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Role of Endothelial Dysfunction Marker along with Toll-like Receptors 4 (TLR-4) Polymorphism in Patients with Type 1 Diabetes Mellitus

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ABSTRACT

Background: *The immune system specifically destroys insulin-producing beta cells in the pancreas in type 1 diabetes mellitus (T1DM), an autoimmune disease. CD4+ T cells plan the attack, while CD8+ T cells kill the beta cells directly, causing insulin insufficiency and uncontrolled blood sugar levels.* ***Objective:*** *The aim of study is to evaluate E-selectin as a diagnostic biomarker for T1D patients. Additionally, evaluate of TLR-4 polymorphisms by PCR-RFLP.* ***Methodology:*** *overall 150 participants included (75 patients and 75 healthy control) the ages between (10 and 35) years. The serum levels of E-selectin estimation by ELISA kits.* ***Results:*** *The present study demonstrates that the level of E-selectin was significantly higher in T1D patients compared with controls. As regards the (TLR4) Asp299Gly (rs4986790) genes in patients with polymorphism, In T1DM patients compared with healthy control, the present results show that the genotypes and allele frequencies significantly (p = 0.001).* ***Conclusion****: According to the Receiver operating characteristic (ROC) analysis, in the present results indicates E-Selectin is considered as a weak prognostic marker to distinguish T1DM patients. Additionally, no such correlation was found into the TLR-4 Asp299Gly (rs4986790) polymorphism in patients with T1D.*

Keywords: E-selectin, TLR-4, Type 1 Diabetes Mellitus.

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INTRODUCTION

The immune system specifically destroys insulin-producing beta cells in the pancreas in type 1 diabetes mellitus (T1DM), an autoimmune disease. CD4+T cells plan the attack, while CD8+T cells kill the beta cells directly; causing insulin insufficiency and uncontrolled blood sugar levels (Mauvais & Van Endert, 2025). Both genetic susceptibility and environmental factors are involved in the development and progression of type 1 Diabetic by impairing immune homeostasis (Quinn *et al.*, 2021). Although type 1 diabetes is typically thought of as having a childhood origin, it can occur at any age and can be difficult to diagnose in adults because type 2 diabetes is more common (Thomas *et al.*, 2018). In recent decades, the incidence of T1DM is rapidly increasing worldwide at a rate of 3–5% per year has been observed, T1DM one of the most common chronic metabolic diseases in childhood and adolescence (Gregory *et al.*, 2022**)**. Recent study in Karbala province, Iraq, The average yearly incidence rate of T1DM was 12.11 per 100,000 (Oleiwi Jasim *et al.,* 2023).

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The aim of current study is to estimation the Serum levels of E- Selectin, it holds promise as prognostic marker of endothelial dysfunction for patients with Type 1 diabetic. Along with detection of single nucleotide polymorphisms (SNPs) of Toll-like Receptors 4 (TLR4) Asp299Gly (rs4986790) gene in patients with Type 1 diabetic.

METHODOLOGY

Participation and spacemen collection

A case control study was designed on a total of 150 subjects divided into Group one contains 75 (39 males and 36 females) patients with T1DM are diagnosed by specialist physicians. Group two comprised of 75 (37 males and 38 females) healthy control persons from volunteers were recruited for this study, the ages of all individuals ranged from (10-35) years. The collection of samples was carried out during the period from January 2024 to the end of June 2024. at Al Najaf Diabetes and Endocrine center in Al-Najaf AL-Ashraf province, Iraq.

Immunological technique

Estimation of Human Serum levels of E-Selectin

The sandwich kit is an Enzyme-Linked Immunosorbent Assay (ELISA). For measuring human serum E- Selectin are used for the accurate quantitative detection in serum in vitro, the manufacturing company Bioassay Technology Laboratory (BT-LAB, China).

Detection of TLR4 Asp299Gly Polymorphism

Amplification Reaction Mixture for TLR4 c.896A > G, Asp299Gly (rs4986790) was used by Primer sequence Forward 5’-GATTAGC ATACTTAGACTACTACCTCCATG-3 ’ Primer sequence Reverse 5’-GATCAACTTCTGAAAAAGCATTCCCAC-3’with Length of digested fragments wild allele: 249 bp, recessive allele: 223 bp +26 bp and NcoI restriction enzyme (Nyati *et al.*, 2010) , in single PCR reaction.

Statistical Analysis

Data were collected, summarized, analyzed and presented using statistical package for social sciences (SPSS) version 26, the independent sample t-test can examine mean differences between any two groups. The Chi-square test ($\chi2$), and the results are given as the correlation coefficient (r) and the significance level (P).In order to detect the cutoff value that predict a positive finding, receiver operator characteristic (ROC) curve analysis was used with its corresponding area under the curve (AUC), accuracy level, sensitivity, specificity and level of significance (*P*). Odds ratio and 95% confidence interval was estimated to measure risk. The P value (≤ 0.05) or ($P \leq 0.01$) P-value was regarded as highly significant.

RESULTS

Demographic Characteristics of Study Population

The mean age of patients was 19.48 ± 5.71 and that of control subjects was 21.42 ± 6.41 years and there was no significant difference between patients and control subjects in mean age (P= 0.119). The frequency distribution of patients and control subjects according to age groups was shown in table (1). Again, there was no significant difference in the frequency distribution of patients and control subjects according to age group (P= 0.249). Patients’ group included 39 (52.0 %) males and 36 (48.0%) females, while, control group included 37 (49.3 %) males and 38 (50.7%) females and there was no significant difference in the frequency distribution of patients and control subjects according to sex (P = 0.744).

Regarding smoking, 11 (14.7%) of T1DM patients with positive smoking, whereas, 12 (16.0%) of healthy control group have positive smoking, and the difference was non-significant in the frequency distribution of patients and control subjects according to smoking (P= 0.821). The present finding, which is the lack of significant difference in distribution of individuals in both groups according to age and sex, are a pre-requisite in order to avoid bias in such case control study.

Table (1): Demographic characteristics of T1DM patients and healthy control subjects.

Characteristic	T1DM patients *n* = 75	Healthy control *n* = 75	*P*
Age (years)			
Mean ±SD	**19.48± 5.71**	**21.42 ± 6.41**	**0.119 † NS**
Range	**10–35years**	**10– 35 years**	
<18, *n* (%)	**36 (48.0%)**	**29 (38.7%)**	**0.249 ¥ NS**
≥ 18, *n* (%)	**39 (52.0%)**	**46 (61.3%)**	
Sex			
Male, *n* (%)	**39 (52.0 %)**	**37 (49.3 %)**	**0.744 ¥ NS**
Female, *n* (%)	**36 (48.0%)**	**38 (50.7%)**	
M:F ratio	**1.08:1**	**1:1.03**	
Smoking status			
Smoking, *n* (%)	**11 (14.7%)**	**12 (16.0%)**	**0.821 ¥ NS**
Non-smoking, *n* (%)	**64 (85.3%)**	**63 (84.0%)**	

Body Mass Index (BMI), in both groups

The frequency distribution of T1DM patients and healthy control according to Body Mass Index (BMI) was shown in table (2). The proportion of T1DM patients with underweight (BMI <18.5 kg/m^2) was 16 (21.3%), the proportion of T1DM patients with normal weight (BMI, 18.5 to <25 kg/m^2) was 45 (60.0%), the proportion of T1DM patients with overweight (BMI, 25 to < 30 Kg/m^2) was 11 (14.7%) and only 3 (4.0%) of T1DM patients have obesity (BMI ≥30 kg/m2).

Healthy control enrolled in the present study was as the followings: 2 (2.7%) of healthy control were underweight, 48 (64.0%) was normal weight, 21 (28.0%) was overweight and only 4 (5.3%) of healthy control have, and there was significant difference in the frequency distribution of both groups according to BMI categories (P= 0.003). Also there was significant difference in the frequency distribution of both group according to BMI and weight (P < 0.05). But there was non-significant difference in the frequency distribution of both group according to Height (P = 0.782).

Table 2: Body Mass Index (BMI) in both groups.

Characteristic	T1DM Patients *n* = 75	Healthy control *n* = 75	*P*
Body Mass Index (BMI)			
Mean ± SD	21.57 ± 4.08	23.71 ± 4.09	0.041 † S
Range	14.8-38.4	18.2-39.6	
Height			
Mean ± SD	155.00 ± 11.3	151.16 ± 13.11	0.782 † NS
Range	103.0-188.0	75.0-173.0	
Weight			
Mean ± SD	43.44 ± 7.6	51.20 ± 10.7	0.022 † S
Range	24.0-110.0	12.0-85.0	
BMI categories			
Under-weight, *n* (%)	16 (21.3%)	2 (2.7%)	0.003 ¥ S
Normal weight, *n* (%)	45 (60.0%)	48 (64.0%)	
Overweight, *n* (%)	11 (14.7%)	21 (28.0%)	
Obese, *n* (%)	3 (4.0%)	4 (5.3%)	

E-Selectin Serum level of in T1DM patients and control.

The comparison of serum E-Selectin level between T1DM patients and healthy control subjects has been carried. Mean levels of serum E-Selectin were (6.99 ± 1.19 and 6.18 ± 1.06) in T1DM patients and healthy control respectively; the level was highly significant higher than in patients group in comparison with healthy control (P < 0.001). As show in **Figure (1)**.

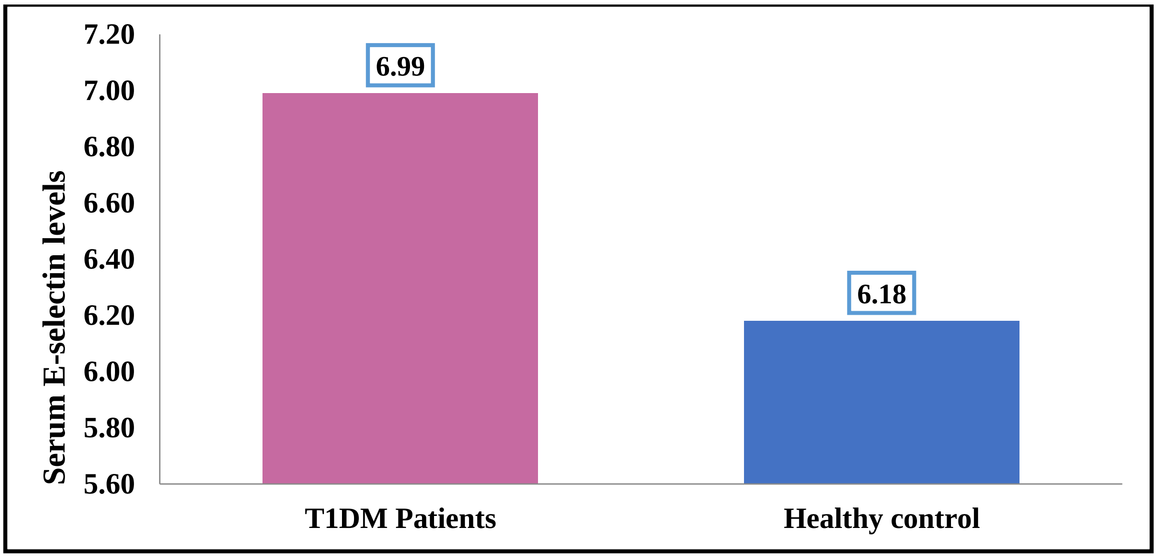

Figure (1): Mean Serum E-Selectin of patients and healthy controls.

Diagnostic Accuracy of E-Selectin level

Receiver operating characteristic (ROC) analysis was performed to reveal the prognostic accuracy of using E-Selectin concentrations to distinguish T1DM patients from healthy control subjects. An optimal E-Selectin cut-off value more than of 6.22 resulted in an AUC value of 0.619 (95% confidence interval [CI], 0.529-0.708, P= 0.003), sensitivity of 61.3%, specificity of 62.7%, PPV of 62.2%, and NPV of 61.8%. The present results indicate E-Selectin is considered as a weak prognostic marker to distinguish T1DM patients from healthy control. as show in **Figure (2)**.

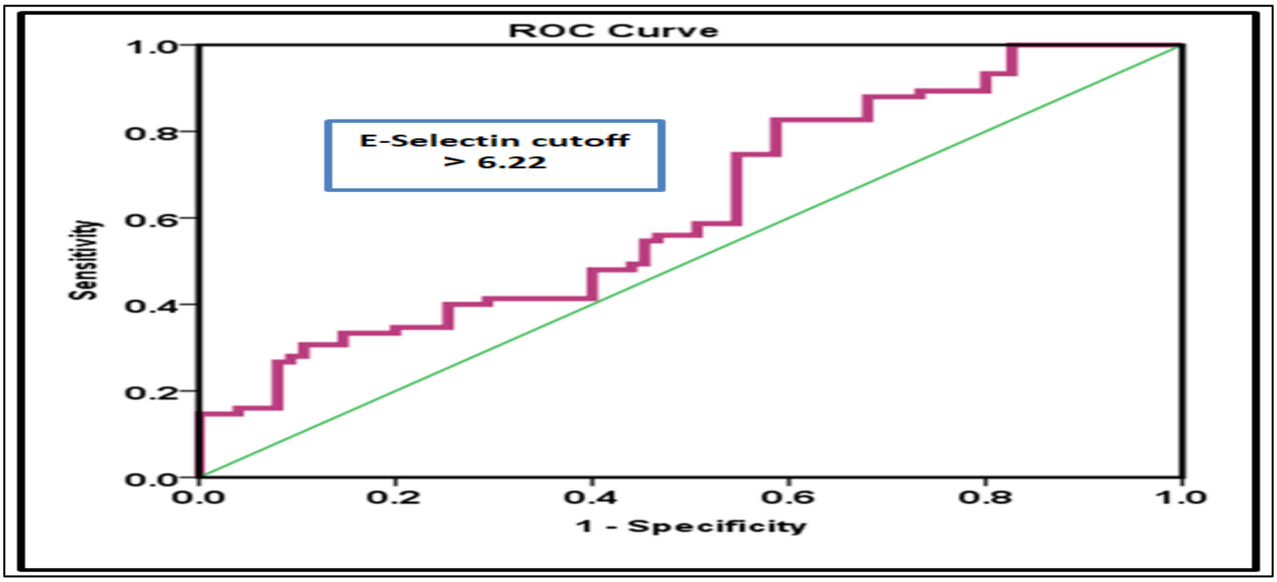

Figure (2): Receiver operator characteristic curve analysis of E-Selectin for the calculation of possible diagnostic cutoff value.

Detection *TLR4 Asp299Gly (rs4986790)* Polymorphism

The distribution of *Asp299Gly (rs4986790)* polymorphism was detected by PCR-RFLP technique; as show in **figure (3).**

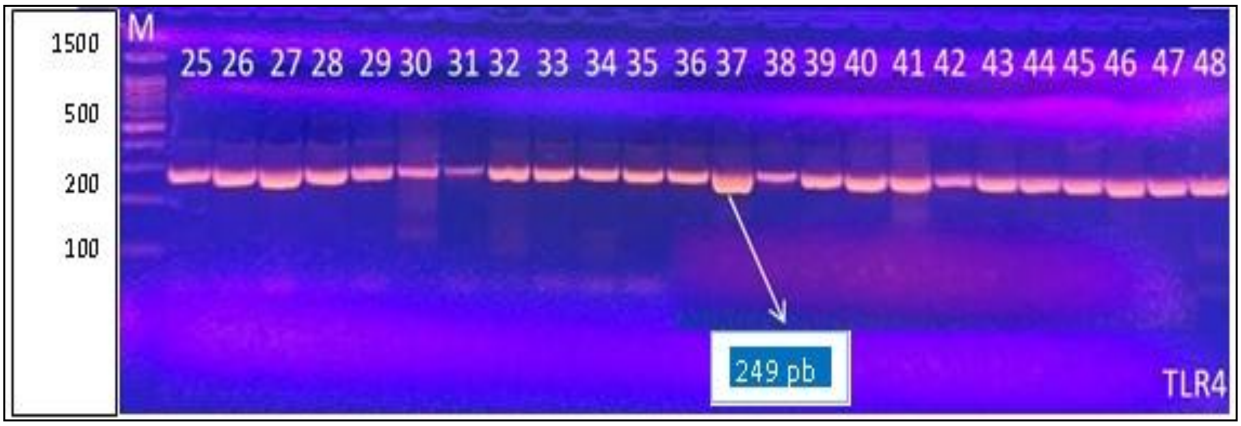

Figure (3): Agarose gel electrophoresis image that showed the PCR-RFLP product analysis of for TLR4 c.896A > G, Asp299Gly *(rs4986790)* genes polymorphism.

Where M: marker (1500-100bp). the lanes (AA) wild type homozygous were showed only A allele at 249bp PCR-RFLP product. Whereas the recessive allele did not find homozygous and heterozygous genotypes were showed as both A and G allele at223bp+26bp PCR-RFLP product. The outer internal control was observed at 249bp PCR-RFLP product.

Genotypic and Alleles Analysis for studied gene in patients and control.

The comparison of genotypes and allele frequencies concerning *Asp299Gly (rs4986790)* SNP between T1DM patients and healthy control, is show in **Table (1)**.

Table (1): *TLR4 Asp299Gly (rs4986790)* genotype frequency in T1DM patients and healthy control.

Asp299Gly (rs4986790)	**Patients** ***n* = 75**	**Control** ***n* = 75**	***P***	**OR**	**95% CI**
Genotype frequency					
Wild type	**75 (100.0%)**	**75 (100.0%)**			
Mutant type	**0**	**0**			
Allele frequency					
G	**0**	**0**			
A	**150 (100.0%)**	**150 (100.0%)**			

DISCUSSION

The globally increase the incidence and prevalence of T1DM approximately 3-5% per year has been reported (Barkai *et al*., 2019). These findings of this study, shown the higher prevalence (52.0%) of T1DM is in (≥ 18) age group. Although, traditionally associated with childhood onset, T1D can develop at any age, posing diagnostic challenges in adults due to the higher prevalence of type 2 diabetes (Thomas *et al*., 2018). The current study shown the results of smokers with T1DM was (14.7 %), compared with the of subjects infected with non-smokers, was (85.3.0%), and this is considered normal because the samples studied are mostly of children or teenager and that the nature of the disease, which affects the ages as autoimmune disease; therefore less percentage of smokers in these ages can be recorded. Regarding the mechanism of passive smoking promoting diabetes metabolism, some studies suggest that nicotine affects the function of islet cells and insulin (Yoshikawa *et al*., 2005).

Thus, children with T1D were more likely to be exposed to passive-smoking. The mean BMI of both group's T1DM and control 21.57 ± 4.08 kg/m2 and 23.71 ± 4.09 kg/m2, respectively. These results agree with another study by Knerr *et al.* patients diagnosed had a mean BMI of 21.6 kg/m2 (Knerr *et al*., 2005). The prevalence of underweight (21.3%) in this study was higher. Nevertheless, underweight as a risk factor for higher mortality has been supported by numerous studies (Conway *et al*., 2010). A cohort study of children with T1DM showed a sharp increase in both overweight patients, from 28.6% to 47%, and obese patients, from 3.4% to 22.7%, over a period of 18 years (Conway *et al* ., 2010).

Given these findings, there is no evidence linking the AG or GG genotype of (rs4986790) SNPs in the TLR4 gene to an increased risk of patients with T1DM. A polymorphism in the TLR4 gene (rs4986790) was linked to a higher incidence of microvascular problems, particularly diabetic retinopathy, in patients with type 2 diabetes (Zhang *et al*., 2022). Another study, demonstrated a strong association between (rs4986790) polymorphism and the risk of infection with (HIV) human immunodeficiency virus (Kim *et al*., 2020). Furthermore, Meliţ *et al*., (2019). Confirmed that (rs4986790) SNPs an increase the risk of infection of *Helicobacter pylori* (Meliţ *et al*., 2019).

CONCLUSION

According to the Receiver operating characteristic (ROC) analysis, in the present results indicates E-Selectin is considered as a weak prognostic marker to distinguish T1DM patients. Additionally, no such correlation was found into the *TLR-4* Asp299Gly (rs4986790) polymorphism in patients with T1D.

Ethical approval

The research was approved by the ethical committee of the Faculty of Medicine /AL-Qadisiyah University, and this research was performed with Al Najaf Diabetes and Endocrine center authorization.

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