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# Vascular endothelial growth factor A with two genetic variants for prediction of mixed microvascular diabetic complications

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## Abstract

**Background:** Vascular endothelial growth factor (VEGF) is a signal protein, induces cell proliferation, and enhances the permeability of the endothelial cells. *VEGF-A* gene is highly polymorphic, with different near-gene variants at varied frequencies linked with altered VEGF protein expression, type 2 diabetes mellitus (T2DM) susceptibility, and associated microvascular complications. The present study aimed to investigate the role of two genetic variants of *VEGF-A*, − 583C > T (rs3025020) and + 936 C/T (rs3025039), for predicting mixed microvascular complications in T2DM. This case–control study was performed on 26 T2DM patients with mixed microvascular complications and 26 apparently healthy individuals, as a control group. Clinical, neurological, funds examinations, and biochemical laboratory investigations were conducted on all groups. The serum level of VEGF-A was measured using ELISA. Genotyping of *VEGF-A* was performed by real-time PCR allelic discrimination system.

**Results:** Serum level of VEGF-A was significantly increased in T2DM with mixed complications. T allele of *VEGF-A* rs3025020 showed higher frequency among T2DM patients with mixed complications than in control group [OR 2.67; 95% CI 1.03–6.91; $p = 0.04$], while CT genotype and T allele of *VEGF-A* rs3025039 had a high frequency in mixed complication group [OR 4.08; 95% CI 1.32–17.44; $p = 0.01$ and OR 4.02; 95% CI 1.52–10.63; $p = 0.004$, respectively].

**Conclusion:** VEGF-A increased the level contributed in the pathogenesis of mixed diabetic microvascular complications. T allele of *VEGF-A* rs3025020, CT genotype, and T allele of *VEGF-A* rs3025039 had the highest frequency in mixed diabetic microvascular complications, so they were considered risk genes for mixed diabetic microvascular complications.

**Keywords:** Type 2 diabetes mellitus, Genetic variants, Mixed microvascular complications, VEGF-A, Real-time PCR

## Background

Microvascular complications are highly prevalent in type 2 diabetes mellitus (T2DM) patients: 38% of the patients present with any stage of chronic kidney disease (CKD) [1], almost 30% have retinopathy (DR) [2], and > 30% have peripheral neuropathy (PN) [3].

Microvascular complications are common in tissues where glucose uptake is independent of insulin activity as these tissues are exposed to glucose levels that directly correlate with blood glucose levels. These metabolic insults result in alterations in blood flow, endothelial permeability, extravascular protein precipitation, and coagulation, leading to organ dysfunction and microvascular complications [4].

Developing microvascular complications is explained by a variety of theories, including generation of reactive oxygen species and oxidative stress, stimulation of polyol pathway, production of advanced glycation end products,

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initiation of flux through the hexosamine pathway, modified expression and action of growth factors such as vascular endothelial growth factor (VEGF), and triggering of protein kinase C (PKC) [5].

VEGF is a mitogen substance in endothelial cells. It is also known as vasopermeable and vasculotropin factor which is a homodimer glycoprotein, about 45 kDa [6]. Derivatives of VEGF including VEGF-A, VEGF-B, VEGF-C, and VEGF-D and two similar compounds of VEGF coded by *Parapoxvirus* (VEGF-E and VEGF-F), and placenta growth factor (PIGF) were involved in the tissue of myocardium, lungs, spleen, and liver. Each VEGF derivative is functionally and structurally a different substance that is coded by a different gene [7].

*VEGF-A* gene encodes VEGF and is situated on chromosome 6 (6p21.3) with seven introns and eight exons and molecular length of 14 kb [8]. Numerous genetic variations in the *VEGF-A* gene have been identified, some of which influence VEGF secretion and modulate VEGF expression [9]. *VEGF-A* gene polymorphism is associated with several diseases including autoimmune disease [10], metabolic syndrome [11], microvascular complications of diabetes [12], recurrent spontaneous miscarriage [13], polycystic ovary [14], necrotizing enterocolitis [15], and cancers [16].

In a hypoxic state, VEGF-A expression rises. It is important in the pathogenesis of diabetes and associated consequences, especially those related to poor vascularization and hypoxia. In this line, it was discovered that inhibiting VEGF-A signaling caused hypertension, proteinuria, and kidney damage in diabetic patients [12].

*VEGF-A* SNPs comprised numerous variants such as rs699947, rs833061, rs1570360, rs699947, rs2010963, rs3025020, and rs3025039. Many studies reported that *VEGF-A* rs3025020 and rs3025039 had a tendency to be a risk factor for developing microvascular complications in diabetic patients.

Therefore, the current study aimed to investigate the role of *VEGF-A*, -583C > T (rs3025020), and + 936 C/T (rs3025039) gene polymorphisms in predicting mixed microvascular complications in T2DM patients.

## Methods

This hospital-based case–control study was conducted in Clinical Pathology Department, Faculty of Medicine Menoufia University from November 2020 to May 2021. Twenty-six T2DM patients with mixed microvascular complications (more than one complication) were selected from the Internal Medicine Outpatient Clinics and Inpatient Departments. Moreover, 26 apparently healthy individuals, age- and gender-matched with patients' group, were included as a control group. Informed written consent was attained from all subjects, and the study was approved by the Research Ethics Committee of Medical Research, Faculty of Medicine, Menoufia University.

All patients enrolled in the study had T2DM (diagnosed based on clinical and laboratory features, as per the 2020 WHO criteria for diabetes classification and diagnosis). Those with other types of diabetes and autoimmune diseases were excluded. None of the patients ever had ketoacidosis. All groups were subjected to the following examinations and testing:

*Clinical Examination*: Neurological and fundus examination were included.

*Laboratory investigations:*

*Blood Sample*: 5 ml of venous blood was aseptically collected and divided as follows: 2 ml was collected into an ethylenediaminetetraacetic acid (EDTA) tube and then divided into two aliquots for HbA1C assay and *VEGF-A* genotyping. The remaining 3 ml was collected in a plain tube for biochemical laboratory investigations.

*Urine Specimen*: Random urine samples were collected to estimate urine creatinine and albumin and calculate albumin/creatinine ratio (ACR).

*Laboratory Tests*: Fasting blood glucose (FBG), lipid profile (triglycerides (TG)), total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), blood urea nitrogen (BUN), creatinine, and uric acid were measured using AU680 analyzers (Beckman Coulter, Indianapolis, USA). Glycated hemoglobin (HbA1C%) was assayed by Roche Diagnostics (Mannheim, Germany). eGFR was calculated according to the Modification of Diet in Renal Disease (MDRD) formula (GFR (mL/min/1.73 $m^2$) = 186 × [serum creatinine] − 1.154 × [age] − 0.203 × [1.212 if black] × [0.742 if female] [17]. For ACR, creatinine was assayed by AU680. Albumin level was determined by an immunoturbidimetric method using the HEALES albumin test kit (HEALES, Shenzhen Huisong Technology Development, China); then, urinary ACR (milligram/gram (mg/g)) was calculated [18].

*Serum VEGF Assay*: Detection of serum VEGF-A levels was performed through the use of a human vascular endothelial growth factor A (VEGF-A) ELISA Kit (Sunlong Biotech Co., Ltd., China) according to the manufacturer's instructions.

### VEGF-A genotyping using real-time PCR technique

1. *Genomic DNA Extraction* [19]: Total genomic DNA was isolated from peripheral blood lymphocytes of all patients using Thermo Scientific Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA). Then, DNA concentration

and purity were measured spectrophotometrically through the use of the Nanodrop (Implen NanoPhotometerTM N60 UV/VIS spectrophotometer, Rödermark, Germany). The extracted DNA was stored at − 80 °C until performing the genotyping.

2. *TaqMan SNP Assay* [20]: Allelic discrimination method was utilized for *VEGF-A* genotyping. TaqMan assays were ordered from TaqMan probes supplied by Thermo Fisher Scientific, USA: rs3025020 (C_1647366_10) fluorescent-labeled probes **[VIC/VAM]** GCCTCTGGAGGGGAGCCCCCTATTC[C/T]GGCCCAACCCATGGCACCCACAGAG and rs3025039 (C_16198794_10) fluorescent-labeled probes **[VIC/VAM]** GCATTCCCGGGCGGGTGACCCAGCA[C/T] GGTCCCTCTTGGAATTGGATTCGCC. The reaction was performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA). 20 µl reaction volume was prepared by adding 1.25 µl of the probe, 10 µl of Master Mix, and 0.75 µl of DNAase-free water to 8 µl of DNA template from each sample and 8 µl of nuclease-free water for the negative control. PCR conditions were as follows: Initial denaturation was done at 95 °C for 15 min, followed by 50 cycles of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min, then primer extension at 74 °C for 2 min, and the final extension step at 72 °C for 1 min. The allele distribution and amplification plots are demonstrated in Figs. 1 and 2.

### Statistical analysis

Statistical Package for Social Sciences, version 19 (SPSS Inc., Chicago, USA), was utilized for analyzing data. Qualitative data (frequency and percentage) were expressed using chi-square test, while quantitative data were expressed using mean, standard deviation (SD), and median. Mann–Whitney test was used for two nonparametric variables between two groups. Difference between more than two groups that are normally distributed and not normally distributed was demonstrated using analysis of variance (ANOVA) and Kruskal–Wallis tests, respectively, followed by post hoc test. Spearman correlation evaluates the strength and direction of association between number of minor alleles of both genetic variants and measured parameters. Odds ratio measures the risk of carrying minor allele as a predictor for complications. Receiver operating characteristic (ROC) curve is created by plotting sensitivity (TP) on *Y* axis versus 1—specificity (FP) on *X* axis at various cutoff values. The diagnostic performance of a test is measured by the area under the

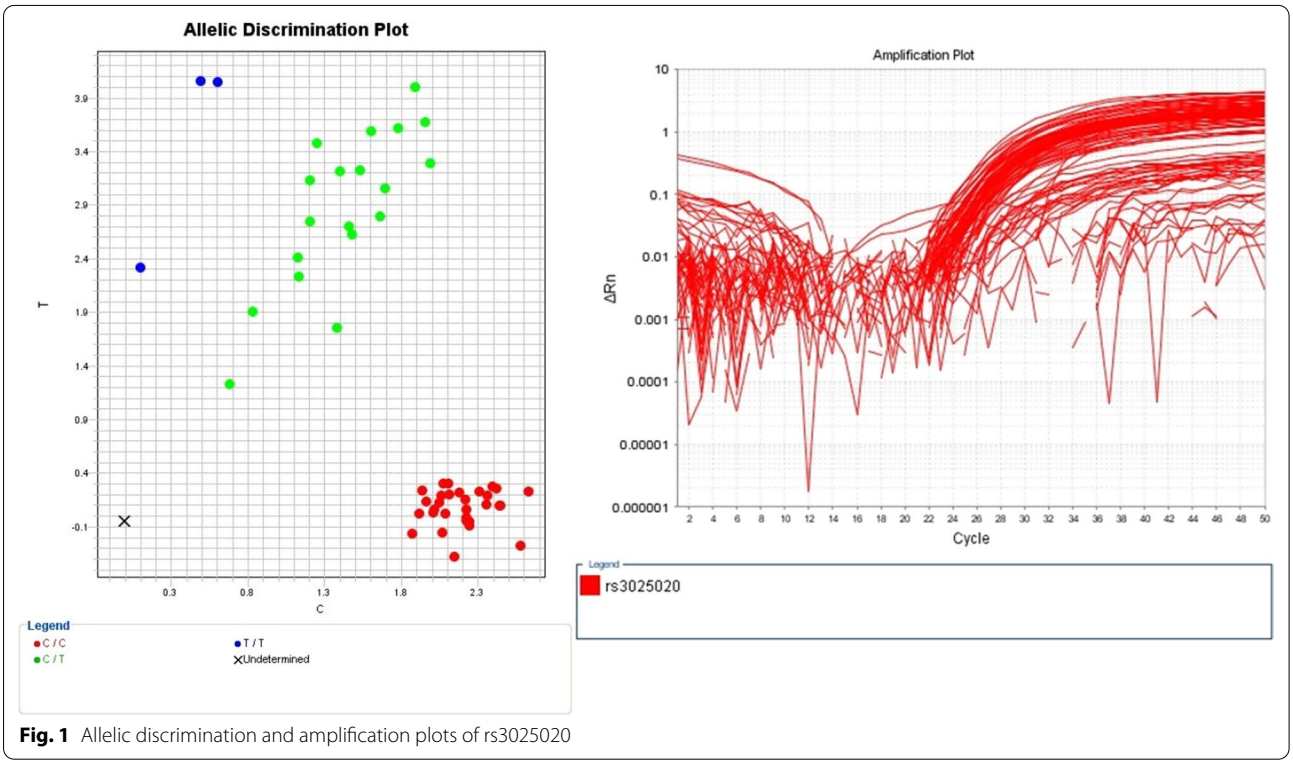


**Fig. 1** Allelic discrimination and amplification plots of rs3025020

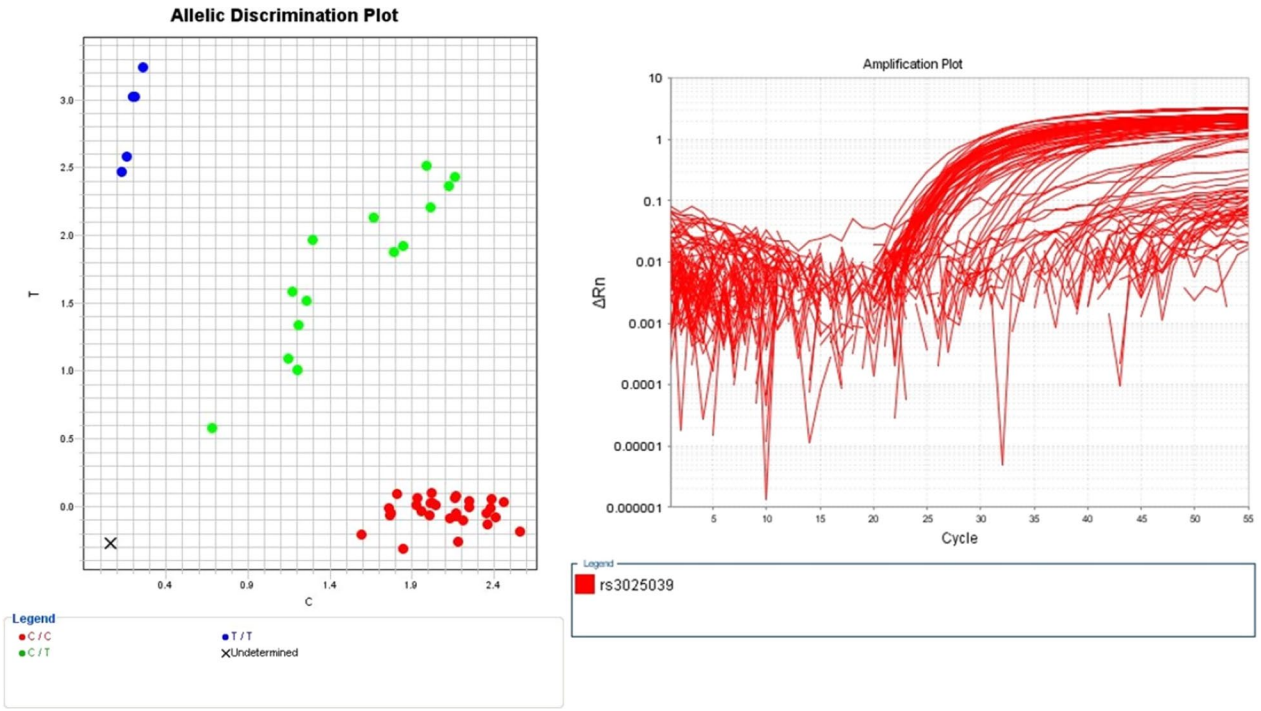


**Fig. 2** Allelic discrimination and amplification plots of rs3025039

**Table 1** Demographic and clinical data of studied groups

| Parameters | Mixed complications $N=26$ | Control $N=26$ | Test | *P* value |
|---|---|---|---|---|
| Age (years) | | | t = | |
| Mean ± SD | 63.31 ± 7.91 | 60.31 ± 4.63 | 1.67 | 0.10 |
| Range | 47–87 | 51–69 | | |
| Sex | | | $X^2$ = | |
| Male | 15 (57.7) | 13 (50.0) | | |
| Female | 11 (42.3) | 13 (50.0) | 0.31 | 0.58 |
| Age of onset of DM (year) | | | | |
| Mean ± SD | 54.88 ± 7.37 | | | |
| Range | 40–71 | – | --- | --- |
| Disease's duration (years) | | | | |
| Mean ± SD | 9.92 ± 4.36 | | | --- |
| Range | 3–20 | – | --- | |
| Neurological examination | | | $X^2$ = | |
| Free | 3 (11.5%) | 26 (100%) | 143.7 | < 0.001 |
| Affected | 23 (88.5%) | 0 (0.0%) | | |
| Fundus examination | | | $X^2$ = | |
| Free | 11 (42.3%) | 26 (100%) | 123.2 | < 0.001 |
| Affected | 15 (57.6%) | 0 (0.0%) | | |

*N*: numbers, *$X^2$ = chi-squared test, *t* = student *t* test, DM: diabetes mellitus

ROC curve (AUC). A $p$ value < 0.05 was considered significant.

## Results

The demographic clinical data of the studied groups (Table 1) demonstrated that the groups were age- and

gender-matched. In T2DM patients, the duration of disease ranged from 3 to 20 years. 88.5% of patients had neurological symptoms, while 57.6% had fundus affection.

Laboratory investigations of the studied groups (Table 2) indicated that FBG, HbA1c%, total cholesterol, LDL-c, albumin/creatinine ratio, creatinine, BUN, and uric acid were statistically significantly higher, while eGFR and HDL-c were statistically significantly lower in T2DM patients with mixed microvascular complications than in control group ($p < 0.001$). Furthermore, VEGF-A was significantly increased in mixed complications group in comparison with control group ($p$ 0.003). Moreover, there was no statistically

**Table 2** Laboratory investigations of studied groups

| Parameters | Mixed complications $N = 26$ | Control $N = 26$ | Test | *P* value |
|---|---|---|---|---|
| FBG (mg/dl) | | | t = | |
| Mean ± SD | 298.0 ± 74.3 | 85.6 ± 15.6 | 14.27 | < 0.001 |
| Median (range) | 297.5 (145–440) | 88 (18–100) | | |
| HbA1c % | | | t = | |
| Mean ± SD | 8.9 ± 1.3 | 5.0 ± 0.6 | 14.19 | < 0.001 |
| Median (range) | 8.7(6.9–12.8) | 5.0 (4–6) | | |
| Cholesterol (mg/dl) | | | t = | |
| Mean ± SD | 205.7 ± 26.4 | 153.5 ± 21.2 | 7.87 | < 0.001 |
| Median (range) | 200 (159–270) | 155.5 (110–180) | | |
| TG (mg/dl) | | | t = | |
| Mean ± SD | 134.2 ± 17.8 | 124.0 ± 22.9 | 1.79 | 0.08 |
| Median (range) | 136.5(73–150) | 121(100–190) | | |
| HDL-c (mg/dl) | | | t = | |
| Mean ± SD | 37.5 ± 2.2 | 40.3 ± 4.0 | 3.05 | 0.004 |
| Median (range) | 37 (35–43) | 40 (34–53) | | |
| LDL-c (mg/dl) | | | t = | |
| Mean ± SD | 135.5 ± 25.3 | 98.2 ± 10.3 | 6.97 | < 0.001 |
| Median (range) | 133 (106–208) | 101(69–112) | | |
| ACR (mg/gm creatinine) | | | | |
| Mean ± SD | | | | < 0.001 |
| Mean ± SD | 730.1 ± 285.5 | 14.9 ± 4.9 | U = | |
| Median (range ) | 781 (110–1150) | 14.5(7–23) | 6.19 | |
| Creatinine (mg/dl) | | | | |
| Mean ± SD | 4.7 ± 2.5 | 0.88 ± 0.12 | U = | < 0.001 |
| Median (range) | 5.55 (0.9–10) | 0.90 (0.6–1.1 | 5.92 | |
| BUN (mg/dl) | | | U = | |
| Mean ± SD | 56.0 ± 17.3 | 13.1 ± 3.8 | | < 0.001 |
| Median (range) | 62 (11–71) | 12.5 (8–20) | 5.6 | |
| Uric acid (mg/dl) | | | t = | |
| Mean ± SD | 7.4 ± 1.8 | 4.5 ± 1.2 | 6.88 | < 0.001 |
| Median (range) | 12.6 (3.5–9.6) | 4.65 (2.7–6.5) | | |
| eGFR (ml/min/1.73m$^2$) | | | U = | < 0.001 |
| Mean ± SD | 20.5 ± 24.5 | 100.2 ± 13.0 | | |
| Median (range) | 7.9 (5.3–99.2) | 4.65 (71.9–133.7) | 5.92 | |
| VEGF-A (pg/ml) | | | | |
| Mean ± SD | 229.1 ± 279.1 | 139.0 ± 61.0 | U = | 0.003 |
| Median (range) | 132.5 (60–1350) | 117.5 (66–290) | 4.53 | |

*FBS* fasting blood sugar, *HbA1c* hemoglobin A1C, *TG* triglyceride, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *BUN* blood urea nitrogen, *ACR* albumin/creatinine ratio, *eGFR* estimated glomerular filtration rate, *VEGF-A* vascular endothelial growth factor A

*$X^2$ = chi-squared test, *t* = Student's t test, *U* = Mann–Whitney test, **P* value ≤ 0 .05 is significant, ***P* value ≤ 0.001 is highly significant

significant difference between the two groups regarding TG ($p$ 0.08).

Regarding *VEGF-A* rs3025020 and rs3025039 polymorphism analysis (Table 3), there was no significant difference in rs3025020 genotypes distribution among the studied groups. T allele had a significantly high frequency in T2DM patients with mixed complications [OR 2.67; 95% CI 1.03–6.91; $p=0.04$]. According to *VEGF-A* rs3025039 distributions, C/T genotype and T allele had a significantly high frequency in T2DM patients with mixed complications [OR 4.08; 95% CI 1.32–17.44; $p=0.01$ and OR 4.02; 95% CI 1.52–10.63; $p=0.004$, respectively] (Fig. 3).

Regarding relationship between rs3025020 and rs3025039 genotypes and different studied parameters in both T2DM patients with mixed diabetic complications (Table 4) and control groups (Table 5), there was a significant difference between both rs3025020 and rs3025039 genotypes in mixed complications group and age of patients ($p=0.01$ in both genes). Moreover, there was a significant difference between rs3025039 genotypes and T2DM duration ($p=0.04$). No significant statistical differences between different genotypes and serum VEGF-A level in different groups were detected.

ROC curve analysis of serum level of VEGF-A (Fig. 4) for differentiation between mixed complication DM cases and controls revealed that AUC was 0.55 and *P* value was 0.53, at cutoff point 117.5, sensitivity 64.5, specificity 50%, and total accuracy 57.7%.

## Discussion

T2DM is a chronic persistent hyperglycemia that results in an increase in glycosylation products. These products cause vascular tissue alterations and promote atherosclerosis by generating inflammation and damage to arterial walls. Atherosclerosis damages smaller blood arteries, leading to microvascular problems such as diabetic peripheral neuropathy, retinopathy, and nephropathy [21].

VEGF is a secreted mitogen that is extremely specific for vascular endothelial cells and has been linked to cell proliferation and migration. VEGF has long been thought to be a key determinant and regulator of angiogenesis, vasculogenesis, and vascular permeability as a multifunctional cytokine. A number of studies have confirmed that *VEGF-A* plays a key role in the pathogenesis of diabetic microvascular complications [22]. In the current study, the role of *VEGF-A* and two genetic variants, -583C>T (rs3025020) and +936 C/T (rs3025039), was investigated for prediction of mixed microvascular complications in type 2 diabetic patients.

This study reported a significant difference in biochemical investigations between diabetic and control groups. T2DM patients with mixed microvascular complications had poor glycemic control as reflected in their high FBG and HbA1c%. Furthermore, diabetic patients had poor lipid control, with high level of kidney functions, ACR, and significant decrease in eGFR and HDL-c. These results were in agreement with those of Omar et al. [23] who reported significant difference in biochemical results

**Table 3** *VEGF-A* rs3025020 and rs3025039 distribution and odds ratio assessment between control and T2DM with mixed complications groups

| Parameters | Mixed complications *N* = 26 No (%) | Control # *N* = 26 No (%) | Test | *P* value | Odds ratio | 95% CI |
|---|---|---|---|---|---|---|
| rs3025020 genotypes | | | | | | |
| CC* | 12 (46.2) | 18 (69.2) | – | – | Ref (1.0) | |
| CT | 11 (42.3) | 8 (30.8) | 1.5 | 0.22 | 2.06 | 0.64–6.63 |
| TT | 3 (11.5) | 0 (0.0) | 1.91 | 0.17 | – | – |
| rs3025020 alleles | | | | | | |
| C* | 35 (67.3) | 44 (84.6) | | | | |
| T | 17 (32.7) | 8 (15.4) | 4.27 | 0.04 | 2.67 | 1.03–6.91 |
| rs3025039 genotypes | | | | | | |
| CC* | 10 (38.5) | 20 (76.9) | – | – | Ref (1) | |
| CT | 12 (46.2) | 5 (19.2) | 6.05 | 0.01 | 4.08 | 1.32–17.44 |
| TT | 4 (15.4) | 1 (3.8) | 2.19 | 0.14 | 8.0 | 0.79–81.33 |
| rs3025039 alleles | | | | | | |
| C* | 32 (61.5) | 45 (86.5) | | | Ref (1) | |
| T | 20 (38.5) | 7 (13.5) | 8.45 | 0.004 | 4.02 | 1.52–10.63 |

Test: chi-squared test, # = reference group, *reference category, CI = confidence interval

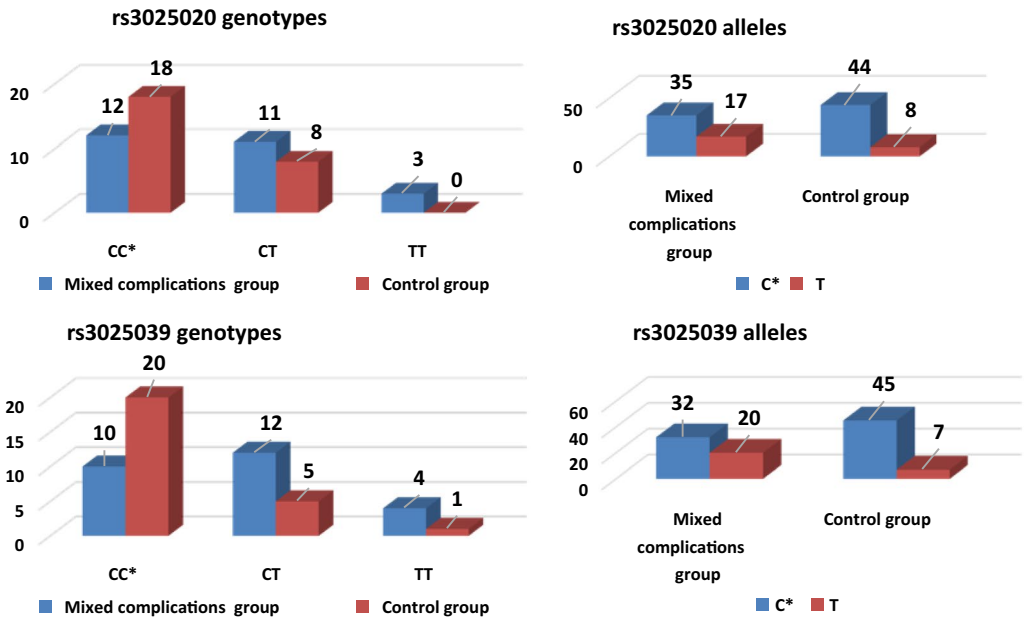


**Fig. 3** *VEGF-A* rs3025020 and rs3025039 genotypes and alleles distribution among the studied groups

**Table 4** Relationship between *VEGF-A* rs3025020 and rs3025039 genotypes and different studied parameters inT2DM with mixed diabetic complications

| Mixed complications | rs3025020 genotypes | | | | rs3025039 genotypes | | | |
|---|---|---|---|---|---|---|---|---|
| | CC *N* = 12 | CT *N* = 11 | TT *N* = 3 | *P* value | CC *N* = 10 | CT *N* = 12 | TT *N* = 4 | *P* value |
| Parameters | Mean ± SD | Mean ± SD | Mean ± SD | *K* | Mean ± SD | Mean ± SD | Mean ± SD | *K* |
| Age (years) | 67.6 ± 8.9 | 60.9 ± 5.5 | 66.7 ± 6.1 | 0.01 | 59.9 ± 8.1 | 69.2 ± 4.2 | 63.0 ± 9.1 | 0.01 |
| Age of onset (years) | 57.8 ± 8.3 | 51.5 ± 5.1 | 56.0 ± 7.2 | 0.09 | 51.2 ± 7.0 | 57.3 ± 5.1 | 56.8 ± 11.6 | 0.12 |
| Disease's duration (Years) | 10.2 ± 4.8 | 6.5 ± 4.7 | 10.7 ± 1.2 | 0.80 | 9.1 ± 5.0 | 11.8 ± 3.3 | 6.3 ± 2.9 | 0.04 |
| FBG (mg/dl) | 290.7 ± 76.4 | 317.3 ± 78.5 | 256.7 ± 34.1 | 0.39 | 297.1 ± 88.6 | 284.3 ± 65.0 | 341.3 ± 60.9 | 0.45 |
| HbA1c % | 9.1 ± 1.4 | 9.0 ± 1.3 | 8.1 ± 0.2 | 0.39 | 9.4 ± 1.5 | 8.6 ± 0.9 | 8.9 ± 1.7 | 0.54 |
| Cholesterol (mg/dl) | 208.2 ± 29.5 | 204.5 ± 27.6 | 200.0 ± 0.0 | 0.77 | 201.5 ± 28.9 | 201.7 ± 20.7 | 228.0 ± 30.6 | 0.27 |
| TG (mg/dl) | 130.3 ± 22.0 | 135.9 ± 14.3 | 143.7 ± 7.8 | 0.49 | 135.2 ± 17.2 | 134.3 ± 21.5 | 131.5 ± 8.3 | 0.64 |
| HDL-C (mg/dl) | 37.5 ± 1.7 | 37.3 ± 2.4 | 38.7 ± 3.5 | 0.70 | 37.3 ± 1.1 | 38.1 ± 2.8 | 36.5 ± 1.9 | 0.53 |
| LDL-C (mg/dl) | 133.0 ± 26.6 | 139.2 ± 28.3 | 132.3 ± 2.3 | 0.82 | 131.0 ± 25.2 | 134.3 ± 18.7 | 150.5 ± 42.3 | 0.58 |
| ACR (mg/gm creatinine) | 764.4 ± 214.0 | 729.1 ± 336.9 | 597.0 ± 407.4 | 0.83 | 729.1 ± 210.5 | 741.4 ± 321.2 | 699.0 ± 408.0 | 0.82 |
| Creatinine (mg/dl) | 5.7 ± 1.7 | 5.8 ± 3.0 | 5.0 ± 3.9 | 0.92 | 6.0 ± 1.9 | 5.5 ± 2.7 | 5.3 ± 3.6 | 0.95 |
| BUN (mg/dl) | 59.9 ± 9.5 | 54.7 ± 20.4 | 45.3 ± 30.1 | 0.69 | 59.2 ± 8.8 | 54.8 ± 21.0 | 52.0 ± 24.1 | 0.98 |
| Uric acid (mg/dl) | 7.7 ± 1.3 | 7.2 ± 2.0 | 6.6 ± 2.6 | 0.83 | 7.4 ± 1.4 | 7.4 ± 2.0 | 7.0 ± 2.2 | 0.92 |
| eGFR (ml/min/1.73 m2) | 12.7 ± 3.5 | 25.5 ± 32.5 | 33.3 ± 37.8 | 0.83 | 12.5 ± 4.8 | 22.7 ± 26.3 | 33.6 ± 44.1 | 0.92 |
| VEGF-A(pg/ml) | 167.9 ± 157.1 | 292.4 ± 393.2 | 241.7 ± 142.2 | 0.40 | 136.0 ± 45.5 | 245.4 ± 219.9 | 412.8 ± 625.2 | 0.83 |

*K* Kruskal–Wallis test, *FBS* fasting blood sugar, *HbA1c* hemoglobin A1C, *TG* triglyceride, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *BUN* blood urea nitrogen, *ACR* albumin/creatinine ratio, *eGFR* estimated glomerular filtration rate, *VEGF* vascular endothelial growth factors

between diabetic and control groups. T2DM patients had a high FBG and HbA1c%, demonstrating that HbA1c is a good indicator of glycemic status. Impaired glycemic control in DM plays a crucial role in rapid progression of microvascular complications caused by variable hemodynamic, metabolic, or endothelial dysfunctions.

**Table 5** Relationship between *VEGF-A* rs3025020 and rs3025039 genotypes and different studied parameters in control group

| Control group | rs3025020 genotypes | | | rs3025039 genotypes | | |
|---|---|---|---|---|---|---|
| | CC *N* = 18 | CT *N* = 8 | *P* value | CC *N* = 20 | CT& TT *N* = 6 | *P* value |
| Parameters | Mean ± SD | Mean ± SD | U | Mean ± SD | Mean ± SD | *U* |
| Age (years) | 59.78 ± 5.22 | 61.5 ± 2.83 | 0.40 | 61.1 ± 4.13 | 57.66 ± 5.61 | 0.18 |
| FBG (mg/dl) | 86.44 ± 18.5 | 83.62 ± 5.45 | 0.09 | 85.95 ± 17.60 | 84.33 ± 5.92 | 0.24 |
| HbA1c % | 4.93 ± 0.50 | 5.28 ± 0.67 | 0.16 | 5.05 ± 0.55 | 5.0 ± 0.69 | 0.98 |
| Cholesterol (mg/dl) | 152.94 ± 24.27 | 154.27 ± 12.81 | 0.81 | 156.15 ± 18.33 | 144.67 ± 28.95 | 0.46 |
| TG (mg/dl) | 126.61 ± 25.35 | 118.13 ± 16.19 | 0.46 | 124.35 ± 24.93 | 122.83 ± 16.42 | 0.88 |
| HDL-C (mg/dl) | 41.05 ± 4.14 | 38.5 ± 3.29 | 0.14 | 40.85 ± 4.26 | 38.33 ± 2.42 | 0.16 |
| LDL-C (mg/dl) | 98.0 ± 11.32 | 98.63 ± 8.07 | 0.77 | 96.20 ± 10.74 | 104.83 ± 4.44 | 0.051 |
| ACR (mg/gm creatinine) | 14.82 ± 5.05 | 14.99 ± 4.79 | 0.98 | 15.10 ± 4.45 | 14.12 ± 6.54 | 0.66 |
| Creatinine (mg/dl) | 0.87 ± 0.14 | 0.90 ± 0.08 | 0.57 | 0.88 ± 0.11 | 0.87 ± 0.18 | 0.88 |
| BUN (mg/dl) | 13.56 ± 3.91 | 12.13 ± 3.40 | 0.34 | 13.25 ± 3.63 | 12.67 ± 4.50 | 0.53 |
| Uric acid (mg/dl) | 4.54 ± 1.23 | 4.44 ± 1.15 | 0.85 | 4.63 ± 1.24 | 4.12 ± 0.98 | 0.39 |
| eGFR (ml/min/1.73 m2) | 100.07 ± 14.41 | 100.36 ± 9.91 | 0.89 | 98.55 ± 12.27 | 105.5 ± 15.07 | 0.49 |
| VEGF-A (pg/ml) | 150.5 ± 66.99 | 113.13 ± 36.07 | 0.24 | 134.2 ± 58.66 | 155.0 ± 71.76 | 0.42 |

*U* Mann–Whitney test, *FBS* fasting blood sugar, *HbA1c* hemoglobin A1C, *TG* triglyceride, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *BUN* blood urea nitrogen, *ACR* albumin/creatinine ratio, *eGFR* estimated glomerular filtration rate, *VEGF-A* vascular endothelial growth factor A

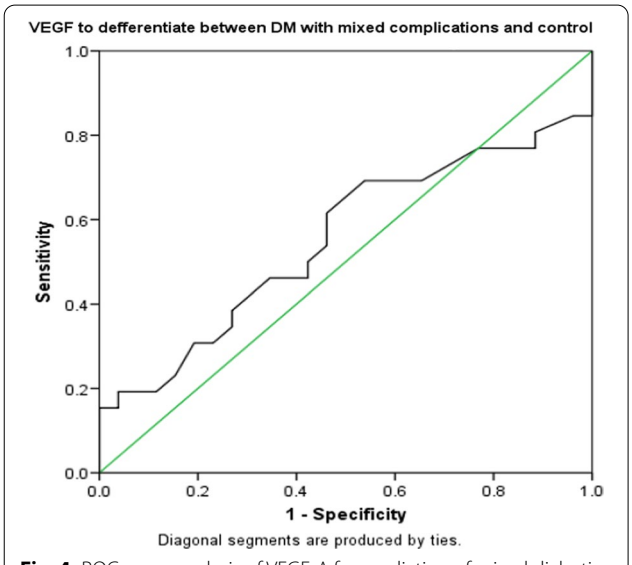


**Fig. 4** ROC curve analysis of VEGF-A for prediction of mixed diabetic complications

Furthermore, Sellami et al. [24] indicated that T2DM patients had higher FBG, HbA1c, total cholesterol, and LDL-C than control group.

Serum level of VEGF-A was significantly increased in T2DM patients with mixed microvascular complications in comparison with the control group. This result was confirmed by Mahdy et al. [25] who found a considerable rise in serum VEGF in diabetic patients with various micro- and macrovascular complications compared to the uncomplicated diabetic patients and control subjects. Biswas et al. [26] discovered that elevated VEGF-A level was a risk factor for the presence and severity of vascular complications in diabetic patients, and VEGF-A concentrations tend to increase with the progression of mixed diabetic microvascular complications. In addition, Zhang et al. [27] reported that hyperglycemia, inflammation, and VEGF have all been associated with microvascular disorders in T2DM patients, and higher circulating levels of VEGF have played a role in the pathogenesis of T2DM microangiopathy. Sharma et al. [28] detected increased serum VEGF-A level in diabetic groups compared to control subjects, and the level further increased with increasing duration of disease.

There was no significant difference in *VEGF-A* rs3025020 genotypes distribution among the studied groups. T allele had a significantly high frequency in T2DM patients with mixed complications. Regarding *VEGF-A* rs3025039 distributions, C/T genotype and T allele had a significantly high frequency in T2DM patients with mixed complications. These results were in agreement with those of Sun et al. [29] who confirmed that *VEGF-A* rs3025039 was associated with susceptibility to diabetic nephropathy in T2DM patients. A possible explanation is that such polymorphism in the VEGF gene boosted its expression, which was hypothesized to play a critical role in podocyte injury by operating in a unique autocrine signaling manner to produce diabetic podocytopathy and the genesis of albuminuria associated

with diabetic nephropathy. Moreover, Arredondo-Garc et al. [30] concluded that the *VEGF-A* rs3025039 genetic variants were related to diabetic neuropathy in Mexican T2DM patients. Kafeel et al. [31] confirmed significant association of C/T *VEGF-A* genetic variant at the + 936 position (rs3025039) with an increased diabetic retinopathy risk among the Asian population. Imbaby et al. [32] reported that T2DM patients showed a statistically significant higher frequency of *VEGF*-936 (rs3025039) C/T genotype compared to controls and lower CC genotype frequency, but this was not statistically significant. Furthermore, the frequency of T allele was higher among patients than controls, but this was not statistically significant. However, Yari et al. [33] studied the relationship between *VEGF* rs3025039 gene variant and the risk of T2DM and showed no significant association between *VEGF* rs3025039 variant and T2DM.

Studying the relationship between rs3025020 and rs3025039 genotypes and different studied parameters in both mixed diabetic complications and control groups showed significant difference between rs3025020 and rs3025039 genotypes in mixed complications group and age of patients. Additionally, there was a significant difference between rs3025039 genotypes in mixed complications group and T2DM duration. However, there was no significant difference with other studied parameters in mixed complications and control groups. Handoko et al. [34] indicated that the length of sickness had an effect on the microvascular complications in T2DM patients. This was in accordance with a study conducted by Abu Al-Halaweh et al. [35] who observed substantial link between microvascular problems and diabetes duration. However, Nanayakkara et al. [36] stated that diabetes duration and age at diagnosis were independently linked to a higher incidence of microvascular problems, and vice versa.

In the present study, ROC analysis of VEGF-A yields minimal clinical significance between groups, as it revealed that AUC was 0.55, statistically insignificant with $p$ value 0.53, and the best cutoff point was 117.5 pg/ml. At this cutoff, the sensitivity was 64.5, specificity was 50%, and total diagnostic accuracy was 57.7%. These results were quite different from those stated by Ahuja et al. [37] as their results indicated that ROC for serum VEGF-A levels was significant in distinguishing between cases and controls and had high accuracy in discriminating between subjects with and without retinopathy. The AUC for discrimination was 0.858 and 0.791 for cases and controls, respectively, with over 90% sensitivity and specificity at various cutoff levels. This agreement could be explained by the smaller number of cases in our study and the other factors that could affect VEGF-A serum level.

In a previous study reported by El-Deeb et al. [38], there was a difficulty in selecting diabetic patient with microvascular complication at specific single organ site like diabetic patient with only diabetic nephropathy or only diabetic retinopathy, etc. We did not face this limitation as our cases were diabetic patients with mixed microvascular complications. However, despite the comprehensive analysis of the association between *VEGF-A* gene polymorphisms and mixed diabetic microvascular complications risk, our analysis still had limitations. First, the mixed diabetic microvascular complications etiology is complex and multifactorial. The relationships between *VEGF-A* gene polymorphisms and other risk factors were not analyzed in our study, such as environmental factors, diet, and exercise. Second, other *VEGF* gene polymorphisms were not analyzed in this study.

## Conclusion

VEGF-A increased the level contributed in the pathogenesis of mixed diabetic complications. Moreover, CT genotype, T allele of rs3025039, and T allele of rs3025020 had a significantly high frequency in T2DM patients with mixed complications. Therefore, VEGF-A serum level and *VEGF-A* rs3025039 and rs3025020 genetic variants could predict mixed microvascular diabetic complications.

Further studies including a larger sample size, haplotyping analysis of *VEGF-A* rs3025039 and rs3025020, and more *VEGF-A* gene SNPs are necessary to achieve more conclusive results about the association between *VEGF-A* gene polymorphisms and mixed diabetic microvascular complications susceptibility.

#### Abbreviations

ACR: Albumin/creatinine ratio; eGFR: Estimated glomerular filtration rate; FBG: Fasting blood glucose; HbA1c: Glycated hemoglobin A1C; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; T2DM: Type 2 diabetes mellitus; VEGF: Vascular endothelial growth factor.

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None.

#### Author contributions

TO designed the protocol of work, wrote the abstract, and discussed the manuscript. GE contributed to selecting idea of study and step-by-step supervision of the research. SK wrote introduction and results section of the manuscript. AD collected samples from endocrine unite of internal medicine department and helped in writing introduction of manuscript. SE edited the manuscript. AS contributed to practical part of the study (real-time PCR). BM helped in methodology writing, editing of manuscript, and final revision. All authors have read and approved the manuscript.

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#### Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

## Declarations

### Ethical approval and consent to participate

This research was approved by the Research Ethics Committee at Menoufia Faculty of Medicine according to 1964 Helsinki Declaration, and informed written consent was taken from every participant in the study; the committee's reference number is 32019PATH51.

### Consent for publication

Consent to publish from the patient had been taken.

### Competing interests

The authors declare that they have no competing interest.

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