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Essa 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 uma breve introdução geral sobre o assunto da dissertação, seguida do manuscrito original.

Artigo Original: "The rs1800469 C/C and rs1800470 T/T genotypes of the *TGFB1* gene confer protection against diabetic retinopathy in a Southern Brazilian population."

SUMÁRIO

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1. Introdução

DM	Diabete mellitus
DM1	Diabetes mellitus tipo 1
DM2	Diabetes mellitus tipo 2
DRC	Doença renal crônica
DRCT	Doença renal crônica terminal
DRD	Doença renal do diabetes
EUA	Excreção urinária de albumina
GWAS	Estudo de associação ampla do genoma (*Genome wide association study*)
HAS	Hipertensão arterial sistêmica
IDF	Federação internacional de diabetes
IMC	Índice de massa corporal
RD	Retinopatia diabética
RDNP	Retinopatia diabética não-proliferativa
RDP	Retinopatia diabética proliferativa
SNPs	Polimorfismo de nucleotídeo único (*Single Nucleotide Polymorphisms*)
TGFB	Fator transformador de crescimento beta (*Transforming Growth Factor Beta*)
TGFB1	Fator transformador de crescimento beta 1 (*Transforming Growth Factor Beta 1*)

TFG	Taxa de filtração glomerular
TFGe	Taxa de filtração glomerular estimada

2. Artigo Original

AH	Arterial hypertension
BMI	Body mass index
CI	Confidence interval
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology collaboration
DKD	Diabetic kidney disease
DM	Diabete mellitus
DR	Diabetic retinopathy
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
SAH	Systemic arterial hypertension
HbA1c	Glycated hemoglobin
HDL	High density lipoprotein
HWE	Hardy-Weinberg equilibrium
LD	Linkage disequilibrium
LDL	Low density lipoprotein
NPDR	Non-proliferative diabetic retinopathy
OR	Odds ratio
PDR	Proliferative diabetic retinopathy
qPCR	Quantitative polymerase chain reaction

SNPs	Single nucleotide polymorphisms
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
TGFB	Transforming growth factor beta
TGFB1	Transforming growth factor beta 1
GFR	Glomerular filtration rate
UAE	Urinary albumin excretion

RESUMO

A retinopatia diabética (RD), uma complicação crônica microvascular do diabetes mellitus (DM), está associada com maior morbi-mortalidade e a uma piora da qualidade de vida dos pacientes. A hiperglicemia crônica associada a outros fatores de risco pode promover danos e falhas em diversos órgãos e tecidos, levando ao aparecimento das complicações do DM. No entanto, existem pacientes que desenvolvem estas complicações apesar de um excelente controle glicêmico e outros que não desenvolvem as complicações mesmo com uma longa duração de DM e hiperglicemia crônica. Desse modo, fatores genéticos podem explicar parte da heterogeneidade restante no desenvolvimento da RD.

Nesse contexto, polimorfismos de nucleotídeo único (*single-nucleotide polymorphisms – SNPs*) em genes que codificam proteínas relacionadas à inflamação e angiogênese, incluindo o Fator Transformador de Crescimento Beta 1 (*TGFB1*), podem ter um papel no desenvolvimento dessa complicação. Embora SNPs no gene *TGFB1* já tenham sido associados com DR, os resultados dos estudos são ainda inconclusivos. Sendo assim, o objetivo do presente estudo foi investigar a associação entre os SNPs rs1800469 e rs1800470 no gene *TGFB1* e a RD.

Para isso, os SNPs rs1800469 e rs1800470 no gene *TGFB1* foram genotipados em 635 pacientes com RD (casos) e 458 pacientes com ≥10 anos de diagnóstico de DM e sem RD (controles), utilizando a técnica de discriminação alélica por PCR em tempo real. Em relação ao SNP rs1800469 (c.-1347 C>T), a frequência do genótipo C/C foi maior nos controles do que nos casos (19,0% *vs*. 13,1%, p= 0,021). A associação deste SNP com proteção para RD também foi observada nos modelos de herança recessivo (p= 0,010) e aditivo (p= 0,042). Da mesma forma, o genótipo T/T do polimorfismo rs1800470

(c.+29 T>C) também foi associado à proteção para RD (25.3% *vs*. 19.2%, p= 0,032). Esta associação foi mantida somente para o modelo de herança recessivo (p= 0,021). Ambos os polimorfismos se mantiveram associados com proteção para a RD após o ajuste para idade, tipo de DM, HbA1c, hipertensão, taxa de filtração glomerular e duração do DM [Razão de Chances (RC)= 0,66; Intervalo de Confiança (IC) 95% 0,45-0,96; p= 0,030; e RC= 0,69; IC 95% 0,49-0,97; p= 0.033; respectivamente]. Também analisou-se as frequências dos haplótipos constituídos pelos SNPs rs1800469 e rs1800470: sujeitos com 0, 1 ou 2 alelos menores nos haplótipos *vs*. sujeitos com 3 ou 4 alelos menores. A frequência de 3 ou 4 alelos menores dos dois SNPs analisados foi menor nos casos com RD em comparação aos controles (17,6% *vs*. 23,4; p = 0,025). Após o ajuste para as mesmas variáveis descritas acima, a presença de ≥3 alelos menores nos haplótipos permaneceu independentemente associada com RD (RC= 0,7; IC 95% 0,49 - 0,99; p= 0,042).

Em conclusão, os SNPs rs1800469 e rs1800470 no gene *TGFB1* estão associados à proteção para RD em pacientes com DM tipo 1 ou 2 de uma população do sul do Brasil.

ABSTRACT

Diabetic retinopathy (DR), a chronic microvascular complication of diabetes mellitus (DM), is associated with increased morbidity and mortality and a worsening in the quality of life of patients. Chronic hyperglycemia associated with other risk factors can promote damage and failure in various organs and tissues, leading to the onset of DM complications. However, there are patients who develop these complications despite excellent glycemic control and others who do not develop complications even with long-term DM and chronic hyperglycemia. Thus, genetic factors may explain part of the remaining heterogeneity in the development of DR.

In this context, single nucleotide polymorphisms (SNPs) in genes that encode proteins related to inflammation and angiogenesis, such as the *transforming growth factor beta 1 (TGFB1)*, may play a role in the development of this complication. Although SNPs in the *TGFB1* gene have already been associated with DR, the results of the studies are inconclusive. Thus, the aim of the present study was to investigate the association between the rs1800469 and rs1800470 SNPs in the *TGFB1* gene and DR.

For this, the rs1800469 e rs1800470 SNPs were genotyped in 635 patients with DR (cases) and 458 patients with ≥10 years of DM and without DR (controls), using allelic discrimination real-time PCR.

Regarding the rs1800469 (c.-1347 C>T) SNP, the frequency of C/C genotype was higher in controls compared to cases (19.0% *vs*. 13.1%, P= 0.021). The association of this SNP with protection for DR was also observed in the recessive (P= 0.010) and additive (P= 0.042) inheritance models. In the same way, the T/T genotype of rs1800470 (c.+29 T>C) SNP was also associated with protection to DR (25.3% *vs*. 19.2%, P= 0.032). This association was only maintained for the recessive model (P= 0.021). Both SNPs remained

associated with protection for DR after adjustment for age, type of DM, type of DM, HbA1c, hypertension, estimated-glomerular filtration rate and DM duration [Odds ratio (OR)= 0.66; 95% Confidence Interval (CI) 0.45-0.96; P= 0.030; and OR= 0.69; 95% CI 0.49-0.97; P= 0.033; respectively]. We also analyzed frequencies of the haplotypes constituted by the rs1800469 e rs1800470 SNPs: subjects with 0, 1 or 2 minor alleles in the haplotypes *vs*. subjects with 3 or 4 minor alleles. The frequency of 3 or 4 minor alleles of the two analyzed SNPs was lower in DR cases compared to controls (17.6% *vs*. 23.4; P= 0.025). After adjusting for the same variables described above, the presence of ≥3 minor alleles remained independently associated with DR (OR= 0.7; 95% CI 0.49 - 0.99; P= 0.042).

In conclusion, the rs1800469 and rs1800470 SNPs in the *TGFB1* gene are associated with protection from DR in patients with type 1 or 2 DM in a population from Southern Brazil.

1. INTRODUÇÃO

1.1 Diabetes mellitus

De acordo com a Federação Internacional de Diabetes (*International Diabetes Federation - IDF*) (1), 463 milhões de pessoas em todo o mundo apresentaram algum tipo de diabetes mellitus (DM) no ano de 2019 (1). Além disso, se nenhuma providência para modificar a trajetória da epidemia for tomada, a prevalência de DM para 2045 é estimada em 700 milhões (1). No Brasil, a prevalência de DM em adultos entre 20-79 anos é de 11,4% (1), contando com aproximadamente 16,8 milhões de casos. Com isso, o Brasil é o quinto país com maior prevalência de DM no mundo (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). O DM tipo 1 (DM1) e o DM tipo 2 (DM2) são as principais formas dessa doença. A incidência de DM1 está aumentando na maioria dos países, especialmente em crianças com idade inferior a 15 anos (1) e representa cerca de 10% dos casos de DM (2). O DM2 é responsável pela maioria dos casos de DM em todo o mundo, representando cerca de 90% dos casos em adultos (1, 2).

O DM1 é uma doença crônica e multifatorial resultante da destruição autoimune das células-beta pancreáticas por linfócitos T e macrófagos. A destruição progressiva das células-beta leva à deficiência total de secreção de insulina fazendo com que a pessoa afetada necessite de terapia com insulina para a sua sobrevivência (2-4). O DM2 é causado por um desequilíbrio entre a ação e secreção de insulina (2). Este tipo de DM ocorre principalmente em indivíduos com mais de 40 anos e com obesidade, que correspondem a 80% dos casos (5). Tanto o DM1 quanto o DM2 são desencadeados por

uma complexa interação entre fatores de risco ambientais e um forte componente genético (6-8).

A hiperglicemia crônica pode provocar danos estruturais no endotélio vascular e no tecido nervoso que causam disfunções em diversos órgãos e tecidos, levando ao aparecimento das complicações crônicas do DM (2), as quais estão associadas com maior morbi-mortalidade e a piora da qualidade de vida dos pacientes (2). Essas complicações podem ser categorizadas em microvasculares [retinopatia diabética (RD), doença renal do diabetes (DRD) e neuropatia periférica (NP)] ou macrovasculares (infarto agudo do miocárdio, acidente vascular cerebral e gangrena) (2). A presença destas complicações geralmente depende do tempo de DM, idade dos pacientes, presença de hipertensão arterial sistêmica (HAS), dislipidemia, suscetibilidade genética e da intensidade e persistência da hiperglicemia (9). No entanto, existem pacientes que desenvolvem estas complicações apesar de um excelente controle glicêmico e outros que não desenvolvem complicações mesmo com uma longa duração dessa doença e hiperglicemia crônica. Desse modo, fatores genéticos podem explicar parte da heterogeneidade restante no desenvolvimento das complicações microvasculares do DM.

1.2 Retinopatia diabética

A RD é uma complicação microvascular do DM e é a principal causa de perda visual irreversível em adultos em idade produtiva no mundo (1, 2, 10, 11). A RD é caracterizada clinicamente pela presença de sinais microvasculares retinais típicos, como microaneurismas, hemorragias, manchas algodonosas, exsudatos duros e neovascularização, as quais são alterações resultantes da quebra da barreira hemato-retiniana e de distúrbios de angiogênese na retina (12). Os principais sintomas são visão

turva e embaçada, perda repentina da visão e distorção das imagens; entretanto, os pacientes podem ser assintomáticos (2).

A RD é diagnosticada por oftalmologista especializado pelo exame de mapeamento de retina sob midríase medicamentosa por oftalmoscopia ou por fotografia da retina (2). A RD é classificada, de acordo com o olho mais afetado, como ausente (sem anormalidades), retinopatia não-proliferativa (RDNP; microaneurismas, hemorragias intrarretinianas, grânulos venosos e anormalidades microvasculares intrarretinianas) ou retinopatia proliferativa (RDP; presença de neovascularização ou hemorragia vítrea/pré-retiniana). Os estágios iniciais da RD (RDNP) são caracterizados por diversas anormalidades microvasculares, incluindo a formação de microaneurismas. Em alguns casos ocorre a diminuição da permeabilidade dos capilares, podendo haver vazamento de fluido para dentro da mácula, causando edema macular (11, 13, 14). À medida que a doença progride para a forma mais grave (RDP), ocorre à perda gradual da microvasculatura da retina, levando à isquemia. Essa isquemia induz a proliferação de vasos sanguíneos anômalos e frágeis (neovascularização) que são propensos a hemorragias. Além disso, também pode haver crescimento de tecido cicatricial que, quando encolhe, forma uma espécie de cicatriz que distorce a retina e pode provocar seu deslocamento ou, ainda, o glaucoma. A hemorragia vítrea, o deslocamento da retina e o tecido fibroso cicatricial contribuem para a perda irreversível da visão (2, 15). As fases da patogenia da RD são representadas na **Figura 1**.

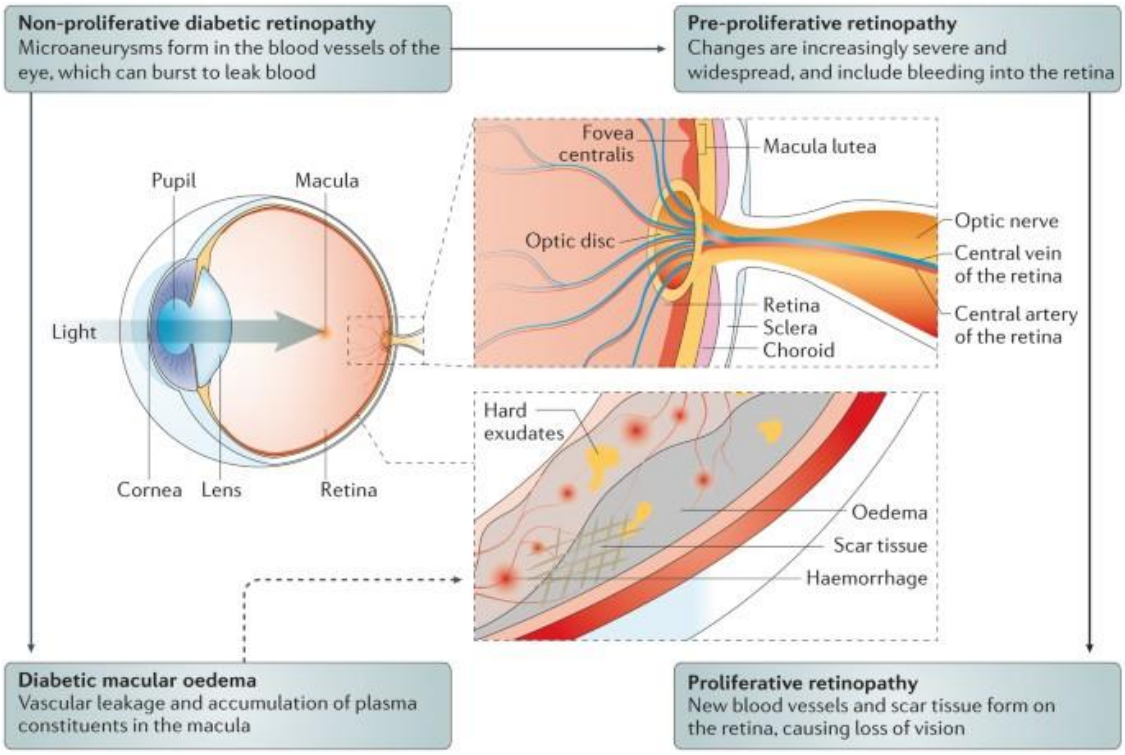

Figura 1. Estágios clínicos e principais eventos patogênicos da retinopatia diabética (15).

1.3 Genética da retinopatia diabética

O desenvolvimento e a progressão da RD dependem de uma interação complexa entre fatores de risco clínicos, ambientais e genéticos (11, 14, 16). Como já mencionado, a prevalência de RD aumenta com a duração do DM, pior controle glicêmico, presença de HAS, dislipidemia e índice de massa corporal (IMC) (2). Parece haver um subgrupo de pacientes com DM que jamais desenvolve RD. Por outro lado, há indivíduos que desenvolvem essa complicação apesar de terem suas glicemias rigidamente controladas, sugerindo que, além dos fatores de risco ambientais tradicionais, existe também um forte componente genético influenciando o seu desenvolvimento (9, 17-20).

A herdabilidade da RD é de 25-50% (18, 19, 21). Estudos mostraram uma alta agregação familiar na ocorrência de RD, sendo o risco de desenvolver essa complicação

3 vezes maior em pacientes com DM e história familiar de RD comparado a pacientes sem história familiar dessa complicação (22). De fato, diversos estudos já identificaram vários *loci* de suscetibilidade para desenvolvimento e/ou progressão da RD (16, 18, 22). Alguns genes candidatos para a RD incluem genes relacionados à inflamação, sistema renina-angiotensina-aldosterona, vias associadas ao metabolismo da glicose e estresse oxidativo, disfunção endotelial e angiogênese (22).

1.4 Polimorfismos no gene TGFB1 e a retinopatia

Evidências crescentes indicam que alguns genes e polimorfismos nesses genes podem ter um papel fundamental nos processos envolvidos no desenvolvimento da RD (19, 22). Nesse contexto, polimorfismo de nucleotídeo único (*single nucleotide polymorphisms* – SNPs) em genes que codificam proteínas relacionadas à inflamação e angiogênese, como o fator transformador de crescimento beta 1 (*transforming growth factor beta 1 – TGFB1*), podem desempenhar papéis importantes no desenvolvimento da RD (23-26).

O TGFB1, a isoforma mais abundante do TGFB, é uma citocina pró-inflamatória e pró-fibrótica (8, 9). O TGFB1 parece estar envolvido na patogênese das complicações microvasculares do DM, de forma multifuncional, devido a sua atividade pró-inflamatória e pró-fibrótica, principalmente através da estimulação da proliferação de fibroblastos e síntese da matriz extracelular (10, 11).

O TGFB1 é codificado pelo gene *TGFB1*, o qual está localizado no cromossomo 19q13 e inclui 7 éxons (12). Estudos de associação ampla do genoma (*genome-wide association study* - GWAS) e estudos de genes candidatos sugerem que SNPs no gene *TGFB1* estão associados ao desenvolvimento e progressão da RD (23-27), bem como DRD (28-30) em diferentes populações. O SNP mais estudado no gene *TGFB1* é o

rs1800470 (c.+29 T>C) (24, 25, 31, 32), que causa uma troca de uma leucina (Leu) por uma prolina (Pro) no códon 10 (23). O alelo T do SNP rs1800470 (c. + 29 T>C, Leu10Pro) no gene *TGFB1* foi associado pela primeira vez ao risco de RD proliferativa (PDR) em pacientes tchecos com DM2 (24). Em contraste, dois outros estudos relataram que o alelo C conferiu risco para RD em pacientes britânicos com DM1 (33) e Poloneses com DM2 (25). Em 2014, a meta-análise de Liu e colaboradores (23) relatou a associação entre dois SNPs no gene *TGFB1*, o rs1800470 e o rs1800469 (c.-1347 C>T) com a DR em pacientes com DM2. Para o SNP rs1800470, os resultados dessa meta-análise mostram que o genótipo T/T foi associado à proteção para esta complicação nos modelos de herança alélico [Razão de Chances (RC) = 1,34; Intervalo de Confiança (IC) 95% = 1,03 - 1,73] e recessivo (RC = 1,70; IC 95 % = 1,13 - 2,56). Já para o SNP rs1800469, a meta-análise de Liu incluiu 3 estudos que investigaram a associação entre este SNP e DR; no entanto, nenhuma associação significativa foi encontrada. Beránek e colaboradores (24) relataram que um haplótipo constituído pelos alelos rs1800470 T e rs1800469 C conferiu risco aumentado para PDR. Devido a esses resultados contraditórios, estudos adicionais são necessários para esclarecer se esses SNPs estão associados à RD.

Os estudos disponíveis relativos à associação entre SNPs no gene *TGFB1* e suscetibilidade à RD são limitados e com resultados conflitantes em pacientes DM2. Nenhum estudo investigou a associação entre SNPs no gene *TGFB1* e RD em pacientes com DM1.

2. JUSTIFICATIVA

A RD é uma complicação microvascular do DM que afeta cerca de 40% dos pacientes com DM1 ou DM2 e está associada com elevada morbidade e mortalidade. A RD é desencadeada por uma complexa interação entre fatores de risco ambientais e um forte componente genético. Neste contexto, o *TGFB1* foi sugerido como um gene candidato para o desenvolvimento da RD por estar envolvido em processos importantes na patogênese dessa complicação, como inflamação e fibrose. Alguns estudos mostraram a associação entre os SNPs rs1800469 (c.-1347 C>T) e rs1800470 (c.+29 T>C) no gene *TGFB1* e a RD; entretanto, os resultados ainda são contraditórios e inconclusivos. Portanto, como parte do esforço contínuo para examinar a hipótese de que SNPs no gene *TGFB1* estão associados com a DR, este estudo teve como objetivos:

3. OBJETIVOS

3.1 Objetivo geral

- Investigar a associação entre os SNPs rs1800469 (c.-1347 C>T) e rs1800470 (c.+29 T>C) no gene *TGFB1* e a RD em pacientes com DM1 ou DM2 de uma população do sul do Brasil.

3.2 *Objetivos específicos*

- Comparar a frequência alélicas e genotípicas dos polimorfismos rs1800469 (c.-1347 C>T) e rs1800470 (c.+29 T>C) no gene *TGFB1* entre pacientes com RD e sem RD.

- Comparar as distribuições dos haplótipos construídos pela combinação dos SNPs rs1800469 e rs1800470 no gene *TGFB1* entre pacientes com RD e controles. Além disso, determinar o desequilíbrio de ligação entre esses dois SNPs.

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Artigo Original

The rs1800469 C/C and rs1800470 T/T genotypes of the *TGFB1* gene confer protection against diabetic retinopathy in a Southern Brazilian population

The rs1800469 C/C and rs1800470 T/T genotypes of the *TGFB1* gene confer protection for diabetic retinopathy in a Southern Brazilian population

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Running title: *TGFB1* SNPs and diabetic retinopathy

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Abstract

Introduction: Diabetic retinopathy (DR) is a common microvascular complication of diabetes mellitus (DM). The transforming growth factor beta 1 (TGFB1) is a pro-inflammatory cytokine that plays a key role in angiogenesis and breakdown of the blood-retina barrier, which are important mechanisms involved in DR pathogenesis. Accordingly, single nucleotide polymorphisms (SNPs) in the *TGFB1* gene have been associated with DR; however, results are still contradictory. Therefore, the aim of this study was to investigate whether two common *TGFB1* SNPs are associated with DR.

Materials and Methods: This case-control study included 1,093 patients with DM: 635 patients with DR (cases) and 458 patients without DR and with ≥10 years of DM (controls). Type 2 DM patients comprised 74.0% of the total sample. The *TGFB1* rs1800469 (c.-1347 C>T) and rs1800470 (c.+29 T>C) SNPs were genotyped by real-time PCR.

Results: Frequency of rs1800469 C/C genotype was higher in controls compared to DR cases (19.0% *vs*. 13.1%, P= 0.021). This genotype remained associated with protection for DR, adjusting for gender, DM type, glycemic control, hypertension, estimated glomerular filtration rate, and DM duration (OR= 0.657; 95% CI 0.45-0.96; P= 0.030). The rs1800470 T/T genotype was observed in 25.3% of the control group and 19.2% of the cases (P= 0.032); thus, being also associated with protection for DR (OR= 0.696; 95% CI 0.49-0.97; P= 0.033), adjusting for the same variables described above.

Conclusions: The *TGFB1* rs1800469 and rs1800470 SNPs are associated with protection for DR in DM patients from a Southern Brazilian population.

Introduction

Diabetic retinopathy (DR) is a common chronic microvascular complication of diabetes mellitus (DM) and is the leading cause of visual impairment and loss in working-aged adults (1-3). DR affects approximately 35% of DM patients, being more frequent in type 1 DM (T1DM) than in type 2 DM (T2DM) patients (4). Its prevalence increases with DM duration, with ≅ 86% of T1DM and 52% of T2DM patients showing some degree of DR after 20 years of DM duration (4). Although the risk of DR increases with poor glycemic control, long-term DM, arterial hypertension (AH), dyslipidemia, and body mass index (BMI), available evidence has suggested its development is also influenced by genetic factors (5-7). In this context, chronic hyperglycemia and other risk factors initiate a cascade of biochemical and physiological changes that might lead to microvascular damage and retinal dysfunction related to retinal ischemia, abnormal angiogenesis, and increased vascular permeability due to breakdown of the blood-retina barrier (1, 2).

The transforming growth factor beta 1 (TGFB1) is a pro-fibrotic and pro-inflammatory cytokine that modulates cell proliferation, differentiation, apoptosis, adhesion, and migration of several cell types and induces the production of extracellular matrix (ECM) proteins (8). Due to its key roles on angiogenesis, endothelial proliferation, deposition of ECM, and blood-retina barrier breakdown, *TGFB1* is a candidate gene for susceptibility for DR as well as other chronic diabetic complications, such as diabetic kidney disease (DKD) (9-11). Accordingly, several studies have associated single nucleotide polymorphisms (SNPs) in the *TGFB1* gene with susceptibility for DR and/or DKD (10-17).

The T allele of rs1800470 (c.+29 T>C, Leu10Pro) SNP in the *TGFB1* gene was first associated with risk for proliferative DR (PDR) in Czech T2DM patients (12). In

contrast, two other studies reported the C allele conferred risk for DR in British T1DM (15) and Polish T2DM (13) patients. In 2014, Liu et al. (10) published a meta-analysis including 3 studies that have investigated the association between the rs1800469 (c.-1347 C>T) SNP and DR; however, no significant association was found. Beránek et al. (12) reported a haplotype constituted by both rs1800470 T and rs1800469 C alleles conferred increased risk for PDR. Due to these contradictory results, additional studies are needed to clarify whether these SNPs are associated with DR.

Therefore, as part of the ongoing effort to examine the hypothesis that *TGFB1* SNPs are associated with DR, this study was designed to investigate whether the rs1800469 (c.-1347 C>T) and rs1800470 (c.+29 T>C) SNPs in the *TGFB1* gene are associated with DR in both T1DM and T2DM from a Southern Brazilian population.

Materials and Methods

DM patients, phenotype measurements, and laboratory analyses

This case-control study was designed following STROBE and STREGA guidelines for reporting of genetic association studies (18, 19). The study population comprised a total of 1,093 DM patients: 635 cases with DR and 458 controls without this complication and with a known DM duration of at least 10 years. Of note, of the total sample with DM, 809 (74.0%) patients had T2DM: 77.4% of the control group and 72.3% of the cases with DR. All included patients were recruited from the outpatient clinic at the Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil) between January 2005 and December 2013 (20, 21). The research protocol was approved by the Ethic Committee in Research from Hospital de Clínicas de Porto Alegre, and all subjects provided assent and written informed consent prior to inclusion in the study.

Patients were diagnosed as having DM according to American Diabetes Association guidelines (22). Assessment of DR was performed by an experienced ophthalmologist using fundoscopy through dilated pupils. DR was classified as 'absent DR' (no fundus abnormalities), non-proliferative DR (NPDR, presence of microaneurysms, intraretinal hemorrhages and hard exudates) or proliferative DR (PDR, newly formed blood vessels and/or growth of fibrous tissue into the vitreous cavity). DR classification was done considering the most severely affected eye, according to the Global Diabetic Retinopathy Group scale (23).

A standard questionnaire was used to collect information regarding age, age at DM diagnosis, type and DM duration, and drug treatment. Moreover, all patients underwent complete physical and laboratory evaluations, as previously reported by our group (20, 24, 25). Ethnicity was defined based on self-classification, and patients were categorized in white and non-white subjects. (25). Briefly, serum and plasma samples were taken after 12 h of fasting for laboratory analyses. Glucose levels were determined using the glucose oxidase method. Glycated hemoglobin (HbA1c) levels were measured by different methods and the results were traceable to the Diabetes Control and Complications Trial (DCCT) method by off-line calibration or using a conversion formulae (26). Creatinine was measured by the Jaffé reaction; total plasma cholesterol, HDL cholesterol and triglycerides by enzymatic methods, and urinary albumin excretion (UAE) by immunoturbidimetry (Sera-Pak immuno microalbuminuria, Bayer, Tarrytown, NY, USA) (27). The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (28). Body mass index (BMI) was calculated as weight (kg)/height (meters)^2. Office blood pressure (BP) was measured in the sitting position, on the left arm, after a 5-min rest by a trained

nurse, with a mercury sphygmomanometer. The mean of two measurements taken 1 min apart was used to calculate systolic and diastolic BP.

Genotyping

Total DNA was extracted from peripheral blood samples using a standardized technique. *TGFB1* rs1800469 (c.-1347 C>T; C-509T) and rs1800470 (c.+29 T>C; T869C; Leu10Pro) SNPs were genotyped using TaqMan SNP Genotyping Assays 20X (Thermo Fisher Scientific, Foster City, CA, USA – Assay ID: C___8708473_10 and C___22272997_10, respectively). Real-Time PCR reactions were performed in 384-well plates, in a total 5 µL volume, using 2 ng of DNA, TaqMan Genotyping Master Mix 1X (Thermo Fisher Scientific) and TaqMan Genotyping Assay 1X. PCR reactions were performed in a real-time PCR thermal cycler (ViiA7 Real-Time PCR System; Thermo Fisher Scientific) with the following protocol: heating for 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s and 62 °C for 90 s.

Haplotype distributions and linkage disequilibrium analysis (LD)

The haplotypes constructed by the combination of the rs1800469 and rs1800470 *TGFB1* SNPs and their frequencies were inferred using the Phase 2.1 program (Seattle, WA, USA), which implements a Bayesian statistical method (29). We also used this program to compare the distributions of different *TGFB1* haplotypes between DR patients and control subjects through permutation analyses of 10,000 random replicates (29). Linkage disequilibrium (LD) between these two SNPs was calculated using Lewontin´s D´|D´| and r^2 measurements (30).

Statistical analyses

Allele frequencies were determined by gene counting, and departures from the Hardy-Weinberg Equilibrium (HWE) were verified using the χ^2 test. Allele and genotype frequencies were compared between groups of subjects using χ^2 tests. Additionally, genotypes were compared between case and control groups under additive, recessive, and dominant inheritance models, categorized as suggested by a previous publication (31).

Clinical and laboratory characteristics were compared between case and control patients and between group of patients categorized according to the different genotypes of the two *TGFB1* SNPs. Variables with normal distribution are shown as mean ± SD and were compared between groups using Student's *t* test. Variables with skewed distribution are shown as median (25^{th} – 75^{th} percentile values) and were compared between groups using Mann-Whitney tests. Categorical data are shown as percentages and were compared between groups using χ^2 tests.

The magnitude of association between *TGFB1* SNPs and DR was estimated using odds ratio (OR) with 95% confidence interval (CI). Multivariate logistic regression analyses were done to evaluate the independent association of each individual *TGFB1* SNP or haplotypes with DR, adjusting for possible confounding factors. Statistical analyses were performed using the SPSS 18.0 software (SPSS, Chicago, IL), and P-*values* < 0.05 were considered significant. Sample size was calculated using the OpenEpi site (http://www.openepi.com) and the minor allele frequencies and ORs observed in the previous studies regarding associations of the rs1800469 and rs1800470 SNPs with DR (12, 32, 33).

Results

Sample description

The clinical and laboratorial characteristics of DR cases and controls are shown in **Table 1.** Males comprised 53.4% of the case group and 39.5% of the control group (P < 0.0001), and the mean age was 60.1 ± 16.2 years in cases and 61.1 ± 19.0 in controls (P = 0.379). The median DM duration was higher in cases compared to controls [23.0 (17.0 – 28.0) *vs*. 19.0 (14.0 – 26.0); P < 0.001]. As expected, median levels of HbA1c, total cholesterol, LDL, and UAE as well as prevalence of AH were significantly higher in cases compared to control subjects. Moreover, eGFR values were lower in DR cases *vs*. controls (P < 0.0001). Ethnic distribution, BMI, HDL cholesterol, and triglycerides levels did not differ significantly between groups (**Table 1**).

Distributions of the TGFB1 rs1800469 and rs1800470 SNPs in case and control groups

Genotype frequencies of the rs1800469 (c.-1347 C>T) and rs1800470 (c.+29 T>C) SNPs in the *TGFB1* gene are in HWE in the case group (P = 0.957 and P = 0.819, respectively). Frequencies of rs1800469 C/C and rs1800470 T/T genotypes did not differ significantly between white and non-white subjects (rs1800469 C/C: 15.5 *vs*. 16.3%, respectively; P = 0.871; rs1800470 T/T: 21.0 *vs*. 26.6%, P= 0.125). Moreover, frequencies of these genotypes did not differ between T1DM and T2DM patients (rs1800469 C/C: 15.5 *vs*. 15.5%, respectively; P > 0.999; rs1800470 T/T: 20.2 *vs*. 22.4%; P= 0.501). Therefore, white and non-white subjects as well as T1DM and T2DM patients were further analyzed together.

Table 2 shows genotype and allele frequencies of the rs1800469 and rs1800470 SNPs in patients with DM (T1DM + T2DM) categorized into DR cases and non-DR

controls. The C/C genotype of the rs1800469 SNP was more frequent in controls compared to cases with DR (19.0% *vs.* 13.1%, P = 0.021). This genotype also conferred protection for DR when considering recessive (P = 0.010) and additive (P = 0.042) inheritance models. However, after adjustment for DM duration, type of DM, gender, AH, HbA1c, and eGFR levels, the C/C genotype only remained associated with protection for DR in the recessive model (OR = 0.657; 95% CI 0.450 – 0.961; P = 0.030). Regarding the rs1800470 SNP, the frequency of T/T genotype was higher in controls compared to cases with DR (25.3% *vs.* 19.2%, P = 0.032). In the same way of rs1800469 SNP, the rs1800470 T/T genotype was also associated with protection for DR in the recessive model, independent of the variables described above (OR = 0.696; 95% CI 0.499 - 0.972; P = 0.033).

Haplotype distributions and linkage disequilibrium

Frequencies of haplotypes produced by the combination of *TFGB1* rs1800469 and rs1800470 SNPs in cases and controls are listed in **Table 3**. Four haplotypes were inferred in both samples and their distributions were not significantly different between case and control groups (P = 0.564). It is noteworthy that the two SNPs of interest are in partial LD in our population ($|D'| = 0.679$ and $r^2 = 0.335$).

Next, we analyzed haplotype frequencies according to the number of minor alleles in haplotypes: a) subjects carrying 0, 1 or 2 minor alleles of rs1800469 and rs1800470 SNPs, and b) subjects carrying 3 or 4 minor alleles (**Figure 1)**. Frequency of 3 or 4 minor alleles of the two analyzed SNPs was lower in DR cases compared to controls (17.6% *vs*. 23.4; P = 0.025; **Figure 1**). Moreover, after adjustment for DM duration, type of DM, gender, AH, HbA1c, and eGFR levels, the presence of ≥3 minor alleles remained independently associated with DR (OR = 0.696; 95% CI 0.490 - 0.987; P = 0.042). This

OR is similar to those observed for each SNP analyzed individually; hence, their effects on DR susceptibility do not seem to be additive.

Discussion

TGFB1 has been recognized as a key factor in the pathogenesis of chronic microvascular complications of DM (10, 11). Accordingly, SNPs in the *TGFB1* gene have been shown to be involved in the susceptibility for DKD due to the role of this gene on tissue fibrosis processes (11, 13, 16, 17, 34). Moreover, *TGFB1* SNPs seem to be associated with susceptibility for DR (10, 14, 33); however, available data is less convincing. Thus, here, we investigated the association of the *TGFB1* rs1800469 and rs1800470 SNPs with DR in T1DM and T2DM patients from a Southern Brazilian population. Our results show both SNPs are associated with protection for DR.

The rs1800469 SNP (c.-1347 C>T; also known as C-509T) is located in the first negative regulatory region of the upstream promoter of the *TGFB1* gene, and the T allele seems to increase both *TGFB1* gene expression and circulating plasma levels in humans (35-37). Interestingly, TGFB1 concentration was higher in T/T homozygous than heterozygous, suggesting a dose-response effect (35). Accordingly, *in vitro* studies using TGFB1 promoter-luciferase reporter plasmids confirmed the T allele increases relative luciferase activity compared to the C allele (36, 37). Increased TGFB1 plasma levels have been linked to the progression of renal disease due to increased ECM production, which leads to glomerulosclerosis and tubulointerstitial fibrosis (8). In the context of DR pathogenesis, augmented TGFB1 circulating levels might increase angiogenesis and endothelial proliferation as well as ECM production and blood-retina barrier breakdown, contributing to the development and progression of DR (9-11). Hence, the rs1800469 T

allele might led to worst outcomes related to the pathogenesis of microvascular diabetic complications.

In agreement with a functional impact of the rs1800469 SNP on TGFB1 levels, here, we found that the C/C genotype was associated with protection for DR. In contrast with our data, the meta-analysis published by Liu et al. (10) did not show any association between this SNP and DR. These discordant findings may be explained by differences in ethnicities since the studies included in the meta-analysis comprised T2DM patients from Czech, Poland, and India populations. Moreover, the meta-analysis included only 3 studies, comprising 521 T2DM patients with DR and 580 controls; thus, there is the possibility of lack of statistical power. Furthermore, Raina et al. (38) showed the T/T genotype of rs1800469 SNP was associated with 5.5-fold risk for end-stage renal disease (ESRD) in T2DM patients from North India. However, other studies were not able to find any association between this SNP and DKD (39-41). Hence, the association of the rs1800469 SNP with diabetic chronic complications should be confirmed in other populations.

The rs1800470 (c.+29 T>C; T869C) SNP causes the replacement of a Leucine (Leu) for a Proline (Pro) in codon 10 (Leu10Pro) of exon 1, which encodes the N-terminal signal peptide of TGFB1 (36). Although it has been speculated that modifications in amino acid composition of the signal peptide will affect its polarity and so lead to different rates of protein export (42), both T and C alleles encode apolar amino acids (36), suggesting they have similar effects on protein function. However, an *in vitro* study showed the C (Pro) allele caused increased TGFB1 secretion compared to the T (Leu) allele (43). Moreover, serum TGFB1 concentration seems to be higher in subjects with the C/C genotype compared to T allele carriers (36, 44, 45). Hence, even though the functional effect of this SNP on *TGFB1* expression is still not clear, it is suggested that

the C allele increases TGFB1 levels (37), which might increase angiogenesis, ECM production, and blood-retina breakdown and, thus, predispose to DR.

Accordingly, we found an association between the rs1800470 T/T genotype and protection for DR. Buraczynska et al. (13) reported the C allele of this SNP conferred risk for DR (OR = 2.22; 95% CI 1.64 – 2.99) in T2DM patients from Poland, which is in agreement with our results. Bazzaz et al. (15) also reported the C allele frequency was increased in T1DM patients with DR compared to controls, although the difference did not reach statistical significance. In contrast, another study showed the T allele was associated with risk for PDR (OR = 2.89; 95% CI 1.6 – 5.1) in T2DM patients from Czech Republic. Moreover, a small study comprising Brazilian T2DM patients (66 cases with DR and 36 controls) was not able to find any association between the rs1800470 SNP and DR (32).

In 2011, Jia et al. (11) published a meta-analysis of 9 studies (1776 cases or 1740 controls) that investigated the association between the rs1800470 SNP and DKD in T1DM or T2DM patients. In agreement with a protective role of T allele, the presence of the C allele was associated with risk for DKD (OR = 1.25, 95% CI 1.05 – 1.48). A recent meta-analysis of 8 Chinese studies (1018 cases with DKD and 941 controls) showed the T/T genotype conferred protection for DKD (OR = 0.55, 95% CI 0.31 – 0.96) in T2DM patients (17). Thus, available evidence suggests that the T allele is associated with decreased TGFB1 levels; thus, being protective for the development of diabetic complications.

This study has a few limitations. First, even though ethnic distributions (white and non-white subjects) were similar between case and control groups, we cannot fully exclude the possibility of population stratification bias when analyzing our samples. Second, although frequencies of the rs1800469 and rs1800470 SNPs were similar

between T1DM and T2DM patients and we have included type of DM in the logistic regression analyses, the sample size precluded any further stratification analysis by DM type. Thus, we cannot exclude the possibility that the strength of association of these SNPs with DR might be different between DM types. Last, this is the first study that demonstrated an associated between the *TGFB1* rs1800469 and rs1800470 SNPs and susceptibility for DR in a T1DM population. However, we did not perform the replication of the observed association in another Brazilian sample.

In conclusion, this study suggests the *TGFB1* rs1800469 and rs1800470 SNPs confer protection for DR in T1DM and T2DM patients from a Southern Brazilian population. Additional studies are needed to confirm the effect possibly played by SNPs in the *TGFB1* gene in the pathogenesis of DR.

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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 DM patients without and with DR.

Characteristics	**Controls (*n* = 458)**	**Cases with DR (*n* = 635)**	**P ***
Age (years)	61.1 ± 19.0	60.1 ± 16.2	0.379
Gender (% males)	39.5	53.4	<0.0001
Ethnicity (% non-white)	16.6	16.6	>0.999
T2DM patients (%)	77.4	72.3	0.061
DM duration (years)	19 (14.0 – 26.0)	23 (17.0 – 28.0)	<0.0001
BMI (kg/m²)	28.0 ± 5.1	27.6 ± 5.0	0.175
HbA1c (%)	7.3 (6.4 – 8.8)	7.7 (6.7 – 9.2)	0.001
Cholesterol total (mg/dL)	193 (162.0 – 230)	197 (165 – 227)	0.048
HDL cholesterol (mg/dL)	47 (38.0 – 56.0)	48 (39.0 – 58.0)	0.829
LDL cholesterol (mg/dL)	110.6 (86.7 – 144.0)	116.2 (89.3 – 145.5)	0.032
Triglycerides (mg/dL)	133.0 (79 – 201.0)	123.5 (82.0 – 184.8)	0.756
Arterial hypertension (%)	72.4	82.1	<0.0001
eGFR (ml/min per 1.73 m^2)	83.0 (62.8 – 98.0)	66.0 (38.0 – 92.0)	<0.0001
UAE (mg/g)	7.9 (3.9 – 22.2)	37.7 (7.6 – 315.3)	<0.0001

Variables are shown as mean ± SD, median (25th-75th percentiles) or %. *P-*values* were computed using Student's *t*, Mann-Whitney, or χ^2 tests, as appropriate. BMI: body mass index; DR: diabetic retinopathy; eGFR: estimated glomerular filtration rate; HbA1c: glycated hemoglobin; T2DM: type 2 diabetes mellitus; UAE: urinary albumin excretion.

Table 2. Genotype and allele frequencies of *TGFB1* rs1800469 and rs1800470 SNPs in DM patients without and with DR.

rs1800469	**Controls (*n* = 453)**	**Cases with DR (*n* = 635)**	**Unadjusted P***	**Adjusted OR (95% CI) / P†**
Genotype				
T/T	182 (40.2)	258 (40.6)	0.021	1
C/T	185 (40.8)	294 (46.3)		1.119 (0.865-1.610) / 0.303
C/C	86 (19.0)	83 (13.1)		0.724 (0.475-1.105) / 0.134
Allele				
T	0.61 (549)	0.64 (810)	0.143	
C	0.39 (357)	0.36 (460)		
Recessive model				
T/T + C/T	367 (81.0)	552 (86.9)	0.010	1
C/C	86 (19.0)	83 (13.1)		0.657(0.450-0.961) / 0.030
Additive model				
T/T	182 (67.9)	258 (75.7)	0.042	1
C/C	86 (32.1)	83 (24.3)		0.730 (0.476-1.119) / 0.149
Dominant model				

T/T	182 (40.2)	258 (40.6)	0.930	1
C/T + C/C	271 (59.8)	377 (59.4)		1.033 (0.772-1.381) / 0.828
rs1800470	**Controls (*n* = 458)**	**Cases with DR (*n* = 633)**	**Unadjusted P***	**Adjusted OR (95% CI) / P†**
Genotype				
C/C	144 (31.4)	196 (31.0)	0.032	1
C/T	198 (43.2)	315 (49.8)		1.192 (0.877-1.620) / 0.262
T/T	116 (25.3)	122 (19.2)		0.718 (0.477-1.081) / 0.113
Allele				
C	0.53 (486)	0.56 (707)	0.212	
T	0.47 (430)	0.44 (559)		
Recessive model				
C/C + C/T	342 (74.7)	511 (80.7)	0.021	1
T/T	116 (25.3)	122 (19.3)		0.696 (0.499-0.972) / 0.033
Additive model				
C/C	144 (55.4)	196 (61.6)	0.152	1
T/T	116 (44.6)	122 (38.4)		0.834 (0.566-1.228) / 0.357
Dominant model				

C/C	144 (31.4)	196 (31.0)	0.919	1
C/T + T/T	314 (68.6)	437 (69.0)		1.144 (0.849-1.541) / 0.377

Data are shown as number (%) or proportion. *P-*values* were calculated using Chi-square tests. † P-*value* and OR (95% CI) obtained using logistic regression analyses adjusting for gender, type DM, HbA1c, arterial hypertension, eGFR and DM duration.

Table 3. Haplotypes of the *TGFB1* SNPs in DM patients without and with DR.

Haplotypes	**Controls (*n* = 940)**	**Cases with DR (*n* = 1,236)**	**P ***
TT	0.086	0.088	
TC	0.522	0.548	0.564
CT	0.379	0.355	
CC	0.013	0.009	

Data are presented as proportion. n = number of chromosomes. The first letter of the haplotypes refers to the rs1800470 polymorphism and the second to the rs1800469 polymorphism. *Permutation P-*value* was computed for comparisons of haplotype frequencies between groups.

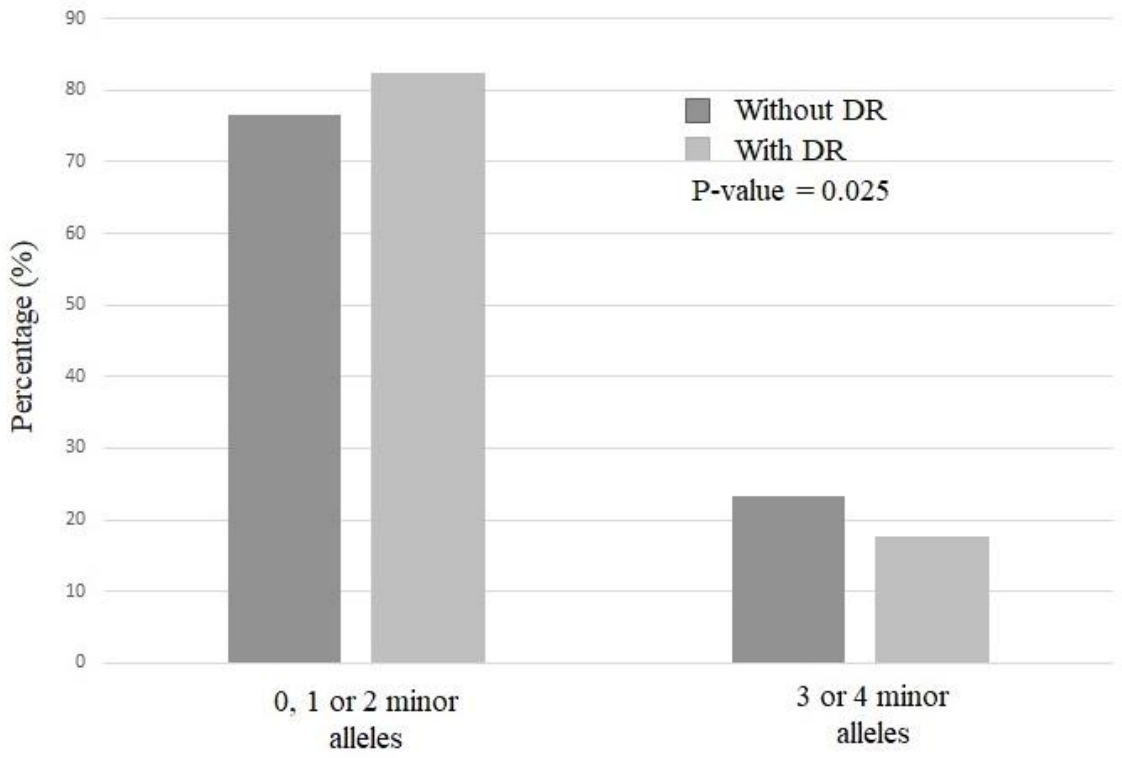

Figure 1. DR patients and non-DR subjects broken down by the number of risk alleles of the analyzed polymorphisms in the estimated haplotypes. Data are presented as percentage. P= 0.025 obtained using χ^2-test.

CONCLUSÕES GERAIS

Os resultados do presente estudo indicam que, isoladamente, os polimorfismos rs1800469 e rs1800470 no gene *TGFB1* conferem proteção para RD em pacientes com DM1 ou DM2 de uma população do sul do Brasil. Essa associação manteve-se mesmo após o ajuste para idade, tipo de DM, HbA1c, hipertensão, taxa de filtração glomerular e duração do DM. Além disso, a frequência de 3 ou 4 alelos menores dos dois SNPs analisados foi menor nos casos de RD em comparação com os controles. Interessantemente, a presença de ≥3 alelos menores permaneceu independentemente associada com RD após o ajuste para as mesmas variáveis descritas acima. Até onde sabemos, este é o primeiro relato de um estudo de polimorfismo no gene *TGFB1* sugerindo proteção para RD em pacientes com DM1. No entanto, mais estudos com amostras maiores são necessários para confirmar o efeito possivelmente desempenhado por polimorfismos no gene TGFB1 na patogênese da RD.

OUTRAS PRODUÇÕES BIBLIOGRÁFICAS NO PERÍODO DO MESTRADO

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

Assmann, Taís S; Recamonde-Mendoza, Mariana; Costa, Aline R; Puñales, Márcia; Tschiedel, Balduíno; Canani, Luís H; Bauer, Andrea C; Crispim, Daisy. Circulating miRNAs in diabetic kidney disease: case-control study and in silico analyses. Acta Diabetologica, v. 56, p. 55-65, 2019.

Dieter, Cristine; Assmann, Taís Silveira; Costa, Aline Rodrigues; Canani, Luís Henrique; De Souza, Bianca Marmontel; Bauer, Andrea Carla; Crispim, Daisy. MiR-30e5p and MiR-15a-5p Expressions in Plasma and Urine of Type 1 Diabetic Patients With Diabetic Kidney Disease. Frontiers in Genetics, v. 10, p. 1, 2019.

Manuscrito desenvolvido no período do mestrado e já aceito para publicação:

Dieter, Cristine; Lemos, Natália E; Corrêa, Nathalia; Costa, Aline R; Canani, Luís H; Crispim, Daisy; Bauer, Andrea C. The rs2442598 polymorphism in ANGPT-2 gene is associated with risk for diabetic retinopathy in patients with type 1 diabetes mellitus from a Brazilian population. Archives of Endocrinology and Metabolism.