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Original article

Impact of the serum epidermal growth factor polymorphism rs4444903 and gene expression in PBMCs on glycemic control in type 2 diabetes mellitus

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Abstract. *Background:* Poor glycemic control is a major concern in type 2 diabetes mellitus (T2DM) patients and is associated with a higher risk of complications and increased mortality. The role of EGF in maintaining pancreatic beta cells is well-established, however, there is a lack of evidence regarding the association between EGF and glycemic control. This study aimed to investigate the association between rs4444903 polymorphism of the EGF gene, EGF gene expression, and EGF levels with T2DM. *Methods:* This was a two-phase case-control study. The first phase included 330 patients with good or poor glycemic control (1:1 ratio). The second phase included 42 patients per group. Serum EGF protein was measured using ELISA. EGF rs4444903 genotype was determined using PCR-RFLP. EGF mRNA in PBMCs was quantified using RT-qPCR. *Results:* Poor glycemic control was associated with lower serum EGF levels. GA genotype of rs4444903 was more frequent among the poor control group (P<0.05). Regression analysis demonstrated that EGF lowered the risk of poor glycemic control (OR=0.99, 95%CI:0.98-0.99, P<0.001) and GA genotype was associated with a two-fold increase in poor control risk (OR=1.96, 95%CI:1.02-3.57, P=0.04). Poor control patients had a 40% reduction in EGF mRNA in their PBMCs (P <0.05). Lower EGF expression was associated with poor glycemic control (OR=3.05, 95%CI:1.10-8.44, P=0.03).*Conclusions*: This study provides evidence that low serum EGF levels, decreased EGF mRNA in PBMCs, and the GA genotype of the rs4444903 are associated with poor glycemic control in T2DM. Further research is warranted to investigate the clinical implications/ applications of this association. (www.actabiomedica.it)

Key words: EGF, rs4444903 SNP, glycemic control, gene expression, peripheral blood mononuclear cells, type 2 diabetes mellitus, case-control study

Introduction

Diabetes mellitus (DM) is a chronic health problem characterized by disturbance in the metabolism of all major classes of macromolecules (carbohydrates, fats, and proteins) (1). The global prevalence of DM has reached an alarming epidemic level (2). A comparative analysis of the worldwide prevalence of DM indicates a clear disparity in its geographic distribution. For example, the Middle East and North Africa (MENA) region has one of the highest prevalence rates of DM worldwide (3). However, prevalence rates are much lower in the United States and Western Europe (3). Unfortunately, epidemiological models

based on current disease trends indicate that by 2045, 136 million individuals inhabiting the MENA region will have DM (3).

Jordan is part of the MENA region (4). The disease trends of DM in Jordan are not different from those in neighboring countries (3). Recent estimates indicate that Jordan has the 6th highest prevalence of DM among the countries in the region (4). Jordan witnessed a steep increase of 6% in the prevalence of DM between 2004 and 2017 (5). These figures clearly indicate that DM represents a public health challenge and a tentative economic burden on Jordan and the region (4).

Despite the complexity of the disease, two major types of DM are recognized: type 1 DM (T1DM) and type 2 DM (T2DM) (6). The latter type is marked by insulin resistance in the context of a gradual decline in the ability of pancreatic β cells to secrete insulin to meet the demands of peripheral target tissues (6). T2DM is the predominant type of the disease (90–95% prevalence rate) (6).

An increase in fat percentage (i.e., obesity) is believed to be the major factor driving insulin resistance and ensuing disease development and progression (7). Nonetheless, a growing body of literature has demonstrated a much more complex etiology and role of a tortuous network of environmental and genetic factors (6).

The complications of DM are a major source of disease morbidity (8). They also contribute to high mortality rates associated with DM (8). Cerebrovascular, peripheral vascular, and coronary artery diseases are the main macrovascular complications of DM (8). Microvascular complications include retinopathy, nephropathy, and neuropathy (8). Notably, peripheral vascular damage and peripheral neuropathy often result in compromised tissue perfusion and impaired wound healing (9); which represents another major source of patient morbidity and mortality (10).

Multiple factors are associated with an increased risk of developing DM-related complications including obesity (7) and lack of adherence to treatment protocols (11). Lack of glycemic control is a major modifiable risk factor for DM complications (12). Despite being the cornerstone of DM treatment guidelines, a considerable percentage of patients with DM fail to maintain blood glucose levels within the reference range indicated by the protocol (13). Accordingly, factors associated with failure to achieve glycemic control remain a rich area for investigation.

Epidermal growth factor (EGF) is a potent mitogenic factor secreted by a variety of cells, including epithelial cells (14), immune cells (15), and platelets (16). The EGF protein can also be detected in plasma, urine, and saliva (14). In addition to its well-established role in promoting cellular growth and differentiation (17), EGF is increasingly being recognized for its role in glucose homeostasis (18).

One important function of the EGF resides in its involvement in preserving the pancreatic β-cell mass (19). This is achieved by regulating the levels of survivin, a protein that safeguards against apoptosis under stressful conditions (19). Similarly, EGF has emerged as a secretagogue that modulates plasma glucose levels by enhancing insulin secretion (20).

Not surprisingly, Tian et al. demonstrated a substantial reduction in the serum levels of EGF among patients with T2DM compared to healthy individuals aged ≤ 45 years (21). Another study showed a reduction in the submandibular EGF protein and mRNA levels in mouse models of DM (22). Interestingly, the role of EGF in modifying glycemic control in patients with T2DM has not yet been investigated.

Genetic variation in the sequence of the *EGF* gene could account for differences in EGF levels among individuals or populations (23). For example, a single nucleotide polymorphism (SNP) in the *EGF* gene at position 61 (A/G) is associated with increased EGF protein expression (23). Given that it is hypothesized, herein, that differences in the EGF protein could modify glycemic control in patients with T2DM, it is conceivable that the above SNP (also known as rs4444903) could also be associated with glycemic control; a hypothesis that will be tested in this report.

Notably, rs4444903 was shown to be associated with the risk of T2DM (but not with glycemic control) in a case-control study performed in an Indian population (24). The same study showed a reduction in *EGF* mRNA levels in the mononuclear cells of patients with T2DM compared with those in disease-free individuals (24).

In this study, we proposed that serum levels of EGF could be reduced in patients with poor glycemic control.

We also hypothesized that these differences could be partially explained by genetic variations in the gene that codes for EGF. We also evaluated whether patients with poor glycemic control had lower *EGF* mRNA expression levels in peripheral mononuclear cells.

Methods

Study design and ethical statement

This study consisted of two phases. The first phase included 330 participants in a 1:1 case-control design. Patients with T2DM and poor glycemic control (n=165) were selected as study cases, whereas patients with T2DM and good glycemic control matched by age, sex, and body mass index (BMI) were selected as controls. The associations of glycemic control with serum EGF and the rs4444903 variant of the *EGF* gene were tested in this phase. This population was previously used to establish an association between serum leptin, the rs2167270 variant of the *LEP* gene, and glycemic control (25). The study design, patient recruitment protocol including eligibility criteria, and glycemic control definition have been described in detail by Alfaqih et al. (25).

In the second phase, 84 participants were recruited in a 1:1 case-control design using the same case definition. The aim of this phase was to examine the transcript levels of the *EGF* gene in relation to glycemic control. Cases and controls in this phase were matched for age and BMI.

The participants in the second phase of the study were recruited from the endocrinology clinic of King Abdullah University Hospital (KAUH) between January and May 2023. The inclusion criteria were as follows: (a) diagnosis of T2DM within the last 15 years according to the American Diabetes Association (ADA) diagnostic criteria, (b) Jordanian descent, and (c) active treatment for T2DM with either metformin or a combination of metformin and another hypoglycemic agent (glipizide, rosiglitazone, or repaglinide). The exclusion criteria were as follows:(a) active treatment with insulin of any form or (b) the presence of any vascular complication, as indicated by the medical history of the patient.

The study protocols were approved by the Institutional Review Board (IRB) of Jordan University of Science and Technology (JUST; Irbid, Jordan; approval ID #25/155/2023). Informed consent was obtained from all study participants before enrollment. All study procedures were in accordance with the Ethical Principles for Medical Research Involving Human Subjects outlined in the Declaration of Helsinki in 1975.

Glycemic control definition

Patients were assigned to either case or control groups based on their HbA1c levels at the time of recruitment. Patients with HbA1c of 7% or above were considered to have poor glycemic control, while those with HbA1c of less than 7% were considered to have good control. This definition is consistent with the American Diabetes Association (ADA) guidelines (26). Note that the same definition was applied in both phases of this study.

Data collection and anthropometric measurements

Details of how demographic data were collected and how anthropometric measurements were made for the patients included in phase one of the study have been previously described (25). The following describes the details related to the second phase of the study. Before enrolling the patients in the second phase, the study objectives were explained to them. Individuals who met the eligibility criteria described in the previous section were included in the study. A short personal interview preceded anthropometric measurements. During the interviews, demographic data such as age, sex, and previous medical and drug histories were collected. Data related to medical and drug histories were checked for validity in the patients' electronic records. Anthropometric measurements included height (measured in centimeters [cm]), weight (measured in kilograms [kg]), and waist circumference (WC) (measured in centimeters [cm]).

For weight measurement, the same digital weighing scale was used for all participants. Participants stood barefoot in the center of a digital scale. A calibrated stadiometer was used to measure height. Participants stood upright with their heels together, and

their back straight. Participants were also instructed to have their feet flat on the floor with heels, buttocks, and shoulders against the stadiometer, and their head aligned so that the Frankfurt Plane is horizontal.

BMI was calculated from height and weight using the following formula: BMI= weight (kg)/ height^2 (m^2). WC was measured using a flexible non-stretchable measuring tape positioned at the midpoint between the inferior costal margin and the superior border of the iliac crest.

Blood sampling and handling

Blood sampling from patients with T2DM enrolled in the first phase of the study has been previously described. In brief, two samples of blood were collected under fasting conditions. One sample was collected in an ethylenediamine tetraacetic acid (EDTA) tube (AFCO, Amman, Jordan). Blood collected from this sample was used for HbA1c measurement and DNA extraction. The other sample was collected in a plain tube with a gel clot activator (AFCO, Amman, Jordan). This sample was used for serum separation. The serum was stored in Eppendorf microcentrifuge tubes at -80 °C. Serum was later used to estimate EGF protein levels by enzyme-linked immunosorbent assay (ELISA) as described in the following sections.

In the second phase, 1.5 mL of blood was collected in an EDTA tube for HbA1c measurement. Another 3 mL was collected in a heparin tube to isolate peripheral blood mononuclear cells (PBMCs). Total RNA was extracted from cells and used for cDNA synthesis (described below). cDNA was then utilized to estimate the mRNA transcript levels of the *EGF* gene.

Biochemical measurements

Several biochemical variables were measured in the first phase of the study. These included HbA1c levels in whole blood, serum glucose, total cholesterol, triglycerides, insulin, and EGF; these measurements were performed as described by Alfaqih et al. (25). Homeostatic model assessment insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR = [fasting insulin (μIU/L) × fasting glucose (mg/dL)]/405.

Serum levels of EGF were measured quantitatively using a sandwich ELISA from serum samples stored at -80 °C. The measurements were performed using a Human EGF Duoset (catalog number: DY236). This kit was purchased from R&D Systems (Minneapolis, MN, USA) and the manufacturer's instructions were followed. Serum samples were used without dilution. The absorbance was measured using a Synergy HTX multimode reader (BioTeK, Winooski, VT, USA) at 450 nm.

The HbA1c level was the only biochemical variable measured in the second phase of the study. HbA1c measurements were performed at the Clinical Chemistry Laboratory of KAUH using an automated analyzer system (Roche Diagnostics, Mannheim, Germany).

DNA extraction and genotyping of rs4444903 SNP of the EGF gene

In the first phase of the study, the rs4444903 SNP of the *EGF* gene was tested for its association with glycemic control. This required the extraction of genomic DNA from whole blood samples, followed by genotyping of patients with T2DM for the different genotypes of rs4444903 using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Extraction of genomic DNA was described previously in Alfaqih et al. (25). DNA samples from the above referenced population were stored at -80 °C in nuclease free and sterile Eppendorf microcentrifuge tubes. Briefly, genomic DNA was purified from whole blood using the QIAamp DNA Mini Blood Kit (Catalog number: 51106; Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration and purity were then assessed using an ND-2000 NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The PCR-RFLP approach used for genotyping the rs4444903 SNP of the *EGF* gene was previously described (25, 27). The above approach was used in this study with slight modifications. The PCR reaction mixture contained 5 μL of 5x FIREPol Master Mix purchased from Solis BioDyne (Tartu, Estonia),17 μL of nuclease-free water, 1 μL of template DNA, and 1 μL of each primer (at a final concentration of 0.2 μM). The final volume of the PCR mixture was 25 μL.

The following thermocycling conditions were used to run the PCR reactions: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The reaction was performed in a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). All the primers were purchased from Integrated DNA Technologies (IDT; Coralville, IA, USA). Forward and reverse EGF primer sequences were 5′ CTAAAGGAAAGGAGGTGGAG 3′ and 5′ AGGGAAGCCACAGGAAAG 3′, respectively. The PCR amplicon was 316 bp in size.

For RFLP, a 20 µL mixture per PCR reaction was prepared. The mixture contained 10 µL of the PCR product, 2 µL of the 10x rCutSmart buffer (New England Biolabs, Ipswich, MA, USA), 7.5 µL of nuclease-free water, and 0.5 µL (equivalent to 5 units) of the Alu I restriction enzyme (New England Biolabs). The mixture was then incubated at 37 °C for 6 h. RFLP products were separated using 3% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light. The products of restriction enzyme digestion corresponding to each genotype of rs4444903 have been described previously (24). The SNP and genotyping information are illustrated in Table 1.

Estimation of EGF mRNA transcript levels from PBMCs

In the second phase of this study, PBMCs were isolated from whole blood. RNA was extracted from the cells and used for cDNA synthesis. *EGF* mRNA transcript levels were estimated using quantitative reverse transcription polymerase chain reaction (RT-qPCR).

Isolation of PBMCs from whole blood

PBMCs were isolated from whole blood samples collected in heparinized tubes. The separation was performed within 2 h of blood collection. Specifically, 2 mL of heparin-treated blood was mixed with an equal volume of phosphate buffered saline (PBS) lacking calcium and magnesium. The mixture was then carefully layered on top of Lymphosep (catalog number: L0560; Biowest, Riverside, MO, USA) in a 15 mL centrifuge tube at room temperature (25 °C). The tube was centrifuged at 400 × *g* at room temperature for 30 min. The supernatant containing the clear plasma was carefully removed using a Pasteur pipette. The PBMC layer was aspirated and transferred to a new 15 mL centrifuge tube. The PBMC layer was washed three times with an equal volume of PBS and centrifuged at 150 × *g* for 10 min. After the last wash, the resulting pellet was resuspended in PBS and added to an equal volume of cryopreservation medium. The medium consisted of 90% fetal bovine serum mixed with 10% dimethyl sulfoxide. The above mix was then transferred to cryovial tubes, frozen in liquid nitrogen, and then stored at -80 °C until later use for RNA extraction.

RNA isolation, cDNA synthesis, and RT-qPCR

Total RNA was isolated from PBMC using Direct-zol RNA MiniPrep Plus Kits (Catalog number: R2071; Zymo Research Corporation, Irvine, CA,

Table 1. SNP Informationa of the EGF Gene with Its Genotyping Strategy.

SNP ID	Location and Base Change	Forward primer Reverse primer	PCR Product Size (bp)	Restriction Enzyme, Incubation Temperature, and Time	RFLP Products (bp)
rs4444903	5' UTR (G/A)	5'CTAAAGGAAAGGAGGTGGAG3' 5' AGGGAAGCCACAGGAAAG 3'	316	Alu I, 37 °C 6 h	GG:272,15,29. GA:272,181,91,15,29 AA:181,91,15,29.

Abbreviations: SNP: single nucleotide polymorphism; UTR: untranslated region; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism. [a]All SNP information was obtained from the NCBI. dbSNP database.

USA). Isolation was performed according to the manufacturer's instructions.

Following RNA isolation, concentration and purity were evaluated spectrophotometrically using a NanoDrop 2000 (Thermo Fisher Scientific). cDNA was synthesized from 1 μg of total RNA using PrimeScript RT Master Mix (catalog number: RR036A; Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The following thermal profile was used: 37 °C for 15 min and 85 °C for 5 s. cDNA was stored at 4 °C until further use.

Prior to its use in RT-qPCR, the cDNA was diluted to 1:5 in nuclease-free water. The TB Green Premix ExTaq TM II kit (catalog number: RR820L; Takara Bio) was used in the RT-qPCR reactions. RT-qPCR reaction mixture included 12.5 μL of TB Green Premix Ex Taq II (Tli RNaseH Plus), 2 μL of the cDNA template, 1 μL of each primer (at a final concentration of 0.4 μM), and 8.5 μL of nuclease free water.

RT-qPCR was performed on a CFX96 Real-time PCR detection system (Bio-Rad) using EGF gene-specific primers, with 36B4 serving as the housekeeping gene. The sequences of the EGF gene specific primers were as the following: 5′ AACTCCTCATTGGCGTGGTC 3' for the forward primer and 5′ AACTCCGAAGCCTCCTGTGT 3′ for the reverse primer whereas the sequences for the 36B4 primers were 5′ TGGCAGCATCTACAACCCTGAAGT 3′ for the forward primer and 5′ AACATTGCGGACACCCTCCAG 3' for the reverse primer. The amplicon sizes of EGF and 36B4 were 170 bp and 83 bp, respectively.

Fold expression was calculated using the $2^{-\Delta\Delta ct}$ method (28). Expression of 36B4 was used for data normalization. Each sample was analyzed in triplicate.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows version 27 (IBM Corp., Armonk, N.Y., USA). The Shapiro-Wilk test was used to examine data normality. Quantitative data were presented as medians with interquartile ranges (IQRs). Categorical data (sex, genotype, and allele frequency) were presented as numbers and percentages. Quantitative variables were compared between groups using the Mann–Whitney U test, and EGF serum levels were compared across rs4444903 genotypes using the Kruskal-Wallis H test. Qualitative variables (sex, allele, and genotype frequencies) were compared across groups using the chi-squared (X^2) test. Spearman's correlation coefficients were used to assess the correlation between two quantitative parameters (EGF levels and glucose, HbA1c, or homeostatic model assessment for insulin resistance [HOMA-IR]).

Logistic regression analysis was used to identify and determine the factors significantly associated with poor glycemic control in patients with T2DM. Binary logistic regression analysis was used to calculate odds ratios (OR) with 95% confidence intervals (CI). For the analysis of the first phase of the study, variables with a *P* value of <0.25 in univariate analysis were included in the multivariate model.

For the second phase analysis, relative gene expression ($2^{-\Delta\Delta ct}$) of *EGF* was initially transformed into a binary variable (high or low) by classifying values above or equal to the mean as "high" gene expression. *EGF* gene expression values below the mean were classified as "low." Afterward, the above variable was included as an explanatory variable, with sex and age as confounding variables. In both regression models (for either phase of the study), the glycemic control status (good versus poor) was selected as the outcome variable. Statistical significance was set at $p < 0.05$.

Results

EGF serum levels and rs4444903 are associated with glycemic control

In this part of the study, 165 patients with T2DM and good glycemic control and 165 with poor glycemic control were recruited. This population was used to test the association between serum EGF levels and the rs4444903 SNP of the *EGF* gene.

Table 2 shows the demographic and clinical characteristics of the study population. The median (IQR) age of the patients with good glycemic control was 60.00 (14.00), which was matched with the

Table 2. Baseline Characteristics and Biochemical Profile of Phase One Study Subjects.

Variables	Glycemic Control		*P* value*
	Good n = 165	Poor n = 165	
Age (years)	60.00 (14.00)	60.00 (11.00)	0.93
Sex Female Male	 90 (54.54%) 75 (45.45%)	 90 (54.54%) 75 (45.45%)	1.000
WC (cm)	105.00 (15.00)	107.00 (17.00)	0.06
BMI (kg/m^2)	28.91 (7.19)	28.93 (6.18)	0.82
HbA1c (%)	6.30 (0.6)	8.65 (1.85)	<0.001
Glucose (mg/dL)	132.30 (41.57)	194.63 (90.94)	<0.001
Cholesterol (mg/dL)	204.27 (71.27)	210.71 (76.17)	0.45
Triglycerides (mg/dL)	128.57 (90.22)	147.43 (101.54)	0.01
Insulin (pmol/mL)	144.81 (122.60)	187.3 (151.20)	0.003
HOMA-IR	7.42 (7.95)	16.40 (17.76)	<0.001
EGF (pg/mL)	158.90 (121.42)	78.26 (54.27)	<0.001

Abbreviations: WC: waist circumference; BMI: body mass index; HbA1c: glycated hemoglobin; HOMA-IR: homeostatic model assessment for insulin resistance; EGF: epidermal growth factor. Data are presented as median (Interquartile range) for continuous variables and n (%) for categorical data (sex). **P* values were calculated using the Mann-Whitney U test for continuous variables and Pearson's chi-squared test for the categorical variable "Sex".

median age of patients with T2DM and poor control [60.00 (11.00)]. Both groups were matched in terms of sex distribution, and the majority of patients in both groups were females (54.6%). Biochemical measurements demonstrated that patients with T2DM and poor glycemic control had significantly higher levels of HbA1c, glucose, triglycerides, insulin, and HOMA-IR ($P < 0.05$). Notably, serum EGF levels were significantly lower in patients with poor glycemic control ($P < 0.001$).

Following the demonstration that EGF serum levels were affected by glycemic control in patients with T2DM, it was tested whether a significant correlation existed between serum levels of EGF and multiple glycemic indices, including fasting serum glucose, HbA1c, and HOMA-IR. In this analysis, a significant negative correlation was observed between serum levels of EGF and glucose (rs = -0.35, P <0.0001; Figure 1A) and HbA1c (rs = -0.31, $P < 0.0001$; Figure 1B). Our data showed no significant correlation between the serum EGF levels and HOMA-IR (Figure 1C).

The GG, GA, and AA genotypes of rs4444903 were compared between patients with good and poor glycemic control. The results of this analysis are shown in Table 3. Genotype frequencies of rs4444903 were significantly different between the two test groups. Specifically, Table 2 shows that the frequency of the GA genotype was higher in patients with poor glycemic control than those with good control (58% compared to 44%) and consequently it increased its risk (OR = 2.06; CI = 1.20 – 3.53, $P = 0.01$). A comparison of the frequencies of the A and G alleles of rs4444903 between the two test groups (Table 4) showed no significant differences ($P = 0.31$).

Considering previous studies have indicated that rs4444903 of the *EGF* gene could significantly modify the expression of the EGF protein, the next analysis tested whether EGF serum levels were significantly different between patients with T2DM, which carry different genotypes of rs4444903 (GG, GA, or AA). The results of this test are shown in Figure 3. These findings demonstrated that patients with T2DM carrying the GA genotype of rs4444903 tended to have

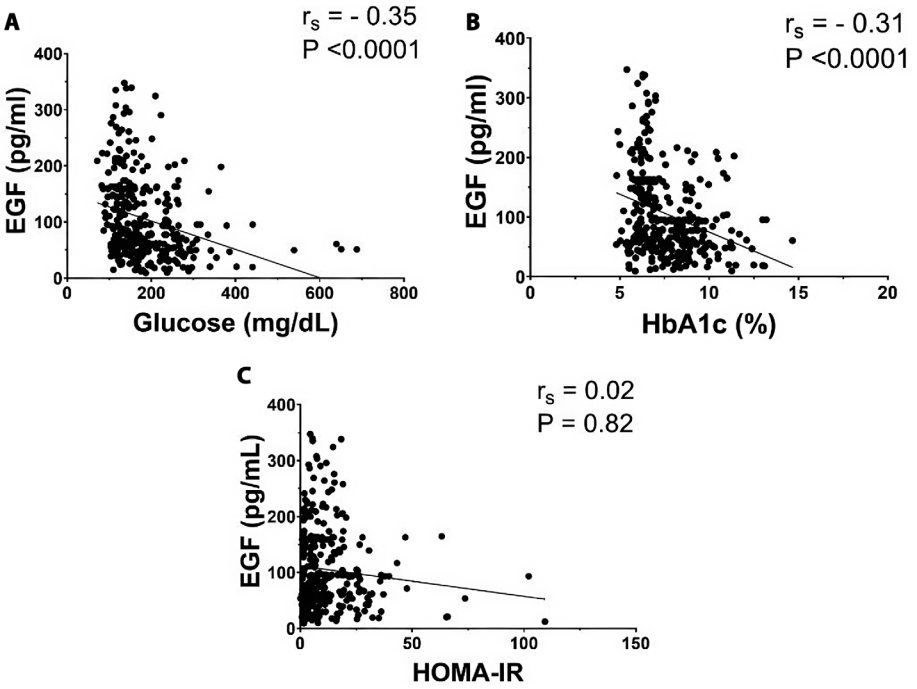

Figure 1. The correlation of glycemic indices with serum EGF levels. Spearman's correlation is used to test the correlation between EGF levels (pg/ml) with each of A) Glucose B) HbA1c, and C) HOMA-IR among study participants of phase one (n=330). r_s represents the Spearman's correlation coefficient. EGF: epidermal growth factor; HbA1c: glycated hemoglobin; HOMA-IR: homeostatic model assessment for insulin resistance.

Table 3. Genotype Frequencies of rs4444903.

		Glycemic Control		OR (95% CI)	*P* value*
		Good (n=165) n (%)	Poor (n=165) n (%)		
Genotype	GG	50 (30.30)	32 (19.40)	1	
	GA	72 (43.63)	95 (57.57)	2.06 (1.20-3.53)	0.01
	AA	43 (26.06)	38 (23.03)	1.38 (0.74-2.57)	0.31

Abbreviations: OR: odds ratio; CI: confidence interval. **P* values were calculated using Pearson's chi-squared test.

Table 4. Allele Frequencies of rs4444903.

		Glycemic Control		*P* value*
		Good (n=165) n (%)	Poor (n=165) n (%)	
Allele	G	172 (52.12)	159 (48.18)	0.31
	A	158 (47.88)	171 (51.82)	

**P* values were calculated using Pearson's chi-squared test.

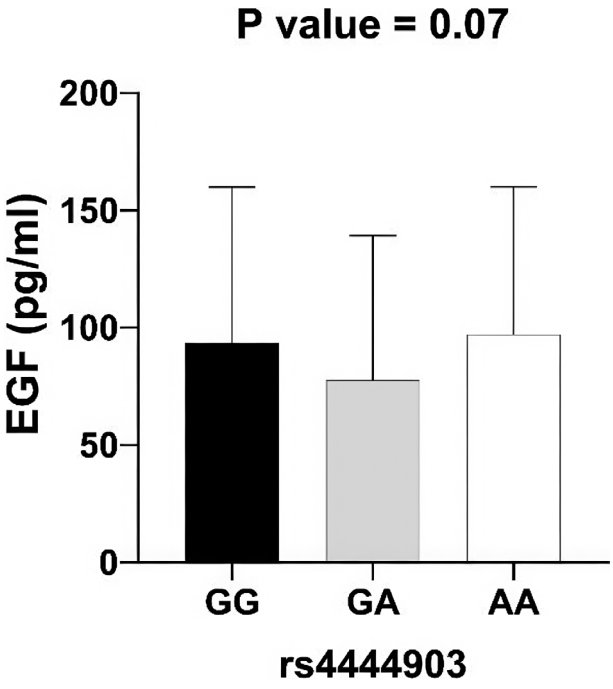

Figure 2. The association between serum EGF levels and the genotype of rs4444903 SNP. Subjects are classified according to their rs4444903 genotype as follows: GG, GA, or AA. Levels of EGF are compared between the different genotypes. The analysis is performed on all patients included in phase one (n=330). The bar represents the median (interquartile range). The Kruskal-Wallis H test is used to assess statistically significant differences.

lower levels of serum EGF than those patients carrying the GG or AA genotypes ($P = 0.07$) (Figure 2).

A multivariate regression model was used to test the independent association between the study variables and glycemic control (Table 5). Using the above model, we found that triglycerides and HOMA-IR were independently associated with poor glycemic control and increased its' risk ($P < 0.05$, OR > 1.00).

Consistent with our finding that serum EGF levels were significantly lower in patients with poor control, the regression model described above indicated that serum EGF was independently associated with a decrease in the risk of poor glycemic control (OR = 0.99, 95% CI: 0.98-0.99, $P < 0.001$). Additionally, the heterozygous GA genotype of rs4444903 was independently associated with an increased risk of poor control compared to the reference GG genotype (OR = 1.96, 95% CI: 1.02 - 3.57, $P = 0.04$).

Table 5. Logistic Regression Analysis of The Study Variables for T2DM Patients Included in The First Phase.

Variable	OR (95%CI)	*P* value*
WC (cm)	1.00 (0.98-1.03)	0.74
Triglycerides (mg/dL)	1.00 (1.00-1.01)	0.01
EGF (pg/mL)	0.99 (0.98-0.99)	<0.001
HOMA-IR	1.06 (1.03-1.09)	<0.001
rs4444903		
GG	Reference	-
GA	1.96 (1.02-3.57)	0.04
AA	1.57 (0.76-3.26)	0.23

Abbreviations: OR: odds ratio; CI: confidence interval; WC: waist circumference; EGF: epidermal growth factor. HOMA-IR: homeostatic model assessment for insulin resistance. **P* values were calculated using binary logistic regression analysis. Variables with P-values<0.25 in the univariate analysis were included in the model.

EGF expression in PBMCs is associated with glycemic control

Several different cell types synthesize and secrete the EGF into the peripheral blood including PBMCs (29). A previous report showed that patients with T2DM had lower levels of *EGF* mRNA in their PBMCs than that of the healthy control group (22).

Considering that we found significantly lower levels of serum EGF in patients with poor glycemic control, the next set of analyses tested whether *EGF* mRNA levels were significantly lower in PBMCs recovered from patients with T2DM and poor glycemic control. To achieve the above goal, an additional 84 patients with T2DM were recruited. This group included 42 patients with good glycemic control and 42 patients with poor glycemic control. The baseline characteristics of the study groups are presented in Table 6. No significant differences were found between the two groups in terms of age, sex, or BMI ($P > 0.05$). Patients with T2DM and poor glycemic control had significantly higher HbA1c levels ($P < 0.001$).

PBMCs were then isolated from the blood of patients in both groups. RNA was extracted from PBMCs, and the mRNA levels of the *EGF* gene were assessed using RT-qPCR. This experiment revealed a 40% reduction in *EGF* mRNA levels in patients with poor glycemic control ($P < 0.05$; Figure 3) compared with that of patients with good glycemic control.

Table 6. Baseline Characteristics of T2DM Patients of The Second Phase.

Variable	Glycemic control		*P* value*
	Good n=42	Poor n=42	
Age (years)	58.50 (14.00)	61.00 (8.00)	0.11
Sex Male Female	 20 (47.62%) 22 (52.38%)	 16 (38.10%) 26 (61.90%)	0.38
BMI (kg/m^2)	30.08 (9.50)	29.06 (8.16)	0.97
HbA1c (%)	6.40 (1.00)	8.30 (1.98)	<0.001

Abbreviations: BMI: body mass index; HbA1c: glycated hemoglobin. Data are presented as median (Interquartile range) or as n (%) for categorical variables (Sex). **P* values were calculated using the Mann-Whitney U test for continuous data and Pearson's chi-square for the categorical variable "Sex".

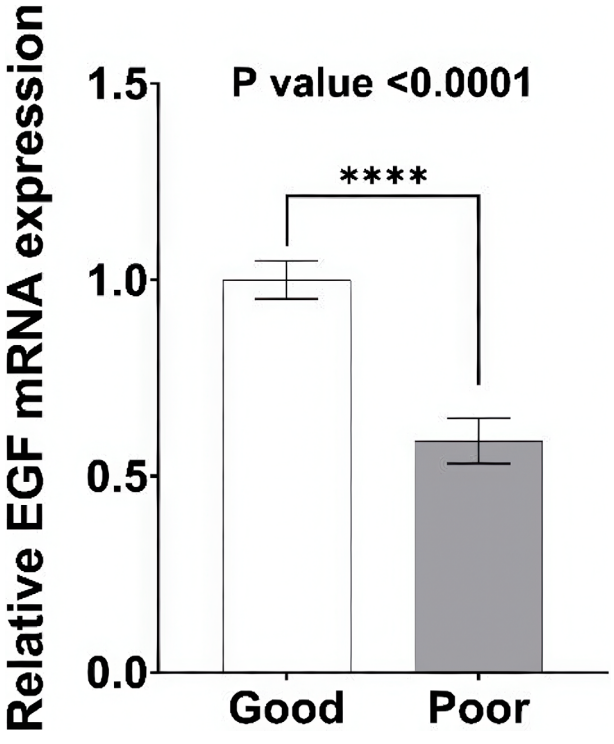

Figure 3. Comparison of EGF mRNA levels between patients with good vs. poor glycemic control of the second phase. T2DM patients with poor control had significantly lower EGF mRNA levels. The expression is determined using RT-qPCR. Fold expression is calculated using the 2−ΔΔct method. The data are expressed as mean ± standard error of the mean of two independent experiments performed in triplicates. The student t-test is utilized to assess the differences between groups. **** denotes $P < 0.0001$. The analysis is performed on all patients included in phase two (n=84) n_{good} =42, n_{poor}=42.

Table 7. Multivariate Logistic Regression Analysis of Variables for T2DM Patients of The Second Phase.

Variables	OR (95%CI)	*P* value*
Age (years)	1.05 (0.99 - 1.10)	0.11
Sex (female vs. male)	1.75 (0.67 - 4.58)	0.26
Relative EGF gene expression (low vs. high)	3.05 (1.10 - 8.44)	0.03

Abbreviations: OR: odds ratio; CI: confidence interval; BMI: body mass index; EGF: epidermal growth factor. [a] *P* values were calculated using binary logistic regression analysis.

Finally, a multivariate logistic regression analysis (Table 7) was performed to determine whether the observed reduction in *EGF* mRNA levels could independently and significantly increase the risk of poor glycemic control after adjusting for age, sex, and BMI. In this analysis, relative gene expression was transformed into a binary variable (high or low). The statistical model described above showed that lower relative expression levels of *EGF* were independently associated with a significantly higher risk of poor glycemic control in patients with T2DM (OR = 3.05; 95% CI: 1.10 - 8.44, $P = 0.03$).

Discussion

Guidelines for the management of adult patients with T2DM emphasize the importance of maintaining blood glucose levels below an HbA1c of 7% or a fasting blood glucose level of 126 mg/dL (30). Evidence supports the notion that such a practice lowers the risk of developing complications (microvascular in particular) in patients with T2DM (31). This, in turn, reduces the morbidity of T2DM and enhances the quality of life of patients (31).

Despite the perceived significance of maintaining glycemic control in patients with T2DM, failure to achieve such control remains a clinical challenge for healthcare workers. Importantly, several studies conducted in different geographic regions have documented a high prevalence of poor glycemic control. This observation highlights the significant impact of this issue on the global scale (32).

A closer look at the prevalence of poor glycemic control in Jordan shows that this problem is of equal

magnitude to global figures. For example, an observational study performed at a major tertiary institution in Northern Jordan reported a 61% prevalence rate of poor glycemic control in a random sample of 237 patients with T2DM (33).

The above figures clearly demonstrate the pressing need to identify the biochemical and genetic markers associated with poor glycemic control. The discovery of such markers should set the stage for a better understanding of the pathophysiological and mechanistic changes associated with poor glycemic control or its complications. This may ultimately help design better therapeutic strategies and management protocols to combat this problem.

EGF is a secretagogue (20). Moreover, a review of the literature demonstrates that the EGF may help to maintain the pancreatic β cell mass (19). An observational case-control study performed in an Indian population reported a reduction in serum EGF levels among patients with T2DM (24). The same study demonstrated that a variant of the *EGF* gene, known for its effect on serum EGF levels, was associated with T2DM (24). EGF is also positively associated with faster wound healing (34). Collectively, the evidence discussed above motivated the research team to investigate the association of EGF and one of its genetic variants (rs4444903) with glycemic control.

One of the major findings of this study was that serum EGF levels were reduced in patients with T2DM and poor glycemic control. This association was independent of the waist circumference, triglyceride level, and insulin resistance status of the population. This finding is consistent with the negative correlation between HbA1c and serum EGF levels described in this report.

Interestingly, Trimal et al. reported a reduction in serum EGF levels in patients with T2DM compared with that of disease-free controls (24). Taken together, the findings of Trimal et al. and the present study may indicate a progressive reduction in serum EGF levels in parallel with disease development and progression. Confirmation of such a relationship requires longitudinal cohort studies that are outside the scope of this report.

The case-control design employed in this investigation did not establish a causal relationship between lower serum EGF levels and a lack of glycemic control. It could, however, be proposed that pharmacological or non-pharmacological interventions that increase serum EGF levels could potentially improve glycemic control in patients with T2DM. The subsequent paragraphs describe some of these interventions.

Pharmacological modalities that could be useful in improving glycemic control include EGF itself or EGF functionally similar ligands that bind to and activate the EGF receptor (EGFR) and could thus induce similar effects to EGF.

Interestingly, the use of the EGF, solely or in combination, has been tested before in the context of increasing the β cell mass in pancreatic islets recovered from adult humans (35). Suarez-Pinzon et al. showed that a combination of gastrin and the EGF could induce an increase in the β cell numbers of human pancreatic islets cultured *ex vivo* or xenografted in immunodeficient mice (35). The use of EGF, alone or in combination, to improve glycemic control has not been tested before in any preclinical models or clinical trials. This remains of future research direction that can be investigated in this field.

In the same line of evidence, the transplantation of pancreatic islets that stably overexpress betacellulin (a ligand similar to the EGF) in a mouse model of DM was shown to improve the glucose sensitivity in experimental mice (36).

Exendin-4 is an antidiabetic drug that improves glucose tolerance in patients with T2DM (37). Exendin-4 primarily induces its therapeutic effects by binding to and activating the glucagon-like-peptide-1 (GLP-1) receptor (37). Recent evidence showed that exendin-4 induced proliferation of the β-cells of the pancreas via binding to the EGFR (38). This indicates that exendin-4 could functionally mimic the effect of the EGF on β cell proliferation and glycemic control.

Given the above relationships, it is conceivable that the administration of exendin-4 could improve glycemic control in patients with T2DM and low serum EGF levels who are not currently receiving exendin-4 as part of their treatment protocol. Confirmation of this hypothesis requires well-designed clinical trials, which is outside the scope of this study.

There are several non-pharmacological lifestyle interventions with reported effects on serum EGF

levels and positive effects on glycemic control. These interventions could potentially improve glycemic control, partially via their impact on serum EGF levels. One observational study found that aerobic exercise positively affected glycemic control (39). Interestingly, aerobic treadmill exercise has been reported to increase serum EGF levels in an Alzheimer mouse model (40). Taken together, these results may indicate that one of the mechanisms by which aerobic exercise improves glycemic control is via its effect on EGF levels.

Given our results that established a relationship between serum EGF levels and glycemic control. It is plausible that genetic variations in the *EGF* gene, associated with a reported effect on its expression could also modify the risk of poor glycemic control. One such genetic variant is the rs4444903 SNP. This SNP was an (A/G) variant (41). Prior evidence supports the association between the A allele of this SNP and reduced *EGF* gene expression (41). Specifically, *EGF* mRNA transcripts carrying the A allele have lower stability and shorter half-life than that of transcripts carrying the G allele (41). To the best of our knowledge, no prior study has explored the potential link between rs4444903 of the *EGF* gene with the risk of poor glycemic control. In this study, we demonstrate the existence of such an association.

In this study, the role of rs4444903 in modifying the risk of poor glycemic control among patients with T2DM was demonstrated using both univariate and multivariate statistical models. Analysis of the genotype frequencies of rs4444903, revealed that the frequency of patients carrying the heterozygous GA genotype was significantly higher among those with poor glycemic control. This difference resulted in a two-fold increase in the risk of poor control among carriers of the GA genotype in the regression model.

Moreover, patients with T2DM carrying the GA genotype had lower levels of EGF protein than patients with the GG genotype. This indicates that genetic polymorphisms in the *EGF* gene could mediate the differences observed in the protein levels of EGF, and consequently, the risk of poor glycemic control. These observations suggest that serum variation in EGF protein levels is a heritable trait. The relative contribution of *EGF* polymorphisms to the heritability of serum EGF levels is an interesting question that represents a future goal for our research team. However, this is beyond the scope of this report.

Trimal et al. reported a four-fold increase in the risk of T2DM in patients with the GA genotype of rs4444903 compared to healthy controls (24). Notably, our findings regarding the effect of the GA genotype of rs4444903 on glycemic control are in agreement with those of Trimal et al. The consistency in the findings of both reports further indicates that EGF may be involved in the regulation of blood glucose, development of T2DM, and T2DM progression.

Several tissues contribute to the EGF levels in the serum of patients with T2DM, including PBMCs (29, 42). Considering the feasibility of collecting PBMCs, their extensive interactions with various body tissues, and their fast turnover, this study assessed the relationship between the transcript levels of *EGF* in PBMCs and glycemic control. PBMCs were used as surrogates for all other tissues (43).

The results of this study showed a 51% reduction in EGF protein levels. In contrast, RT-qPCR analysis indicated a 40% reduction in *EGF* mRNA levels in isolated PBMCs. Considering the experimental errors and perceived differences in the sensitivity of RT-qPCR and ELISA-based methods, it appears that EGF secretion by PBMCs is one of the major sources of serum EGF.

Cell cycle regulation is one of the many functions of EGF (44). A lower level of *EGF* expression in the PBMCs of patients with T2DM and poor glycemic control matches the findings of Slieker et al. (45) who reported a correlation between HbA1c levels and the expression of genes related to the cell cycle in the blood of patients with T2DM.

Low levels of EGF may compromise the ability of tissues to adequately respond to stress, and thus impede their regenerative process upon exposure to injury (14). Unsurprisingly, dysregulation of EGF expression has been implicated in the development of DM complications. For example, *EGF* mRNA expression is reduced in the renal interstitium of patients with diabetic nephropathy (46).

Given the association between poor glycemic control and an increased risk of DM complications, including delayed wound healing (47), and the findings of this investigation which demonstrated low

levels of serum EGF in patients with poor glycemic control. This report may have uncovered an axis that explains the higher percentage of complications in patients with T2DM and poor glycemic control. This axis connects poor control with an increased risk of DM complications due to reduced EGF levels.

These insights highlight the potential utility of targeted interventions that aim to optimize EGF levels to improve healing outcomes in patients with T2DM. Further research is warranted to fully elucidate and validate the axis discussed above.

The A allele of rs4444903 has been reported to reduce *EGF* mRNA stability (48). This effect presumably reduces the levels of *EGF* mRNA transcripts in patients who are either heterozygous (GA) or homozygous (AA), compared to those in patients who are homozygous (GG). Unfortunately, data correlating the rs4444903 genotype with the *EGF* mRNA levels are not available. This represents one limitation of this report.

Another limitation is that all patients with T2DM participating in this study were exclusively recruited from one institution located in the northern part of Jordan. This limits the geographical diversity of the sample. Third, the sample size was relatively small and therefore a limitation of the study. Our report demonstrates the downregulation of *EGF* mRNA expression in one sample. Lower EGF protein levels have also been observed in a different sample from the same population. This sampling arrangement reinforces the validity of our results, which associate EGF expression with glycemic control and is considered a strength of this report.

Conclusions

In conclusion, our study provides strong evidence that a reduction in serum EGF levels, a decrease in *EGF* mRNA transcripts in PBMCs, or the GA genotype of the rs4444903 SNP serve as independent markers for the increased risk of poor glycemic control in patients with T2DM. However, further studies are required to validate these findings. Future investigations are recommended to assess if interventional approaches that increase serum EGF levels would improve glycemic control potentially via an effect on the β cell mass.

Ethic Approval: Institutional Review Board (IRB) of Jordan University of Science and Technology (JUST; Irbid, Jordan; approval ID #25/155/2023).

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Authors Contribution: M.A.A., O.F.K. and M.S.S contributed to the conception and design of the study; J.M. collected data, M.A.A and J.M. performed the experiments. MAA and E.A analyzed the data; M.A.A. and J.M. wrote the original draft of the manuscript, M.A.A, M.S.S and E.A wrote and substantially revised the final draft of the manuscript, M.A.A., and O.F.K supervised the work. All authors approved the final version of the manuscript.

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