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# Investigation of CTLA-4 gene polymorphisms in a sample of Iraqi children with newly diagnosed type 1 diabetes mellitus

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**ABSTRACT**

**Introduction:** Type 1 diabetes mellitus (T1DM) is a complicated autoimmune disorder that is marked by the destruction of pancreatic islands by T cells. It is widely believed that genetic factors play a crucial role in the development of T1DM and other autoimmune diseases. At present, the contribution of the gene cytotoxic T-lymphocyte antigen-4 (CTLA4) to type 1 diabetes has not been fully determined.

**Objectives:** The primary objective of this research was to examine the relationship between the CTLA-4 [-1722T/C (rs733618) and -318 C/T (rs5742909)] polymorphisms and the onset of T1DM in Iraqi children.

**Patients and Methods:** In this case-control study, a total of one hundred children were examined. Specifically, fifty children diagnosed with T1DM and fifty age and sex-matched non-diabetic children were recruited as controls. The polymerase chain reaction (PCR) technique was employed using the allele-specific PCR method to analyze the CTLA-4 [-1722T/C (rs733618)] and [-318 C/T (rs5742909)] polymorphisms. The levels of glutamic acid decarboxylase antibody (anti-GAD Ab), anti-islet antigen-2 antibody (anti-IA-2 Ab), and insulin were determined using the enzyme-linked immunosorbent assay (ELISA) method.

**Results:** Children with T1DM showed significantly higher levels of anti-IA2 and anti-GAD than healthy controls, while patients had significantly lower levels of insulin than healthy controls. However, there was no statistically significant relationship between CTLA-4 polymorphisms -1722T/C (rs733618) and -318 (rs5742909) and anti-GAD Ab, anti-IA-2 Ab, with insulin levels in T1DM patients and controls.

**Conclusion:** The findings of the present investigation indicate that neither CTLA-4 polymorphism -1722T/C (rs733618) nor CTLA-4 polymorphism -318 (rs5742909) are associated with genetic predisposition to T1DM.

*Implication for health policy/practice/research/medical education:*
Type 1 diabetes mellitus (T1DM) is a serious autoimmune condition that arises from the destruction of insulin-producing beta cells in the pancreas. It is a prevalent chronic disease among children if left untreated with lifelong insulin therapy, it can be fatal. Despite advancements in treatment, a definitive way to prevent or cure the disease remains elusive. Recent studies from Iraq have shown a rise in T1DM cases among children, which has been attributed to environmental and economic changes in the country. Furthermore, genetic factors are believed to significantly contribute to the development of T1DM. The current study aimed to determine if there was any association between T1DM and polymorphisms in the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) gene in Iraqi children.

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## Introduction

Type 1 diabetes mellitus (T1DM), previously referred to as insulin-dependent diabetes mellitus, is an autoimmune disorder that commonly emerges during childhood and adolescence. It constitutes approximately ten percent of all diabetes cases and is one of the most prevalent chronic

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illnesses in childhood. This condition is distinguished by an insulin shortage resulting from the destruction of pancreatic beta cells, which necessitates lifelong insulin therapy for survival (1,2).

Type 1 diabetes mellitus is an increasingly complex disease influenced by a variety of factors, including genetic susceptibility and environmental interactions (3-6). The genetic component of the disease is linked to the major histocompatibility complex (MHC), which includes human leukocyte antigen (HLA) classes I and II. In addition, cytotoxic T-lymphocyte antigen-4 (CTLA4), is a co-stimulatory receptor on CD4+ T cells that inhibits activation by binding to B7. This antigen is located on chromosome 2q33 and has four exons and three introns. Variants in CTLA4 have been linked to the development of type 1 diabetes and other autoimmune conditions, likely due to decreased intracellular production of the protein, which impairs cell activation and proliferation, leading to an unregulated immune response and autoimmune imbalance (7-9).

The human CTLA-4 gene (CTLA4) possesses a thymine single-base substitution polymorphism in its promoter at position 318 (C−318/T−318) (10). Moreover, this gene features a T→C mutation at position -1722, which may influence the transcriptional regulation of CTLA-4, according to the study by Hudson et al, who stated that the 733618 (-1772T) allele was present in the promoter and reduced the transcription level of the CTLA-4 gene by affecting the binding of transcription factors (11). In a case-control study involving a North African population, the analysis of two single nucleotide polymorphisms (SNPs) in the CTLA4 promoter region (-1722 and -318) demonstrated a strong correlation between the CTLA-4 region and the disease (12). Another study conducted in South Morocco discovered a substantial increase in the -318 C/T allele among T1DM patients (13), and a more recent study reported that the CTLA-4 region is implicated in the pathogenesis of T1DM (14).

## Objectives

The present investigation seeks to determine whether there exists a correlation between the polymorphisms of the CTLA-4 gene (-1722T/C [rs733618]) and (-318 C/T [rs5742909]) and the onset of T1DM in children, as well as their association with the presence of glutamic acid decarboxylase antibody (anti-GAD Ab), anti-islet antigen-2 antibody (anti-IA-2 Ab), and insulin. This knowledge is essential for understanding the genetic predisposition to T1DM and developing better treatments and vaccines in the future. Additionally, early diagnosis of T1DM can significantly benefit from this research by reducing the suffering caused by diabetic complications.

## Patients and Methods

A case-control study (1:1) was executed between 50 newly diagnosed children with T1DM and 50 non-diabetic controls. The study period was from October to February 2023, since the leftover samples were collected from the central teaching hospital of pediatrics in Baghdad.

### *Specimen collection*

Five milliliters of the specimen were divided into 3 mL, which were placed in a sterile gel tube and allowed to clot. The serum was then separated from the clotted sample by centrifugation at 4000 revolutions per minute (rpm) for a period of 15 minutes. The serum was stored at a temperature -20 °C and was subsequently utilized for the estimation of insulin, anti-glutamic acid decarboxylase antibodies (anti-GAD Ab), and anti-islet antigen-2 antibody (IA-2A) markers. The remaining 2 mL of blood were collected in ethylenediaminetetraacetic acid (EDTA) tubes for the determination of hemoglobin A1c (HbA1c) and genomic DNA extraction.

### *DNA extraction*

Blood samples were retrieved from the deep freeze at a temperature of -20 °C and subsequently thawed. The nucleic acid extraction kit II, manufactured by Promega (USA) was utilized for the purpose of isolating and purifying DNA from the aforementioned samples. The extraction process was carried out in accordance with the manufacturer's instructions.

### *T1DM diagnosis*

The diagnostic process was carried out in accordance with the World Health Organization's (WHO's) established criteria in 2002, which involved assessing random blood sugar (RBS, Randox, United Kingdom) and HbA1c (Finecare, China) levels. The testing procedure was conducted in accordance with the manufacturer's instructions.

### *Serological test*

The quantitative measurement of human insulin levels in serum samples from patients and controls was done using the enzyme-linked immunosorbent assay (ELISA) technique, supplied by SunLong Biotechnology (China). For the assessment of anti-GAD Ab and anti-IA-2 Ab levels, the GAD65 test kit, sourced from ELK biotechnology (China), and the islet antigen-2 antibody kit, obtained from SunLong Biotechnology (China), were employed, respectively. The respective procedures were executed in accordance with the manufacturer's instructions.

### *Detection of CTLA-4 gene (-1722T/C, rs733618) and (-318 C/T, rs5742909) SNPs*

Genomic DNA was extracted from 200 microliter blood samples using a whole blood DNA extraction kit for each sample. The CTLA-4 polymorphic regions (rs733618) and (rs5742909) were amplified using the polymerase chain reaction (PCR) with an allele-specific PCR technique, as illustrated in Table 1 (15,16).

**Table 1.** Primers of PCR

| SNP name, size of PCR products | Primers | Oligonucleotide sequences |
|---|---|---|
| -1722 T/C (rs733618)<br>Allele T: 237bp<br>Allele C: 237 bp | T-allele forward primer | 5′-ATGATCATGGGTTTAGCTGT-3′ |
| | C-allele forward primer | 5′-GTGATCATGGGTTTAGCTGC-3 |
| | Reverse primer | 5′-CCATGTTGGTGGTGATGCAC-3′ |
| -318 C/T (rs733618)<br>Allele T:185 bp<br>Allele C:185 bp | T-allele forward primer | 5′-ACTTAGTTATCCAGATCCAC-3′ |
| | C-allele forward primer | 5′-ACTTAGTTATCCAGATCCAT-3′ |
| | Reverse primer | 5′-AGGCTCTTGAATAGAAAGC-3′ |

### *Interpretation of the results*

The PCR amplification products were subjected to electrophoresis on a 2% agarose gel and visualized under ultraviolet (UV) transilluminator. The molecular weights of the expected PCR products were 185 and 237 base pairs (bp) for the -318 (C/T) and -1722 (T/C) dimorphisms, respectively. Concerning insulin, (GADA 65), and (IA-2A), known concentrations and their corresponding reading optical density are plotted on a log scale (x-axis) and a log scale (y-axis), respectively. The concentration of the marker in the sample was determined by placing the optical density of the sample on the y-axis. The original concentration is calculated by multiplying the dilution factor.

### *Statistical analysis*

Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS) software (version 25.0; SPSS, Chicago, IL, USA). The Shapiro-Wilk test was applied to normalize continuous data, and normally distributed data were expressed as the mean and standard deviation and were analyzed using the student T-test and analysis of variance (ANOVA). Moreover, the Mann-Whitney U test (for two-group comparisons) and the Kruskal-Wallis test (for three-group comparisons) were conducted to analyze non-normally distributed data, which were presented as medians and ranges.

The risk of association between different SNPs in the CTLA-4 gene and T1DM was estimated by calculating the odds ratio (OR) and its corresponding 95% confidence intervals (CIs) using binary logistic regression. Patients who were homozygous for the wild-type genotype were considered as the dependent variable, and different variants were entered into the model as independent variables. Categorical variables were expressed as numbers and percentages and were analyzed using the chi-square ($\chi 2$) test. Fisher's exact test was also conducted for categorical variables when the sample sizes were small, which was valid for all sample sizes. Differences were considered statistically significant when the $P$ value was $< 0.05$.

## Results

### *Study population demographics and clinical features*

Table 2 presents the demographic and clinical characteristics of the 100 children involved in the study, comprising 50 children with T1DM and 50 healthy controls. The results show that there were no significant differences in the mean age, sex, and weight between the two groups ($P > 0.05$). However, there were statistically significant differences in family history of T1DM and place of residence between the T1DM patients and healthy controls ($P < 0.001$).

### *Biochemical tests related to T1DM*

The RBS and HbA1c levels of patients were found to be significantly higher (217.20±87.7 mg/dL and 10.27±1.89%, respectively) than those of controls (88.7±7.3 mg/dL and 4.92±0.5%, respectively), with statistically significant differences ($P < 0.05$) observed between the two groups. Additionally, patients displayed lower insulin levels than

**Table 2.** Study population demographics and clinical features

| Variables | T1DM Patients (n=50) | Controls (n=50) | *P* value |
|---|---|---|---|
| Age, years | | | |
| Mean ± SD | 8.08±4.65 | 7.88±4.42 | |
| Median | 8 | 8 | 0.974* |
| Range | < 1-15 | 1-15 | |
| Gender | | | |
| Male | 20 (42.6%) | 27 (57.4%) | 0.161** |
| Female | 30(56.6%) | 23 (43.4%) | |
| Weight, kg | | | |
| Mean ± SD | 28.88±14.02 | 33.24±18.40 | |
| Median | 27 | 31 | 0.381* |
| Range | 8-62 | 8-78 | |
| Residence | | | |
| Urban | 35 (70%) | 48 (96%) | **< 0.001**** |
| Rural | 15 (30%) | 2 (4%) | |
| Family history | | | |
| No | 18 (36%) | 37 (74%) | **< 0.001**** |
| Yes | 32 (64%) | 13 (26%) | |
| Disease duration, day | | | |
| Mean ± SD | 3.98±3.30 | - | - |
| Range | 1-23 | | |
| Insulin using | | | |
| Yes | 31 (62%) | - | - |
| No | 19 (38%) | | |
| Type of insulin | | | |
| Self-titrated | 13 (43%) | - | - |
| Premixed | 17 (56.7%) | | |

*Mann Whitney U test, ** $\chi^2$ test.

controls (1.99±0.66 mU/L versus 2.60±1.52 mU/L), with a statistically significant difference ($P<0.001$) noted in the results (Table 3).

### *Autoantibody tests related to T1DM and controls*

Table 4 indicates that the median levels of anti-IA2 and anti-GAD Ab in patients were 43.8 pg/mL and 27.7 ng/mL, respectively, which were significantly higher than the control group's median levels of 15.3 pg/mL and 13 ng/mL ($P<0.001$).

### *Molecular assay*

Two specific SNPs in the CTLA-4 gene (-1722T/C, rs733618 and -318C/T, rs5742909) were examined to determine their potential role in the onset of T1DM. Figures 1 and 2 illustrate gel electrophoresis of the PCR products, which possess predicted lengths of 237 and 185 base pairs (bp), respectively.

**Table 3.** Biochemical tests related to the study population

| Tests | Patient population (n=50) | Control population (n=50) | *P* value* |
|---|---|---|---|
| RBS, mg/dL | | | |
| Mean±SD | 217.20±87.7 | 88.7±7.3 | < 0.001* |
| Median | 207.5 | 89.0 | |
| Range | 75-539 | 73-100 | |
| HbA1c, % | | | |
| Mean±SD | 10.27±1.89 | 4.92±0.5 | 0.004* |
| Median | 10.0 | 4.9 | |
| Range | 5.7-15 | 4.0-6.1 | |
| Insulin, mU/L | | | |
| Mean±SD | 1.99±0.66 | 2.60±1.52 | < 0.001** |
| Median | 1.9 | 2.6 | |
| Range | 0.6-3.5 | 0.1-8.4 | |

RBC; random blood sugar, HbA1c; hemoglobin A1c.
Normal values: RBS <200 mg/dL, HbA1c 5.7-6.4%.
*Mann-Whitney U test, **Student T-test.

**Table 4.** T1DM and controls related auto-antibodies

| Tests | Patient population (n=50) | Control population (n=50) | *P* value* |
|---|---|---|---|
| Anti-IA2, pg/mL | | | |
| Mean±SD | 43.7±15.43 | 15.77±6.19 | <0.001* |
| Median | 43.8 | 15.3 | |
| Range | 11.3-73.8 | 7.4-37.7 | |
| GAD, ng/mL | | | |
| Mean±SD | 22.5±9.18 | 13.2±5.92 | <0.001* |
| Median | 27.7 | 13 | |
| Range | 1.6-32.2 | 1.8-23.1 | |

Anti-IA2 Ab; Anti-islet antigen-2 antibody, GAD; glutamic acid decarboxylase antibody.
*Mann-Whitney U test, **Student T-test.

### *Genotype distribution and allele frequency of -1722T/C, rs733618*

The wild-type TT genotype was more prevalent in control subjects (42%) than in patients (30%), though this difference was not statistically significant. On the other hand, the mutant genotype (CC) was more frequently observed in patients (2%) than in controls (0%), but this difference was also not statistically significant. Additionally, there were no statistically significant variations between patients and controls with regard to dominance, recessive models, or allele frequency (Table 5).

### *Genotype distribution and allele frequency of (-318C/T, rs5742909)*

There were no statistically significant differences observed in the wild-type CC genotype or mutant genotype (TT)

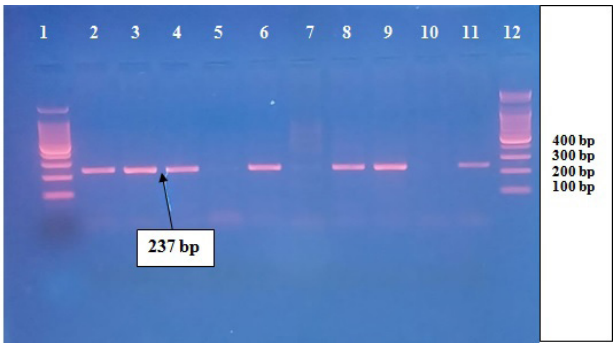


**Figure 1.** 2% agarose gel electrophoresis for allele specific PCR for CTLA 4 CT-1722 (T/C). Lane 1 and 12: 100 bp DNA ladder, Lane11: positive control for amplification, Lane 10: negative control for amplification, Lanes 2, 4, 6 and 8: PCR products upon using allele specific T primer. Lane 3, 5, 7 and 9: PCR products upon using allele specific C primer. Heterozygous genotype will give positive reaction upon using both allele specific primers. However, homozygous genotype will give positive reaction upon using only one of these allele specific primers. Lane from4 to 7: homozygous wild genotype, Lane 2, 3 and 8, 9: Heterozygous genotype.

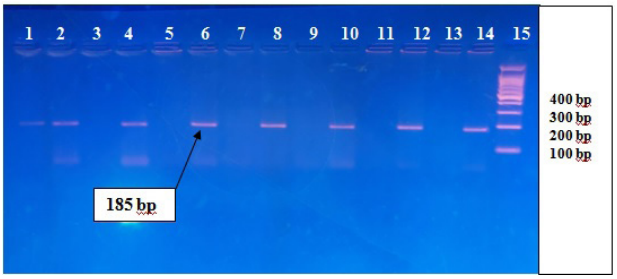


**Figure 2.** 2% agarose gel electrophoresis for allele specific PCR for CTLA 4 CT-318 (C/T). Lane15: 100 bp DNA ladder, Lane14: positive control for amplification, Lane 13: negative control for amplification, Lanes 1, 3, 5, 7, 9 and 11: PCR products upon using allele specific T primer. Lane2, 4, 6, 8, 10 and 12: PCR products upon using allele specific C primer. Heterozygous genotype will give positive reaction upon using both allele specific primers. However, homozygous genotype will give positive reaction upon using only one of these allele specific primers. Lane from 4 to 12: homozygous wild genotype, Lane 1, 2: Heterozygous genotype.

between patients and controls, as well as no statistically significant variations in the dominance, recessive models, or allele frequencies (Table 6).

The outcomes of laboratory tests were consistent across various -1722T/C (rs733618) and -318C/T (rs5742909) genotypes of SNPs in both patients and controls, and no statistically significant disparity was observed ($P > 0.05$) (Tables 7-10).

## Discussion

Many research studies have identified numerous crucial risk loci that are closely associated with the development of diabetes. Among these, CTLA-4 has been identified as a susceptibility gene for T1DM. Research studies have been conducted among different populations in several countries to investigate the correlation between CTLA-4 polymorphisms and T1DM (17,18). In our study, no significant differences between T1DM patients and healthy controls regarding age, sex, and weight was detected (Table 2). The findings of the present study are consistent with those of previous studies conducted by Ho et al in Canada (19), Saygili et al in Turkey (20), and Lakshmanan et al in Qatar (21). However, the findings of the present study are in disagreement with those of Boboc et al in Romania (22).

In the current study, a statistically significant difference was identified in the residence and family history of T1DM between T1DM patients and healthy controls (P <0.001; Table 2), which aligns with the findings of previous researches conducted in Egypt by Awadalla et al (23) and in Nigeria by Amadi et al (24). However, contrasting results were reported in studies conducted in Turkey by Saygili et al (20) and in Malawi by Msekandiana et al (25), differing from the findings of the present study.

**Table 5.** Frequency of different genotypes and alleles of the polymorphism (-1722T/C, rs733618) among the study groups

| -1722T/C (rs733618) | Patients (n=50) | Controls (n=50) | *P* value | OR (95% CI) |
|---|---|---|---|---|
| Genotypes | | | | |
| TT | 15 (30.0%) | 21 (42%) | 0.302* | 1.0 |
| TC | 34 (68.0%) | 29 (58.0%) | | 1.60 (0.26-1.39) |
| CC | 1 (2.0%) | 0 (0%) | | Xero (0 to infinity) |
| Dominant model | | | | |
| TT+TC | 49 (98.0%) | 50 (100.00%) | 1.000** | 1.0 |
| CC | 1 (2.0%) | 0 (0%) | | Zero (0 to infinity) |
| Recessive model | | | | |
| TT | 15 (30.0%) | 21 (42%) | 0.298** | 1.0 |
| TC+CC | 35 (70.0%) | 29 (58%) | | 0.59 (0.25-1.35) |
| Alleles | | | | |
| T | 64 (64.00%) | 71 (71.0%) | 0.144** | 1.0 |
| C | 36 (36.0%) | 29 (29.0%) | | 0.72 (0.40-1.31) |

T: Thymine, C: Cytosine, TT: Mutant homozygous, CT: mutant heterozygous, CC: Wild-type homozygous, OR: Odds ratio, CI: Confidence interval. *$\chi^2$- test, **Fisher's exact test.

**Table 6.** Frequencies of different genotypes and alleles of the polymorphism -318C/T, rs5742909 among the study groups

| -318C/T (rs5742909) | Patients (n=50) | Controls (n=50) | *P* value | OR (95% CI) |
|---|---|---|---|---|
| Genotypes | | | | |
| CC | 47 (94%) | 48 (96%) | | 1.0 |
| CT | 3 (6%) | 1 (2%) | 0.366* | 0.32 (0.03-3.25) |
| TT | 0 (0%) | 1 (2%) | | Infinity (2 to infinity) |
| Recessive model | | | | |
| TT+CT | 3 (6.00%) | 2 (4.0%) | 1.000** | 1.0 |
| CC | 47 (94.0%) | 48 (96%) | | 1.53 (0.24-9.58) |
| Dominant model | | | | |
| TT | 0 (0%) | 1 (2.0%) | 1.000** | Zero |
| CT+CC | 50 (100%) | 48 (98%) | | Zero (0 to infinity) |
| Alleles | | | | |
| T | 3 (3%) | 3 (3%) | 0.242* | 1.0 |
| C | 97 (97%) | 97 (97%) | | 1 (0.19-5.07) |

T: Thymine, C: Cytosine, TT: Wild-type homozygous, CT: Mutant heterozygous, CC: mutant homozygous, OR: Odds ratio, CI: Confidence interval. *$\chi^2$- test, **Fisher's exact test.

**Table 7.** Association of numerous genotypes of the rs733618 SNP with laboratory test results in patients

| Laboratory tests | TT (n=15) | TC (n=34) | CC (n=1) | *P* value |
|---|---|---|---|---|
| RBS, mg/dL<br>Median<br>Range | 215<br>75-539 | 206<br>88-462 | 231 | 0.466[a] |
| HbA1c, %<br>Mean±SD | 10.53±1.377 | 10.12±2.20 | 10.0 | 0.556[b] |
| Anti-IA2, pg/mL<br>Median<br>Range | 50.49<br>23.6-98.3 | 42.38<br>11.3 -99.0 | 53.03 | 0.460[a] |
| Insulin, mU/L<br>Median<br>Range | 1.81<br>0.75-2.53 | 2.14<br>0.66-3.50 | 2.671 | 0.753[a] |
| GAD, ng/mL<br>Median<br>Range | 29.2<br>1.64-31.81 | 26.52<br>10.4-34.0 | 28.24 | 0.674[a] |

Normal values: RBS <200 mg/dL, HbA1c 5.7-6.4%.
[a] Kruskal Wallis test, [b] Analysis of variance.

**Table 8.** Association of numerous genotypes of the rs733618 SNP with laboratory test results in controls

| Laboratory tests | TT (n=21) | TC (n=29) | CC (n=0) | *P* value |
|---|---|---|---|---|
| RBS, mg/dL<br>Median<br>Range | 90<br>73-100 | 89<br>73 - 100 | - | 0.187[a] |
| HbA1c, %<br>Mean±SD | 4.93±0.65 | 4.91±0.57 | - | 0.874[b] |
| Anti-IA2, pg/mL<br>Median<br>Range | 15.81<br>8.0-37.7 | 15.39<br>7.48– 50.49 | | 0.888[a] |
| Insulin, U/mL<br>Median<br>Range | 2.76<br>1.19-5.05 | 2.42<br>0.15- 8.46 | - | 0.777[a] |
| GAD, ng/mL<br>Median<br>Range | 13.47<br>1.82-23.1 | 14.0<br>1.82 – 23.1 | - | 0.273[a] |

Normal values: RBS <200 mg/dL, HbA1c 5.7-6.4%.
[a] Mann Whitney U test, [b] Student *t* test.

According to the results of the present study, individuals with T1DM exhibited significantly higher levels of several markers, including RBS, HbA1c, anti-islet cell antibodies (anti-IA2 Ab), and anti-GAD Ab, compared to healthy controls. Additionally, patients had lower levels of insulin compared to healthy controls (Tables 3 and 4). The findings of the current study are in line with previous studies conducted by Madhurima et alin India (26), Belhiba et al in Morocco (27), and Bravis et al in the United Kingdom (28). Autoantibodies that arise due to islet destruction are likely to target insulin as their primary target and may be employed for the diagnosis of T1DM. Typically, individuals diagnosed with T1DM exhibit the presence of one or more autoantibodies at the time of diagnosis (5).

The current study did not find a statistically significant relationship between the distribution of genotypes (-1722T/C, rs733618) and (-318C/T, rs5742909) in individuals with T1DM and healthy controls, as evidenced in Tables 5 and 6. Furthermore, no disparities were observed between the SNPs of the CTLA-4 gene and other clinical laboratory tests, such as RBS, HbA1c, anti-IA2 Ab, insulin, and anti-GAD Ab, in T1DM patients and controls, as indicated in Tables 7, 8, 9, and 10, with P values greater than 0.05.

The outcomes reported in this study align with those found in previous researches (29,30). However, this finding contrasts with the results of a study conducted by Saleh et al in Egypt, which demonstrated an association between the (-1722 T/C) CTLA-4 polymorphism and T1DM in the Egyptian population and identified distinct susceptibility haplotypes in this cohort of Egyptian origin (7). The discrepancy in these findings may be due to variations in the expression of the CTLA4 gene, differences in ethnic and environmental factors, geographic region,

**Table 9.** Association of numerous genotypes of the rs5742909 SNP with laboratory test results in patients

| Laboratory tests | TT (n=0) | CT (n=3) | CC (n=47) | *P* value |
|---|---|---|---|---|
| RBS, mg/dL<br>Median<br>Range | - | 190<br>141-231 | 208<br>75-539 | 0.513[a] |
| HbA1c, %<br>Mean±SD | - | 10.36±0.63 | 10.26±1.95 | 0.931[b] |
| Anti-IA2, ng/mL<br>Median<br>Range | - | 32.7<br>31.9 -68.30 | 44.66<br>11.30-83.86 | 0.854[a] |
| Insulin, U/mL<br>Median<br>Range | - | 2.09<br>0.75±2.53 | 1.94<br>0.66-3.50 | 0.838[a] |
| GAD, pg/mL<br>Median<br>Range | - | 32.75<br>31.91-68.3 | 26.05<br>1.64-32.2 | 0.096[a] |

[a] Mann Whitney U test, [b] Student *t* test.

**Table 10.** Association of numerous genotypes of the rs5742909 SNP with laboratory test results in controls

| Laboratory tests | TT (n=1) | CT (n=1) | CC (n=48) | *P* value |
|---|---|---|---|---|
| RBS, mg/dL<br>Median<br>Range | 91 | 90 | 89<br>73-100 | 0.903 ‡ |
| HbA1c, %<br>Mean±SD | 5.0 | 4.1 | 4.9±0.602 | 0.392 * |
| Anti-IA2, ng/mL<br>Median<br>Range | 18.02 | 16.76 | 15.39<br>7.48-50.4 | 0.565‡ |
| Insulin, mU/L<br>Median<br>Range | 3.21 | 1.82 | 2.64<br>0.15-8.46 | 0.463‡ |
| GAD, pg/mL<br>Median<br>Range | 15 | 20.21 | 13.04<br>1.82-23.10 | 0.433‡ |

Normal values: RBS <200 mg/dL, HbA1c 5.7-6.4%.
[a] Kruskal Wallis test, [b] Analysis of variance.

and limited sample size, which can significantly impact incompatibility. Furthermore, methodological issues may also contribute to the divergent results between this study and other research.

## Conclusion

The present study investigated the association of two SNPs of the CTLA-4 gene, namely -1722T/C (rs733618) and -318C/T (rs5742909), with new-onset T1DM in Iraqi children. Three genotypes were observed for each SNP, including TT, TC, and CC for -1722T/C (rs733618) and CC, CT, and TT for -318C/T (rs5742909). For -1722T/C (rs733618), the wild-type TT genotype was more prevalent in healthy controls than in patients, while the mutant genotype (CC) was more common in patients than in controls, but without any significant difference. In addition, no significant differences were observed between patients and controls for the CTLA-4 (-318C/T, rs5742909) polymorphism. The results suggest that the CTLA-4 (-1722T/C, rs733618) and (-318C/T, rs5742909) polymorphisms may not be associated with T1DM. To further understand the role of the CTLA-4 gene in T1DM, additional SNPs should be studied on a larger sample size, and epigenetic research must be conducted.

## Limitations of the study

This research was constrained by a small sample size, resulting from difficulties in obtaining parental consent for the utilization of leftover samples, and some samples had insufficient quantity for all necessary tests, thereby necessitating their exclusion from the study. Furthermore, the investigation was conducted in a single location, Baghdad city, the capital of Iraq, which might have influenced the outcomes. Therefore, additional research on a larger scale with different SNPs is essential. Nevertheless, this study, in our judgment, presented insights into the relationship between the CTLA-4 gene SNPs under investigation and T1DM in an Iraqi pediatric population and showed a significant difference in family history of T1DM and place of residence between the T1DM patients and healthy controls.

## Authors' contribution

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**Formal analysis:** Ealaf Abbas Khudair.
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**Methodology:** Arwa Mujahid Al-Shuwaikh, Ealaf Abbas Khudair.
**Resources:** All authors.
**Visualization:** All authors.
**Software:** Ealaf Abbas Khudair.
**Supervision:** Arwa Mujahid Al-Shuwaikh.
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**Visualization:** All authors.
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## Conflicts of interest

The authors declare that they have no competing interests.

## Ethical issues

The present study was approved by the institutional review board of Al-Nahrain University College of Medicine (No.202207/76 at 14 November, 2022). This study was also conducted based on the ethical standards stipulated in the Declaration of Helsinki. Before any intervention. All participants provided written informed consent. Ethical issues (including plagiarism, data fabrication, and double publication) have been completely observed by the authors.

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