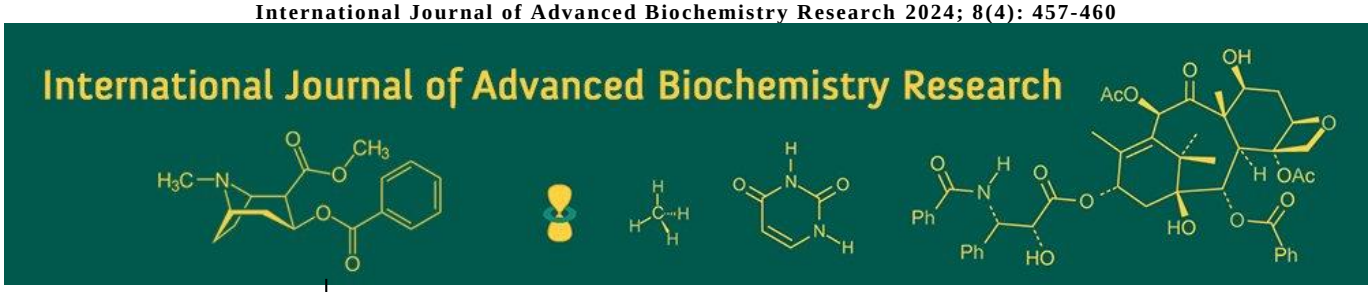

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Doaa H Alasady
College of Biotechnology, Al-Qasim Green University, Babylon, Iraq

Rawaa SA AL-Azawi
Collage of Science, Al-Qasim Green University, Babylon, Iraq

Hamzah H Kzar
Veterinary Medicine Collage, Al-Qasim Green University, Babylon, Iraq

Noora H Ali
Veterinary Medicine Collage, Al-Qasim Green University, Babylon, Iraq

Zainab A Radhi
Veterinary Medicine Collage, Al-Qasim Green University, Babylon, Iraq

Corresponding Author:
Hamzah H Kzar
Veterinary Medicine Collage, Al-Qasim Green University, Babylon, Iraq

Biochemical and genetic study on Iraqi patients with diabetes mellitus with and without diabetic foot ulcer

Doaa H Alasady, Rawaa SA AL-Azawi, Hamzah H Kzar, Noora H Ali and Zainab A Radhi

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Abstract
One of the main complications of diabetic mellitus (DM) is diabetic foot ulcer (DFU) which leads to significant morbidity and mortality among affected individuals worldwide. This study was designed to assessment of DFU with some of the biochemical markers and genetic variants, in Iraqi patients with DM. The current study involved 135 subjects that were subdivided into 45 patients with (DFU), 45 diabetics without (NDFU), and 45 subjects as controls (CONT). The genotyping analysis using restriction enzymes *HaeIII* and *NcoI* (PCR-RFLP) for estimation of genotypes of VEGF (rs3025039) and TNF-α (rs1800629) and the results reveal the association with increased susceptibility to DFU in diabetic patients. The results of this study showing statistical differences in the biochemical markers, HbA1c and CRP in the DFU group compared to both NDFU and CONT group. On the other hand, the genetic analyses showed significant differences in the SNP of VEGF and TNF-α genotypes between the DFU group and CONT group. Odds ratios were calculated for each genotype to investigate the genetic polymorphism differences in all study groups. In conclusion, the results show the main role of VEGF (rs3025039) and TNF-α (rs1800629) gene polymorphism in the incidence of DFU in Iraqi population.

Keywords: Diabetic foot ulcers, HbA1c, CRP, VEGF, TNF-α diabetes mellitus

Introduction
A metabolic disorder that consists of high blood glucose levels (hyperglycemia) due to faults with the secretion of insulin and/or its actions has been referred to as diabetes mellitus (DM). Diabetes-related morbidity currently is a global issue as it was shown that DM is one of the most common diseases throughout the world with more than 537 million adults aged 20-79 affected by diabetes in 2021 that was projected to reach more than 643 million by 2030 [1]. The diabetes patients are also not excluded from among the common affected disease, which is the DFU (5-15%) during their life time [2]. For developing the therapy of DFU in Iraqi patients with DM and designing such interventions that can can some complications of these problems cannot be ignored.

These studies have also lead to the identification of some genes that are considered target genes in DFU and some others are the TNF-αgene and VEGF gene. Explore these genes for instances where they are varied may block angiogenesis, inflammatory responses, and healing of the wound, all three of which contribute to the vulnerability of Iraqi patients with DM to the development of diabetic foot ulcers. A term that is used to define that is considered a complication of DM as the area that does not heal in the lower extremities affected by the non-healing ulcers; the major complication is also a risk factor for severe problems, lower limb amputation and more deaths [3, 4].

Based on one study, up to 25% of persons with diabetes might count themselves among those affected by foot ulcers after some time [5]. Additionally, other studies have centered on the possibility that pathogenesis of DFU may have multiple causes, with an intricate series of vascular abnormalities, neuropathy, impaired wound healing, and immune system irregularities [6]. In the healthcare, DFU presents another area of high burden that translates into economical costs and as well adversely affects patients' quality of life [7]. Nerve damage, bad management of sugar to the body, stiffness of the arteries and foot deformity are the main risk factors for DFU [8]. To some extend the genetic information that is leading to hosts susceptibility is not understood [9].

The biochemical markers like HbA1c, CRP, IL-6, and TNF-α have been reported to be involved in the inflammatory response, oxidative stress, and endothelial dysfunctions, resulting in DFU establishment [10]. The levels of HbA1c, the chronic hyperglycemia marker, confirm that the HbA1c levels are high which may be due to elevated risk of developing DFU and ineffective wound healing outcomes [11]. In parallel, two findings have been made the CRP, IL-6, and TNF-α levels obtained from the PDU patients, which attend that systemic inflammation is involved in the disease progression [12]. But not the biochemical markers only, the genetic factors also constitute an important classifiers of DFU risk with a couple of common gene variant constituting a leading role such as genetic polymorphism in genes responsible for angiogenesis, inflammation and wound healing pathways [13].

Materials and Methods

Study design

An experimental design of a cross-sectional type was applied in the research for biochemical markers and genetic variants, which are related to Iraqi patients with the diabetes mellitus experienced foot ulcers. In the study, 135 participants were recruited and they were divided into 3 groups which consistsing from DFU Group (45 Iraqi patients who diagnosed with diabetic foot ulcers), Diabetic Non-DFU Group from NDFU (45 Iraqi patients with diabetes except the cases of foot ulcers) and the Control Group from CONT (45 persons having neither diabetes or foot ulcers) The Exclusion Criteria are these other significant comorbidities that may affect wound healing process and biochemical markers, pregnancy and lactation, and any other condition than that normally could disturb the study participation or results evaluation.

Biochemical analysis

In this process, the analysis has been done at biochemistry laborotory/ Al-Qasim Green University. Blood samples were drawn from all the participants in the study on an overnight fast and the selected biomarkers which include HbA1c (glycated hemoglobin) and CRP (C-reactive protein) were determined qualitatively by the standard laboratory kits.

Genetic analysis

The pure, human genomic DNA was extracted from the peripheral blood samples using manufacturer's instructions in the human-specific kits. Polymorphisms linked to DFU, such as the VEGF genes of VEGF gene and the TNF-α gene, were PCR-based analyzed and the PCR-RFLP genotyping method were used to characterize the genotypes. Specific primers targeting the single nucleotide polymorphisms (SNPs) of interest in the VEGF and TNF-α genes were designed based on previously published papers [14]. The PCR reaction was carried out in a thermal cycler (bioneer/Korea) using the following conditions:

Table 1: PCR Conditions and primers for genotyping analysis

Gene	SNP	Primer Sequence (5' to 3')	Denaturation (°C)	Annealing (°C)	Extension (°C)	Number of Cycles	Product Size (bp)
VEGF	rs3025039	F: 5'-3' TCTGGCCTCGGCCCCAAGGCA R:5'-3' CCTGGGCAAGGCGGTGAGGTT	95 °C	61 °C	72 °C	40	187
TNF-α	rs1800629	F:5'-3' GAAAGCATGATCCCAAAGTAGAC R:5'-3' TGGTGGTTTGCTACGACGTGG	95 °C	59 °C	72 °C	40	107

The genomic DNA was extracted from peripheral blood samples using a DNA extraction kit according to the manufacturer's instructions. PCR amplification was performed using specific primers targeting the single nucleotide polymorphisms (SNPs) of interest in the VEGF and TNF-α genes. The PCR reaction mixture (25 µl) contained [components] (provide details of PCR components such as DNA template, primers, dNTPs, buffer, and DNA polymerase). PCR products were digested with a specific restriction enzyme RE targeting the SNP site of interest. The digestion reaction mixture (20 µl) contained (provide details of digestion components such as PCR product, restriction enzyme (For VEGF Gene: rs3025039 used restriction enzyme for this SNP is *HaeIII*. For TNF-α Gene: rs1800629: used restriction enzyme for this SNP is *NcoI*), buffer, and incubation conditions). Digestion was carried out at 37°C for 4 hours. Digested PCR products were separated by agarose gel electrophoresis. The gel was stained with save stain and visualized under UV light. Different genotypes were identified based on the presence or absence of restriction enzyme cleavage sites and the size of DNA fragments on the gel.

Statistical analysis

Statistical analysis was conducted using SPSS software (version 22). Descriptive statistics were used to summarize demographic and clinical characteristics of the study groups. Continuous variables were compared between groups using t-tests or non-parametric tests, as appropriate. Categorical variables were compared using chi-square tests. A p-value < 0.05 was considered statistically significant.

Ethical approval

This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Institutional Review Board/Ethics Committee of Al-Qasim Green University. Informed consent was obtained from all participants before enrollment in the study.

Results

Table 2: Demographic and clinical characteristics of study participants

Characteristic	DFU N=45	DNFU N=45	CONT N=45
Age (Y), Mean ± SD	57.3 ± 9.8	59.1 ± 8.5	58.5 ± 7.9
Gender (M/F)	28/17	27/18	26/19
Diabetes Duration (Y), Mean ± SD	10.4 ± 6.2	9.9 ± 5.1	N/A
HbA1c (%), Mean ± SD	9.8 ± 1.05**	7.6 ± 1.02	5.3 ± 0.07
CRP (mg/L), Mean ± SD	12.7 ± 1.9**	7.5 ± 1.2	3.2 ± 0.5

The results showing significant differences were observed in HbA1c levels between the DFU group and both the DNFU

group and the CONT group (p<0.001). Also the results suggested that the CRP levels were significantly higher in the DFU group compared to both the DNFU group and the CONT group (p<0.001). The frequency of certain genetic variants, particularly in the VEGF and TNF-α genes, differed significantly between the DFU group and the control groups (p<0.05).

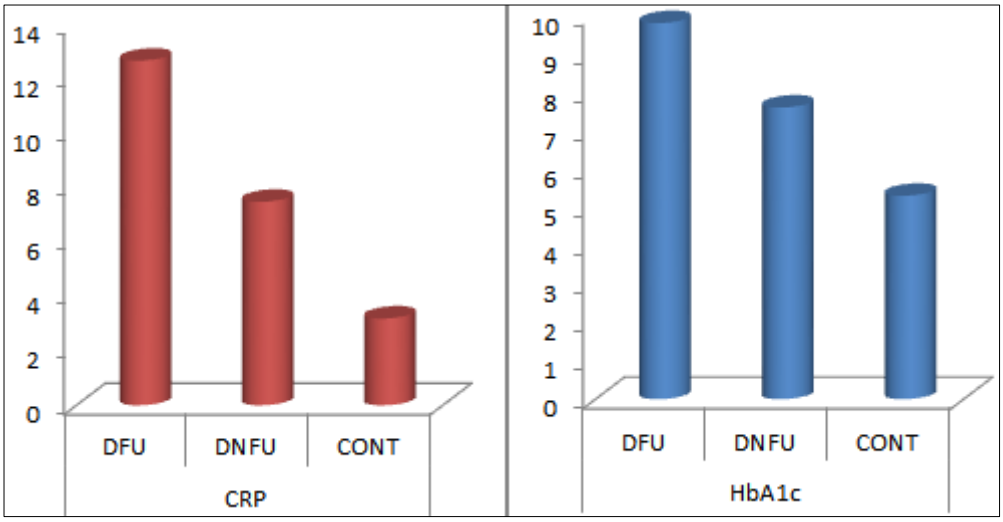

Fig 1: Comparison between the biochemical markers in study groups

In the Table 3, the frequencies of specific genotypes (GG, GA, AA for VEGF; CC, CG, GG for TNF-α) are presented for all study groups.

Table 3: The frequency of genetic SNPs in study groups

Genetic Variant	DFU (n=45), n (%)	DNFU (n=45), n (%)	CONT(n=45), n (%)
VEGF Genotype	GG: 15 (33.3%)	GG: 10 (22.2%)	GG: 8 (17.8%)
	GA: 22 (48.9%)	GA: 20 (44.4%)	GA: 15 (33.3%)
	AA: 8 (17.8%)	AA: 15 (33.3%)	AA: 22 (48.9%)
TNF-α Genotype	CC: 14 (31.1%)	CC: 18 (40.0%)	CC: 20 (44.4%)
	CG: 20 (44.4%)	CG: 15 (33.3%)	CG: 18 (40.0%)
	GG: 11 (24.4%)	GG: 12 (26.7%)	GG: 7 (15.6%)

In figure 2, the allele frequencies of the VEGF and TNF-α genes are presented for each study group.

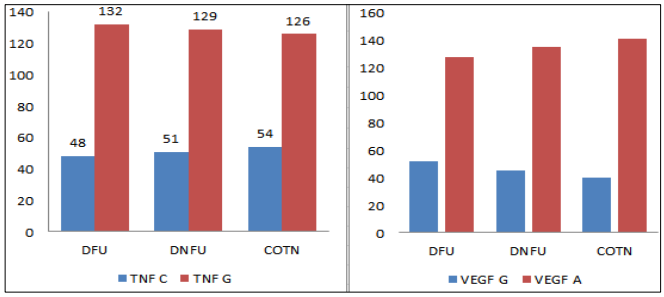

Fig 2: Allele frequencies of VEGF and TNF-α SNPs in study props

Discussion

Diabetic foot ulcer (DFU) is currently reported as the most dangerous complications of diabetes mellitus which yields in high morbidity and mortality rates of patients around the world. The present research tried to verify the connection between biochemical indicators (HbA1c and CRP), and genetic SNPs (VEGF and TNF-a) in the DFU presence in Iraqi patients with DM, the study findings suggested a probable role of these biomarkers and genetic variants in the pathogenesis of DFU, and the study provides background information for future study and clinical improvement The data collection and analysis proved that the level of biochemical indicators: HbA1c and CRP were definitely differed among the three groups which were DFU, DNFU and control groups. alternatively, the previously established relationships between the increase in levels of HbA1c and CRP and DFU development risk and poor wound healing outcomes are ones that should not be unnoticed [15]. Moreover, the interleukin-6 [IL-6] and tumor necrosis factor-alpha [TNF-α] biochemical markers which mentioned in other studies were found to be related to DFU pathogenesis and such other biomarkers could be useful at risk stratification and monitoring of the disease in addition to the biomarkers analyzed by this study [16]. In combination between the biochemical markers that studied in our investigation suggested the association between genetic SNPs of VEGF and TNF-a genes and DFU susceptibility. this study observed significant differences in the distribution of VEGF and TNF-α genotypes between the DFU group and the control groups [17]. Specifically, certain genotypes of VEGF and TNF-α were more prevalent in patients with DFU compared to DNFU individuals and healthy controls. The results of the current study suggest a potential relationship between genetic predisposition to DFU development and highlight the importance of genetic factors in the disease pathogenesis. The p-values that were estimated for each genotype make further points on the correlations between genetic variants and DFU risk. Notably, individuals carrying specific genotypes of VEGF and TNF-α exhibited a significant P-value (less than 0.05) of developing DFU compared to those with different genotypes. On the other hand, the findings support the hypothesis that reported by other reference on genetic polymorphisms in genes involved in angiogenesis, inflammation, and wound healing pathways may influence DFU susceptibility [18]. Although, it's essential to acknowledge the limitations of this study. for example, the sample size was relatively small, which may limit the generalizability of our findings. Future studies with larger cohorts are warranted to validate our results and identify additional genetic and biochemical factors associated with

DFU. on the other hand, the cross-sectional design of the study precludes causal inference, and longitudinal studies are needed to assess the temporal relationship between biomarkers, genetic variants, and DFU development. In conclusion, our study provides valuable insights into the role of biochemical markers and genetic factors in DFU pathogenesis among Iraqi patients with DM.

Conclusion

This study revealed strong correlation between higher levels of HbA1c and CRP and propensity to DFU formation.

Conflict of interest: No.
Funding: Non.

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