Universidade Federal do Rio Grande do Sul

Programa de Pós-graduação em Ciências Médicas: Endocrinologia

Investigação de polimorfismos no gene *UCP2* e avaliação da expressão de microRNAs que bloqueiam esse gene em pacientes com diabetes mellitus tipo 1 e doença renal do diabetes

Dissertação de Mestrado

Cristine Dieter

Porto Alegre, novembro de 2018.

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Cristine Dieter

Orientadora: Profa. Dra. Daisy Crispim Moreira

Coorientadora: Profa. Dra. Bianca Marmontel de Souza

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“Que os nossos esforços desafiem as impossibilidades.

Lembrai-vos de que as grandes proezas da história foram conquistadas daquilo que parecia impossível.”

Charles Chaplin

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Esta dissertação de mestrado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia da Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de uma breve introdução geral sobre o assunto da dissertação e na sequência são apresentados dois artigos originais. Após, são apresentadas as considerações finais.

Artigo original 1: -866G/A and Ins/Del polymorphisms in the *UCP2* gene and diabetic kidney disease: case-control study and meta-analysis

Artigo original 2: MiR-15a-5p and miR-30e-5p expressions in plasma and urine from type 1 diabetes patients are associated with diabetes kidney disease

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1. Introdução

3'UTR	Região 3' não traduzida
5'UTR	Região 5' não traduzida
CKD-EPI	*Chronic Kidney Disease Epidemiology Collaboration*
CRM	Cadeia respiratória mitochondrial
DM	Diabetes mellitus
DM1	Diabetes mellitus tipo 1
DM2	Diabetes mellitus tipo 2
DRC	Doença renal crônica
DRCT	Doença renal crônica terminal
DRD	Doença renal do diabetes
EROs	Espécies reativas de oxigênio
EUA	Excreção urinária de albumina
GSIS	Secreção de insulina estimulada por glicose
HAS	Hipertensão arterial sistêmica
IDF	*International Diabetes Federation*
Ins/Del	Inserção/Deleção
KDIGO	*Kidney Disease/ Improving Global Outcomes*
MDRD	*Modification of Diet for Renal Disease*

miR	microRNA
miRNAs	microRNAs
qPCR	Real-time quantitative PCR
RC	Razão de chances
RD	Retinopatia diabética
TFG	Taxa de filtração glomerular
TFGe	Taxa de filtração glomerular estimada
TFG-β1	*Transforming growth fator beta 1*
UCP	*Uncoupling protein*
UCP2	*Uncoupling protein 2*

2. Artigos

3'UTR	3' untranslated region
ACE	angiotensin-converting-enzyme
AGE	Advances glycation end-products
BINGO	Biological Networks Gene Ontology
BMI	Body mass index
BP	Blood pressure
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
DCCT	Diabetes Control and Complications Trial
DKD	Diabetic kidney disease
DM	Diabetes mellitus

DR	Diabetic retinopathy
ECM	Extracellular matrix
eGFR	Estimated glomerular filtration rate
EMT	Epithelial-mesenchymal transition
ESRD	End-stage renal disease
FDR	False Discovery Rate
FEM	Fixed effect model
GFR	Glomerular filtration rate
GO	Gene Ontology
HbA1c	Glycated hemoglobin
HG	High glucose
Ht	Haplotype
HWE	Hardy-Weinberg equilibrium
Ins/Del	Insertion/Deletion
KDIGO	Kidney Disease Improving Global Outcomes
LD	Linkage disequilibrium
MESH	Medical subject heading
miR	MicroRNA
miRNA	MicroRNA
MOOSE	Meta-analysis of Observational Studies in Epidemiology
MTI	miRNA-target gene interactions
NOS	*New-Castle-Ottawa Scale*
OR	Odds ratio

PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
qPCR	Real-time quantitative PCR
RAGEs	Advances glycation end-products receptors
REM	Random effect model
ROS	Reactive oxygen species
RTECs	Renal tubular epithelial cells
SUMO	Small ubiquitin-like modifier
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
U6snRNA	Small nuclear RNA U6
UAE	Urinary albumin excretion
UCPs	Uncoupling proteins

RESUMO

A doença renal do diabetes (DRD) é uma complicação microvascular comum do diabetes mellitus (DM) que afeta cerca de 40% dos pacientes com DM tipo 1 (DM1) ou DM tipo 2 (DM2). A DRD é a principal causa de doença renal crônica terminal em pacientes que iniciam terapia de substituição renal e está associada com o aumento da mortalidade cardiovascular. Essa complicação é uma doença progressiva, clinicamente caracterizada por albuminúria e diminuição gradual da taxa de filtração glomerular (TFG). Estudos vêm demonstrando que além dos fatores de risco ambientais, fatores genéticos e epigenéticos também influenciam o desenvolvimento DRD.

A proteína desacopladora 2 (UCP2) é uma proteína presente na membrana mitocondrial interna que atua diminuindo a produção de espécies reativas de oxigênio (EROs) pela cadeia respiratória mitocondrial. Como a produção aumentada de EROs é um mecanismo chave pelo qual a hiperglicemia leva as complicações crônicas do DM, o gene *UCP2* é um gene candidato para essas complicações, incluindo a DRD. De fato, diferentes polimorfismos comuns nesse gene têm sido associados às complicações microvasculares do DM; entretanto, os estudos demonstram resultados conflitantes quanto à associação destes polimorfismos com o desenvolvimento da DRD. Sendo assim, realizou-se um estudo de caso-controle e uma meta-análise com o objetivo de investigar a associação dos polimorfismos -866G/A (rs659366) e Ins/Del no gene *UCP2* com DRD em pacientes diabéticos.

O estudo de caso-controle incluiu 385 pacientes com DM1 (223 pacientes sem DRD e 162 pacientes com DRD) coletados no Hospital de Clínicas de Porto Alegre. A genotipagem do polimorfismo Ins/Del foi realizada pela separação direta dos produtos de

PCR em gel de agarose 2,5% corado com GelRed™ e a do polimorfismo -866G/A, por PCR em tempo real. As frequências alélicas e genotípicas dos dois polimorfismos estudados, bem como dos haplótipos formados por eles, não diferiram significativamente entre casos e controles. Da mesma forma, as distribuições destes polimorfismos entre casos e controles foi similar quando se analisou os modelos de herança aditivo, dominante e recessivo. Na meta-análise foram incluídos três artigos (quatro estudos) da literatura que investigaram a associação dos polimorfismos -866G/A e Ins/Del no gene *UCP2* com DRD, além dos dados do nosso estudo de caso-controle descrito acima. De acordo com o encontrado no nosso estudo de caso-controle, os resultados da meta-análise não demonstraram associação entre os polimorfismos estudados e a DRD, mesmo após análise para os diferentes modelos de herança genética.

Como já mencionado, diversos estudos vêm demonstrando a associação de fatores epigenéticos, como os microRNAs (miRNAs), com o desenvolvimento da DRD. Os miRNAs são pequenas moléculas de RNA não-codificantes que regulam a expressão gênica, estando, assim, envolvidos em vários processos biológicos e patológicos. MiR-15a-5p e miR-30e-5p têm como um dos seus alvos o gene *UCP2*; portanto, podem regular a expressão de *UCP2*, influenciando os níveis de EROS e, consequentemente, podendo estar associados à DRD. Assim, realizou-se um estudo de caso-controle com o objetivo de avaliar a expressão dos miR-15a-5p e miR-30e-5p no plasma e urina de pacientes com DM1 e DRD e pacientes com DM1 sem essa complicação crônica.

Neste estudo de caso-controle, a expressão dos dois miRNAs de interesse foi avaliada no plasma e urina de 40 pacientes com DM1 (17 sem DRD e 23 com DRD) usando-se a técnica de qPCR. Análises de bioinformática também foram realizadas buscando-se encontrar as vias patogênicas nas quais os miR-15a-5p e miR-30e-5p estão

envolvidos. Como resultado, encontrou-se uma diminuição desses miRNAs no plasma e urina de pacientes com DM1 e DRD quando comparado com pacientes com DM1 sem DRD. Análises de bioinformática demonstraram que os dois miRNAs regulam diversos genes que participam de vias relacionadas ao TGF-β, angiogenêse, apoptose, hipóxia e estresse oxidativo.

Em conclusão, tanto no nosso estudo de caso-controle como na meta-análise não observamos nenhuma associação entre os polimorfismos -866G/A e Ins/Del no gene *UCP2* e a DRD. Entretanto, nosso estudo de caso-controle que investigou a expressão dos miR-15a-5p e miR-30e-5p no plasma e urina de paciente com DM1 com e sem DRD, demonstrou que a expressão desses dois miRNAs está diminuída nos pacientes com DRD quando comparado ao grupo controle.

ABSTRACT

Diabetes kidney disease (DKD) is a common microvascular complication of diabetes mellitus (DM) that affects approximately 40% of patients with type 1 DM (T1DM) or type 2 DM (T2DM). DKD is the leading cause of end-stage renal disease in patients initiating renal replacement therapy and is associated with increased cardiovascular mortality. This complication is a progressive disease, clinically characterized by albuminuria and a gradual decrease in the glomerular filtration rate (GFR). Studies have shown that in addition to the environmental risk factors, genetic and epigenetic factors also influence DKD development.

Uncoupling protein 2 (UCP2) is located in the mitochondrial internal membrane and decreases the production of reactive oxygen species (ROS) by the mitochondrial respiratory chain. Taking into account that increased ROS production is a key mechanism by which hyperglycemia leads to chronic diabetic complications, the *UCP2* gene is a candidate gene for these complications, including DKD. In fact, different common polymorphisms in this gene have been associated with diabetic microvascular complications; however, studies have shown conflicting results regarding the association of these polymorphisms with the development of DKD. Thus, a case-control study and a meta-analysis were carried out to investigate the association of -866G/A (rs659366) and Ins/Del polymorphisms in *UCP2* gene with DKD.

The case-control study comprised 385 T1DM patients (223 patients without DKD and 162 patients with DKD) recruited at the Hospital de Clínicas de Porto Alegre. Genotyping of Ins/Del polymorphism was performed by direct separation of PCR products in 2.5% agarose gel stained with GelRed ™, while genotyping of the -866G/A polymorphism was done by real-time PCR. Allele and genotype frequencies of the two

analyzed polymorphisms, as well as the haplotypes constituted by them, did not differ significantly between case and control groups. Likewise, distributions of these polymorphisms between cases and controls were similar when analyzing additive, dominant and recessive inheritance models. Our meta-analysis included three articles (four studies) that investigated the association of *UCP2* -866G/A and Ins/Del polymorphisms with DKD plus the data from our present case-control study. According to the results found in our case-control study, meta-analysis showed no association between both analyzed polymorphisms and DKD, even after analysis of the different genetic inheritance models.

As already mentioned, several studies have demonstrated the association of epigenetic factors, such as microRNAs (miRNAs), with the development of DKD. MiRNAs are small non-coding RNAs that regulate gene expression; hence, being involved in several biological and pathological processes. MiR-15a-5p and miR-30e-5p have as one of their targets the *UCP2* gene; therefore, they might regulate *UCP2* expression, influencing ROS levels and, consequently, be associated with DKD. Thus, a case-control study was conducted aiming to evaluate miR-15a-5p and miR-30e-5p expressions in plasma and urine of T1DM patients with DKD and T1DM patients without this chronic complication.

In this case-control study, expression of the two miRNAs of interest was evaluated in plasma and urine of 40 patients with T1DM (17 without DKD and 23 with DKD) by qPCR. Bioinformatics analyses were also performed to find pathogenic pathways in which miR-15a-5p and miR-30e-5p are involved. As a result, these two miRNAs were downregulated in plasma and urine of T1DM patients with DKD compared to T1DM patients without this complication. Bioinformatics analyses showed that these miRNAs regulate several genes

that participate in pathways related to TGF-β, angiogenesis, apoptosis, hipoxia, and oxidative stress.

In conclusion, both case-control and meta-analysis results were not able to demonstrate any association between the *UCP2* -866G/A and Ins/Del polymorphisms with DKD. However, our case-control study that investigated the expression of miR-15a-5p and miR-30e-5p in plasma and urine of T1DM patient with and without DKD demonstrated that these two miRNAs are downregulated in patients with DKD when compared to the group control.

1. INTRODUÇÃO

O diabetes mellitus (DM) é um conjunto de distúrbios metabólicos que apresentam em comum à hiperglicemia, a qual pode ser resultante de defeitos na secreção de insulina, ação da insulina, ou ambos. De acordo com a Federação Internacional de Diabetes (*International Diabetes Federation* - IDF) (1), 425 milhões de indivíduos em todo o mundo apresentam algum tipo de DM. Estatísticas mostram que o número de indivíduos afetados continua a aumentar e que, se não forem tomadas providências para modificar a trajetória dessa epidemia, esse número poderá chegar a 629 milhões de indivíduos até 2045 (1).

Atualmente, a prevalência do DM no Brasil é de 8,68% (2), sendo que cerca de 10-15% do total de casos apresenta DM tipo 1 (DM1), o qual está associado ao desenvolvimento de complicações crônicas de elevada morbidade e mortalidade em indivíduos jovens em idade produtiva (1). O DM1 é causado pela destruição autoimune das células-beta pancreáticas mediadas por linfócitos T e macrófagos, levando a uma deficiência total na secreção de insulina (3). Como consequência, os indivíduos necessitam de administração de insulina exógena para a sobrevivência e prevenção das complicações crônicas do DM (3-6). O curso da destruição das células-beta e sua progressão para o DM1 clínico depende de uma complexa interação entre componentes genéticos, epigenéticos e uma variedade de fatores ambientais (6-9).

A hiperglicemia crônica associada ao DM causa danos, disfunções e falhas de vários órgãos e tecidos, especialmente rins, olhos, nervos, coração e vasos sanguíneos, causando as chamadas complicações crônicas (3). As complicações crônicas do DM podem ser divididas em dois grandes grupos: microvasculares [doença renal do diabetes (DRD), retinopatia diabética (RD) e neuropatia diabéticas] e macrovasculares (doença vascular

periférica e acidente vascular cerebral). De modo geral, a presença destas complicações depende do tempo de DM, idade do paciente, hipertensão arterial sistêmica (HAS), dislipidemia, suscetibilidade genética do paciente ao tipo de complicação e intensidade e persistência da hiperglicemia (10, 11).

1.1 Doença Renal do Diabetes

A DRD é uma importante complicação crônica do DM, sendo a principal causa de doença renal crônica (DRC) e doença renal crônica terminal (DRCT), que requer tratamento dialítico ou transplante renal (11, 12). Ainda, a DRD é a maior responsável por transplantes renais em diversos países (11). Nos Estados Unidos, cerca de 200.000 pacientes são tratados para DRCT por apresentarem DRD, sendo que, a cada ano, 50.000 novos pacientes iniciam diálise. A taxa de diálise poderia ser ainda maior se fossem considerados os casos de pacientes com DRD que morrem devido às complicações cardiovasculares antes de atingirem a DRCT. Pacientes com DRD que apresentam DRCT se deparam com uma taxa de mortalidade de 20% após o primeiro ano de diálise, o que é maior que a taxa de mortalidade conhecida para muitos cânceres de órgãos sólidos, como os cânceres de próstata e de mama (13).

A DRD é caracterizada como uma série de alterações estruturais que afetam a função renal, as quais ocorrem em decorrência do ambiente diabético e iniciam por hiper-filtração renal e hipertrofia glomerular, seguida de albuminúria progressiva com declínio da taxa de filtração glomerular (TFG) (11, 14). A microalbuminúria ocorre geralmente depois de 10-15 anos do início do DM, seguida de macroalbuminúria que se desenvolve de 15-25 anos após o início do DM (15).

Dentre as alterações fisiopatológicas encontradas na DRD, pode-se destacar o espessamento da membrana basal glomerular com aumento da deposição de colágeno pelas células mesangiais na matriz extracelular e diminuição da quantidade de podócitos. Em consequência, ocorre a excreção de proteínas de alto peso molecular na urina, como por exemplo, a albumina. Outras regiões do rim também são afetadas, como o túbulo proximal, onde ocorre atrofia do epitélio tubular com perda de microvilosidades, infiltração inflamatória e diminuição de capilares **(Figura 1)** (13).

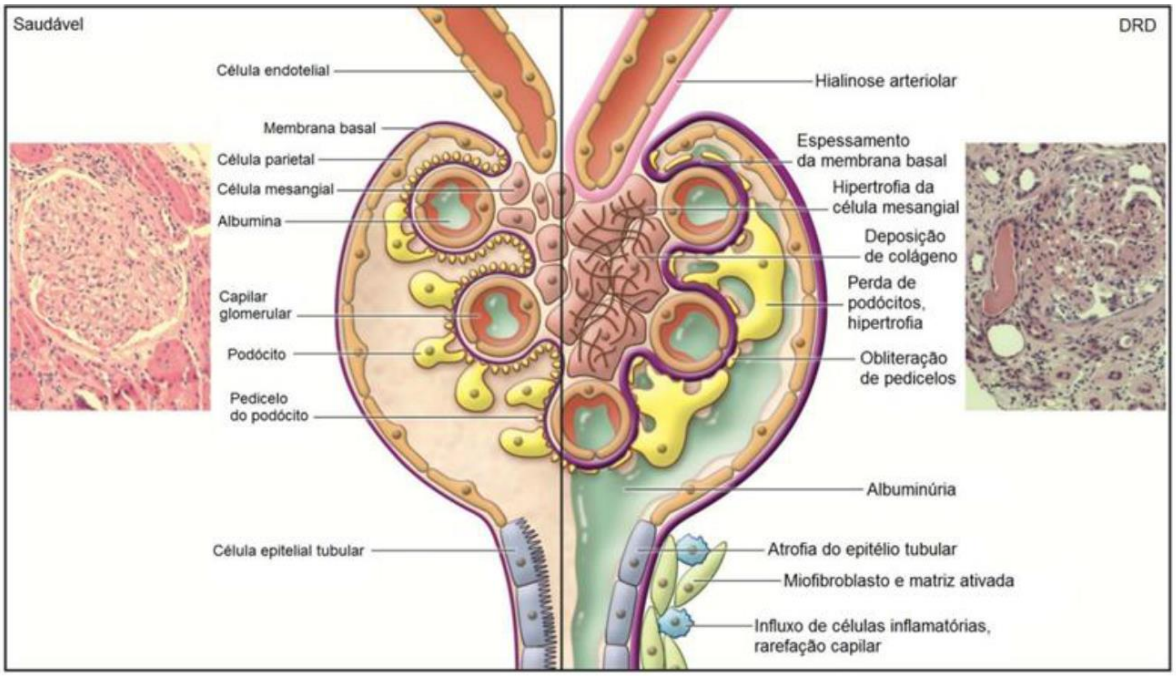

Figura 1. Lesões histopatológicas da Doença Renal do Diabetes. O glomérulo de um paciente saudável inclui arteríola aferente, capilares glomerulares, células endoteliais, membrana basal, podócitos, células epiteliais parietais, células túbulo-epiteliais e é impermeável à albumina. Em contraste, o glomérulo de um paciente com diabetes apresenta hialinose arteriolar, expansão mesangial, deposição de colágeno, espessamento da membrana basal, perda e hipertrofia de podócitos, albuminúria, atrofia do epitélio tubular, acúmulo de matriz e miofibroblastos ativados,

influxo de células inflamatórias e rarefação de capilares. Também é mostrado tecido renal de glomérulo saudável e de paciente com DRD (corado com ácido periódico de Schiff). Adaptado de Reidy *et al.* (13)

Os processos envolvidos no desenvolvimento das lesões e alterações renais são complexos e pouco conhecidos. Sem dúvida a hiperglicemia está relacionada ao desenvolvimento e progressão da DRD, afetando diversas células, como por exemplo, células endoteliais e mesangiais renais, células inflamatórias, podócitos, além do sistema tubular renal e ductos coletores. Como consequência da hiperglicemia, ocorre o aumento da produção de espécies reativas de oxigênio (EROs) pela mitocôndria, as quais ativam fatores de transcrição e moléculas de sinalização, aumentando assim a expressão de citocinas, fatores de crescimento e proteínas de matriz extracelular. Além disso, a hiperglicemia também aumenta a expressão celular de *transforming growth fator beta 1* (TGF-β1), que estimula a produção de matriz extracelular, contribuindo para a hipertrofia celular e síntese de colágeno (16).

Para avaliação da gravidade da disfunção renal utiliza-se atualmente os valores de albuminúria, a qual é classificada em: 1) albuminúria normal ou levemente aumentada; 2) albuminúria moderadamente aumentada (anteriormente conhecida como microalbuminúria) ou, 3) albuminúria severamente aumentada (anteriormente chamada de macroalbuminúria) (17), combinada com o uso da estimativa da TFG. É importante que se avalie esses dois aspectos, pois alguns pacientes com valores normais de albumina já podem apresentar uma diminuição na TFG (18). Assim as medidas de albuminúria e TFG estimada (TFGe) são consideradas os principais critérios diagnósticos de doença renal (**Figura 2**), auxiliando o rastreamento, classificação e o tratamento desta patologia (17).

Prognósticos da DRC por categorias da TFG e albuminúria

Categorias TFG* (ml/min/1,73m²)				Categoria de albuminúria persistente		
				A1	A2	A3
				Normal ou pouco aumentado < 30 mg/g < 3mg/mmol	Aumento moderado 30-300 mg/g 3-30 mg/mmol	Aumento severo >300 mg/g >30 mg/mmol
	G1	Normal ou aumentado	≥90			
	G2	Pouco diminuído	60-89			
	G3a	Pouco ou moderadamente diminuído	45-59			
	G3b	Moderado a severamente diminuído	30-44			
	G4	Severamente diminuído	15-29			
	G5	Falência renal	<15			

Verde: baixo risco; Amarelo: risco moderadamente aumentado; Laranja: alto risco; Vermelho: altíssimo risco. * TGF: Taxa de filtração glomerular

Adaptado: Guias KDIGO, 2012

Figura 2. Valores para a classificação dos diferentes graus de doença renal considerando-se a albuminúria e a taxa de filtração glomerular (TFG), de acordo com as diretrizes da *Kidney Disease/Improving Global Outcomes* (KDIGO) (17). DRC: Doença renal crônica.

Para o cálculo da TFGe, duas equações matemáticas atualmente são mais utilizadas: 1) equação MDRD (*Modification of Diet for Renal Disease*) (19); e 2) equação CKD-EPI (*Chronic KidneyDisease Epidemiology Collaboration*) = 141 x min (SCR/κ, 1)$^{\alpha}$ x max (SCR/κ, 1)$^{-1,209}$ x 0,993^{idade} x 1,018 [se feminino] x 1,159 [se negro] (20). A equação CKD-EPI utiliza as mesmas variáveis que a equação do MDRD; no entanto, foi validada em uma coorte que compreendia indivíduos saudáveis e indivíduos com DRC, e, por isso, apresenta melhor desempenho e previsão de desfechos adversos (20).

1.1.1 Genética da Doença Renal do Diabetes

Sabe-se que o controle glicêmico dos pacientes diabéticos está muito relacionado com o desenvolvimento ou não das complicações crônicas do DM; entretanto, parece haver um subgrupo de pacientes diabéticos que jamais desenvolve DRD mesmo tendo um controle metabólico ruim. De modo contrário, há indivíduos que desenvolvem essa complicação apesar de terem suas glicemias rigidamente controladas, sugerindo que, além dos fatores de risco ambientais tradicionais (tabagismo, HAS, controle glicêmico inadequado e tempo de exposição à hiperglicemia), existe também um forte componente genético influenciando o seu desenvolvimento.

Nesse contexto, estudos de famílias mostraram uma alta agregação familiar na ocorrência de DRD, onde, o risco de desenvolver essa complicação aumenta de 25% para 43% em pacientes diabéticos com história familiar de DRD quando comparado a pacientes sem história familiar dessa complicação (21, 22). De fato, diversos estudos já identificaram vários *loci* de suscetibilidade para desenvolvimento ou progressão da DRD, dentre os quais, pode-se destacar os localizados nos genes *ACE, AFF3*, *FRMD3*, *CARS* e *UCP2* (11, 23-27).

1.1.1.1 Proteína Desacopladora 2 - UCP2

As proteínas desacopladoras (*uncoupling proteins* - UCPs) estão presentes na membrana mitocondrial interna e fazem parte de uma superfamília de proteínas transportadoras. De modo geral, estas proteínas apresentam estruturas e funções similares; entretanto, possuem uma expressão tecidual diferente. A UCP1 é principalmente expressa na gordura marrom (28). Já, a UCP2 tem uma distribuição tecidual bastante ampla, sendo

expressa nos tecidos adiposos marrom e branco, músculo esquelético, coração, rins, fígado, ilhotas pancreáticas, macrófagos, células endoteliais da retina, entre outros (29-32). A UCP3 é basicamente restrita ao músculo esquelético e as UCP4 e 5 são expressas no cérebro (31, 33).

Estudos têm demonstrado que através do transporte de prótons do espaço intermembrana para a matriz mitocondrial, a UCP2 é capaz de desacoplar a oxidação dos substratos da síntese de ATP, dissipando assim a energia do potencial de membrana e, consequentemente, diminuindo a produção de ATP pela cadeia respiratória mitocondrial (CRM) **(Figura 3)**. Esse desacoplamento está associado a diversos mecanismos relacionados com a patogênese do DM e de suas complicações crônicas, como por exemplo, a regulação do metabolismo de ácidos graxos livres, regulação negativa da secreção de insulina pelas células-beta pancreáticas e diminuição da formação de EROs pela mitocôndria (32-34). A produção aumentada de EROs é um mecanismo chave pelo qual a hiperglicemia crônica no DM ativa as diversas rotas que levam as complicações crônicas do DM, incluindo a DRD (35). Dessa forma, o gene *UCP2* é um gene candidato para a DRD e as outras complicações crônicas do DM.

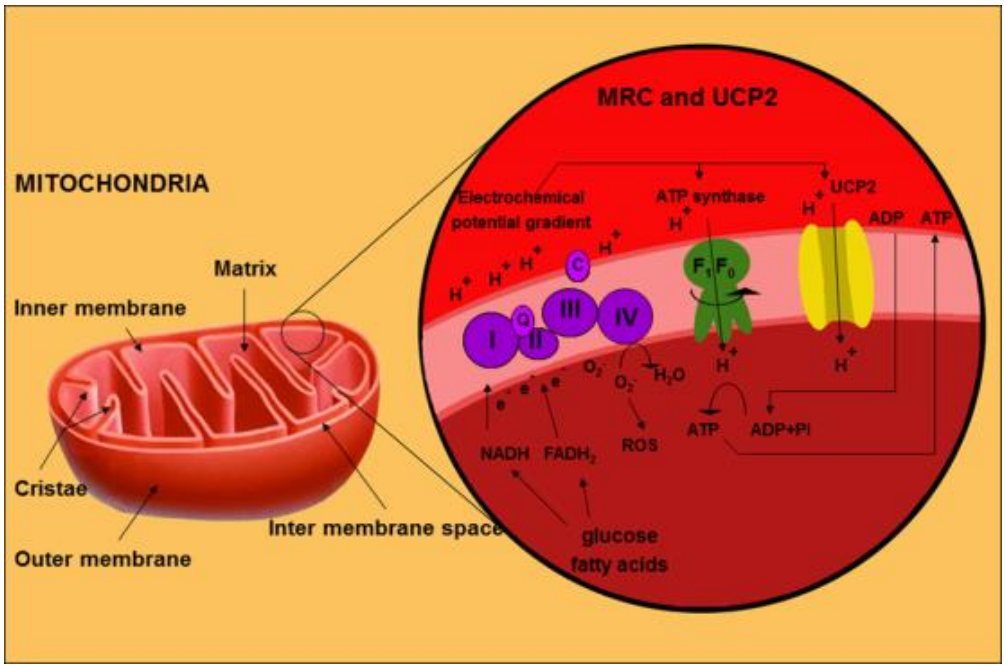

Figura 3. Dissipação de energia mediada pela UCP2 (32). A UCP2 desacopla a oxidação dos substratos da síntese de ATP, dissipando assim a energia do potencial de membrana e, consequentemente, e diminuindo a produção de ATP pela cadeia respiratória mitocondrial. MRC: cadeira respiratória mitocondrial. ROS: espécies reativas de oxigênio.

Neste contexto, estudos funcionais vêm demonstrando uma importante atuação da *UCP2* no tecido renal. Qiu *et al.* (36) evidenciaram que a administração oral de genipina, um inibidor de *UCP2*, é capaz de adiar a progressão para DRD em camundongos C57BL/6J. Ainda, os autores demonstraram que a inibição de *UCP2* reduziu a albuminúria e as lesões dos podócitos. De modo contrário, Chen *et al.* (37) demonstraram que o uso de genipina para inibição de UCP2 levou ao aumento de estresse oxidativo, diminuição da capacidade antioxidante e aumento da apoptose em células tubulares renais de ratos cultivadas com alta concentração de glicose. Além disso, o silenciamento de *UCP2* em

células renais mesangiais de ratos levou ao aumento da apoptose, inflamação e geração de EROs, além do aumento de necrose e redução da viabilidade celular (38).

Além dos estudos funcionais, até o momento, três polimorfismos comuns no gene *UCP2* foram relatados como estando associados com DM (27, 39) e suas complicações crônicas, dentre elas a DRD (26, 39): o polimorfismo -866G/A (rs659366), que ocorre na região promotora do gene; o polimorfismo Ala55Val (rs660339), presente no éxon 4, e o polimorfismo de inserção/deleção (Ins/Del) de 45bp na região 3' não traduzida do gene (3'UTR). Sabe-se que o polimorfismo -866G/A é funcional, alterando uma região importante de ligação de fatores de transcrição na região promotora do gene, com isso aumentando ou diminuindo a expressão de *UCP2* de acordo com diferentes tecidos e ligação de diferentes fatores de transcrição (40-42).

Em relação às complicações crônicas do DM, um estudo do nosso grupo demonstrou que o haplótipo -866A/55Val/Ins (constituído pelos polimorfismos -866G/A, Ala55Val e Ins/Del no gene *UCP2*) foi associado com risco para RD proliferativa em pacientes com DM1 [Razão de Chances (RC) = 2,68; p = 0,014] ou DM2 (RC = 2,75; p = 0,00001) (43). Posteriormente, demonstramos que esse haplótipo estava associado a menor expressão de *UCP2* na retina de portadores do haplótipo mutado (-866A/55Val/Ins) comparado a homozigotos para o haplótipo de referência (-886G/55Ala /Del) (8,4 ± 7,6 vs. 18,8 ± 23,7 unidades arbitrárias; p = 0,046) (44).

Recentemente, relatamos que o haplótipo -866A/55Val/Ins também parece ser um fator de risco independente para DRD (RC = 2,14; p = 0,040) em pacientes com DM2, ajustando-se para idade, sexo, tratamento com inibidores da enzima conversora de angiotensina, triglicerídeos e TFGe. Além disso, pacientes com DM2 portadores do haplótipo mutado mostraram uma diminuição na TFGe quando comparados com indivíduos

com o haplótipo de referência (p = 0,035). Ao analisar amostras de biópsia renal, encontramos uma diminuição significativa da expressão do gene *UCP2* nos portadores do haplótipo mutado comparado aos indivíduos com o haplótipo de referência (média e desvio padrão: 0,32 ± 1,20 *vs*. 1,85 ± 1,16; p < 0,001) (26).

Dessa forma, a UCP2, bem como polimorfismos nesse gene, parece ter um papel importante no desenvolvimento da DRD, principalmente devido a importante função desta proteína na proteção contra o estresse oxidativo. Entretanto, estudos adicionais são necessários para confirmar a associação de polimorfismos nesse gene com DRD, visto que os dados disponíveis, até o momento, são escassos e conflitantes. Da mesma forma, considerando que o gene *UCP2* é regulado diferencialmente entre diferentes tecidos, o que influencia suas funções, são necessários estudos que investiguem os reguladores da expressão dessa proteína e suas associações com DRD, incluindo o estudo de fatores epigenéticos, como os microRNAs (miRNAs).

1.1.2 Epigenética da Doença Renal do Diabetes

A epigenética representa as alterações químicas e estruturais que regulam a atividade gênica sem que ocorram alterações na sequência de nucleotídeos do DNA. Dentre os principais mecanismos epigenéticos estão a metilação do DNA, as modificações de histonas e os RNA não-codificadores (**Figura 4**) (45, 46).

Os miRNAs são uma classe de RNA fita simples não-codificadores de proteínas, de 19–25 nucleotídeos, que agem como potentes reguladores pós-transcricionais da expressão gênica em plantas e animais. Até o momento, 2.693 miRNAs diferentes foram identificados

em humanos, os quais regulam aproximadamente 60% dos genes codificadores de proteínas (47-50).

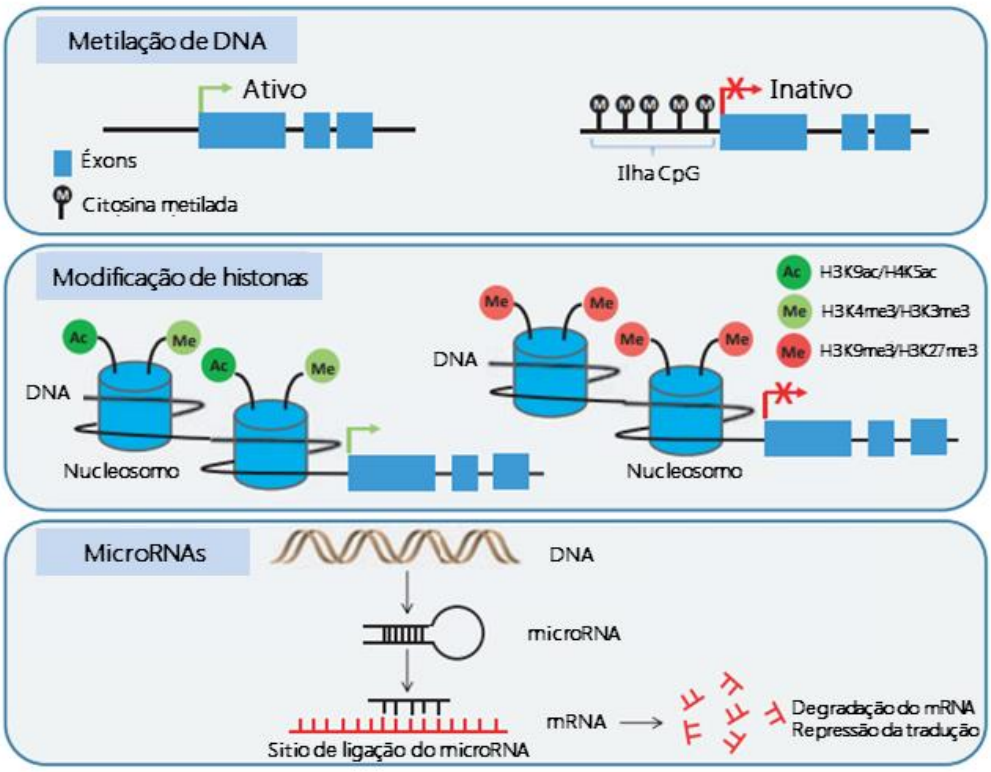

Figura 4. Mecanismos epigenéticos. A metilação do DNA é uma modificação covalente de citosinas em regiões de ilhas CpG dentro de sequências de genes e leva ao silenciamento transcricional. Nas modificações das histonas as caudas de histonas N-terminais podem sofrer uma variedade de modificações covalentes pós-transacionais, incluindo metilação e acetilação, as quais podem levar a ativação ou repressão da transcrição do gene, dependendo de quais resíduos são modificados e quais modificações ocorrem. Os miRNAs regulam a expressão de genes através do silenciamento pós-transcriptional de genes alvo. Por meio da complementariedade de bases entre as sequências de miRNAs com a região 3' UTR ou 5'UTR de RNAs mensageiros, ocorre a degradação ou inibição de tradução do mesmo. Adaptado de D'Assario *et al.* 2013. (46)

Os miRNAs exercem seus efeitos regulatórios ligando-se às regiões 3'UTR ou 5'UTR de RNAm alvos, por meio da complementariedade de pares de bases. Evidências sugerem que a especificidade do miRNA esteja ligada a uma pequena sequência de nucleotídeos, de aproximadamente 8 pares de bases, conhecida como *seed sequence*. A variação significativa no grau de complementariedade dessas sequências permite que um único miRNA se ligue a diversos RNAm, e, da mesma forma, cada RNAm pode ser regulado por vários miRNAs (51, 52). Em mamíferos, estes pequenos RNAs foram associados à regulação da proliferação, apoptose, diferenciação e hematopoiese, entre outras funções biológicas importantes (53-55).

Estudos recentes demonstraram alterações na expressão de miRNAs em diferentes patologias humanas, inclusive DM e suas complicações crônicas (56-61), enfatizando a importância dessas moléculas em processos patológicos. Kato *et al.* (62) demonstraram que os miRNAs podem ser induzidos em células renais *in vivo* e *in vitro* em condições hiperglicêmicas e podem promover a acumulação de proteínas de matriz extracelular relacionadas à fibrose e à disfunção glomerular. Além disso, estudos vêm demonstrando que diversos miRNAs circulantes estão desregulados em diferentes estágios da DRD tanto em amostras de sangue como urina (63-74). Entretanto, os resultados desses estudos são variados e inconclusivos. Dessa forma, visando sintetizar os resultados desses estudos, nosso grupo realizou uma revisão sistemática da literatura sobre os 27 estudos que investigaram a expressão de miRNAs em pacientes com DRD e em indivíduos controles (59). Esses estudos avaliaram a expressão de 1 miRNA até 1066 miRNAs no plasma, soro, urina, exosomos urinários ou biópsias renais de pacientes diabéticos (DM1 ou DM2) com DRD e de indivíduos controles (DM sem DRD ou indivíduos saudáveis). A DRD foi diagnosticada usando-se diferentes critérios diagnósticos [Excreção urinária de albumina

(EUA), TFGe, biópsia renal ou razão EUA/creatinina]. Como resultado, mostramos que 6 miRNAs estavam consistentemente desregulados em pacientes com diferentes graus de DRD comparados aos controles; isto é, eram diferencialmente expressos entre casos e controles em pelo menos 3 estudos da literatura. Entre esses miRNAs, miR-21-5p, miR-29a-3p, miR-126, miR-214 e miR-342 tinham a expressão aumentada, enquanto que o miR-192 tinha a expressão diminuída em pacientes com DRD comparado aos controles (59).

Ainda, ao investigar a expressão de 48 miRNAs no plasma de pacientes com >10 anos de DM1 (controles DM1), pacientes com diferentes graus de DRD (casos) e indivíduos saudáveis, através de análise de *macroarray*, e posterior validação dos resultados, nosso grupo demonstrou também a expressão aumentada dos miR-21-3p e miR-378a-5p e a expressão diminuída dos miR-16-5p e miR-29a-3p nos pacientes com DRD comparado aos controles DM1. Em posterior análise *in silico,* demonstrou-se que esses miRNAs regulam genes das vias PI3K/Akt, TGF-β1 e relaxina, o que indica que esses miRNAs podem ter um papel importante na patogênese da DRD (75).

1.1.2.1 MiRNAs e o Gene UCP2

Conhecer a função dos miRNAs na regulação de genes envolvidos na patogênese da DRD, como a *UCP2*, é fundamental para a compreensão desta complicação crônica do DM. Neste contexto, um estudo recente destacou o papel do miR-15a na síntese de insulina por meio da regulação do gene *UCP2* (76). Esse estudo demonstrou um aumento nos níveis de miR-15a em ilhotas expostas a alta concentração de glicose por 1h e uma diminuição de seus níveis após 3 dias de tratamento. Além disso, os níveis de miR-15a foram associados com a expressão de *UCP2*, inibindo significativamente os níveis proteicos de UCP2 em

células MIN6 (uma linhagem de células β pancreáticas) (76). Já é bem conhecido o papel da UCP2 como um regulador negativo da secreção de insulina (32), sendo que camundongos *knockout* para Ucp2 (*Ucp2*$^{-/-}$) apresentam níveis mais elevados de ATP nas ilhotas, com consequente aumento da secreção de insulina após estimulação com glicose. Por outro lado, a super-expressão de *UCP2* em linhagens de células-beta reduz os níveis de ATP e secreção de insulina estimulada por glicose (GSIS) (77, 78). Sendo assim, a diminuição da expressão de *UCP2* causada pelo miR-15a aumentaria a secreção de insulina após a estimulação com glicose; entretanto, teria uma efeito negativo na proteção contra o estresse oxidativo, podendo predispor à DRD e as outras complicações crônicas do DM (79).

Jiang *et al.* (80) demonstraram que o miR-30e é capaz de regular de forma direta a UCP2 em células tubulares renais. Esses autores relataram que a UCP2 é aumentada em células epiteliais tubulares dos rins após 3 dias de obstrução ureteral unilateral em camundongos. Camundongos com o gene *UCP2* bloqueado se tornaram resistentes à fibrose renal induzida pela obstrução ureteral. Além disso, foi evidenciado o papel da UCP2 na produção de matriz extracelular nas células tubulares renais por meio do aumento da expressão de TGF-β1, uma importante citocina envolvida na fibrose renal. Buscando compreender melhor esses dados, os autores realizaram uma análise de bioinformática em bancos de dados e descobriram a existência de sítios de ligação conservados para o miR-30e na região 3'UTR do RNAm *UCP2*. A transfecção de uma linhagem de células renais (NRK-52E) com um pre-miR-30e diminuiu a expressão de *UCP2* e TGF-β1, diminuindo a fibrose renal e confirmando que este miRNA regula diretamente a UCP2 (80). Poucos

estudos, até o momento, investigaram a associação desses miRNAs com DRD, e nenhum deles em uma população brasileira.

2. JUSTIFICATIVA

A DRD é uma importante complicação crônica do DM, sendo a principal causa de DRC terminal, que requer tratamento dialítico ou transplante renal. A DRD afeta cerca de 40% dos pacientes com DM e está associada com elevada morbidade e mortalidade em indivíduos em idade produtiva. Dentre os principais fatores associados ao desenvolvimento da DRD, destaca-se a hiperglicemia crônica decorrente do ambiente diabético que o individuo se encontra. Como consequência desta hiperglicemia, ocorre o aumento da produção de EROs pela mitocôndria, o que pode levar a danos no DNA como também aumento de estresse celular.

Nesse contexto, estudos vêm demonstrando o papel do gene *UCP2*, bem como polimorfismos nesse gene, com o desenvolvimento do dano renal e progressão para DRD, uma vez que está proteína é capaz de desacoplar a cadeia respiratória mitocondrial, diminuindo assim a produção de EROs. Entretanto, estudos adicionais são necessários para confirmar a associação de polimorfismos nesse gene com DRD, visto que os dados disponíveis, até o momento, são escassos e conflitantes. Da mesma forma, considerando que o gene *UCP2* é regulado diferencialmente entre diferentes tecidos, o que influencia suas funções, são necessários estudos que investiguem os reguladores da expressão dessa proteína e suas associações com DRD, incluindo o estudo de fatores epigenéticos, como os miRNAs.

MiRNAs são moléculas de RNA pequenos e não-codificantes que regulam negativamente a expressão gênica. Mudanças na expressão de miRNAs foram observadas em diversas situações patológicas, incluindo o DM1 e a DRD. Assim, o estudo de miRNAs capazes de regular genes envolvidos no desenvolvimento da DRD é fundamental para

melhor compreender a patogênese desta importante complicação do DM. Neste contexto, recentemente demonstrou-se que os miR-15a-5p e miR-30e-5p regulam a expressão de *UCP2* afetando a secreção de insulina e fibrose renal, respectivamente. Até o momento, nenhum estudo avaliou polimorfismos no gene *UCP2* em pacientes com DM1 com e sem DRD na população brasileira, da mesma forma que a expressão dos miR-15a e miR-30e no plasma e na urina ainda não foi avaliada em pacientes com e sem DRD, nesta mesma população.

3. OBJETIVOS

3.1 Objetivos gerais

- Avaliar a associação entre os polimorfismos -866G/A e Ins/Del no gene *UCP2* e a DRD em pacientes com DM1.

- Avaliar a expressão dos miR-15a-5p e miR-30e-5p no plasma e urina de pacientes com DM1 com e sem DRD.

3.2 Objetivos Específicos

- Comparar as frequências do polimorfismo -866G/A e Ins/Del no gene *UCP2* em pacientes com DM1 com e sem DRD.

- Realizar uma meta-análise de todos os estudos que avaliaram a associação dos polimorfismos -866G/A e Ins/Del no gene *UCP2* com DRD, visando se estes polimorfismos estão associados a esta doença.

- Comparar as expressões plasmáticas dos miR-15a-5p e miR-30e-5p entre pacientes com DM1 com e sem DRD.

- Comparar as expressões urinárias dos miR-15a-5p e miR-30e-5p entre pacientes com DM1 com e sem DRD.

- Correlacionar os níveis de expressão dos miR-15a-5p e miR-30e-5p com características laboratoriais relacionadas à DRD, tais como albuminúria e TFG.

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ARTIGO ORIGINAL 1

-866G/A AND INS/DEL POLYMORPHISMS IN THE *UCP2* GENE AND THE DIABETIC KIDNEY DISEASE: CASE-CONTROL STUDY AND META-ANALYSIS

POLIMORFISMOS -866G/A E INS/DEL NO GENE *UCP2* E A DOENÇA RENAL DO DIABETES: ESTUDO DE CASO CONTROLE E META-ANÁLISE

-866G/A and Ins/Del polymorphisms in the *UCP2* gene and diabetic kidney disease: case-control study and meta-analysis

Cristine Dieter[1,2], Taís Silveira Assmann[1,2], Natália Emerim Lemos[1,2], Eloísa Toscan Massignam[1], Bianca Marmontel de Souza[1,2], Daisy Crispim*[1,2].

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

2 – Postgraduation Program in Medical Sciences: Endocrinology, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

* Correspondence to: Dr. Daisy Crispim. Rua Ramiro Barcelos 2350; prédio 12; 4° andar. Zip Code 90035-003. Porto Alegre, Rio Grande do Sul, Brazil. E-mail: dcmoreira@hcpa.edu.br

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ABSTRACT

Introduction: Uncoupling protein 2 (UCP2) decreases reactive oxygen species (ROS) formation by mitochondria. ROS overproduction is a key contributor to the pathogenesis of diabetic microvascular complications, including diabetic kidney disease (DKD). Thus, *UCP2* gene polymorphisms are candidate risk factors for DKD; however, their associations with this complication are still inconclusive. Here, we describe a case-control study and a meta-analysis conducted to investigate the association between the *UCP2* -866G/A and Ins/Del polymorphisms and DKD.

Materials and Methods: The case-control study comprised 385 patients with type 1 diabetes mellitus (T1DM): 223 patients without DKD (controls) and 162 with DKD (cases). *UCP2* -866G/A (rs660339) and *UCP2* Ins/Del polymorphisms were genotyped by real-time PCR and conventional PCR, respectively. For the meta-analysis, a literature search was conducted to identify all studies that investigated associations between *UCP2* polymorphisms and DKD. Pooled odds ratios (OR) were calculated for different inheritance models.

Results: In the case-control study, allele and genotype frequencies of the *UCP2* -866G/A and Ins/Del polymorphisms did not differ significantly between case and control groups ($P > 0.05$). Frequencies of the different haplotypes constituted by them were also similar between groups ($P = 0.892$). Three articles (4 studies) plus the present case-control study were eligible for inclusion in the meta-analysis. In agreement with case-control data, meta-analysis results showed that the -866G/A and Ins/Del polymorphisms were not associated with DKD under allele contrast, dominant, recessive and additive inheritance models.

Conclusions: Our case-control and meta-analysis studies did not indicate an association between the analyzed *UCP2* polymorphisms and DKD.

Keywords: UCP2; polymorphisms; diabetic kidney disease.

INTRODUCTION

Diabetic kidney disease (DKD) is a common microvascular complication that affects 40% of patients with diabetes mellitus (DM) (1, 2). DKD is the leading cause of end-stage renal disease (ESRD) in subjects starting renal replacement therapy and is associated with increased cardiovascular mortality (1, 3). This complication is a progressive disease, characterized by pathophysiological changes resulting from the diabetic milieu, which begin with glomerular hypertrophy and hyperfiltration and might progress to albuminuria and a gradual decline in the glomerular filtration rate (GFR) (4, 5). The main risk factors for DKD are duration of chronic hyperglycemia, arterial hypertension, dyslipidemia, and genetic susceptibility (6, 7).

Chronic hyperglycemia causes renal damage through five main mechanisms: increased formation of advanced glycation end-products (AGEs); increased expression of the receptor for AGEs; activation of protein kinase C isoforms; increased flux of glucose through the polyol pathway; and upregulation of the hexosamine pathway (8, 9). Several lines of evidence have shown that the mitochondrial overproduction of reactive oxygen species (ROS) is the unifying upstream mechanism by each hyperglycemia activates all these five pathways (8, 10, 11).

Uncoupling protein 2 (UCP2) is a mitochondrial anion carrier protein expressed in a number of tissues, including adipose tissue, liver, kidney, and retina (12, 13). This protein mildly uncouples the oxidative phosphorylation from ATP synthesis by dissipating the proton gradient generated across the mitochondrial inner membrane; thereby, decreasing ATP production. The uncoupling then leads to tissue-specific functions, such as regulation of glucose and lipid metabolism and immune cell activation and, importantly, decreasing ROS formation by mitochondria (12, 14).

Consistent with the role of UCP2 in decreasing oxidative stress, several studies have suggested that polymorphisms in the *UCP2* gene are associated with the development of DM and its chronic complications (15-19). To date, three common *UCP2* polymorphisms have been well studied: the functional -866G/A polymorphism (rs659366) in the promoter region; the Ala55Val polymorphism (rs660339) in exon 4, and the 45bp insertion/deletion (Ins/Del) polymorphism in the 3' untranslated region (3' UTR) (19, 20).

Our group previously showed that the polymorphic *UCP2* -866A/55Val/Ins haplotype (constituted by the -866G/A, Ala55Val, and Ins/Del polymorphisms) was associated with risk for proliferative diabetic retinopathy (DR) in type 1 and type 2 diabetic patients (15). The -866G/A and the Ala55Val polymorphisms were in almost complete linkage disequilibrium (LD) in our population from South Brazil (15). Recently, we reported that the polymorphic -866A/55Val/Ins haplotype was also an independent risk factor for DKD (OR = 2.14, 95% CI 1.04 – 4.40) in patients with type 2 DM (T2DM) (18). Moreover, T2DM patients carrying the polymorphic haplotype showed lower estimated GFR (eGFR) compared with patients carrying the reference haplotype (-866G/Ala55/Del). Interestingly, the polymorphic haplotype was associated with decreased *UCP2* gene expression in human kidney biopsy samples (18).

Therefore, here we performed a case-control study to investigate if the *UCP2* -866G/A and Ins/Del polymorphisms were also associated with DKD in patients with type 1 DM (T1DM). Additionally, we conducted a systematic review and meta-analysis of the literature on the subject as part of the ongoing effort to evaluate if *UCP2* polymorphisms are associated with DKD in T1DM or T2DM patients.

MATERIALS AND METHODS

Case-control study

Subjects, phenotype measurements, and laboratory analyses

This case–control study was designed in agreement with STROBE and STREGA guidelines for reporting of genetic association studies (21, 22). The sample population comprised 162 T1DM patients with DKD (cases) and 223 T1DM patients without this complication and with at least 10 years of DM duration (T1DM controls). All T1DM patients were recruited from the outpatient clinic at Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil). Patients were diagnosed as having T1DM according to American Diabetes Association guidelines (23). A standard questionnaire was used to collect information on age, age at T1DM diagnosis, T1DM duration, and drug treatment, in addition all patients underwent physical and laboratory evaluations, as previously described (24, 25). The ethnic group was defined based on self-classification.

Serum and plasma samples were taken after 12 h of fasting for laboratory analyses (24, 25). Glucose levels were determined using the glucose oxidase method. Glycated hemoglobin (HbA1c) levels were measured by different methods and the results were traceable to the Diabetes Control and Complications Trial (DCCT) method by off-line calibration or using a conversion formulae (26). 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) (27).

The diagnosis of DKD was based on the urinary albumin excretion (UAE) 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 UAE (UAE <30 mg/24h, **control group**), moderately increased UAE (UAE 30 – 299 mg/24h) or severely increased UAE (UAE >300 mg/24h) (28). Therefore, the **case group** comprised patients who have moderately to severely increased UAE (moderate to severe DKD). Patients with other causes of albuminuria or renal diseases were excluded from the study. Estimated GFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation: $eGFR = 141 \times \min(SCR/\kappa, 1)^{\alpha} \times \max(SCR/\kappa, 1)^{-1,209} \times 0,993^{age} \times 1,018$ [if female] x 1,159 [if black] (29).

In addition, we also included a third group constituted of 489 healthy blood donors recruited from the same hospital, and who did not have diabetes or family history of this disease. These patients were used as non-diabetic controls; thus, only subjects with HbA1c <5.7% were included in this group (23). All subjects gave assent and written informed consent prior to participation. The study protocol was approved by Ethic Committee in Research from Hospital de Clínicas de Porto Alegre.

Genotyping

DNA was extracted from peripheral blood leucocytes by a standardized salting-out procedure (30). *UCP2* -866G/A polymorphism (rs659366) was genotyped using primers and probes contained in the TaqMan SNP Genotyping Assay 20× (Thermo Fisher Scientific, Foster City, CA, USA – assay ID: C___8760350_10). Real-Time PCR reactions were performed in 384-well plates, in a total of 5 µl volume, using 2 ng of DNA, TaqMan Genotyping Master Mix 1× (Thermo Fisher Scientific) and TaqMan Genotyping Assay 1×. Then, plates were placed in a real-time PCR thermal cycler

(ViiA7 Real- Time PCR System; Thermo Fisher Scientific) and heated for 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s and 63 °C for 90 s. Genotyping of the *UCP2* 45 bp Ins/Del polymorphism was performed by direct separation of the PCR products on 2.5% agarose gel stained with GelRed ™, as previously described (16).

As already described, the -866G/A polymorphism is in almost complete LD with the Ala55Val polymorphism (|D'| = 0.991, r^2 = 0.905) in our population. Therefore, only the *UCP2* -866G/A and Ins/Del polymorphisms were analyzed in the present case-control study (15).

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 the χ^2 test. Allele and genotype frequencies were compared between groups of subjects using χ^2 tests. Between all pairs of biallelic loci, we examined widely used measures of LD, Lewontin's D' |D'| and r^2 (31). Haplotypes constructed with the combination of the two *UCP2* polymorphisms and their frequencies were inferred using the PHASE 2.1 program, which implements a Bayesian statistical method (32).

Clinical and laboratory characteristics were compared between group of patients categorized according to the different genotypes of the two *UCP2* polymorphisms using unpaired Student's *t* test, One-Way ANOVA or χ^2 test, as appropriate. Variables with normal distribution are shown as mean ± SD or percentage. Variables with skewed distribution were log-transformed before analyses and are shown as median (25^{th} – 75^{th} percentile values). Multivariate logistic regression analyses were done to evaluate the independent association of each individual *UCP2* polymorphism or haplotypes with DKD, adjusting for possible confounding factors. Variables with significant

associations with DKD in the univariate analysis or with an important biological association with this complication were chosen for inclusion in the multivariate model. T1DM duration was not included as an independent variable in these analyses since T1DM control group was selected based on this characteristic. Statistical analyses were performed using the SPSS 18.0 software (SPSS, Chicago, IL), and P values <0.05 were considered significant.

Systematic review and meta-analysis

Search strategy and eligibility criteria

This study was designed and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) and Meta-analysis of Observational Studies in Epidemiology (MOOSE) statements (33, 34). PubMed and Embase repositories were searched to retrieve all articles that investigated associations between DKD and at least one of the two polymorphisms of interest. The medical subject headings (MeSH) used for this search are shown in the **Supplementary Material 1**. The search was restricted to human studies and English, Portuguese, or Spanish language articles, and was completed on September, 2018. References from all articles identified were searched manually to find other relevant studies.

Eligibility evaluation was made by title and abstracts review and when abstracts did not provide adequate information, the full text of the paper was retrieved for evaluation. This was done independently in a standardized manner by two investigators (C.D. and N.E.L.), as previously described (17, 35). Discrepancies were solved by discussion between them and when necessary a third reviewer (D.C.) was accessed. Observational studies that compared the -866G/A or Ins/Del polymorphisms between

patients with and without DKD were included in the meta-analysis. Articles were excluded from the analysis if genotype frequencies in the control group deviated from those predicted by the HWE, or if they did not have enough data to estimate an OR with 95% CI. If results were duplicated and had been published more than once, the most complete study was chosen.

Data extraction and quality control assessment

Necessary information from each study was independently extracted by two investigators (C.D. and N.E.L.) using a standardized extraction form (17, 35), and consensus was sought in all extracted items. When consensus could not be achieved, differences in data extraction were decided by reading the original publication or by consulting a third reviewer (D.C.). Data extracted from each study was as follows: (1) characteristics of each study and its samples (including name of first author, publication year, number of subjects in case and control groups, mean age, gender, ethnicity, age at T1DM diagnosis); (2) case and control definitions; (3) polymorphism frequencies and OR (95% CI). When data were not available, the authors were contacted by email.

Two investigators (C.D. and N.E.L.) independently evaluated the quality of each selected study using the Newcastle-Ottawa Scale (NOS) (36). The NOS contains eight items divided into three dimensions: selection, comparability, and exposure. For each item, a sequence of answer options is provided. A star scoring system is used to allow a semi-quantitative evaluation of paper quality, such that the highest quality studies are given a maximum of one star for each item, with exception of the item related to comparability, which allows two stars to be given. Therefore, the final NOS score varies from 0 to 9 stars.

Statistical analysis for meta-analysis

Genotype distributions in the control group were tested for conformity with HWE using a goodness-of-fitness χ^2 test. Polymorphism-DKD associations were analyzed using OR (95% CI) calculation based on allele contrast, dominant, recessive and additive models of inheritance (37). Heterogeneity was tested using χ^2-based Cochran's Q statistic and inconsistency was assessed with the I^2 metric (38, 39). Heterogeneity was considered statistically significant at P <0.10 for the Q statistic and/or I^2 >50% for the I^2 statistics. Where significant heterogeneity was detected, the DerSimonian and Laird random effect model (REM) was used to calculate OR (95% CI) for each study and for the pooled effect; where heterogeneity was not significant, the fixed effect model was used. Sensitivity analyses were performed to recognize important studies with a considerable impact on inter-study heterogeneity. All statistical analyses were performed using Stata 11.0 software (StataCorp, College Station, TX, USA).

RESULTS

Case-control study

Comparisons of clinical and laboratorial characteristics between T1DM case and control groups, categorized according to UAE values, are show in **Table 1**. As expected, HbA1c, triglycerides, total cholesterol, LDL cholesterol, and creatinine levels were increased in patients with DKD compared to T1DM controls. Prevalence of arterial hypertension and DR were also increased in the DKD group. eGFR was decreased in patients with DKD compared to T1DM controls. The ethnic proportion did not differ significantly between case and control groups: 10.5% of black subjects in the case group *vs*. 5.4% of black subjects in the control group (P= 0.093). Frequencies of the

minor alleles of the -866G/A and Ins/Del polymorphisms in white and black subjects were: 40.5% *vs.* 44.8% for the -866A allele (P= 0.814), and 30.7% *vs.* 20.3% for the Ins allele (P= 0.386).

Table 2 shows genotype and allele frequencies of the -866G/A and Ins/Del polymorphisms in T1DM patients with UAE >30 mg/24h (DKD cases) and T1DM patients with UAE <30 mg/24h (T1DM controls). Genotype distributions of the two analyzed polymorphisms were in agreement with those predicted by HWE in both groups (P ≥0.05), and they were similar between DKD cases and T1DM controls (**Table 2**). Of note, this result did not change after adjustment for ethnicity, HbA1c, serum creatinine, and triglycerides (**Table 2**). Accordingly, allele distributions of the -866G/A and Ins/Del polymorphisms did not differ between case and control groups, and these polymorphisms were also not associated with DKD when assuming different genetic inheritance models (**Table 2**). It is worth noting that when we stratified patients according to the UAE severity (T1DM controls *vs*. patients with moderate UAE *vs*. severe UAE), the -866G/A and Ins/Del frequencies also did not differ significantly among groups (**Table S1**).

The -866G/A polymorphism is in moderate LD with the Ins/Del polymorphism (|D'|= 0.711, r^2= 0.311) in our population. Four haplotypes (Ht) produced by the combination of these two polymorphisms were inferred in the total sample of T1DM patients: -866G/Del (Ht1; 52.7%), -866A/Del (Ht2; 17.2%), -866G/Ins (Ht3; 6.5%) and -866A/Ins (Ht4; 23.6%). Distributions of these haplotypes were similar between T1DM controls and cases with DKD (P= 0.892) (**Table 3**). Moreover, frequency of 3 or 4 minor alleles of the -866G/A and Ins/Del polymorphisms (Ht3/Ht4 or Ht4/Ht4) were similar between T1DM controls and patients with DKD (17.2% *vs*. 15.3%, adjusted P= 0.604; **Table 2**). These frequencies were also similar among groups according to the

severity of DKD (T1DM controls *vs*. moderate UAE *vs*. severe UAE; P= 0.805; **Table S1**)

In an exploratory analysis, all clinical and laboratories characteristics showed in **Table 1** were then compared between all T1DM patients (control + case subjects) broken down by the presence of the -866G/A and Ins/Del polymorphisms. Frequency of DR was not significantly different among the -866G/A genotypes (G/G: 49.2%; G/A: 53.8% and A/A: 65.1%; P= 0.117). In contrast, presence of DR was increased in patients carrying the Ins/Ins genotype (81.8%) compared to patients with the Del/Del or Ins/Del genotypes (48.5% and 57.0%, respectively; P= 0.002). Frequency of DR was 70.4% in patients carrying 3 or 4 minor alleles of the -866G/A and Ins/Del polymorphisms, 49.7% in patients with 0/1 minor allele, and 54.1% in patients with 2 minor alleles (P= 0.029). No other characteristic described in **Table 1** differed among the genotypes of the two analyzed polymorphism (data not shown). Genotype and allele frequencies of the -866G/A and Ins/Del polymorphisms were similar between T1DM patients (T1DM controls + DKD patients) and non-diabetic subjects (**Table S2**), suggesting that these two polymorphisms are not associated with T1DM risk.

Systematic review and meta-analysis

Figure 1 shows a flow diagram illustrating the strategy used to identify and select articles for inclusion in our meta-analysis. A total of 182 possible relevant citations were retrieved from PubMed and Embase, and 178 of them were excluded during the review of titles and abstracts. Four articles remained to be fully evaluated. Nevertheless, following careful analysis of their full texts, one article was excluded because it did not have a control group. Therefore, three articles (18, 40, 41) plus the present case-control study were included in our meta-analyses, totalizing four articles (five studies). In total,

717 controls without DKD and 648 cases with this complication were analyzed for the -866G/A polymorphism, and 937 controls and 857 cases for the Ins/Del polymorphism. The article by Tiwari *et al*. (41) analyzed the two *UCP2* polymorphisms in two different populations from South India and North India, and, because of that, their results are shown separately.

With exception of the present case-control study, the other three articles included only T2DM patients. Two studies comprised Caucasian populations (18, 40), the present study investigated a mixed population, while Tiwari *et al*. (41) analyzed two Asian populations. All studies investigated the Ins/Del polymorphism, while the study by Lindholm *et al*. (40) was the only one that did not investigate the -866G/A polymorphism. Two studies (18, 40) plus the present case-control classified DKD using the UAE; while one study (41) classified DKD using serum creatinine levels. Genotype and allele distributions of the *UCP2* polymorphisms in case and control samples from the different studies analyzed, as well as their respective ORs (95 CI%) for association with DKD, are shown in **Table S3**. Quality assessment using the NOS scale showed that most studies were considered as having good quality since 8 stars were given for the studies by Lindholm *et al*. (40) and Souza *et al*. (18), and 7 stars for the study by Tiwari *et al*. (41).

Table 4 summarizes the results of quantitative pooled analyses for associations between -866G/A and Ins/Del polymorphisms and susceptibility to DKD. Our results showed no significant associations between these polymorphisms and DKD under allele contrast, additive, recessive, or dominant inheritance models. A significant heterogeneity was observed among studies of the -866G/A polymorphism considering the dominant model of inheritance (**Table 4**). Thus, sensitivity analyses were performed to evaluate the effect of each individual study on the meta-analysis performed for this

model. This was carried out by repeating the meta-analysis excluding a different study at a time. These analyses showed that the study by Tiwari *et al*. (41) explained the observed heterogeneity in the meta-analysis of the -866G/A polymorphism under a dominant model. However, after exclusion of this study from the respective meta-analysis, the pooled OR remained not significant (OR= 0.91, 95% CI 0.71 – 1.16).

DISCUSSION

ROS overproduction is one of the main mechanisms by which hyperglycemia leads to chronic diabetic complications, including DKD (8, 10, 11). Although UCP2 has a recognized role in reducing oxidative stress, to date, only few studies have evaluated the association between polymorphisms in the *UCP2* gene and DKD. Therefore, aiming to better understand the relationship between the *UCP2* -866G/A and Ins/Del polymorphisms and the development of this chronic diabetic complication, we performed a case-control study and a meta-analysis of genetic association studies on this subject.

Our case-control study suggested that both analyzed polymorphisms and the haplotypes constituted by them are not associated with DKD in T1DM patients. In contrast, our previous study showed that the polymorphic -866A/55Val/Ins haplotype was associated with DKD in Brazilian T2DM patients after adjustment for age, gender, treatment with ACE-inhibitors, triglycerides, and eGFR levels (18). In both studies, DKD was classified using UAE levels. Souza *et al*. (18) also reported that T2DM patients carrying the -866A/55Val/Ins haplotype (dominant model) showed lower eGFR compared to patients with the reference haplotype, which was not observed in the present study. These discrepancies may be explained by differences in DKD pathophysiology between T1DM and T2DM **(Figure 2)** (42). Also, we cannot fully

exclude the possibility of type II error when analyzing associations between the *UCP2* polymorphisms and DKD. Although we had more than an 80% power ($\alpha = 0.05$) to detect an OR= 2.0 for the association with the -866G/A and Ins/Del polymorphisms, we cannot rule out the possibility that these polymorphisms would be individually associated with DKD with lower ORs. There is also a possibility that these two polymorphisms are only associated with DR, an association observed in both T1DM and T2DM patients (15). Considering that the majority of DKD patients have some degree of DR (43), it is plausible that the association with DKD in T2DM patients (18) was not independent from DR.

Meta-analysis has been regarded as a powerful method for pooling data from different studies because it could overcome the problem of small sample sizes as well as insufficient statistical power of genetic association studies for common diseases (34). Therefore, trying to overcome the problem of small sample size, we also performed a meta-analysis including 3 published studies from different populations plus the results from the present case-control study. Meta-analysis results indicated that the -866G/A and Ins/Del polymorphisms are not associated with DKD. Among the studies included in our meta-analysis, only the study by Tiwary *et al*. (41) showed an association between the -866G/A polymorphism and DKD in South Indians. These authors did not observe an association between this polymorphism and DKD in North Indians. The other studies were not able to show an association of the -866G/A or Ins/Del polymorphisms with DKD, including the study by Souza *et al*. (18) that, as already mentioned, only observed an association with the disease when analyzing the haplotypes constituted by the two polymorphisms.

Rudofsky *et al*. also observed that frequency of DKD was similar among German T1DM (44) and T2DM (44, 45) patients carrying the different genotypes of the

-866G/A polymorphism. These two studies were not included in our meta-analysis because they did not include an appropriate control group. In addition, Tripathi *et al*. (46) reported an association between the Ins/Del polymorphism and risk for ESRD in non-diabetic subjects from North India; however, genotype distributions of this polymorphism were not in HWE in the control group. Thus, this study could not be included in our meta-analysis and should be interpreted with caution.

Therefore, to date, most studies indicated that the -866G/A and Ins/Del polymorphisms are not risk factors for DKD. We also acknowledge that certain factors unrelated to the *UCP2* polymorphisms could have interfered with the present findings. First, meta-analysis is prone to publication bias, and although we have attempted to trace unpublished observations, we cannot be sure that small negative studies were overlooked. Second, although the meta-analysis increased the statistical power, the total sample power might still not be enough to show associations with lower ORs. Third, heterogeneity is potentially a significant problem when interpreting the results of any meta-analysis, and our meta-analysis showed significant inter-study heterogeneity when analyzing the -866G/A polymorphism in the dominant model of inheritance. The exclusion of the study by Tiwari *et al.* (41) was able to reduce heterogeneity; however, this exclusion did not change the association with DKD. Therefore, we could not fully exclude the possibility that the heterogeneity observed might reduce our power to detect true associations.

Although these negative results regarding associations between *UCP2* polymorphisms and DKD, functional studies have suggested that UCP2 plays an important role in the development of renal damage. Qiu *et al.* (47) reported that oral administration of genipin, an UCP2 inhibitor, partially prevented the progression of DKD in C57BL/6J mice by reducing glucose-induced albumin leakage through

podocytes monolayer, consequently improving podocyte function. Accordingly, Jiang *et al*. (48) showed that UCP2 was induced in kidney tubular epithelial cells after unilateral ureteral obstruction in mice, while those mice with ablated *UCP2* resisted obstruction-induced kidney fibrosis. Moreover, *UCP2* knockdown in NRK-52E tubular cells abolished the effect of TGF-β1 treatment, decreasing extracellular matrix production (48). In contrast, Chen *et al.* (49) demonstrated that inhibition of UCP2 by genipin increased oxidative stress in rat proximal tubular cells treated with high glucose medium, and this led to increased cell apoptosis. *UCP2* knockdown in renal mesangial cells of rats also increased oxidative stress, inflammation and apoptosis *in vitro* (50). Therefore, whether UCP2 has a protective or deleterious effect in renal function remains to be clarified.

In conclusion, data reported here suggest that the *UCP2* -866G/A and Ins/Del polymorphisms are not important risk factors for DKD, classified according to UAE values. Further additional studies with large sample sizes are necessary to elucidate the effects possibly played by *UCP2* polymorphisms in the pathogenesis of DKD.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure legend

Figure 1. Flowchart illustrating the search strategy used to identify association studies of *UCP2* polymorphisms and DKD for the meta-analysis study.

Figure 2. Differences in DKD pathophysiology between T1DM and T2DM.

Supplemental material list

Supplementary material 1: Mesh's used for the search for articles for meta-analyses.

Table S1. Genotype and allele frequencies of *UCP2* Ins/Del polymorphism in T1DM patients with UAE <30 mg/24h (T1DM control), T1DM patients with UAE 30-300 mg/24h (moderate DKD), and T1DM with UAE >300 mg/24h (severe DKD).

Table S2. Genotype and allele frequencies of *UCP2* -866G/A and Ins/Del polymorphisms in T1DM patients and nondiabetic subjects.

Table S3. Genotype and allele distributions of the *UCP2* -866G/A and Ins/Del polymorphisms in T1DM patients with (cases) and without (controls) DKD.

Table 1. Clinical and laboratory characteristics of T1DM patients with UAE >30 mg/24h (DKD cases) and T1DM patients with UAE <30 mg/24h (T1DM controls).

Characteristics	T1DM controls (n = 223)	DKD cases (n = 162)	P*
Age (years)	36.8 ± 12.8	37.7 ± 13.6	0.478
Gender (% male)	47.5	48.8	0.892
Ethnicity (% black)	5.4	10.5	0.093
HbA1c (%)	8.4 ± 1.7	9.5 ± 2.2	0.0001
BMI (kg/m^2)	24.2 ± 3.6	23.9 ± 3.6	0.413
Hypertension (%)	31.8	46.0	0.012
Age at diagnosis (years)	15.4 ± 10.0	15.4 ± 10.6	0.993
T1DM duration (years)	20.7 ± 8.2	20.6 ± 10.5	0.956
Systolic BP (mmHg)	121.1 ± 15.7	123.4 ± 19.3	0.244
Diastolic BP (mmHg)	77.2 ± 10.6	78.3 ± 13.5	0.423
Triglycerides (mg/dL)	70.0 (51.7 – 98.5)	100.0 (70.2 – 159.5)	< 0.001
Total cholesterol (mg/dL)	177.7 ± 42.1	193.0 ± 58.0	0.007
LDL cholesterol (mg/dL)	100.8 ± 30.6	111.5 ± 48.0	0.031
HDL cholesterol (mg/dL)	57.7 ± 16.7	56.2 ± 19.0	0.429
Diabetic retinopathy (%)	44.8	66.9	< 0.001
Serum creatinine (µg/dL)	0.9 (0.7 – 1.0)	1.0 (0.8 – 1.6)	< 0.001
eGFR	104.0 (87.2 – 121.0)	87.0 (46.0 – 117.0)	< 0.001
UAE (mg/g)	5.5 (3.3 – 10.7)	86.9 (39.0 – 353.8)	-

Data are shown by mean ± standard deviation, median (25^{th} – 75^{th} percentile values) or %. BMI: body mass index; BP: blood pressure; DKD: diabetic kidney disease; eGFR: estimated glomerular

filtration rate; HbA1c: glycated hemoglobin; T1DM: type 1 diabetes mellitus; UAE: urinary albumin excretion. *P values were computed using Student's *t* tests, or Chi-square tests, as appropriate.

Table 2. Genotype and allele frequencies of *UCP2* -866G/A and Ins/Del polymorphisms in T1DM patients with UAE >30 mg/24h (DKD cases) and in T1DM patients with UAE <30 mg/24h (T1DM controls).

Polymorphisms	T1DM controls	DKD cases	OR (95% CI)/ Unadjusted P value*	Adjusted OR (95% CI) /† P value
-866G/A	n = 223	n = 162		
Genotype				
G/G	77 (34.5)	61 (37.7)	1	1
G/A	107 (48.0)	72 (44.4)	0.849 (0.542 - 1.332)/ 0.477	0.779 (0.447 – 1.359)/ 0.379
A/A	39 (17.5)	29 (17.9)	0.939 (0.522 – 1.687)/ 0.832	1.263 (0.628 – 2.541)/ 0.513
Allele				
G	0.59	0.60	0.706	-
A	0.41	0.40		
Recessive model				
G/G + G/A	184 (82.5)	133 (82.1)	1	1
A/A	39 (17.5)	29 (17.9)	1.029 (0.606 – 1.747)/ 0.917	1.449 (0.771 – 2.723)/ 0.249
Additive model				
G/G	77 (66.4)	61 (67.8)	1	1

A/A	39 (33.6)	29 (32.2)	0.939 (0.522 – 1.687)/ 0.832	1.313 (0.634 – 2.717)/ 0.463
Dominant model				
G/G	77 (34.5)	61 (37.7)	1	1
G/A + A/A	146 (65.5)	101 (62.3)	0.873 (0.573 – 1.330)/ 0.528	0.900 (0.538 – 1.506)/ 0.689
Ins/Del	n = 222	n = 156		
Genotype				
Del/Del	107 (48.2)	82 (52.6)	1	1
Ins/Del	93 (41.9)	59 (37.8)	0.828 (0.536 – 1.279)/ 0.394	0.710 (0.411 – 1.225)/ 0.219
Ins/Ins	22 (9.9)	15 (9.6)	0.890 (0.435 – 1.822)/ 0.749	1.453 (0.616 – 3.551)/ 0.393
Allele				
Del	0.69	0.71	0.492	-
Ins	0.31	0.29		
Recessive model				
Ins/Del + Del/Del	200 (90.1)	141 (90.4)	1	1
Ins/Ins	22 (9.9)	15 (9.6)	0.967 (0.485 – 1.930)/ 0.924	1.705 (0.749 – 3.881)/ 0.204
Additive model				
Del/Del	107 (82.9)	82 (84.5)	1	1

Ins/Ins	22 (17.1)	15 (15.5)	0.890 (0.435 – 1.822)/ 0.749	1.276 (0.545 – 2.990)/ 0.574
Dominant model				
Del/Del	107 (48.2)	82 (52.6)	1	1
Ins/Del + Ins/Ins	115 (51.8)	74 (47.4)	0.840 (0.557 – 1.265)/ 0.403	0.821 (0.493 – 1.367)/ 0.448
Presence of the *UCP2* mutated haplotype	(n = 209)	(n = 150)		
0 or 1 mutated allele	110 (52.6)	83 (55.3)	1	1
2 mutated alleles	63 (30.2)	44 (29.4)	0.926 (0.573 – 1.494)/ 0.752	0.751 (0.411 – 1.372)/ 0.352
3 or 4 mutated alleles	36 (17.2)	23 (15.3)	0.847 (0.467 – 1.536)/ 0.584	1.207 (0.593 – 2.458)/ 0.604

Data are shown as number (%) or proportion. DKD: diabetic kidney disease; T1DM: type 1 diabetes mellitus; UAE: urinary albumin excretion. *P-values were calculated using Chi-square tests. † P-values and OR (95% CI) obtained using logistic regression analyses adjusting for ethnicity, HbA1c, serum creatinine (logarithmic scale), and triglycerides (logarithmic scale).

Table 3. Haplotypes of the *UCP2* polymorphisms in T1DM patients with and without DKD.

Haplotypes	**T1DM controls (n = 418)**	**DKD cases (n = 300)**	**P *value***
Ht 1 (-866G/Del)	0.518	0.540	0.892
Ht 2 (-866A/Del)	0.171	0.173	
Ht 3 (-866G/Ins)	0.069	0.060	
Ht 4 (-866A/Ins)	0.242	0.227	

Data are presented as proportion. n = number of chromosomes. DKD: diabetic kidney disease; T1DM: type 1 diabetes mellitus. The first letter of the haplotypes refers to the -866G/A polymorphism and the second to the Ins/Del polymorphism. P values for the comparisons of haplotype frequencies between patients with or with DKD were calculated using permutations tests.

Table 4. Pooled measures for associations between the *UCP2* -866G/A and Ins/Del polymorphisms and susceptibility to DKD.

Inheritance model	*n* studies	*n* controls	*n* cases	I^2 (%)	Pooled OR (95% CI)
***UCP2* -866 G/A**					
Allele contrast[b]	4	717	648	46.0	1.03 (0.88-1.21)
Additive[b]	4	390	351	0.0	1.04 (0.75-1.45)
Recessive[b]	4	717	648	0.0	1.05 (0.78-1.42)
Dominant[a]	4	717	648	53.2	1.04 (0.74-1.45)
***UCP2* Ins/Del**					
Allele contrast[b]	4	719	641	0.0	0.96 (0.81-1.14)
Additive[b]	4	444	413	0.0	1.08 (0.71-1.63)
Recessive[b]	4	719	641	13.3	1.11 (0.74-1.65)
Dominant[b]	5	937	857	0.0	0.89 (0.74-1.08)

[a] If significant heterogeneity was detected (I^2 >50%), the DerSimonian and Laird random effect model (REM) was used to calculated OR (95% CI); **[b]** if heterogeneity was not significant, the fixed effect model (FEM) was used for this calculation. DKD: diabetic kidney disease.

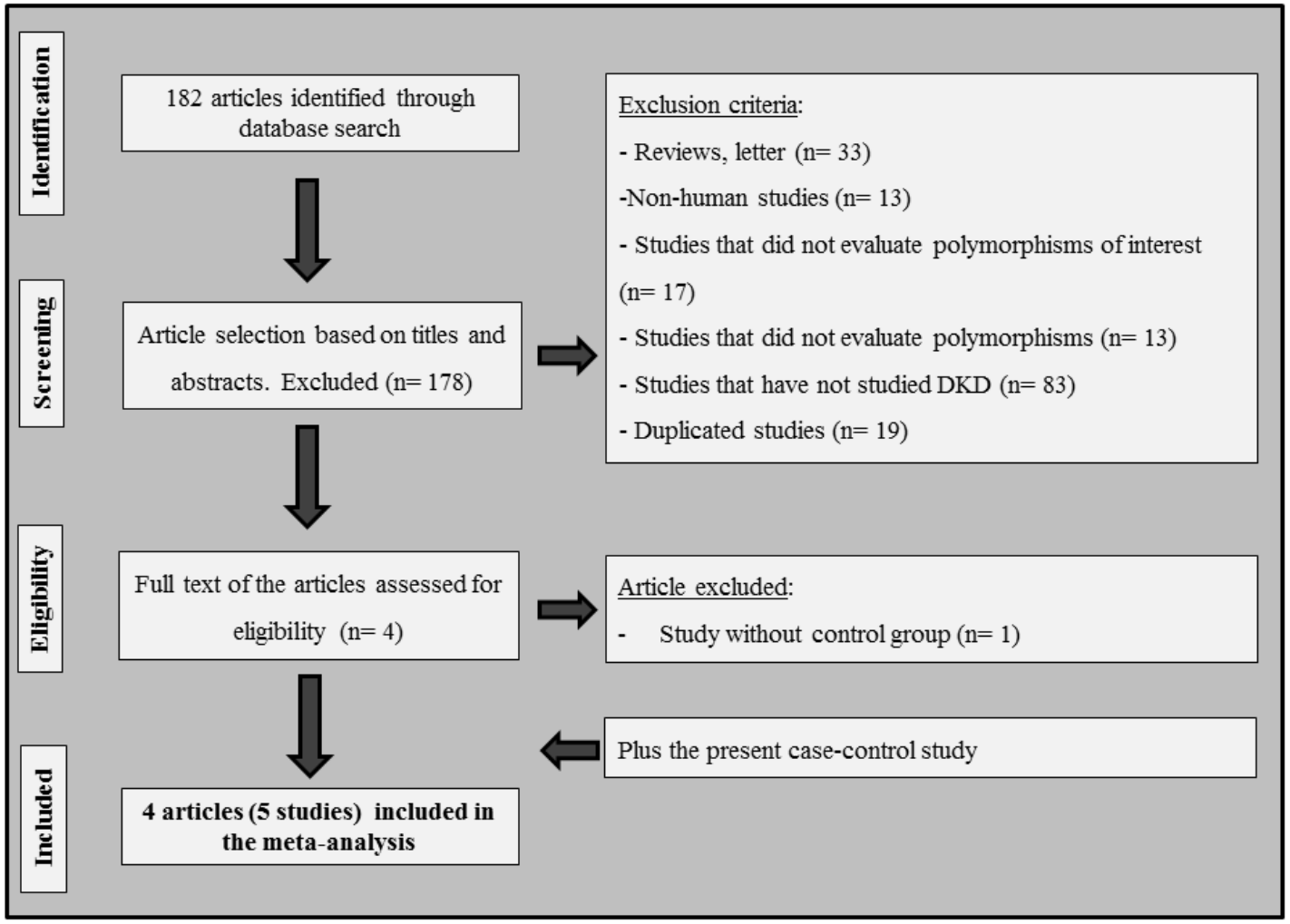

Figure 1. Flowchart illustrating the search strategy used to identify association studies of *UCP2* polymorphisms and DKD for the meta-analysis study.

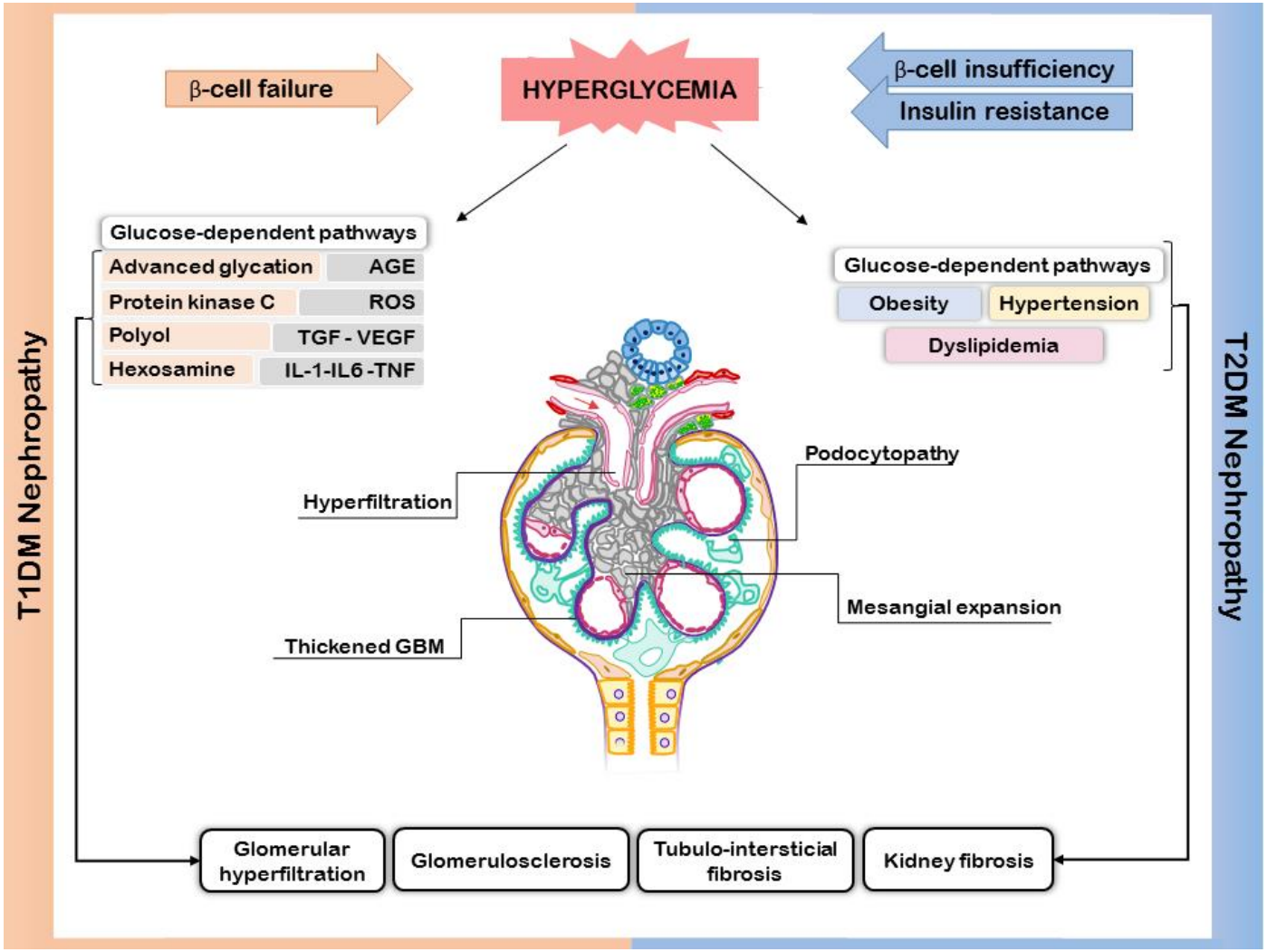

Figure 2. Differences in DKD pathophysiology between T1DM and T2DM.

Supplementary material 1: The MeSH used for meta-analyses search: ("diabetes mellitus" OR "diabetes mellitus, type 1" OR "diabetes mellitus, type 2" OR "diabetic nephropathy" OR "diabetes complications") AND ("polymorphism, genetic" OR "polymorphism, single-stranded conformational" OR "polymorphism, single nucleotide" OR "polymorphism, restriction fragment length" OR "amplified fragment length polymorphism analysis" OR "DNA copy number variations" OR "mutation" OR "frameshift mutation" OR "mutation rate" OR "INDEL mutation" OR "mutation, missense" OR "point mutation" OR "codon, nonsense") AND ("uncoupling protein 2").

Table S1. Genotype and allele frequencies of *UCP2* -866G/A and Ins/Del polymorphisms in T1DM patients with UAE <30 mg/24h (T1DM control), T1DM patients with UAE 30-300 mg/24h (moderate DKD), and T1DM with UAE >300 mg/24h (severe DKD).

Polymorphisms	T1DM control group	Moderate DKD	Severe DKD	P*
- 866G/A (rs659366)	n = 223	n = 110	n = 52	
Genotype				
G/G	77 (34.5)	42 (38.2)	19 (36.5)	0.962
G/A	107 (48.0)	48 (43.6)	24 (46.2)	
A/A	39 (17.5)	20 (18.2)	9 (17.3)	
Allele				
G	0.59	0.60	0.60	0.929
A	0.41	0.40	0.40	
Recessive model				
G/G + G/A	184 (82.5)	90 (81.8)	43 (82.7)	0.985
A/A	39 (17.5)	20 (18.2)	9 (17.3)	
Additive model				
G/G	77 (66.4)	42 (67.7)	19 (67.9)	0.978
A/A	39 (33.6)	20 (32.3)	9 (32.1)	
Dominant model				
G/G	77 (34.5)	42 (38.2)	19 (36.5)	0.803
G/A + A/A	146 (65.5)	68 (61.8)	33 (63.5)	
Ins/Del (rs763034008)	n = 222	n = 103	n = 53	
Genotype				
Del/Del	107 (48.2)	57 (55.3)	25 (47.2)	0.768
Ins/Del	93 (41.9)	36 (35.0)	23 (43.4)	

Ins/Ins	22 (9.9)	10 (9.7)	5 (9.4)	
Allele				
Del	0.69	0.73	0.69	0.608
Ins	0.31	0.27	0.31	
Recessive model				
Ins/Del + Del/Del	200 (90.1)	93 (90.3)	48 (90.6)	0.994
Ins/Ins	22 (9.9)	10 (9.7)	5 (9.4)	
Additive model				
Del/Del	107 (82.9)	57 (85.1)	25 (83.3)	0.929
Ins/Ins	22 (17.1)	10 (14.9)	5 (16.7)	
Dominant model				
Del/Del	107 (48.2)	57 (55.3)	25 (47.2)	0.442
Ins/Del + Ins/Ins	115 (51.8)	46 (44.7)	28 (52.8)	
Presence of the *UCP2* mutated haplotype	**n = 209**	**n = 101**	**n = 49**	
0 or 1 mutated allele	110 (52.6)	59 (58.4)	24 (49.0)	0.805
2 mutated alleles	63 (30.1)	27 (26.7)	17 (34.7)	
3 or 4 mutated alleles	36 (17.2)	15 (14.9)	8 (16.3)	

Data are shown as number (%) or proportion. DKD: diabetic kidney disease; T1DM: type 1 diabetes mellitus; UAE: urinary albumin excretion. *P-*values* were calculated using Chi-square tests.

Table S2. Genotype and allele frequencies of *UCP2* -866G/A and Ins/Del polymorphisms in T1DM patients and nondiabetic subjects.

Polymorphisms	Nondiabetic subjects	T1DM patients	P value*	Adjusted OR (95% CI) /† P value
-866G/A	n = 489	n = 385		
Genotype				
G/G	187 (38.2)	138 (35.8)	0.754	1
G/A	221 (45.2)	179 (46.5)		1.050 (0.748 – 1.476)/ 0.777
A/A	81 (16.6)	68 (17.7)		1.123 (0.713 – 1.770)/ 0.617
Allele				
G	0.61	0.59	0.459	-
A	0.39	0.41		
Recessive model				
G/G + G/A	408 (83.4)	317 (82.3)	0.735	1
A/A	81 (16.6)	68 (17.7)		1.094 (0.722 – 1.657)/ 0.673
Additive model				
G/G	187 (69.8)	138 (67.0)	0.584	1
A/A	81 (30.2)	68 (33.0)		1.117 (0.708 – 1.762)/ 0.634
Dominant model				
G/G	187 (38.2)	138 (35.8)	0.511	1
G/A + A/A	302 (61.8)	247 (64.2)		1.069 (0.777 – 1.471)/ 0.681
Ins/Del	n = 374	n = 378		
Genotype				
Del/Del	194 (51.9)	189 (50.0)	0.837	1

Ins/Del	147 (39.3)	152 (40.2)		1.111 (0.787 – 1.568)/ 0.550
Ins/Ins	33 (8.8)	37 (9.8)		1.173 (0.660 – 2.087)/ 0.586
Allele				
Del	0.72	0.70	0.545	-
Ins	0.28	0.30		
Recessive model				
Ins/Del + Del/Del	341 (91.2)	341 (90.2)	0.742	1
Ins/Ins	33 (8.8)	37 (9.8)		0.121 (0.643 – 1.954)/ 0.687
Additive model				
Del/Del	194 (85.5)	189 (83.6)	0.682	1
Ins/Ins	33 (14.5)	37 (16.4)		1.169 (0.660 – 2.071)/ 0.593
Dominant model				
Del/Del	194 (51.9)	189 (50.0)	0.660	1
Ins/Del + Ins/Ins	180 (48.1)	189 (50.0)		1.123 (0.811 – 1.555)/ 0.486

Data are shown as number (%) or proportion. T1DM: type 1 diabetes mellitus. *P-values were calculated using Chi-square tests. † P-values and OR (95% CI) obtained using logistic regression analyses adjusting for hypertension and ethnicity.

Table S3. Genotype and allele distributions of the *UCP2* -866G/A and *UCP2* Ins/Del polymorphisms in DM patients with (cases) and without (controls) DKD.

-866G/A polymorphism		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]
Tiwari, *et al*. 2009[a]	Asian	106	43	52	11	146	80	56	10	65.1	74.0	1.524 (1.037 – 2.239)
Tiwari, *et al.* 2009[b]	Asian	90	43	37	10	75	30	36	9	68.3	64.0	0.824 (0.521 – 1.302)
Souza, *et al*., 2015	Caucasian	287	101	134	52	278	99	131	48	58.5	59.2	1.027 (0.810 – 1.301)
The present case-control study	Caucasian	165	62	74	29	218	74	104	40	60.0	59.0	0.913 (0682 – 1.221)
Ins/Del polymorphism		Cases (n) by total and genotype				Controls (n) by total and genotype				Del allele frequency (%)		
Reference	Ethnicity	Total	Del/Del	Ins/Del	Ins/Ins	Total	Del/Del	Ins/Del	Ins/Ins	Cases	Controls	OR (95% CI)[a]
Lindholm, *et al.,* 2004	European	216	NS	NS	NS	218	NS	NS	NS	NS	NS	NA
Tiwari, *et al.*, 2009[a]	Asian	105	73	30	2	149	108	38	3	83.8	85.2	1.115 (0.685 – 1.815)
Tiwari, *et al.*, 2009[b]	Asian	90	61	26	3	75	45	24	6	82.0	76.0	0.685 (0.400 – 1.169)
Souza, *et al.*, 2015	Caucasian	287	144	110	33	278	132	124	22	70.0	69.8	1.021 (0.792 – 1.316)
The present case-control study	Caucasian	159	82	62	15	217	106	89	22	71.0	69.0	0.921 (0.671 – 1.265)

[a] Calculated from the available genotypes. DM: diabetes mellitus; DKD: diabetic kidney disease; NS, not shown; NA, not available.

ARTIGO ORIGINAL 2

MIR-15A-5P AND MIR-30E-5P EXPRESSIONS IN PLASMA AND URINE FROM TYPE 1 DIABETES PATIENTS ARE ASSOCIATED WITH DIABETIC KIDNEY DISEASE

EXPRESSÕES DOS MIR-15A-5P E MIR-30E-5P NO PLASMA E URINA DE PACIENTES COM DIABETES MELLITUS TIPO 1 ESTÃO ASSOCIADAS COM DOENÇA RENAL DO DIABETES

MiR-15a-5p and miR-30e-5p expressions in plasma and urine from type 1 diabetes patients are associated with diabetic kidney disease

Cristine Dieter[1,2], Taís Silveira Assmann[1,2], Aline Costa Rodrigues[1], Luís Henrique Canani[1,2], Bianca Marmontel de Souza[1,2], Andrea Carla Bauer[1,2], and Daisy Crispim*[1,2].

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

2- Postgraduation Program in Medical Sciences: Endocrinology, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

* Correspondence to: Dr. Daisy Crispim. Ramiro Barcelos Street 2350; building 12; 4° floor. Zip Code 90035-003. Porto Alegre, Rio Grande do Sul, Brazil. E-mail: dcmoreira@hcpa.edu.br

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ABSTRACT

Introduction: Diabetic kidney disease (DKD) is a common microvascular complication that affects 40% of patients with diabetes mellitus (DM). Emerging evidence suggests a role for several microRNAs (miRNAs) in the development of DKD. In this context, miR-15a-5p and miR-30e-5p have been shown to regulate the expression of the uncoupling protein 2 (UCP2), a mitochondrial protein that decreases reactive oxygen species (ROS) formation by mitochondria. Since ROS overproduction is a key contributor to the pathogenesis of DKD, dysregulation of these two miRNAs may be involved in DKD pathogenesis. Thus, the aim of this study was to compare the expression of miR-15a-5p and miR-30e-5p in type 1 DM (T1DM) patients with DKD (cases) or without this complication (controls). **Methods:** MiR-15a-5p and miR-30e-5p expressions were analyzed in plasma and urine of 17 T1DM controls and 23 DKD cases using qPCR. Bioinformatics analyses were performed to determine in which pathways the two analyzed miRNAs were involved. **Results:** MiR-15a-5p and miR-30e-5p expressions were downregulated in plasma of patients with DKD compared to T1DM controls [miR-15a-5p: 0.272 (0.039 – 0.484) *vs*. 0.466 (0.223 – 3.243), P= 0.024; miR-30e-5p: 0.534 (0.147 – 0.943) *vs*. 2.416 (0.514 – 4.330), P= 0.006]. These miRNAs were also significantly downregulated in urine of DKD cases. Our bioinformatics analyses indicated that these two miRNAs regulate several genes that participate in pathways related to angiogenesis, apoptosis, cell differentiation, oxidative stress, and hypoxia. **Conclusions:** Our results suggests that miR-15a-5p and miR-30e-5p are downregulated in patients with DKD.

Keywords: microRNA expression, diabetic kidney disease, bioinformatics analysis.

INTRODUCTION

Diabetic kidney disease (DKD) is a common microvascular complication that occurs in approximately 40% of patients with diabetes mellitus (DM), and it is caused by kidney dysfunction, which can lead to end-stage renal disease (ESRD) (1). DKD is clinically characterized by albuminuria and a gradual reduction in the glomerular filtration rate (GFR) (2). Pathological changes in renal cells from DKD patients include glomerular hypertrophy, mesangial expansion, and tubulointerstitial fibrosis due to the accumulation of extracellular matrix (ECM) proteins, thickening of basement membrane, and podocyte dysfunction (3). At the cellular level, chronic hyperglycemia leads to reactive oxygen species (ROS) overproduction by mitochondria, which then triggers key pathways related to DKD: increased formation of advanced glycation end-products (AGEs) and overexpression of their receptors (RAGEs); activation of protein kinase C isoforms; increased flux of glucose through the polyol pathway; and upregulation of the hexosamine pathway (4).

The main risk factors for DKD are duration of chronic hyperglycemia, arterial hypertension, dyslipidemia, and genetic and epigenetic components (3, 5). Regarding epigenetic factors, emerging evidence has suggested an important role for microRNAs (miRNAs) in the pathogenesis of DKD (3, 6-8). MiRNAs are small (≅ 21-23 nucleotides) non-coding RNAs that regulated gene expression of 60% of protein coding genes (9); thus, regulating many cellular functions and influencing the development and progression of a number of diseases (3, 6, 9).

In this context, miR-15a-5p and miR-30e-5p were reported as having the uncoupling protein 2 (*UCP2*) as a target gene (10, 11). UCP2 is a mitochondrial protein

that mildly uncouples the oxidative phosphorylation from ATP synthesis by dissipating the proton gradient generated across the mitochondrial inner membrane, consequently decreasing ATP production, and reducing ROS formation by mitochondria (12). Taking into account the recognized role of UCP2 in decreasing oxidative stress, and that *UCP2* gene polymorphisms have been associated with DKD and other diabetic complications (12-16), dysregulation of miR-15a-5p and miR-30e-5p might also be involved in DKD pathogenesis.

Accordingly, experimental studies have linked both miRNAs to podocyte injury, epithelial-mesenchymal transition (EMT) in tubular epithelial cells, and kidney fibrosis, which are features related to chronic kidney disease (CKD) and DKD (11, 17-21). In humans, miR-15a-5p was reported as being downregulated in urine of patients with CKD or DKD compared to healthy controls (22, 23). Expression of miR-30e-5p was also downregulated in urinary exosomes of DKD patients compared to healthy subjects or type 2 DM patients without this complication (24). Moreover, expression of this miRNA in urine was correlated with proteinuria levels in DKD patients (25). Even though these studies have associated dysregulation of miR15a-5p and miR-30e-5p with DKD, their exact roles and clinical relevance remain unknown. Therefore, in the present study, we analyzed miR-15a-5p and miR-30e-5p expressions in plasma and urine of type 1 DM (T1DM) patients with and without DKD.

METHODS

Sample and phenotype measurements

This case-control study was designed following STROBE guidelines for reporting of association studies (26). The sample comprised 40 T1DM patients, who were divided in 17 patients without DKD (control group) and 23 DKD cases (12 with moderate DKD and 11 with severe DKD). All T1DM patients included in the study were from outpatient clinics at Hospital de Clínicas de Porto Alegre or Instituto da Criança com Diabetes at Grupo Hospitalar Conceição (Rio Grande do Sul, Brazil), and were recruited between August 2014 and July 2018. T1DM diagnosis was based on the American Diabetes Association criteria (27).

DKD was classified following the Kidney Disease Improving Global Outcomes (KDIGO) guidelines (28). T1DM patients were divided into 3 groups according to their renal function: 1) patients with ≥10 years of T1DM and without any degree of DKD [urinary albumin excretion (UAE) <30mg/g and estimated GFR (eGFR) ≥60 ml/min/1.73 m²; **T1DM control**]; 2) patients with moderate DKD (UAE 30-300mg/g and/or eGFR 30-59 ml/min/1.73 m²); and 3) patients with severe DKD (UAE >300 mg/g and/or eGFR 1-29 ml/min/1.73 m²). Therefore, the **case group** was constituted by patients with moderate or severe DKD. Exclusion criteria were any febrile illness during the last 3 months, chronic inflammatory or rheumatic diseases, hepatitis, HIV-positivity, glucocorticoid treatment, liver or cardiac failure, kidney transplantation, hereditary dyslipidemia, and inborn or acquired errors of metabolism excepting DM.

A standard questionnaire was used to collect information on age, age at diagnosis, T1DM duration, drug treatment, and ethnicity. The ethnic group was defined based on self-

classification. All patients underwent physical and laboratory evaluations, as previous described (29). Serum creatinine was measured by the Jaffé reaction and UAE by immunoturbidimetry (Sera- Pak immuno microalbuminuria, Bayer, Tarrytown, NY, USA) (30). Estimated GFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation: $eGFR = 141 \times \min(SCR/\kappa, 1)^{\alpha} \times \max(SCR/\kappa, 1)^{-1,209} \times 0,993^{age} \times 1,018$ [if female] x 1,159 [if black] (31). The study protocol was approved by Ethic Committees in Research from Hospital de Clínicas de Porto Alegre and Grupo Hospitalar Conceição/Instituto da Criança com Diabetes, and all patients gave their informed consent in writing.

RNA extraction and quantification of miRNA expressions by qPCR

Peripheral blood samples of all subjects were collected in the morning with at least 8 hours of fasting, in EDTA-coated tubes. Midstream 20 ml voided urine samples were also collected from all patients. Immediately after collection, blood and urine samples were centrifuged at 3500 rpm for 15 min at 4°C, and their aliquots were stored at -80°C until quantification of miRNA expressions. Total RNA was isolated from 450 µl of plasma or urine using the MiRVana PARIS miRNA Isolation Kit (Ambion, Thermo Fisher Scientific, DE, USA), according to the manufacturer's instructions. Purity and concentration of RNA samples were measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Only RNA samples that achieved adequate purity ratios (A260/A280 = 1.9-2.1) were used for subsequent analyses (32).

Real-time quantitative PCR (qPCR) was performed in two separate reactions: first, the total RNA was reverse-transcribed into cDNA and, second, the cDNA was amplified by qPCR. Reverse transcription of 2 ng/μl of RNA into cDNA was carried out using TaqMan miRNA RT assays (Thermo Fisher Scientific) specific for each miRNA of interest (assay reference number: 000389 for hsa-miR-15a-5p, and 002223 for hsa-miR-30e-5p). The *small nuclear RNA U6* (*U6snRNA*) gene was used as reference gene (assay reference number: 001973).

Next, qPCR experiments were carried out in a ViiA™ 7 Fast Real-Time PCR System. PCR reactions were performed using 0.5 μl TaqMan miRNA Assays 20x (Thermo Fisher Scientific) for target miRNAs or *U6snRNA*, 5 μl TaqMan Universal PCR Master Mix II no UNG 2x, and 1 μl of cDNA (10 ng/μl), in a total volume of 10 μl. Each sample was assayed in triplicate and a negative control was included in each experiment. Cycling conditions for these genes were an initial cycle of 95°C for 10min, followed by 50 cycles of 95°C for 15s and 60°C for 90s. Quantification of the two target miRNAs was performed using the $2^{-\Delta\Delta Cq}$ method and the *U6snRNA* gene as the reference and are shown as n-fold changes in relation to the calibrator sample (32).

Bioinformatics analyses

To better understand the functional involvement of miR-15a-5p and miR-30e-5p in DKD, these miRNAs were submitted to bioinformatics analyses to investigate their putative target genes and to find possible biological pathways under their regulation. Bioinformatics analyses were performed in Cytoscape v. 3.2.1 software (33) using two plugin tools: 1) CyTargetLinker (34), and 2) Biological Networks Gene Ontology (BiNGO) (35).

The CyTargetLinker v3.0.1 was used to search for validated and predicted miRNA-target gene interactions (MTI) and visualize them in a graphical way. For this study, we obtained Homo sapiens MTIs from one experimentally validated database (miRTarBase v.4.4) and from two predicted miRNA databases (MicroCosm v.5.0 and TargetScan v.6.2).

Next, functional enrichment analysis of miRNA-target genes was performed to retrieve gene ontology (GO) annotations for miR-15a-5p and miR-30e-5p target genes that were identified with the CyTargetLinker, using the BiNGO plug-in on Cytoscape environment. This investigation was performed for targets of each individual miRNA, as well as for targets of the two miRNAs analyzed together. Significance for GO pathways enrichment was estimated with a hypergeometric test and adjusted for multiple hypotheses using the Benjamini & Hochberg False Discovery Rate (FDR) test. Pathways with a q-value <0.05 were considered strongly enriched for the genes targeted by the two analyzed miRNAs.

Statistical analyses

Normal distribution was checked using the Kolmogorov Smirnov and Shapiro-Wilk tests. Variables with normal distribution are presented as mean ± SD. Variables with skewed distribution were log-transformed before analyses and are presented as median (25–75th percentiles). Categorical data are shown as percentages. Clinical and laboratory characteristics and miRNA expressions were compared among groups using Student's t-test or χ^2 tests, as appropriate. Correlations between quantitative variables were analyzed using Pearson's correlation tests. All statistical analyses were performed using the SPSS statistical package (v.18.0) for Windows (SPSS Inc, Chicago, IL), and P values <0.05 were considered statistically significant.

RESULTS

Characteristics of the T1DM patients

Clinical and laboratorial characteristics of T1DM controls and DKD cases included in this study are summarized in **Table 1**. Age, gender, ethnicity, BMI, age at T1DM diagnosis, duration of T1DM, prevalence of hypertension, and total cholesterol levels did not differ significantly between case and control groups. As expected, HbA1c levels were higher in DKD cases compared to controls (P= 0.003). Triglycerides and HDL cholesterol levels also seem to be higher in DKD patients (P= 0.046 and P= 0.062, respectively). Moreover, prevalence of diabetic retinopathy was increased in DKD cases compared to controls (P= 0.043) (**Table 1**).

Expressions of miR-15a-5p and miR-30e-5p in plasma and urine of T1DM patients with or without DKD

Expressions of miR-15a-5p and miR-30e-5p were investigated in plasma and urine of T1DM controls and DKD cases (**Fig. 1**). Both analyzed miRNAs were significantly downregulated in plasma of DKD cases compared to T1DM controls [miR-15a-5p: 0.272 (0.039 – 0.484) *vs.* 0.466 (0.223 – 3.243), P= 0.024; miR-30e-5p: 0.534 (0.147 – 0.943) *vs.* 2.416 (0.514 – 4.330), P= 0.006; respectively; **Fig. 1A and 1B**]. Regarding the expression of these two miRNAs in plasma of DKD cases broken down by the degree of this complication, no significance difference was found between patients with moderate or severe DKD [miR-15a-5p: 0.239 (0.039 – 1.015) *vs.* 0.286 (0.026 – 0.472), P= 0.404; miR-30e-5p: 0.473 (0.098 – 1.443) *vs.* 0.534 (0.245 – 0.839), P= 0.711; respectively].

In agreement to the expression profile found in plasma, expressions of miR-15a-5p and miR-30e-5p were also decreased in urine of DKD cases compared to T1DM controls [miR-15a-5p: 0.493 (0.190 – 0.862) *vs.* 1.647 (0.687 – 4.511), P= 0.032; miR-30e-5p: 0.613 (0.126 – 1.653) *vs.* 5.851 (2.265 – no value >50th percentile), P= 0.001, respectively; **Fig. 1C and 1D**]. Urine expressions of the two analyzed miRNAs also did not differ between patients with moderate or severe DKD (P > 0.05).

We next evaluated possible correlations between miR-15a-5p and miR-30e-5p expressions in plasma and DKD-related measurements (eGFR, creatinine, and UAE levels) and HbA1c. MiR-15a-5p and miR-30e-5p expressions showed a significant negative correlation with UAE levels (r= -0.469, P= 0.009 and r= -0.490, P= 0.003, respectively). MiR-30e-5p was also negatively correlated with HbA1c levels (r= -0.393, P= 0.008). No significant correlation was found between the two analyzed miRNAs and eGFR values or creatinine levels.

Target prediction and functional enrichment analysis for miR-15a-5p and miR-30e-5p

Target prediction of the miR-15a-5p and miR-30e-5p was performed using distinct bioinformatics resources in Cytoscape environment. Using the strategy described in the Methods Section, 2197 genes were identified as putative targets of the miR-15a-5p and 2208 as targets of the miR-30e-5p, being 314 of these genes modulated by both miRNAs (**Fig. 2A** and **Supplementary Fig. 1**). After that, we analyzed only the experimentally validated target genes of these two miRNAs (**Fig. 3**). As shown in **Fig. 3A**, 23 validated target genes were found for miR-15a-5p and only 2 validated targets for miR-30e-5p. Among the validated target genes found for miR-15a-5p, some genes were already reported

as being associated with kidney dysfunction or DKD pathogenesis, including *BCL2*, *VEGFA*, *UCP2*, *BMI1*, and *NFKB1* and its inhibitor *CHUK* (*IKKA*) (**Fig. 3A**). **Fig. 3B** shows those targets of miR-15a-5p and miR-30e-5p that were found in all 3 analyzed databases of MITs (one database of experimentally validated targets and two of computationally predicted targets). As can be observed in this figure, *UCP2* is a predicted and a validated target of miR-15a-5p, being present in all the 3 analyzed databases (**Fig. 3B**). Of note, most validated MTIs shown in **Fig. 3A** were not computationally predicted (**Fig. 3B**), demonstrating the importance of using distinct databases for target gene analysis.

To explore the biological pathways possibly affected by the two analyzed miRNAs, we carried out functional enrichment analysis of their target genes using pathways maps from the BiNGO Database. GO pathways were investigated for biological, cellular, and molecular processes associated with the set of predicted and validated target genes found for miR-15a-5p and miR-30e-5p in the previous analysis. A total of 250 unique significant pathways were enriched for miR-15a-5p, being 183 pathways involved in biological processes, 63 in cellular components, and 20 in molecular functions (**Supplementary Table 1**). Of note, some of these pathways participate in more than one biological category of BiNGO. For miR-30e-5p, a total of 142 unique significant pathways were enriched, being 109 pathways involved in biological processes, 20 in cellular components, and 15 in molecular functions (**Supplementary Table 2**).

Moreover, a total of 81 unique pathways were enriched for both miRNAs, being 64 pathways involved in biological process (**Fig. 2B**), 14 in cellular components (**Fig. 2C**), and 3 in molecular functions (**Fig. 2D**). Many of these pathways are well stablished to be involved in DKD pathogenesis, such as transforming growth factor beta receptor, oxidative stress, apoptosis, VEGF and angiogenesis, endoplasmic reticulum stress, hypoxia, and

mitochondrial transport pathways (**Fig. 4A** for pathways derived from predicted + validated targets, and **Fig. 4B** for pathways derived only from validated targets).

DISCUSSION

Chronic hyperglycemia induces cellular damage through increased production of ROS, which then triggers the main pathways related to microvascular diabetic complications, including DKD (4). Recent studies have shown that miR-15a-5p and miR-30e-5p are associated with podocyte injury, EMT in tubular epithelial cells, and kidney fibrosis (11, 17-21), and also seem to be dysregulated in urine of patients with DKD or CKD (22-25). Interestingly, both miRNAs target *UCP2* (10, 11), a mitochondrial protein with a key role in decreasing oxidative stress (12). Based on these findings, we therefore analyzed miR-15a-5p and miR-30e-5p expressions in T1DM patients according to DKD presence. Our results indicate that miR-15a-5p and miR-30e-5p are downregulated in plasma and urine of T1DM patients with DKD compared to patients without this complication.

MiR-15a-5p regulates several genes involved in cell division, metabolism, stress response, apoptosis, and angiogenesis (36). Moreover, this miRNA is an important tumor suppressor gene, and its downregulation has being associated with various types of cancer, including renal cell carcinoma (37, 38). MiR-15a-5p is abundantly expressed in human and mouse renal tissue (36), although only few studies evaluated its function regarding CKD and DKD. In agreement with our results, miR-15a-5p expression was also decreased in urinary exosomes of patients with DKD or CKD compared to controls (22, 23). In addition, Sun *et al*. (17) reported that this miRNA was downregulated by high glucose (HG) in human renal tubular epithelial cells (RTECs). Treatment of RTECs with a miR-15a-5p

mimic was able to reverse HG-induced EMT in these cells, since it inhibited *α-SMA* and *collagen I* expressions, and restored *E-cadherin* expression (17).

Our bioinformatics analysis using only an experimentally validated database showed that miR-15a-5p regulates genes from several pathways involved in mechanisms related to kidney dysfunction and DKD development (39): *VEGFA*, *BCL2*, *NFKB1* and its inhibitor CHUK (IKKA), *UCP2*, and BMI1 (Fig. 3A). When we used both experimentally validated and computationally predicted tools, *UCP2*, *CCNE1*, *TSPYL2*, *DMTF1*, and *MYB* remained as significant targets of miR-15a-5p (Fig. 3B).

As already mentioned, UCP2 decreases oxidative stress, being a candidate gene for DKD. Liang *et al*. (10) confirmed experimentally in MIN-6 cells that miR-15a-5p directly targets *UCP2*, decreasing mitochondrial uncoupling and, thus, increasing insulin biosynthesis in this beta-cell line, since UCP2 is a negative regulator of insulin secretion. Regarding kidney dysfunction, Qiu *et al.* (40) reported that oral administration of genipin (an UCP2 inhibitor) partially prevented the progression of DKD in C57BL/6J mice by improving podocyte function. Accordingly, UCP2 was induced in RTECs after unilateral ureteral obstruction in mice, while those mice with ablated *UCP2* resisted obstruction-induced EMT and kidney fibrosis (11). Additionally, *UCP2* knockdown in NRK-52E tubular cells abolished the effect of TGF-β1 treatment, decreasing extracellular matrix production (11). In contrast, Chen *et al*. (41) showed that inhibition of UCP2 by genipin increased oxidative stress in rat RTECs treated with HG medium, leading to increased apoptosis. *UCP2* knockdown in renal mesangial cells of rats also increased oxidative stress, inflammation and apoptosis *in vitro* (42). Therefore, it is still not clear if UCP2 has a protective or deleterious effect in renal function.

CCNE1, a member of the cyclin E family, regulates cell cycle G1/S transition, consequently promoting cell proliferation. Overexpression of this gene has been observed in many tumors, resulting in chromosome instability and tumorigenesis (43-45). MiR-15a-5p is downregulated in breast cancer specimens compared to normal tissue (46, 47), and its overexpression causes cell growth inhibition, suppression of migration and G1 phase arrest by directly targeting *CCNE1* (46). *DMTF1* (cyclin-D binding myb-like transcription factor 1) is a transcription factor that functions as a tumor suppressor by activating ARF-p53 pathways to arrest cell growth or induce apoptosis (48, 49). To date, no study has linked *CCNE1* and *DMTF1* to DKD or CKD.

TSPYL2 (testis-specific protein Y-encoded like 2), also known as CDA1, acts in chromatin remodeling and as inhibitor of cell proliferation in response to DNA damage (50). Interestingly TSPYL2 is a regulator of cell-cycle arrest induced by TGF-β1 (51), which is a major player in DKD pathogenesis, mainly because of its potent pro-fibrotic actions (3). Accordingly, *TSPYL2* expression was upregulated in the aorta of a murine diabetic model of atherosclerosis (52). *In vitro* studies in vascular cells showed that TGF-β1 treatment increased TSPYL2 protein, which then amplified TGF-β1 signaling leading to upregulation of ECM genes (52). Chai *et al*. (53) reported that TSPYL2 knockout in diabetic mice reduced expression of TGF-β1 receptors in the kidney as well as reduced renal matrix accumulation and attenuated glomerular and tubulointerstitial injury. Therefore, this gene might be a new candidate gene for DKD.

MYB proto-oncogene is a transcription factor involved in normal adult hematopoiesis, and its aberrant expression has been found in several solid cancer and human leukemia (54). MiR-15a-5p is an experimentally validated target of MYB (55, 56). Hence, transfection of K562 myeloid leukemia cells or nasal NK/T-cell lymphoma with a

miR-15a-5p mimic decreased MYB expression and blocked the cells in the G1 phase of the cell cycle, decreasing cell proliferation (55, 56). No study has evaluated MYB expression in the context of kidney dysfunction and DKD.

In agreement with our present results, miR-30e-5p expression was also downregulated in urinary exosomes of type 2 DM patients with DKD compared to healthy controls or diabetic patients without this complication (24). This association is biologically plausible since all miR-30 family members seem to be essential for structural and functional homeostasis of podocytes, where they are abundantly expressed (18, 19, 21, 57). TGF-β1 treatment downregulates miR-30 expression in glomerular podocytes *in vivo* and *in vitro*, and the sustained expression of miR-30 inhibits TGF-β1-induced apoptosis of podocytes, while this knockdown aggravates podocyte injury (18, 57). Wu *et al*. (18) showed that miR-30s exert their protective roles by direct inhibition of *Notch1* and *p53*, which mediate podocyte injury. Moreover, miR-30 inhibits the excessive activation of calcium/calcineurin signaling, preventing cytoskeletal damage and apoptosis of podocytes (19, 58). Zhao *et al*. (20) reported that in renal tissue of over-week-old db/db mice and in RTECs cultured for 6h in HG, miR-30e-5p was downregulated while its target *GLIPR-2*, involved in EMT, was upregulated. Besides, miR-30e-5p overexpression in RTECs promoted proliferation of these cells and inhibited EMT, which could avoid renal fibrosis in DKD (20). Accordingly, Jiang *et al*. (11) showed that miR-30e-5p was also downregulated in RTECs from mice with ureteral occlusion-induced kidney fibrosis and in TGF-β1-treated NRK-52E renal cells. Transfection of a miR-30e-5p mimic in NRK-52E cells reduced TGF-β1-induced *UCP2* expression, inhibiting EMT, whereas a miR-30e inhibitor promoted epithelial cell phenotype changes through a loss of E-cadherin, induction of α-

SMA, and fibrinogen expression (11). Thus, the downregulation of miR-30e-5p seems to be involved in renal fibrosis.

Our bioinformatics analyses indicated that *UBE2I* (ubiquitin-conjugating enzyme E2 I) and *MUC17* (mucin 17) genes are validated target genes of miR-30e-5p (Fig. 3A). UBE2I, also known as UCB9, constitute a core machinery in the sumoylation pathway. Sumoylation is a process in which a small ubiquitin-like modifier (SUMO) is covalently attached to other proteins, modifying their functions (59). Important roles for sumoylation were shown in heterochromatin configuration, and sumoylation of core histones negatively regulates transcription (59). MiR-30e-5p-induced downregulation of *UBE2I* inhibited the proliferation and migration of vascular smooth muscle cells (60). MUC17 is a glycoprotein characterized as a membrane-bound mucin that provides protection to gut epithelial cells (61, 62). Although UBE2I and MUC17 have not been studied in the context of kidney dysfunction or DKD, a polymorphism in the *MUC17* gene was previously associated with protection for CKD in a exome-wide association study (63).

In conclusion, we demonstrated that miR-15a-5p and miR-30e-5p are downregulated in plasma and urine of DKD patients. Bioinformatics analyses suggested that these miRNAs regulate genes involved in key mechanisms related to DKD pathogenesis, such as TGF-β receptor, angiogenesis, apoptosis, and hypoxia. Moreover, they also are involved in oxidative stress pathway (probably by targeting *UCP2*), which is the main mechanism linking hyperglycemia to diabetic chronic complications. Our study also suggests that *CCNE1*, *DMTF1*, *TSPYL2*, *MYB* and *UBE2I* might constitute new potential candidate genes for DKD.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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LEGENDS OF FIGURES

Figure 1. Expression of miR-15a-5p and miR-30e-5p in plasma and urine of T1DM controls and DKD cases. Relative expressions of (**A**) miR-15-5p and (**B**) miR-30e-5p and plasma, and (**C**) miR-15-5p and (**D**) miR-30e-5p in urine between T1DM controls and DKD cases were evaluated using qPCR. Results are expressed as n-fold changes in relation to the calibrator sample ($\Delta\Delta C_q$ method), using the *U6 snRNA* as the reference gene, and are shown as median (25_{th} – 75_{th} percentiles). P-values were obtained using Student's *t*-test. * P <0.050, and ** P <0.010.

Figure 2. Venn diagrams showing interactions between miR-15a-5p and miR-30e-5p and their target genes and gene ontology pathways: (**A**) target genes, (**B**) biological process pathways, (**C**) cellular component pathways, and (**D**) molecular function pathways shared between the two analyzed miRNAs.

Figure 3. Interactions between miR-15a-5p and miR-30e-5p and their target genes. (**A**) Experimentally validated target genes of the analyzed miRNAs. Colored genes are those previously associated with DKD, (**B**) Significant target genes of the analyzed miRNAs found in the three analyzed databases (validated + computationally predicted). Lines in red means interaction retrieved from miRTarBase, in blue from microcosm, and in purple from TargetScan. Squares represent miRNAs and the circles represent their target genes.

Figure 4. Significant enriched pathways related to DKD pathogenesis and regulated by target genes of miR-15a-5p and miR-30e-5p. (**A**) Top 10 selected pathways of predicted and validated target genes of both miRNAs, and (**B**) Selected pathways of validated target genes of both miRNAs.

SUPPLEMENTAL MATERIAL LIST

Supplementary Figure 1. Target analysis of miR-15a-5p and miR-30e-5p.

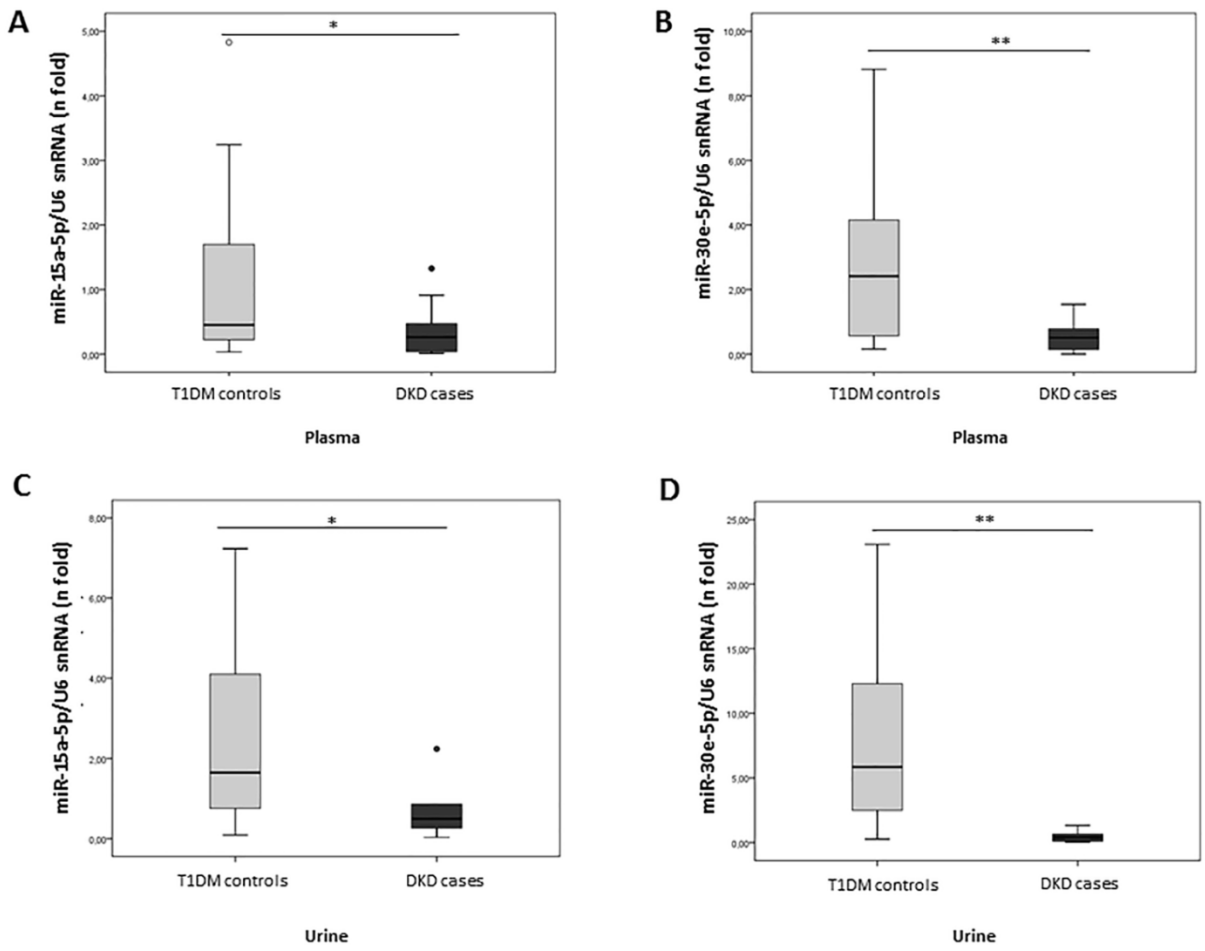
Supplementary Table 1. Enriched pathways for miR-15a-5p. (*Tabela em formato excel encaminhada em arquivo digital anexo a dissertação*)

Supplementary Table 2. Enriched pathways for miR-30e-5p. (*Tabela em formato excel encaminhada em arquivo digital anexo a dissertação*)

Table 1. Clinical and laboratory characteristics of T1DM controls and DKD cases.

Characteristics	T1DM controls (n = 17)	DKD cases (n = 23)	P *
Age (years)	24.2 ± 5.5	26.3 ± 6.5	0.288
Gender (% male)	52.9	47.8	> 0.999
Ethnicity (% black)	5.9	13.0	0.831
BMI (kg/m²)	23.2 ± 3.3	23.0 ± 2.9	0.802
HbA1c (%)	8.6 ± 0.9	10.2 ± 1.9	0.003
Hypertension (%)	11.8	36.4	0.169
Age at diagnosis (years)	9.0 (2.0 – 12.5)	6.0 (5.0 – 8.0)	0.918
Duration of diabetes (years)	15.6 ± 5.0	18.8 ± 7.5	0.132
Cholesterol total	168.3 ± 34.4	183.6 ± 60.0	0.454
Triglycerides	69.0 (45.0 – 107.0)	114.0 (64.0 – 145.5)	0.046
HDL Cholesterol	46.5 ± 11.9	59.4 ± 19.3	0.062
Creatinine (μg/dl)	0.7 (0.6 – 0.9)	1.1 (0.8 – 4.3)	-
eGFR (mL/min per 1.73m2)	123.0 (112.5 – 126.0)	95.5 (17.6 – 120.7)	-
UAE (mg/g)	6.1 (3.3 – 9.3)	132.8 (44.8 – 597.9)	-
Diabetic retinopathy (%)	5.9	40.0	0.043

Variables are shown as mean ± SD, median (25th-75th percentiles) or %. *P value was computed using Student's *t* tests or χ^2 tests, as appropriate. BMI: body mass index; DKD: diabetic kidney disease; eGFR: estimated glomerular filtration rate; HbA1c: glycated hemoglobin; T1DM: type 1 diabetes mellitus; UAE: urinary albumin excretion.



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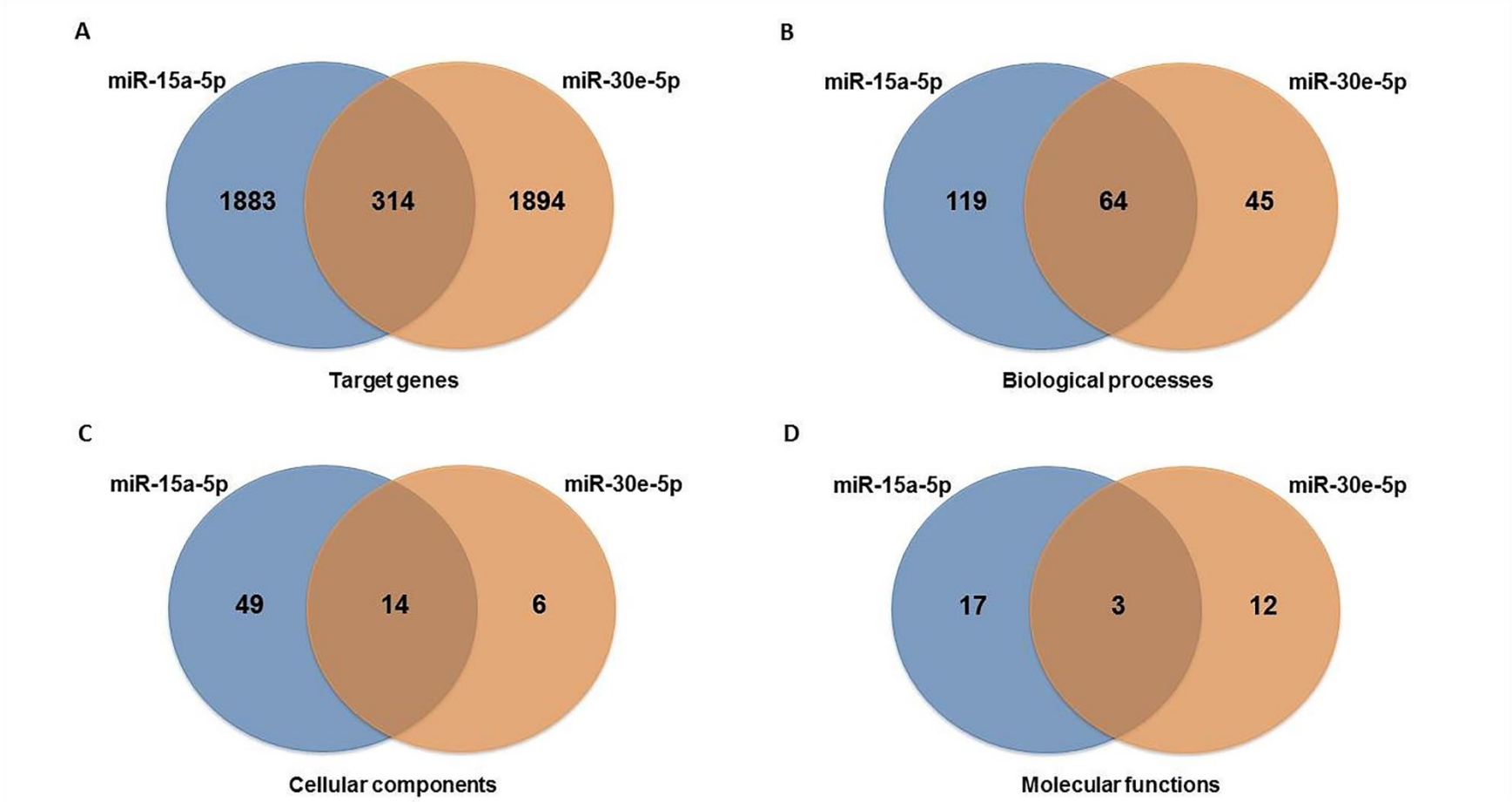

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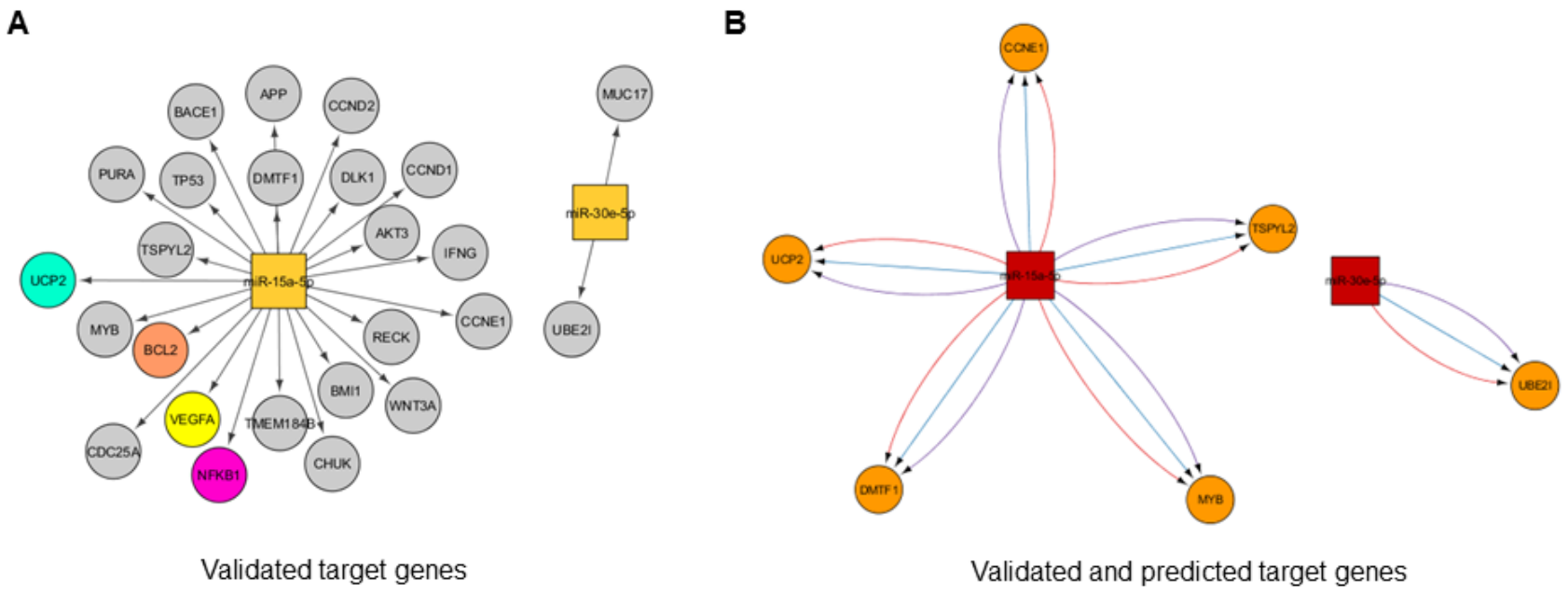

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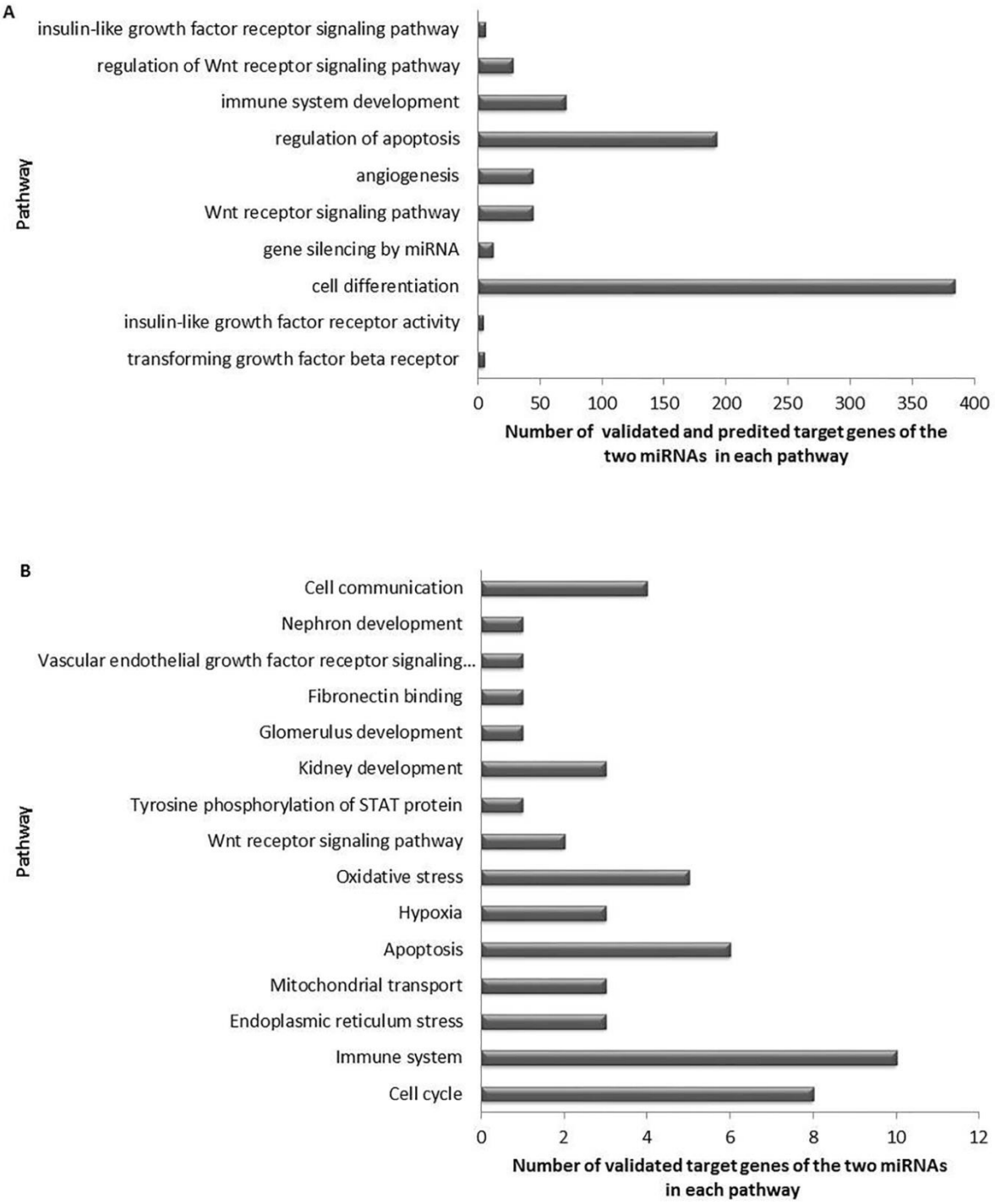

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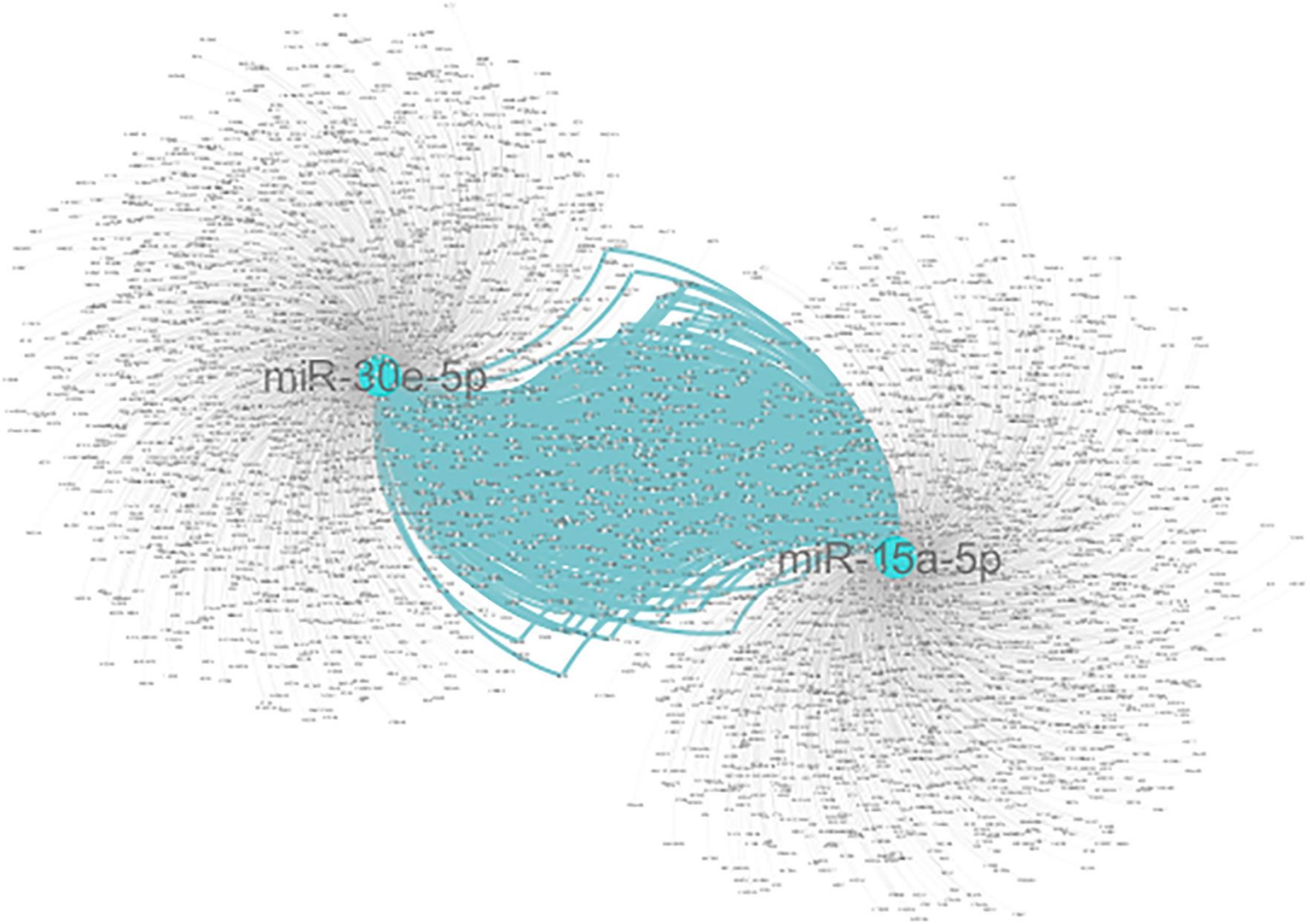

Supplementary Figure 1. Target analysis of miR-15a-5p and miR-30e-5p.

CONSIDERAÇÕES FINAIS

As principais conclusões deste estudo são:

- Os polimorfismos -866G/A e Ins/Del no gene *UCP2* não estão associados com a DRD em pacientes com DM1 da nossa população. Vale ressaltar que este foi o primeiro estudo brasileiro que investigou esses polimorfismos em pacientes com DM1 e DRD.
- Ainda em relação aos polimorfismos no gene *UCP2* e a DRD, a meta-análise realizada não demonstrou associação entre os mesmos e a DRD. Até o momento, nenhuma meta-análise sobre o assunto havia sido realizada.
- Demonstrou-se que a expressão dos miR-15a-5p e miR-30e-5p estão diminuídas no plasma e na urina dos pacientes com DRD quando comparados ao grupo de pacientes com DM1 sem DRD.
- Análises de bioinformática demonstraram que os miR-15a-5p e miR-30e-5p regulam diversos genes que participam de vias relacionadas a patogênese da DRD, tais como TFG-β, angiogênese, apoptose, hipóxia e estresse oxidativo.

Desta forma, embora não se tenha encontrado uma associação entre os polimorfismos -866G/A e Ins/Del no gene *UCP2* com DRD, a presente dissertação demonstrou que miRNAs que possuem como genes alvo o gene *UCP2* estão desregulados em pacientes com DM1 e DRD.

OUTRAS PRODUÇÕES BIBLIOGRÁFICAS NO PERÍODO DO MESTRADO

Além dos artigos que fazem parte da presente dissertação, ao longo do período do mestrado foram desenvolvidos os seguintes manuscritos:

1. Lemos NE, **Dieter C**, Dorfman LE, Assmann TS, Duarte GCK, Canani LH, et al. The rs2292239 polymorphism in *ERBB3* gene is associated with risk for type 1 diabetes mellitus in a Brazilian population. **Gene**. 2018;644:122-8.

2. Duarte GCK, Assmann TS, **Dieter C**, de Souza BM, Crispim D. *GLIS3* rs7020673 and rs10758593 polymorphisms interact in the susceptibility for type 1 diabetes mellitus. **Acta diabetologica**. 2017.

3. Lemos NE, de Almeida Brondani L, **Dieter C**, Rheinheimer J, Boucas AP, Bauermann Leitao C, et al. Use of additives, scaffolds and extracellular matrix components for improvement of human pancreatic islet outcomes in vitro: A systematic review. **Islets**. 2017;9(5):73-86.

Manuscritos desenvolvidos no período do mestrado e em fase de finalização:

1. Lemos NE, **Dieter C**, Carlessi R, Rheinhemer J, de Almeida Brondani L, Leitão CB, Bauer AC, Crispim D. Effect of exendin-4 in renal protection of rats submitted to brain death.

2. Lemos NE, **Dieter C**, Carlessi R, Rheinhemer J, de Souza BM, Leitão CB, Bauer AC, Crispim D. Comparison of two techniques for evaluation of pancreatic islet viability: flow cytometry and FDA/PI staining.

3. Massignam ET, **Dieter C**, Pellenz FM, Assmann TS, Canani LH, Crispim D. Rs4636297 (G / A) polymorphism in the miR-126 gene is associated with protection for diabetic retinopathy in patients with type 1 diabetes mellitus

4. **Dieter C**, Lemos NE, Dorfmann LE, Assmann TS, Duarte GCK, Canani LH, Bauer AC, Crispim, D. rs11755527 polymorphism in *BACH2* gene and the Type 1 Diabetes Mellitus: case control study in a Brazilian population

5. Lemos NE, **Dieter C**, Carlessi R, Rheinhemer J, de Souza BM, Leitão CB, Bauer AC, Crispim D. Exendin-4 prevents inflammatory damage affecting the function of rat pancreatis islets.

6. Bouças AP, de Souza BM, da Silva LPA, **Dieter C,** Nique PS, Sortica DA, Leitão CB, Bauer AB, Crispim D. *IFIH1* Knockdown Reduces the Expression of Inflammation-, Endothelial Dysfunction, and Hypertension-Related Genes Induced by Viral Infection in a Human Endothelial Cell Line.