Universidade Federal do Rio Grande do Sul
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Papel do VEGF e seus Receptores na Retinopatia Diabética: estudo de expressão gênica e de associação.

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Tese apresentada como requisito para a obtenção do título de Doutor em Medicina junto a Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Ciências Médicas: Endocrinologia.

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Dedico esta tese ao meu pai Enio Vailati (*in memorian*) por ser o entusiasta silencioso do mundo que se abria diante das minhas próprias escolhas.

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FIGURAS E TABELAS

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ABREVIATURAS E SIGLAS

χ^2: Chi-square

AMD: age-related macular degeneration

ANOVA: analysis of variance

AU: arbitrary units

BMI: body mass index

BP: blood pressure

CATT: Comparison of AMD Treatments Trials

cDNA: complementary desoxiribonucleic acid

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico

DM: diabetes mellitus

DME: diabetic macular edema

DNA: desoxiribonucleic acid

DR: diabetic retinopathy

EMD: edema macular diabético

FIPE: Fundo de Incentivo ao Pesquisador

FLK1-: fetal liver kinase 1

FLT-1: fms-like tyrosine kinase 1

FLT-4: fms-like tyrosine kinase 4

HbA1c: glycated hemoglobin

HCPA: Hospital de Clínicas de Porto Alegre

HSCM: Hospital Santa Casa de Misericórdia

HWE: Hardy-Weinberg equilibrium

IDF: International Diabetes Federation

IVAN: Alternative Treatments to Inhibit VEGF in Patients with Age-Related Choroidal Neovascularisation

KDR: kinase domain region

LD: linkage disequilibrium

Log: logarithm

mRNA: messenger ribonucleic acid

PCR: polymerase chain reaction

PDR: proliferative diabetic retinopathy

PlGF: placental growth factor

RD: Retinopatia Diabética

RDP: Retinopatia Diabética Proliferativa

RNA: ribonucleic acid

RS: Rio Grande do Sul

RT- qPCR: reverse transcription-quantitative polymerase chain reaction

RT-PCR: real time polymerase chain reaction

SD: standard deviation

SE: standard error

SNP: single nucleotide polymorphism

SPSS: Statistical Package for the Social Sciences

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

UA: unidades arbitrárias

UFRGS: Universidade Federal do Rio Grande do Sul

VEGF: vascular endothelial growth factor / fator de crescimento do endotélio vascular

VEGFR1: vascular endothelial growth factor receptor 1

VEGFR2: vascular endothelial growth factor receptor 2

VEGFR3: vascular endothelial growth factor receptor 3

VPF: vascular permeability factor / fator de permeabilidade vascular

LISTA DE SÍMBOLOS

μg/μl: micrograma por microlitro

μg: micrograma

μL: microlitro

kDa: kilodaltons

Kg/m^2: kilograma por metro quadrado

mg/dL: miligrama por decilitro

mg: miligrama

mL: mililitro

mmHg: milímetros de mercúrio

ng: nanograma

SUMÁRIO

APRESENTAÇÃO DA TESE

A presente tese apresenta uma introdução contextualizando epidemiologicamente a retinopatia diabética (RD) e a base fisiopatológica da neovascularização retiniana na evolução para RD proliferativa. A introdução se segue de dois artigos na língua inglesa em formato de artigos originais. O artigo 1, publicado na revista *Investigative Ophthalmology & Visual Science*, sobre a associação do polimorfismo do *VEFGA* -634G/C e a expressão deste gene em retina humana de doadores de córnea não portadores de diabetes mellitus (DM). O artigo 2, ainda não publicado, é um estudo de associação entre polimorfismos dos genes *FLT-1* e *KDR* e a RD proliferativa em pacientes com DM tipo 2. Posteriormente, apresentamos nossas considerações finais, procurando demonstrar a importância dos estudos genéticos na investigação e compreensão da RD.

Desejo uma boa leitura.

Fabiana Vailati

INTRODUÇÃO

Diabetes mellitus (DM) é uma doença que causa importante perda da qualidade de vida. Em 2014, a *World Health Organization* estimava 422 milhões de pessoas portadoras de DM no mundo, sendo uma prevalência de 8,5% da população adulta[1]. Estima-se que essa prevalência aumente para 642 milhões em 2040[2]. O número de indivíduos com DM tipo 2 (DMT2) vem crescendo em todos os países. No Brasil, a *International Diabetes Federation* (IDF)[2] projeta um aumento na prevalência de DM de 14,3 milhões em 2015 para 23,3 millhões em 2040. Ainda de acordo com o IDF, o Brasil é o quarto país com o maior número de portadores de DM[2].

A retinopatia diabética (RD) é uma complicação frequente da DM. Aproximadamente 30-45% dos portadores de DM terão algum grau de RD após 15 anos de doença, sendo que 10% terão perda visual importante e 2% serão cegos [1]. Uma metanálise recente de 35 estudos publicados entre 1980 e 2008 reuniu dados de 22.896 pessoas com DM tipo 1 (DMT1) e DMT2 e demonstrou uma prevalência de 34,6% de qualquer grau de RD, 10,2% de risco de perda visual, 6,96% de RD proliferativa (RDP) e 6,81% de edema macular diabético (EMD)[3]. A severidade da RD é influenciada pelo tempo de DM, hemoglobina glicada (Hb1Ac) e nível de pressão arterial [3].

A RD é a mais comum complicação vascular da DMT1 e DMT2 sendo caracterizada pelo aumento da permabilidade vascular, anormalidades hemodinâmicas, isquemia retiniana e neovascularização. A neovascularização é o fator que define a RDP.

Na década de 40, começaram as primeiras publicações sobre a investigação de angiogênese normal e patológica[4], mas Judah Folkman, na década de 70, foi o precursor das pesquisas da angiogênese em tecido tumoral tendo identificado o que chamou de fator de angiogênese tumoral[5]. Em 1989, duas publicações apresentaram um dos principais fatores envolvidos na neovascularização e que são estudados até hoje. Leung *et al*[6] relataram a identificação de um fator mitógeno endotelial a partir de células foliculares pituitárias, chamado fator de crescimento do endotélio vascular (VEGF). Keck *et al*[7] descreveram uma citocina capaz de induzir o aumento da permeabilidade vascular, nomeando-a fator de permeabilidade vascular (VPF). Mais tarde, foi demonstrado que os dois fatores tratavam da mesma citocina então denominada VEGF.

O subtipo VEGFA é a mais potente e multifuncional citocina estudada até hoje que age no endotélio vascular e, desde sua identificação, seu papel na angiogênese fisiológica e patológica tem

sido investigado. Esta descoberta teve importante influência na compreensão e tratamento das doenças neovasculares, incluindo doenças retinianas como a RD.

A angiogênese é o desenvolvimento de novos vasos sanguíneos e o VEGFA é um fator determinante como mediador da angiogênese fisiológica (crescimento ósseo, funções reprodutivas e embriogênese) e patológica (psoríase, crescimento tumoral, doenças neovasculares da retina) tendo em vista que promove o crescimento das células endoteliais. O VEGFA também é considerado como fator de sobrevivência endotelial [8,9] e inibidor da apoptose[9].

O VEGFA, especialmente a isoforma VEGFA 165, é considerado o principal fator envolvido na neovascularização na RDP. Embora sendo importante fator de doenças neovasculares retinianas mediadas por isquemia, o VEGFA é expresso em retinas normais e tem papel endógeno na preservação da retina em adultos[10,11]. O VEGFA é produzido predominantemente por uma variedade de células endoteliais, hematopoiéticas e estromais em resposta ao processo isquêmico e ao estímulo de outros fatores de crescimento como *transforming growth factor beta*, interleucinas e *platelet-derived growth factors*[12]. São descritos três receptores de tirosina kinase, FLT-1 (*fms-like tyrosine kinase-1*, VEGFR-1), KDR/FLK-1 (*kinase domain region/fetal liver kinase-1*, VEGFR-2) e FLT-4 (*fms-like tyrosine kinase-4*, VEGFR-3), os quais apresentam sete domínios *imunoglobulin-like* no domínio extracelular, uma única região transmembrana e uma sequência comum de tirosina kinase interrompida por um domínio *kinase-insert*[13]. O VEGFA se liga ao FLT-1 e ao KDR presente principalmente no endotélio vascular. Mesmo sendo um receptor tirosina kinase, o FLT-4 é restrito ao endotélio linfático, não sendo um receptor de VEGFA, unindo-se ao VEGFC e VEGFD[14]. (Figura 1)

Ao longo dos últimos 20 anos, muitas pesquisas têm sido realizadas considerando o VEGFA como fator determinante na regulação da angiogênese normal e patológica, mas o interesse pela interação entre o VEGFA e seus receptores tem crescido em estudos mais recentes, encorajados pelos avanços nas pesquisas genéticas.

Dessa forma, o presente estudo tem por objetivo avaliar polimorfismos nos genes que codificam o VEGFA e seus receptores, bem como investigar se esses polimorfismos podem influenciar a suscetibilidade a doenças neovasculares de retina.

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ARTIGO 1

The C Allele of -634G/C Polymorphism in the *VEGFA G*ene is Associated with Increased *VEGFA* Gene Expression in Human Retinal Tissue.

ORIGINAL ARTICLE

The C Allele of -634G/C Polymorphism in the *VEGFA* Gene Is Associated with Increased *VEGFA* Gene Expression in Human Retinal Tissue

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RESUMO

OBJETIVO: O objetivo deste estudo foi analisar o efeito do polimorfismo -634G/C na expressão do gene do fator de crescimento do endotélio vascular A (VEGFA) em retina humana.

MÉTODOS: Estudo transversal realizado em 190 doadores de córnea analisando o polimorfismo -634G/C (rs2010963) e a expressão gênica do *VEGFA* na retina. Indivíduos com diabetes mellitus ou doença retiniana não foram incluídos neste estudo.

RESULTADOS: 53 amostras de retina foram analisadas (18 GG, 17 GC e 18 CC). A expressão do gene *VEGFA* foi medida através da reação em cadeia da polimerase via transcriptase reversa. Doadores tinham entre 13 e 79 anos (média 55.8 ± 15.8 anos) e 49.1% (n = 26) eram do sexo masculino. Indivíduos com o alelo C [genótipos CC (5.15 ± 4.47 unidades arbitrárias) or GC (3.72 ± 3.25 unidades arbitrárias)] apresentaram maior expressão do gene *VEGFA* quando comparados a individuos com genótipo GG (2.62 ± 2.56 unidades arbitrárias, $P = 0.045$).

CONCLUSÃO: Este estudo demonstra que a presença do alelo C do polimorfismo -634G/C está associado a maior expressão do *VEGFA* em retina humana. (Invest Ophthalmol Vis Sci. 2012;53:6411–6415)

PALAVRAS-CHAVE: fator de crescimento do endotélio vascular, retina, polimorfismo, expressão gênica.

ABSTRACT

PURPOSE: The purpose of this study was to evaluate the effect of the -634G/C polymorphism on vascular endothelial growth factor A (VEGFA) gene expression in the human retina.

METHODS: A cross-sectional study was performed to analyze the frequency of the -634G/C polymorphism (rs2010963) in 190 cadaveric cornea donors. Individuals with diabetes mellitus (DM), retinal disease, or both were not included in this study.

RESULTS: A total of 53 retinal samples were analyzed (18 GG, 17 GC, and 18 CC). *VEGFA* gene expression was measured by reverse transcription–quantitative polymerase chain reaction (RT-qPCR). Donor age ranged from 13 to 79 years (mean, 55.8 ± 15.8 years), and 49.1% (n = 26) were male. Subjects carrying the C allele (CC or GC genotypes, 5.15 ± 4.47 arbitrary units [AU] or 3.72 ± 3.25 AU, respectively) presented higher *VEGFA* expression than subjects with the GG genotype (2.62 ± 2.56 AU; $P = 0.045$).

CONCLUSIONS: This study indicates that the C allele of the -634G/C polymorphism is associated with higher *VEGFA* expression in the human retina. (*Invest Ophthalmol Vis Sci.* 2012;53:6411–6415)

KEYWORDS: vascular endothelial growth factor, retina, polymorphism, gene expression.

INTRODUCTION

Vasculogenesis, or the spontaneous formation of blood vessels during embryogenesis, as well as angiogenesis, a complex sequence of events leading to the formation of new blood vessels from preexisting vessels,[1–6] plays a fundamental role in specific physiological processes. However, vascular turnover is extremely low, and angiogenesis rarely occurs in healthy adults. In fact, the etiology and pathogenesis of some diseases are determined by a persistent angiogenic response, as in diabetic retinopathy (DR).[7,8]

VEGF belongs to a group of dimeric glycoproteins, which includes the placental growth factor (PlGF), VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, and VEGFF.[6,9] VEGFA is a potent, multifunctional cytokine that acts on the endothelium.[5,10]

The gene that encodes VEGFA in humans is organized into eight exons and seven introns located in chromosome 6p21.3.[11] The alternative splicing process results in four main isoforms containing 121, 165, 189, and 206 amino acids, respectively: VEGFA 121, VEGFA 165, VEGFA 189, and VEGFA 206.[5,12–14] VEGF165, the main VEGFA isoform,[5,6] is a 45-kDa homodimeric glycoprotein, which acts directly and selectively through receptors FLT-1 (fms-like tyrosine kinase-1, VEGFR-1) and KDR/FLK-1 (kinase domain region/fetal liver kinase-1, VEGFR-2) expressed in the vascular endothelium (Figure 1).[5] It causes increased vascular permeability, promotes angiogenesis, and stimulates endothelial cell proliferation and migration in a variety of physiological and pathologic processes.[5,13]

VEGFA promotes the growth of vascular endothelial cells from arteries, veins, and lymphatic vessels, prevents endothelial apoptosis induced by nutrient deprivation, and is considered an endothelial survival factor.[10,15] Although it is a major mediator of ischemia-mediated ocular neovascularization, *VEGFA* is expressed in normal retinas and has an endogenous role in retinal preservation in adults.[16,17]

In a previous study from our group, the CC genotype of the -634G/C polymorphism (rs2010963) in the *VEGFA* gene was associated with PDR in type 2 DM (T2DM) patients.[18] The C allele was observed more often in patients with DR[19–22] or with diabetic macular edema (DME)[20] in some studies, but not in others.[23,24] Furthermore, serum and vitreous levels of VEGFA were elevated in CC genotype carriers, independent of the presence of DR.[25]

We therefore hypothesized that the -634C allele is associated with risk of PDR owing to an increased *VEGFA* gene expression in C allele carriers. Thus, the purpose of this study was to evaluate the effect of -634G/C polymorphism on *VEGFA* gene expression in retinas from cadaveric cornea donors.

SUBJECTS AND METHODS

Samples

One hundred and ninety eyes were obtained from cornea donors identified through the *Central de Transplantes do Rio Grande do Sul* (a Brazilian organization that regulates organ donations in Rio Grande do Sul [RS], Brazil). The collection took place at two hospitals in Porto Alegre (RS), *Hospital de Clínicas de Porto Alegre* (HCPA), and *Hospital Santa Casa de Misericordia* (HSCM), from May 2009 to May 2010. A standard questionnaire was used to collect information from medical records about age, sex, presence of arterial hypertension, and DM, smoking status, occurrence of other diseases, and cause of death. Individuals with DM, ocular/retinal disease, or both, and those without sufficient information were not included in the study.

The CC genotype, minor genotype, for the -634G/C polymorphism was present in 18 subjects. Also, the eye tissue of 18 subjects with GG and 17 with GC was included in the *VEGFA* gene expression study.

After enucleation and separation of the corneas for donation, retinas were visually separated from the remaining intraocular structures, immediately snap-frozen in liquid nitrogen, and then conserved at -80ºC until analyses. The mean duration ± standard deviation (SD) from death of the donor to dissection and conservation of the retinal tissue was 5.54 ± 2.2 hours. A 10 mL sample of peripheral blood was also collected from each donor for DNA extraction and genotyping of the *VEGFA* -634G/C polymorphism. Following genotyping, a subset of subjects were divided into groups according to the presence of the different genotypes of the analyzed polymorphism (18 CC homozygous, 17 heterozygous, and 18 GG homozygous), and the *VEGFA* gene expression in retinal tissue from this group was measured as described below.

The relatives of the donors signed a Letter of Informed Consent authorizing the use of the material that would otherwise have been discarded. The project was approved by the Committee of Ethics in Research at HCPA and HSCM. All subjects were treated in accordance with the Declaration of Helsinki.

Genotyping of VEGFA -634G/C (rs2010963) Polymorphism

DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. Genotyping of the -634G/C polymorphism located in the promoter region of the *VEGFA* gene was performed using primers and probes contained in the Human Custom TaqMan Genotyping Assay 40X (Life Technologies – Applied Biosystems – ABI, Foster City, CA). The following sequences of primers and probes were used: forward primer 5'-GAGAGAAGTCGAGGAAGAGAGAGA-3', reverse primer 5' -CCCAAAAGCAGGTCACTCACTT-3', VIC-5'-CCTGTCGCTTTCGC-3', and FAM-5'-CCTGTCCCTTTCGC-3'. Real-time PCR (RT-PCR) reactions were performed on 96-well plates, with a total volume of 5 µL containing 2 ng genomic DNA, TaqMan Genotyping Master Mix 1X (ABI), and Custom TaqMan Genotyping Assay 1X (ABI). RT-PCR experiments were performed in a 7500 Fast Real Time PCR System (ABI). The thermocycling condition was as follows: an initial cycle of 95ºC for 10 minutes, followed by 45 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Fluorescence data of each plate were analyzed by the System Sequence Detection Version 2.1 program (ABI). All amplification reactions were performed in duplicate, with an error rate of 0.01% based on the results of the duplicates.

RNA Isolation

Retinal tissues (250 mg) were homogenized in phenol-guanidine isothiocyanate (Invitrogen Life Technologies, Foster City, CA). RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000g) at 4ºC. RNA pellet was washed twice with 75% ethanol and resuspended in 10 to 50 µL diethylpyrocarbonatetreated water. The concentration and quality of total RNA samples were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific Inc., Waltham, MA). Only total RNA samples that achieved adequate purity ratios (A260/A280 = 1.9–2.1) were used for subsequent analyses.[26] In addition, RNA integrity and purity were also checked using agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA).

Quantification of VEGFA Gene Expression by Quantitative RT-PCR

Real-time reverse transcription-PCR was performed in two separate reactions: first, RNA was reverse transcribed into cDNA, then cDNA was amplified by quantitative real-time PCR (RT-

qPCR). Reverse transcription of 1 µg RNA into cDNA was carried out using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies), following the manufacturer's protocol for oligo (dT) method.

RT-qPCR experiments were performed in a 7500 Fast Real-Time PCR System Thermal Cycler with 7500 Fast System Sequence Detection 1.4 Software (ABI). Experiments were performed by monitoring, in real time, the increase in fluorescence of the SYBR Green dye.[27] PCR reactions were performed using 10 µL of 2X Fast SYBR Green Master Mix (ABI), 1 µL (1 ng/µL) forward and reverse primers for *VEGFA* or *β-actin*, and 1 µL cDNA template (0.25 µg/µL), in a total volume of 20 µL. Each sample was assayed in triplicate, and a negative control was included in each experiment. The thermocycling conditions for these genes were as follows: an initial cycle of 95ºC for 20 seconds, followed by 45 cycles of 95ºC for 3 seconds and 60ºC for 30 seconds, each cycle. The specificity of the RT-qPCR was determined using melting-curve analyses, and all primers generated amplicons that produced a single sharp peak during the analyses.

Quantification of the *VEGFA* mRNA was performed using the relative standard curve method,[26,28] and *β-actin* as the reference gene. Relative standard curves were generated for both target and reference genes by preparing serial dilutions of the same cDNA sample with a known relative quantity. Then, relative amounts of each *VEGFA* mRNA sample were obtained by normalizing their results using *β-actin* and were presented as arbitrary units (AU).

Primers for *VEGFA* and *β-actin* genes were designed using published human sequences and the Primer Express 3.0 Software (ABI), and they were projected to target two consecutive exons of a gene in order to prevent the amplification of any contaminated genomic DNA. The following primer sequences were used: *VEGFA* forward primer 5'-GGCGAGGCAGCTTGAGTTAA-3', reverse primer 5'-CACCGCCTCGGCTTGTC-3', *β-actin* forward primer 5'-GCGCGGCTACAGCTTCA-3', reverse primer 5'-CTTAATGTCACGCACGATTTCC-3'.

Statistical Analyses

Data are described as mean ±SD or as percentages. Clinical characteristics and *VEGFA* mRNA abundance were compared between genotypes and alleles using Student's *t*-test, one-way ANOVA, or χ^2 test, as appropriate. Pearson's correlation test was used to assess the correlation between *VEGFA* gene expression and age. Variables with skewed distribution were logarithmically transformed before analyses. A linear regression was performed to evaluate the independence of

the association between -634G/C polymorphism and *VEGF* expression. The mRNA levels (log transformed) were entered as the dependent variable. Age, smoking status, sex, and hypertension were the independent variables. Statistical analysis was performed using statistical software, SPSS 16.0 (Statistical Package for Social Sciences, Armonk, NY).

RESULTS

Fifty-three retina tissue samples from cornea donors were included in the gene expression analyses. Donor age ranged from 13 to 79 years (mean, 55.8 ± 15.8 years), and 49.1% (n = 26) were male. Among these subjects, 18 (34%) were CC homozygous, 18 (34%) were GG homozygous, and 17 (32%) were heterozygous. The clinical characteristics of the three genotype groups were similar regarding age, sex, proportion of hypertensive individuals, and smokers (Table 1).

When comparing the CC (5.15 ± 4.47 AU) and GC genotypes (3.72 ± 3.25 AU), both had similar gene expression of *VEGFA* ($P = 0.87$), while GG (2.62 6 ±2.56 AU) had lower expression when compared with CC ($P = 0.025$) and GC ($P = 0.04$).

No significant differences were observed when gene expression was analyzed by sex (men, 3.97 ± 3.69 AU, versus women, 3.7 ± 3.6 AU; $P = 0.87$), hypertensive status (hypertensives, 3.09 ± 3.28 AU, versus normotensives, 4.9 ± 4.2 AU; $P = 0.237$), or smoking status (smokers, 3.9 ± 4.1 AU, versus nonsmokers, 5.71 ± 4.08 AU; $P = 0.259$). *VEGFA* gene expression did not correlate with age ($r = 0.293$; $P = 0.911$).

VEGFA gene expression in retinal samples analyzed according to the presence of different genotypes of the -634G/C polymorphism is depicted in Table 1 and the Figure 2. *VEGFA* gene expression was higher in both CC and GC genotype groups when compared with the GG genotype group ($P = 0.045$). Assuming a dominant model, the *VEGFA* mRNA levels were higher in -634C allele carriers (CC and GC) when compared with GG carriers (4.46 ± 3.94 AU versus 2.62 ± 2.56 AU; $P = 0.031$).

In a multilinear regression model, the presence of the C allele remained associated with higher *VEGF* gene expression ($P = 0.045$). The only other factor statistically associated with VEGF mRNA levels was smoker status ($P = 0.02$) (Table 2).

DISCUSSION

In this study, we observed that in nondiabetic individuals without retinal disease, the *VEGFA* -634C allele is associated with increased retinal expression of the *VEGFA* gene. This finding was independent of age, sex, smoking status, or hypertension status.

It is well known that functional polymorphisms can influence gene expression and regulate the final amount of protein in a given disease. The growing interest in researching the *VEGFA* -634G/C polymorphism is justified by its association with DR.[18,29] Previous studies suggested that the C allele of the *VEGFA* -634G/C polymorphism is a risk factor for DR[18,19,21,22] or DME, independent of the presence of DR,[20] and the presence of the C allele in homozygosity is an independent risk factor for the development of PDR in T2DM patients.[18] In addition, the C allele was also associated with elevated serum and vitreous levels of VEGFA in patients with type 2 DM.[25] These reports are in agreement with the results presented here.

Although much is known about changes involving VEGFA in eye diseases, and the inhibition of this protein through the therapeutic use of intravitreous anti-VEGFA drugs, little is known about its role in the normal human retina. In experimental studies of rats and monkeys, *VEGFA* was shown to be constitutively expressed in vascularized tissues of normal eyes.[16,30] In rats, retinal *VEGFA* expression is associated with age and is found to be higher in older animals.[30] This suggests greater susceptibility to neovascularization and may be related to the etiology, for instance, of the choroidal neovascular membrane and more severe DR in older patients. However, in the present study, no association was seen between *VEGFA* gene expression and donor age.

The increased *VEGFA* gene expression in the retina of donors carrying CC or GC genotypes is compatible with the role of this protein in DME and DR, because it is an important factor in increased vascular permeability, stimulus of mitosis of endothelial cells and in angiogenesis.[5,31] Our findings suggest that this polymorphism should be investigated as a risk factor for greater VEGFA production in the retina of DM patients and consequent greater predisposition to revascularization and increased vascular permeability from exposure to low oxygen tension and ischemia.[5,6]

A functional effect of the -634G/C polymorphism cannot be inferred from this study. It should be noted that we genotyped and analyzed the retina of individuals without DM and/or eye/retinal disease—there was no hypoxia inducing VEGFA production. Therefore, a greater effect

on VEGFA expression might be supposed in subjects exposed to DM or eye/retinal disease. Previous functional data regarding the -634G/C polymorphism indicate that this variant could be directly associated with increased *VEGFA* gene expression.[18,19,32] We also cannot rule out the possibility that this polymorphism is not itself responsible for the observed changes in *VEGFA* gene expression, but rather a surrogate that is in linkage disequilibrium with an unknown causative polymorphism in a distal regulatory site.

Although our sample may seem small, it is adequate for the purposes of this study. Human retinal tissue is hard to obtain, which complicates gene expression research. We believe that our results provide a new and original insight for the investigation of retinal neovascular diseases, especially DR. The identification of putative functional DNA variants will support the development of new treatment options in the future.

We must be careful and critical in the interpretation of genetic association data. Therefore, additional genotype studies measuring VEGF levels in retinal disease are required to confirm the present findings. Even though this is a difficult task, owing to the variety of clinical and genetic factors involved in DM complications such as DR, the recognition of genetic predisposing factors to increased VEGF production could help in the identification of susceptibility to ischemic damage. The *VEGFA* -634G/C polymorphism might be one such factor.

In conclusion, the C allele of the *VEGFA* -634G/C polymorphism is associated with increased *VEGFA* gene expression in retinas of nondiabetic cornea donors. This provides evidence of increased risk of DR in DM patients carrying this allele. Further studies will be necessary in DM patients to elucidate our findings.

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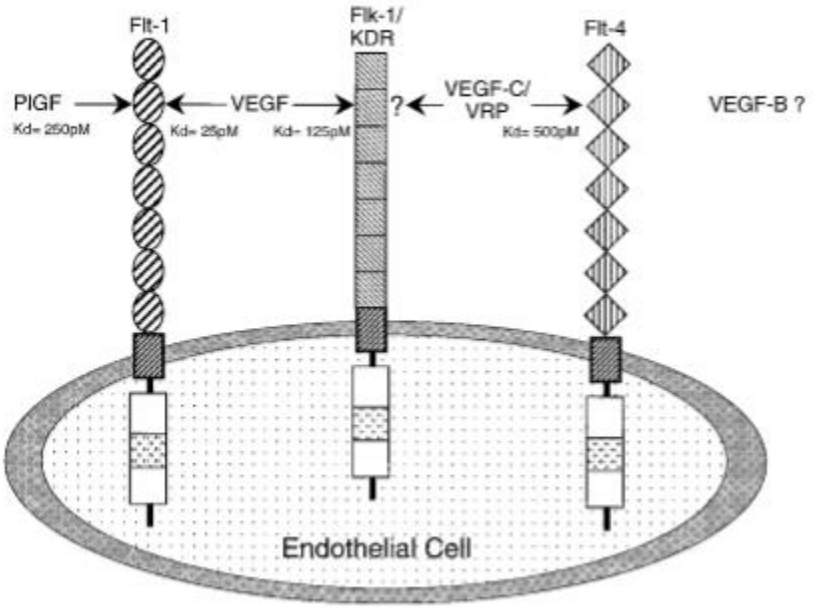
Figure 1 – The VEGF and VEGF receptors family



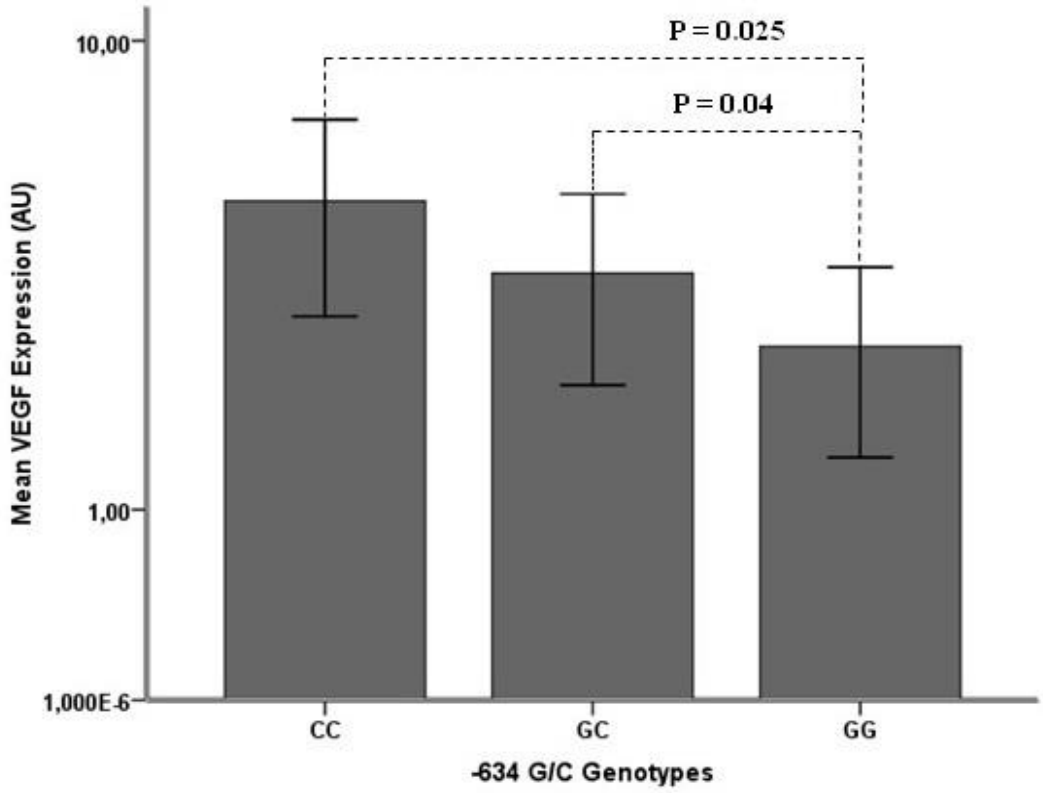
Adapted from Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev. 1997;18:4-25.[5]

Table 1 - Clinical characteristics and *VEGFA* gene quantification in retina from cadaveric cornea donors according to the *VEGFA* -634G/C polymorphism

	-634G/C genotype			***P***
	CC (n = 18)	**GC (n = 17)**	**GG (n = 18)**	
Age (years)	60.6 ± 10.42	53.71 ± 16.53	53.44 ± 19.24	0.375
Male sex - n (%)	12 (66.67)	5 (29.41)	9 (50)	0.088
Arterial hypertension - n (%)	2 (11.11)	3 (17.65)	6 (33.33)	0.251
Smoker Status- n (%)	7 (38.89)	4 (23.53)	4 (22.22)	0.354
VEGFA (AU)	5.15 ± 4.47*	3.72 ± 3.25*	2.62 ± 2.56	0.045

Data are expressed in mean ± SD or number of subjects (percentage). *P* values were computed by ANOVA One-way test or by χ^2 test. * $P \leq 0.05$ in relation to the GG genotype group (LSD post hoc test). AU = arbitrary units.

Figure 2 - *VEGFA* gene expression in human retinal samples stratified according to different genotypes of the *VEGFA* -634G/C polymorphism.



P = 0.045 (One-Way ANOVA test). Data are presented as mean ± 2 SD. AU = arbitrary units.

Table 2 – Multiple Linear Regression and C allele of -634 VEGF gene expression

	Unstandardized coefficients		
	BETA	**SE**	***P***
C allele	-0.317	0.155	0.045
Age	0.003	0.004	0.530
Male sex	-0.081	0.151	0.596
Arterial hypertension	0.134	0.128	0.302
Smoker status	-0.267	0.133	0.02
Constant	0.555	0.272	0.049

VEGF expression (arbitrary units) as the dependent variable.

SE = standard error

ARTIGO 2

Polymorphisms in the *FLT-1* and *KDR* genes and proliferative diabetic retinopathy in patients with type 2 diabetes mellitus: an association study.

ORIGINAL ARTICLE

Polymorhisms in the *FLT-1* and *KDR* genes and proliferative diabetic retinopathy in patients with type 2 diabetes mellitus: an association study.

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RESUMO

INTRODUÇÃO: Diabetes mellitus (DM) é uma doença de alta morbidade e mortalidade com importante perda na qualidade de vida. O fator de crescimento vascular endotelial (VEGF) é determinante na regulação da angiogênese e permeabilidade vascular de doenças oculares que envolvem isquemia e neovascularização, como a retinopatia diabética (RD). O receptores tirosina quinases e seus ligantes são essenciais para este processo. Neste estudo, avaliamos a associação de polimorfismos dos receptores *fms-like tyrosine kinase-1* (FLT-1, VEGFR1) e *kinase domain region/fetal liver kinase-1* (KDR/FLK-1, VEGFR2) com RD proliferativa (RDP) em indivíduos brancos portadores de DM tipo 2 (DMT2).

OBJETIVO: avaliar a associação de polimorfismos de *FLT-1* e *KDR* a RDP.

MATERIAL E MÉTODOS: Estudo de caso-controle, baseado em estudo transversal, de 490 portadores brancos de DMT2. Indivíduos com RDP foram considerados casos e indivíduos sem RD com 10 anos de doença ou mais foram considerados controles. Quatro polimorfismos incluindo um no *FLT-1* (rs9508034) e três no *KDR* (rs2071559, rs6828477 e rs7667298) foram analisados por PCR em 265 casos e 225 controles. Análise de haplótipos foi realizada por método Bayesiano e os alelos, genótipos e frequência de haplótipos comparados entre casos e controles. O equilíbrio de Hardy-Weinberg de cada polimorfismo foi analisado pelo teste do χ^2.

RESULTADOS: Os pacientes com RDP foram significativamente diferentes dos pacientes controle em relação ao sexo, IMC, creatinina sérica e nefropatia. Todos os genótipos estavam em equilíbrio de Hardy-Weinberg. As frequências genotípicas e alélicas foram semelhantes entre os casos e controles para os polimorfismos *FLT-1* - rs9508034 (P = 0,719) e *KDR* - rs2071559 (P = 0,381), rs6828477 (P = 0,973) e rs7667298 (P = 0,729). Na análise de regressão logística, não foi observada associação entre os SNPs estudados e RDP após controle para sexo, idade no momento do diagnóstico, idade, duração do DM, IMC, HbA1c, hipertensão arterial ou tabagismo. A análise estatística Bayesiana da combinação dos três polimorfismos *KDR* não foram diferentes entre os casos e controles (P = 0,772).

CONCLUSÃO: Não houve associação de SNPs analisados e presença de RDP.

PALAVRAS-CHAVE: polimorfismo, retinopatia diabética, fator de crescimento do endotélio vascular, receptor do fator de crescimento do endotélio vascular, DM tipo 2.

ABSTRACT

BACKGROUND AND AIMS: Diabetes mellitus (DM) is a high morbidity and mortality disease, with significant loss of quality of life. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and vascular permeability in ischemic and neovascular eye diseases, including diabetic retinopathy (DR). VEGF tyrosine kinase receptors and their ligands are essential for these processes. In the present study, the association of proliferative DR (PDR) with single nucleotide polymorphisms (SNP) in *fms-like tyrosine kinase-1* receptor (FLT-1, VEGFR1) and *kinase domain region/fetal liver kinase-1* receptor (KDR/FLK-1, VEGFR2) genes was evaluated in patients with type 2 DM (T2DM).

PURPOSE: to determine the association of *FLT-1* and *KDR* polymorphisms and PDR.

MATERIALS AND METHODS: A case-control nested to a cross-sectional study conducted in 490 white patients with T2DM. Patients with PDR were considered cases and patients without DR and T2DM for 10 years or more were considered controls. Four SNPs including one in the *FLT-1* gene, namely rs9508034, and three in the *KDR* gene including rs2071559, rs6828477 and rs7667298, were genotyped using PCR-based assays in 265 Caucasian cases and 225 controls. Haplotype analyses were performed by Bayesian statistical method and the alleles, genotypes and haplotype frequencies were compared between cases and controls. The Hardy-Weinberg equilibrium of each SNP was tested by χ^2 test.

RESULTS: Patients with PDR differed significantly from control patients regarding proportion of male sex, BMI, serum creatinine and nephropathy. All genotypes were in Hardy-Weinberg equilibrium. The genotypic and allelic frequencies were similar among cases and controls for the *FLT-1* - rs9508034 ($P = 0.719$) and *KDR* polymorphisms – rs2071559 ($P = 0.381$), rs6828477 ($P = 0.973$), and rs7667298 ($P = 0.729$). In logistic regression analysis, no association of the studied SNPs and PDR was observed after controlling for gender, age at diagnosis, age, DM duration, BMI, HbA1c, arterial hypertension or smoking. The Bayesian statistical analysis of the combination of the three *KDR* polymorphisms was not different between cases and controls ($P = 0.772$).

CONCLUSION: In the present study, we did not observe an association of analyzed SNPs and PDR.

KEYWORDS: polymorphism, diabetic retinopathy, vascular endothelial growth factor, vascular endothelial growth factor receptor, diabetes mellitus type 2.

INTRODUCTION

Diabetes mellitus (DM) is disease with significant loss of quality of life, and its prevalence is increasing worldwide [1,2]. Diabetic retinopathy (DR) is a frequent complication of DM. It is estimated that 30-45% of affected individuals will have some degree of retinopathy after 15 years of disease, 10% will have severe visual impairment and 2% will be blind[1].

DR is characterized by increased vascular permeability, hemostatic abnormalities, increased tissue ischemia and neoangiogenesis. Retinal neovascularization is a hallmark of proliferative DR (PDR). The most established factors associated with the presence and severity of DR are blood pressure levels, DM duration and glycated hemoglobin[3]. However, these factors alone do not explain all cases of DR. We have previously described genetic polymorphisms in the vascular endothelial cell growth factor (VEGF) gene as an independent risk factor for the development of PDR in patients with DM type 2 (T2DM)[4].

The subtype A of the VEGF (VEGFA), originated from alternative splicing of the *VEGF* gene, is the main cytokine involved in the induction of vascular permeability and angiogenesis, and is implicated in the pathogenesis of DR. The VEGFA acts mainly through two tyrosine kinase receptors: FLT-1 (fms-like tyrosine kinase, VEGFR-1), and FLK-1/KDR (fetal liver kinase-1/kinase domain region, VEGFR-2)[5,6]. These receptors are expressed mainly in the blood vascular endothelium. Hypoxia upregulates FLT-1 expression which binds VEGFA, placental growth factor (PlGF) and VEGFB. The KDR is the main mediator of the angiogenic, mitogenic and permeability effect of VEGFA. It is expressed mostly on endothelial cells and regulates physiological and pathological angiogenesis. While bound to VEGFA, it performs dimerization and tyrosine phosphorylation resulting in a cascade of protein activation, mitogenic, chemotactic and prosurvival/antiapoptotic signal and inducing migration, endothelial proliferation and increasing vascular permeability[5-11].

There are few reports about the genetic variations in the VEGF receptors and retinal diseases and even less about DR. The aim of the present study was to extend our previous observation of the role of the VEGF pathway in the development of DR, evaluating common single nucleotide polymorphisms (SNPs) in *FLT-1* and *KDR* genes in patients with T2DM.

SUBJECTS AND METHODS

Subjects, phenotype measurements, and laboratory analyses

This case-control study was designed in accordance with the STROBE and STREGA guidelines for the reporting of genetic association studies[12,13].

This study was carried out in 490 unrelated white Brazilian T2DM patients who are participating in a multicenter study in the Brazilian state of Rio Grande do Sul that started recruiting patients in 2002. All subjects were self-designated as white. The ethics committees of the participating institutions approved this study and all procedures were performed, including drawing blood samples only after the informed consent was signed.

A standard questionnaire was used to collect information on gender, age, age at diagnosis, DM duration, smoking habit and drug treatment. All patients underwent a clinical and laboratory evaluation. They were weighed without shoes, wearing light outdoor clothes and their height was measured. Weight and height were used to calculate body mass index (BMI). Blood pressure (BP) was measured twice, in the sitting position, after 5 minutes rest, and with a 2 minute interval between measurements, using a mercury sphygmomanometer. The mean of both measurements was used to calculate systolic and diastolic BP. Arterial hypertension was defined as BP $\geq$ 140/90 mmHg or use of antihypertensive drugs. Retinal status was based on a fundoscopy examination by a single experienced ophthalmologist through dilated pupils. DR was classified as absent (no fundus abnormalities), nonproliferative DR (microaneurysms, and hemorrhage), or PDR (neovascularization)[14]. Patients classified as no DR (control group) or PDR (cases) based on the most severe degree of retinopathy in the worst-affected eye were enrolled in the study. Additionally, to be included in the control group, the subjects had to have T2DM for ten years or more.

Serum sample was collected after a 12h fast for laboratory analyses, DNA extraction and genotyping. Serum creatinine level was determined using the Jaffe's reaction; glycated hemoglobin (HbA1c) was quantified by ion-exchange HPLC procedure (Merck-Hitachi L-9100 GhB Analyser, Merck, Darmstadt, Germany reference range: 4.7–6.0%).

Genotyping

The DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure[15]. All analyzed polymorphisms were genotyped using primers and probes contained in the Human Custom TaqMan Genotyping Assay 20x (Thermo Fisher -Life Technologies, Foster City, CA, USA), and described in **Table 1**. Reactions were conducted in 384-well plates, in a total 5 µl reaction volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Thermo Fisher - Life Technologies) and Custom TaqMan Genotyping Assay 1x. The plates were then positioned in a real-time PCR thermal cycler (ViiA7 Real Time PCR System; Thermo Fisher - Life Technologies) and heated for 10 min at 95ºC, followed by 50 cycles of 95ºC for 15s and 62ºC for 1 min. Fluorescence data files from each plate were analyzed using automated allele-calling software (Thermo Fisher – Life Technologies).

Selection of Candidates Genes

KDR polymorphisms were selected from the International HapMap Project and searching the available literature. Considering the *KDR* gene, due to linkage disequilibrium (LD) between some of the common polymorphisms in this gene, at least three polymorphisms had to be genotyped to estimate all haplotypes with more than 10% frequency and that would cover 81% of all possible *KDR* haplotypes. The rs2071559 polymorphism in *KDR gene* and rs9508034 polymorphism in *FLT-1* gene were also selected based on preceding studies[16,17].

Statistical Analysis

Data are described as mean ± standard deviation (SD) or percentages. Clinical characteristics were compared between genotypes and alleles using Student's t-test or χ^2 test, as appropriate. Statistical analysis was performed using statistical software *SPSS 20.0* (*Statistical Package for Social Sciences*).

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 the χ^2 test.

Considering the polymorphisms in *KDR* gene, between all pairs of biallelic loci, we examined widely used measures of linkage disequilibrium (LD), Lewontin's D' $|D'|$ and r^2 [18].

The haplotypes constructed from the combination of the three *KDR* polymorphisms and their frequencies were inferred using the Phase 2.1 program (Seatle, WA, USA), which implements a Bayesian statistical method[19]. We also used this program to compare the distributions of different *KDR* haplotypes between cases and controls through permutation analyses of 10,000 random replications[19].

RESULTS

The main clinical and laboratory characteristics of patients according to the retinal status are summarized in **Table 2**. T2DM patients with PDR differed significantly from control patients for sex, BMI, serum creatinine, and diabetic kidney disease.

The distributions of the *FLT-1* and *KDR* polymorphisms in T2DM patients with and without PDR are presented in **Table 3**. All genotype frequencies were in agreement with those predicted by HWE. The genotypic and allelic frequencies were similar among cases and controls for the *FLT-1* (rs9508034) and *KDR* (rs2071559, rs6828477 and rs7667298) polymorphisms. Assuming recessive or dominant inheritance models did not disclose an association of the studied polymorphisms and PDR.

In logistic regression analysis, no association of the studied SNPs and PDR was observed after controlling for gender, age at diagnosis, age, DM duration, BMI, HbA1c, arterial hypertension or smoking.

We used a Bayesian statistical method to estimate the frequency of different haplotypes produced by the combination of the three *KDR* polymorphisms in cases and controls. Eight haplotypes were inferred in both samples and are shown in **Table 4**. Permutation analysis showed that the distributions of all inferred haplotypes were not different between cases and controls (P = 0.772).

It is noteworthy that considering both $|D'|$ and r^2 measurements, we did not find any significant LD among all pairs of combination of the three analyzed polymorphisms (Table 5).

DISCUSSION

In the present study we evaluate SNPs that covered the most common genetic variation of FLT-1 and KDR genes. None of the SNPs evaluated, nor the haplotypes showed association with proliferative DR.

Limited data are available on FLT-1 and KDR genetic variation in the population, its ethnic differences and its effect on gene function or retinal expression. We only found two studies regarding the rs2071559 and DR. Yang *et al*[16] described the association of the minor allele (C alelle) and any DR stage in an investigation of 500 T2DM Chinese patients (P=0.034). However, after Bonferroni correction, it did not sustain the significance. Choudhuri *et al*[20] did not verify significant differences among PDR, non-proliferative DR, absence of DR and healthy control individuals in 372 Eastern Indians.

Some SNPs of the *FLT-1* and *KDR* genes have been associated with age-related macular degeneration (AMD). AMD has a similar DR pathogenesis involving the VEGF pathways and they share the same treatment with antiangiogenic drugs. The rs9508034 was shown to increase the risk of neovascular AMD 2.3-fold under a dominant genetic model in different ethnic groups[17].

Hermann *et al*[21] identified the SNP rs6828477 as independent predictor of a better visual outcome in 366 eyes with neovascular AMD who were treated with ranibizumab for at least 1 year. It suggests that genetic variation could partially explain the differences in the response to antiangiogenic treatment. However, researchers in the Comparison of AMD Treatments Trials (CATT) Research Group and Alternative Treatments to Inhibit VEGF in Patients with Age-Related Choroidal Neovascularisation (IVAN) Study Investigators gathered, respectively, 835 and 512 participants and could not replicated the outcomes of Hermann *et al*[22,23].

Lazzeri *et al*[24] observed that the allele rs2071559 A was less frequent in the AMD patients than in the control subjects, while Cruz-Gonzalez F *et al*[25] did not find any difference concerning the response of AMD treatment with ranibizumabe and the rs2071559 polymorphism. The rs2071559 was studied in many non retinal associations like susceptibility to coronary heart disease[26], coronary colateral circulation[27], glioblastoma survival[28], ovarian hyperstimulation syndrome[29], maternal BMI and risk of spontaneous preterm birth[30]. Merlo *et al*[31] demonstrated an association between the rs2071559 of *KDR* and carotid intima-media thickness in subjects with T2DM. However, no data regarding PDR is available.

Many genes and genetic variants have been studied but it is difficult to reproduce its outcomes. Nowadays no VEGF receptors gene has conferred a high risk for DR, as we can see in AMD, so we can not conclude about associations or causality.

Although no association was found among VEGF receptors SNPs studied and PDR, the role of these receptors in the process of neovascularization and increase of vascular permeability is well-established[5,6,8-10]. Many clinical trials are focused on neutralizing the VEGFA effects using VEGFA inhibitors such as bevacizumabe, ranibizumabe and aflibercept. However, there are no anti-FLT-1 or anti-KDR antibodies to modify the signal transduction of these receptors. A greater understanding of the roles of the VEGFA receptors may show new and interesting clinical implications. The analyses of VEGFR SNPs may represent a clinical tool to better identify patients more likely to benefit from anti-angiogenic treatment and, maybe in the future, the possible benefits of gene therapy for DR. The gene therapy could better target the molecular mechanisms involved in the DR making it more effective and specific.

A limitation of the present study is the restricted sample size. However, the two previous reports about the rs2071559 had a similar sample size, and applied the analysis to other stages of DR besides the proliferative DR. On the other hand, due to the lack of information on SNPs of VEGF receptors associated with PDR, this could be considered as an exploratory study.

In conclusion, in the present study, we did not observe an association of analyzed SNPs and PDR. However, the pathogenesis of DR is complex, most likely multifactorial, including environmental and polygenic influences, and probably cannot be explained by a simple genetic association. However, the suspicion around the candidate genes associations would lead to investigations that elucidate the metabolic pathways. Due to the angiogenic implication in the pathology of DR, assessment of VEGF pathway variants that correlate with the neovascular retina status may allow for greater understanding of genetic risk factors and overall pathophysiology. As we collect further information about genotype–phenotype correlations, our understanding regarding the pathophysiology of DR might improve. Ultimately, this information may help to stratify patients into different risk groups, which may positively impact clinical management decisions. In addition, this information may lead to the investigation of future drug targets.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Table 1 – Characteristics of the Genetic Loci, SNPs, primers and probes used for the genotyping of the analyzed polymorphisms

	Chr	**Nucleotides**	**Position in the gene**	**Primer**	**Probes**
FLT -1					
rs9508034	13	AC	intronic	F: 5'GGAGAGAGAATTAAACTGGACTGTGA3' R: 5'GCCTGCAGCCTTTGATTACTG3'	VIC: TGTGCTCTCAAAACAAA FAM: TGCTCTCCAAACAAA
KDR					
rs7667298	4	CT	5'-UTR	F: 5'CGCAGGCAGAGGAAACG3' R: 5'TCCCACCCTGCACTGAGT3'	VIC: AGAGCGGTCAGTGTGTG FAM: AGCGGTCAATGTGTG
rs2071559	4	CT	upstream variant	F: 5'AACTTGGAGCCGCCAAATATTTTG3' R: 5'TTGCTCTTAATCAGAAAACGCACTTG3'	VIC: TCGCCAGCATTCC FAM: TTCGCCAACATTCC
rs6828477	4	CT	intronic	F: 5'GCGGATTTGCCCTAAAACATGAAT3' R: 5'GCTTATTAGCATTTCATAAACTACACTTGCA3'	VIC: CCATGGCGGTTTTG FAM: CCATGGCAGTTTTG

Chr: Chromossome; 5'-UTR: untranslated region 5' – UTR;

Table 2 - Clinical and laboratory characteristics of patients with T2DM according to retinal status

	Controls (n = 225)	**Cases (n = 265)**	***P****
Male	96 (43)	159 (60)	<0.001
Age at diagnosis (years)	44.14 ± 11.27	42.2 ± 13.00	0.083
Age (years)	61 ± 9.02	59.93 ± 10.18	0.226
BMI (Kg/m²)	28.80±5.18	27.75±5.18	0.031
HbA1c (%)	7.2±2.23	7.11±1.8	0.698
DM duration (years)	16.89±7.77	17.66±10.07	0.346
Arterial hypertension (%)	164 (72.89)	197 (73.34)	0.208
Serum creatinine (mg/dL)	1.33±1.67	3.36±3.32	<0.001
Nephropathy	80 (41)	208 (83.2)	<0.001
Smoking	104 (49)	115 (47.5)	0.781

Data are expressed as mean ± SD or (percentage). **P* values were computed by χ^2 or Student's t-test, as appropriate.

Table 3 - Genotypic and allelic frequencies of *KDR* and *FLT-1* polymorphisms in white T2DM patients according to retinal status

	Genotype Frequencies			Allele Frequencies			
	Controls	**Cases**	***P****		**Controls**	**Cases**	***P****
FLT -1							
rs9508034	**n = 214**	**n = 256**					
AA	**14 (6.5)**	**19 (7.5)**	**0.719**	A	**0.27**	**0.26**	**0.757**
AC	**87 (40.5)**	**95 (37.0)**		C	**0.73**	**0.74**	
CC	**113 (53.0)**	**142 (55.5)**					
KDR							
rs2071559	**n = 215**	**n = 255**					
CC	**58 (27.0)**	**55 (21.5)**	**0.381**	C	**0.52**	**0.49**	**0.348**
CT	**108 (50.0)**	**140 (55.0)**		T	**0.48**	**0.51**	
TT	**49 (23.0)**	**60 (23.5)**					
rs6828477	**n = 216**	**n = 254**					
CC	**41 (19.0)**	**48 (19.0)**	**0.973**	C	**0.43**	**0.41**	**0.869**
CT	**104 (48.0)**	**120 (47.0)**		T	**0.57**	**0.59**	
TT	**71 (33.0)**	**86 (34.0)**					
rs7667298	**n = 214**	**n = 257**					
CC	**57 (26.5)**	**76 (29.5)**	**0.729**	C	**0.53**	**0.48**	**0.703**
CT	**113 (53.0)**	**127 (49.5)**		T	**0.47**	**0.52**	
TT	**44 (20.5)**	**54 (21.0)**					

Genotype data are expressed as number (percentage) and data of allelic frequencies expressed as proportions. * *P* values were computed by χ^2 test.

Table 4 - Haplotype of the *KDR* gene in T2DM patients without (controls) and with PDR (cases).

Haplotypes	Controls (n= 434)	Cases (n= 512)	Frequency in the total sample
Ht1 (C C C)	0.060	0.058	0.059
Ht2 (C C T)	0.162	0.186	0.175
Ht3 (C T C)	0.187	0.160	0.172
Ht4 (C T T)	0.016	0.024	0.020
Ht5 (T C C)	0.024	0.034	0.030
Ht6 (T C T)	0.286	0.268	0.276
Ht7 (T T C)	0.246	0.242	0.244
Ht8 (T T T)	0.019	0.028	0.024

Data are presented as proportion. n, number of chromosomes. The first letter of haplotypes refers to the rs6828477 polymorphism, the second letter to the rs7667292 polymorphism and the third letter to the rs2071559 polymorphism. **P* value = 0.772 for comparisons of haplotype frequencies between groups.

Table 5 - Pairwise linkage disequilibrium values for the three analyzed polymorphisms in *KDR* gene.

Genome position	dsSNP ID	rs6828477	rs7667298	rs2071559	
55100634	rs6828477		0.144362	0.29264	
55125564	rs7667298	0.017729		0.702482	|D'|
55126199	rs2071559	0.059633	0.66671		
		r^2			

Pairwise linkage disequilibrium (LD) values, |D'| (right) and r² (left), are shown.

CONCLUSÕES

A retinopatia diabética (RD) tem sido uma importante causa de perda visual e cegueira em todo o mundo. A identificação do VEGFA e seu papel na regulação da angiogênese através dos receptores tirosina kinase trouxeram novas perspectivas acerca das investigações da patofisiologia da RD. Os dois estudos apresentados na presente tese de doutorado avaliaram aspectos genéticos tanto do VEGFA quanto de seus receptores FLT-1 e KDR.

No primeiro artigo, publicamos a associação entre o polimorfismo do VEGFA -634G/C (rs2010963) e a expressão do gene *VEGFA* em retina humana de doadores de córnea não portadores de diabetes mellitus (DM). Em estudo transversal analisando 53 amostras de retina, os indivíduos com o alelo C apresentaram maior expressão do gene *VEGFA*.

No segundo artigo, fundamentados na diferença entre a resposta da ligação do VEGFA aos seus receptores, buscamos identificar polimorfismos genéticos dos receptores FLT-1 e KDR que pudessem estar associados a RD proliferativa. Baseado em estudo transversal do nosso grupo de pesquisa em DM, realizamos estudo de caso-controle com 490 portadores brancos de DM tipo 2. Indivíduos sem RD com 10 anos de doença ou mais foram considerados controles e aqueles com RDP foram considerados casos independente do tempo de doença. Analisamos quatro polimorfismos sendo um no *FLT-1* (rs9508034) e três no *KDR* (rs2071559, rs6828477 e rs7667298), mas não encontramos associação de SNPs analisados e presença de RDP.

A busca de genes candidatos envolvidos na patogênese da RD são sempre terreno fértil para novas pesquisas científicas e, apesar de nossos resultados não terem sido significativos na investigação de polimorfismos nos receptores de VEGF, acreditamos que estudos de expressão gênica possam trazer maiores esclarecimentos e futuramente elucidar questionamentos como o porquê alguns pacientes portadores de DM de longa duração ou com controle glicêmico inadequado não desenvolvem RD enquanto outros com melhor controle ou menor tempo de doença apresentam RD.