notwithstanding these limitations, taking into account that the frequencies of the *UCP* polymorphisms are very similar between T2DM patients and non-diabetic subjects, it seems unlikely that these variants could play an important role in the pathogenesis of T2DM in our population.

Meta-analysis has been considered a powerful tool for pooling the results from different studies that can overcome the problem of small sample sizes as well as inadequate statistical power of genetic association studies of complex traits [49]. Therefore, to further investigate the effects of the *UCP1* -3826A/G, *UCP2* -866G/A, *UCP2* Ala55Val, *UCP2* Ins/Del and *UCP3* -55C/T polymorphisms on T2DM susceptibility, we conducted a meta-analysis of 22 published articles from different populations and also included the results from our case-control study. The results suggest that *UCP1* -3826A/G, *UCP2* -866G/A and *UCP2* Ins/Del polymorphisms are not associated with risk of T2DM in the populations investigated. In contrast, when evaluating allele contrast and dominant models, the *UCP2* 55Val allele was associated with increased risk of T2DM in Asian samples but not in Europeans. Furthermore, the *UCP3* -55T allele also was associated with risk to T2DM in Asian populations under allele contrast and dominant models.

Table 2. Pooled measures for associations between the *UCP1* -3826A/G, *UCP2* -866G/A, *UCP2* Ala55Val, *UCP2* Ins/Del and *UCP3* -55C/T polymorphisms and susceptibility to T2DM.

Inheritance model	*n* studies	*n* cases	*n* controls	I^2 (%)	Pooled OR (95% CI)
***UCP1* -3826A/G**					
Allele contrast overall	7	3,071	2,561	58.0	0.93 (0.82–1.06)
Allele contrast Asian	2	1,130	1,240	81.6	0.99 (0.74–1.33)
Allele contrast European	4	1,391	1,085	10.5	0.98 (0.85–1.14)
Additive	7	3,071	2,561	55.8	0.88 (0.66–1.16)
Recessive	7	3,071	2,561	44.5	0.94 (0.88–1.01)
Dominant	7	3,071	2,561	34.7	0.96 (0.91–1.00)
Co-dominant	7	3,071	2,561	0.0	0.99 (0.94–1.04)
***UCP2* -866G/A**					
Allele contrast overall	12	4,487	4,229	54.8	1.03 (0.93–1.14)
Allele contrast Asian	4	1,159	1,300	0.0	0.97 (0.86–1.09)
Allele contrast European	7	2,788	2,271	70.9	1.03 (0.87–1.22)
Additive	11	4,356	4,111	49.0	1.02 (0.96–1.09)
Recessive	11	4,356	4,111	42.1	1.02 (0.96–1.08)
Dominant	11	4,356	4,111	54.4	1.01 (0.87–1.17)
Co-dominant	11	4,356	4,111	46.2	1.01 (0.97–1.05)
***UCP2* Ala55Val**					
Allele contrast overall	5	2,243	1,959	93.4	1.09 (0.89–1.34)
Allele contrast Asian	3	1,197	1,270	86.5	1.25 (1.02–1.51)
Allele contrast European	2	915	571	97.5	0.90 (0.63–1.27)
Additive	4	1,981	1,723	79.5	1.41 (0.92–2.16)
Recessive	4	1,981	1,723	80.9	1.24 (0.82–1.87)
Dominant	4	1,981	1,723	61.4	1.27 (1.03–1.57)
Co-dominant	4	1,981	1,723	43.2	1.06 (1.00–1.12)
***UCP2* Ins/Del**					
Allele contrast overall	3	1,010	699	44.6	1.01 (0.95–1.08)
Allele contrast European *	2	910	579	68.3	1.12 (0.77–1.63)
Additive	2	879	581	0.0	0.96 (0.84–1.11)
Recessive	2	879	581	0.0	0.96 (0.84–1.09)
Dominant	2	879	581	0.0	1.00 (0.92–1.09)
Co-dominant	2	879	581	0.0	1.02 (0.94–1.11)
***UCP3* -55C/T**					
Allele contrast overall	8	3,370	3,695	63.1	1.17 (1.02–1.34)
Allele contrast Asian	2	986	1,051	76.0	1.22 (1.04–1.44)
Allele contrast European	5	1,839	2,195	59.7	1.12 (0.91–1.38)
Additive	8	3,370	3,695	54.1	1.32 (1.01–1.72)
Recessive	8	3,370	3,695	44.5	1.11 (1.00–1.24)
Dominant	8	3,370	3,695	54.8	1.18 (1.02–1.37)
Co-dominant	8	3,370	3,695	9.5	1.05 (1.00–1.10)

Where significant heterogeneity was detected (I^2>50%), the DerSimonian and Laird random effect model (REM) was used to calculate OR (95% CI) for each individual study and for the pooled effect; where heterogeneity was not significant, the fixed effect model (FEM) was used for this calculation.
*Stratification analysis was only performed for Europeans for the *UCP2* Ins/Del polymorphism (allele contrast model), since only one study of Asians was identified.
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Subjects carrying the Val/Val genotype of the *UCP2* Ala55Val polymorphism appear to have a lower degree of uncoupling of the mitochondrial internal membrane, lower energy expenditure [50], higher exercise energy efficiency [51], higher metabolic rate, increased susceptibility to obesity and T2DM [16,45,52,53] and greater weight loss [54] than subjects with the Ala allele. Notwithstanding, other studies have reported that this polymorphism is not individually associated with basal metabolic rate, metabolic syndrome, BMI, obesity, insulin secretion or T2DM [27,35,38,55,56,57,58]. The T allele of the *UCP3* -55C/T polymorphism has been associated with increased incidence of T2DM [59], higher BMI [60,61], lower BMI [62,63], higher HDL cholesterol levels [62], larger waist circumference [64], and high total cholesterol and LDL cholesterol levels and reduced risk of

T2DM [31]. However, other studies have not found associations between this polymorphism and metabolic rate, obesity, BMI, insulin secretion or T2DM [55,57,65,66,67]. The inconsistencies in the results reported by the studies cited here may be at least partially explained by differences in study design, sample size, ethnicity, age, sex and environmental factors and also by synergetic effects with other polymorphisms in the *UCP* genes and in other genes associated with T2DM and/or obesity.

It is well known that functional polymorphisms can influence gene expression, regulating the final quantity of protein in a given tissue, or promote changes in protein activity. The -55C/T polymorphism is located 55 bp upstream of the most commonly used transcription initiation site of skeletal muscle [68]. This polymorphism is potentially interesting because it is located at 6 bp from the TATA box and 4 bp downstream of a putative peroxisome proliferator-activated receptor (PPAR) responsive element and could modify the PPAR responsiveness of the *UCP3* gene [69,70]. For this reason, it has been suggested that the *UCP3* gene could be one of the PPAR-γ targets involved in the modulation of lipid metabolism and insulin sensitivity [63]. In male Pima Indians, subjects carrying the -55T allele had significantly higher *UCP3* mRNA expression than homozygotes for the -55C allele [71]. Moreover, reduced muscle *UCP3* mRNA levels have been associated with increased BMI in Pima Indians [72]. The increased *UCP3* mRNA expression in -55T allele carriers could explain the association between this polymorphism and lower BMI [62,63]; however, as already mentioned, other studies have reported an association between this polymorphism and higher BMI [60,61]. Further studies are still needed to elucidate the functional effects of the *UCP3* -55C/T polymorphism on *UCP3* expression.

The *UCP2* Ala55Val polymorphism causes a conservative amino acid change (alanine/valine) at position 55 of exon 4 and, until now, there had been no evidence that this alteration generates a functional change in the protein [5]. It is therefore possible that this polymorphism may not be a true disease-causing variant, but could simply be reflecting the effects of a functional polymorphism. Taking into account that the Ala55Val polymorphism is tightly linked to the *UCP2* -866G/A polymorphism ($|D'| = 0.991$) [17], which has a demonstrable effect on *UCP2* gene expression in a number of different tissues (reviewed in [4,5]), one could hypothesize that the -866G/A polymorphism should be the candidate for the functional polymorphism in the *UCP2* gene. However, our meta-analysis results did not detect any association between the *UCP2* -866G/A polymorphism and T2DM; ruling out a role in T2DM in the populations analyzed. Some other, currently unknown, *UCP2* causative polymorphisms in linkage disequilibrium with the *UCP2* Ala55Val polymorphism must be responsible for the associations reported.

Despite all our efforts, we are aware that certain factors unrelated to the *UCP* polymorphisms analyzed could have interfered with the findings of our meta-analysis. First, meta-analysis is notoriously prone to publication bias, and although we have attempted to trace unpublished observations, we cannot be sure that small negative studies were overlooked. Second, one of the studies identified [57] was not included in the meta-analysis because genotype and allele frequencies for the *UCP2* Ala55Val, *UCP2* -866G/A and *UCP3* -55C/T polymorphisms were not reported for incident diabetic cases and were not provided by the authors. Hsu *et al.* [57] analyzed 14 tag SNPs of the *UCP2-UCP3* cluster in an ethnically diverse cohort of postmenopausal women aged >50 years and showed that none of the polymorphisms analyzed remained significantly associated with risk of T2DM after adjustment for multiple testing. Notwithstanding, they reported that a *UCP2-UCP3* haplotype (rs591758–rs668517–rs647126–rs1800006) was significantly associated with a greater risk of T2DM especially among overweight Caucasians. This haplotype was not constituted by any of the polymorphisms evaluated in our study. Third, because of the difficulty in getting the full texts of articles published in several languages, we only included studies published in English and Spanish. Fourth, heterogeneity is potentially a significant problem when interpreting the results of any meta-analysis of genetic association studies, and our meta-analysis showed significant inter-study heterogeneity in almost all genetic inheritance models tested. To investigate this issue in greater depth, meta-regression analyses were performed and showed that age, sex, BMI, and ethnicity did not made significant contributions to inter-study heterogeneity. The heterogeneity observed could be due to differences in sample selection, genotyping methods or gene-environment interactions and without detailed information on the metabolic and clinical characteristics of the studies reviewed we could not fully exclude the possibility that the heterogeneity observed might reduce our power to detect true associations. However, sensitivity analyses omitting one study at time did not significantly change the results for associations between the *UCP* polymorphisms and T2DM. Fifth, we also cannot rule out the possibility of type II error when analyzing associations between the *UCP* polymorphisms and T2DM after stratifying by ethnicity. For the whole sample, we had at least 80% power ($\alpha = 0.05$) to detect even modest ORs (1.17–1.2) for almost all analyzed polymorphisms under the allele contrast model, which is an evidence that our results are robust. However, after stratification by ethnicity, we had 80% power to detect only OR $\geq$1.3 for all *UCP* polymorphisms (allele contrast model).

It is worth noting that our results are consistent with a previous meta-analysis conducted by Xu *et al.* [73], who analyzed associations between T2DM and *UCP2* Ala55Val, *UCP2* -866G/A and *UCP3* -55C/T polymorphisms in 17 studies, but did not analyze neither the *UCP1* -3826A/G and *UCP2* Ins/Del polymorphisms nor the allele contrast or co-dominant inheritance models. These authors also failed to detect an association between the *UCP2* -866G/A polymorphism and T2DM. In common with our meta-analysis, they also reported that the *UCP3* -55C/T polymorphism was associated with T2DM in Asians when assuming an additive inheritance model (OR = 1.15; 95% CI 1.03–1.28), and in the overall population when assuming dominant (OR = 1.33; 95% CI 1.02–1.73) and recessive (OR = 1.19; 95% CI 1.04–1.36) models. Similarly, they found that the *UCP2* Ala55Val polymorphism was associated with T2DM in Asians for the additive model (OR = 1.23; 95% CI 1.12–1.36), and in the overall population for dominant (OR = 1.42; 95% CI 1.10–1.84) and recessive (OR = 1.39; 95% CI 1.16–1.66) models.

The association between the *UCP2* Ala55Val and *UCP3* -55C/T polymorphisms with T2DM only in Asian populations may be explained in part by the known differences in lifestyle and body weight distributions between Asian and Caucasian populations as well as differences in the genotype frequencies of the analyzed polymorphisms. Luan *et al.* [74] found that the effects of genetic polymorphisms on obesity and related-diseases could be changed by nutritional characteristic of the population. It may be possible that different diet pattern between Caucasian and Asian populations could modulate the effect of *UCP* polymorphism on obesity and diabetes susceptibility.

In conclusion, our results indicate that the *UCP1* -3826A/G, *UCP2* Ins/Del and *UCP2* -866G/A polymorphisms are not important risk factors for T2DM. However, our results strongly

suggest that the *UCP2* Ala55Val and *UCP3* -55C/T are associated with susceptibility to T2DM, mainly in the Asian population. The absence of any associations of the five *UCP* polymorphisms and T2DM in our case-control study is in agreement with our meta-analysis results showing important associations of the *UCP2* Ala55Val and *UCP3* -55C/T polymorphisms with T2DM only in Asians. Further additional studies with larger samples are necessary to elucidate the roles possibly played by *UCP* polymorphisms in the pathogenesis of T2DM; particularly studies that analyze the effects of gene-gene and gene-environment interactions.

Supporting Information

Figure S1 Funnel plot for contrast allele model for *UCP1* -3826A/G and *UCP3* -55C/T polymorphisms.
(TIF)

Figure S2 Funnel plot for contrast allele model for *UCP2* -866G/A, Ala55Val and Ins/Del polymorphisms.
(TIF)

Table S1 Characteristics of the eligible studies included in the meta-analysis.
(DOCX)

Table S2 Genotype and allele distributions of the *UCP1* -3826A/G, *UCP2* -866G/A, *UCP2* Ala55Val, *UCP2* Ins/Del and *UCP3* -55C/T polymorphisms in patients with type 2 diabetes mellitus and nondiabetic subjects.
(DOC)

Table S3 Newcastle-Ottawa quality assessment scale for the studies included in the meta-analysis.
(DOC)

Author Contributions

Conceived and designed the experiments: BMS LAB DC. Performed the experiments: BMS LAB APB DAS CKK. Analyzed the data: BMS LAB CKK DC. Contributed reagents/materials/analysis tools: LHC CBL. Wrote the paper: BMS LAB APB DAS CKK CBL LHC DC.

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Table S1. Characteristics of the eligible studies included in the meta-analysis.

Author [Ref.]	Year	Ethnicity	Polymorphism	Age (years)	Male (%)	BMI (kg/m^2)
Kubota et al. [27]	1998	Asian	Ala55Val	59.8 ± 13.1	44.3	24.5 ± 4.6
Shiinoki et al. [28]	1999	Asian	Ins/Del	57.0 ± 9.0	48.0	ND
Sivenius et al. [46]	2000	European	-3826A/G	60.1 ± 5.8	54.0	30.5 ± 5.2
Heilbronn et al. [30]	2000	European	-3826A/G	ND	ND	ND
Meirhaeghe et al. [31] [a]	2000	European	-55C/T	56 ± 6.0	47.0	28.1 ± 4.8
Meirhaeghe et al. [31] [b]	2000	European	-55C/T	56.0 ± 7.0	47.0	31.5 ± 6.6
Dalgaard et al. [32]	2001	European	-55C/T	61.0 ± 11.0	58.0	ND
Mori et al. [33]	2001	Asian	-3826A/G	62.9 ± 11.8	56.3	23.1 ± 3.5
Krempler et al. [34]	2002	European	-866G/A	56.2 ± 12.9	48.0	36.9 ± 6.0
Cho et al. [35]	2004	Asian	Ala55Val; -55C/T	59.0 ± 10.0	48.0	ND
D`Adamo et al. [36]	2004	European	-866G/A	62.0 ± 11.0	50.0	30.1 ± 6.0
Ji et al. [37]	2004	Asian	-866G/A	60.0 ± 10.0	46.2	22.4 ± 3.4
Sasahara et al. [15]	2004	Asian	-866G/A	65.5 ± 10.2	60.0	27.4 ± 4.4
Wang et al. [38]	2004	European	-866G/A; Ala55Val; Ins/Del	62.3 ± 12.3	79.0	27.6 ± 18.9
Bulotta et al. [39]	2005	European	-866G/A	60.4 ± 8.7	51.0	30.9 ± 5.6
Pinelli et al. [40]	2006	European	-866G/A; -55C/T	58.0 ± 8.3	42.0	31.6 ± 6.1
Sramkova et al. [41]	2007	European	-3826A/G	58.8 ± 7.0	38.0	30.5 ± 5.5
Franco-Hincapié et al. [29]	2009	Mixed	-3826A/G; -866G/A; -55C/T	58.0 ± 10.7	35.0	27.0 ± 4.6
Beitelshees et al. [42]	2010	European	-866G/A	62.0 ± 12.5	65.0	29.4 ± 5.9
Heidari et al. [43]	2010	Asian	-866G/A	ND	54.7	ND
Vimaleswaran et al. [44]	2010	Asian	-3826A/G	43.0 ± 13.0	43.6	26.1 ± 4.2
Vimaleswaran et al. [45]	2011	Asian	-866G/A; Ala55Val; -55C/T	49.0 ± 12.0	ND	25.2 ± 4.2
The present case-control study	-	Caucasian -Brazilian	-3826A/G; -866G/A; Ala55Val; Ins/Del; -55C/T	59.6 ± 10.5	42.7	28.8 ± 5.4

Data are shown as mean ± SD or %; [a] case-control study; [b] MONICA cohort study; ND, no data.

Table S2. Genotype and allele distributions of the *UCP1* -3826A/G, *UCP2* -866G/A, *UCP2* Ala55Val, *UCP2* Ins/Del and *UCP3* -55C/T polymorphisms in patients with type 2 diabetes mellitus and nondiabetic subjects

***UCP1* -3826A/G**			**Cases (n) by total and genotype**				**Controls (n) by total and genotype**				**A allele frequency (%)**		
Reference	Year	Ethnicity	Total	A/A	A/G	G/G	Total	A/A	A/G	G/G	Cases	Controls	OR (95% CI)[a]
Sivenius et al. [46]	2000	European	70	43	26	1	123	81	40	2	80.0	82.0	0.87 (0.51, 1.48)
Heilbronn et al. [30]	2000	European	45	22	19	4	99	59	36	4	70.0	78.0	0.67 (0.38, 1.17)
Mori et al. [33]	2001	Asian	320	83	156	81	250	58	116	76	50.3	46.4	1.17 (0.93, 1.48)
Sramkova et al. [41]	2007	European	295	157	124	14	120	61	49	10	74.2	71.3	1.18 (0.84, 1.64)
Franco-Hincapié et al. [29]	2009	Mixed	550	163	267	120	445	162	211	72	53.9	60.1	0.78 (0.65, 0.94)
Vimaleswaran et al. [44]	2010	Asian	810	292	372	146	990	396	446	148	59.0	62.5	0.86 (0.75, 0.99)
The present study	2012	European	981	489	370	122	534	263	211	60	68.7	69.0	0.99 (0.84, 1.16)
***UCP2* -866G/A**			Cases (n) by total and genotype				Controls (n) by total and genotype				G allele frequency (%)		
Reference	Year	Ethnicity	Total	G/G	G/A	A/A	Total	G/G	G/A	A/A	Cases	Controls	OR (95% CI)[a]
Krempler et al. [34]	2002	European	201	65	106	30	391	186	156	49	58.7	67.5	0.68 (0.53, 0.87)
Sasahara et al. [15]	2004	Asian	413	116	205	92	172	50	90	32	52.9	55.2	0.92 (0.71, 1.18)
Ji et al. [37]	2004	Asian	184	53	94	37	134	37	69	28	54.3	53.4	1.04 (0.76, 1.43)
Wang et al. [38]	2004	European	131	ND	ND	ND	118	ND	ND	ND	67.0	58.0	1.48 (1.03, 2.13)
D`Adamo et al. [36]	2004	European	483	222	197	64	563	247	266	50	66.3	67.5	0.96 (0.80, 1.15)
Bulotta et al. [39]	2005	European	746	374	317	55	327	142	144	41	71.4	65.4	1.32 (1.08, 1.60)

Table S3. Newcastle-Ottawa quality assessment scale for the studies included in the meta-analysis

Author [Ref.]	Year	Selection	Comparability	Exposure
Kubota et al. [27]	1998	**	**	**
Shiinoki et al. [28]	1999	**	**	**
Sivenius et al. [46]	2000	****	**	***
Heilbronn et al. [30]	2000	**	**	**
Meirhaeghe et al. [31] [a]	2000	***	**	***
Meirhaeghe et al. [31] [b]	2000	***	**	***
Dalgaard et al. [32]	2001	***	*	***
Mori et al. [33]	2001	***	*	***
Krempler et al. [34]	2002	****	**	***
Cho et al. [35]	2004	***	*	***
D`Adamo et al. [36]	2004	***	**	***
Ji et al. [37]	2004	**	**	***
Sasahara et al. [15]	2004	***	**	**
Wang et al. [38]	2004	***	*	***
Bulotta et al. [39]	2005	****	*	***
Pinelli et al. [40]	2006	***	**	***
Sramkova et al. [41]	2007	***	*	***
Franco-Hincapié et al. [29]	2009	**	*	***
Beitelshees et al. [42]	2010	*	**	***
Heidari et al. [43]	2010	****	**	**
Vimaleswaran et al. [44]	2010	****	*	***
Vimaleswaran et al. [45]	2011	****	*	***
The present case-control study	2012	****	*	***

[a] case-control study; [b] MONICA cohort study.

Pinelli et al. [40]	2006	European	342	167	145	30	305	147	124	34	70.0	68.5	1.07 (0.85, 1.36)
Franco-Hincapié et al. [29]	2009	Mixed	540	213	251	76	449	144	229	76	62.0	58.0	1.18 (0.99, 1.42)
Beitelshees et al. [42]	2010	European	107	37	56	14	341	132	151	58	60.7	60.9	1.01 (0.70, 1.46)
Heidari et al. [43]	2010	Asian	75	29	38	8	75	27	41	7	64.0	63.3	1.03 (0.64, 1.65)
Vimaleswaran et al. [45]	2011	Asian	487	185	239	63	919	358	432	129	62.5	62.5	0.96 (0.82, 1.13)
The present study	2012	European	778	272	372	134	435	152	211	72	58.9	59.2	0.99 (0.83, 1.17)
***UCP2* Ala55Val (C/T)**				Cases (n) by total and genotype				Controls (n) by total and genotype				C allele frequency (%)	
Reference	Year	Ethnicity	Total	C/C	C/T	T/T	Total	C/C	C/T	T/T	Cases	Controls	OR (95% CI) [a]
Kubota et al. [27]	1998	Asian	210	60	107	43	218	64	97	57	54.0	51.6	1.10 (0.84, 1.44)
Cho et al. [35]	2004	Asian	500	158	227	115	133	30	76	27	54.3	51.1	1.12 (0.86, 1.47)
Wang et al. [38]	2004	European	131	ND	ND	ND	118	ND	ND	ND	37.0	45.0	0.72 (0.50, 1.03)
Vimaleswaran et al. [45]	2011	Asian	487	264	198	25	919	408	412	99	74.5	66.8	1.45 (1.22, 1.73)
The present study	2012	European	784	265	371	148	453	142	229	82	57.5	56.6	1.03 (0.88, 1.22)
***UCP2* Ins/Del**				Cases (n) by total and genotype				Controls (n) by total and genotype				Del allele frequency (%)	
Reference	Year	Ethnicity	Total	Del/Del	Ins/Del	Ins/Ins	Total	Del/Del	Ins/Del	Ins/Ins	Cases	Controls	OR (95% CI) [a]
Shiinoki et al. [28]	1999	Asian	100	66	30	3	120	76	38	6	81.0	79.2	1.18 (0.74, 1.91)
Wang et al. [38]	2004	European	131	ND	ND	ND	118	ND	ND	ND	75.0	68.0	1.42 (0.96, 2.09)
The present study	2012	European	779	379	314	86	461	226	191	44	68.8	69.7	0.96 (0.80, 1.14)
***UCP3* -55C/T**				Cases (n) by total and genotype				Controls (n) by total and genotype				C allele frequency (%)	

Reference	Year	Ethnicity	Total	C/C	C/T	T/T	Total	C/C	C/T	T/T	Cases	Controls	OR (95% CI) [a]
Meirhaeghe et al. [31] [b]	2000	European	171	116	49	6	124	70	46	8	82.2	75.0	1.54 (1.03, 2.29)
Meirhaeghe et al. [31] [c]	2000	European	49	36	13	0	894	542	312	40	86.7	78.1	1.84 (1.01, 3.33)
Dalgaard et al. [32]	2001	European	455	252	169	34	521	280	192	49	74.0	72.2	1.10 (0.90, 1.35)
Cho et al. [35]	2004	Asian	499	251	204	44	132	62	59	11	70.7	69.3	1.07 (0.80, 1.44)
Pinelli et al. [40]	2006	European	342	240	94	8	305	224	78	3	83.9	86.2	0.84 (0.62, 1.15)
Franco-Hincapié et al. [29]	2009	Mixed	545	425	109	11	449	319	112	13	88.0	84.5	1.40 (1.08, 1.80)
Vimaleswaran et al. [45]	2011	Asian	487	278	180	29	919	460	377	82	75.6	70.6	1.29 (1.08, 1.54)
The present study	2012	European	822	559	231	32	351	239	99	13	82.1	82.2	0.99 (0.79, 1.25)

[a] Calculated from the reported genotypes, [b] case-control study; [c] MONICA cohort study; ND, no data (no genotype data available).

Figure S1. Funnel plot for contrast allele model for UCP1 -3826A/G and UCP3 -55C/T polymorphisms.

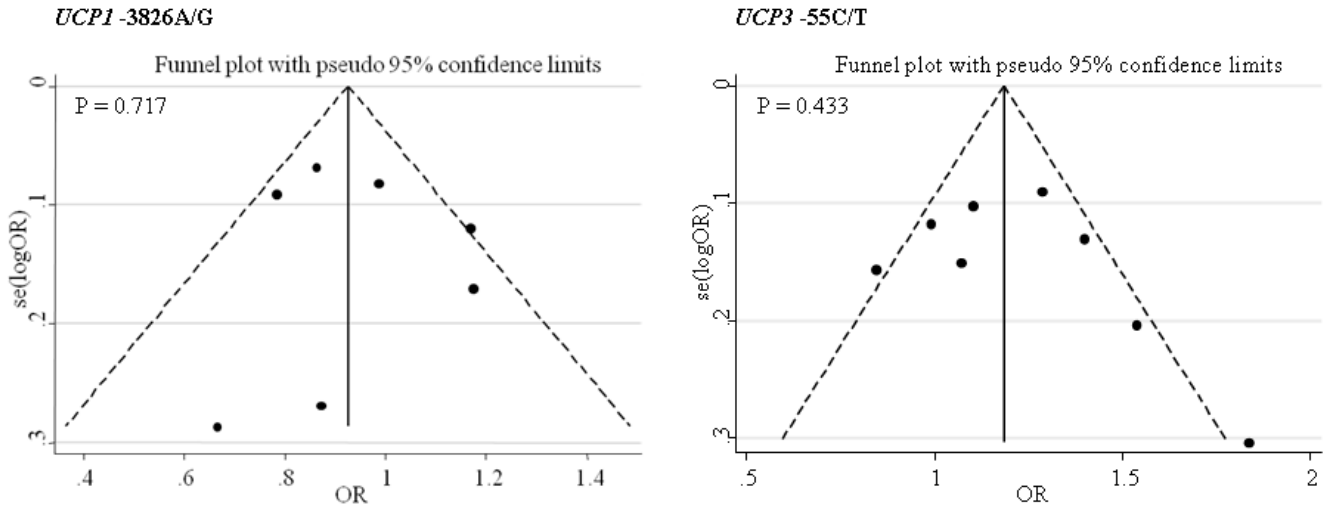
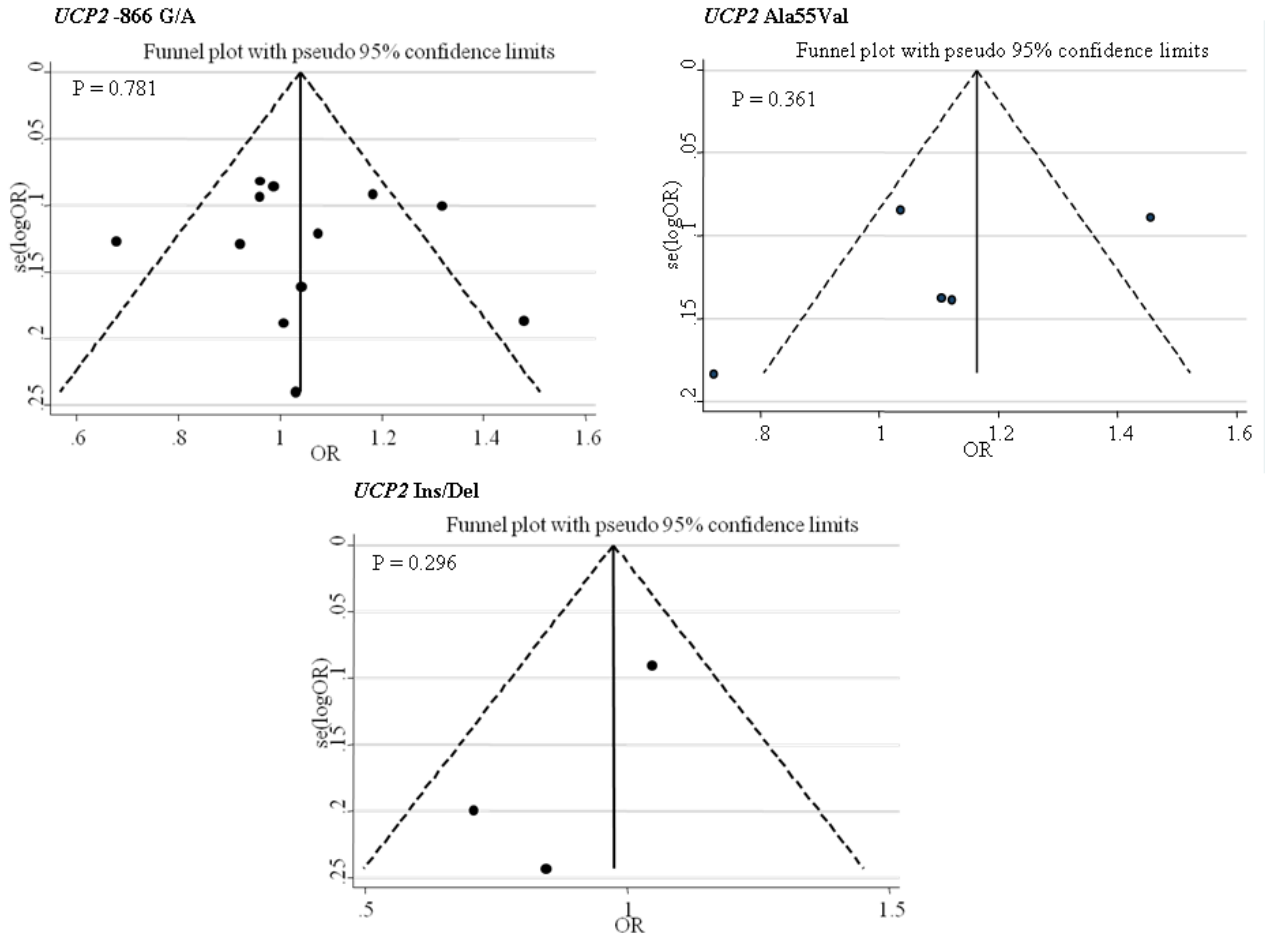


Figure S2. Funnel plot for contrast allele model for UCP2 -866G/A, Ala55Val and Ins/Del polymorphisms.



PARTE II

Polymorphisms of the *UCP2* gene are associated with diabetic kidney disease and glomerular filtration rate in type 2 diabetic patients and with decreased *UCP2* gene expression in human kidney

Polymorphisms of the *UCP2* gene are associated with diabetic kidney disease and glomerular filtration rate in type 2 diabetic patients and with decreased *UCP2* gene expression in human kidney

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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. In the T2DM group, 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 ± 1.20 *vs*. 1.85 ± 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 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 in 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 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. Arterial hypertension (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 Assay 40x (Life Technologies, Foster City, CA, USA). Primer and probe sequences can be found elsewhere [21]. The reactions were conducted in 96-well plates, in a total 5 μL reaction volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1 x (Life Technologies), and Custom TaqMan Genotyping Assay 1x. Plates were then positioned in a real-time PCR thermal cycler (7500 Fast Real PCR System; Life Technologies) and heated for 10 min at 95°C, followed by 45 cycles of 95°C for 5s and 62°C for 1min. Fluorescence data files from each plate were analyzed using automated allele-calling software (SDS 2.1; Life Technologies).

RNA isolation

Kidney tissue biopsies (250 mg) were homogenized in phenol-guanidine isothiocyanate (Invitrogen - Life Technologies). RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000 x g) at 4°C. RNA pellet was washed twice with 75% ethanol and ressuspended in 10-50 μL of diethylpyrocarbonate treated water.

Concentration and quality of total RNA samples were assessed using a NANODROP 2000 spectrophotometer (Thermo Scientific Inc., DE, USA). Only RNA samples which achieved adequate purity ratios (A260/A280 = 1.9–2.1) were used for subsequent analyses [29]. In addition, RNA integrity and purity were also checked on agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium, Inc.). The mean RNA concentration (± SD) isolated was 16.8 ± 31.4 μg / 250 mg kidney tissue biopsy.

Quantification of UCP2 gene expression by Real-Time qPCR

Real-time reverse transcription-PCR was performed in two separate reactions: first, RNA was reverse transcribed into cDNA, then cDNA was amplified by quantitative real-time PCR (RT-qPCR). Reverse transcription of 5 μg of RNA into cDNA was carried out using the SuperScript™ VILO Master Mix for RT-PCR (Invitrogen - Life Technologies), following the manufacturer's protocol for the random primer method.

RT-qPCR experiments were performed in a 7500 Fast Real-Time PCR System Thermal 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%, $\alpha = 0.05$).

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 ($P \geq 0.20$, data not shown).

As already described, the -866G/A polymorphism is in almost complete linkage disequilibrium (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, AH 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 ± 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 ± SD *UCP2* mRNA concentration in the whole kidney tissue group was 0.88 ± 1.39 n fold change (logarithmic scale). No significant difference was observed when *UCP2* gene expression was analyzed by gender (men: 0.84 ± 1.59 *vs*. women: 0.91 ± 1.21 n fold change; P = 0.879), AH status (normotensive: 0.59 ± 1.57 *vs*. hypertensive: 0.97 ± 1.31 n fold change; P = 0.442), or presence of DM (non-diabetic patients: 0.76 ± 1.44 *vs*. DM patients: 0.93 ± 1.30; P = 0.762 n fold change). *UCP2* gene expression did not correlated with age (r^2 = -0.054, P = 0.739).

UCP2 gene expression in kidney samples stratified by the presence of the selected *UCP2* haplotypes is depicted in **Figure 2**. *UCP*2 gene expression was decreased in kidneys from *UCP2* mutated haplotype carriers when compared to kidneys from patients with the reference haplotype (0.32 ± 1.20 *vs*. 1.85 ± 1.16 n fold change, respectively; P < 0.0000001). *UCP2* gene expression was similar between patients heterozygous or homozygous for the mutated haplotype (P = 0.750 from Tukey's post hoc test; **Figure 2**). After linear regression analysis, the presence of the mutated haplotype remained significantly associated with decreased UCP2 gene expression after controlling for age, gender and presence of DM (β = -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 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 homozygosis, showed a 5-fold decrease in *UCP2* gene expression when compared to kidneys from patients with the reference haplotype. This finding is biologically plausible since both -866G/A and Ins/Del polymorphisms have been reported as functional polymorphisms [21, 38-42].

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

Further studies are necessary to better define if the -866G/A and Ins/Del polymorphisms have a synergistically effect on *UCP2* gene expression or if one of them has a

major effect on it. Alternatively, there is a possibility that the three analyzed *UCP2* polymorphisms are not themselves responsible for the observed association with DKD, only being in LD with a still unknown functional polymorphism. Nevertheless, previous functional studies indicate that the -866G/A and Ins/Del polymorphisms could be directly leading to changes in *UCP2* gene expression [43, 44]. Moreover, the -866A allele was reported as being associated with lower plasma total antioxidant status (increase oxidative stress) in DM patients with coronary heart disease [45], which could explain the association of the mutated haplotype containing this allele with risk for 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 ($\alpha = 0.05$) 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 polymorphism. Thus, we cannot rule out the possibility that these polymorphisms would be associated with DKD with lower ORs. The results of our *UCP2* gene expression study in kidney biopsies also should be interpreted with caution as most of our sample was constituted by patients who undergone nephrectomy, and for whom we did not have information about DKD diagnosis. Thus, further studies will be necessary to confirm whether the -866A/55Val/Ins haplotype is also associated with changes in *UCP2* gene expression in kidneys from DM patients with different degrees of DKD and eGFR.

In conclusion, data reported here suggest that the *UCP2* -866A/55Val/Ins haplotype is associated with an increased risk of DKD and with a lower eGFR in T2DM patients. Furthermore, this mutated haplotype was associated with decreased *UCP2* gene expression in human kidneys. Further additional studies will be necessary to confirm the association

between the *UCP2* -866A/55Val/Ins haplotype and DKD as well as to elucidate how this haplotype increases the risk of this diabetic complication. Moreover, therapeutic strategies to counteract ROS through reinforcing the action of UCP2 should be explored.

Conflict of interest

Nothing to declare.

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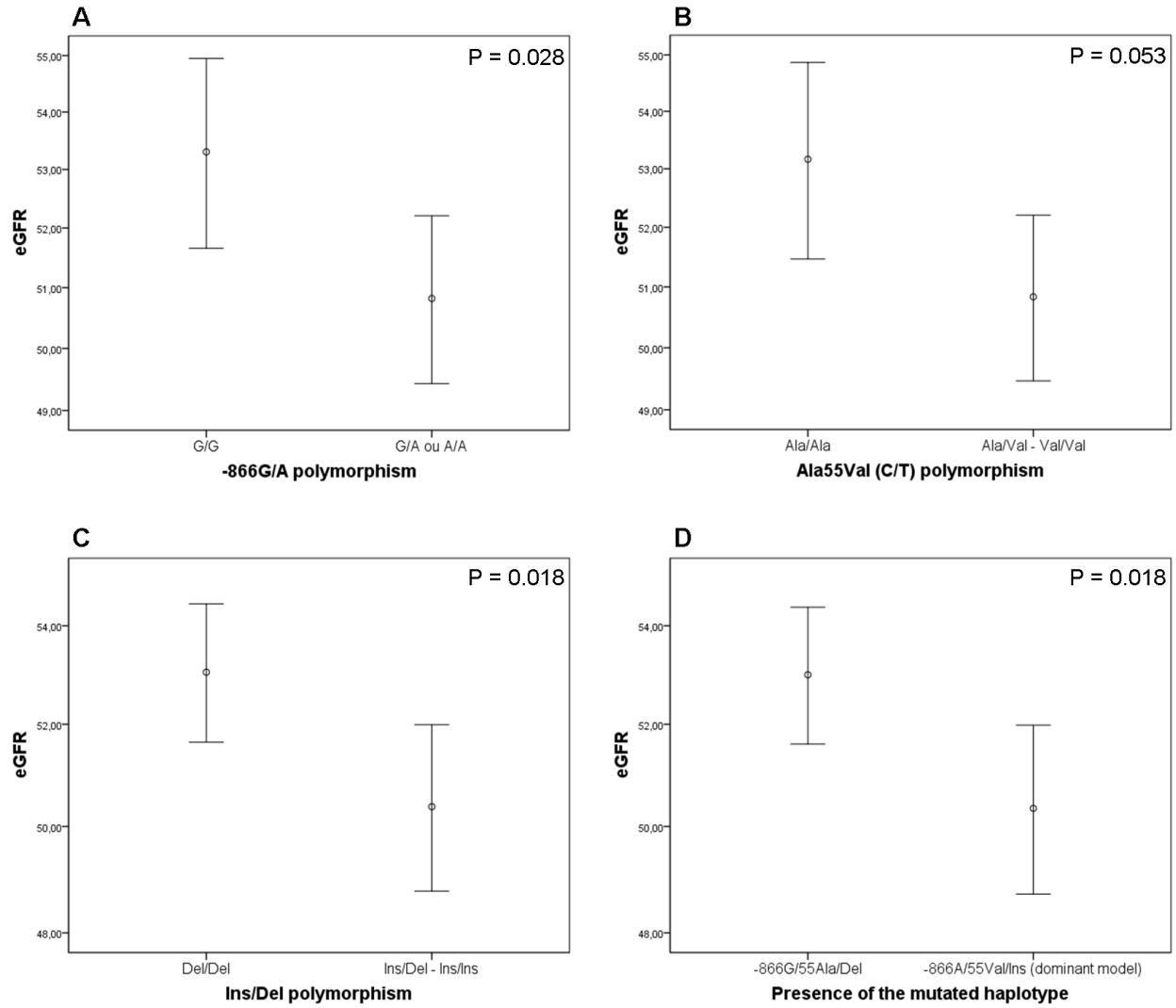

Figure 1: Estimated glomerular filtration rate (eGFR) in T2DM patients according to different *UCP2* polymorphisms and presence of the mutated *UCP2* haplotype. **A)** eGFR in patients stratified according to the presence of the A allele of the -866G/A polymorphism (dominant model). **B)** eGFR in patients 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).

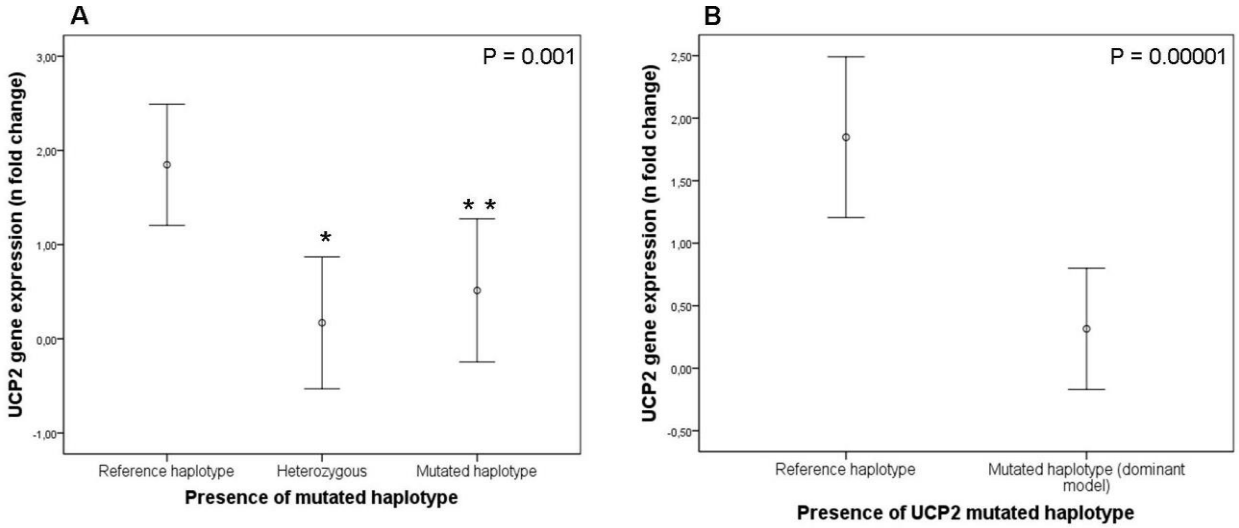

Figure 2: *UCP2* gene expression in human kidney biopsies according to the presence of the *UCP2* mutated haplotype. **A)** *UCP2* gene expression in subjects homozygous for the reference haplotype (-866G/55Ala/Del), heterozygous (reference/mutated haplotypes) or homozygous for the mutated haplotype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

Table 1. Clinical and laboratory characteristics of T2DM patients broken down by the presence 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

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

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	n = 281	n = 287		
Ala/Ala	93 (33.1)	102 (35.5)	0.828	1
Ala/Val	135 (48.0)	133 (46.3)		0.700 (0.407-1.202) / 0.196
Val/Val	53 (18.9)	52 (18.2)		0.793 (0.408-1.542) / 0.494
Ala	0.571	0.587	0.629	-
Val	0.429	0.413		
Dominant Model				
Ala/Ala	93 (33.1)	102 (35.5)	0.600	1
Ala/Val + Val/Val	188 (66.9)	185 (64.5)		0.726 (0.436-1.209) / 0.219

45 bp Ins/Del	n = 278	n = 287		
Del/Del	132 (47.5)	144 (50.5)	0.181	1
Ins/Del	124 (44.6)	110 (38.3)		0.753 (0.459-1.235) / 0.261
Ins/Ins	22 (7.9)	33 (11.5)		1.218 (0.539-2.752) / 0.636
Del	0.698	0.700	0.978	-
Ins	0.302	0.300		
Dominant model				
Del/Del	132 (47.5)	144 (50.2)	0.578	1
Ins/Del + Ins/Ins	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	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).

CONCLUSÃO

Os nossos resultados demonstram que os polimorfismos -3826A/G (*UCP1*), -866G/A e Ins/Del no gene *UCP2* não estão associados com risco para o DM2 em indivíduos brancos do Sul do Brasil. Por outro lado, através de uma metanálise dos estudos disponíveis na literatura e mais o nosso estudo de caso-controle, verificamos que os polimorfismos Ala55Val (*UCP2*) e -55C/T (*UCP3*) conferem uma maior suscetibilidade para o DM2 em asiáticos, mas não em europeus.

Além disso, verificamos que a presença do haplótipo mutado -866A/55Val/Ins (*UCP2*) está associado com aumento do risco para DRD e com a diminuição da TFGe em pacientes com DM2. Este haplótipo também foi associado com uma menor expressão gênica da UCP2 em biópsias de rins humanos em comparação a rins obtidos de indivíduos homozigotos para o haplótipo de referência. Estes resultados reforçam o possível papel de polimorfismos na *UCP2* na patogênese das complicações crônicas do DM, uma vez que em estudos prévios também demonstramos que o haplótipo -866A/55Val/Ins estava associado com risco aumentado de DR proliferativa e a uma menor expressão de UCP2 em amostras de retina humana.

Talvez, polimorfismos nos genes *UCP*s possam não estar fortemente associados com a patogênese do DM2 na nossa população, mas parecem ter um papel importante nas complicações crônicas do DM. Essa associação com DRD e RD é biologicamente plausível visto que, a hiperglicemia proveniente do DM leva a um aumento do estresse oxidativo, causando danos no endotélio vascular destes tecidos e, que a UCP2 tem importantes propriedades na redução da produção de EROs. Entretanto, estudos adicionais são necessários para verificar se mudanças na expressão desse gene também ocasionam mudanças nos níveis

de proteína, assim como, confirmar esta associação com estudos funcionais para elucidar se este haplótipo é realmente um fator risco para a DRD.

Além dos artigos já citados, ao longo do período do doutorado foram desenvolvidos os seguintes manuscritos, em colaboração:

- Meta-Analysis Reveals the Association of Common Variants in the Uncoupling Protein (UCP) 1–3 Genes with Body Mass Index Variability. Brondani LA, Assmann TS, de **Souza BM**, Bouças AP, Canani LH, Crispim D. PloS one 9 (5), e96411, 2014.

- Association of the UCP polymorphisms with susceptibility to obesity: case–control study and meta-analysis. Brondani LA, **de Souza BM**, Assmann TS, Bouças AP, Bauer AC, Canani LH, Crispim D. Molecular biology reports, 1-15, 2014.

- The A Allele of the rs1990760 Polymorphism in the IFIH1 Gene Is Associated with Protection for Arterial Hypertension in Type 1 Diabetic Patients and with Expression of This Gene in Human Mononuclear Cells. Bouças AP, Brondani LA, **Souza BM**, Lemos NE, de Oliveira FS, Canani LH, Crispim D. PloS one 8 (12), e83451, 2013.

- The UCP1− 3826A/G polymorphism is associated with diabetic retinopathy and increased UCP1 and MnSOD2 gene expression in human retina. Brondani LA, **de Souza BM**, Duarte GCK, Kliemann LM, Esteves JF, Marcon AS, Gross JL, Canani LH, Crispim D. Investigative ophthalmology & visual science 53 (12), 7449-7457, 2012.