ORIGINAL ARTICLE

Genetic predictors associated with diabetic retinopathy in patients with diabetic foot

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KEY WORDS

diabetic foot, diabetic retinopathy, polymorphisms, type 2 diabetes

ABSTRACT

INTRODUCTION Early detection of diabetic retinopathy (DR) is crucial for preventing irreversible blindness. Recent studies identified some of the genetic factors involved in the pathology of DR, although their precise underlying mechanisms remain unclear.

OBJECTIVES This pilot study aimed to determine genetic predictors of DR among patients with type 2 diabetes (T2D) and diabetic foot (DF) based on pathogenetic pathways.

PATIENTS AND METHODS The study included 114 patients with T2D and DF (64 with DR, 50 without DR). Genetic analysis was performed for each patient and the following alterations were analyzed: rs759853 (*AKR1B1*), rs1800469 (*TGFB1*), rs2073618 and rs3134069 (*TNFRSF11B*), rs6330 and rs11466112 (*NGF*), rs1801133 (*MTHFR*), rs8192678 (*PPARGC1A*), rs1799983 (*NOS3*), rs1553005 (*CALCA*), and rs121917832 (*CDKN1B*).

RESULTS Correlations with DR were identified for the following single nucleotide variants (SNVs): rs759853, rs2073618, and rs3134069. Carriers of the G allele of the rs759853 variant had a higher risk of DR in the dominant model (odds ratio [OR], 3.0; 95% confidence interval [CI], 1.15–7.81; $P = 0.02$). We analyzed 2 SNVs of the osteoprotegerin gene (rs3134069 and rs2073618), and found that the A allele of the rs3134069 variant decreased the risk of DR in both the recessive and additive models (OR, 3.33; 95% CI, 1.07–10.3; $P = 0.04$). Conversely, there were fewer carriers of the C allele of the rs2073618 variant in patients with DR in the dominant model (OR, 0.28; 95% CI, 0.09–0.92; $P = 0.04$).

CONCLUSIONS The results of our study suggest that the SNVs rs759853, rs3134069, and rs2073618 may be involved in the development of DR in patients with T2D and DF.

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INTRODUCTION Diabetic retinopathy (DR) is one of the most common causes of blindness in developed countries.[1] Owing to its asymptomatic onset and progressive course, DR is typically diagnosed at a late stage when treatment options are limited and often results in irreversible blindness.[2] A previous study showed that there is a genetic predisposition for the severity of DR,[3] and in patients with long-lasting type 2 diabetes (T2D) and poor glycemic control, diabetic complications often did not occur. Therefore, it appears that, in addition to known metabolic and hemodynamic factors, genetic factors also affect the course of DR, though the exact underlying mechanisms are still being explored.

Risk factors for the development of DR (poor control of blood glucose, hypertension, hyperlipidemia, obesity) are related to factors that contribute to the development of neuropathy, which is the main cause of diabetic foot (DF).[4]

Advances in knowledge of pathogenetic pathways leading to microangiopathy suggest that

many genes may be involved in its development. The known biochemical mechanisms underlying DR include formation of advanced glycation end products, activity of polyol and hexosamine pathways, activation of protein kinase C isoforms, and oxidative stress. Impaired activity of those pathways leads to hypoxia of the retinal tissue, dysfunction of endothelial cells, impairment of vasodilation, hyperactivation of angiogenic factors, and changes in extracellular matrix.[5]

The development of methods that would allow an early identification of patients with a genetic predisposition to DR in those with T2D and DF could slow the disease progression. After reviewing the literature, we identified a number of single nucleotide variants (SNVs) that are possibly associated with DR in patients with T2D and DF, including variants of the following genes: *AKR1B1* (aldose reductase; rs759853), *TGFB1* (transforming growth factor β1; rs1800469), *TNFRSF11B* (tumor necrosis factor receptor superfamily, member 11b), also known as *OPG* (osteoprotegerin; rs2073618, rs3134069), *NGF* (nerve growth factor; rs6330, rs11466112), *MTHFR* (methylenetetrahydrofolate reductase; rs1801133), *PPARGC1A* (peroxisome proliferator-activated receptor gamma; rs8192678), *NOS3* (nitric oxide synthase 3; rs1799983), *CALCA* (calcitonin-related polypeptide alpha; rs1553005), and *CDKN1B* (cyclin-dependent kinase inhibitor 1B; rs121917832).

This preliminary study aimed to investigate the possible involvement of different genetic variants in the risk of developing DR in a population of patients with T2D and DF, based on the similarity of the pathogenetic processes of diabetic complications.

PATIENTS AND METHODS This pilot retrospective study was conducted in the Department of Diabetology and Internal Diseases and the Department of Medical Genetics, Medical University of Warsaw, Poland, between December 2010 and September 2013. We included 114 patients with DF and T2D that were divided into 2 groups: 64 patients with DR (DR group) and 50 patients without DR (control group). DR was diagnosed by an ophthalmologist using a slit lamp and Haag-Streit noncontact lens (Haag-Streit, Harlow, Essex, United Kingdom) during hospitalization. DR was staged according to the Early Treatment Diabetic Retinopathy Study (ETDRS) classification as either nonproliferative DR or proliferative DR.[6] The nonproliferative type included background retinopathy and preproliferative retinopathy. Owing to the relatively small size of the study group, patients were not subdivided according to the stage of DR.

DF was diagnosed according to the International Consensus on the Diabetic Foot and Practical Guidelines on the management and Prevention of the Diabetic Foot 2007, which defined DF as: "ulceration, infection, or destruction of deep tissues located in the lower limbs below the ankles in patients with diabetes and neuropathy and/or peripheral arterial disease."[7] All patients underwent physical examination and their medical history was taken. The physical examination included assessment of the foot ulceration and deformation, reflexes of the knee and Achilles tendon, and pulses on the posterior and dorsal tibial arteries. The stage of neuropathy was assessed using a tip-therm type device (temperature discrimination), monofilament (sense of touch), neurotips (discrimination of pain), and Semmes–Weinstein tunnel fork (discrimination of vibration). Painless ulcerations were assessed as neuropathic DF. The neuropathy was identified according to the Toronto Clinical Neuropathy Scoring System. The ankle-brachial index was measured in each patient. In patients with a score below the norm, a Doppler ultrasound was performed. The study was approved by the Bioethics Committee fo the Medical University of Warsaw.

The genetic material was isolated from the whole blood samples using the salting-out method.[8] Genotyping of selected SNVs of the following genes: *MTHFR* (rs1801133), *NGF* (rs6330), *PPARGC1A* (rs8192678), *AKR1B1* (rs759853), *NOS3* (rs1799983), *TNFRSF11B* (rs2073618 and rs3134069), *CALCA* (rs1553005), *TGFB1* (rs1800469) was performed with the Sequenom MassARRAY system (Sequenom iPLEX assay, San Diego, California, United States). All genetic tests were performed with negative control. The possible interactions between selected gene variants and DR in patients with T2D are shown in Supplementary material, *Table S1* and *Figure S1*.

Statistical analysis Statistical analysis was performed using the Statistica 13.1 software (StatSoft, Inc., 2017, Tulsa, Oklahoma, United States). The normality of the distribution was tested by the Shapiro–Wilk and Lilliefors tests. Normally distributed continuous variables were presented as mean, and nonnormally distributed variables were presented as median values. Categorical variables were presented as numbers and percentages of the total. The χ^2 test was used to assess intergroup significance for categorical variables and the *t* test and Mann–Whitney test were used to determine differences in means and medians, respectively.

The genotype distribution of all SNVs in both study groups was tested for the Hardy–Weinberg equilibrium (HWE) using the χ^2 test. The genotype distribution of 2 SNVs, rs759853 and rs2073618, in the control group deviated from the HWE (P = 0.03 and P = 0.02, respectively). Since both groups that were under investigation in the present study were not selected from the general population but consisted of patients with diabetes, the above SNVs were not excluded from analyses.[9] Moreover, the high quality of assays (call rate >95%, unambiguous allelic discrimination plots) suggested a violation of HWE assumptions in the study groups rather than technical genotyping errors. The associations of genotypes with DR were conducted under the

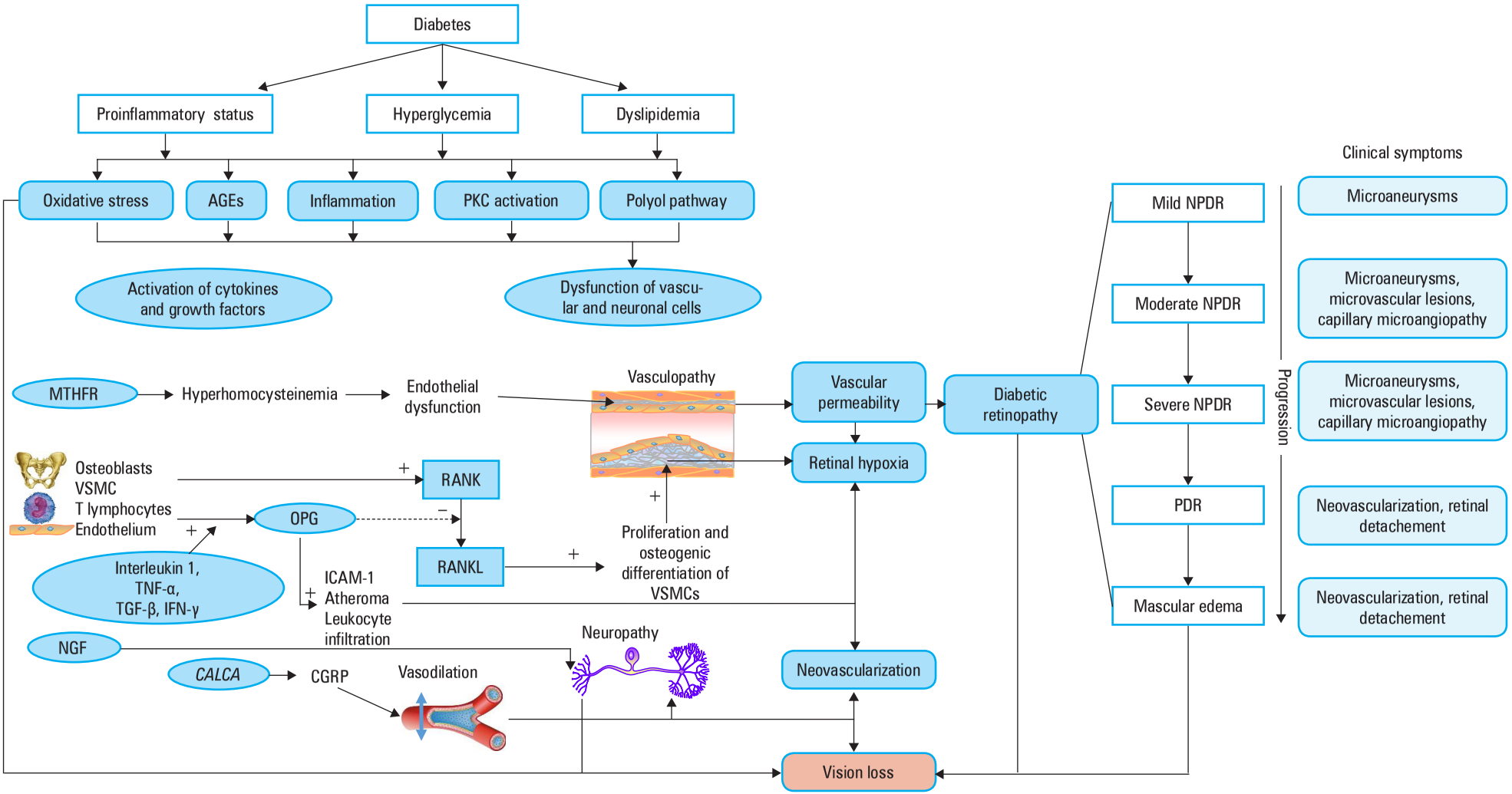

FIGURE 1 The role of studied gene products in the etiology of diabetic retinopathy
Abbreviations: AGEs, advanced glycation end products; *CALCA*, calcitonin-related polypeptide α; CGRP, calcitonin gene-related peptide; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon γ; MTHFR, methylenetetrahydrofolate reductase; NGF, nerve growth factor; NPDR, nonproliferative diabetic retinopathy; OPG, osteoprotegerin; PDR, proliferative diabetic retinopathy; PKC, protein kinase C; RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; VSMCs, vascular smooth muscle cells

TABLE 1 Characteristics of the diabetic retinopathy group compared with the control group

Parameter		DR (n = 64)	Control group (n = 50)	*P* value	OR	95% CI
Sex[a], n (%)	Female	15 (23)	23 (46)	0.01	2.78	1.25–6.21
	Male	49 (77)	27 (654)			
Age[b], y		62.8 (9.7)	65.7 (9.7)	0.12	–	–
Diabetes duration[b], y		16.97 (9.2)	17.1 (9.48)	0.81	–	–
Age at time of diabetic foot diagnosis[b], y		55.98 (10.49)	60.62 (9.48)	0.03	0.95	0.92–0.99
DF duration[c], y		6.82 (5.81)	4.53 (3.47)	0.02	1.12	1.02–1.24
Hip circumference[b], cm		112.49 (12.51)	107.71 (11.54)	0.12	–	–
Waist circumference[b], cm		107.71 (16.5)	99.68 (13.87)	0.07	–	–
WHR[b]		0.95 (0.18)	0.93 (0.11)	0.86	–	–
Weight[b], kg		96.77 (10.3)	87.11 (17.94)	0.01	1.03	1.01–1.06
Height[b], cm		173.0 (8.8)	169.0 (9.0)	0.03	1.05	1.00–1.09
BMI[b], kg/m^2		32.24 (5.0)	30.24 (5.42)	0.05	1.0	1.00–1.00
Patients with/without nephropathy[a], n (%)		34 (53)/30 (47)	11 (22)/36 (72)	0.002	3.71	1.61–8.55

Data are presented as mean (SD) unless stated otherwise.

a χ^2 test; **b** Mann–Whitney test; **c** *t* test

Abbreviations: BMI, body mass index; CI, confidence interval; DF, diabetic foot; DR, diabetic retinopathy; OR, odds ratio; WHR, waist-to-hip ratio

TABLE 2 Allele distribution and association with diabetic retinopathy

Chr.	SNV	Gene	Risk allele	RAF		Statistics			HWE in DR+		HWE in DR–	
				DR+	DR–	OR	95% CI	*P* value	χ^2	*P* value	χ^2	*P* value
1	rs1801133	*MTHFR*	T	0.67	0.73	0.76	0.43–1.35	0.39	0.26	0.61	0.21	0.64
1	rs11466112	*NGF*	C	NA	NA	NA	NA	NA	NA	NA	NA	NA
1	rs6330	*NGF*	C	0.57	0.51	1.28	0.75–2.16	0.42	2.64	0.10	1.29	0.26
4	rs8192678	*PPARGC1A*	G	0.70	0.78	0.64	0.35–1.18	0.18	0.31	0.58	1.70	0.19
7	rs759853	*AKR1B1*	G	0.63	0.53	1.48	0.87–2.51	0.18	0.28	0.59	5.04	0.03
7	rs1799983	*NOS3*	G	0.73	0.75	0.89	0.49–1.61	0.76	1.94	0.16	0.01	0.93
8	rs2073618	*TNFRSF11B*	C	0.52	0.60	0.71	0.42–1.21	0.23	0.0001	0.99	5.56	0.02
8	rs3134069	*TNFRSF11B*	A	0.96	0.89	3.04	1.02–9.06	0.06	NA	NA	NA	NA
11	rs1553005	*CALCA*	G	0.65	0.64	1.04	0.60–1.79	NA	2.87	0.09	0.87	0.35
12	rs121917832	*CDKN1B*	G	NA	NA	NA	NA	NA	NA	NA	NA	NA
19	rs1800469	*TGFB1*	T	0.27	0.34	0.70	0.40–1.24	0.25	2.53	0.11	1.26	0.26

Abbreviations: chr., chromosome; DR+, patients with DR; DR-, control group; HWE, Hardy–Weinberg equilibrium; RAF, risk allele frequency; NA, not available; SNV, single nucleotide variant; others, see TABLE 1

additive, dominant, or recessive models using the PLINK 1.9 software (http://www.cog-genomics.org/plink2/).[10] The logistic regression analyses were adjusted for the following covariates: sex, body mass index, age of onset of DF, duration of DF, and diabetic nephropathy. All *P* values lower than 0.05 were considered significant.

RESULTS The anthropometric and clinical characteristics of the study groups are presented in TABLE 1. There were significantly more men in the DR group than in the control group. The DR group was also characterized by higher weight and height, a longer duration of DF, and they were younger at T2D diagnosis than the control group. We observed nephropathy, ischemic heart disease, and heart failure more frequently in the DR group than in the control group (TABLE 1).

In the 2 SNVs of *TNFRSF11B* (rs3134069 and rs2073618), we found that the A allele of rs3134069 decreased the risk of DR in both the recessive and additive models (OR, 3.33; 95% CI, 1.07–10.3; *P* = 0.04; TABLE 2). In contrast, there were fewer C carriers of the rs2073618 variant in the DR group in the dominant model (OR, 0.28; 95% CI, 0.09–0.92; *P* = 0.04; TABLE 3). The SNVs rs1801133 (*MTHFR*), rs759853 (*AKR1B1*), as well as rs2073 618 and rs3134069 (2 variants of the *TNFRSF11B* gene) correlated with DR (TABLE 3). Carriers of the G allele of rs759853 (*AKR1B1*) had a higher risk of DR in the dominant model (odds ratio [OR], 3.0; 95% confidence interval [CI], 1.15–7.81; *P* = 0.02; TABLE 3).

TABLE 3 Genotype distribution and association with diabetic retinopathy

Chr.	SNV	Gene	Risk allele	Genotype distribution RR, RA, AA		Model OR (95% CI); *P* value			Model[a] OR (95% CI); *P* value		
				DR+	DR−	Recessive	Additive	Dominant	Recessive	Additive	Dominant
1	rs1801133	*MTHFR*	T	28, 30, 6	26, 21, 3	1.62 (0.38–6.82); 0.51	1.35 (0.74–2.44); 0.33	1.39 (0.66–2.93); 0.38	5.50 (0.46–66.2); 0.18	3.15 (1.27–7.81); 0.01[b]	3.49 (1.24–9.83); 0.02[b]
1	rs6330	*NGF*	C	24, 25, 15	11, 29, 10	2.13 (0.92–4.92); 0.08	1.26 (0.75–2.12); 0.34	0.82 (0.33–2.01); 0.66	4.52 (1.39–14.7); 0.01[b]	2.07 (1.00–4.31); 0.05	1.34 (0.40–4.48); 0.63
4	rs8192678	*PPARGC1A*	G	30, 29, 5	32, 14, 4	0.50 (0.23–1.06); 0.07	0.65 (0.36–1.19); 0.16	1.03 (0.26–4.04); 0.97	0.64 (0.25–1.69); 0.37	0.81 (0.39–1.70); 0.58	4.64 (0.25–6.55); 0.76
7	rs759853	*AKR1B1*	G	24, 32, 8	18, 17, 15	1.07 (0.50–2.30); 0.87	1.42 (0.86–2.37); 0.17[b]	3.0 (1.15–7.81); 0.02[b]	0.76 (0.29–2.04); 0.59	1.30 (0.68–2.49); 0.42	3.59 (1.06–4.27); 0.04[b]
7	rs1799983	*NOS3*	G	36, 21, 7	28, 19, 3	1.01 (0.48–2.13); 0.98	0.90 (0.51–1.58); 0.70	0.52 (0.13–2.12); 0.36	1.05 (0.40–2.77); 0.93	1.03 (0.48–4.46); 0.94	1.02 (0.17–6.49); 0.99
8	rs2073618	*TNFRSF11B*	C	17, 32, 15	14, 32, 4	0.93 (0.41–2.13); 0.86	0.97 (0.38–1.19); 0.17[b]	0.28 (0.09–0.92); 0.04[b]	1.70 (0.53–5.48); 0.37	0.95 (0.43–2.11); 0.90	0.39 (0.01–1.72); 0.21[b]
8	rs3134069	*TNFRSF11B*	A	59, 5, 0	39, 11, 0	3.33 (1.07–10.3); 0.04[b]	3.33 (1.07–10.3); 0.04[b]	NA	4.56 (1.09–19.0); 0.04[b]	4.56 (1.09–19.0); 0.04[b]	NA
11	rs1553005	*CALCA*	G	30, 23, 11	22, 20, 8	1.12 (0.53–2.36); 0.76	1.03 (0.62–1.71); 0.90	0.92 (0.34–2.49); 0.87	1.23 (0.47–3.24); 0.67	1.15 (0.60–2.20); 0.68	1.18 (0.33–4.16); 0.80
19	rs1800469	*TGFB1*	T	7, 20, 37	4, 26, 20	1.41 (0.39–5.12); 0.60	0.71 (0.40–1.25); 0.23	0.49 (0.23–1.03); 0.06	1.56 (0.30–4.44); 0.60	0.76 (0.37–1.58); 0.47	0.53 (0.20–1.41); 0.21

Models: recessive *RR* vs *RA+AA*; additive *RR* vs *RA* = *RA* vs *AA;* dominant *RR+RA* vs *AA*.

a Adjusted for sex, BMI, age of onset of DF, duration of DF, and diabetic nephropathy

b Statistically significant data

Abbreviations: AA, second allele homozygote; RR, risk allele homozygote; RA, heterozygote; others, see TABLES 1 and 2

Moreover, we observed that the distribution of the C and G alleles frequencies in the whole population for the rs114661112 variant of the *NGF* gene and the rs121917832 variant of the *CDKN1B* gene was 100% (TABLE 2).

The analysis of the frequencies of the following alterations: rs1553005 (*CALCA*), rs1799983 (*NOS3*), rs1801133 (*MTHFR*), rs8192678 (*PPARGC1A*), rs121917832 (*CDKN1B*), rs6330 and rs1466112 (*NGF*), and rs1800469 (*TGFB1*) showed no association with DR in recessive, additive, or dominant models (TABLE 3).

The analysis of the above genetic models revealed that the association of DR with rs3134069 (*TNFRSF11B*) in recessive and additive models and with rs759853 (*AKR1B1*) in the dominant model was more evident (TABLE 3). However, the relationship with rs2073618 was no longer present in the dominant model after adjustment. Furthermore, we found relationships with rs1801133 in both the recessive and dominant models and with rs6330 in the recessive model that were not present before the adjustment.

DISCUSSION

The present study is the first to investigate common genetic variants associated with DR in a population of patients with T2D and DF. The last study showed an association between the rs2274907 variant of the *ITLN1* gene and the occurrence of DF in patients with T2D.[11] Our study demonstrated that genetic predisposition to DR in patients with T2D and DF may be due to the presence of the rs2073618 and rs3134069 variants of the *TNFRSF11B* gene, and the rs759853 variant of the *AKR1B1* gene.

In patients with diabetes, a strong association between elevated serum OPG concentrations and microvascular complications was identified.[12] Recent studies investigating the relationship between the genetic variability of *TNFRSF11B* for OPG and diabetic complication yielded similar outcomes: an Italian study conducted by Pitocco et al[13] suggested a protective role of the C and T alleles of the SNVs rs2073618 and rs3134069 in patients with Charcot neuroarthropathy, and Korzon-Burakowska et al[14] confirmed the associations of the above variants in a similar small study group of patients with Charcot neuroarthropathy. In a Polish study by Mrozikiewicz-Rakowska et al,[15] the C allele of rs3134069 was found to have a protective role in T2D patients with chronic kidney disease and DF. Meanwhile, another study published by the same authors showed a correlation between DF and rs2073618 (*TNFRSF11B*) in patients with diabetes, irrespective of the type of DF, but failed to show any association with the frequency of the SNV rs3134069 (*TNFRSF11B*).[16] The first study demonstrating an association between the variants of the *OPG* gene and DR was conducted in Caucasians with T2D. In this Slovenian population, the minor C allele of the rs2073618 variant of the *TNFRSF11B* gene occurred more frequently (*P* = 0.004) in patients with diabetes with DR, while

rs3134069 was not associated with DR; however, the combination of both SNVs (rs2073618 and rs3134069) conferred an increased risk of DR.[17] In our study, we demonstrated a correlation between DR and the rs3134069 and rs2073618 variants of the *TNFRSF11B* gene; however, we showed these alleles to have different roles. The presence of the C allele in rs3134069 and rs2073618 was associated with a lower risk of DR in patients with T2D and DF. Our contrary results might have been caused by environmental factors and the smaller size of the study group. Nevertheless, these findings indicate that the OPG/RANKL/RANK system and the analyzed genetic alterations are probably involved in the pathogenesis of microangiopathy, leading to DR and nephropathy in patients with DF; however, further studies exploring this association are needed.

The correlation of the promoter rs759853 SNV with susceptibility to DR has been difficult to determine, with conflicting results reported.[18] In a meta-analysis by Abhary et al,[19] the T allele of the rs759853 variant of the *AKR1B1* gene was suggested to protect against DR in type 1 diabetes, but not in T2D. In the present study, we observed that the G allele of the rs759853 variant of the *AKR1B1* gene increased the risk of developing DR in patients with T2D and DF. Although these results should be considered with caution due to the heterogeneity of the studies and different environmental factors, the frequencies of the alleles in *AKR1B1* suggest that this gene does contribute to the development of DR. As further evidence, Toyoda at al[20] demonstrated a neuroprotective effect of ranirestat, a new aldose reductase inhibitor, in spontaneously diabetic Torii rats. Specifically, they showed that rs759853 (*AKR1B1)* could be a target for genetically oriented pharmacotherapy in humans.

Many experimental studies suggested that *NGF* plays an important role in the pathogenesis of DR.[21] Our study provided the first assessment of the potential role of the SNVs of the *NGF* gene (rs6330 and rs11466112) in the development of DR in patients with T2D and DF. We did not find the rs6330 (*NGF*) variant to be protective against DR, and we found no association between DR and the frequency of the rs11466112 (*NGF*) variant. Indeed, the distribution of the C allele of the rs11466112 variant frequencies in the whole population was 100%. Our pilot study indicates the need for further studies examining the role of the analyzed genetic alterations in the development of DR.

Variants in the gene encoding TGFB1 may lead to pericyte apoptosis in the retina, but the data do not explain this genetic background. In our study, the rs1800469 variant of the *TGFB1* gene was not found to protect against the development of DR in patients with DF in T2D. Indeed, after conducting a meta-analysis, Liu et al[22] concluded that the rs1800469 variant may not be associated with DR risk.

The C677T alteration of the *MTHFR* gene leads to impaired enzyme activity, resulting in elevated plasma homocysteine levels that contribute to macro- and microangiopathy. In a previous study conducted by Maeda et al,[23] which included 190 Japanese patients with T2D, the frequency of TT *MTHFR* homozygous patients with DR was higher than that of the other 2 genotypes. The role of the *MTHFR* genotype in susceptibility to DR was significant under hyperglycemic conditions, which modified the risk of DR. These conclusions corresponded with the results obtained by Yigit et al,[24] who demonstrated that the rs1801133 variant is related to diabetic peripheral neuropathy and to DR. In their study, a higher frequency of the TT genotype was found in patients with a positive history of DR than in those with a negative history. Furthermore, in a meta-analysis by Niu et al,[25] the *MTHFR* 677TT genotype was suggested to confer a moderately augmented risk for DR. The results of the present study are consistent with this conclusion, as the frequency of the TT genotype was associated with DR. The analyzed variant was associated with homocysteine levels in a genome-wide association study.[26,27]

It is probable that *PPARGC1A* affects angiogenesis in the retina by upregulating the expression of the *VEGF* gene, which plays a key role in the development of proliferative DR. Petrovic et al[28] found that an increased risk of DR was associated with the AA genotype of the rs8192678 variant (14.4% vs 5.9%; $P = 0.035$) in a study involving Slovenian patients with T2D (160 with and 101 without DR). Meanwhile, our results did not confirm the above association with the frequency of the rs8192678 (*PPARGC1A*) variant.

Activation of protein kinase C reduces nitric oxide production, which in turn affects microcirculation and may impact diabetic vascular complications. Indeed, the recent study by Li et al[29] showed that alterations of *NOS3*, including the rs1799983 variant, might contribute to the development of T2D in the Han Chinese population. Similarly to the previous studies, we found no correlation between rs1799983 (*NOS3*) and DR.[30,31]

Finally, the results of the present study did not verify the impact of the *CALCA* (rs1553005) or *CDKN1B* (rs121917832) gene alterations on the risk of DR. Specifically, we found no association between these variants and DR in patients with DF and T2D.

The main limitation of our study was the small sample size, which resulted from very strict inclusion criteria. DF is a rare complication and there are few publications examining genetic factors in the DF population. The individual risk factors leading to the development of DR and DF are similar; therefore, the selection of such a group of patients could allow for a better identification of genetic factors that predispose a patient to DR. A meta-analysis conducted by Orlewski et al[32] indicated that the combination of the alterations of the glutathione-S-transferase *(GST)* genes should be investigated rather than individual variants.

Indeed, the main cause of the diabetes epidemic is the interaction between a variety of environmental and genetic risk factors. Although our research is preliminary, the potential of future studies with a larger population is undeniably great. The role of studied gene products in the etiology of DR is presented in Supplementary material, *Figure S1*.[33] In this study, we did not correct the obtained results for multiple testing because we investigated the already established associations in a general diabetic population. The study population of patients with diabetes was stratified for the presence of its clinical phenotypes.[34]

In summary, we selected several genetic variants possibly engaged in the pathogenetic pathways leading to DR among T2D patients with DF. Among them, 3 seem to play a significant role in the development of DR, namely, rs759853 (*AKR1B1*), rs3134069, and rs2073618 (*TNFRSF11B)*. The present study showed the possible directions for future investigation of the genetic background of DR in patients with DF. However, our results need to be recapitulated on a larger scale. Knowledge about genetic factors that predispose to DR might broaden our understanding of the pathologic pathways leading to this complication of long-lasting diabetes.

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