

ORIGINAL ARTICLE

Toll-like receptor 4 and cytotoxic T cells CD8+ are prognostic markers in type 1 Diabetes Mellitus

Noor AlHuda Ghanem Hadi Al-Fatlawi

Abstract

Objective: To determine the correlation between toll-like receptor 4 and cytotoxic T cells in patients with type-1 diabetes mellitus.

Method: The cross-sectional, case control study was conducted at Al-Manatharah Hospital in the Al-Najaf province of Iraq from June 2021 to December 2021, and comprised patients of either gender aged 20-69 years having type-1 diabetes mellitus in group A, and healthy subjects in control group B. From all the subjects, 4ml blood was collected by vein puncture. Fasting blood glucose and lipid profile were estimated using a precipitant kit. Also measured were toll-like receptor 4 and cytotoxic T cell levels using an enzyme-linked immunosorbent assay kit. Data was also noted on age, gender and body mass index. Data was analysed using SPSS version 23

Results: Of the 100 subjects, 60(60%) were cases; 30(50%) males and as many females with mean age 59.21 ± 18.092 years. The remaining 40(40%) were controls; 20(50%) males and as many females with mean age 42.076 ± 11.019 years. BMI values were not significantly different between the groups ($p > 0.05$), and all lipid profile values were significantly higher in group A compared to group B ($p < 0.05$) except high-density lipoprotein which was higher in group B compared to group A ($p < 0.05$). Group A patients had significantly higher levels of toll-like receptor 4 and cytotoxic T cell than group B subjects ($p < 0.05$).

Conclusion: A significant relationship was found between type-1 diabetes and higher levels of toll-like receptor 4 and cytotoxic T cells.

Keywords: Diabetes mellitus, Toll-like receptor 4, Cytotoxic T cells, CD8. DOI: <https://doi.org/10.47391/JPMA.IQ-04>

Introduction

In type-1 diabetes mellitus (T1DM), pancreatic beta cells that produce insulin are specifically damaged, an autoimmune disease, without affecting other Langerhans cells.¹ It has been shown that both cellular and humoral immunity are involved in T1DM's pathophysiology.² The most prevalent theories suggest that environmental triggers, infections, nutrition and synthetic drugs can initiate self-targeting immune cascades in the early years of life.³

Anderson et al. showed that both central and peripheral immune tolerance mechanisms contribute to the emergence of autoreactive T cells in non-obese mice with diabetes.⁴

The most common hypothesis is that microbial infections initiate and/or exacerbate islet inflammation in genetically susceptible individuals.⁵ For instance, enteroviruses, such as coxsackievirus B1, have been linked to T1DM.⁶ Cells infected by viruses may experience direct cytolysis and/or localised inflammation that initiates or worsens the disease and may fuel autoimmune disease.⁷ Some stomach microbiota can either progress or prevent cell destruction

in a mouse model of non-obese diabetes, which is an unrestricted model of T1DM.⁸ It was noted that bacterial components and metabolites may influence the current state of activation and/or differentiation of innate and adaptive immune effectors.^{9,10}

Toll-like receptor 4 (TLR4) is a transmembrane protein and a member of the pattern recognition receptor (PRR) family. Its activation triggers the production of inflammatory cytokines and the nuclear factor kappa B (NF- κ B) intracellular signalling pathway, which together activate the innate immune system. TLRs play a crucial role in immunological and inflammatory responses.¹¹

The activation of TLRs 2 and 4 results in the production of cytokines and chemokines. Therefore, ligand restriction by TLRs may result in an inflammatory reaction.¹²

TLR4-expressing cells of the immune system, both innate and adaptive, are involved in inflammation and immune system processes that cause T1DM.¹³

Cluster of differentiation 8 (CD8) is a transmembrane glycoprotein that serves as a co-receptor for the T-cell receptor (TCR). Naive cytotoxic T lymphocytes (CD8+) require antigen-specific activation and fundamental cytokine signalling to become effector cells.¹⁴ Major histocompatibility complex (MHC) class I atoms, which are

LAB Investigations, University of Kufa, Iraq.

Correspondence: : Noor AlHuda Ghanem Hadi Al-Fatlawi

email: noorulhudag.hadi@uokufa.edu.iq

present on both completely nucleated cells and platelets, are used to provide peptide antigens to CD8+ T lymphocytes. Endogenous peptides are delivered to cytotoxic T lymphocytes by MHC class I receptors, but it can also be possible for CTLs to be exposed to exogenous peptides through a mechanism known as cross-presentation.¹⁵ Dendritic cells in secondary lymphoid organs deliver antigens to naive CD8+ T cells during illness. The peptide/MHC I complex requires the optimal restriction of TCR. Using the perforin-granzyme route and death receptor pathway, cytotoxic T lymphocytes exhibit killer effector functions.¹⁶

T1DM in both mouse models and humans, have autoreactive CD8+ T lymphocytes, which are activated by peptide antigens supplied by MHC class I proteins, which are the main initiators of beta-cell destruction.¹⁷

The current study was planned to determine the relationship between TLR4 and CD8 in T1DM.

Patients and Methods

The cross-sectional, case control study was conducted at Al-Manatharah Hospital in the Al-Najaf province of Iraq from June 2021 to December 2021. After approval from the ethics review board of the University of Kufa, Iraq, the sample size was calculated in the light of an earlier study¹⁸ using the formula:

$$n = \left(\frac{z^2 p(1-p)}{d^2} \right)$$

The sample comprised patients of either gender aged 20-69 years having T1DM for at least one year in group A, and healthy subjects in control group B. Patients outside the age range and those having an acute infection were excluded. The patients were separated into subgroups based on gender, age and body mass index (BMI). The patients were diagnosed by an expert physician. Demographic and clinical data was recorded using a questionnaire.

After taking informed consent from the participants, simple random blood samples were collected from all the subjects using disposable needles and plastic syringes for venepuncture of the antecubital veins between 9am and 11am.

Venous blood 4mL was collected in gel tubes and coagulated for 10min at room temperature. After 15min of centrifugation at 3000 runs per minute (rpm), the serum was separated, 1ml of which was used for enzyme-linked immunosorbent assay (ELISA) tests.

BMI was calculated and subjects were classified as per using the standard definition.¹⁹

The level of human fragment crystallizable gamma receptors (FCGR) IIb in serum was measured using a kit provided by Medical LLC / China (Catalogue Np. E-EL-H0063/96T)

The concentration of CD8 in the serum was measured using a kit purchased from Elabscience / China (Catalogue No. E-EL-H 2359/96T).

Lipid profile, including total cholesterol (TC), high-density lipoprotein (HDL) and triglyceride (TG), was measured using a precipitant kit (Biolabo SA, France).

Data was analysed using SPSS version 23 Descriptive and inferential statistics were employed and $p < 0.05$ was taken as significant for all comparisons.

Results

Of the 100 subjects, 60(60%) were cases; 30(50%) males and as many females with mean age 59.21 ± 18.092 years. The remaining 40(40%) were controls; 20(50%) males and as many females with mean age 42.076 ± 11.019 years. BMI values were not significantly different between the groups ($p > 0.05$), while all lipid profile values were significantly higher in group A compared to group B ($p < 0.05$) except HDL which was higher in group B compared to group A ($p < 0.05$) (Table 1).

There was a significant increase in the serum TLR4 level in group A (7.561 ± 2.431 ng/ml, interquartile range [IQR]: 6.98) compared to group B (3.851 ± 1.323 ng/ml, IQR: 3.35) (Figure 1).

There was a significant increase in serum CD8 level in group A (6.5 ± 1.06 ng/ml, IQR: 6.3) compared to group B (0.973 ± 0.31 ng/ml, IQR: 1.3) (Figure 2).

There was a non-significant decrease in serum TLR4 levels in females compared to males in group A (7.092 ± 2.782 ng/ml, IQR: 7.3 vs. 7.709 ± 1.007 ng/ml, IQR: 7.12) (Figure 3).

There were significantly higher serum CD8 levels in females (6.061 ± 2.464 ng/ml, IQR: 6.22) than males in group A (1.462 ± 1.313 ng/ml, IQR: 1.61) (Figure 4).

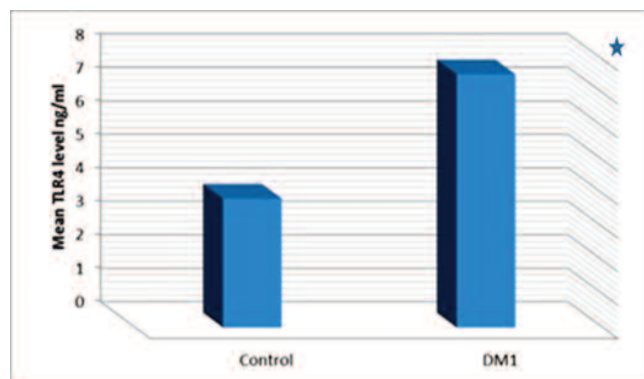
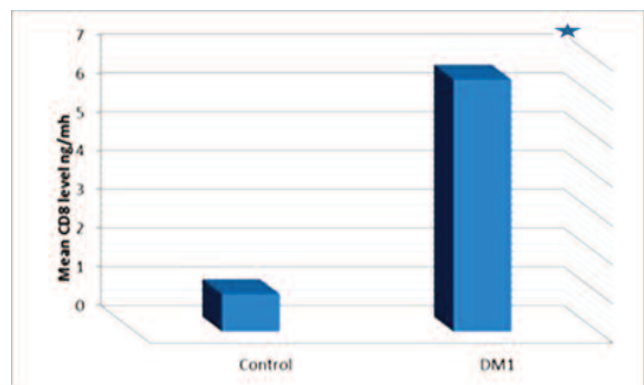
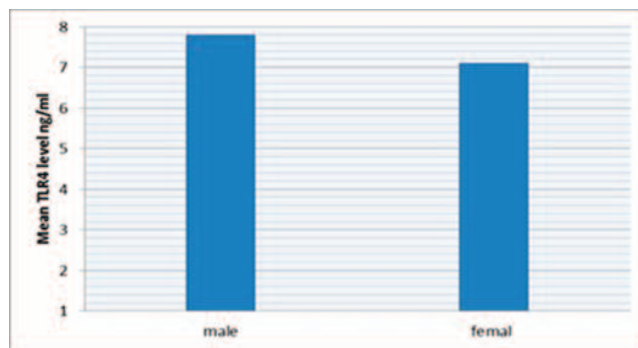
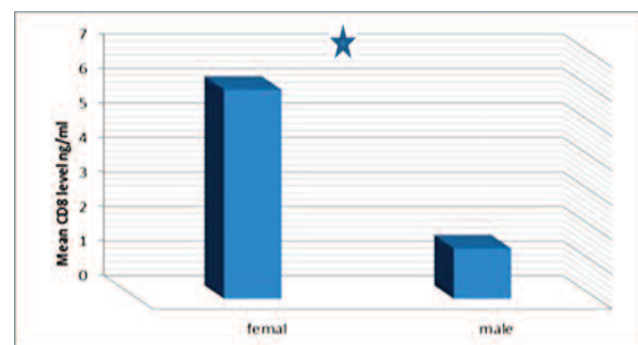
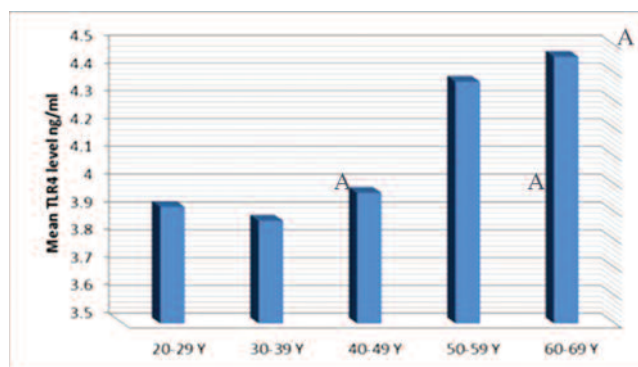
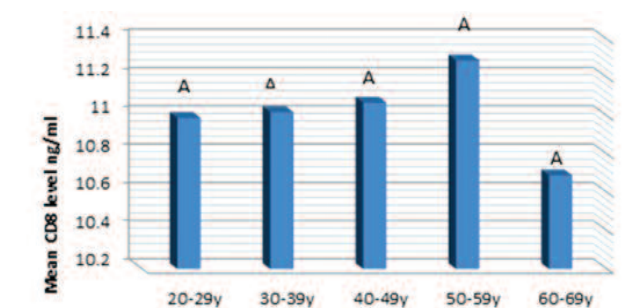
Patients in group A aged 20-29 years, 30-39 years, 40-49 years, 50-59 years, and 60-69 years had serum TLR4 levels of 3.92 ± 0.89 ng/ml, IQR: 3.81, 3.87 ± 1.21 ng/ml, IQR: 3.78, 3.97 ± 1.18 ng/ml, IQR: 4.01, 4.37 ± 1.05 , IQR: 4.32 and 4.46 ± 2.18 , IQR: 4.12), respectively, with no significant differences (Figure 5).

Patients in group A aged 20-29 years, 30-39 years, 40-49 years, 50-59 years, and 60-69 years had serum CD8 levels of 11.099 ± 3.87 ng/ml, IQR: 10.93, 10.902 ± 3.77 ng/ml, IQR:

Table: Baseline characteristics and demographics of adult diabetes patients and controls.

Groups Characteristics	*Control* (n=40)	*Patients* (n=60)
Age (year), Mean $\pm$ SD	42.076 $\pm$ 11.019	59.21 $\pm$ 18.092
Median (Range), IQR:	50 (22-69), 43.32	52 (20-69): 57.12
Gender N. (%)		
Males	20 (50%)	20 (50%)
Females	30 (50%)	30 (50%)
BMI (Kg/m ²), Mean $\pm$ SD IQR:		
Normal weigh	22.019 $\pm$ 1.019, 22.01	23.074 $\pm$ 1.040, 22.67
Overweight	25.049 $\pm$ 1.050, 25.35	27.009 $\pm$ 0.099, 26.5
Obese weight	30.089 $\pm$ 1.014, 30.15	33.23 $\pm$ 2.002, 31.08
FBG mg/dl, Mean $\pm$ SD IQR:	95.095 $\pm$ 17.046, 98.21	218.02 $\pm$ 87.083 *, 222.78
Chole. mmol/L, Me $\pm$ SD IQR:	3.099 $\pm$ 1.022, 2.78	5.060 $\pm$ 0.034 *, 5.12
TG mmol/L, Me $\pm$ SD IQR:	1.971 $\pm$ 0.741, 1.89	3.101 $\pm$ 1.901 *, 3.56
LDL mmol/L, Me $\pm$ SD IQR:	2.033 $\pm$ 1.009, 2.45	3.006 $\pm$ 1.029 *, 3.19
VLDL mmol/L, Me $\pm$ SD IQR:	0.911 $\pm$ 0.301, 1.07	1.55 $\pm$ 0.791 *, 1.89
HDL mmol/L, Me $\pm$ SD IQR:	1.301 $\pm$ 0.141, 1.23	0.991 $\pm$ 0.491 *, 1.002

N: Numbers, Me: Mean, FBG: Fasting blood glucose, Chole: Cholesterol, TG: Triglycerides, LDL-C: low-density lipoprotein, BMI: Body mass index, VLDL: Very low-density lipoprotein, HDL: High-density lipoprotein, SD: Standard deviation, IQR: Interquartile range. * Mean significant difference at ($P < 0.05$).

**Figure 1:** Toll like receptor 4 (TLR4) levels in type-1 diabetes mellitus (T1DM) and control groups.
* Significant difference ($p < 0.05$).**Figure 2:** Cytotoxic T. cells (CD8) level in type-1 diabetes mellitus (T1DM) and control groups.
* Significant difference ($p < 0.05$).**Figure 3:** Toll-like receptor 4 (TLR4) levels in male and female type-1 diabetes mellitus (T1DM) patients.**Figure 4:** Cytotoxic T. cell (CD8) level in male and female type-1 diabetes mellitus (T1DM) patients.
* Significant difference ($p < 0.05$).**Figure 5:** Effect of age on Toll-like receptor 4 (TLR4) levels in type-1 diabetes mellitus (T1DM) patients. Non-significant differences ($p > 0.05$).**Figure 6:** Effect of age on Cytotoxic T. cell (CD8) level in type-1 diabetes mellitus (T1DM) patients. Non-significant differences can be seen in the same letters.

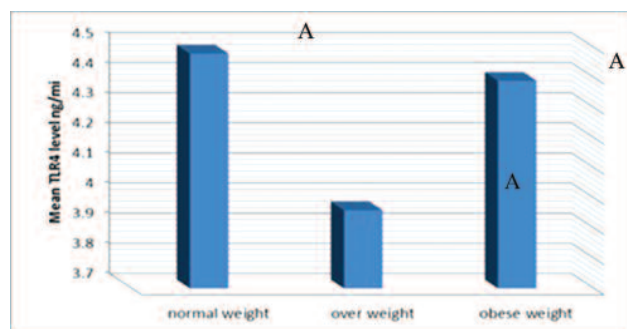


Figure 7: Effect of body mass index on Toll-like receptor 4 levels in patients with T1DM. Non-significant differences can be seen in the same letters.

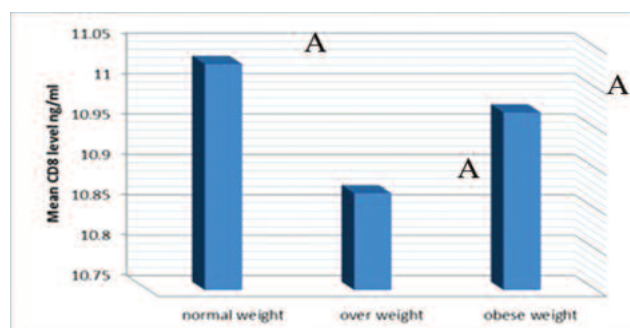


Figure 8: Effect of body mass index (BMI) on Cytotoxic T. cells (CD8) level in type-1 diabetes mellitus (T1DM) patients. Non-significant differences can be seen in the same letters

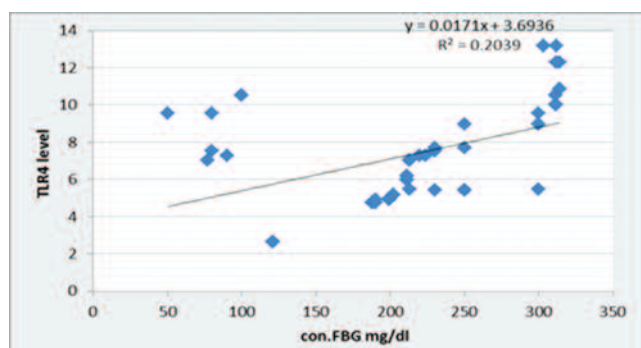


Figure 9: Correlation between Toll-like receptor 4 (TLR4) and fasting blood glucose (FBG) concentrations in type-1 diabetes mellitus (T1DM).

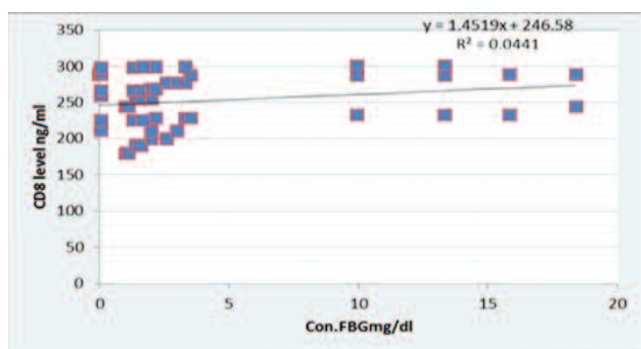


Figure 10: Correlation between Cytotoxic T. cells (CD8) and fasting blood glucose (FBG) concentrations in type-1 diabetes mellitus (T1DM) patients.

10.67, 10.907 ± 1.99 ng/ml, IQR: 11.01, 11.329 ± 3.62 ng/ml, IQR: 11.39 and 10.70 ± 2.04 ng/ml, IQR: 11.12, respectively, and the difference was non-significant (Figure 6).

Group A patients in the three BMI categories had serum TLR4 levels of 4.448 ± 1.013 ng/ml, IQR: 4.39, 4.096 ± 1.112 ng/ml, IQR: 4.45 and 3.939 ± 0.109 ng/ml, IQR: 4.17, respectively, with the differences being non-significant (Figure 7).

Group A patients in the three BMI categories had serum CD8 levels of 11.103 ± 2.08 ng/ml, IQR: 10.23, (11.011 ± 2.19) ng/ml, IQR: 10.91 and 10.90 ± 0.78 ng/ml, IQR: 11.31., respectively, with the differences being non-significant (Figure 8).

Correlation and linear regression analysis between TLR4 and FBG in group A patients showed a non-significant positive association (Figure 9) and a non-significant positive association between CD8 and FBG (Figure 10).

Discussion

The study revealed a significant increase in FBG levels in T1DM patients compared to the controls. The outcomes were expected because the main characteristic of DM is hyperglycaemia. Blood glucose is firmly controlled by two key processes: insulin secretion by pancreatic β -cells in response to nutrients, and insulin activity on significant objective organs, that is, skeletal muscle, liver and adipose tissue. T1DM is often associated with obesity and results from insufficient insulin production/secretion and insulin resistance (IR).²⁰

Data showed a significant decrease in HDL and an increase in TG, low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) levels in patients compared to the controls. In diabetes, blood glucose is not utilised by tissues, resulting in hyperglycaemia, and fatty acids from adipose tissues are mobilised for energy purposes. Excess fatty acids accumulate in the liver, which is then converted to TG and affects lipid profile levels in general.²¹

The results showed a significant increase in serum TLR4 levels in the patient group compared to the control group.

T1DM is a proinflammatory state characterised by increased levels of circulating biomarkers of monocyte activity. Increased inflammation in T1DM might be partially mediated by the activation of the innate immune pathway by TLRs.²²

The involvement of TLR2 and TLR4 in hyperglycaemia and diabetes in humans is poorly understood. TLR4 messenger ribonucleic acid (mRNA) expression is increased in fat tissues of db/db mice (mice express mutations in leptin receptor that leads to obesity).²³

Mohammad et al. showed increased TLR4 expression in type 1 diabetic non-obese mice, and this is related to increased NFK (Nuclear factor kappa-light-chain-enhancer of activated B cells) activation in response to the TLR4 ligand, LPS (Lipopolysaccharide), resulting in increased proinflammatory cytokines.²⁴ TLRs are characterised by an extracellular ligand binding domain, a single transmembrane domain, and an intracellular domain.²⁵ Upon ligand binding, TLR subunits associate, prompting the arrangement of the toll-interacting region domain-containing adaptor proteins of the MyD88 family (Myeloid differentiation primary response 88). Subsequent downstream signal transduction events leading to the activation of MAPKs (mitogen-activated protein kinase) and NFK and transcription of proinflammatory chemokines, such as monocyte chemoattractant protein-1, and cytokines, such as interleukin-1 (IL-1), IL-6, and tumour necrosis factor (TNF).²⁶ MyD88 is also involved in NFK activation by every TLR identified so far, except TLR3 leading to increased expression of proinflammatory cytokines IL-1 and IL-6.²⁷

T1DM is a pro-inflammatory state and increased TLR4 expression may contribute to the pro-inflammatory state of diabetes. Increased TLR4 levels in T1DM are similar to those observed in other immune diseases, such as immune thrombocytopenia.²⁸

The current results showed a significant increase in the serum CD8 levels of the patients compared to the control group.

CD8-mediated autoimmune diseases result from the breakdown of self-tolerance mechanisms in autoreactive CD8 T cells. How autoimmune T cell populations emerge and are supported, and the molecular projects characterising immune system T cells are obscure. In previous studies in mice, researchers followed the fate of β -cell-specific CD8+ T cells in non-obese diabetic mice, and showed that type 1 diabetes is a stem-like autoimmune progenitor population in the pancreatic lymph node (pLN), which self-renews and gives rise to pLN autoimmune mediators. The pLN autoimmune mediators migrate to the pancreas, where they further differentiate into β -cells.^{29,30} In type 1 diabetes, β -cell-specific CD8 T cells destroy insulin-producing β -cells. The main causes of autoreactive CD8+ T cells are a critical component of beta-cell destruction in both human patients and the non-fat diabetic NOD animal model of T1DM. Deficiency of CD8+ T-cell populations protects mice from insulinitis and diabetes. T cells that have been activated in pLNs and are specific for beta-cell antigens enter the inflamed islets.³¹ The islets become more pro-inflammatory and open to additional immune infiltration once the T cells infiltrate the islets. Insulinitis

represents the dynamic immune cell invasion of islets, the most common of which are CD8+ T cells, followed by macrophages and a much smaller population of CD4+ T cells.^{30,31}

Compared to the male patient group, the results showed a considerable increase in serum CD8 levels in the female patient group in the current study. Sex hormones have a significant impact on the shape of T-cell reactions. Sex hormones also indirectly affect the transcriptional patterns of T cells, and also have an impact on T cell responses by influencing innate immune cells and thymic epithelial cell gene expression. The role of oestrogen in autoimmune diseases is quite complex.³²

Conclusion

The importance of targeting CD8+ T cells in therapeutic approaches was found to be of critical value. Increased TLR4 and CD8 levels in T1DM patients were crucial prognostic indicators of the disease.

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Conflict of Interest: None.

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