# A case-control study of promoter and 5'UTR VEGF polymorphisms in diabetic retinopathy

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**Aims.** Angiogenesis is activated in the retina of diabetic patients with retinopathy complications. Vascular endothelial growth factor (VEGF) plays an essential role as a mediator of the retinal induced neovascularization. VEGF levels are increased significantly in the vitreous and aqueous ocular fluids in the patients with proliferative diabetic retinopathy. The aim of this study is to investigate whether *VEGF* promoter/5'UTR polymorphisms are associated with diabetic retinopathy (DR) development and/or severity.

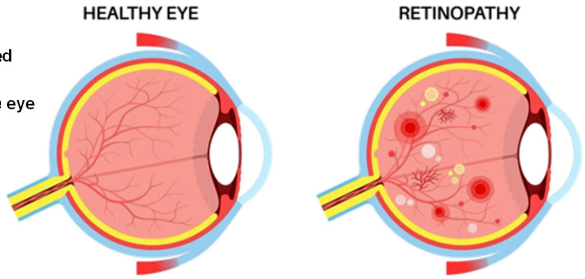
**Methods.** In this case-control study, Type 2 diabetic patients with or without DR, and normal controls were recruited. DR was classified based on the presence or absence of proliferative changes. The genotyping was analysed by polymerase chain reaction followed by restriction fragment length polymorphism.

**Results.** A total of 259 subjects (172 with diabetes±DR, 87 healthy controls; male:female, 125:134) were recruited. The average age was 59.5±9.6 years, diabetes duration was 8.5±4.5 years, body mass index was 30.6±6.0 kg/m2, and HbA1c was 7.37±1.2% (57 mmol/mol). Significant differences in the distribution of genotypes in T(-1,498)C, G(-1,190)A and G(-1,154)A polymorphism in diabetic patients versus controls, and DR patients versus DM without DR patients were shown. No association between DR severity and polymorphisms was detected. Non-significant differences in allele frequency were found. The haplotypes TGAGC, TGACT and CAGCT were significantly increased in DM patients, whereas CGGCC was the most common haplotype in DR patients.

**Conclusions.** Multiple *VEGF* polymorphisms and CGGCC haplotype are significantly correlated with a higher DR risk in Type 2 diabetic patients.

**A Case-control Study Of Promoter And 5'UTR VEGF Polymorphisms In Diabetic Retinopathy**

**Background:**
- Diabetic retinopathy (DR) is a devastating ocular microvascular complication of diabetes mellitus (DM) and is a major cause of blindness in diabetic patients worldwide.
- Angiogenesis is activated in the retina of DR patients.
- Vascular endothelial growth factor (VEGF) is a key mediator of the retinal induced neovascularization.
- VEGF levels are significantly increased in the vitreous and aqueous fluids of the eye in patients with proliferative DR.




**Aim:**
To determine whether *VEGF* promoter/5'UTR polymorphisms are associated with DR development and/or severity.

**Methodology:**
- Type 2 diabetic patients with or without DR, and normal controls were recruited.
- Genotyping was analysed by polymerase chain reaction followed by restriction fragment length polymorphism.

**Main outcomes:**
1. The distribution of T(-1,498)C, G(-1,190)A and G(-1,154)A polymorphism genotypes in DM patients versus controls and in DM patients with DR versus without DR was significantly different.
2. The haplotypes TGAGC, TGACT and CAGCT were significantly increased in DM patients, while CGGCC haplotype was the most common in DR patients.

Significant associations between multiple *VEGF* polymorphisms and CGGCC haplotype with an increased DR risk in Type 2 diabetic patients were detected.

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**Graphical Abstract**
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## INTRODUCTION

The number of individuals affected by type 2 diabetes mellitus (DM) is increasing worldwide rendering it a major global public health challenge. Diabetic retinopathy (DR) is the most frequent vascular complication in type 2 DM patients occurring in around 75% of patients within 15 years of diagnosis with DM (ref.[1]). DR is a key cause of vision loss among diabetic patients with about one third of patients ending up with blindness due to this complication[2-4]. According to the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR), around 30% of DM patients develop early DR, while around a quarter do not, regardless of their glycemic exposure. These findings suggest that genetic mutations/variations could affect the risk[5]. The frequency of DR varies for different ethnic groups; however, DM is very common worldwide. Hence, studying diabetic complications and potential candidate genes that could contribute to increasing their risk would be useful at both the local and global level.

Multiple features characterize DR including, hemostatic abnormalities, high vascular permeability, and tissue ischemia followed by neovascularization. The exposure of cells to chronic hyperglycemia and other metabolic dysregulations in diabetic patients results in the development of microangiopathy, including microaneurysms, hemorrhages, and basement membrane thickening[6]. These changes lead to increased vascular permeability resulting in leakage and macular edema[4]. Vascular endothelial growth factor (VEGF) is a key mediator of the pathological, ischemia-associated retinal neovascularization[4,6,7]. Many ocular cells produce VEGF and the level of VEGF is significantly increased in the vitreous and aqueous fluids of eyes affected by proliferative diabetic retinopathy (PDR) (ref.[8,9]). The expression of retinal VEGF is high in DR patients[10,11]. Additionally, VEGF-induced vascular permeability may have a role in the development of non-proliferative DR (non-PDR) (ref.[7]). These findings support the well-established role of VEGF in the development and progression of DR making the *VEGF* gene a focus of research in DR patients.

Otherwise, due to the major contribution of DR to vision loss in diabetic patients, its etiology is under intense investigation. As mentioned above, only some diabetic patients develop this complication while chronic hyperglycemia induces DR in most patients with long DM duration but this is mostly non-severe[12,13]. Therefore, it is inadequate to cause severe forms of DR in most patients[12,13]. These findings suggest a possible role of genetic factors as indicated by the findings of the Diabetes Control and Complications Trial (DCCT) that found a robust familiar retinopathy transmission in patients with severe DR compared to those with mild DR (ref.[14]). Due to the vascular modifications that occur in diabetes and the upregulation of VEGF levels in the affected patients, the *VEGF* gene could be a candidate gene that affects the risk. To date, genetic studies on VEGF have identified polymorphisms associated with DR (ref.[5,15,16]), but produced variable findings[17-19]. Older studies found higher VEGF serum levels in type 1 diabetes and DR patients[20-23].

As far as we know, there are no studies to date on *VEGF* polymorphisms in the Jordanian population or Middle Eastern populations in general, hence, we performed this case-control study, to assess their potential role in DR by investigating the frequency of *VEGF* polymorphisms in diabetic patients and whether these polymorphisms are associated with the development of severe DR in a sample of Jordanians with Type 2 diabetes.

## MATERIALS AND METHODS

### Subject recruitment

Subjects were recruited at the Ophthalmology Clinic of the National Center of Diabetes, Endocrinology and Genetics (NCDEG). Inclusion criteria were; age range 30–88 years, diagnosis with type 2 diabetes by standard means illustrated by the American Diabetes Association, DM duration <15 years, and no previous retinal problems before DM diagnosis. The standards of the American Diabetes Association used to establish and confirm diabetes diagnosis included using one of four tests: (a) the most common test is fasting plasma glucose (FPG) level >6.9 mmol/L (>125 mg/dL); (b) random plasma glucose level ≥11.1 mmol/L (≥200 mg/dL) in addition to diabetes symptoms such as polyuria, polydipsia, fatigue, or weight loss; (c) oral glucose tolerance test using 75 gm sugar with a two-hour post-load glucose ≥11.1 mmol/L (≥200 mg/dL); (d) or HbA1c test ≥48 mmol/mol (≥6.5%). After using any of the aforementioned tests, the standard requires a confirmation using a second test, either the same test or a different test. HbA1c testing was performed only for diabetic patients included in this study, while we relied on medical history records to exclude diabetes in the control group. We collected demographic data (age and sex), clinical data such as height, weight, and history of diabetes, hypertension, ischemic heart disease and/or dyslipidemia. Complete data was collected for each subject. We considered consistent sex distribution in each group.

For diagnosing retinopathy, an experienced ophthalmologist detected the following features by slit lamp biomicroscopy through dilated pupils, hemorrhages, exudates,

new vessels, and fibrous proliferation. The evidence-based International Clinical DR Disease Severity Scale agreed on by the American Academy of Ophthalmology (AAO) in 2001 and the International Council of Ophthalmology (ICO) in 2002 was applied to determine DR severity. Based on this scale, DR severity can be classified into the following classes: (1) no retinopathy when ophthalmoscopic abnormalities are absent; (2) mild non-proliferative DR (NPDR) when microaneurysms (MA) only are present; (3) moderate NPDR when more than just MA appears but the changes are less than the severe form; (4) severe NPDR when more than 20 intraretinal (IR) hemorrhages in each of the 4 quadrants, definite venous beading in 2 or more quadrants, or prominent intraretinal microvascular abnormalities (IRMA) in one or more quadrants are present without any signs of proliferative retinopathy; and (5) proliferative DR when neovascularization and/or vitreous/preretinal hemorrhage are detected. No Large Language Models (LLMs) were used at any stage of this research.

**Ethical approvals**

This study was approved by the Deanship of Scientific Research of the University of Jordan and the Institutional Review Board (IRB) of the NCDEG, approval number 10/671/9/MS. This study was performed in consistence with the ethical principles of the Declaration of Helsinki. All subjects signed a written informed consent before blood withdrawal. Subject names were confidential, and coding numbers were given to them.

**DNA extraction and genotyping**

We withdrew blood samples from all participants and preserved them in EDTA tubes for DNA extraction. We extracted DNA from whole blood samples using QIAGEN Puregene Blood Core Kit B (QIAGEN Sciences, Maryland, USA) and referring to the manufacturer's instructions. Genomic DNA was amplified by polymerase chain reaction (PCR) using the forward and reverse primers in Table S1. Bio-Rad, S1000 Thermal cycler™, USA was used to run PCR and the conditions applied for all polymorphisms were: initial denaturation for five minutes at 95 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C, and final extension for seven min at 72 °C (Bio-Rad, S1000 Thermal cycler™, USA). PCR products were digested at 37 °C for 4 h with restriction endonucleases shown in Table S1 and they were run on a 2.5% agarose gel. The detected fragment sizes were shown in Table S1 for each studied polymorphism. Validation of PCR findings was done by 1) including a negative control that had all the components in the PCR tubes except the DNA template in every PCR run; 2) reproducing about 15% of all the studied samples by other lab personnel.

**Statistical analysis**

We used the Statistical Package for Social Sciences (SPSS) software, version 22 (Chicago, Illinois, USA) for statistical analysis. The presented values were either shown as mean ± standard deviation (SD) or counts and percentages (%). The sample size was selected to identify differences in DR risk between the groups. Analysis of variance (one-way ANOVA) was used to detect differences in quantitative variables between the studied groups, while Chi-square'($\chi 2$¨) test was applied to assess differences between groups in categorical variables. The association of the *VEGF* polymorphisms with DR was determined using Chi-square $\chi 2$ test. For significant results, a *P* value of less than 0.05 was defined as a significant value. We also analysed genotype and allele frequencies for agreement with the Hardy-Weinberg equilibrium. We followed the Strengthening the Reporting of Observational Studies in Epidemiology (*STROBE*) guideline to report the results of this study.

**Haplotype analysis**

The interaction between genetic polymorphisms at the five loci was assessed by haplotype analysis. We measured the haplotype frequencies of the studied five single nucleotide polymorphisms for patients affected by diabetes with or without DR, and these were compared with those of the controls. We also compared the haplotypes and their frequencies in patients affected by DR to those with DM but not DR. Using SNPStats software, haplotype frequencies were calculated and linkage disequilibrium was represented by D prime (D') (ref.[24]).

## RESULTS

A total of 259 subjects were recruited in this study (78 were affected by diabetes and DR (DR), 94 were affected by diabetes without DR (DWR), and 87 were healthy controls). The clinical characteristics of the recruited 259 subjects are given in Table 1. After amplifying DNA using PCR, we performed RFLP analysis to identify the genotypes of the five *VEGF* polymorphisms C(-7)T and C(-634)G in the 5' UTR and T(-1,498)C, G(-1,190)A and G(-1,154)A in the promoter region of *VEGF* gene, Fig. 1. The details of the restriction enzymes and sites, and the generated fragments are shown in Table S1.

**Genotype and allele frequency**

Comparing genotype frequencies among DM patients versus controls revealed a significant difference in genotype distribution for T(-1,498)C, G(-1,190)A, and G(-1,154)A polymorphisms, Table 2. Additionally, allele frequency analysis showed no significant difference for

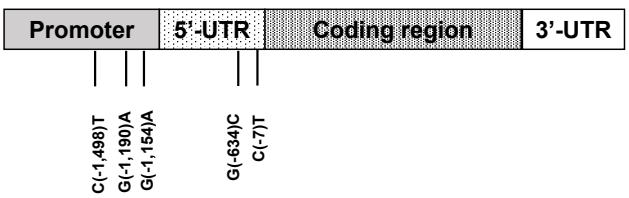


**Fig. 1.** *VEGF* promoter and 5'UTR polymorphisms investigated in this study. The location of the studied single nucleotide polymorphisms relative to *VEGF* gene.
UTR, untranslated region.

**Table 1.** Clinical data of the participants in this study.

| Clinical characteristics | Group | | | *P* |
|---|---|---|---|---|
| | DR n=78 | DM n=94 | Control n=87 | |
| Sex (M:F) | 48.7:51.3 | 38.3:61.7 | 58.6:41.4 | 0.026 |
| Mean age (years) | 61.6+8.2 | 59.8+7.9 | 56.9+11.9 | 0.008 |
| BMI (kg/m$^2$) | 32.2+5.7 | 31.6+6.3 | 27.8+4.9 | <0.001 |
| $HbA_{1C}$ (%) | 7.8+1.1 | 7.0+1.2 | UA | <0.001 |
| $HbA_{1C}$ (mmol/mol) | 62.2+12.4 | 52.6+15.2 | UA | <0.001 |
| Duration of DM (years) | 10.6+4.0 | 6.7+4.1 | NA | <0.001 |
| HTN (%) | 70.1 | 66.0 | 19.5 | <0.001 |
| IHD (%) | 18.2 | 21.3 | 2.4 | 0.001 |
| DLP (%) | 66.7 | 79.8 | 12.6 | <0.001 |

BMI, body mass index; DLP, Dyslipidemia; DM, Diabetes Mellitus; DR, diabetic retinopathy; F, female; $HbA_{1C,}$ hemoglobin A1c; HTN, Hypertension; IHD, Ischemic Heart Disease; M, male; NA, not applicable; UA, unavailable.
Qualitative results are shown as percentages.
Quantitative results are shown as mean ± standard deviation.
HbA1c was presented as a percentage and in mmol/mol.
*P* value <0.05 was considered significant.
Analysis of variance (one-way ANOVA) was applied for detecting differences in quantitative variables between the studied groups.
Chi-square $\chi 2$ test was applied to detect differences between the studied groups in categorical variables.

any polymorphism between diabetic patients and controls, Table 2. When comparing *VEGF* polymorphisms in DR patients with those in DWR patients, significant differences in the genotype frequencies of T(-1,498)C, G(-1,190)A, and G(-1,154)A polymorphisms were detected, Table 3. Allele frequency analysis in DR versus DWR group revealed an insignificant difference for all polymorphisms of *VEGF*, Table 3.

We also analysed the relevance of selected clinical parameters (age, sex, BMI, hypertension, ischemic heart disease, dyslipidemia, HbA1C, and DM duration) to *VEGF* polymorphisms genotypes among DR patients as shown in Table S2. Multiple logistic regression revealed non-genetic parameters such as DM duration (*P*<0.001) and age (*P*=0.035), in addition to genetic mutations such as, T(-1,498)C, G(-1,190)A and G(-1,154)A polymorphisms as significant predictors of DR. In Table S3, we tested whether the studied polymorphisms were correlated with DR severity and found insignificant results.

**Haplotype analysis**

Haplotype analysis was performed and shown in Table 4. After identifying the major haplotypes, we compared them in DR versus DWR using the Monte Carlo simulation test. Our findings showed that the TGAGC, TGACT and CAGCT haplotypes were significantly elevated in patients with DM (*P*<0.05), whereas TAGGC, CGGGT and CGACC haplotypes were significantly less among DM patients when compared to controls. When we compared haplotypes between patients affected by DR to those without DR, we found TGAGC and TGGGC haplotypes to be significantly less common in DR patients. On the other hand, CGGCC haplotype was significantly more in DR patients than in patients with uncomplicated diabetes. Overall, our results suggest an association of *VEGF* polymorphisms with DR development.

The studied polymorphisms showed consistency to Hardy-Weinberg equilibrium based on D' shown in Table S4.

## DISCUSSION

In diabetic retinopathy, retinal hypoxia was observed. During hypoxia, VEGF expression is induced leading to stimulation of angiogenesis[25,26]. However, the arteriovenous saturation difference showed a negative correlation with VEGF levels[27]. Most of the hypoxia responsive elements are found in the 5' UTR, promoter region of *VEGF* gene, hence we focused on the polymorphisms in this region in our study. These polymorphisms have been under investigation in different ethnicities but not in the Jordanian population or other populations of the Middle East. The findings of this study are a valuable contribution to the understanding of the genetic risk factors for DR in the Middle Eastern population. Adding this information about Middle Eastern population genetics to the databases may assist in customizing treatment plans to the patients of these populations based on their genetic background. Additionally, finding significant correlations between VEGF genetic polymorphisms and DR may support testing these polymorphisms as biomarkers for diabetic microvascular complications. We investigated 3 polymorphisms in the promoter region, T(-1,498)C, G(-1,190)A, and G(-1,154)A, and 2 in the 5'UTR of *VEGF* gene C(-7)T, and C(-634)G. Genotypes of T(-1,498)C, G(-1,190)A and G(-1,154)A polymorphisms were significantly different in DM patients with or without DR versus normal controls. G and A alleles of G(-1,154)A polymorphism were significantly different between DM patients versus controls. When we compared the genotypes of T(-1,498)C, G(-1,190)A and G(-1,154)

**Table 2.** Genotype and allele distribution and frequency of VEGF polymorphisms in diabetic patients versus control aubjects.

| Genotype/Allele | Controls (n=87) | DM Patients (n=172) | *P* |
|---|---|---|---|
| 1. T(-1,498)C | | | **<0.001** |
| TT | 33 (37.9) | 64 (37.2) | |
| TC | 16 (18.4) | 70 (40.7) | |
| CC | 38 (43.7) | 38 (22.1) | |
| T | 82 (47.1) | 198 (57.6) | 0.238 |
| C | 92 (52.9) | 146 (42.4) | 0.259 |
| 2. G(-1,190)A | | | **0.002** |
| GG | 13 (14.9) | 61 (35.5) | |
| GA | 42 (48.3) | 65 (37.8) | |
| AA | 32 (36.8) | 46 (27.7) | |
| G | 68 (39.1) | 187 (54.4) | 0.120 |
| A | 106 (60.9) | 157 (45.6) | 0.147 |
| 3. G(-1,154)A[a] | | | **0.001** |
| GG | 51 (58.6) | 62 (36.9) | |
| GA | 10 (11.5) | 16 (9.5) | |
| AA | 26 (29.9) | 90 (53.6) | |
| G | 112 (64.4) | 140 (41.7) | **0.033** |
| A | 62 (35.6) | 196 (58.3) | **0.023** |
| 4. C(-634)G[b] | | | |
| CC | 14 (17.1) | 38 (22.4) | 0.306 |
| CG | 27 (32.9) | 64 (37.6) | |
| GG | 41 (50.0) | 68 (40.0) | |
| C | 55 (33.5) | 140 (41.9) | 0.842 |
| G | 109 (66.5) | 200 (58.1) | 0.648 |
| 5. C(-7)T | | | 0.056 |
| CC | 54 (62.1) | 124 (71.1) | |
| CT | 27 (31.0) | 45 (26.2) | |
| TT | 6 (6.9) | 3 (1.7) | |
| C | 135 (77.6) | 293 (85.2) | 0.583 |
| T | 39 (22.4) | 51 (14.8) | 0.250 |

DM, Diabetes Mellitus; VEGF, vascular endothelial growth factor.
Data was presented as counts and (percentages).
[a] The genotype of four samples of diabetic patients was undetected (n=168).
[b] The genotype of five samples of controls and two samples of diabetic patients were undetected (n=82 and 170, respectively).
*P* value <0.05 was considered significant.
Chi-square $\chi 2$ test was applied to detect differences between the studied groups.

A polymorphisms in diabetic patients with DR to those without it, we found a significantly different distribution and frequencies, whereas C(-634)G and C(-7)T showed no significant difference. Additionally, allele frequencies were not significantly different. We also found no significant association between DR severity and any of the polymorphisms. Multiple logistic regression revealed non-genetic parameters such as DM duration and age, and genetic parameters in *VEGF* gene promoter like T(-1,498)C, G(-1,190)A and G(-1,154)A polymorphisms as significant predictors of DR. In the Japanese population, C(-7)T, T(-1,498)C, G(-1,154)A, C(-634)G, and G(-1,190)A polymorphisms were identified in *VEGF* (ref.[28]). They showed a significant association of the CC genotype of the C(-634)G polymorphism with DR, while an Indian study found a significant association of the CG genotype of the same polymorphism with DR (ref.[28,29]). The genotypes of CT in the C(-7)T region and TC in the T(-1,498)C region of *VEGF* were also found to be statistically significant in the Indian population with DR but not in the Japanese population[28,29]. A Pakistani case-control study that investigated G(-1,154)A polymorphism in *VEGF* revealed no significant association of genotypes or allele frequencies between DR patients in comparison to those without it[30]. A meta-analysis found that G(-634)C polymorphism increased the risk to DR in overall populations and in Caucasians in addition to a significant association between this polymorphism and increased NPDR risk[31]. Additionally, C(-1,498)T also was significantly associated with DR risk in Caucasians[31]. Moreover, G(-1,154)A played an essential role in PDR etiology in Caucasians and C(-1,498)T was significantly associated with NPDR risk in most populations and Asians. When comparing the detected haplotype frequencies between DR and DWR groups and between DM and control groups, significant differences were detected.

**Table 3.** Genotype and allele distribution and frequency of VEGF polymorphisms in diabetic retinopathy patients versus diabetic patients without retinopathy.

| Genotype | DWR (n=94) | DR (n=78) | *P* |
|---|---|---|---|
| 1. T(-1,498)C | | | **<0.001** |
| TT | 34 (36.2) | 30 (38.5) | |
| TC | 44 (46.8) | 26 (33.3) | |
| CC | 16 (17.0) | 22 (28.2) | |
| T | 112 (59.6) | 86 (55.1) | 0.641 |
| C | 76 (40.4) | 70 (44.9) | 0.588 |
| 2. G(-1,190)A | | | **0.007** |
| GG | 36 (38.3) | 25 (32.0) | |
| GA | 31 (33.0) | 34 (43.6) | |
| AA | 27 (28.7) | 19 (24.4) | |
| G | 103 (54.8) | 84 (53.8) | 0.942 |
| A | 85 (45.2) | 72 (46.2) | 0.917 |
| 3. G(-1,154)A[a] | | | **0.005** |
| GG | 32 (34.8) | 30 (39.5) | |
| GA | 7 (7.6) | 9 (11.8) | |
| AA | 53 (57.6) | 37 (48.7) | |
| G | 71 (38.6) | 69 (45.4) | 0.513 |
| A | 113 (61.4) | 83 (54.6) | 0.577 |
| 4. C(-634)G[b] | | | 0.424 |
| CC | 18 (19.2) | 20 (26.3) | |
| CG | 38 (40.4) | 26 (34.2) | |
| GG | 38 (40.4) | 30 (39.5) | |
| C | 74 (39.4) | 66 (43.4) | 0.659 |
| G | 114 (60.6) | 86 (56.6) | 0.713 |
| 5. C(-7)T | | | 0.101 |
| CC | 70 (74.5) | 54 (69.2) | |
| CT | 24 (25.5) | 21 (26.9) | |
| TT | 0 (0) | 3 (3.9) | |
| C | 164 (87.2) | 129 (82.7) | 0.759 |
| T | 24 (12.8) | 27 (17.3) | 0.465 |

DR, diabetic retinopathy; DWR, diabetic without retinopathy; VEGF, vascular endothelial growth factor.
Data was presented as counts and (percentages).
[a] The genotype of two samples of diabetic patients without DR and two samples of diabetic patients with DR were undetected (n=92 and 76, respectively).
[b] The genotype of two samples of diabetic patients with DR was undetected (n=76).
*P* value <0.05 was considered significant.
Chi-square $\chi 2$ test was applied to detect differences between the studied groups.

Due to the role that VEGF plays in ocular neovascularization, anti-VEGF therapies were adopted for treating DR such as bevacizumab, ranibizumab, and aflibercept[32,33]. They are less destructive than laser, thus, they can be a promising treatment for DR (ref.[34, 35]). Although anti-VEGF therapeutic agents showed a promising effect in the restoration of visual acuity in PDR patients, this success was confined to patients affected by both DR and diabetic macular edema (DME) (ref.[36]). These findings imply a possible role for genetic polymorphisms in DR development. Former studies identified *VEGF* polymorphism C(-634)G as a genetic risk factor for DME that exhibited a good response to anti-VEGF therapy when present[37]. Additionally, the American Academy of Ophthalmology (AAO) Preferred Practice Pattern committee revealed that the treatment of DR with anti-VEGF treatment is based on sufficient evidence[38]. However, follow-up studies are required to confirm that *VEGF* polymorphism C(-634)G can be considered as a pharmacogenetic marker. Moreover, studies of *VEGF* polymorphisms in DR patients produced varying results among different population groups[31,39-43].

Overall, DR pathogenesis is a highly complicated multistage process during which angiogenesis acts as the most important event suggesting an essential role for VEGF (ref.[44-46]). Many retinal cells including retinal astrocytes, Muller, microglia, and pigment epithelial cells express VEGF (ref.[47, 48]) This would add to the complexity of studying VEGF's role in DR development.

The pathophysiological mechanisms by which VEGF may lead to DR development and progression are not yet well-understood, however, there are several feasible expla-

**Table 4.** Haplotype percentage distribution of VEGF polymorphisms in DM and DR patients, and controls.

| Condition | Haplotype | DM | Control | Odds Ratio | (95% CI) | *P* |
|---|---|---|---|---|---|---|
| DM | | | | | | **0.0001** |
| | TGGCC | 13.3 | 7.5 | 1.0 | Reference[#] | NA |
| | TGACC | 12.8 | 1.9 | 2.03 | 0.61–6.83 | 0.25 |
| | CAGGC | 8.3 | 7.5 | 1.23 | 0.40–3.79 | 0.72 |
| | TGAGC | 1.1 | NA | 20.25 | 1.0–3500.0 | **0.0001** |
| | CAAGC | 10.1 | 5.4 | 1.36 | 0.45–4.10 | 0.59 |
| | CAAGT | 9.4 | 3.6 | 1.52 | 0.5–4.69 | 0.46 |
| | TGGGC | 6.3 | 3.8 | 0.78 | 0.24–2.58 | 0.68 |
| | CAGGT | 5.3 | 7.9 | 0.67 | 0.26–1.75 | 0.41 |
| | TAAGC | 1.7 | 4.4 | 0.36 | 0.09–1.41 | 0.14 |
| | TAGGC | 1.0 | 7.5 | 0.10 | 0.02–0.49 | **0.005** |
| | TAAGT | 1.8 | 4.6 | 0.38 | 0.10–1.41 | 0.15 |
| | CAACC | 2.0 | 2.1 | 2.39 | 0.35–16.48 | 0.38 |
| | CGGCC | 1.0 | 5.2 | 0.01 | 0.00–2.64 | 0.11 |
| | TAGCC | 1.0 | 5.3 | 0.03 | 0.00–3.10 | 0.14 |
| | TAGGT | 1.0 | 4.7 | 0.13 | 0.01–1.12 | 0.065 |
| | TGACT | 3.8 | NA | 1.09 | 0.2–1995.0 | **0.0001** |
| | CGGGT | 0.3 | 3.6 | 0.07 | 0.01–0.73 | **0.027** |
| | CGACC | 1.0 | 3.6 | 0.05 | 0.01–0.39 | **0.0048** |
| | TGAGT | 1.0 | 2.1 | 0.28 | 0.05–1.45 | 0.13 |
| | CGAGC | 1.0 | 2.7 | 0.00 | -∞–∞ | 1 |
| | TAGCT | 1.0 | 2.9 | 0.00 | -∞–∞ | 1 |
| | CAGCT | 1.4 | 1.0 | 20.2 | 0.2–2970.0 | **0.0001** |
| | CAACT | 1.4 | 2.5 | 0.00 | -∞–∞ | 1 |
| | CAGCC | 1.3 | 1.8 | 17.04 | 0.10–2956.67 | 0.28 |
| | TGGGT | 0.3 | 2.4 | 0.64 | 0.01–28.66 | 0.82 |
| | Rare* | 4.0 | 6.1 | 0.12 | 0.02–0.66 | **0.016** |
| DR | | DR | DM | | | **0.05** |
| | TGGCC | 16.7 | 10.3 | 1.0 | Reference[#] | NA |
| | TGACC | 12.6 | 13.3 | 0.65 | 0.31–1.36 | 0.25 |
| | TGAGC | 8.6 | 13.5 | 0.39 | 0.17–.91 | **0.03** |
| | CAAGC | 13.0 | 6.3 | 1.27 | 0.51–3.19 | 0.61 |
| | CAAGT | 9.2 | 10.0 | 0.61 | 0.27–1.37 | 0.23 |
| | CAGGC | 8.0 | 8.8 | 0.63 | 0.25–1.53 | 0.31 |
| | TGGGC | 3.7 | 8.4 | 0.32 | 0.10–0.99 | **0.05** |
| | CAGGT | 5.5 | 5.3 | 0.69 | 0.26–1.80 | 0.45 |
| | TGACT | 3.6 | 3.6 | 0.47 | 0.10–2.32 | 0.36 |
| | CAACC | NA | 5.1 | 0.00 | -∞–∞ | 1 |
| | TAAGC | 1.0 | 3.9 | 0.56 | 0.01–1.19 | 0.07 |
| | CAGCC | 1.4 | 1.0 | 2.97 | -∞–∞ | 1 |
| | TGGCT | 1.7 | 1.6 | 2.61 | 0.07–4.37 | 0.58 |
| | TAAGT | 2.6 | 1.0 | 0.97 | 0.28–31.79 | 0.37 |
| | CAACT | 2.3 | 1.0 | 1.04 | 0.17–40.75 | 0.5 |
| | CGGCC | 2.6 | NA | 0.97 | 1.02–995.0 | **0.0001** |
| | Rare* | 8.5 | 7.71 | 0.97 | 0.22–1.71 | 0.35 |

DM, diabetes mellitus; DR, diabetic retinopathy; NA, not available; VEGF, vascular endothelial growth factor.
Data was presented as percentages, *P* values <0.05 were considered significant.
*Rare haplotypes include those with a frequency of less than 1% for both studied groups (DM and control or DM and DR).
[#]Reference haplotype contains the unmutated alleles of the five studied polymorphisms (T(-1,498), G(-1,190), G(-1,154), C(-634), and C(-7)).
Monte Carlo simulation test was applied to compare haplotype distribution between the studied groups.

nations such as VEGF induction of inflammation that plays an essential role in DR development[21,46]. Another possible explanation includes the effect of induced VEGF expression under chronic hyperglycemic conditions in DM patients. This can lead to increased vascular permeability, increased pro-apoptotic protein damage to vascular homeostasis, and then retinal neovascularization[48]. An additional potential mechanism is mediated by the role of VEGF in inducing matrix metalloproteinase (MMP) expression leading to remodeling of the extracellular matrix, inducing apoptosis in retinal cells, and in promoting their neovascularization[48,49].

The limitations of this study include a relatively small sample. Additionally, we studied 5 polymorphisms in the promoter and 5'-UTR but did not include 3'-UTR or *VEGF* gene polymorphisms, hence, we cannot exclude the effect of other *VEGF* or *VEGF*-related polymorphisms on DR development. However, our study included a relatively large number of polymorphisms compared to studies from other populations. Among the limitations was the absence of measuring VEGF levels in the vitreous fluid or serum of the participants. On the advantageous side, to the best of our knowledge, this is the only study that investigated these polymorphisms not only in Jordanian population but also in the populations of the surrounding Middle Eastern and Arab countries. Prospective studies including more participants should be performed to clearly identify the role of *VEGF*-related polymorphisms in DR development. Further studies should also include a complete scan of the *VEGF* gene and its flanking regulatory regions in diabetic patients, together with measuring VEGF levels in serum and vitreous fluid in a larger sample to elucidate the potential roles of the different known polymorphisms in DR development and to help in the selection of potential candidates to receive anti-VEGF therapy for DR.

## CONCLUSION

Our understanding of the role of genetics in DR should be expanded using new approaches and research techniques. The correlation of *VEGF* polymorphisms with DR needs more investigation, therefore, more future studies are needed for a solid establishment of the link between the different genotypes and DR risk. Our findings suggest a potential and significant role of *VEGF* promoter and 5'-UTR polymorphisms in DR development in the Jordanian population. Additionally, our results give a new insight into the pathogenesis of DR, particularly among Middle Eastern populations. Targeting *VEGF* polymorphisms may be promising as a therapeutic strategy to treat DR.

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**Conflict of interest statement:** The authors state that there are no conflicts of interest regarding the publication of this article.

**Availability of data and materials**: Supporting and raw data of this study are available on request from the corresponding author, DAH.

**Ethical approval and informed consent statements:** This study was approved by the Deanship of Scientific Research and the Institutional Review Board (IRB) at the National Center of Diabetes, Endocrinology and Genetics, Amman, Jordan. Performing this study was in consistence with the ethical standards of the Declaration of Helsinki. All participants signed a written informed consent before taking part in this study.

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**Table S1.** PCR and RFLP details for testing *VEGF* polymorphisms in this study.

| PM | Forward primer 5'-3' | Reverse primer 5'-3' | PCR band size (bp) | Restriction enzyme* | RFLP bands and genotypes |
|---|---|---|---|---|---|
| T(-1,498)C | GTGTGTGCGTGTGGGGTTGGCGG | CGACCCCCACCAAGGTTCACAG | 301 | Fnu4HI | -TT, 301<br>-TC, 301, 266, 35<br>-CC, 266, 35 |
| G(-1,190)A | TCCTGCTCCCTCCTCGCCAATG | TCCACAGTGATTTGGGGAAGTAGA | 404, 188 | DdeI | -GG, 404, 188<br>-GA, 404, 188, 145, 43<br>-AA, 404, 145, 43 |
| G(-1,154)A | TCCTGCTCCCTCCTCGCCAATG | GGCGGGGACAGGCGAGCCTC | 184, 22 | MnlI | -GG, 150, 34, 22<br>-GA, 184, 150, 34, 22<br>-AA, 188, 22 |
| C(-634)G | TTGCTTGCCATTCCCCACTTGA | CCGAAGCGAGAACAGCCCAGAA | 470 | BsmFI | -CC, 470<br>-CG, 470, 247, 196<br>-GG, 247, 196 |
| C(-7)T | GAGGAGGGGGAGGAGGAAGAA | AAGACAGCAGAAAGTTCATGGTCTC | 283 | DdeI | -CC, 283<br>-CT, 283, 263, 20<br>-TT, 263, 20 |

PCR, polymerase chain reaction; PM, polymorphism; RFLP, restriction fragment length polymorphism; VEGF, vascular endothelial growth factor.



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**Table S2.** Clinical characteristics of diabetic patients according to VEGF polymorphisms genotypes.

| | Clinical Characteristics | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Genotype n=78 | Sex (M:F) | Age (years) | BMI (kg/m$^2$) | HTN (%) | IHD (%) | DLP (%) | $HbA_{1C}$ (%) | Duration of DM (years) |
| 1. T(-1,498)C | | | | | | | | |
| TT | 15:15 | 59.6± 7.3 | 31.3± 5.7 | 73.3 | 10.0 | 46.7 | 7.8±1.6 | 10.9±3.8 |
| TC | 14:12 | 63.0± 8.0 | 32.2± 6.3 | 72.0 | 16.0 | 81.0 | 7.8±1.1 | 11.0±4.0 |
| CC | 9:13 | 62.7± 9.3 | 33.3 ± 5.0 | 63.6 | 31.8 | 77.3 | 7.6±1.0 | 9.7±4.6 |
| *P* | 0.660 | 0.243 | 0.501 | 0.729 | 0.124 | **0.012** | 0.807 | 0.484 |
| 2. G(-1,190)A | | | | | | | | |
| GG | 12:13 | 59.7± 7.1 | 33.1± 6.4 | 72.0 | 12.0 | 48.0 | 8.1± 1.1 | 10.4 ± 3.9 |
| GA | 17:17 | 63.3± 8.2 | 31.6 ± 5.5 | 69.7 | 15.2 | 79.4 | 7.6± 1.1 | 11.3 ±4.0 |
| AA | 9:10 | 60.9± 9.3 | 32.2 ± 5.2 | 68.4 | 31.6 | 68.4 | 7.6 ± 1.2 | 9.5 ± 4.5 |
| *P* | 0.980 | 0.231 | 0.634 | 0.965 | 0.208 | **0.040** | 0.266 | 0.312 |
| 3. G(-1,154)A | | | | | | | | |
| GG | 15:15 | 61.6± 8.5 | 31.9 ± 5.7 | 76.7 | 20.0 | 73.3 | 8.0± 1.0 | 10.8 ± 4.0 |
| GA | 5:4 | 61.7± 8.7 | 34.4 ± 7.3 | 66.7 | 11.1 | 44.4 | 8.1 ± 1.0 | 8.2 ± 4.1 |
| AA | 17:20 | 61.1± 7.9 | 32.1 ± 5.3 | 66.7 | 19.4 | 64.9 | 7.5 ± 1.2 | 10.9± 4.2 |
| *P* | 0.860 | 0.966 | 0.505 | 0.468 | 0.824 | 0.237 | 0.151 | 0.200 |
| 4. C(-634)G | | | | | | | | |
| CC | 11:9 | 62.0± 6.4 | 31.1 ± 5.3 | 85.0 | 20.0 | 70.0 | 8.1± 1.1 | 10.7± 4.1 |
| CG | 14:12 | 59.9± 9.3 | 33.5 ± 6.1 | 61.5 | 19.2 | 73.1 | 7.8± 1.2 | 10.3 ± 4.1 |
| GG | 13:17 | 61.7± 7.6 | 31.8 ± 5.7 | 66.7 | 16.7 | 63.3 | 7.5± 1.1 | 10.6 ± 4.2 |
| *P* | 0.642 | 0.619 | 0.340 | 0.205 | 0.948 | 0.725 | 0.259 | 0.940 |
| 5. C(-7)T | | | | | | | | |
| CC | 27:27 | 60.6± 8.0 | 32.9 ± 5.8 | 68.5 | 13.0 | 64.8 | 7.9± 1.1 | 10.5± 4.1 |
| CT | 10:11 | 64.4± 7.1 | 30.9 ± 5.6 | 80.0 | 35.0 | 76.2 | 7.5± 1.2 | 11.0 ± 4.0 |
| TT | 1:2 | 60.0± 1.7 | 29.3 ± 2.8 | 33.3 | 0 | 33.3 | 7.6± 0.7 | 9.0 ± 5.2 |
| *P* | 0.848 | 0.175 | 0.275 | 0.230 | 0.065 | 0.295 | 0.511 | 0.713 |

BMI, body mass index; DLP, dyslipidemia; DM, diabetes mellitus; DR, diabetic retinopathy; $HbA_{1C,}$ hemoglobin A1c; HTN, hypertension; IHD, ischemic heart disease; VEGF, vascular endothelial growth factor.

Data are represented as means ± SD or as counts and (percentages).

HbA1c was presented as a percentage.

*P* value <0.05 was considered significant.

Analysis of variance (one-way ANOVA) was applied for detecting differences in quantitative variables between the studied groups.

Chi-square $\chi 2$ test was applied to detect differences between the studied groups in categorical variables.

**Table S3.** The association of VEGF polymorphisms with the severity of retinopathy in DR patients.

| Genotype | Retinopathy Severity | | | | *P* |
|---|---|---|---|---|---|
| | Mild | Moderate | Severe | Proliferative | |
| 1. T(-1,498)C * | | | | | 0.760 |
| TT | 10 (34.5) | 8 (27.6) | 6 (20.7) | 5 (17.2) | |
| TC | 4 (15.4) | 9 (34.6) | 4 (15.4) | 9 (34.6) | |
| CC | 5 (22.7) | 6 (27.3) | 6 (27.3) | 5 (22.7) | |
| 2. G(-1,190)A | | | | | 0.405 |
| GG | 9 (37.5) | 6 (25.0) | 5 (20.8) | 4 (16.7) | |
| GA | 4 (11.8) | 11 (32.4) | 8 (23.4) | 11 (32.4) | |
| AA | 6 (31.6) | 6 (31.6) | 3 (15.8) | 4 (21.0) | |
| 3. G(-1,154)A * | | | | | 0.317 |
| GG | 6 (19.6) | 7 (27.4) | 7 (21.6) | 10 (31.4) | |
| GA | 0 (35.0) | 4 (40.0) | 2 (10.0) | 3 (15.0) | |
| AA | 13 (36.1) | 11 (30.5) | 6 (16.7) | 6 (16.7) | |
| 4. C(-634)G | | | | | 0.397 |
| CC | 4 (21.1) | 5 (26.3) | 7 (36.8) | 3 (15.8) | |
| CG | 7 (26.9) | 8 (30.8) | 2 (7.7) | 9 (34.6) | |
| GG | 8 (26.7) | 8 (26.7) | 7 (23.3) | 7 (23.3) | |
| 5. C(-7)T | | | | | 0.711 |
| CC | 14 (26.5) | 13 (24.5) | 13 (24.5) | 13 (24.5) | |
| CT | 4 (19.0) | 8 (38.1) | 3 (14.3) | 6 (28.6) | |
| TT | 1 (33.3) | 2 (66.7) | 0 (0) | 0 (0) | |

DR, diabetic retinopathy; VEGF, vascular endothelial growth factor.
Data was presented as counts and (percentages).
*P* value <0.05 was considered significant.
Chi-square $\chi 2$ test was applied to detect differences between the studied groups.

**Table S4.** The consistency of the studied VEGF polymorphisms with Hardy-Weinberg Equilibrium.

| DM[a] | D' |
|---|---|
| T(-1,498)C with G(-1,190)A | 0.552 |
| T(-1,498)C with G(-1,154)A | 0.042 |
| T(-1,498)C with C(-634)G | 0.357 |
| T(-1,498)C with C(-7)T | 0.292 |
| G(-1,190)A with G(-1,154)A | 0.004 |
| G(-1,190)A with C(-634)G | 0.491 |
| G(-1,190)A with C(-7)T | 0.411 |
| G(-1,154)A with C(-634)G | 0.093 |
| G(-1,154)A with C(-7)T | 0.089 |
| C(-634)G with C(-7)T | 0.349 |
| DR[b] | |
| T(-1,498)C with G(-1,190)A | 0.827 |
| T(-1,498)C with G(-1,154)A | 0.012 |
| T(-1,498)C with C(-634)G | 0.570 |
| T(-1,498)C with C(-7)T | 0.403 |
| G(-1,190)A with G(-1,154)A | 0.038 |
| G(-1,190)A with C(-634)G | 0.585 |
| G(-1,190)A with C(-7)T | 0.464 |
| G(-1,154)A with C(-634)G | 0.095 |
| G(-1,154)A with C(-7)T | 0.151 |
| C(-634)G with C(-7)T | 0.258 |

DM, diabetes mellitus; DR, diabetic retinopathy.
[a] diabetic patients to control.
[b] patients with diabetic retinopathy to diabetic patients but not retinopathy.