MODERN MEDICINE AND PRACTICE
https://inovatus.es/index.php/ejmmp

EUROPEAN JOURNAL OF MODERN MEDICINE AND PRACTICE
Vol. 4 No. 7 (Jul - 2024) EJMMP ISSN: 2795-921X

EVALUATION THE EXPRESSION OF THE CTLA-4 AND MIR-21 IN CHILDREN AND ADOLESCENTS WITH TYPE 1 DIABETES MELLITUS NAJAF CITY, IRAQ

Haider. A. Alluhaiby, Prof. Dr. Manal. K. Mohammad
Department of Microbiology, College of Medicine, University of Al-Qadisiyah, Iraq

Abstract: Background: Type 1 diabetes mellitus (T1DM) is a chronic condition and is an autoimmune disease, the body's immune system mistakenly attacks and destroys the insulin-producing beta cells in the pancreas. **Aim of study:** This study aims to look to evaluation the expression of the miR-21 and evaluation the expression of the CTLA-4 in serum of children and adolescents with T1DM. **Materials Methods:** This study included 100 patients with type 1 diabetes mellitus who were admitted to hospital from the period September 2023 and December 2024 and other group consist of 100 healthy individuals used as a control Samples were collected in a gel and EDTA tubes, and significant results were shown for the samples, evaluation the expression of the miR-21 identified by the quantitive real time PCR (qRTpcr). Then the and enzyme-linked immunosorbent assay (ELISA) method was used to perform to evaluation the expression of the CTLA-4 according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. **Results:** The level of serum MicRNA21 was highly significantly higher than in patients' group in comparison with healthy control subjects and the level of serum CTLA4 was highly significantly lower than in patients' group in comparison with healthy control subjects. **Conclusion:** Both CTLA4 and MiRna 21, can be used as predictive and diagnostic biomarkers for type 1 diabetes mellitus.

Key Words: type 1 diabetes mellitus, CTLA4, MiRna 21.

Introduction:

Type 1 diabetes is an autoimmune disorder where the body's immune system targets and destroys pancreatic beta cells, leading to absolute insulin deficiency and resulting in hyperglycemia (1). As previously mentioned, the action of autoantibodies leading to β-cell destruction commences at an initial stage, with approximately 90% of cells being destroyed prior to the stage where symptoms become evident (2). T1DM accounts for about 5-10% of all diabetes cases globally and the incidence of T1DM is increasing worldwide, recent estimates suggest an annual increase of about 3-4% in many countries, the prevalence of T1DM varies significantly by region, Northern Europe and North America have higher prevalence rates, while Asia has lower rates. (3). The incidence of T1DM has been increasing over the past few decades, possibly due to environmental changes and improved diagnostic capabilities. (4). The number of T1DM cases is expected to continue rising, with a significant increase in developing countries as they adopt more Westernized lifestyles. (5). Mortality rates have decreased due to advancements in insulin therapy and diabetes management, but T1DM still carries a higher risk of premature death compared to the general population. (6). T1DM is primarily an autoimmune disorder. The immune system mistakenly targets and destroys the beta cells in the pancreatic islets of Langerhans. (7). The presence of autoantibodies against beta cell antigens, such as glutamic acid decarboxylase (GAD65), insulin, and insulinoma-associated antigen-2 (IA-

2), is a hallmark of T1DM and can be detected years before clinical onset. (8). Both CD4+ helper T cells and CD8+ cytotoxic T cells are involved in the immune attack on beta cells. CD8+ T cells directly kill beta cells, while CD4+ T cells help orchestrate the immune response. (9). In T1DM, defective CTLA-4 function can contribute to the failure of immune tolerance, allowing autoreactive T cells to escape regulation and target pancreatic beta cells for destruction. (10). Impaired CTLA-4 signaling may result in inadequate suppression of autoreactive T cells, facilitating their activation and proliferation. This leads to increased immune-mediated destruction of beta cells. (11). Tregs play a key role in maintaining self-tolerance and preventing autoimmunity. CTLA-4 is critical for the suppressive function of Tregs. Defective CTLA-4 in Tregs can reduce their ability to control autoreactive T cells, contributing to the autoimmune attack on beta cells. (12). Differences in miRNA expression profiles between individuals can contribute to varying susceptibilities to T1DM. Individuals with a family history of T1DM may exhibit distinct miRNA expression patterns that predispose them to the disease. (13). MiRNAs can serve as potential biomarkers for early detection and diagnosis of T1DM. Circulating miRNAs in blood or other body fluids can reflect ongoing autoimmune processes and beta cell destruction. (14). Targeting dysregulated miRNAs offers a potential therapeutic approach for T1DM. Strategies include using miRNA mimics to restore the function of downregulated miRNAs or using miRNA inhibitors (antagomirs) to block the effects of upregulated miRNAs. (15). Elevated levels of miR-21 have been associated with increased beta cell apoptosis. It targets various pro-survival genes and can enhance the susceptibility of beta cells to autoimmune destruction. (16). MiR-21 is known to be upregulated in response to inflammatory cytokines such as IL-1β and TNF-α, which are involved in the autoimmune attack on pancreatic beta cells. Elevated miR-21 levels contribute to the pro-inflammatory environment that promotes beta cell destruction. (17). MiR-21 modulates the activity and differentiation of T cells, including the balance between regulatory T cells (Tregs) and effector T cells. Dysregulation of miR-21 can lead to a reduction in Treg function and an increase in autoreactive T cells, exacerbating the autoimmune response against beta cells. (18). MiR-21 is implicated in the endoplasmic reticulum (ER) stress response, which is a critical pathway leading to beta cell apoptosis in T1DM. Upregulation of miR-21 contributes to ER stress-induced apoptosis, further reducing beta cell mass. (19). MiR-21 influences the expression of chemokines and their receptors, which are involved in the recruitment of immune cells to the pancreatic islets. Increased miR-21 levels can enhance the infiltration of autoreactive immune cells into the islets, promoting the destruction of beta cells. (20). Environmental influences, including diet and lifestyle factors, can impact miR-21 expression and contribute to the development of T1DM. For instance, dietary components that affect inflammation and immune function may modulate miR-21 levels. (21). Circulating levels of miR-21 in blood or other body fluids can serve as a potential biomarker for the early detection of T1DM. Elevated miR-21 levels may reflect ongoing beta cell destruction and inflammation. (22). MiR-21 expression levels could help predict disease progression and the risk of complications. Monitoring miR-21 levels might provide insights into the effectiveness of therapeutic interventions. (23). Targeting miR-21 with specific inhibitors (antagomirs) offers a potential therapeutic approach for T1DM. By reducing miR-21 levels, it may be possible to alleviate beta cell apoptosis and modulate immune responses, thereby preserving beta cell function. (24). Combining miRNA21-based therapies with other immunomodulatory treatments may offer synergistic effects in preserving beta cell function and preventing autoimmune destruction. (25). Understanding individual miRNA expression profiles can enable personalized treatment strategies for T1DM. Personalized medicine approaches can optimize therapeutic interventions based on specific miRNA dysregulation patterns. (26).

Material and method:

Study design:

The case-control study was conducted in the Al-Najaf governorate. The participants included in this study were 100 patients (55 males and 45 females), all of whom were diagnosed with T1DM and aged between 3

and 18 years. The study also included a healthy control group of 100 individuals (55 males and 45 females), also ranging from 3 to 18 years in age. They were attended the consultant clinic for Diabetes and Endocrinology Center in Al-Sadr teaching hospital in the period from 10 September 2023 to 20 December 2024 under the supervision of medicine physician. Patients were diagnosed with diabetic T1DM according to the history, laboratory examination (Hba1c test). The data concerning patients were gathered from their personal records and direct patient-questionnaire, which included information such as patient number, family medical history and the treatment they're undergoing. Every participant gave their informed consent verbally. four milliliters of venous blood were drawn by disposable syringe under aseptic technique.

Ethical approval:

Before beginning sample collection for this study, the project received preliminary permission. Furthermore, the Scientific Research Methodology Ethics Committee of Al-Qadisiyah University's Medicine College, Microbiology Branch approved the study's design. by the document numbered 30/5121 in the date of 10 September, 2023.

Laboratory work:

Two ml of blood were collected in ethylinditetracitic acid (EDTA) tube, and stored at ¯20°C for DNA extraction for detection of MiRna21 by RT-PCR. Three ml of blood were collected in gel tube, and stored at ¯4°C for detection of CTLA-4 by ELISA method.

Statistical analysis:

The data was collected, summarised, analysed, and presented using statistical software such as SPSS version 20 and Microsoft Office Excel 2019. The data was analysed by calculating the mean and standard deviation. To determine if the variables were normally distributed, a Kolmogorov-Smirnov normality test was performed. For studying the difference in mean between any two groups, the independent sample t-test is commonly used, assuming that the variable follows a normal distribution. The One-way ANOVA test is commonly used to analyse the difference in mean between multiple groups, assuming that the variable follows a normal distribution. A chi-square test was utilised to examine the relationship between two categorical variables. Using receiver operator characteristic (ROC) curve analysis and its corresponding area under the curve (AUC), we were able to determine the cutoff value that predicts a positive finding. Estimates were made of the odds ratio and 95% confidence interval to assess risk, accuracy level, sensitivity, specificity, and level of significance (P). The level of significance was set at a P-value of less than 0.05, indicating statistical significance. An even higher level of significance was considered at 0.01 or less, indicating a highly significant result.

Results:

Subjects Immunological Analysis Results:

Serum (CTLA-4) level in patients with type 1 diabetic mellitus and healthy control:

The serum levels of CTLA-4 were compared among patients with T1DM and healthy subjects. The findings from this comparison are illustrated in table 1 and Figure1. The average levels of serum CTLA-4 are also provided, were 461.7 ± 94.52 ng/ml and 1057.39 ± 173.96 ng/ml, in patients with T1DM and healthy control respectively; the level was highly significantly higher than in patients' group in comparison with healthy control subjects ($P < 0.0001$).

Table (1): Serum (CTLA-4) level in patients with type 1 diabetic Meletus and healthy control.

			P
	Patients *n* = 100	Healthy control *n* = 100	
(CTLA-4) level (ng/ml)			
Mean± SD	461.7 ± 94.52	1057.39 ± 173.96	< 0.0001 † HS
Range	378.42-687.33	798.43-1296.74	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; NS: not significant at P > 0.05
HS: Highly significant at P ≤ 0.0001.

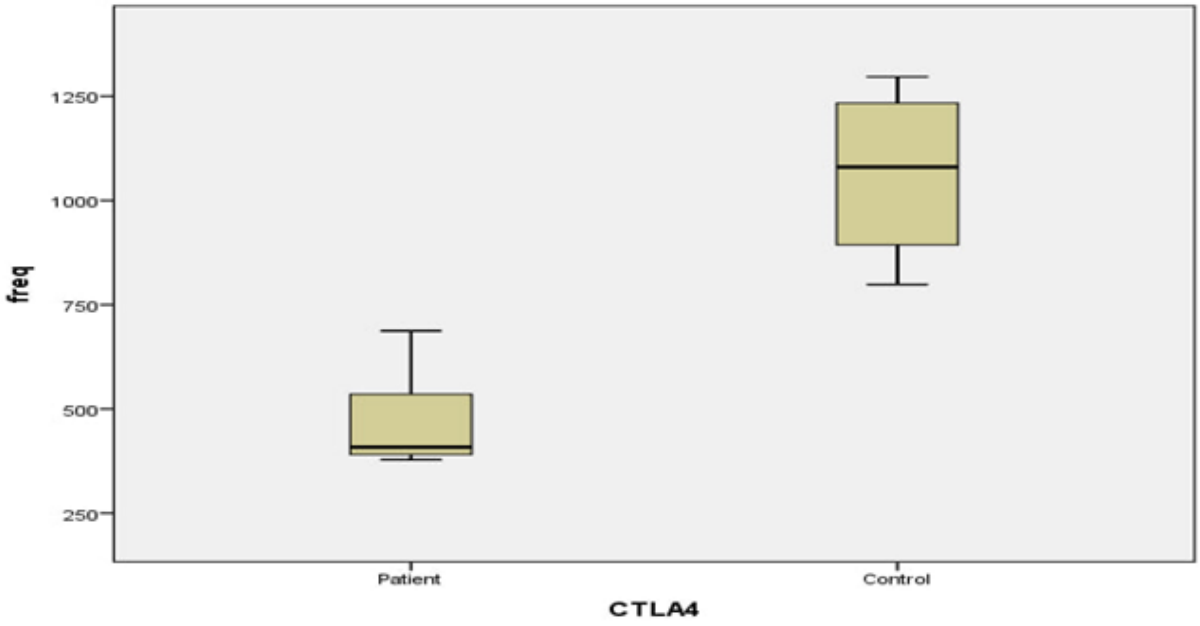

Figure (1): Serum (CTLA-4) level of patients and healthy controls.

Evaluation of (CTLA-4):

In order to assess the cutoff value of CTLA-4 and its predictive potential for T1DM diagnosis, a Receiver Operator Characteristic (ROC) curve analysis was conducted. The outcome of this analysis is presented in the following table (2), and figure (2).

Table (2): Sensitivity and specificity of (CTLA-4) level (> 742.8785-fold) in type 1 diabetic mellitus disease.

(CTLA-4) level	**patients *n* = 100**	**Healthy control *n* = 100**
≥742.8785	0 (%)	100 (%)
< 742.8785	100 (%)	0 (%)
Sensitivity %	100 %	
Specificity %	100%	
PPV %	100 %	
NPV %	100 %	
AUC (95% CI)	0.1 (1-1)	

CI: Confidence interval, AUC: Area under curve. PPV= positive patient value. NPV= negative patient value.

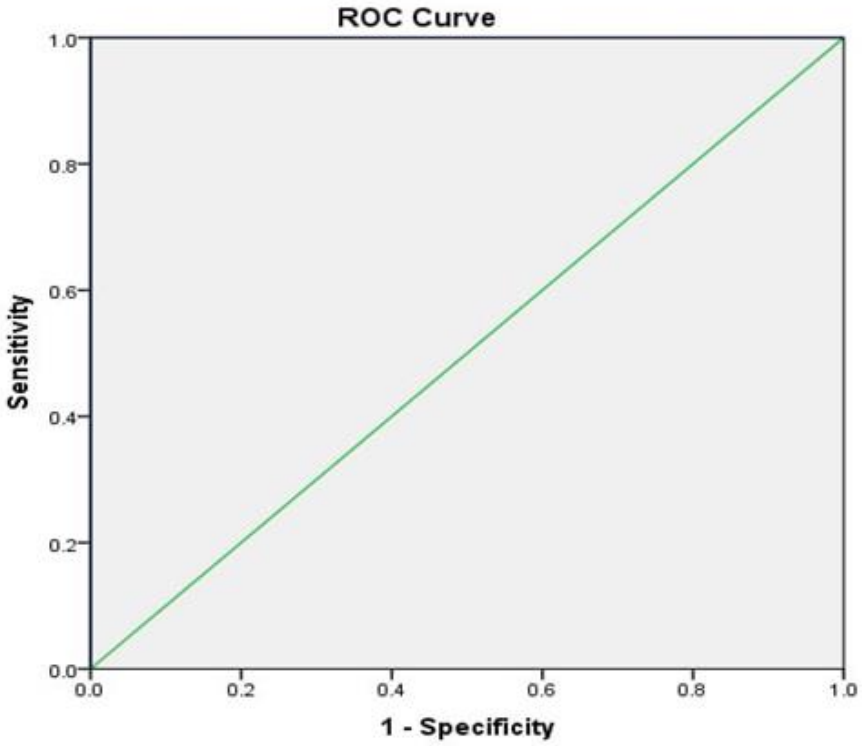

Figure (2): Receiver operator characteristic curve analysis of (CTLA-4) for the calculation of possible diagnostic cutoff value.

Frequency distribution of (CTLA-4) levels according to sex:

The comparison of (CTLA-4) patient levels according to gender has been carried out and the results were demonstrated in table (3). whereas Mean levels of serum in male and female (CTLA-4) were 666 ± 166.55 ng/ml, and 544.7 ± 97.27 ng/m respectively.

Table (3): Frequency distribution of (CTLA-4) levels according to gender.

	Male/Female comparison		
	Male *n* = 52	Female *n* = 48	*P*
(CTLA-4)			
Mean± SD	666 ± 166.55*	544.7 ± 97.27	0.002 † S
Range	478.45 – 977.7	408.43- 960.56	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; S: significant at P < 0.05 NS: not significant at P > 0.05.

Frequency distribution of (CTLA-4) patients serum levels according to Family history:

The comparison of (CTLA-4) patient levels according to family history the analysis has been conducted and the findings of this study are visually represented in Table (4) whereas Mean levels of serum in positive family history and negative family history (CTLA-4) were 581.19 ± 103.82 ng/ml, and 618.01 ± 187.43 ng/ml respectively.

Table (4): Frequency distribution of (CTLA-4) in patients serum levels according to family history.

	Family history comparison		
	Positive *n* = 27	Negative *n* = 73	*P*
(CTLA-4)			
Mean± SD	581.19 ± 103.82	618.01 ± 187.43	0.36 † NS
Range	448.38 – 797.6	408.42- 977.75	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; S: significant at P < 0.05 NS: not significant at P > 0.05

Detection of (MiRNA 21) Gene expression:

The relative expression of the miRNA-21 gene in the T1DM patient and control groups was established using quantitative real-time PCR (qRT-PCR). The data reported identified a significant variation in the miR-21 expression level between individuals with T1DM and the normal controls. As depicted in table (5) and Figure (3), the average expression level of miR-21 came to 0.74 in controls and 1.09 in T1DM patients. This outcome indicates a notable elevation of the miR-21 gene in patients with T1DM in comparison to the control group (P= 0.035)

Table (5): Comparison of serum level *miRNA 21* between patient with Type 1 Diabetes Mellitus and healthy control.

Parameter	**Mean ± SD**		**P-value † S**
	Patient (n=100)	**Control (n=100)**	
miRNA 21	1.09 ± 0.19	0.74 ± 0.06	0.035

n: number of cases; **SD**: standard deviation; †: independent samples t-test; NS: not significant at P > 0.05 S: significant at P < 0.05.

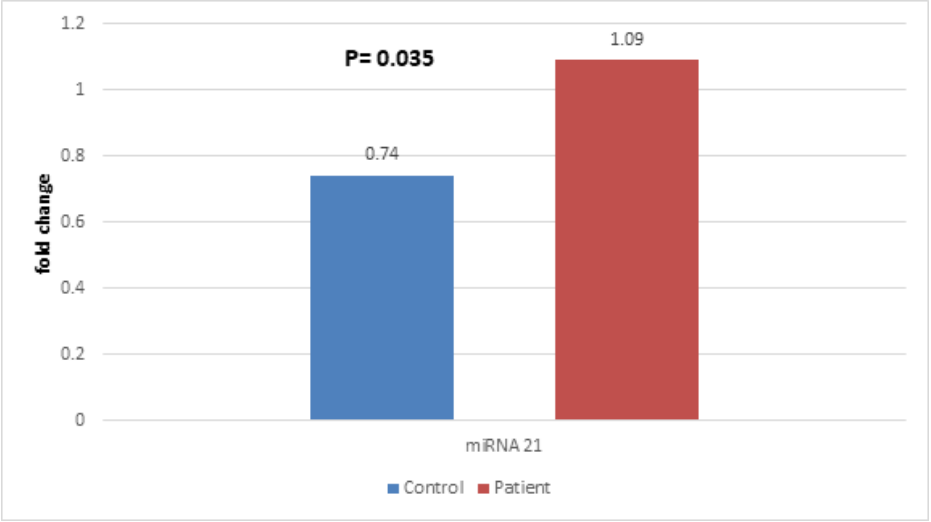

Fig (3) comparison of *miRNA 21* between patient with Type1 Diabetes mellitus and healthy control in serum.

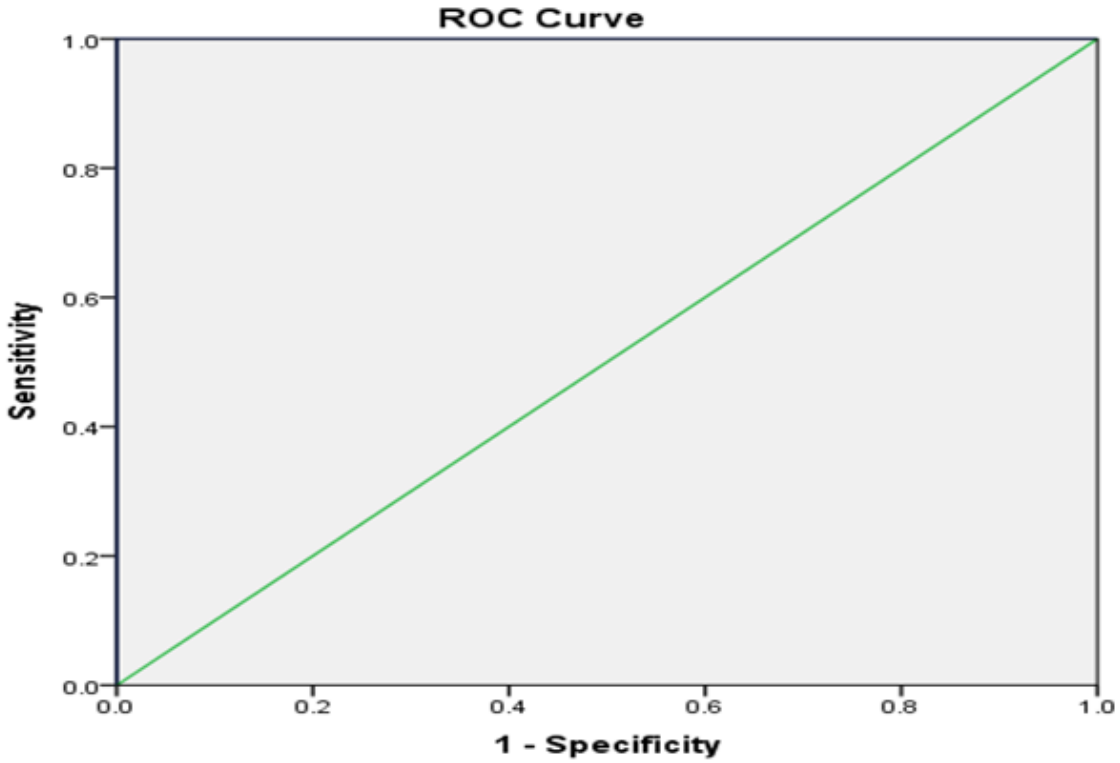

Figure (4): Receiver operator characteristic curve analysis of (miRNA21) for the calculation of possible diagnostic cutoff value.

Table (6): The cut off, Sensitivity and Specificity of *miRNA21* in patient with Type1 Diabetes mellitus and healthy control.

Area Under Curve	Cut off	P-value	Sensitivity	Specificity S
1.000	0.886	0.007	100%	100%

NS: not significant at P > 0.05 S: significant at P < 0.05.

Table (7): **The interaction between patient with Type1 Diabetes mellitus and healthy control.**

miRNA21			Patient	Control
Cutoff_0.886	Control	Count	0	100
		% within miRNA21	0%	100%
	Patient	Count	100	0
		% within miRNA21	100%	0%

Correlation between miRNA-21 expression and serum CTLA4 levels in patient with type1 diabetic Mellitus:

The Association between miR-21gene expression and serum CTLA4 levels in patient with T1DM was summarized in table (8). The present results show significantly strong negative relationship between *miRNA21* and CTLA4 levels (P=0.02).

Table (8): Association between miR-21gene expression and serum CTLA4 levels in patient with type1 diabetic Mellitus.

Parameter		Correlation Coefficient	P-value
CTLA4 level	*miRNA-21*	(-0.78)	0.02 ¥ S

¥: correlation test; NS: not significant at P > 0.05. S: significant at P ≤ 0.05.

Results:

The findings of this current study, as illustrated in table (1) and figure (3-6), elucidated that Mean levels of serum (CTLA-4) were 461.7 ± 94.52 ng/ml and 1057.39 ± 173.96 ng/ml, in patients with T1DM and healthy control respectively; the level was highly significantly higher than in patients' group in comparison with healthy control subjects (P < 0.0001). In this matter, (Hossen *et al*., 2023) (27), Explain the Regulatory T cells, including natural regulatory T cells and peripheral-induced regulatory T cells, are coordinated to maintain immune homeostasis, and a reduction in frequency and/or function of Treg cells is one of the main reasons for breaking and the immune tolerance of the immune system to b cells and causing T1DM, that CTLA-4 plays a key role in controlling Treg cell-mediated immunological tolerance and in case blocking CTLA-4 in healthy human leaded to develop T1DM more quickly, therefor This result showed that CTLA-4 is essential for Treg cell differentiation and function. The results of the current study are in agreement with the results of the study conducted by the researcher (Wang *et al*., 2011) (28), in UK CTLA-4 is strongly upregulated in regulatory T cells infiltrating the target tissue in a patient with T1DM and expression of this protein is critical for effective regulation. other study agreed with this result achieved by (Zóka *et al*., 2014) (29), revealed that the lower CTLA-4 expression found on the T lymphocyte population in patients with T1DM indicates an impaired tolerogenic signaling that might result in a lower level of suppressive stimuli towards the effector T-cells. Another study conducted by (Kavvoura & Ioannidis, 2005) (30), showed that the CTLA-4 has a greater affinity for the B7 molecule than does CD28, and it downregulates T-cell function, Therefore, it plays a crucial role in T-cell-mediated autoimmunity and thus in susceptibility to autoimmune diseases, including T1DM, CTLA-4-deficient patient rapidly develop beta cell destruction, it is considered the most likely candidate gene for T1DM susceptibility for the IDDM12 locus on chromosome 2q33.

According to the table (2) and figure (2) the result shows that the CTLA4 cutoff value was ≥742.8785-fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under curve of 100%, 100%, 100%, 100% and 0.1 respectively. The present results indicate that the CTLA4 is considered as an excellent diagnostic marker. Moreover, the present study results revealed that serum CTLA4 could be used in early diagnosis of T1DM when its level above the cutoff value with high sensitivity specificity and accuracy rates. Moreover, it can be used as a prognostic marker for T1DM as sensitivity and specificity of serum CTLA4 values are high, and this study completely agreed with (Al-Isawi *et al*., 2022) (31), In Iraq, and with (Atandi Batchy *et al*., 2023) (32), In Congo, and with (Ebrahim *et al*., 2022) (33), in Ethiopia.

Distribution of serum CTLA4 levels according to sex as it mentioned in table (3) demonstrate that the mean levels were higher in male groups in comparison with female groups, and the difference was significant (P=0.002). additionally, according to the family history, the result comparing between positive family history, and patients with negative family history mentioned in table (4); the mean levels were slightly higher in patients with negative family history in comparison with patients with positive family history, but the difference was non-significant (P=0.36), these results agreed with (Jasim *et al*., 2020) (34), In Iraq, and with (Lo Preiato *et al*., 2021) (35) In Italy.

According to the table (5), (6) and (7) and figure (3) and (4), the result agreed with (Bayat *et al*., 2021) (36), which showed the average expression level of miR-21 was observed to be 1 in the control subjects and 2.34 in the T1DM patients, respectively. This outcome mandates that the miR-21 gene expression had a nearly twofold significant increase in the T1DM patients when contrasted with the control group ($p < 0.0001$). A study by (Mostahfezian *et al*., 2019) (37), also showed that the ROC curve analysis was performed for miR-21 which suggests the potential role of miR-21 as a biomarker in T1DM patients and the expression level of this miRNA has been analyzed in different tissues of T1DM patients, miRNA blocks the death of pancreatic β-cells by means of PDCD4 (an apoptosis inducer) inhibition and prevents T1DM in the pancreas. The expression levels of miR-21 were also significantly upregulated in T1DM patients. This upregulation may promote autoimmunity through the inhibition of T-cells apoptosis, this results compatible with result in current study. Compelling evidence in a study by (Brown *et al*., 2020) (38), Studies have indicated that the increased regulation of miR-21 might expose β-cells to pro-inflammatory cytokines. Regrettly, this occurrence fosters cell death, therefore possibly instigating T1DM. An escalated regulation of miR-21 in T1DM patients is also documented by (Fouad *et al*., 2019) (39), While investigating the plasma of T1DM patients, the researchers traced elevated levels of miR-21. They further asserted that within the early five years of the disease onset, the increased levels of this miRNA can be identified.

The results in table (8), show significantly strong negative relationship (-0.78), between miRNA21 expression and CTLA4 levels (P=0.02). This result was completely compatible to gene polymorphism result in current study and agreed with (Skafi *et al*., 2020) (40), results which showed that miRNAs can directly impact the expression of CTLA-4. For example, miR-21 has been shown to manage the expression of CTLA-4 when the mutant allele C replaces T at the SNP (rs1292037), by binding directly to its 3'UTR. However, it is unable to connect to the wild-type allele T at the same SNP (rs1292037). This particular miRNA has been identified as overactive in peripheral blood mononuclear cells (PBMCs) of T1DM patients, implying that it could play a role in this autoimmune condition due to T cell overactivation. Additionally, miR-21 is known to directly target CTLA-4, and it was shown to be underrepresented in Treg. This lowered expression may be crucial for the increased presence of CTLA-4 in these cells. Indeed, if this miRNA is overexpressed, it can result in a decline of CTLA-4 expression within Treg cells. Interestingly, the majority of miRNAs indirectly influencing CTLA-4 expression do this by impacting the level of fork head box P3 (FOXP3), a crucial factor for CTLA-4 transcription.

Conclusion:

There is highly positive significant association between the levels of MiRNA21 and the presence or development and severity of type 1 diabetes Meletus in both genders.

There is highly negative significant association between the levels of CTLA4 and the presence or development and severity of type 1 diabetes Meletus in both genders.

There is a no significant correlation between family history and presence of type 1 diabetes Meletus in both genders.

Acknowledgement:

I am grateful to medical college of Al-Qadisiya university and the Diabetes and Endocrinology Center in Al-Sadr teaching hospital, for meeting all of my study needs.

References:

1. Kumar, R., Saha, P., Kumar, Y., Sahana, S., Dubey, A., & Prakash, O. (2020). A review on diabetes mellitus: type1 & Type2. World Journal of Pharmacy and Pharmaceutical Sciences, 9(10), 838-850.

2. Dariya, B., Chalikonda, G., Srivani, G., Alam, A., & Nagaraju, G. P. (2019). Pathophysiology, etiology, epidemiology of type 1 diabetes and computational approaches for immune targets and therapy. Critical Reviews™ in Immunology, 39(4).
3. Katsarou, A., Gudbjörnsdottir, S., Rawshani, A., Dabelea, D., Bonifacio, E., Anderson, B. J., ... & Lernmark, Å. (2017). Type 1 diabetes mellitus. Nature reviews Disease primers, 3(1), 1-17.
4. Tuomilehto, J. (2013). The emerging global epidemic of type 1 diabetes. Current diabetes reports, 13(6), 795-804.
5. Diamond, J. (2016). The double puzzle of diabetes. In Health Psychology (pp. 465-473). Routledge.
6. Katz, M., & Laffel, L. (2015). Mortality in type 1 diabetes in the current era: two steps forward, one step backward. Jama, 313(1), 35-36.
7. Kopan, C., Tucker, T., Alexander, M., Mohammadi, M. R., Pone, E. J., & Lakey, J. R. T. (2018). Approaches in immunotherapy, regenerative medicine, and bioengineering for type 1 diabetes. Frontiers in immunology, 9, 1354.
8. Knip, M., Siljander, H., Ilonen, J., Simell, O., & Veijola, R. (2016). Role of humoral beta-cell autoimmunity in type 1 diabetes. Pediatric diabetes, 17, 17-24.
9. Martinov, T. (2018). Programmed death-1 regulates islet-specific lymphocytes in type 1 diabetes (Doctoral dissertation, University of Minnesota).
10. Garyu, J. W., Meffre, E., Cotsapas, C., & Herold, K. C. (2016). Progress and challenges for treating Type 1 diabetes. Journal of autoimmunity, 71, 1-9.
11. Rowshanravan, B., Halliday, N., & Sansom, D. M. (2018). CTLA-4: a moving target in immunotherapy. Blood, The Journal of the American Society of Hematology, 131(1), 58-67.
12. Liberal, R., Grant, C. R., Longhi, M. S., Mieli-Vergani, G., & Vergani, D. (2015). Regulatory T cells: mechanisms of suppression and impairment in autoimmune liver disease. IUBMB life, 67(2), 88-97.
13. Åkerman, L. (2016). Aspects of the Pre-Diabetic Period in Type 1 Diabetes (Doctoral dissertation, Linköping University Electronic Press).
14. Vasu, S., Kumano, K., Darden, C. M., Rahman, I., Lawrence, M. C., & Naziruddin, B. (2019). MicroRNA signatures as future biomarkers for diagnosis of diabetes states. Cells, 8(12), 1533.
15. Hashimoto, N., & Tanaka, T. (2017). Role of miRNAs in the pathogenesis and susceptibility of diabetes mellitus. Journal of human genetics, 62(2), 141-150.
16. Sims, E. K. (2018). Microrna 21 targets B Cell Lymphoma 2 (Bcl2) Mrna to increase beta cell apoptosis and exosomal Microrna 21 could serve as a biomarker of developing Type 1 Diabetes Mellitus.
17. Alshanwani, A. R. M. (2016). Role of microRNA-21 in the regulation of human saphenous vein smooth muscle cell function and vascular remodelling (Doctoral dissertation, University of Leeds).
18. Rodríguez-Galán, A., Fernández-Messina, L., & Sánchez-Madrid, F. (2018). Control of immunoregulatory molecules by miRNAs in T cell activation. Frontiers in immunology, 9, 2148.
19. Qadir, M. M. F., Klein, D., Álvarez-Cubela, S., Domínguez-Bendala, J., & Pastori, R. L. (2019). The role of microRNAs in diabetes-related oxidative stress. International journal of molecular sciences, 20(21), 5423.
20. Thiel, J. (2018). The impact of microRNAs 96/183 and neuroplin-1 on t cell activation and autoimmunity (Doctoral dissertation, Dissertation, Duisburg, Essen, Universität Duisburg-Essen, 2018).

21. Cerna, M. (2019). Epigenetic regulation in etiology of type 1 diabetes mellitus. International journal of molecular sciences, 21(1), 36.
22. Lakhter, A. J., Pratt, R. E., Moore, R. E., Doucette, K. K., Maier, B. F., DiMeglio, L. A., & Sims, E. K. (2018). Beta cell extracellular vesicle miR-21-5p cargo is increased in response to inflammatory cytokines and serves as a biomarker of type 1 diabetes. Diabetologia, 61, 1124-1134.
23. La Sala, L., Mrakic-Sposta, S., Tagliabue, E., Prattichizzo, F., Micheloni, S., Sangalli, E., ... & Ceriello, A. (2019). Circulating microRNA-21 is an early predictor of ROS-mediated damage in subjects with high risk of developing diabetes and in drug-naïve T2D. Cardiovascular diabetology, 18(1), 1-12.
24. Xu, W. D., Pan, H. F., Li, J. H., & Ye, D. Q. (2013). MicroRNA-21 with therapeutic potential in autoimmune diseases. Expert opinion on therapeutic targets, 17(6), 659-665.
25. Wen, D. (2017). Delivery of small molecule and RNA for the treatment of type 1 diabetes and prostate cancer.
26. Seyhan, A. A. (2015). microRNAs with different functions and roles in disease development and as potential biomarkers of diabetes: progress and challenges. Molecular BioSystems, 11(5), 1217-1234.
27. Hossen, M. M., Ma, Y., Yin, Z., Xia, Y., Du, J., Huang, J. Y., ... & Huang, Z. (2023). Current understanding of CTLA-4: from mechanism to autoimmune diseases. Frontiers in immunology, 14, 1198365.
28. Wang, C. J., Schmidt, E. M., Attridge, K., Kenefeck, R., Wardzinski, L., Chamberlain, J. L., ... & Walker, L. S. (2011). Immune regulation by CTLA-4—relevance to autoimmune diabetes in a transgenic mouse model. Diabetes/metabolism research and reviews, 27(8), 946-950.
29. Zóka, A., Barna, G., Somogyi, A., Műzes, G., Oláh, Á., Al-Aissa, Z., ... & Firneisz, G. (2015). Extension of the CD4+ Foxp3+ CD25−/low regulatory T-cell subpopulation in type 1 diabetes mellitus. Autoimmunity, 48(5), 289-297.
30. Kavvoura, F. K., & Ioannidis, J. P. (2005). CTLA-4 gene polymorphisms and susceptibility to type 1 diabetes mellitus: a HuGE Review and meta-analysis. American journal of epidemiology, 162(1), 3-16.
31. Al-Isawi, H. R., Safi, F., & Muhammed, H. A. (2022). Association of CTLA-4 (+ 49A/G) gene Polymorphism with type 1 diabetes mellitus in Iraqi children. Cellular and Molecular Biology, 68(5), 1-6.
32. BATCHY, A., BOUMBA, L., & ELENGA-BONGO, C. L. (2023). CTLA4 Gene Expression in Type 1 Diabetes Patients with CMV Infection in Pointe-Noire. Int J Diabetes Metabolic Synd, 3(1), 1-6.
33. Ebrahim, E., Teklu, T., Tajebe, F., Wondmagegn, T., Akelew, Y., & Fiseha, M. (2022). Association of cytotoxic T-lymphocyte antigen-4 gene polymorphism with type 1 diabetes mellitus: in silico analysis of biological features of CTLA-4 protein on ethiopian population. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy, 2733-2751.
34. Al-Issawi, H. A. The Role of Cytotoxic T Lymphocyte Associated Protein-4 Gene (-318 C/T) Polymorphism in Type 1 Diabetes Patients in Iraqi Children.
35. Lo Preiato, V., Salvagni, S., Ricci, C., Ardizzoni, A., Pagotto, U., & Pelusi, C. (2021). Diabetes mellitus induced by immune checkpoint inhibitors: type 1 diabetes variant or new clinical entity? Review of the literature. Reviews in Endocrine and Metabolic Disorders, 22, 337-349.
36. Bayat, R., Salehi, Z., Dalili, S., & Bahreini, F. (2021). Influence of rs1292037 Genetic Variant on miR-21 Gene Expression in Patients with type 1 Diabetes Mellitus.

37. Mostahfezian, M., Azhir, Z., Dehghanian, F., & Hojati, Z. (2019). Expression pattern of microRNAs, miR-21, miR-155 and miR-338 in patients with type 1 diabetes. Archives of Medical Research, 50(3), 79-85.

38. Brown, C. Y., Sadlon, T., Hope, C. M., Wong, Y. Y., Wong, S., Liu, N., ... & Barry, S. C. (2020). Molecular insights into regulatory T-cell adaptation to self, environment, and host tissues: Plasticity or loss of function in autoimmune disease. Frontiers in Immunology, 11, 1269.

39. Fouad, M., Salem, I., Elhefnawy, K., Raafat, N., & Faisal, A. (2020). MicroRNA-21 as an early marker of nephropathy in patients with type 1 diabetes. Indian journal of nephrology, 30(1), 21-25.

40. Skafi, N., Fayyad-Kazan, M., & Badran, B. (2020). Immunomodulatory role for MicroRNAs: Regulation of PD-1/PD-L1 and CTLA-4 immune checkpoints expression. Gene, 754, 144888.