Universidade Federal do Rio Grande do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

Investigação da associação dos polimorfismos rs705708 no gene *ERBB3* e rs773120 no gene *PA2G4* com o diabetes mellitus tipo 1 e parâmetros clínicos e laboratoriais relacionados

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Dissertação apresentada como requisito parcial à obtenção do título de mestra em Endocrinologia pelo Programa de Pós-graduação em Ciências Médicas: Endocrinologia da Faculdade de Medicina da Universidade Federal do Rio Grande do Sul.

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“Ciência e vida cotidiana não podem e não devem ser separadas.”

Rosalind Franklin

RESUMO

Introdução: O diabetes mellitus tipo 1 (DM1) é uma doença multifatorial causada pela destruição autoimune das células-beta pancreáticas. O DM1 é considerado um grave problema de saúde pública, uma vez que possui taxas de morbidade e mortalidade elevadas, além de apresentar um aumento crescente de sua prevalência na população. Sendo assim, uma melhor compreensão das bases genéticas do DM1 poderá levar à identificação de pacientes predispostos para o seu desenvolvimento. Nesse sentido, o gene *ERRB3 (Erb-b2 Receptor Tyrosine Kinase 3)* está envolvido em mecanismos celulares relacionados à patogênese do DM1, como apresentação de antígenos, autoimunidade e apoptose de células-beta induzida por citocinas. Assim, alguns estudos sugerem que polimorfismos de nucleotídeo único (SNPs) nesse gene podem conferir risco para o desenvolvimento de DM1. O SNP rs705708 (G/A) é um SNP possivelmente funcional e que parece estar associado ao risco de DM1; no entanto, os poucos estudos na literatura apresentam resultados conflitantes. O *PA2G4* (*Proliferation-associated protein 2G4*) também é um gene candidato para essa doença, pois, além de ter um papel importante na regulação da proliferação celular e na imunidade adaptativa, atua regulando o ERBB3. Ainda não há estudos relacionando SNPs nesse gene e o DM1.

Objetivo: Avaliar a associação dos SNPs rs705708 (G/A) no gene *ERBB3* e do rs773120 (C/T) no gene *PA2G4* com o DM1 e suas características clínicas e laboratoriais.

Métodos: Este estudo foi aprovado pelo CEP-HCPA (2019-0392) e seguiu um delineamento do tipo caso-controle. Foram incluídos 976 indivíduos brancos do sul do Brasil, categorizados em 501 casos com DM1 e 475 controles sem a doença. Os SNPs estudados foram genotipados através de ensaio de discriminação alélica por PCR em tempo real.

Resultados: As frequências alélicas e genotípicas dos SNPs rs705708 (*ERBB3*) e rs773120 (*PA2G4*), não diferiram entre casos e controles; não se observando uma associação desses

SNPs com o DM1 mesmo após ajuste para covariáveis (idade, sexo e presença de haplótipo *HLA DR/DQ* de alto risco para DM1). No entanto, os pacientes com DM1 portadores do alelo A do SNP rs705708 tinham uma menor idade de diagnóstico dessa doença comparados com os pacientes com o genótipo G/G [13,5 (8,0 – 21,0) *vs*. 16,5 (10,0 – 23,0) anos; p= 0,027]. Interessantemente, o alelo A desse SNP também foi associado com proteção para hipertensão arterial, independente da taxa de filtração glomerular estimada (TFGe) e da idade [Razão de Chances (RC)= 0,605, Intervalo de Confiança (IC) 95% 0,37 – 0,98; p= 0,041]. O alelo A também foi associado com melhor função renal [TFGe mais alta e valores mais baixos de excreção urinária de albumina (p= 0,003 e 0,020, respectivamente)] comparado a pacientes com o genótipo G/G.

Conclusões: Embora não tenha sido observada associação entre os SNPs estudados e o DM1, o alelo A do SNP rs705708 no gene *ERBB3* parece estar associado com baixo risco de hipertensão arterial e com melhor função renal na população estudada. Mais estudos são necessários para confirmar esses resultados e para melhor elucidar os efeitos do SNP rs705708 na hipertensão e nas complicações crônicas do DM1.

Palavras-chave: Diabetes mellitus; Polimorfismo de DNA; *ERBB3*; *PA2G4*.

ABSTRACT

Introduction: Type 1 diabetes mellitus (T1DM) is a multifactorial disease caused by an autoimmune destruction of pancreatic beta-cells. T1DM is considered a serious public health problem due to its high morbidity and mortality rates, in addition to showing a growing increase in its prevalence in the population. Thus, a better understanding of the genetic basis of T1DM could lead to the identification of patients predisposed to its development. In this context, the *ERRB3* gene (*Erb-b2 Receptor Tyrosine Kinase 3*) is involved in cellular mechanisms related to the pathogenesis of T1DM, such as antigen presentation, autoimmunity, and cytokine-induced beta-cell apoptosis. Accordingly, some studies suggest single nucleotide polymorphisms (SNPs) in this gene confer risk for the development of T1DM. The rs705708 (G/A) SNP is a possibly functional SNP that appears to be associated with risk of T1DM; however, the few studies in the literature show conflicting results. *PA2G4* (*Proliferation-associated protein 2G4*) is also a candidate gene for this disease because, in addition to playing an important role in regulating cell proliferation and adaptive immunity, it acts by regulating ERBB3. There are still no studies relating SNPs in this gene and T1DM.

Objective: To evaluate the association of rs705708 (G/A) SNP in the *ERBB3* gene and rs773120 (C/T) SNP in the *PA2G4* gene with T1DM and its clinical and laboratory characteristics.

Methods: This study was approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre (2019-0392) and followed a case-control design. We included 976 white individuals from Southern Brazil, categorized into 501 cases with T1DM and 475 non-diabetic controls. The studied SNPs were genotyped using real-time PCR – allelic discrimination assay.

Results: Both allele and genotype frequencies of *ERBB3* rs705708 and *PA2G4* rs773120 SNPs did not differ between cases and controls. Thus, no association of these SNPs with DM1 was observed, even after adjustment for covariates (age, gender and presence of a high risk *HLA DR/DQ* haplotype for T1DM). However, T1DM patients carrying the A allele of the rs705708 SNP had a lower age of T1DM diagnosis compared to patients with the G/G genotype [13.5 (8.0 – 21.0) *vs.* 16.5 (10.0 – 23.0) years; P= 0.027]. Interestingly, the A allele of this SNP was also associated with protection against arterial hypertension, which was independent of the estimated glomerular filtration rate (eGFR) and age [Odds ratio (OR)= 0.605, 95% Confidence Interval (CI) 0.37 – 0.98; P= 0.041]. The A allele was also associated with better renal function [higher eGFR and lower urinary albumin excretion values (P= 0.003 and 0.020, respectively)] compared to patients with the G/G genotype.

Conclusions: Although no association between the studied SNPs and T1DM was observed, the A allele of rs705708 SNP in the *ERBB3* gene seems to be associated with a low risk of arterial hypertension and with better renal function in the studied population. More studies are needed to confirm these results and to better elucidate the effects of rs705708 SNP on hypertension and chronic complications of T1DM.

Keywords: Diabetes mellitus; DNA Polymorphism; ERBB3; PA2G4.

LISTA DE ABREVIATURAS

Introdução

AKT	*Protein kinase B*
APC	Células apresentadoras de antígenos
BACH2	*BTB domain and CNC homolog 2*
BAD	*BCL2 associated agonist of cell death*
BCL2	*BCL2 apoptosis regulator*
CTLA4	*Cytotoxic T-lymphocyte associated protein 4*
DAISY	*Diabetes Autoimmunity Study in the Young*
DM	Diabetes mellitus
DM1	Diabetes mellitus tipo 1
DM2	Diabetes mellitus tipo 2
DRD	Doença renal do diabetes
DNAr	Ácido desoxirribonucleico ribossômico
EBP1	*ERBB3-binding protein 1*
EGFR/ERBB1	*Epidermal growth factor receptor*
ERBB2/HER2	*Erb-b2 receptor tyrosine kinase 2*
ERBB3/HER3	*Erb-b2 Receptor Tyrosine Kinase 3*
ERBB4/HER4	*Erb-b2 receptor tyrosine kinase 4*
GAD65	Ácido glutâmico descarboxilase-65
GLIS3	*GLIS family zinc finger 3*
GRB2	*Growth factor receptor bound protein 2*
GSK3β	*Glycogen synthase kinase 3β*

GTP	Guanosina trifosfato
GTPase	Enzimas hidrolases
GWAS	*Genome Wide Association Studies*
HLA	*Human leukocyte antigen*
IA-2	Tirosina fosfatase
IC 95%	Intervalo de confiança de 95%
IDF	Federação Internacional de Diabetes
IL27	*Interleukin 27*
IL2RA	*Interleukin 2 receptor subunit alpha*
INS	*Insulin*
MAPK	*Mitogen activated protein kinases*
MEK	*Mitogen-activated protein kinase*
MSK2	*Ribosomal protein S6 kinase A4*
MYC6	Miosina
NFκB	*Nuclear factor-κB*
OBFC2B	Proteína de ligação ao DNA
ORMDL3	*ORMDL sphingolipid biosynthesis regulator 3*
PA2G4	*Proliferation-associated protein 2G4*
PB1	Polimerase viral
PCNA	Antígeno nuclear de proliferação celular
PDK1	*Pyruvate dehydrogenase kinase 1*
PI3K	*Phosphatidylinositide 3-kinases*
PIP2 e 3	*Phosphatidylinositol biphosphate e triphosphate*
PPIn	Polifosfoinositídeo
PTPN22	*Protein tyrosine phosphatase non-receptor type 22*

Raf	*Proto-oncogene serine/threonine-protein kinase*
Ras	*Small GTPases subfamily proteins*
RC	Razão de chances
RD	Retinopatia diabética
RNAm	Ácido ribonucleico mensageiro
RNAr	Ácido ribonucleico ribossômico
RNLS	*Renalase, FAD dependent amine oxidase*
RPS26	Proteína ribossomal S26
RSK1	*Ribosomal protein S6 kinase A1*
SMARCC2	*SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily c member 2*
SNP	*Single nucleotide polymorphism*
TFG	Taxa de filtração glomerular
TIF-IA	Fator de iniciação de transcrição I
tRNALeu	RNA transportador com uma leucina
VEGFA	*Vascular endothelial growth factor A*
ZnT8	Molécula transportadora de zinco

Artigo original

ALCAM	*Activated leukocyte cell adhesion molecule*
ASP	*Affected sibling pair*
BMI	*Body mass index*
BP	*Blood pressure*
CI	*Confidence Interval*

CKD	*Chronic kidney disease*
CKD-EPI	*Chronic Kidney Disease Epidemiology Collaboration*
CXCL-16	*C-X-C motif chemokine ligand 16*
DBP	*Diastolic blood pressure*
DKD	*Diabetic kidney disease*
DR	*Diabetic retinopathy*
EBP1	*ERBB3-binding protein 1*
eGFR	*Estimated glomerular filtration rate*
EGFR/ERBB1	*Epidermal growth factor receptor*
ERBB2/HER2	*Erb-b2 receptor tyrosine kinase 2*
ERBB3/HER3	*Erb-b2 Receptor Tyrosine Kinase 3*
ERBB4/HER4	*Erb-b2 receptor tyrosine kinase 4*
HbA1c	*Glycated hemoglobin*
HBDI	*Human Biological Data Interchange*
HDL	*High Density Lipoproteins*
HLA	*Human leukocyte antigen*
HNF4α	*Hepatocyte nuclear factor 4 alfa*
HWE	*Hardy–Weinberg Equilibrium*
KDIGO	*Kidney Disease: Improving Global Outcomes*
LDL	*Low Density Lipoproteins*
OBFC2B	*Nucleic acid binding protein 2*
OR	*Odd ratio*
PA2G4	*Proliferation-associated protein 2G4*
PCR	*Polymerase chain reaction*
RPS26	*Ribosomal protein S26*

RVLM	*Rostral ventrolateral medulla*
SHR	*Spontaneously hypertensive rat*
SMARCC2	*SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily c member 2*
SNP	*Single nucleotide polymorphism*
STREGA	*STrengthening the REporting of Genetic Association Studies*
STROBE	*STrengthening the Reporting of OBservational studies in Epidemiology*
T1DM	*Type 1 diabetes mellitus*
T2DM	*Type 2 diabetes mellitus*
T1DGC	*Type 1 Diabetes Genetics Consortium*
TGF-β	*Transforming growth-factor beta*
UAE	*Urinary albumin excretion*

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 é apresentado o artigo original. Após, são apresentadas as considerações finais.

Artigo original: ***The relantionship between polymorphisms in the ERBB3 and PA2G4 genes and type 1 diabetes mellitus and related-features: a case-control study in a Southern Brazilian population***

SUMÁRIO

1 INTRODUÇÃO

1.1 DIABETES MELLITUS TIPO 1

O diabetes mellitus (DM) vem se tornando, cada vez mais, um sério problema de saúde pública devido ao aumento de sua prevalência na população. De acordo com a Federação Internacional de Diabetes (*International Diabetes Federation* - IDF), atualmente 463 milhões de adultos em todo o mundo apresentam algum tipo de DM e a estimativa é que esse número aumente para 700 milhões de indivíduos afetados por essa doença em 2045 (1). Estima-se que 90 – 95% dos casos de DM correspondem ao DM tipo 2 (DM2) e 5 – 10% ao DM tipo 1 (DM1) (1, 2). Com relação ao DM1, atualmente existem 2,58 bilhões de crianças e adolescentes (<20 anos de idade) com essa doença e cerca de 130 mil novos casos por ano (1). O Brasil é o 3º país com maiores prevalência e incidência de DM1 em todo o mundo, com 51.500 pessoas acometidas pela doença e 7.300 novos casos por ano (em crianças com <15 anos de idade) (1).

O DM é um grupo de desordens metabólicas de etiologia múltipla caracterizado pela hiperglicemia crônica resultante de defeitos na secreção e/ou ação da insulina (2). Essa hiperglicemia crônica pode provocar lesões estruturais no endotélio vascular e no tecido nervoso que causam danos, disfunções e falhas de vários órgãos e tecidos, levando ao aparecimento das complicações crônicas do DM (2). Estas complicações são divididas em microvasculares [doença renal do diabetes (DRD), retinopatia diabética (RD) e neuropatia diabética] e macrovasculares (doença arterial coronariana, doença vascular periférica e acidente vascular cerebral) e são as causas mais comuns de morbidade e mortalidade em pacientes com DM (2).

A DRD é caracterizada como uma série de alterações estruturais que afetam a função renal, as quais iniciam por hiperfiltração renal e hipertrofia glomerular, seguida de albuminúria progressiva com declínio da taxa de filtração glomerular (TFG) (3, 4). A RD se caracteriza por anormalidades microvasculares do olho, incluindo a formação de microaneurismas. Em estágios mais avançados da doença pode ocorrer a perda gradual da microvasculatura da retina, levando à isquemia, seguida de proliferação de vasos sanguíneos anômalos e frágeis (neovascularização) que são propensos a hemorragias. Também pode haver crescimento de tecido cicatricial, que pode provocar o deslocamento da retina (5-7).

A classificação atual do DM compreende 4 categorias: DM1, DM2, DM gestacional e outros tipos específicos (2). O DM1 acomete principalmente crianças e adultos jovens e é causado pela destruição autoimune das células-beta pancreáticas mediada por linfócitos T e macrófagos, o que leva a uma deficiência total na secreção de insulina e faz com que os indivíduos afetados necessitem de tratamento com insulina para sobrevivência (2, 8-11). O ataque autoimune contra as células-beta é evidenciado pela presença de autoanticorpos contra antígenos dessas células, como contra a insulina, ácido glutâmico descarboxilase-65 (GAD65), tirosina fosfatase chamada de IA-2 e uma molécula transportadora de zinco (ZnT8). Geralmente os indivíduos com DM1 apresentarão pelo menos um desses autoanticorpos no momento do diagnóstico (12, 13).

Sendo uma doença multifatorial, o DM1 é causado pela complexa interação entre diversos fatores de risco ambientais, imunológicos, genéticos e epigenéticos (14-16). Estudos de varredura de genoma (*Genome Wide Association Studies* – GWAS) já associaram o DM1 a mais de 50 *loci*. Dentre eles, o *loci HLA* (*human leukocyte antigen*) *de classe II DR/DQ* é o principal *locus* de suscetibilidade [razão de chances (RC) > 7], sendo responsável por 30-50% do risco genético para o DM1 (15, 17, 18). No entanto, polimorfismos em outros genes também mostraram associação com risco para o DM1; como nos genes *INS* (*insulin*), *PTPN22*

(*protein tyrosine phosphatase non-receptor type 22*), *CTLA4* (*cytotoxic T-lymphocyte associated protein 4*) e *ERBB3* (*erb-b2 receptor tyrosine kinase 3*) (19-23). Nesse sentido, estudos com modelos de predição mostraram que a combinação de polimorfismos *HLA* e polimorfismos não-HLA pode melhorar a predição da doença e influenciar no desenvolvimento de autoanticorpos das ilhotas pancreáticas e na progressão para o DM1 (16, 24-28). Dentre os polimorfismos não-HLA, uma das principais regiões associadas à doença é a região 12q13, no cromossomo 12, onde está localizado o *ERBB3* (19, 29-31).

1.2 GENE *ERBB3*

O gene *ERBB3 (Erb-b2 Receptor Tyrosine Kinase 3)* codifica o receptor *epidermal growth factor receptor 3* pertencente à família de receptores tirosina quinases que consistem em 4 receptores de fatores de crescimento epidérmicos: ERBB1 (EGFR), ERBB2 (HER2), ERBB3 (HER3) e ERBB4 (HER4) (32).

Como o ERBB3 não possui um domínio quinase ativado, ele precisa sofrer heterodimerização com outros membros da família de fatores de crescimento epidérmico para poder ativar diversas vias de sinalização que estão envolvidas no ciclo celular, na proliferação, diferenciação, sobrevivência ou morte celular (29). A proteína codificada pelo gene *ERBB3* é expressa nos sistemas gastrointestinal, reprodutivo, respiratório, trato urinário, pele, sistema nervoso e sistema endócrino. No sistema endócrino, estudos sugerem que a deficiência de insulina aumenta a expressão gênica e proteica de ERBB3 (33).

O ERBB3 apresenta duas vias de ativação principais: a primeira corresponde à via da PI3K (*phosphatidylinositide 3-kinases*) / PDK1 (*pyruvate dehydrogenase kinase 1*) / AKT (*protein kinase B*). Nela, a PI3K é ativada após a formação de um dímero entre o ERBB3 e ERBB1/2. Uma vez ativada, a PI3K acumula PDK1/AKT na membrana celular, o que resulta em fosforilação e sinalização celular. Esta via é responsável por regular o crescimento celular,

apoptose e invasão de células tumorais. Já a segunda via de ativação se dá por meio da MAPK (*mitogen activated protein kinases*). A ativação do ERBB3 e a fosforilação da tirosina quinase resulta na ativação de MAPK, que uma vez ativada é capaz de traduzir estímulos extracelulares para a célula, regulando fatores de transcrição no núcleo, e induzindo a proliferação celular (**Figura 1**) (34, 35).

Alterações no *locus* do *ERBB3* já foram associadas com doenças autoimunes, tais como vitiligo e artrite reumatoide (30, 36, 37). Sabe-se ainda que o aumento da expressão de *ERBB3* desempenha um papel importante na progressão de várias formas de tumores (35). Como demonstrado anteriormente, o receptor codificado por esse gene é fundamental para a ativação da via PI3K/AKT que, por sua vez, vai desencadear outras vias relacionadas com a sobrevivência e proliferação das células (29, 38). A deficiência ou a inibição da via PI3K pode resultar na diminuição do número de células T regulatórias e desencadear autoimunidade (39).

Wang et al. (39) demonstraram que esse gene é expresso na superfície de células CD11+ (monócitos e células dendríticas), sugerindo que o ERBB3 desempenhe um papel na resposta imune. Esse mesmo estudo sugere também que a porcentagem de ERBB3 nas células está diretamente relacionada com a capacidade das células apresentadoras de antígenos (APC) estimularem a proliferação de células T.

Acredita-se que o ERBB3 esteja indiretamente envolvido na função das APCs, pois essas podem influenciar na suscetibilidade à autoimunidade através de diversos mecanismos, como eliminação de células T autorreativas e ativação de células T regulatórias. A literatura sugere que esse gene possa ter um papel molecular de grande importância relacionado tanto à infecção quanto à resposta imune, os quais são fatores que podem contribuir para a patogenia e suscetibilidade genética ao desenvolvimento do DM1 (39). Além disso, o *ERBB3* é um forte

candidato ao risco para o DM1 por ser expresso em células-beta pancreáticas e células ductais (40).

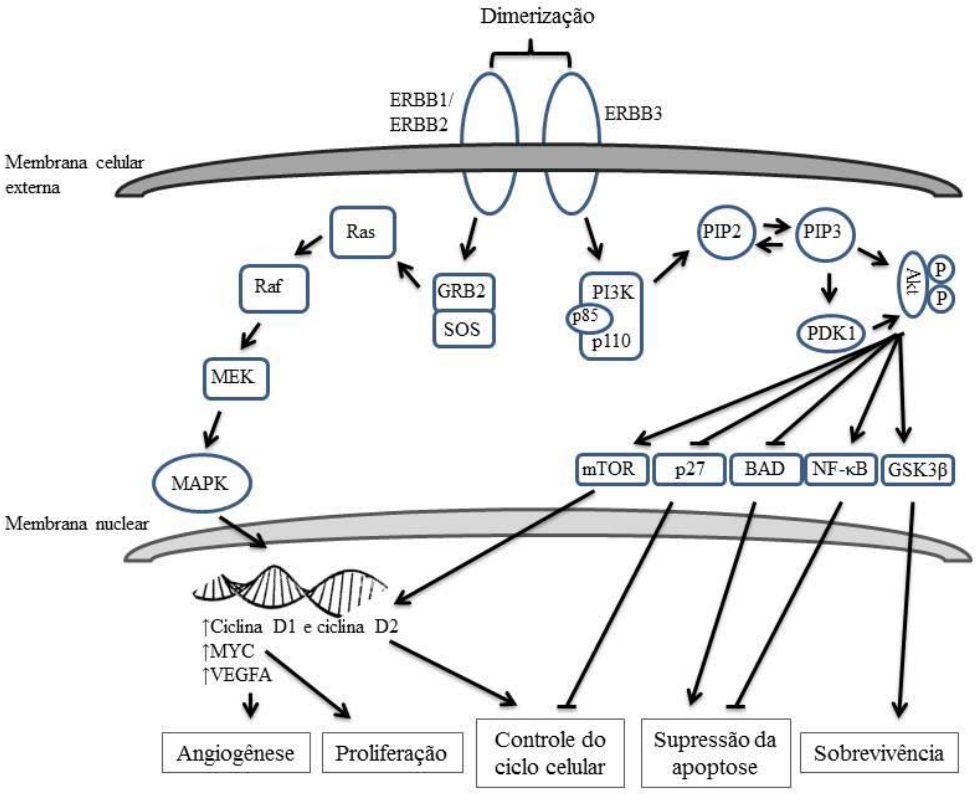

Figura 1 – **Ativação das vias PI3K e MAPK pelo gene *ERBB3.*** Legenda: →: ativação; ⊣ : inibição; GRB2: *growth factor receptor bound protein 2*; Ras: *small GTPases subfamily proteins*; Raf: *proto-oncogene serine/threonine-protein kinase*; MEK: *mitogen-activated protein kinase*; MAPK: *mitogen activated protein kinases*; VEGFA: *vascular endothelial growth factor A*; PI3K: *phosphatidylinositide 3-kinases*; PDK1: *pyruvate dehydrogenase kinase 1*; PIP2: *phosphatidylinositol biphosphate*; PIP3: *phosphatidylinositol triphosphate*; Akt: *protein kinase B*; GSK3β: *glycogen synthase kinase 3β*; NF-κB: *nuclear factor-κB*; BAD: *BCL2 associated agonist of cell death*. Fonte: Adaptada de Baselga; Swain, (2009); Li; Yuan; Cao, (2013).

Nesse sentido, Frohnert et al. (41) avaliaram o desempenho de um modelo de predição constituído por polimorfismos em 10 genes [*HLA, PTPN22, INS, IL2RA* (*interleukin 2 receptor subunit alpha*) , *ERBB3, ORMDL3* (*ORMDL sphingolipid biosynthesis regulator 3*),

BACH2 (*BTB domain and CNC homolog 2*), *IL27* (*interleukin 27*), *GLIS3* (*GLIS family zinc finger 3*) e *RNLS* (*renalase, FAD dependent amine oxidase*)] para prever o desenvolvimento de DM1 na coorte prospectiva do *Diabetes Autoimmunity Study in the Young* (DAISY). Os resultados demonstraram que o modelo com 10 polimorfismos, incluindo no *ERBB3*, apresentou melhor desempenho na predição de DM1 do que o modelo contendo apenas polimorfismos no *HLA*.

Além disso, estudos recentes mostram que o desenvolvimento de autoanticorpos contra a insulina pode estar relacionado a diversos SNPs (*single nucleotide polymorphism*) em genes associados com o risco para o DM1, entre eles o rs2292239, no *ERBB3* (42, 43).

O SNP rs2292239 (A/C), localizado no íntron 7 do gene *ERBB3*, é o SNP mais estudado nesse gene em relação ao DM1 (24, 29, 36). Esse SNP já foi associado com o DM1 em diversas populações, sendo que a maioria dos estudos mostra uma associação do alelo A com risco para o DM1, conforme a **Tabela 1** (19, 21, 25, 26, 39, 44-49). Essa associação do rs2292239 com risco para o DM1 também foi replicada na nossa população, em um estudo conduzido por nosso grupo, no qual foram avaliados cerca de mil indivíduos (incluindo casos e controles) de uma população caucasiana brasileira e foi encontrada uma associação do alelo A com risco para o DM1 (47). Assim, a associação desse SNP com risco para o DM1 parece já estar bem consolidada na literatura.

Outro SNP no *ERBB3* que também vem sendo estudado em relação à suscetibilidade ao DM1 é o rs705708 (G/A), localizado no íntron 15 desse gene. Conforme demonstrado na **Tabela 1**, esse SNP foi associado com risco para DM1 em uma população norte-americana (29) e em um GWAS que incluiu indivíduos asiáticos, britânicos, europeus, dinamarqueses e norte-americanos (30). Sun et al. (26) identificaram o rs705708 como associado com DM1 em uma análise de haplótipo (rs2292239, rs705708 e rs2292238) em uma população chinesa. Além disso, em um estudo feito por Kaur et al. (36) o genótipo G/G desse SNP foi associado

com diminuição da expressão do *ERBB3*, sugerindo que esse SNP é funcional. Na população brasileira, ainda não existem estudos relacionando o rs705708 com o DM1.

Tabela 1 – Resumo dos estudos que avaliaram a associação entre os SNPs rs2292239 e rs705708 no gene *ERBB3* e o DM1

Estudo	Desenho do estudo	SNP estudado no *ERBB3*	População estudada	Resultado
Awata et al., 2009 (44)	Caso-controle	rs2292239	Japonesa	O alelo A foi associado com risco para DM1 (RC= 1,38; IC 95% 1,16-1,65).
Espino-Paisan et al., 2011 (45)	Caso-controle	rs2292239	Espanhola	O alelo A foi associado com risco para DM1 (RC= 1,21; IC 95% 1,02-1,43).
Kiani et al., 2015 (46)	Caso-controle	rs2292239	Paquistanesa	O alelo T foi associado com risco para DM1 (p< 0,001).
Lemos et al., 2018 (47)	Caso-controle	rs2292239	Brasileira	O alelo A foi associado com risco para DM1 (RC= 1,62; IC 95% 1,02-2,59).
Maziarz et al., 2015 (25)	Caso-controle	rs2292239	Sueca	A associação entre o DM1 e os genótipos A/A + A/C foi mais forte entre pacientes positivos para o autoanticorpo IA-2.
Nikitin et al., 2010 (48)	Caso-controle	rs2292239	Russa	Não foi encontrada associação desse SNP com DM1.
Reddy et al., 2011 (21)	Caso-controle	rs2292239	Caucasianos da Geórgia	O alelo A foi associado com risco para DM1 (RC= 1,3; IC 95% 1,1-1,4).
Sun et al., 2016 (26)	Caso-controle	rs2292239	Chinesa	O alelo A foi associado com risco para DM1 (RC= 1,55; IC 95% 1,26-1,90).
Todd et al., 2007 (19)	Caso-controle	rs2292239	Europeia	O alelo A foi associado com risco para DM1 (RC= 1,28; IC 95% 1,21-1,35).
Wang et al., 2010 (39)	Caso-controle	rs2292239	Caucasianos da Geórgia	O alelo A foi associado com risco para DM1 (RC= 1,3; IC 95% 1,2-1,4).

Continua.

Conclusão.

Estudo	Desenho do estudo	SNP estudado no *ERBB3*	População estudada	Resultado
Yamashita et al., 2011 (49)	Caso-controle	rs2292239	Japonesa	O alelo A foi associado com risco para DM1 (RC= 1,5; IC 95% 1,3-1,8).
Cooper et al., 2009 (30)	GWAS	rs705708	Asiática, britânica, europeia, dinamarquesa e norte-americana	O alelo A foi associado com risco para DM1 (p< 0,001).
Kaur et al., 2016 (36)	Transversal	rs705708	*HapMap cell lines (CEU population)*	O genótipo G/G foi associado à menor expressão do gene *ERBB3*.
Keene et al., 2012 (29)	GWAS	rs705708	Norte-americana	O alelo A foi associado com risco para DM1 (RC= 1,31; IC 95% 1,13-1,51).
Sun et al., 2016 (26)	Caso-controle	rs705708	Chinesa	Esse SNP foi associado com DM1 em uma análise de haplótipo (rs2292239, rs705708 e rs2292238): o haplótipo G-G-A foi menos frequente nos pacientes com DM1 do que nos controles (RC= 0,71; IC 95% 0,55-0,92).

Fonte: Elaborada pela autora. DM1: diabetes mellitus tipo 1; GWAS: *genome-wide association study*; IC 95%: intervalo de confiança de 95%; RC: razão de chances; SNPs: *single nucleotide polymorphism*.

1.3 GENE *PA2G4*

O gene *PA2G4* (*Proliferation-associated protein 2G4*), também chamado de *EBP1* (*ERBB3-binding protein 1*), codifica uma proteína (EBP1) de ligação ao RNA que pode ser expressa nos mais diversos tecidos do corpo humano e ser encontrada tanto no citoplasma como no núcleo ou nucléolo. Essa proteína está envolvida na regulação do crescimento e proliferação celular, estando presente nos complexos ribonucleoproteicos (50-52). A EBP-1

interage com o domínio citosólico do receptor ERBB3, participando da inibição da via de transdução do sinal desse receptor, após ser fosforilada pela PKC (proteína quinase C) (50, 53). Nesse sentido, a heregulina, pertencente à família dos EGFs (fatores de crescimento epidérmicos) que ativam o ERBB3, promove a dissociação do complexo ERBB3-EBP1 e contribui na translocação da EPB1 do citoplasma para o núcleo (54). Outro fator que interfere na relação EBP1/ERBB3 é a superexpressão do tRNALeu livre, que perturba a interação entre ERBB3 e EBP1, reforçando a via de sinalização ERBB2/ERBB3 e resultando na fosforilação de RSK1/MSK2, o que aumenta a proliferação e a resistência à morte celular (55).

No núcleo, a EBP1 atua como co-repressora da transcrição de genes regulados por receptores andrógenos e outros genes envolvidos no ciclo celular, interagindo com histonas desacetilases (56). Além disso, ela se liga diretamente a vários polifosfoinositídeos (PPIns), que são fosfolipídios da membrana presentes no núcleo e, além de serem componentes estruturais, participam do remodelamento da cromatina, transcrição e processamento do RNAm. A interação da EBP1 com os PPIns permite à EBP1 alternar sua expressão entre diferentes compartimentos e funções celulares; sendo que sua presença no nucléolo contribui para o seu papel na supressão da proliferação celular (50, 57).

Por outro lado, a EBP1 também pode atuar na indução da proliferação de diversos tipos celulares, através da regulação negativa da proteína p53 (uma proteína que controla o ciclo celular), do aumento de expressão do PCNA (antígeno nuclear de proliferação celular) ou da supressão da apoptose, via inibição da fragmentação do DNA, interagindo com a Akt (proteína quinase B) fosforilada nuclear (58, 59). Nguyen et al. (58) demostraram em células T que a depleção de GTP (guanosina trifosfato) inibe a síntese de RNAr nessas células, pela inibição do TIF-IA (fator de iniciação de transcrição I), uma proteína de ligação à GTP que recruta RNA polimerase I para o promotor de DNAr, diminuindo a proliferação das células;

porém, quando não há essa depleção, o TIF-IA-GTP liga-se à EBP1 e, juntos, aumentam a transcrição do PCNA, aumentando a proliferação de células T.

A expressão do *PA2G4*, assim como da proteína EBP1, vêm sendo investigadas em muitos estudos relacionados ao câncer. O aumento dessa expressão se mostra associado com a inibição do crescimento e a indução da diferenciação de células cancerígenas humanas (60, 61). Já a expressão diminuída desse fator é relacionada com a presença de tumores ou com um pior prognóstico (62-64).

Corso et al. (65) propuseram que a EBP1 está presente em um complexo proteico que regula a expressão de genes do *locus* HLA de classe II, especialmente HLA-DRA, HLA-DRB1 e HLA-DQA1; estando envolvida na resposta imune adaptativa. Outro estudo demostrou que a infecção pelo vírus *Influenza* induz a expressão de *PA2G4*, ao mesmo tempo em que a EBP1 interage com a proteína viral PB1 e inibe a síntese de RNA e a replicação do vírus (66).

Além disso, o estudo de Shen et al. (67) mostrou que a diminuição da expressão de EBP1 aumentou a capacidade de proliferação de células R7T1 (linhagem de células-beta pancreáticas de camundongo). Da mesma forma, Squatrito et al. (50) relataram que a sua superexpressão inibiu a proliferação de fibroblastos humanos.

Desse modo, pode-se notar que o *PA2G4*, assim como a proteína codificada por ele (EBP1), possui várias funções regulatórias, incluindo funções relacionadas à proliferação celular e imunidade, as quais estão relacionadas à patogênese do DM1. Entretanto, até o momento, nenhum estudo avaliou a associação de SNPs nesse gene com o desenvolvimento do DM1.

Um SNP presente nesse gene é o rs773120 (C/T), que está localizado no íntron 5 do *PA2G4*. Esse SNP já foi associado em GWAS com a expressão de diversos outros genes, como o *RPS26*, que codifica uma proteína ribossomal, em células linfoblásticas (68) e em

pacientes com a doença de Alzheimer (69); o *MYC6*, que codifica uma miosina, o *SMARCC2*, que codifica uma helicase/ATPase, a qual participa do remodelamento da cromatina, em células sanguíneas (70); e o *OBFC2B*, que codifica uma proteína de ligação ao DNA, em osteoblastos, com interação terapêutica (71).

Além disso, esse SNP também pode interferir na regulação epigenética do *PA2G4*, perturbando, por exemplo, a ligação de RNAs não-codificantes longos (como o NONHSAG011351) nesse gene; podendo afetar as funções do *PA2G4* e sua rota de sinalização (36).

2 JUSTIFICATIVA

O DM1 é uma doença autoimune e multifatorial que caracteriza um grave problema de saúde pública, uma vez que possui acentuada morbidade e mortalidade e importantes repercussões econômicas e sociais decorrentes do impacto de suas complicações crônicas. Sendo assim, uma melhor compreensão das bases genéticas e moleculares do DM1 poderá levar à identificação de pacientes que apresentam maior predisposição para o seu desenvolvimento.

O gene *ERRB3* vem sendo relacionado com o DM1 por estar presente nas células-beta pancreáticas e células do sistema imune e, também, por estar envolvido em vias celulares associadas à patogênese do DM1, como proliferação celular e resposta imune. Nesse sentido, estudos mostram que SNPs no *ERBB3* parecem conferir risco para essa doença. Entretanto, até o momento, o SNP rs705708 ainda não foi estudado na população brasileira, embora seja importante fazer a replicação dos resultados dos estudos, uma vez que as frequências alélicas e genotípicas podem variar entre populações diferentes.

Além disso, outro gene que pode estar envolvido na patogênese do DM1 é o *PA2G4*, que, além de ter um papel importante na regulação da proliferação celular e na imunidade adaptativa, atua regulando o ERBB3; tornando-se, portanto, um gene candidato para essa doença. No entanto, ainda não existem estudos relacionando SNPs nesse gene e o DM1.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a associação dos SNPs rs705708 (G/A) no gene *ERBB3* e do rs773120 (C/T) no gene *PA2G4* com o DM1.

3.2 OBJETIVOS ESPECÍFICOS

a) Comparar as frequências dos SNPs em estudo no gene *ERBB3* e no gene *PA2G4* entre indivíduos com DM1 (casos) e indivíduos sem a doença, doadores do banco de sangue (controles);

b) Verificar se esses SNPs estão associados com alguma característica clínica ou laboratorial relacionada ao DM1, como idade de diagnóstico, perfil glicêmico e lipídico, dentre outras.

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4 ARTIGO ORIGINAL

The relantionship between polymorphisms in the ERBB3 and PA2G4 genes and type 1 diabetes mellitus and related-features: a case-control study in a Southern Brazilian population

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Short running title: *ERBB3* and *PA2G4* genes in the T1DM context.

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ABSTRACT

Introduction: The Erb-b2 receptor tyrosine kinase 3 (*ERBB3*) is involved in autoimmune processes related to type 1 diabetes mellitus (T1DM) pathogenesis. Accordingly, some studies have suggested that single nucleotide polymorphisms (SNPs) in the *ERBB3* gene confer risk for T1DM. Proliferation-associated protein 2G4 (*PA2G4*) is also a candidate gene for this disease because it regulates cell proliferation and adaptive immunity. Moreover, it is known that PA2G4 regulates ERBB3. To date, no study has evaluated the association of *PA2G4* SNPs and T1DM or related-features.

Objective: To evaluate the association of *ERBB3* rs705708 (G/A) and *PA2G4* rs773120 (C/T) SNPs with T1DM and its related-characteristics.

Methods: This case-control study included 976 white subjects from Southern Brazil, categorized into 501 cases with T1DM and 475 non-diabetic controls. The *ERBB3* and *PA2G4* SNPs were genotyped by allelic discrimination-real-time PCR.

Results: Genotype and allele frequencies of the *ERBB3* rs705708 and *PA2G4* rs773120 SNPs were not associated with T1DM controlling for age, gender, and *HLA-DR/DQ* haplotypes of high-risk for this disease. However, T1DM patients carrying the *ERBB3* rs705708 A allele had an early age at T1DM diagnosis compared to G/G patients. Interestingly, in T1DM group, the rs705708 A allele was also associated with protection against arterial hypertension and with better renal function (higher estimated glomerular filtration rate and lower urinary albumin excretion values) *vs*. G/G patients.

Conclusions: Although no association was observed between the *ERBB3* rs705708 and *PA2G4* rs773120 SNPs and T1DM, the rs705708 A allele seems to be associated with low risk of hypertension and with better renal function in this Southern Brazilian population.

Key-words: Type 1 diabetes mellitus; Polymorphism; *ERBB3*; *PA2G4*; Hypertension.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) accounts for 5-10% of all diabetic cases, and is characterized by the cellular-mediated autoimmune destruction of pancreatic beta cells, causing an absolute insulin deficiency (1, 2). The resulting chronic hyperglycemia then damages various organs and tissues, leading to the development of diabetic chronic complications, which are associated with high morbidity and mortality rates (2).

As a multifactorial disease, T1DM is caused by a complex interaction between several environmental, immunological, genetic, and epigenetic factors (3, 4). Regarding genetic factors, single nucleotide polymorphisms (SNPs) in more than 60 loci are known to predispose to T1DM, with *HLA class II DR/DQ* region showing the greatest effect on susceptibility for this disease (3, 5). Studies on genetic models of risk prediction have shown the combination of *HLA DR/DQ* and non-*HLA* SNPs can improve T1DM prediction (6, 7). Among the non-*HLA* SNPs, one of the main loci associated with T1DM is the 12q13 region, where the *Erb-b2 receptor tyrosine kinase 3* (*ERBB3*) and *Proliferation-associated protein 2G4* (*PA2G4*) genes are located (8, 9).

The *ERBB3* gene encodes a tyrosine kinase receptor, which is a member of the epidermal growth factor receptor (EGFR) family (10). ERBB3 has an impaired tyrosine kinase activity; consequently, it needs to undergo heterodimerization with other members of the EGFR family in order to activate downstream signaling pathways, such as cell survival, differentiation, and proliferation (8, 11). Moreover, ERBB3 plays a key role in modulating antigen presenting cell function, autoimmunity, and cytokine-induced beta-cell apoptosis, which are mechanisms involved in T1DM pathogenesis (12-14). Accordingly, a number of studies have reported the association between SNPs in the *ERBB3* gene and T1DM risk (8, 9, 15), with the the rs2292239 (A/C) SNP being the most studied in different populations (16-20). Recently, a meta-analysis of 9 case-control studies confirmed the rs2292239 A allele

confers risk for T1DM (OR = 1.29, 95% CI 1.22-1.36) (19). The rs705708 (G/A) SNP, located in intron 15 of *ERBB3* gene, has also been associated with risk for T1DM; however, in a few studies (8, 9, 15, 17). The rs705708 G/G genotype was associated with decreased *ERBB3* expression, suggesting this SNP is functional (13). To date, no study evaluated the association between the rs705708 SNP and T1DM in a Brazilian population.

The *PA2G4* gene, also called *EBP1* (*ERBB3-binding protein* 1), encodes an RNA-binding protein (EBP1) that is expressed in several tissues, being found both in the cytoplasm and nucleolus (21, 22). EBP-1 interacts with the cytosolic domain of the ERBB3 receptor, modulating the transduction pathway of this receptor (23, 24). This protein also appears to be involved in the adaptive immune response, increasing T cells proliferation and regulating the expression of *HLA class II* genes (25, 26). Moreover, decreased expression of EBP1 seems to increase the proliferation of R7T1 cells (a murine beta cell line) (27). Additionally, EBP1 can interact with the hepatocyte nuclear factor 4 alfa (HNF4α) and may influence insulin secretion (28). The rs773120 (C/T) SNP located in intron 5 of *PA2G4* gene seems to be functional since it has been associated with modifications in the expression of other genes, *RPS26*, *OBFC2B*, and *SMARCC2* (29-32). However, until this date, no study has evaluated the association between *PA2G4* SNPs and the development of T1DM.

Within this context, the aim of this study was to investigate the association of the *ERBB3* rs705708 (G/A) and *PA2G4* rs773120 (C/T) SNPs with T1DM and its clinical and laboratory characteristics.

MATERIALS AND METHODS

Samples, phenotype measurements and laboratory analyses

A total of 976 participants were enrolled in this case-control study, which was designed following the STROBE and STREGA guidelines (33, 34). Cases (n = 501) were defined by the presence of T1DM, being recruited from the outpatient clinic at the Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil), between January 2005 and December 2013, and at the Instituto da Criança com Diabetes/Hospital Nossa Senhora da Conceição (Porto Alegre, Rio Grande Do Sul, Brazil), between August 2014 and September 2016. T1DM was diagnosed accordingly to the American Diabetes Association guidelines (2).

Controls (n = 475) were defined as non-diabetic blood donors recruited from the blood bank of the Hospital de Clínicas de Porto Alegre, between January 2005 and December 2015. Only subjects with glycated hemoglobin (HbA1c) values < 5.7% and without family history of DM were included in the control group (2). For both case and control groups, individuals for whom data on ethnicity, HbA1c values, and *HLA DR/DQ* genotypes were missing were excluded from the study. All subjects included in the study were self-declared as white.

Data on age, age at T1DM diagnosis, and drug treatment were collected using a standard questionnaire. All T1DM patients underwent physical examinations and laboratory tests, as previously reported by our group (35-37). Serum, whole blood and urine samples were collected for laboratory analyses (37). Assessment of diabetic retinopathy (DR) was performed by an experienced ophthalmologist using fundoscopy through dilated pupils, and the diagnosis of diabetic kidney disease (DKD) was based on KDIGO guidelines, using both urinary albumin excretion (UAE) and estimated glomerular filtration rate (eGFR; calculated with the CKD-EPI equation), as previously reported (37, 38).

For the control group, a simplified questionnaire was used to collect information on age and familial history of DM or other diseases, and blood was collected for HbA1c measurement. From both case and control groups, peripherial blood was also collected for DNA extraction.

The study was approved by the Ethic Committee in Research from Hospital de Clínicas de Porto Alegre, and all patients and non-diabetic controls provided assent and written informed consent prior to inclusion in the study.

Genotyping

A standardized salting-out technique was used to extract total DNA from blood leukocytes, and the DNA samples were kept at -20ºC until genotyping experiments (39). *ERBB3* rs705708 and *PA2G4* rs773120 SNPs were selected based on previous studies that reported an association between these SNPs and T1DM susceptibility and/or the involvement of these genes in key pathways associated with the pathogenesis of T1DM (as referred in the Introduction section), and also considering the novelty and originality criteria. Both, *ERBB3* rs705708 (G/A) and *PA2G4* rs773120 (C/T) SNPs were genotyped using TaqMan SNP Genotyping Assays 20x (Thermo Fisher Scientific, Foster City, CA, USA; assays IDs: C___8340587_10 and C___8340080_10, respectively). Real-time PCR reactions were run in 384-well plates, using 2 ng of DNA, Mastermix TaqPath ProAmp 1x (Thermo Fischer Scientific) and TaqMan SNP Genotyping Assay 1x, in a final volume of 5 µl. Plates were placed in the ViiA7 Real-Time PCR System (Thermo Fischer Scientific) and heated for 10 min at 95 ºC, followed by 50 cycles of 95 ºC for 15 s and 62 ºC for 1 min. Ten percent of samples were genotyped twice in order to calculate the genotyping error rate for each SNP. The calculated error rate for PCR duplicates was less than 0.5%, and the genotyping success was more than 95% for both SNPs.

Knowing that *HLA DR/DQ* haplotypes influence the association between non-*HLA* SNPs and T1DM, frequencies of *HLA* high-risk haplotypes were also investigated in our samples aiming to control a possible association of the studied SNPs with T1DM for thes *HLA* haplotypes. For this, three SNPs (rs3104413, rs2854275, and rs9273363) near to the *HLA class II* loci were genotyped using Custom TaqMan Genotyping Assays 20x (Thermo Fisher Scientific), and used for prediction of the *HLA* T1DM high-risk haplotypes, as previously described (5, 40). Then, we calculated the predicted frequencies of the following *HLA-DR/DQ* haplotypes: low-risk haplotypes (*DRx/DRx* or *DR4/DQ7*), intermediate risk haplotypes (*DR3/DRx*), and high-risk haplotypes (*DR4/DQ8* or *DR3/DR4-DQ8* or *DR3/DR3*), where *x* can be different non-risk alleles (40).

Statistical analysis

Allele frequencies were calculated by gene counting and departures from the Hardy–Weinberg Equilibrium (HWE) were analyzed using goodness of fitness χ^2 tests. Allele and genotype frequencies were compared between groups of subjects with χ^2 tests. Genotypes were also compared between groups considering different inheritance models, categorized as previously suggested (41). Normal distribution of the quantitative clinical and laboratorial variables was checked using Kolmogorov-Smirnov and Shapiro–Wilk tests. Quantitative variables are shown as median (25^{th} – 75^{th} percentile values) and were compared between groups of patients using the unpaired Mann-Whitney *U* test. Categorical data are shown as percentages and were compared between groups using χ^2 tests.

Logistic regression analyses were performed to estimate Odd ratios – ORs with (95% Confidence Intervals – CIs) and P values for the independent association of each SNP with T1DM or related features under the different inheritance models, adjusting for covariates. Those covariates with $P \leq 0.250$ in the univariate analysis were chosen for inclusion in the

multivariate logistic regression model. Statistical analyses were performed using the SPSS 18.0 software (SPSS, Chicago, IL), and P values <0.050 were considered significant.

RESULTS

Sample description

Table 1 shows the main characteristics of T1DM patients (cases) and non-diabetic subjects (controls) included in the study. As expected, frequencies of T1DM high-risk *HLA DR/DQ* haplotypes and HbA1c values were higher in cases compared to controls (P ≤0.001). Age and body mass index (BMI) values were lower in the case group compared to controls (P ≤0.001). Moreover, the percentage of males was higher in the control group compared to T1DM patients (57.0% vs. 50.6%; P = 0.048). The median of T1DM duration was 18 years (12.0 – 24.2), 60.7% of these patients had DR, 40.6% had DKD, and 39.3% had arterial hypertension.

Genotype and allele frequencies in case and control groups

Table 2 shows allele and genotype frequencies of the *ERBB3* rs705708 (G/A) and *PA2G4* rs773120 (C/T) SNPs in T1DM patients and non-diabetic controls. *ERBB3* rs705708 A allele frequency was 46.5% in both case and control groups (P = 0.971). Accordingly, genotype frequencies of this SNP did not differ between groups (P = 0.863), and this result did not change after adjustment for age, gender, and presence of T1DM high-risk *HLA DR/DQ* haplotypes. Moreover, the rs705708 A allele remained not associated with T1DM under dominant, additive, and recessive inheritance models, even after adjustment for the same covariates mentioned above (**Table 2**).

PA2G4 rs773120 T allele frequency was 9.5% in cases and 10.5% in controls (P = 0.534). Genotype frequencies of this SNP did not differ between groups (P = 0.499). In

addition, this SNP was not associated with T1DM considering the dominant model (P = 0.410), and the adjustment for age, gender and high-risk *HLA DR/DQ* haplotypes did not change the result (**Table 2**). Of note, additive and recessive models were not analyzed for this SNP due to the low frequency of the T allele.

Frequencies of T1DM high-risk *HLA DR/DQ* haplotypes were similar between case and control subjects carrying the A allele of the *ERBB3* rs705708 SNP and G/G subjects (P = 0.855). In contrast, these frequencies were lower in subjects carrying the T allele of the *PA2G4* rs773120 SNP than in C/C subjects (28.7 *vs*. 39.2%, P = 0.025). Thus, in order to confirm that high-risk *HLA DR/DQ* haplotypes did not influence the association between the *PA2G4* rs773120 SNP and T1DM, we also excluded subjects carrying the high-risk *HLA DR/DQ* haplotypes from samples. Considering only subjects without high-risk *HLA DR/DQ* haplotypes, the *PA2G4* rs773120 T allele frequency was 12.5% in cases and 11.2% in controls (P = 0.649), and this allele remained not associated with T1DM.

Comparison of clinical and laboratory characteristics of T1DM patients between different genotypes of ERBB3 and PA2G4 SNPs

Clinical and laboratory characteristics related to T1DM were compared between T1DM patients stratified by the presence of the *ERBB3* rs705708 A allele analyzed under the dominant model (**Table 3**). Gender, T1DM duration, frequencies of high-risk *HLA DR/DQ* haplotypes, BMI, diastolic blood pressure (DBP), HbA1c, triglycerides, HDL, LDL, and total cholesterol did not differ significantly between patients carrying the A allele (G/A or A/A genotypes) and patients with the G/G genotype (P >0.050). Patients carrying the A allele were younger than G/G patients (P = 0.002), and they showed a lower age at diagnosis compared to the G/G group [13.5 (8.0 – 21.0) *vs*. 16.5 (10.0 – 23.0); P = 0.027]. Interestingly, prevalence of arterial hypertension was lower in patients carrying the rs705708 A allele compared to G/G

patients (34.7% *vs*. 50.8%; P = 0.003). This association of the A allele with protection for hypertension was independent of age and eGFR (OR = 0.605, 95% CI 0.374 – 0.979; P = 0.041).

Regarding the diabetic chronic complications, prevalence of DR was also lower in patients carrying the rs705708 A allele compared to the G/G group (57.0% *vs*. 69.5%; P = 0.029). However, the association between this allele and DR was not independent of covariates (OR = 0.74, 95% CI 0.43 – 1.28; P = 0.279, adjusting for age, hypertension, HbA1c, and eGFR). Although DKD prevalence did not differ significantly between rs705708 genotype groups (P = 0.372), eGFR values were higher while UAE values were lower in the G/A + A/A group compared to G/G carriers (P = 0.003 and 0.020, respectively; **Table 3**).

To further clarify the protective association of the rs705708 A allele with hypertension and with renal damage, T1DM patients were dichotomized accordingly to eGFR values (42) (**Table 4**). Interestingly, when patients were categorized according to normal renal function [≥90 mL/min per 1.73 m^2, stage 1 of chronic kidney disease (CKD)] *vs*. any degree of altered renal function (stages 2 to 5), the A allele only remained independently associated with protection for hypertension in those patients with normal renal function (OR = 0.45, 95% CI 0.24 – 0.84; P = 0.013), adjusting for age, but not in those patients with altered renal function (P = 0.541). A similar result was obtained after stratifying patients accordingly to eGFR values ≥60 mL/min per 1.73 m^2: the A allele was only associated with protection for hypertension in those patients with normal or mild loss of renal function (≥60 mL/min per 1.73 m^2, stages 1 and 2) but not in those patients with eGFR values <60 mL/min per 1.73 m^2 (states 3 to 5, **Table 4**).

The same characteristics present in Table 3 were also compared between T1DM patients stratified by the presence of the *PA2G4* rs773120 T allele analyzed under the

dominant model (**Supplementary Table 1**). None of these clinical and laboratory characteristics differed significantly between groups (all P >0.050).

DISCUSSION

ERBB3 and *PA2G4* genes seem to be involved in pathways and mechanisms related to T1DM pathogenesis (12-14, 23-28). However, to date, only a few studies have investigated the association between the *ERBB3* rs705708 SNP and T1DM (8, 9, 15, 17), while no study analyzed the association of *PA2G4* SNPs with this disease. Thus, here, we investigated the association of *ERBB3* rs705708 (G/A) and *PA2G4* rs773120 (C/T) SNPs with T1DM and related-features. Our results showed no association between these SNPs and T1DM susceptibility; however, in T1DM patients, the *ERBB3* rs705708 A allele seems to be associated with an early age at T1DM diagnosis, protection against arterial hypertension and better renal function (higher eGFR and lower UAE values). The *PA2G4* rs773120 SNP was not associated with T1DM and related-features.

The rs705708 SNP is located in intron 15 of the *ERBB3* gene and it seems to be functional since Kaur et al. (13) showed the G/G genotype of this SNP was associated with lower *ERBB3* expression in HapMap cell lines (from CEU populations) (13); however, the functionality of this SNP was not confirmed in other studies. Keene et al. (8) reported the rs705708 A allele was associated with risk for T1DM (OR = 1.31, 95% CI 1.13 – 1.51) in affected sibling pair (ASP) families from the Type 1 Diabetes Genetics Consortium (T1DGC) and Human Biological Data Interchange (HBDI) repository from North America. Two other studies confirmed the association between the A allele of this SNP and risk for T1DM in ASP familes from cohorts assembled by the T1DGC (9, 15), which are in contrast with our present findings.

Despite the lack of association of the rs705708 SNP with T1DM in our case-control study, T1DM patients who carried the A allele had an earlier age at T1DM diagnosis, suggesting this allele is associated with a more aggressive development of T1DM in our population. This association is biologically plausible considering that *ERBB3* is expressed on the surface of CD11+ cells (monocytes and dendritic cells), playing a key role on immune regulation and T cell proliferation (12). *ERBB3* is also expressed in pancreatic beta cells, and insulin deficiency increases its expression (43, 44), while pro-inflammatory cytokines downregulate its expression (13, 14). *ERBB3* knockodown is able to decrease basal and cytokine-induced apoptosis, suggesting ERBB3 is required for apoptosis of beta-cells (13). Taking these findings into account, the presence of the rs705708 A allele could upregulate *ERBB3* expression; consequently, increasing the immune attack and beta cell death, thus leading to an early T1DM onset.

Besides the association of the *ERBB3* rs705708 A allele with an early T1DM diagnosis, this allele was associated with protection against hypertension. Diabetes and hypertension have a 60-65% coexistence rate. In contrast to type 2 diabetes mellitus (T2DM), in T1DM patients, BP is not usually altered at the beginning of the disease, thus the appearance of hypertension in these patients is mainly related to the progressive appearance of kidney damage (45). Here, 39% of T1DM patients had hypertension and most of them showed mild to moderate CKD. Hence, to better clarify the observed association, we stratified T1DM patients according to eGFR. This analysis showed the association of the rs705708 A allele with protection for hypertension was observed only in those patients with normal to mild loss of renal function but not in those with worst renal function. A possible explanation for these results is that in patients with moderate to severe renal dysfunction (eGFR <60 mL/min per 1.73 m^2; states 3 to 5 of CKD), hypertension would be more influenced by renal dysfunction *per ser* and other features, such as age and insulin resistance, than by genetic

polymorphisms. Therefore, in this group of patients, the effect of the rs705708 SNP in hypertension would be less important than in T1DM patients with normal renal function.

To our knowledge no other study has evaluated the association between *ERBB3* SNPs and hypertension; however, a previous study showed that ERBB3 plasma levels were associated with a reduced risk of hypertension in Chinese men with overweight. Moreover, ERBB3 levels were negatively correlated with DBP and BMI in men (46). Interestingly, Panagodage et al. (47) reported that treatment with low-dose acetylsalicylic acid reversed preeclampsia-related abnormalities in trophoblasts through decreasing the expression of *ERBB3*, *ALCAM*, and *CXCL-16*.

Hypertension is a common complex disorder of uncertain etiology. However, over recent years, it has been recognized that besides vascular and renal dysfunction, components of both innate and adaptive immune systems and low-grade inflammation play an important role in this condition (48-51). Even though the roles of ERBB3 on hypertension or vascular and renal dysfunction have not been well described; several lines of evidence have shown the involvement of other members of EGFR family on hypertension and related-conditions (52-54). In addition to modulating basal vascular tone and tissue homeostasis, EGFR (ERBB1) seems to be involved in proinflammatory, proliferative, migratory and remodeling processes, with enhanced deposition of extracellular matrix components, thus leading to vascular diseases, such as hypertension, atherosclerosis, and diabetic vascular chronic complications (52). EGFR-induced vasoconstriction was shown in spontaneously hypertensive rats (SHRs) and Dahl salt-sensitive rats as well as in experimental models of hypertension caused by infusion of angiotensin II, endothelin-1, aldosterone, and deoxycorticosterone acetate (52, 53, 55). Under these experimental conditions, EGFR blockade attenuated elevated BP (52). EGFR may also affect BP through its actions on abnormal regulation of vascular tone, inflammation, and renal sodium handling, which are majors factors involved in the regulation

of BP (52, 53). Moreover, in rostral ventrolateral medulla (RVLM), a major vasomotor center, microinjection of ERBB2 or ERBB4 inhibitors increased BP and renal sympathetic nerve activity (56). Accordingly, *Erbb2* expression in the brainstem was lower in SHRs than in normotensive rats (56).

Here, the *ERBB3* rs705708 A allele was also associated with better renal function (lower UAE values and higher eGFR *vs.* G/G genotype group) and protection for DR, although this last association was not independent of the presence of arterial hypertension. EGFR family members play key roles in nephrogenesis and in renal electrolyte homeostasis. They also promote glomerular hypertrophy, which is involved in the development of DKD (54, 57). Accordingly, treatment of diabetic rats with EGFR inhibitors decreased glomerular hypertrophy and tubular epithelial cell proliferation, preserved podocyte mass, and decreased albuminuria; thus, attenuating kidney damage (57, 58). Moreover, Akhtar et. al. (59) showed that the treatment with angiotensin-(1-7) prevented the development of hyperglycemia-induced vascular complications in diabetic rats partly by inhibiting EGFR, ERBB3, and ERBB4 transactivations. Hence, the upregulation of EGFR members seems to have a detrimental effect in the development of DKD and other renal diseases. However, EGFR signaling is also involved in mechanisms of repair, indicating a role in renal protection against injuries (54, 60). Thus, depending on the type, severity, and localization of the environmental stimulus, the same EGFR signaling mechanism might cause beneficial or detrimental consequences on kidneys (54, 60). Among the numerous stimuli that can activate the EGFR family members, the epidermal growth factor (EGF), transforming growth-factor beta (TGF-β), chronic hyperglycemia, and angiotensin II are well known to be involved in DKD development (60).

To date, no other study has analyzed the association between the *ERBB3* rs705708 SNP and diabetic chronic complications. Nevertheless, the T allele of the *ERBB3* rs2292239

SNP was previously associated with risk for DR in a T1DM Danish cohort of Pediatric Diabetes (61) and in Chinese Hui DM patients (62). Moreover, the rs12671550 and rs2072454 SNPs in the *EGFR* (*ERBB1*) gene were associated with susceptibility for DKD in T2DM patients from Taiwan (63). Furthermore, the A allele of the *ERBB4* rs7588550 SNP confered protection against DKD in a T2DM Japanese population (64) and in T1DM patients from the GENIE consortium (65). Therefore, taking into account the role of ERBB3 in hypertension, vascular and renal function and that other SNPs in *ERBB3* gene or in other members of the EGFR family have been associated with diabetic chronic complications, our present results suggesting associations between the *ERBB3* rs705708 SNP and hypertension and laboratorial markers of renal function seem to be biologically plausible albeit not yet completely understood.

The present study has some limitations. First, we cannot rule out the possibility of population stratification bias when analyzing our samples, even though only white subjects were included in the study and both T1DM patients and nondiabetic subjects were recruited from the same hospital, 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 associations of the *ERBB3* rs705708 and *PA2G4* rs773120 SNPs with T1DM. Although we had more than 80% power ($\alpha = 0.050$) to detect an OR $\geq$1.5 for the *ERBB3* rs705708 SNP and $\geq$1.7 for the *PA2G4* rs773120 SNP (for the minor alleles), we cannot exclude the possibility that these SNPs would be associated with T1DM with lower ORs. Of note, the *PA2G4* rs773120 is a very rare SNP, being the frequency of T allele quite low in the world population (https://www.ncbi.nlm.nih.gov/snp/rs773120; accessed in June 20th, 2021); thus, studies in larger samples are necessary to better elucidate the relationship between this SNP and T1DM.

In conclusion, although the present study suggests no association between *ERBB3* rs705708 and *PA2G4* rs773120 SNPs and T1DM in a Southern Brazilian population, the

rs705708 A allele seems to be associated with early age at T1DM diagnosis, arterial hypertension, and better renal function in T1DM patients. Replication of these findings in other populations might help elucidate the effects of rs705708 SNP on hypertension and diabetic chronic complications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. Clinical and laboratory characteristics of T1DM patients (cases) and non-diabetic subjects (controls).

Characteristics	**Cases (n = 501)**	**Controls (n = 475)**	**P***
Age (years)	34.0 (25.0 – 44.0)	38.0 (31.0 – 46.0)	≤0.001
Gender (% male)	50.6	57.0	0.048
T1DM duration (years)	18.0 (12.0 – 24.2)	-	-
HbA1c (%)	8.6 (7.3 – 9.9)	5.3 (5.1 – 5.5)	≤0.001
BMI (kg/m^2)	23.3 (21.6 – 26.0)	26.3 (24.0 – 29.6)	≤0.001
Arterial hypertension (%)	39.3	-	-
Triglycerides (mg/dL)	80.0 (57.0 – 121.2)	-	-
Total cholesterol (mg/dL)	174.0 (150.0 – 203.0)	-	-
LDL cholesterol (mg/dL)	97.1 (80.1 – 118.0)	-	-
HDL cholesterol (mg/dL)	54.0 (44.0 – 66.2)	-	-
eGFR (mL/min per 1.73 m^2)	98.0 (75.0 – 118.0)	-	-
UAE (mg/g)	10.7 (4.9 – 48.4)	-	-
Diabetic retinopathy (%)	60.7	-	-
Diabetic kidney disease (%)	40.6	-	-
T1DM high-risk *HLA DR/DQ* haplotypes (%)	58.4	18.6	≤ 0.001

Data are shown as median (25-75th percentiles) or %. BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; T1DM, type 1 diabetes mellitus; UAE, urinary albumin excretion. T1DM high-risk HLA DR/DQ haplotypes: DR4/DQ8 or DR3/DR4-DQ8 or DR3/DR3. *P values were obtained using $\chi 2$ or Mann-Whitney U tests, as appropriate.

Table 2. Genotype and allele frequencies of *ERBB3* rs705708 and *PA2G4* rs773120 SNPs in T1DM patients (cases) and non-diabetic subjects (controls).

	Cases	**Controls**	**P***	**Adjusted OR (95% CI) / †P**
***ERBB3* rs705708 SNP**	**n = 501**	**n = 475**		
Genotypes				
G/G	154 (30.7)	150 (31.6)		1
G/A	228 (45.5)	208 (43.8)		0.878 (0.595 – 1.296) / 0.513
A/A	119 (23.8)	117 (24.6)	0.863	0.882 (0.563 – 1.380) / 0.582
Alleles				
G	0.535	0.535		
A	0.465	0.465	0.971	-
Recessive model				
G/G + G/A	382 (76.2)	358 (75.4)		1
A/A	119 (23.8)	117 (24.6)	0.806	0.949 (0.643 – 1.402) / 0.794
Additive model				
G/G	154 (56.4)	150 (56.2)		1

A/A	119 (43.6)	117 (43.8)	≥ 0.999	0.897 (0.573 – 1.404) / 0.633
Dominant model				
G/G	154 (30.7)	150 (31.6)		1
G/A + A/A	347 (69.3)	325 (68.4)	0.830	0.879 (0.615 – 1.257) / 0.481
***PA2G4* rs773120 SNP**	**n = 491**	**n = 475**		
Genotype				
C/C	404 (82.3)	380 (80.0)		
C/T	80 (16.3)	90 (18.9)		
T/T	7 (1.4)	5 (1.1)	0.499	-
Allele				
C	0.905	0.895		
T	0.095	0.105	0.534	-
Dominant model				
C/C	404 (82.3)	380 (80.0)		1
C/T + T/T	87 (17.7)	95 (20.0)	0.410	0.954 (0.623 – 1.461) / 0.829

Data are shown as number (%) or proportion. *P values were calculated using χ^2 tests. †P values and ORs (95% CIs) were obtained using logistic regression analyses adjusting for age, gender and T1DM high-risk *HLA DR/DQ* haplotypes. Genotype distributions of *PA2G4* rs773120 (C/T) SNP were in agreement with the HWE in controls ($P \geq 0.050$). *ERBB3* rs705708 (G/A) genotypes were not in HWE in controls, only in the case group.

Table 3. Clinical and laboratory characteristics of T1DM patients broken down by the presence of the A allele of the *ERBB3* rs705708 SNP (dominant model).

Characteristics	**G/A + A/A (n = 343)**	**G/G (n = 153)**	**P***
Age (years)	33.0 (24.0 – 43.0)	38.5 (28.0 – 47.0)	0.002
Gender (% male)	49.9	51.0	0.893
Age at diagnosis (years)	13.5 (8.0 – 21.0)	16.5 (10.0 – 23.0)	0.027
T1DM duration (years)	17.0 (12.0 – 23.2)	19.0 (12.0 – 26.0)	0.365
HbA1c (%)	8.6 (7.3 – 9.9)	8.6 (7.4 – 10.0)	0.513
BMI (kg/m^2)	23.2 (21.4 – 26.0)	23.2 (21.8 – 25.9)	0.585
Arterial hypertension (%)	34.7	50.8	0.003
Systolic BP (mmHg)	120.0 (110.0 – 130.0)	120.0 (110.0 – 130.0)	0.015
Diastolic BP (mmHg)	80.0 (70.0 – 80.0)	80.0 (70.0 – 80.0)	0.486
Triglycerides (mg/dL)	80.0 (56.2 – 124.0)	83.0 (58.5 – 118.5)	0.557
Total cholesterol (mg/dL)	174.0 (150.0 – 202.2)	172.0 (148.7 – 209.5)	0.635
LDL cholesterol (mg/dL)	97.0 (79.6 – 118.0)	97.8 (80.2 – 119.0)	0.531
HDL cholesterol (mg/dL)	54.0 (44.0 – 67.0)	54.0 (43.0 – 66.0)	0.599
eGFR (mL/min per 1.73 m^2)	103.0 (76.0 – 121.7)	93.0 (61.0 – 110.0)	0.003
UAE (mg/g)	9.3 (4.5 – 45.3)	13.6 (5.7 – 82.9)	0.020
Diabetic retinopathy (%)	57.0	69.5	0.029
Diabetic kidney disease (%)	39.3	45.5	0.372
T1DM high-risk *HLA DR/DQ* haplotypes (%)	58.2	57.0	0.927

Data are shown as median (25-75th percentiles) or %. BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; T1DM,

type 1 diabetes mellitus; UAE, urinary albumin excretion. T1DM high-risk *HLA DR/DQ* haplotypes: *DR4/DQ8* or *DR3/DR4-DQ8* or *DR3/DR3*. *P values were obtained using χ^2 tests or Mann-Whitney *U* tests, as appropriate.

Table 4. Prevalence of arterial hypertension in T1DM patients stratified by the presence of the *ERBB3* rs705708 A allele and eGFR values.

Characteristics	**G/A + A/A (%)**	**G/G (%)**	**OR (95% CI) / P***
Arterial hypertension (%)			
eGFR <60 mL/min per 1.73 m^2	65.6	66.7	0.86 (0.28 – 2.63) / 0.797
eGFR ≥60 mL/min per 1.73 m^2	31.8	49.5	0.57 (0.33 – 0.97) / 0.038
Arterial hypertension (%)			
eGFR <90 mL/min per 1.73 m^2	55.9	62.7	0.80 (0.39 – 1.65) / 0.541
eGFR ≥90 mL/min per 1.73 m^2	24.2	46.3	0.45 (0.24 – 0.84) / 0.013

eGFR, estimated glomerular filtration rate; T1DM, type 1 diabetes mellitus. *OR (95% CI) / P values were obtained from logistic regression analyses adjusting for age.

Supplementary Table 1. Clinical and laboratory characteristics of T1DM patients broken down by the presence of the T allele of the *PA2G4* rs773120 SNP (dominant model).

Characteristics	**C/T + T/T (n = 86)**	**C/C (n = 400)**	**P***
Age (years)	35.0 (26.0 – 46.0)	35.0 (25.0 – 44.0)	0.770
Gender (% male)	52.3	50.3	0.818
Age at diagnosis (years)	16.0 (10.0 – 23.2)	14.0 (8.0 – 21.2)	0.217
T1DM duration (years)	17.0 (12.0 – 21.0)	19.0 (13.0 – 26.0)	0.125
HbA1c (%)	9.0 (7.4 – 10.0)	8.6 (7.3 – 9.8)	0.381
BMI (kg/m^2)	24.0 (21.5 – 26.8)	23.2 (21.6 – 26.0)	0.381
Arterial hypertension (%)	39.4	39.9	≥ 0.999
Systolic BP (mmHg)	120.0 (110.0 – 130.0)	120.0 (110.0 – 130.0)	0.886
Diastolic BP (mmHg)	80.0 (70.0 – 82.0)	80.0 (70.0 – 80.0)	0.783
Triglycerides (mg/dL)	77.5 (58.0 – 118.0)	82.0 (55.8 – 124.0)	0.926
Total cholesterol (mg/dL)	168.0 (144.0 – 191.0)	175.5 (151.0 – 205.2)	0.085
LDL cholesterol (mg/dL)	87.2 (77.1 – 107.3)	98.8 (81.9 – 118.3)	0.074
HDL cholesterol (mg/dL)	50.0 (42.7 – 62.0)	55.0 (44.2 – 67.0)	0.052
eGFR (mL/min per 1.73 m^2)	97.0 (79.0 – 119.5)	97.0 (72.0 – 118.0)	0.553
UAE (mg/g)	14.0 (5.0 – 103.5)	10.5 (4.9 – 43.5)	0.138
Diabetic retinopathy (%)	56.9	62.3	0.500
Diabetic kidney disease (%)	47.1	39.3	0.382
T1DM high-risk *HLA DR/DQ* haplotypes (%)	48.4	60.6	0.105

Data are shown by median (25-75th percentiles) or %. BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; T1DM,

type 1 diabetes mellitus; UAE, urinary albumin excretion. T1DM high-risk *HLA DR/DQ* haplotypes: *DR4/DQ8* or *DR3/DR4-DQ8* or *DR3/DR3*. *P values were obtained using χ^2 tests or Mann-Whitney *U* tests, as appropriate.

5 CONSIDERAÇÕES FINAIS

O presente estudo não demonstrou associação entre os SNPs rs705708 no gene *ERBB3* e rs773120 no gene *PA2G4* com o DM1 na população estudada. No entanto, o alelo A do SNP rs705708 parece estar associado com menor idade de diagnóstico de DM1, proteção para hipertensão arterial e melhor função renal nesta população do sul do Brasil. Este resultado é biologicamente plausível, considerando o envolvimento do gene *ERBB3* em mecanismos celulares relacionados à patogênese do DM1 e suas complicações.

Nesse sentido, os presentes dados contribuem para a busca de possíveis marcadores genéticos de suscetibilidade a essas doenças, os quais poderão ser utilizados em futuros escores preditivos. No entanto, estudos genéticos adicionais, maiores e em populações diferentes, são necessários para confirmar os resultados encontrados e para melhor elucidar os efeitos dos SNPs nos genes *ERBB3* e *PA2G4* na patogênese do DM1 e suas complicações crônicas.

6 OUTRAS PRODUÇÕES BIBLIOGRÁFICAS NO PERÍODO DO MESTRADO

Além do artigo que faz parte da presente dissertação, ao longo do período do mestrado foram desenvolvidos os seguintes manuscritos:

1) **Massignam ET**, Dieter C, Pellenz FM, Assmann TS, Crispim D. Involvement of *miR-126* rs4636297 and *miR-146a* rs2910164 polymorphisms in the susceptibility for diabetic retinopathy: a case-control study in a type 1 diabetes population. Acta Ophthalmol. 2021 Jun;99(4):e461-e469. doi: 10.1111/aos.14638. Epub 2020 Oct 29. PMID: 33124182.

2) Dieter C, Assmann TS, Lemos NE, **Massignam ET**, Souza BM, Bauer AC, Crispim D. -866G/A and Ins/Del polymorphisms in the *UCP2* gene and diabetic kidney disease: case-control study and meta-analysis. Genet Mol Biol. 2020 Mar 27;43(2):e20180374. doi: 10.1590/1678-4685-GMB-2018-0374. PMID: 31479096; PMCID: PMC7198021.