Universidade Federal do Rio Grande do Sul

Programa de Pós-graduação em Ciências Médicas: Endocrinologia

ESTUDO DA ASSOCIAÇÃO DE POLIMORFISMOS NO GENE *ABCA1* E A DOENÇA RENAL DO DIABETES

Dissertação de Mestrado

Fabiana Goes Nogueira

Porto Alegre, outubro de 2015

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, da Universidade Federal do Rio Grande do Sul (UFRGS) como requisito parcial para obtenção do título de Mestre em Endocrinologia.

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Esta dissertação de mestrado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição da Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de um artigo de revisão e de um artigo original sobre o tema da dissertação.

- Artigo de revisão: "ATP-BINDING CASSETTE TRANSPORTER A1 (*ABCA1*) E A DOENÇA RENAL DO DIABETES"

- Artigo original: "POLIMORFISMOS NO GENE ATP-BINDING CASSETTE TRANSPORTER A1 (*ABCA1*) E A DOENÇA RENAL DO DIABETES"

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1. Introdução

ABCA1	*ATP-binding cassette transporter A1*
Apo-A1	Apolipoproteína A1
$ApoE^{-/-}$	Apolipoprotein E knock-out
$BTBR^{ob/ob}$	Black and tan brachyuric mouse obese
CHO	Cholesterol
DCV	Doença Cardiovascular
DM 1	Diabetes Mellitus tipo 1
DM 2	Diabetes Mellitus tipo 2
DM	Diabetes Mellitus
DRCT	Doença Renal Crônica Terminal
DRD	Doença renal do diabetes
EUA	Excreção urinária de albumina
$FVB^{db/db}$	Loss of function mutation of the leptin receptor mice
GWA	*Genome wide association*
GWS	*Genome wide scan*
HDL	*High density lipoprotein*
TFG	Taxa de filtração glomerular
TRS	Terapia Renal Substitutiva

2. Artigo de revisão e artigo original

Apo A-I	Apolipoproteina A-I
$ApoE^{-/-}$	Apolipoprotein E knock-out
$BTBR^{ob/ob}$	Black and tan brachyuric mouse obese
CHO	Cholesterol
DCV	Doença cardiovascular
DM1	Diabetes mellitus tipo 1
DM2	Diabetes mellitus tipo 2
DRCT	Doença renal crônica terminal
DRD	Doença renal do diabetes
EUA	Excreção urinária de albumina
FC	Free cholesterol
$FVB^{db/db}$	Loss of function mutation of the leptin receptor mice
GWA	Genome wide association
GWS	Genome wide scan
HDL	High density lipoprotein
LD	Linkage disequilibrum
PL	Phospholipids
RCT	Reverse cholesterol transport
mRNA	messenger RNA
SR-B1	Scavenger receptor class B type 1

TFG	Taxa de filtração glomerular
TRS	Terapia renal substitutiva
PCR	Polymerase Chain Reaction
OR	Odds ratio
SNPs	Single Nucleotide Polymorphisms

RESUMO

Introdução A doença renal do diabetes (DRD) é a principal complicação clínica do Diabetes Mellitus tipo 1 e 2 (DM1 e DM2). A relação entre o dano renal e os lipídios tem sido investigada há décadas e as evidências demonstram que o acúmulo de lipídios intra renal está associado com o desenvolvimento de glomerulosclerose, fibrose túbulo intersticial e progressão da DRD. Além disso, os fatores genéticos ampliam o risco para o desenvolvimento da DRD. Evidências sugerem que o gene *ATP-binding cassette transporter A1 (ABCA1)* possa estar envolvido nos danos causados pelo acúmulo do colesterol intracelular. O *ABCA1* possui papel central no efluxo do colesterol celular para as moléculas aceptoras pobre em lipídios presentes no plasma. O conhecimento sobre a relação dos polimorfismos do *gene ABCA1* e a DRD ainda é escasso. Dessa forma, o presente trabalho buscou investigar a associação entre os polimorfismos no gene *ABCA1* e a presença de proteinúria em indivíduos com DM2.

Métodos As frequências alélicas e genotípicas dos polimorfismos rs1800977 (C/T), rs2230806 (G/A), rs2066715 (G/A), rs4149313 (A/G) e rs2030808 (G/A) no gene *ABCA1* foram analisadas em 365 pacientes com DM2 e proteinúria ou doença renal crônica terminal (DRCT) (casos) e 322 pacientes com DM2 e valores normais de excreção urinária de albumina (controles) em uma população do sul do Brasil. Os haplótipos construídos a partir da combinação dos cinco polimorfismos estudados e suas frequências foram inferidos utilizando o programa Phase 2.1, o qual implementa o método estatístico bayesiano.

Resultados O polimorfismo rs1800977 (C/T) foi associado com proteinúria em indivíduos com DM2 segundo o modelo de herança dominante (C/T + T/T vs. C/C) (P = 0.038). Da mesma forma, a presença do alelo T desse polimorfismo foi associada com

proteção para proteinúria (RC = 0,61; IC 95% 0,410 – 0,902; P = 0,013). Os demais polimorfismos estudados não foram associados com DRD em pacientes com DM2. As análises dos haplótipos demonstraram diferença nas distribuições dos haplótipos entre os casos e os controles (P = 0.004). Os polimorfismos estudados não se apresentaram em desequilíbrio de ligação.

Conclusão O polimorfismo rs1800977 (C/T) está significativamente associado com proteção para DRD em uma população branca do sul do Brasil.

ABSTRACT

Introduction Diabetic Kidney Disease (DKD) is a major complication of Diabetes Mellitus. The relationship between renal disease and lipids has been investigated for decades and the evidences demonstrate that lipid accumulation in kidney is associated with the development of glomerulosclerosis, tubulointerstitial fibrosis and progression of DKD. Also, the genetic susceptibility is a relevant target on progression of DKD. The ATP- binding cassette transporter A1 (*ABCA1*) gene plays a central role in cholesterol efflux from cells to lipid-free receptors in bloodstream. Data regarding *ABCA1* genetic variants and DKD are very scarce. Therefore, the aim of the present study was to investigate whether *ABCA1* polymorphisms are associated with presence of proteinuria in T2DM.

Methods Frequencies of the *ABCA1* rs1800977 (C/T), rs2230806 (G/A), rs2066715 (G/A), rs4149313 (A/G) and rs2030808 (G/A) were analyzed 365 T2DM patients with proteinuria or end-stage renal disease (ESRD) (cases) and 322 T2DM patients with normal albumin excretion rate (controls) subjects from Brazil. Haplotypes constructed from the combination of these polymorphism were inferred using a Bayesian statistical method.

Results The rs1800977 (C/T) polymorphism was associated with proteinuria in T2DM patients under a dominant inheritance model (C/T+T/T *vs.* C/C) (P = 0.038). The presence of the T allele was associated with proteinuria protection (OR = 0.61 95% CI 0.41- 0.902; P = 0.013). The others four polymorphisms analyzed were not associated with proteinuria. Permutations analysis showed that the distributions of inferred haplotypes were statistically different between case and controls groups (P = 0.004).

The |D'| and r^2 measurements did not demonstrated any significant LD between all pairs of combination of the five analyzed polymorphisms.

Conclusions The *ABCA1* rs1800977 (C/T) polymorphism is significantly associated with protection to DKD in white Brazilian T2DM subjects.

INTRODUÇÃO

A doença renal do diabetes (DRD) é a complicação microvascular crônica mais frequente do diabetes mellitus tipo 1 e tipo 2 (DM1 e DM2), com prevalência e incidência crescente em todo mundo (1). A DRD é a causa mais observada entre indivíduos com doença renal crônica terminal (DRCT) que iniciam a terapia renal substitutiva (TRS) (2). O aumento de excreção urinária de albumina (EUA) e a redução da taxa de filtração glomerular (TFG) são fatores independentes e aditivos associados à mortalidade por doença cardiovascular (DCV) entre indivíduos com DRD (3).

Tradicionalmente, a DRD é diagnosticada com base na medida da EUA e conceituada em estágios. Inicialmente, o primeiro é formalmente descrito como nefropatia incipiente ou microalbuminuria (EUA 30-300mg/24h). Já o estágio de macroalbuminúria, ou nefropatia clínica, o valor de EUA correspondente é ≥300 mg/24h ou proteinúria ≥500 mg/24h, que podem estar acompanhados ou não por redução da taxa de filtração glomerular (TFG <60mL/min/1.73m^2) (4). Segundo recomendações atuais, a classificação de micro e macroalbuminuria deve ser evitada e substituída por albuminúria de 30-299 mg/24h e albuminúria ≥300mg/24h, respectivamente (5). No entanto, evidências baseadas em estudos de coorte reforçam a associação entre o risco de progressão da DRD à DRCT, mortalidade e doença cardiovascular de acordo com a classificação da EUA (6, 7).

Curiosamente, nem todos os indivíduos com DM desenvolvem DRD. Aqueles que não evoluem para DRD, mesmo em condições adversas de tratamento, parecem ser protegidos por questões ainda desconhecidas.

As evidências sobre o papel dos fatores genéticos na progressão da DRD baseiam-se em estudos de agregação familiar que mostram uma importante

concordância para o desenvolvimento da DRD em algumas famílias e reforçam a existência de genes suscetíveis à gênese da DRD (8-10). A contribuição da genética na progressão da DRD também é confirmada a partir de estudos de associação com base em genes candidatos, em que se incluem análises de polimorfismos, alterações na sequência de DNA que ocorrem com uma frequência maior ou igual a 1% na população, (11). Mais recentemente, em estudos *genome-wide scan* (GWS) as evidências genéticas se ampliaram ao se identificar regiões cromossômicas contendo genes potencialmente envolvidos na doença, assim como descrito pelo estudo *Joslin Study of Genetics of Nephropathy in Type 2 Diabetes Family Collection* o qual demonstrou importante associação entre o locus 9q21.32 e o aumento no risco de microalbuminuria, proteinuria e DRCT entre pacientes com DM (12).

Considerando que a DRD possui patologia de origem multifatorial, acredita-se que a interação entre fatores genéticos e fatores de risco, como hiperglicemia, hipertensão e dislipidemia, contribuam para o surgimento da DRD (11, 13, 14).

Em 1936, Kimmelstiel & Wilson (15) identificaram pela primeira vez, a presença de conteúdo lipídico em biopsias renais de pacientes com DRD. Posteriormente, estudos utilizando modelos experimentais de DRC apontam que, assim como na doença aterosclerótica, o acúmulo de lipídios celulares parece ser um importante alvo na patogênese das glomerulopatias (16-19). Paralelamente, os estudos envolvendo o gene *ABCA1* (ATP- binding cassette transporter A1) e o efluxo de colesterol tem ampliado o conhecimento sobre os possíveis mecanismos responsáveis pelo acumulo do colesterol nos glomérulos e túbulos renais associados à DRD (20).

O ABCA1 é um dos transportadores de membrana da família dos ABC transportadores, os quais são responsáveis pelo deslocamento de uma série de substâncias incluindo esteróis, produtos do metabolismo e drogas através da membrana

celular (21). O ABCA1 promove a remoção do excesso de colesterol intracelular para moléculas aceptoras com baixo gradiente de colesterol como a apolipoproteína A-I (apo A-I) no plasma (22).

O gene *ABCA1* está localizado no cromossomo 9q.31.1 e possui 50 éxons, sendo que 49 codificam os 2261 aminoácidos da proteína ABCA1 (210KDa). O gene é altamente conservado entre as espécies, compartilhando 90 % da identidade entre humanos e ratos (21).

Um ensaio experimental realizado por Tang *et al.* (20) demonstrou redução significativa da expressão do transportador ABCA1 e de seu RNAm em macrófagos e rins isolados de ratos com DM induzido por estreptozotocina. Recentemente, Herman-Edelstein *et al.* (23) demonstraram importante presença do acúmulo de colesterol intracelular em biopsias renais de pacientes com e sem DRD, e este acúmulo estava relacionado com uma redução importante da expressão do RNAm do ABCA1. O estudo também demonstrou significativa correlação entre a redução no RNAm e TFG e proteinúria destes pacientes. Corroborando tais achados, Tsun *et al.* (24) demonstraram que a exposição de células mesangiais e tubulares de humanos à hiperglicemia promoveu redução da expressão do ABCA1, revelando prejuízo na capacidade de mediar o efluxo de colesterol celular para apo-A1 e HDL colesterol.

Com base nas evidências clínicas e biológicas mencionadas que envolvem o gene *ABCA1*, sugere-se que este seja um relevante gene candidato e que a investigação de seu papel na genética da DRD possa contribuir para o conhecimento da patogênese desta complicação.

Inicialmente, mutações no gene *ABCA1* foram identificadas em duas formas de doenças por herança genética envolvendo o HDL colesterol. Em homozigose, a Doença de Tangier e em heterozigose a Hipoalfalipoproteinemia familiar, ambas caracterizadas

por deficiências severas nos valores de HDL plasmáticos e prejuízos importantes no efluxo de colesterol celular resultando em grave acúmulo de colesterol em diversos órgãos, assim como aterosclerose precoce e proteinúria (25, 26).

Mais recentemente, apesar da heterogeneidade dos estudos, diversos polimorfismos genéticos no gene *ABCA1* entre eles R219K, I883M, V825I, R1587K e o promotor -565C/T, tem sido associados ao risco de doença aterosclerótica (27-32). Entretanto, dados sobre a associação destes polimorfismos no gene *ABCA1* e a DRD são escassos e algumas evidências são baseadas no risco para o desenvolvimento do DM em diferentes grupos étnicos.

Inicialmente, Daimon *et. al* (33, 34) demonstraram associação entre haplótipos formados por polimorfismos do gene *ABCA1* e o risco para DM [RC = 2,58 (IC 95% 1,62 – 4,12)], independentes de alterações no perfil lipídico, o que sugere que diferentes mecanismos envolvendo o gene *ABCA1* sejam responsáveis pelo risco de DM e pelas alterações plasmáticas das lipoproteínas.

Em uma população mexicana, o estudo do sequenciamento do gene *ABCA1* mostrou que o alelo C do polimorfismo R230C, em homozigose, estava presente em 20,1% desses indivíduos, enquanto que os genótipos R230C e C230C foram mais frequentes entre os indivíduos DM2 quando comparados com os indivíduos sem DM2 (41,2 *vs.* 11,1%), mostrando um risco de 4.527 vezes maior de desenvolvimento de DM2 nesta população [(IC 95% 2,474 – 8,499), p = 0,003] (35). Posteriormente, o mesmo grupo confirmou estes achados com o estudo do polimorfismo R230C em uma coorte de mexicanos com e sem DM2 (36).

Em Turcos, o alelo T do polimorfismo C97T no gene *ABCA1* esteve associado com proteção para o DM2 e sua frequência foi maior entre turcos não diabéticos quando

comparados aos diabéticos [(34% *vs.* 21%, p = 0,020); RC = 0,52 (IC 95% 0,30 – 0,88)]. O perfil lipídico não esteve associado ao polimorfismo C97T neste estudo (37).

Outra investigação a cerca da associação do polimorfismo C97T no gene *ABCA1* e o risco de DM2 foram estudados em população Saudi. O alelo T também se mostrou protetor para o desenvolvimento do DM2 [RC = 0,46 (IC 95% 0,37 – 0,57)] (38).

Em caucasianos, a contribuição dos polimorfismos no gene *ABCA1* e o DM2 ainda é desconhecida. Em um estudo *Genome Wide Association* (GWA) o polimorfismo R230C não foi identificado como um lócus de risco para o DM2 (39, 40). Recentemente, um grande estudo envolvendo 27 polimorfismos no gene *ABCA1* não mostrou associação destes polimorfismos e o DM2 (41).

Até o momento, apenas o estudo de Yoshida *et.al* (42), em japoneses, mostrou associação entre o polimorfismo I823M no gene *ABCA1* e proteção para a DRC em indivíduos com síndrome metabólica, em modelos de herança recessivo e aditivo [RC = 0,76 (IC 95% 0,60 – 0,95)] e [RC = 0,66 (IC 95% 0,47 – 0,92)], respectivamente. No mesmo estudo, outro polimorfismo no gene *ABCA1*, o -14C/T, foi associado com risco para a DRC segundo os modelos de herança recessivo e aditivo [RC = 1,79 (IC 95% 1,18 – 2,67) e RC = 1,86 (IC 95% 1,22 – 2,80), respectivamente]. Nesse contexto, o objetivo do presente estudo foi investigar a associação entre os polimorfismos -565 C/T [rs1800977 (C/T)], R219K [rs2230806 (G/A)], V825I [rs2066715 (G/A)], I883M [rs2066714 (A/G)], R1587K [rs2030808 (G/A)] no gene *ABCA1* e a presença de proteinúria em pacientes com DM2 em uma população branca do Sul do Brasil.

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ARTIGO DE REVISÃO

ATP-BINDING CASSETTE TRANSPORTER A1 (*ABCA1*) E A DOENÇA RENAL DO DIABETES

ATP-BINDING CASSETTE TRANSPORTER A1 (*ABCA1*) IN DIABETIC KIDNEY DISEASE (DKD)

ATP- BINDING CASSETTE TRANSPORTER A1 E A DOENÇA RENAL DO DIABETES (DRD)

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SUMMARY

Diabetic Kidney Disease (DKD) is one of the most important complication of Diabetes mellitus (DM). Between 30-40 % of patients with DM may develop DKD. The reason why some patients will evolve this complication and others will not is unknown, but smoking, chronic hyperglycemia, hypertension, genetic predisposition, lipid profile and age are some of the risk factor. It has been reported DKD between diabetic siblings and clustering of DKD within families and specific ethnic group supporting the genetic contribution to the disease. Different approaches have been done to investigate DKD genes. Clinical and experimental studies have provided insights into the role of ATP-binding cassette transporter A1 (*ABCA1)* gene as a relevant target in the pathogenesis of DKD. Excessive intracellular cholesterol (CHO) deposition may result from impaired cholesterol efflux due to downregulation of ABCA1 transporter which has been associated with GFR, proteinuria, podocyte injury and glomerusclerosis process on mesangial cells. Cardiovascular complication still account as an evident manifestation of DKD and different genetic variants of the *ABCA1* gene have been reported as strongly associated with coronary artery disease. As such, parallels between mechanism that underpin atherosclerosis and glomerulosclerosis provide further support for investigation of DKD. This study focused on the contribution of the different genetic variants of the *ABCA1* as a relevant target in the pathogenesis of DKD.

Keywords: ATP-binding cassette transporter A1 (ABCA1), Diabetic Kidney Disease (DKD), Cholesterol

SUMÁRIO

A Doença renal do diabetes (DRD) é umas das principais complicações do Diabetes mellitus (DM). Cerca de 30 a 40% dos pacientes com DM desenvolverão DRD. As causas ainda são pouco conhecidas, entretanto, tabagismo, hiperglicemia, hipertensão, predisposição genética, perfil lipídico e idade são os fatores de risco conhecidos para o desenvolvimento da DRD. Os estudos tem relatado a agregação da DRD entre familiares e irmãos diabéticos, bem como entre grupos étnicos específicos, justificando a contribuição da genética no surgimento da DRD. Diferentes abordagens têm sido feitas para o estudo de genes envolvidos na DRD. Ensaios clínicos e experimentais têm fornecido informações a respeito do papel do ATP-binding cassette transporter A1 *(ABCA1)* como um alvo relevante na patogênese da DRD. O excesso de colesterol intracelular é uma consequência do prejuízo no efluxo do colesterol intracelular mediado pela reduzida expressão do transportador ABCA1. Tal fato tem sido associado com diferentes fenótipos da DRD como baixa taxa de filtração glomerular, proteinúria, lesão dos podócitos e das células mesangiais nos rins. As complicações cardiovasculares são prevalentes na DRD e diferentes variantes genéticas do *ABCA1* têm sido associadas com a doença arterial coronariana. Desta forma, a investigação a cerca dos parâmetros envolvidos nos processos de aterosclerose e glomerulosclerose contribuem para o conhecimento da DRD. Este estudo tem como objetivo, focar as diferentes variantes genéticas do *ABCA1* que contribuem para o esclarecimento da patogênese da DRD.

Descritores: ATP-binding cassette transporter A1 (*ABCA1*), Doença renal do diabetes (DRD), colesterol

INTRODUCTION

According to World Health Organization (WHO) in 2012, 347 million people suffered from Diabetes mellitus (DM) and the deaths related to DM will double from 2005 to 2030 (1). The long-term damage of DM is associated with microangiopathic complications such diabetic kidney disease, neuropathy and retinopathy. Patients with DM also have an increased incidence of macroangiopathic complications such as atherosclerosis, coronary artery disease (CAD), and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in patients with DM (4, 5).

DIABETIC KIDNEY DISEASE (DKD)

DKD is one of the most important complication of DM and together with hypertension is the leading cause of chronic kidney disease (CKD) a worldwide public health problem defined by structural or function abnormalities of the kidney with or without decreased of glomerular filtration rate (GRF; <60ml/min/1.73m^2) over a period of at least three months (6).

DKD is the mainly cause of patients requiring dialysis or kidney transplantation in developed and emerging nations (7). Between 30-40 % of patients with DM may develop DKD (8). So far, the reason why some subjects will evolve this complication and others will not is unknown (8), but smoking, chronic hyperglycemia, hypertension, genetic predisposition, lipid profile and age are some of the risk factors(9).

The morphologic lesion in DKD is defined by thickening of the glomerular basement membrane, increased fractional mesangial volume, and podocyte abnormalities (10). DKD can be classified according to the urine albumin excretion

(UAE) and glomerular filtration rate (GFR). Regarding UAE, DKD could be divided into stages, being the first one formerly named as incipient nephropathy or microalbuminuria (UAE of 20-199 μg/min or 30 - 300mg/24h). However, not all patients with microalbuminuria will progress to a more advance stage, also formerly named as overt nephropathy or macroalbuminuria (UAE ≥199 μg/ min; >300mg/24h and proteinuria ≥500 mg/24h) (11, 12) that could be accompanied by a decrease in GFR or not. Although more recent guidelines suggest avoiding the terms micro- and macroalbuminuria (13), there is a clear increased risk of progressing to end-stage renal disease (ESRD), death and cardiovascular events according to UAE levels (14).

In the 80th decade studies suggested that approximately 80% of microalbuminuric type 1 DM patients progressed to proteinuria over a period of 6-14 years (15). However, reviewing some of the studies, the worsening of renal function seems not be so determined as initially assumed (16). Some determinant factors may affect this incidence, as in some patients microalbuminuria remained stable, whereas in other microalbuminuria regress to normoalbuminuria (16). In a well-documented study, The Joslin cohort identified that 19% of type 1 DM patients followed-up with microalbuminuria went on to have proteinuria, whereas the majority, 60%, presented a regression to normal albumin excretion levels (17). As reported in a 7.3 years follow-up study performed by The European Diabetes (EURODIAB) Prospective Complications Study in type 1 DM, 13.9% of the microalbuminuric patients progressed to macroalbuminuria, 35.5% remained microalbuminuric and 50.6% reverted to normoalbuminuria (18).

The incidence and prevalence of DKD varies. Data from Developing Education on Microalbuminuria for Awareness of renal and cardiovascular risk in Diabetes study (DEMAND) that evaluated 32.208 people from 33 countries with known type 2 DM,

demonstrated that 39% had microalbuminuria (19). According to the Danish study, in type 2 DM, up to 33% of patients presented DKD after an 18-year follow-up and this may differ depending on the ethnic origin from 20 to 50%. As published by the UK Prospective Diabetes Study (UKPDS), the annual incidence of proteinuria was 2.0%, and the prevalence after 10 years of type 2 DM course was 25% (20). Pima Indians presented probably one of the most elevated incidence of DKD. In a longitudinal study, 1715 Pima Indians with type 2 DM and no proteinuria at the baseline were followed-up during 4.7 to 6.5 years, among them, 366 incident cases of proteinuria occurred during this period (21). In other study, the prevalence of DKD was 65% in Pima Indians aged between 45-74 years old (22).

A variety of mechanisms contribute to the development and outcomes of the kidney injury, such as hyperglycemia and hemodynamic (23) and metabolic sets, genetic predisposition (24) and blood pressure levels (25) . In addition, elevated levels of serum lipids, smoking, and the amount and source of dietary protein also appear to be risk factors to DKD (9).

In this context, DKD is associated with a high incidence of cardiovascular death in patients with type 2 DM even in earlier stages of the kidney disease, before decrease in GFR (20). Bruno and Gross evaluated 685 patients starting dialysis in the metropolitan area of Porto Alegre, Brazil. Out of these 685, 182 (26.5%) had DM (6). In this cohort, 111 patients with DM were followed by an average of 3.5 years. The mean survival after starting dialysis for the diabetic subjects were 25 months, and the main death cause was cardiovascular disease (7).

GENETIC OF THE DIABETIC KIDNEY DISEASE

Evidences to support a genetic component to DKD have been long investigated. Primarily, it comes from the observation of DKD between diabetic siblings and clustering of DKD within families and specific ethnic groups (26).

The DKD offspring have three to four time increased incidence of renal disease compared with children of diabetic parents without renal disease (5). In 1990, analyses of serum creatinine and urine protein concentration from diabetic members of 316 Pima Indians families, in two successive generation, showed that proteinuria occurred among 14.3% of the diabetic offspring if neither parent had proteinuria, 22.9% if at least one diabetic parent had proteinuria, and 45.9% if both parents had diabetes and proteinuria (27). Also, diabetic siblings from parents with hypertension or cardiovascular disease are more likely to develop DKD (5, 11, 26, 28, 29).

Recent, genome-wide scans have identified candidate loci for DKD. In a family-based association study, 66 extended families with European ancestry and type 2 DM and non-type 2 DM from the Joslin Study of Genetics of Nephropathy in Type 2 Diabetes Family Collection, showed clear association between the locus 9q21.32 and the risk of high microalbuminuria, proteinuria, and ESRD among diabetic individuals, strengthening the evidence of this loci on diabetic nephropathy susceptibility (30).

In a longitudinal cohort study conducted in 429,918 veterans baseline differences in prevalence of early DKD were analyzed. The prevalence of DM was 56.2% in Caucasians, 15.3% in African Americans, 0.5% in Asians and 0.4 % in Native Americans. After adjustment for age, sex and economic status, African Americans and Native Americans were more likely to have early DKD than Caucasians (31). On the other hand, Gerchman et al. did not observe a higher prevalence of early DKD among Brazilian Africans. However, African descendants had a more advanced DKD (32).

Together, these observations reinforce the role of genetic contributions to DKD. Also, the development of a complex disease such as DKD probably depends on the effect of many genetic variables acting synergistically and additively with each other and with environmental factors (11).

There are different approaches to identifying DKD genes. An often used strategy is the candidate gene. This approach is based on previous information regarding the gene of interest and evaluates its role based on the biologically plausibility. Clinical and experimental studies have provided insights into the role of lipids and lipid-modulating proteins as key determinants on kidney disease. This study focused on the contribution of the ATP-binding cassette transporter A1 gene (*ABCA-1*) as relevant target in the pathogenesis of DKD.

THE ABCA-1 TRANSPORTER A1 AND THE CHOLESTEROL CONTENT ON DIABETIC KIDNEY DISEASE

The relationship between renal disease and lipids has been investigated for decades. Kimmelstiel and Wilson first identified the presence of lipid droplets in kidney biopsies from patients with DKD (33). In 1982 several studies were encouraged by the "lipid nephrotoxicity hypothesis" that precipitate or aggravate glomerular and tubulointerstitial disease (34-36).

The exact mechanism is not fully understood, but experimental evidences suggest that excessive intracellular cholesterol (CHO) deposition may result from impaired cholesterol efflux due to downregulation of ABCA1 transporter expression independently from the increased levels on plasma(37).

The ATP-binding cassette transporter A1 (ABCA1) is a 2261-amino-acid sterol-induced membrane protein, member of super-family of ABC transporters that is a

crucial player in the initiation of reverse cholesterol transport (RCT) from peripheral cells back to the liver for excretion. In this process, the ABCA1 protein controls the efflux of intracellular cholesterol to lipid-poor apolipoprotein A-I (Apo A-I), the major apolipoprotein of HDL. Patients with incipient or overt DKD have impaired serum capacity to induce ABCA1 cholesterol efflux (38).

In a study with kidney biopsies from patients with DKD, Herman-Edelstein and col. found a highly significant correlation between decreased mRNA expression of genes that regulate cholesterol efflux such as *ABCA1* and eGFR and proteinuria, supporting the possible role of abnormal lipid metabolism in the pathogenesis of DKD (39).

According to Zuo and col., in the apo E $^{-/-}$ knockout mice mouse model of atherosclerosis mild renal dysfunction perturbs macrophage lipid homeostasis by inhibiting cholesterol efflux mediated by decreased ABCA1 transporter (40) .

In experimental models of mice with type 1 DM the ABCA1 protein was decreased and accompanied by an increased cholesterol content from the isolated kidney (37). Also, in Akita and OVE26 mice, two genetic models of type 1 DM, the expression of ABCA1 is decreased, resulting in decreased cholesterol efflux (41).

Similar findings were observed in FVB$^{db/d}$ mice, a model of type 2 DM with DKD, which shown an important increase of triglycerides and cholesterol content in glomeruli and tubules cells (35).

Furthermore, lipotoxicity and lipid accumulation cause podocyte dysfunction and apoptosis (42). Podocytes express genes and proteins that modulate cellular cholesterol homeostasis, such as low density lipoprotein receptor (LDLR), ABC transporters, and apolipoproteins involved in maintaining cellular cholesterol levels

(43). Changes in podocyte structure and density are present at the early stages of DKD and might contribute to renal injury in type 2 DM (44).

Recently, normal human podocytes treated with serum from patients with DKD demonstrated an increased cholesterol accumulation, consequent from impaired in reverse cholesterol transport associated with a strong downregulation of ABCA1 mRNA that was independent of circulating cholesterol (45). In BTBR $^{ob/ob}$ mice, a type 2DM model of DKD, cholesterol accumulation occurs in kidneys, a phenotype that was prevented by cholesterol depletion with cyclodextrin (45).

In this context, it might be postulated that cholesterol overload due to impaired reverse cholesterol transport occurs in glomerular podocytes, and this may initiate the onset of albuminuria and the podocyte injury both contributing to the pathogenesis of DKD.

In addition to the investigation of the cholesterol content on different kidney cells, some studies have also evaluated the role of mesangial cells, a glomerular pericyte cell that share many properties with the macrophages and could mimic the atherosclerosis process on the kidney (46).

Protein expression and the ability to mediate cholesterol efflux of ABCA1 were determined in human renal mesangial and proximal tubular cells under diabetic conditions. Under high glucose condition, it was demonstrated a significant decreased in the capacity of the ABCA1 to mediate cholesterol efflux (47). Moreover, the histological examination revealed that ABCA1 was mainly expressed in renal tubules especially in the nephropathy group (47).

Besides that, the cardiovascular complication still account as an evident manifestation of DKD (48) and different genetic variants of the *ABCA1* have been reported as strongly associated with the risk of coronary artery disease. The capacity of

cholesterol efflux in macrophages is also compromised in DKD and it could be a link between the two conditions. As such, parallels between mechanisms that underpin atherosclerosis and glomerulosclerosis provide support for investigation of the parameters that contribute to DKD (35, 49, 50).

POLYMORPHISMS IN THE *ABCA1* GENE ASSOCIATED TO TYPE 2 DIABETES MELLITUS

The gene *ABCA1* gene has 50 exons and is located on chromosome 9q31.1. The *ABCA*1 is highly conserved between species, showing over 90% identity with mouse ABCA1 at the protein level (51).

Mutations in *ABCA1* are responsible for two forms of heritable HDL disorders, in homozygosis, they are associated with Tangier Disease, and in heterozygosis with familial hypoalphalipoproteinemia. Both are characterized by HDL deficiency, defective apolipoprotein-mediated phospholipid and cholesterol efflux from cells and the accumulation of macrophage foam cells in various tissues, including arteries. A consistent phenotype with the proposed function of the antiatherogenic ABCA1 cholesterol transporter (52).

A number of common genetic variants in *ABCA1* have been associated with type 2 DM across multiple ethnic groups. The mechanism is not clear, but the role of *ABCA1* in type 2 DM was first propose with the observation that knock-out mice for Abca1 have significantly impaired glucose tolerance (53). Daimon et.al reported an *ABCA1* haplotype association with DM in Japaneses [OR 2.58 (95% CI 1.62-4.12)] (54). This was independent of abnormalities of serum lipid levels and may indicate different

mechanisms which link *ABCA1* gene to DM or abnormalities of serum lipids levels (54, 55).

Screening the *ABCA1* coding sequence among Mexican-Mestizos, showed that the C allele of the nonsynonymous variant R230C, was present in 20.1% of these individuals, and that the R230C or C230C genotypes were significantly more frequent in type 2 DM than in non-type 2 DM individuals (41.2 vs. 11.1%), showing a 4.527–fold increased risk for type 2 DM (95% CI 2.474–8.499, P = 0.003) (56).

In a large cohort of Mexican individuals the *ABCA1* 230C allele was significantly associated with type 2 DM. The R230C/C230C genotypes were statistically more frequent in the early-onset type 2 DM than in control subjects. Also, the R230C variant was not in linkage disequilibrium (LD) with any other polymorphism tested in the haplotype, suggesting that this SNP is a significant risk genetic variant for type 2 DM (57).

The R230C variant was also seen in the Oji-Cree population as a causing of familial hypoalfalipoproteinemia (58). Once this population has the highest reported frequency of type 2 DM, almost 40% of adults, (59) it may raise the possible association of the R230C variant to predispose DM in this population (60).

The investigation of the *ABCA1* C97T variant in Turkish type 2DM patients showed a higher frequency of TT genotype in non-diabetic patients compared to those with type 2DM (14% vs. 3% ; P=0.008). Also the frequency of T allele was higher in non-diabetic patients [(34% vs.21%, P=0.020) OR 0.52 (95% CI 0.30-0.88)] suggesting a protective factor against type 2DM (61). No association with lipid concentrations was observed (61).

Among the Saudi population, the frequency of the T allele of the *ABCA1* C97T SNP was significantly higher in health subjects compared to type 2DM patients, (0.28

vs. 0.45; P <0.0001); suggesting that the T allele may be a protective factor against type 2 DM [OR = 0.46 (95% CI 0.37 – 0.57)] (62).

The role of ABCA1 in type 2 DM is not so clear among Caucasian populations. The R230C was not identified as a risk locus for type 2 DM in genome-wide association studies in this population (63, 64). Recently, a large study evaluated 27 *ABCA1* genetic variants in white subjects (65). None of the genetic variants in *ABCA1* was associated with increased risk or protection for type 2 DM (65).

Data regarding *ABCA1* genetic variants and DKD are very scarce. In a case-control study, key genetic loci previously associated with dyslipidemia, including *ABCA1* SNPs, were assessed in order to detect a possible association to DKD. No gene related to dyslipidemia evaluated showed to be associated with DKD in type 1 DM individuals after multivariate analysis (66). In a Japanese population, Yoshida and cols reported in a case-control association study, genetic variants that conferred susceptibility to CKD in individuals with metabolic syndrome. The results demonstrated that recessive and additive models of 2583A/G (Ile823Met) *ABCA1* polymorphism [OR = 0.76 (95% CI 0.60 - 0.95)] and [OR = 0.66 (95% CI 0.47 - 0.92) respectively] were protective to CKD. On the other hand, the recessive and additive models of *ABCA1* -14 C/T polymorphism [OR = 1.79 (95% CI 1.18 - 2.67) and OR = 1.86 (95% CI 1.22 - 2.80)] were associated with increased risk of CKD (67). Further studies are needed in order to understand the role of this gene in the predisposition of DKD. Due to the strong association of dyslipidemia, cardiovascular disease and DKD in patients with type 2 DM, the evaluation of *ABCA1* polymorphisms in our population is mandatory, unfortunately unavailable at the moment.

CONCLUSION

There have been described several abnormalities in lipid accumulation in DKD, including cholesterol accumulation and ABCA1 downregulation. Changes that are thought to contribute to DM and that are associated with glomerulosclerosis.

As the contribution of the genetic variation in *ABCA1* is well established as a cause of Tangier disease, an extremely rare recessive disorder associated to accelerate atherosclerosis, further studies are important to examine *ABCA1* genetic variants that may confer risk to renal injury, especially among subjects with type 2 DM.

Table 1. Studies of the association between *ABCA1* polymorphism and type 2 DM or associated characteristics

Study population	Results
Japanese population with type 2DM (54)	Association between ABCA1 haplotype in the 5`region with type 2 DM (OR = 2.58; 95% CI = 1.62-4.12)
Mexican-Mestizo population with type 2DM (56)	Association between the R230C polymorphism and type 2 DM (95% CI = 2.474 - 8.499; P= 0.003)
Mexican population with type 2DM (57)	Association between R230C/C230C genotype with early-onset of type 2 DM (OR = 3.77; 95%CI = 2.121 - 6.748)
Caucasians with type 2DM (63)	No association between the R230C polymorphism with type 2 DM
Turkish with type 2 DM (61)	Association between the T allele of C97T polymorphism with type 2 DM (OR = 0.52; 95% CI = 0.30 - 0.88)
Saudi population with type 2 DM (62)	Association between the T allele of C97T polymorphism with type 2 DM (OR = 0.46; 95% CI = 0.3732 – 0.5729)
Caucasians with type 2 DM (65)	No association between the 27 polymorphisms with type 2DM
Japanese population with Metabolic Syndrome (67)	Association between recessive (OR = 0.76; 95% CI = 0.60 - 0.95) and additive models (OR = 0.66; 95% CI = 0.47 - 0.92) of I883M polymorphism with CKD
Japanese population with Metabolic Syndrome (67)	Association between recessive (OR = 1.79; 95% CI = 1.18-2.67) and additive models (OR = 1.86; 95% CI 1.22-2.80) of 14C/T polymorphism with CKD

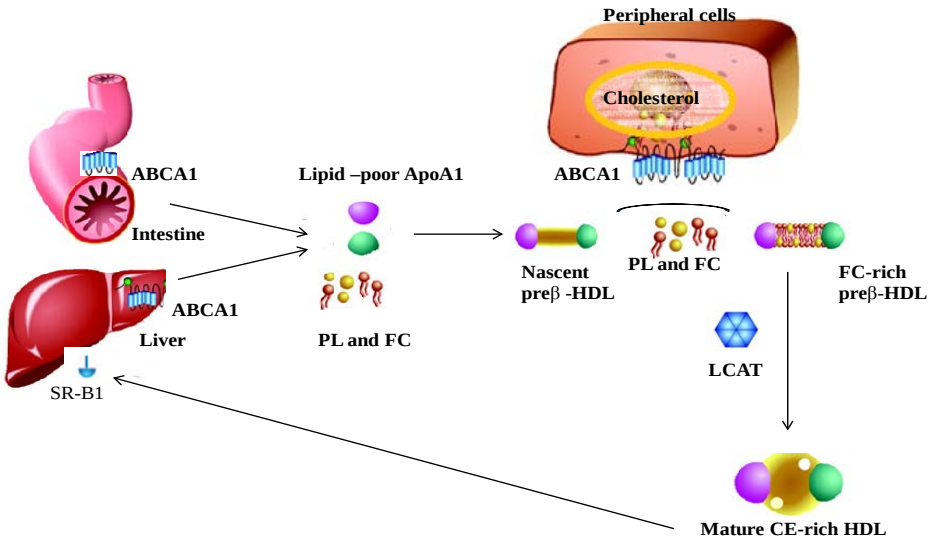

Figure 1. Reverse cholesterol transport (RCT) pathway. Both intestine and liver secrete apolipoprotein A-I (ApoA-I), which acquires free cholesterol (FC) and phospholipids (PL) via ATP-binding cassette transporter 1 (ABCA1) from these tissues, forming nascent preβ-HDL. Also, in peripheral cells preβ-HDL acquires more FC and PL via ABCA1 and turns into FC-rich preβ-HDL. On plasma the enzyme lecithin cholesterol acyl-transferase (LCAT) esterified the enriched lipoprotein resulting in mature HDL that can be removed by hepatocyte scavenger receptor class B type 1 (SR-B1). Adapted from Lewis & Rader (2) and Qasim & Rader (3).

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ARTIGO ORIGINAL

POLIMORFISMO NO GENE ATP-BINDING CASSETTE TRANSPORTER A1 (*ABCA1*) ESTÁ ASSOCIADO COM PROTEÇÃO PARA DOENÇA RENAL DO DIABETES

POLYMORPHISM IN ATP-BINDING CASSETTE TRANSPORTER A1 (*ABCA1*) IS ASSOCIATED WITH PROTECTION TO DIABETIC KIDNEY DISEASE (DKD)

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ABSTRACT

Introduction In kidney accumulation of lipids without abnormalities in serum lipid profile is associated with the development of glomerulosclerosis, tubulointerstitial fibrosis and progressiom of DKD. The involvement of genetic factors in the development of DKD has been investigated for decades. ATP- binding cassette trasnporter A1 (*ABCA1*) gene plays a central role in cholesterol efflux from cells to lipid-free receptors in bloodstream. Therefore, the aim of the present study was to investigate whether *ABCA1* polymorphisms are associated with presence of proteinuria in T2DM.

Methods Frequencies of the *ABCA1* rs1800977 (C/T), rs2230806 (G/A),rs2066715 (G/A),rs4149313 (A/G), and rs2030808 (G/A) were analyzed 365 T2DM patients with proteinuria or end-stage renal disease (ESRD) (cases) and 322 T2DM patients with normal albumin excretion rate (controls) subjects from Brazil. Haplotypes constructed from the combination of these polymorphism were inferred using a Bayesian statistical method.

Results The rs1800977 (C/T) polymorphism was associated with proteinuria in T2DM patients in dominant inheritance model (C/T+T/T vs.C/C). The presence of the T allele was associated with proteinuria protection (OR = 0.61 95% CI 0.41- 0.902; P = 0.013). The others four polymorphisms were not associated with proteinuria in T2DM patients. Permutations analysis showed that the distributions of inferred haplotypes were statistically different between case and controls groups (P = 0.004). The |D'| and r^2 measurements, did not demonstrated any significant LD between all pairs of combination of the five analyzed polymorphisms.

Conclusions The rs1800977 (C/T) polymorphism of *ABCA1* gene is significantly associated with protection to DKD in white Brazilian T2DM subjects.

Keywords: Type 2 Diabetes, Diabetic Kidney Disease, DNA polymorphism and ATP-binding cassette transporter A1

INTRODUCTION

Diabetic kidney disease (DKD) is one of the major complication of diabetes mellitus (DM) (1) and accounts for a large proportion of all patients requiring renal substitution treatment (2). DKD was originally described as a glomerular disease, leading to increase protein excretion in urine. More recently, DKD definition embraces not only proteinuria, but also decrease glomerular filtration rate (GFR). Approximately 40% of type 2 DM (T2DM) patients are affected by DKD, which is an evident cause of increase morbidity and mortality (3, 4).

Despite all improvements in the management of the risk factors for DKD, including hyperglycemia, hypertension, and dyslipidemia, a relevant proportion of subjects shows progression of renal injury (5). One possible explanation to this could be the involvement of genetic susceptibility in the pathogenesis of this complication (4). In fact, an inherited susceptibility to DKD probably exists, and great effort has been made to detect genes and genetic polymorphisms that harbor DKD susceptibility (6-8). However, results are still inconclusive with different variants associated with small effects in different populations (9).

The relationship between renal disease and lipids has been investigated for decades. Kimmelstiel and Wilson first identified the presence of lipid droplets in kidney biopsies from patients with DKD (10). After that, clinical and experimental studies have reported an accumulation of lipids in the kidney without abnormalities in serum lipid profile but associated with the development of glomerulosclerosis, tubulointerstitial fibrosis, and progression of DKD (11, 12). Also, normal human podocytes treated with serum from patients with DKD demonstrated an increased cholesterol accumulation,

resulting from an impairment in reverse cholesterol transport (RCT), which was associated with a strong downregulation of ATP-binding cassette transporters A1 (*ABCA1*) mRNA that was independent of circulating cholesterol (13).

ABCA1 is a 2261 amino-acid sterol-induced membrane protein, member of super-family of ABC transporters, and has a major role in the initiation of RCT from peripheral cells back to the liver for excretion. In this process, the ABCA1 protein controls the efflux of intracellular cholesterol to lipid-free apoA-I, the major apolipoprotein of HDL (14)

In Japanese population, some *ABCA1* variants have been described to be associated with chronic kidney disease (CKD) in individuals with metabolic syndrome (15). To date, no data regarding this gene and DKD in Caucasians or in non-Japanese are available. Therefore, studies are needed to investigate the association between *ABCA1* polymorphisms and DKD in different populations. Within this context, the aim of this study was to investigate the association of genetic variants in *ABCA1* gene with DKD in white T2DM from South Brazil.

MATERIALS AND METHODS

Subjects, phenotype measurements, and laboratory analyses

A total of 687 unrelated white T2DM patients were enrolled in the study. The sample population comprised 365 T2DM patients with proteinuria or end-stage renal disease (ESRD) (cases) and 322 T2DM patients with normal albumin excretion rate (controls). T2DM patients were participating in a multicenter study that recruited patients in South Brazil. A detailed description of the study can be found elsewhere(16). T2DM was defined as a diagnosis of DM after the age of 35 years, with no insulin

therapy during the first year after diagnosis and no previous episodes of ketoacidosis (17).

A standard questionnaire was used to collect clinical and anthropometric data such as gender, age, age at T2DM diagnosis, T2DM duration, and drug treatment. All patients had physical and laboratory evaluations. In summary, they were weighed bare feet, wearing light outdoor clothes and their height was measured. Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$. Office blood pressure (BP) was measured in sitting position, on the left arm, after a 5-min rest with mercury sphygmomanometer. The mean of two measurements taken 1 min apart was used to calculate systolic and diastolic BP. Arterial hypertension was defined as BP levels ≥140/90 mmHg.

Patients were classified according to urine albumin excretion rate (AER) in at least two out of three consecutive 24-h timed samples in a 6-month period-as controls (AER <30 mg/24h) or cases (AER ≥300 mg/24h or dialysis).

Serum and plasma samples were taken after 12h of fasting for laboratory analysis. Fasting plasma glucose was measured using the glucose-peroxidase colorimetric method. HbA1c measurements were performed by different methods and the results were traceable to the Diabetes Control and Complications Trial (DCCT) method by off-line calibration or through conversion formula (18). Serum creatinine was determined by the Jaffé reaction; total plasma cholesterol, HDL cholesterol and triglycerides by enzymatic methods, and albuminuria by immunoturbidimetry (Sera-Pak immune microalbuminuria, Bayer, Tarrytown,NY, USA; mean intra and interassay coefficients of variance of 4.5% and 11% respectively) (19). Patients interrupted the use of angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists for at least one week before having their albuminuria measured.

The study protocol was approved by Ethic Committee in Research from Hospital de Clinicas de Porto Alegre and all patients gave their informed consent in writing.

Genotyping

DNA was extracted from peripheral blood leukocytes by a standardized salting-out procedure (20). *ABCA1* polymorphisms were genotyped using primers and probes contained in the Human Custom TaqMan Genotyping Assay 20X (Life Technologies), and described in **table 1**. Reactions were conducted in 384-well plates, in a total 5µl reaction volume using 2ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Life Technologies), and Custom TaqMan Genotyping Assay 1x. The plates were then positioned in a real-time PCR thermal cycler (ViiA7 Real-Time PCR System; Life Technologies) and heated for 10 min at 95°C for 15 and 60°C for 1 min. Fluorescence data files from each plate were analyzed using automated allele-calling software (Life Technologies).

The location of the -565 C/T [rs1800977 (C/T)], R219K [rs2230806 (G/A)], V825I [rs2066715 (G/A)], I883M [rs2066714 (A/G)], and R1587K [rs2030808 (G/A)] polymorphisms in the *ABCA1* gene is shown in **figure 1**. Once the data from *ABCA1* polymorphisms and DKD are very scarce, polymorphisms were selected from the International HapMap Project (21). Also, *ABCA1* polymorphisms were selected based on their potential regulatory role, and influence on lipid levels or cardiovascular events (22). In order to cover possible linkage disequilibrium (LD) between some of the common polymorphism in this gene, at least five polymorphisms had to be genotyped with a Minor Allele Frequency (MAF) > 10% to cover 80% of the gene variability.

Statistical analysis

Allele frequencies were determined by gene counting and departures from the Hardy-Weinberg equilibrium (HWE) were verified using χ^2 tests. 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 (23). The haplotypes constructed from the combination of the five *ABCA1* polymorphisms and their frequencies were inferred using the Phase 2.1 program (Seattle,WA,USA), which implements a Bayesian statistical method (24).

Clinical and laboratory characteristics were compared between groups by using unpaired Student's t-test, one-way ANOVA or χ^2 test, as appropriate. Variables with normal distribution are presented as mean ± SD or percentage. Variables with skewed distribution were log-transformed before analyses and are presented as medians (minimum-maximum values).

Multivariate logistic regression analyses were performed to assess the independent association of *ABCA1* polymorphisms or haplotype with DKD, as well as to control for possible confounding factors whenever a statistically significant association was found in univariate analyses. A *P* value of <0.05 was considered statically significant. These statistical analyses were done using SPSS, version 18.0.

RESULTS

Sample description

The main clinical and laboratory characteristics of patients according to the analyzed groups are described in t**able 2**. Case subjects differed from controls for

gender, T2DM duration, systolic BP, LDL cholesterol, HDL cholesterol, total cholesterol, triglycerides, creatinine and albuminuria levels (P <0.05) **(table 2)**.

Genotype and allele distributions

Genotype and allele frequencies of the *ABCA1* rs1800977 (C/T), rs2230806 (GA), rs2066715 (GA), rs2066714 (AG) and rs2030808 (GA) polymorphisms in case and control groups are depicted in **table 3**. The genotypes of rs2030808, rs2066715, rs2066714 and rs1800977 polymorphisms were in agreement with those predicted by the HWE in controls (P >0.05); however, the rs2230806 polymorphism deviated from HWE (P = 0.025). Because no genotyping errors were detected this Single Nucleotide Polymorphisms (SNP) was kept in the analysis.

Genotype and allele frequencies of the rs2066715, rs2066714 and rs2030808 polymorphisms did not differ between case and control groups (P >0.05). Moreover, frequencies of these three polymorphisms also did not differ when assuming dominant, recessive or additive inheritance models (data not show).

Genotype frequencies of the rs2230806 (GA) polymorphism differ between controls (33.2% G/G, 41.6% A/G, 25.2% A/A) *vs*. cases (42.4% G/G, 38.4% A/G, 18.9% A/A) (P = 0.028). The minor A allele frequency among controls was 0.459 compared to 0.381 in cases; however, this difference did not reach formal statistical significance (P = 0.051). When we analyzed the different models of inheritance (dominant, recessive and additive), after adjustment for age, gender, T2DM duration and systolic BP, no association was found (data not show).

Genotype and allele frequencies of the rs1800977 (C/T) polymorphism did not differ significantly between case and control subjects **(table 3)**. However, analyzing the models of inheritance, a statistical significance was observed under the dominant model (C/T + T/T *vs.* C/C), showing that the presence of the T allele was associated with

proteinuria protection (controls: 41% C/C and 59% C/T + T/T vs. cases: 49.3% C/C and 50.7% C/T + T/T; P = 0.038). In multivariate analysis adjusted for age, gender, T2DM duration and systolic BP, the presence of T allele (C/T + T/T) was associated with a 39% reduction in proteinuria (OR = 0.61 95% CI 0.41– 0.902; P = 0.013).

Haplotype distribution and LD

We used a Bayesian statistical method to estimate the frequency of different haplotypes produced by the combination of the five *ABCA1* polymorphisms in T2DM patients with and without DKD. Twenty-four haplotypes were inferred in both samples. **Table 4** describes eleven haplotypes with frequencies higher than 1%, which altogether accounted for 90.6% of the observed haplotypes, the remaining 9.43% being among the rare haplotypes. The most frequent haplotypes were Ht1, Ht2, Ht3, Ht4, and Ht16; however, only Ht1, Ht2 and Ht4 had frequencies higher than 10%. Permutation analysis showed that the distributions of inferred haplotypes were statistically different between case and control groups (P = 0.004) due to differences in Ht1, Ht2, Ht4 and Ht12 frequencies. While Ht2 was more frequent in cases, Ht1, Ht4 and Ht12 showed the opposite result, being more frequent in controls (**Table 4**). It is noteworthy that taking into account both |D'| and r^2 measurements, we did not find any significant LD between all pairs of combination of the five analyzed polymorphisms (**Figure 2**).

DISCUSSION

ABCA1 is a major intracellular cholesterol transporter that has a central role to cholesterol homeostasis by mediating efflux to lipid-free apolipoprotein acceptors in bloodstream (25). Many polymorphisms have been described in the *ABCA1* gene, most of them have been associated with HDL cholesterol levels and atherosclersosis.

However, the results are excessively heterogeneous as HDL levels may not correlate to cholesterol efflux (26-32).

Previous studies reported that mutations in *ABCA1* are responsible for two forms of heritable HDL disorders: in homozygosis, for the Tangier Disease, and in heterozygosis, for Familial hypoalphalipoproteinemia, both related to premature coronary artery disease and proteinuria (33).

There is growing evidence about the role of *ABCA1* and cholesterol accumulation in kidney disease. Herman-Edelstein *et al*. (34) showed a highly significant correlation between decreased mRNA expression of *ABCA1* and eGFR and proteinuria. Tsun *et al.* (35) demonstrated that *ABCA1* are expressed in human mesangial cells and proximal tubular epithelial cells, and the induction of DM in rats treated with streptozotocin significantly reduced renal expression of *ABCA1*, which preceded the development of DKD. Recently, Merscher-Gomez *et al.* (13) reported that podocytes *in vitro* exposed to serum of patients with DKD showed cholesterol accumulation due to a strong downregulation of *ABCA1* mRNA and, consequently, an impairment of RCT not linked to intracellular cholesterol synthesis or cholesterol levels from plasma. In agreement, Zhou *et al.* (36) showed an impairment in the capacity of serum to induce cholesterol efflux mediated by *ABCA1* in T2DM patients with proteinuria. Altogether, these observations suggest that *ABCA1* should be considered as a candidate gene for DKD.

Nevertheless, data from the association of *ABCA1* polymorphism with proteinuria are very scarce. A genome-wide-scan study from Joslin Study Genetics of Nephropathy in Type 2 Diabetes Family Collection showed an association between the locus 9q21.32 and risk of proteinuria and ESRD among patients with DM (37), suggesting the an association of the *ABCA1* loci with DKD. In this context, in the

present study we have demonstrated an association of *ABCA1* polymorphisms and DKD in white T2DM patients. The rs1800977 CT polymorphism, in the promoter region of *ABCA1* gene, was significantly associated with DKD. Genotype frequencies of rs2230806 polymorphism trended toward a difference between case and control subjects; however, the rs2230806 association was not maintained after adjustment for covariables (P = 0.063). This might be explained by the small sample size, which could not confer enough statistical power to the analysis of this polymorphism.

Yoshida *et al.* evaluated the association between *ABCA1* polymorphism and CKD. These authors reported that the rs2066714 polymorphism, under recessive and additive models of inheritance, was significantly associated with protection for CKD in Japaneses with metabolic syndrome (15). We were not able to replicate the rs2066714 results in our study probably due to ethnic differences between the two populations as the genotype frequency are differently distributed in both studies. In our results the frequency of the minor G allele was 0.193 and in Japanese study, the frequency of the G allele was 0.597. Further studies are needed to confirm this data in CKD subjects.

Recently, a meta-analysis of 12,551 individuals with atherosclerosis and 19,548 controls without atherosclerosis pooled from 42 studies that analyzed the *ABCA1* rs2230806 polymorphism revealed that this SNP was associated with atherosclerosis in different ethnic groups. Individuals with A/A genotyped had a significantly lower risk for developing atherosclerosis compared to the other genotypes (OR = 0.69; 95% CI = 0.60 – 0.80; P >0.01) (38). The R219K polymorphism results in the substitution of an arginine by a lysine at the 219 residue in an extracellular domain of the protein that may result in an enhanced interaction between *ABCA1* and the acceptors in bloodstream (39). The potential protective effect of the A allele of the rs2230806 is consistent with a

hypothesized increase in activity of *ABCA1* gene associated with this polymorphism (40).

Furthermore, our findings demonstrated that the presence of minor T allele of polymorphism rs1800977 (C/T), under a dominant inheritance model, was protective against DKD (39% reduction). This association hold after controlling for possible confounding factors at multivariate analysis (P = 0.013). The rs1800977 (C/T) polymorphism is located at the promoter region of the *ABCA1* gene and, therefore, could have a functional significance. Kyriakou *et al.* reported that the expression of the *ABCA1* mRNA was lower in atherosclerotic plaque of patients carrying the T allele. The presence of the T allele at the rs1800977 (C/T) locus has been associated with increased severity of atherosclerosis (41). In the same way, in a Chinese sample, Qi *et al.* demonstrated that monocytes from individuals with acute coronary syndrome carrying the TT genotype exhibited significant lower cellular cholesterol efflux levels as well the lowest levels of ABCA1 mRNA and protein expression (42). Benton *et al.* also demonstrated that the T allele of rs1800977 (C/T) polymorphism may increase the risk for cardiovascular disease in multiple ethnic sample (43). Although our results showed the opposite: allele T of rs1800977 (C/T) polymorphism was associated with protection to DKD, the role of *ABCA1* in vascular and renal injury remain yet to be explored (44).

Regarding to haplotype analysis, twenty-four haplotypes were constructed from the rs1800977 (C/T), rs2230806 (G/A), rs2066715 (G/A), rs2066714 (A/G) and rs2030808 (G/A) polymorphisms to improve the power of conventional single locus analysis (45). Considering the most frequent haplotypes, the Ht1 frequency, which comprises the T allele of rs1800977 (C/T) polymorphism, was higher in T2DM patients without DKD. On the other hand, the Ht2, which comprises the allele C of rs1800977

(C/T) polymorphisms, was more frequent in T2DM patients with DKD. In the same way, the Ht4 was more frequent among controls.

Some factors may have interfered with the findings of the present study. First, we cannot rule out the possibility of population stratification bias, even though only white subjects were studied and both cases and controls were recruited by the same way, thus reducing the risk of false-positive/negative associations due to this bias. Second, we cannot fully exclude the possibility of a type II error when analyzing the association between the analyzed polymorphism and T2DM with and without DKD.

In conclusion, the present results suggest that the rs1800977 (C/T) polymorphism of *ABCA1* gene is significantly associated with protection to DKD in white Brazilian T2DM subjects.

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CONFLIT OF INTEREST

The authors declare that they have no competing interests

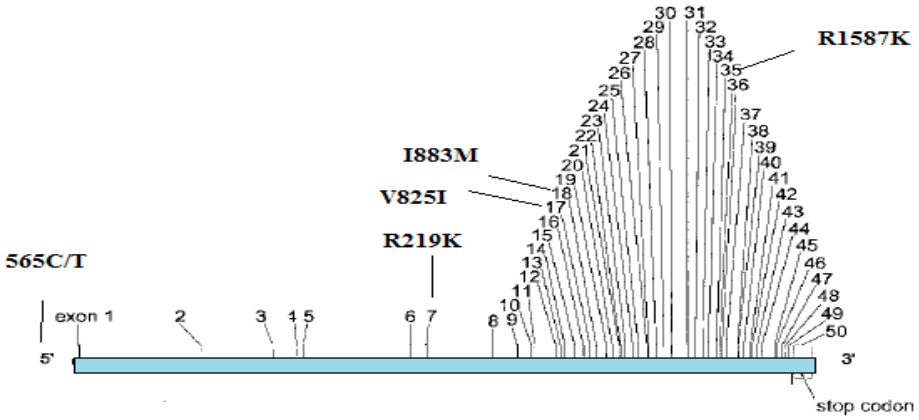

Figure 1. Map of *ABCA1* locus on chromosome 9. The fifty exons are numbered from the left to the right according to the transcriptional region. Polymorphisms analyzed are described according to the exons belonged. Figure adapted from Santamarina-Fojo *et al.* (46)

Figure 2. Pairwise linkage disequilibrium values for the five analyzed polymorphisms in *ABCA1* gene.

dsSNP ID	rs1800977 (C565T)	rs2230806 (R219K)	rs2066715 (V825I)	rs4149313 (I833M)	rs2230808 (R1587K)
rs1800977		0.195	0.081	0.696	0.311
rs2230806	0.013		0.1595	0.467	0.8565
rs2066715	0.012	0.011		0.159	0.155
rs4149313	0.434	0.229	0.011		0.159
rs2230808	0.013	0.089	0.040	0.115	

|D'|

r^2

Pairwise linkage disequilibrium (LD) values, |D'| (right) and r^2 (left), are shown.

Table 1. Primers and probes used for the genotyping of the analyzed *ABCA1* polymorphisms.

Polymorphisms	Primers	TaqMan MGB Probes
rs1800977 (C/T)	F: 5'- CCAAGAGAAAGCATCACTCTCTATTTTTG- 3'	VIC: 5'- CCCTTACACATATTCAACC-3'
	R: 5' – GTTTGCGAACTTTGACAAATGAAACATT-3'	FAM: 5'- CCTTACACATACTCAACC-3'
rs2230806 (G/A)	F: 5' – GCAACGGAAAAGGCAATCTAGAAGA -3'	VIC: 5'- AACCTCCCATTTTGCTC -3'
	R: 5' – AAGTCTTGAAGTTCAGTGAGCGA- 3'	FAM: 5' – ACCTCCCATCTTGCTC -3'
rs2066715 (G/A)	F: 5'- CATTGGTGTCATCTGAGA- 3'	VIC: 5' – TCTCCCGACCTCTCC- 3'
	R: 5' – GCAGGGCGGCAGAGT -3'	FAM: 5'- TCTCCCAACCTCTCC- 3'
rs4149313 (A/G)	R: 5' – CTCTGCATTTTCACATACAGGTTTGT- 3'	VIC: 5' – TGTATTTTACACTAATTTTG – 3'
	R: 5' – GGATTTCCAGTAGTCTTATAGCCTGGA- 3'	FAM: 5' – TGTATTTTACACTCATTTTG – 3'
rs2230808 (G/A)	F: 5' – TTCCCCACTATTTTTATGTTGCTGTCT -3'	VIC: 5'- AAAACAATTACGAATGGACC – 3'
	R: 5'- GCATCTAACTCTGGAGCTCCAAAAT – 3'	FAM: 5'- ATAAAACAATTACTAATGGACC-3'

Table 2. Clinical and laboratory characteristics of type 2 diabetes mellitus patients

Characteristics	Control group n= 322	Case group n= 365	*P** value
Gender (% males)	37.1	61.9	< 0.0001
Age (years)	61.4 ± 9.7	59.7 ± 11.3	0.054
Diabetes duration (years)	14.7 ±7.6	16.6 ± 10.1	0.011
Body Mass Index (kg/m^2)	28.6 ± 5.0	27.9 ± 6	0.145
HbA1c (%)	7.8 ± 1.8	8.0 ± 2.1	0.541
Systolic Blood Pressure (mmHg)	140.1 ± 20.9	149.1 ± 26.6	< 0.0001
Diastolic Blood Pressure (mmHg)	83.9 ± 11.8	85.5 ± 15.4	0.205
LDL-c (mg/dL)	128.0 ± 42.0	114.2 ± 48.3	0.002
Cholesterol (mg/dL)	207.9 ± 43.8	192.2 ± 52.5	0.001
HDL-c (mg/dL)	46.3 ± 10.8	40.4 ± 12.6	< 0.0001
Tryglycerides (mg/dL)	151 (26 -1359)	160 (27 - 1265)	< 0.0001
Creatinine (mg/dL)	0.8 (0.5 - 2.1)	1.1 (0.4 - 14.0)	< 0.0001
Albuminuria (mcg/min)	5.2 (0.1 - 26.1)	520 (42.6 - 7680)	< 0.0001

Data are mean± SD, median (min-max) or %. * *P* values were obtained from Student's *t*-test or χ^2-test. Only *P* values lower than the 0.05 were considered statistically significant

Table 3. Genotype and allele frequencies of *ABCA1* polymorphisms in controls and cases.

Polymorphism	Control	Case	Unadjusted P Value	Adjusted OR (95%CI)/P value
rs1800977 (C/T)	n= 278	n= 351		
CC	114 (41.0)	173 (49.3)	0.108	1
CT	125 (45.0)	139 (39.6)		0.59 (0.386 – 0.109)/ 0.114
TT	39 (14.0)	39 (11.1)		0.67 (0.373 – 1.190)/ 0.176
C	0.634	0.690	0.161	
T	0.365	0.309		
rs2230806 (G/A)	n= 322	n= 328		
GG	107 (33.2)	140 (42.7)	0.028	1
AG	134 (41.6)	126 (38.4)		0.70 (0.446 – 1.091)/ 0.115
AA	81 (25.2)	62 (18.9)		0.65 (0.390 – 1.090)/ 0.103
G	0.540	0.618	0.051	
A	0.459	0.381		
rs2066715 (G/A)	n= 221	n= 323		
GG	201 (91.0)	286 (88.5)	0.515	1
AG	20 (9.0)	36 (11.1)		1.65 (0.834 – 3.263)/ 0.150
AA	0 (0)	1 (0.3)		-
G	0.954	0.941	0.616	
A	0.045	0.058		

rs4149313 (A/G)	n= 283	n= 365		
AA	194 (68.5)	245 (67.1)	0.877	1
AG	75 (26.5)	99 (27.1)		1.01 (0.655 – 1.572)/ 0.948
GG	14 (5.0)	21 (5.8)		1.49 (0.641 – 3.469)/ 0.354
A	0.818	0.806	0.806	-
G	0.181	0.193		
rs2030808 (G/A)	n= 297	n= 344		
GG	184 (61.9)	213 (61.9)	0.908	1
AG	106 (35.7)	121 (35.2)		1.03 (0.691 – 1,542)/ 0.877
AA	7 (2.4)	10 (2.9)		1.96 (0.554 – 6.903)/ 0.297
G	0.797	0.795	0.969	
A	0.202	0.204		

Data are presented as number (%) or proportion. *P values were computed by the χ^2-test comparing T2DM patients with and without proteinuria. **P after adjustment of gender, age, T2DM duration and systolic BP; OR, odds ratio

Table 4. The most frequent haplotypes of the *ABCA1* gene in T2DM according to the absence or presence of proteinuria

Haplotypes	Controls* (n= 418)	Cases* (n= 640)	Frequency in the total sample
Ht1 [T G A G G] †	0.1444	0.1215	0.1321
Ht2 [C G A G G] †	0.2327	0.2816	0.2591
Ht3 [T G A G A]	0.0883	0.1109	0.0987
Ht4 [C G A G A] †	0.1903	0.1578	0.1728
Ht8 [C G G G G]	0.0297	0.0213	0.0258
Ht9 [T G G G A]	0.0281	0.0271	0.0276
Ht10 [C G G G A]	0.0431	0.0447	0.0438
Ht12 [C G G A G] †	0.0187	0.0076	0.0136
Ht15 [T A A G G]	0.0315	0.0386	0.0347
Ht16 [C A A G G]	0.0629	0.0570	0.0602

n = number of chromosomes. Data are presented as proportion. The first letter of the haplotypes refers to the C565T (rs1800977) polymorphism, the second to the R219K (rs2230806) polymorphism, the third to V825I (rs2066715) polymorphism, the forth to the I883M (rs2066714) polymorphism and the fifth to the R1587K (rs2030808). * Permutation P value = 0.004 for comparisons of haplotypes frequencies between groups. † Adjusted residuals which deviated from expected values (P values < 0.05).

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CONCLUSÕES GERAIS

O presente estudo indica que entre os polimorfismos estudados no gene *ABCA1*, apenas o polimorfismo rs1800977 (C/T) foi significativamente associado com proteção para DRD em pacientes com DM2 segundo análises do modelo de herança dominante (C/T + T/T vs. C/C). A presença do alelo T foi associada com 39% de redução da proteinúria em pacientes DM2 que apresentavam valores normais de excreção urinária de albumina (RC = 0.61 95% CI 0.41 – 0.902; P = 0.013).

Embora mais estudos sejam necessários para avaliar o efeito do polimorfismo rs1800977 no gene *ABCA1* e a progressão da DRD, acredita-se que os mecanismos envolvendo o transporte reverso do colesterol mediado pelo *ABCA1* estejam conferindo proteção às células renais e consequentemente prevenindo a progressão da DRD.