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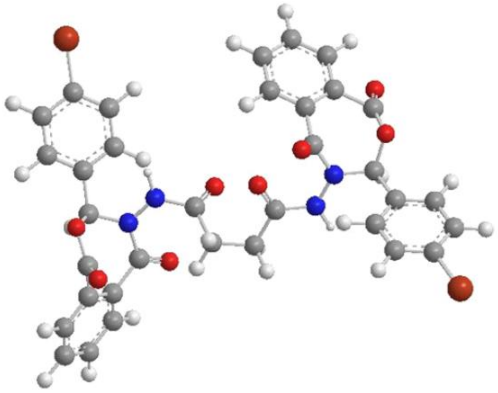

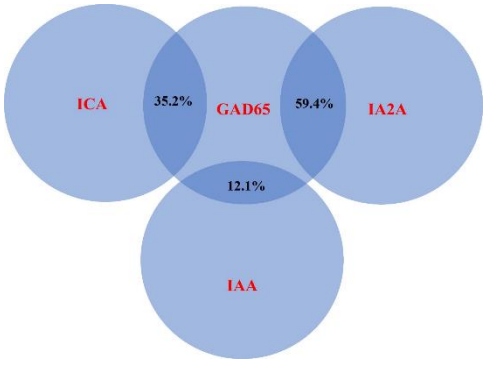

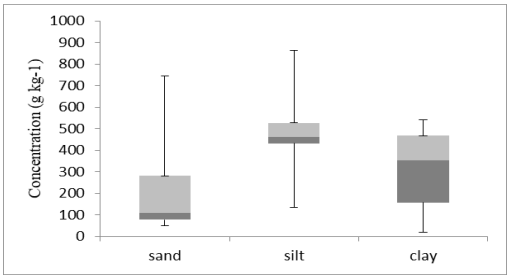

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Type I Diabetes Mellitus among Children and Adolescent in Sulaimaniyah City, Iraq

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Abstract

Diabetes mellitus type I is an autoimmune disorder in which pancreatic β cell autoantibodies are the most significant immunological markers. In this study, we investigated the prevalence of antibodies GADA, IAA, IA2A, and ICA. Seventy-seven patients were selected for the study and another 93 healthy controls were studied. Autoantibodies were measured in the serum samples obtained from both patients and the control group using enzyme-linked immunosorbent assay (ELISA). According to the results of this study, there was a significant difference in the level of GAD65 when the patient group was compared to the control. The mean value for the GAD in the control group was 2.095±0.89, while in patient groups it was 3.56±3.95 ng/ml, and they were significantly different ($p<0.01$). A qualitative measurement for both antibodies ICA and IA2A showed a positive result in more than 50% of the patients while ICA was positive in 12% of the control groups and IA2A was positive in 1.3% in the control group. Qualitative assessment of the IAA antibodies revealed that 32.8% were positive, while all healthy subjects were negative. Fasting C-peptide level in the patient group was 0.745±0.12 ng/ml while it was 2.12±0.48 ng/ml in healthy subjects. HbA1C level in patients was 10.46±2.27 while it was 5.38±0.24 in healthy subjects. The risk factors, maternal status, and children's status effect on the development of diabetes were studied and it was found that a significant difference ($p<0.05$) when a family history of DM was compared with patient and control groups, while there was not significant difference found between both groups when a family history of other autoimmune diseases was compared. Neonatal diseases between both groups were compared and no significant difference was observed as well. From the result of this study, it can be concluded that environmental risk factors such as obesity, family history of DM may play a significant role in triggering the immune system and leading to beta cells destruction, while ethnic background, geography, maternal obesity, maternal diseases, infections during pregnancy, neonatal diseases such as jaundice, thyroid, vitiligo, and celiac are not regarded as a potential risk factor in developing the disease. More than 90% of T1D individuals tested positive for autoantibodies. The most often found autoantibodies were IA2A and GADA. Antibodies were much more prevalent in female children.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder that causes hyperglycemia resulting from insulin deficiency, impaired insulin action, or both **[1]**. The two main subtypes of DM are type 1diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), with T2DM being the more prevalent and affecting 90–95% of DM patients **[2]**. The pathologies and comorbidities associated with DM, including

Cardiovascular Disease (CVD), renal failure, amputations, and blindness, are first brought on by chronic hyperglycemia, a hallmark of both T1DM and T2DM **[2,3]**. T1DM, also known as autoimmune or idiopathic diabetes, is characterized by the destruction of pancreatic β cells **[4]**.

It is crucial to distinguish T1DM from other types of diabetes as it aids in managing the condition since it helps with drug selection, disease prognosis assessment, and identifying family members who may be at risk for getting diabetes **[5]**. These markers are essential diagnostic tools for distinguishing between T1DM, T2DM, diabetes of monogenic origin, and other kinds of diabetes. Additional characteristics that attempt to distinguish the type of diabetes include genetics, age at onset, clinical presentation at illness onset, associations with ketoacidosis, obesity, and acanthosis nigricans. About 0.39% of the general population and 2.8% of tested Hispanic American children exhibited autoantibodies for T1DM, respectively. Islet autoantibodies are well-known and increasingly important in the diagnosis of many types of diabetes **[6]**.

The measurement of pancreatic β cell activity using C-peptide is effective and common **[7]**. The mature, functional insulin hormone is produced by β cells' prohormone convertase enzymes by cleaving the proinsulin's 31-amino acid C-peptide linker chain **[8]**. The plasma concentration of C-peptide in healthy persons is 0.3-0.6 nmol/l in the fasting state, rising to 1-3 nmol/l after meals **[9]**. The liver extracts very little C-peptide, and its peripheral clearance is steady. It circulates at amounts around five times greater in the systemic circulation because it has a longer half-life than insulin (20-30 vs. 3-5 min) **[10]**.

In T1DM autoimmunity erupts and the autoimmune process lasts for years. The eruption of the autoimmune process is determined by genetic polymorphism **[11]**, and triggered by environmental factors **[12]**. One of the environmental triggers that have been explored the most in-depth is infectious pathogens. Viral infections are believed to have a role in an etiology of T1DM according to epidemiological, serological, and histological investigations, as well as those conducted on experimental animals. Theoretical possibilities include β cell autoantigens being exposed to adjacent inflammation or T-cell cross-reactivity between viruses and islet autoantigens **[13]**. Diet is another environmental element that influences how islet autoimmunity progresses to clinical T1DM. Early exposure to cow's milk is linked to quicker development of T1DM. The chemical similarity of albumin to ICA, a protein on the surface of pancreatic β cells, is one theory explaining the part cow's milk plays in the development of disease **[14]**.

Autoantibodies are beneficial for T1DM diagnostic assistance. These include protein tyrosine phosphatase autoantibodies (IA-2A-ab), islet cell antibodies (ICA-ab), glutamic acid decarboxylase autoantibodies (GAD-ab), and insulin autoantibodies (IAA-ab) **[15]**. ICA was initially introduced by Bottazzo and colleagues in 1974 **[16]**. Using immunofluorescence tests, it was discovered that slices of the human pancreas treated with sera from diabetic patients who both had Addison's disease and myxedema had cytoplasmic fluorescence in the islets of Langerhans about 40 years ago. Cytoplasmic islet cell antibodies (ICA) were given the name for this reaction **[16,17]**.

Autoantibodies against a 64 kDa interacted with GAD at low titers in 1990, according to research from the DeCamilli and Baekkeskov groups **[18]**, and a subset of the anti-GAD autoantibodies in newly diagnosed diabetics was identical to those in stiff-man syndrome (SMS). In the past, Baekkeskov and colleagues **[19]**, showed that employing metabolically tagged human islets, serum from T1DM may immunoprecipitated a 64-kDa molecule. Testes, the ovary, and neurons all express the two GAD isoforms known as GAD65 and GAD67 **[20]**. Gamma-aminobutyric acid (GABA), a significant inhibitory neurotransmitter in the central nervous system, is biosynthesized in large part by the enzyme GAD65. A pyridoxal 5′-phosphate (PLP) cofactor binding site is located in the middle domain of the GAD65 structure, which also has a COOH terminal catalytic domain **[21]**.

GAD65 is only found in high concentrations in pancreatic islets of the adult human pancreas, where it is mostly located in cells and a small number of cells **[22]**. Although the possibility of producing antibodies against these "foreign" peptides, such as bovine insulin, during treatment with exogenous

forms of insulin had long been understood, the discovery of anti-insulin antibodies (in T1DM patients prior to the administration of exogenous insulin) was made in 1983 **[23]**.

Accordingly, a vast number of studies have shown that anti-IAA exist for years before the onset of T1DM and that they have an inverse relationship with the age at which T1DM manifests **[24]**. Genetics are involved because greater IAA levels have been linked to the DR4 **[25]**, and DQ8 genotypes **[26]**. Tyrosine phosphatase is mostly found on the surface of secretory granules in neuroendocrine cells, and IA-2A can identify it **[27]**.

Although it is found in extra-pancreatic tissues as well, the zinc transporter family member 8 (ZnT8) is a member of the cation diffusion facilitator family and is highly expressed in pancreatic β cells **[28]**. The formation of numerous islet autoantibodies is a crucial stage in the pathophysiology of T1DM, and prospective studies in the relatives of patients with the disease have demonstrated that this is a reliable and early indication of the risk of diabetes progression. Since more than ten years ago, it has been recognized that finding two or more islet autoantibodies is substantially more likely to result in T1DM than finding only one autoantibody **[29]**.

At the time of T1DM start, the majority of patients have at least one of the aforementioned antibodies positive **[30]**. In 85% of cases, ICA can be seen **[31]**. GAD-ab is present in 50–80% of people. At the time of diagnosis, 40–70% of children have IAA **[32]**. At the start of T1DM, IA2-ab is documented in 32–75% of individuals **[33]**. Numerous additional antibodies, such as those for thyroid disease (ALTD), idiopathic Addison's illness, and celiac disease are also linked to the antibodies connected to TIDM. Numerous investigations have been conducted to determine if these antibodies might be used as a diagnostic marker for the onset of DM in children **[34]**. In recent decades, a variety of techniques have emerged for the detection of diabetes-related autoantibodies. For the detection of autoantibodies, enzyme-linked immunosorbent assays (ELISA), immunoblotting assays, and indirect immunofluorescence (IIF) are often utilized, with progressively increased sensitivity and specificity **[35]**.

However, it will take a long time and a lot of samples to identify multiple targets. In this study, we created a multiplexed Array-ELISA technique to analyze diabetes-related autoantibodies simultaneously. Later we set out to measure the levels of ICA, GAD, IAA and IA-2A antibodies in the serum of children and adolescents with type I diabetes in Sulaimaniyah city and then associate them with several environmental factors.

Materials and Methods

Study samples

In the present study, the blood samples have been collected from those cases who their ages under 18-years old (children and adolescence), including 77 patients (40 females and 37 males) with T1DM and 93 control cases (46 females and 47 males) that were free from signs and symptoms of T1DM. All samples have been collected from Dr. Jamal Ahmad Rashid pediatric teaching hospitals from As-Sulaymaniyah governorate.The clinical characteristics and information were collected from the registered persons according to special data (Questionnaires) information prepared for these purposes (S1). The relationship between the level of different antibodies and age, gender, BMI, and environmental factors were studied. Measurement of the antibodies was performed using an ELISA washer (Bio Tek), model ELx508 and reader ELISA reader (Bio Tek), model Elx800. The kits used throughout the research were Human Islet Cell Antibody (Anti-IC) ELISA kits. Cat.No: MBS013703, Human Glutamic Acid Decarboxylase (GAD) ELISA kit Cat.No: MBS261840, Human insulin autoantibodies (IAA) ELISA Kit Catalog Number: MBS9424584, and Human Islet Antigen-2 Antibody

(IA-2A) ELISA Kit Catalog Number：MBS285091, while Cobas e411 was used for the measurement of c-peptide level.

Experimental design

The study was designed to investigate the frequency of diabetes autoantibodies GADA ICA, IA2A and IAA autoantibodies and the serum level of fasting C-Peptide in children diagnosed with T1DM in comparison to healthy children having neither significant medical illness as T1DM. Blood collection in different age groups was performed in (Dr. Jamal Ahmad Rashid Pediatric Teaching Hospitals) in Sulaymaniyah Governorate.

Statistical analysis

All the statistical analyses performed in this research were done using GraphPad Prism 9. The difference has been considered as significant, when the $p< 0.05$.

RESULTS

Age at diagnosis of type 1 diabetes

Table 1 is showing the age of the patients at which diabetes was diagnosed. The majority of the patients were diagnosed as diabetic in their early age and the minority were diagnosed at age 15-17.

Table 1: Age at diagnosis of type 1 diabetes.

Age (Years)	Frequency	Percent
1-4	15	19.5
5-9	38	49.35
10-14	21	27.25
15-17	3	3.9

Distribution of patients and healthy control groups according to age, BMI, and sex

Figure 1 A shows the distribution of all subjects based on their age, BMI, and sex depending on the questionnaire that was filled out for all cases participated in this study. The patients and control subjects were grouped based on their ages into 4 levels. Most TIDM patients were between 10-14 years and the least were between 1-4 years. The females and males are almost equally distributed between the two groups. BMI measurement was done using the equation mass (kg/m^2). The patient group were having higher BMI (18.22±3.97) compared to the control (15.37±3.52) and there was a significant difference between the groups according to Welch's test ($p=0.001$) (Figure 1B).

Figure 1: Distribution of patients and healthy control groups according to age (A) and BMI (B).
Statistical analysis of BMI between the patient and control groups (unpaired t-test was used for the comparison).

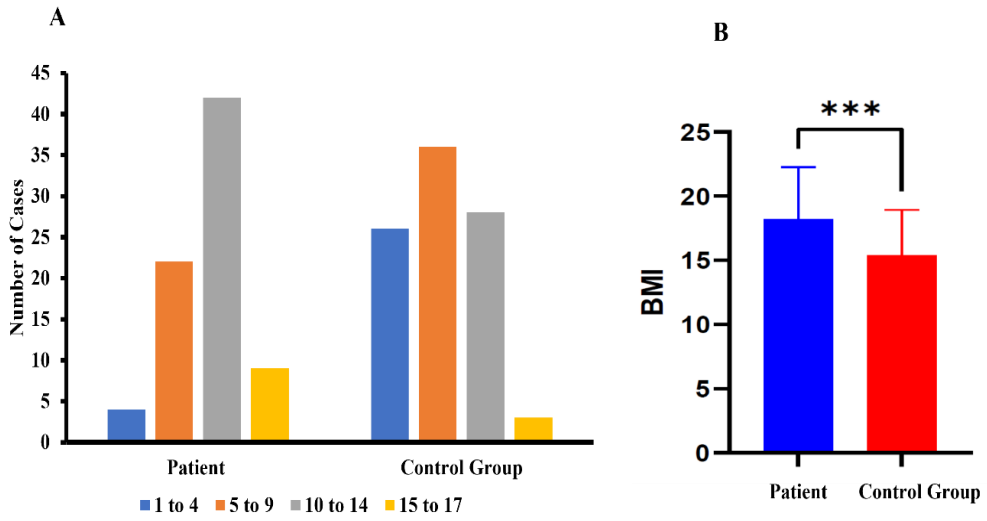

Figure 1: Distribution of patients and healthy control groups according to age (A) and BMI (B).
Statistical analysis of BMI between the patient and control groups (unpaired t-test was used for the comparison).

Family history of DM and autoimmune among patients and control groups

Figure 2 is showing the family history of DM and other autoimmune diseases. According to the questionnaire filled in the hospital, all healthy participants in this study were having no family history of the TIDM while only 3 subjects were T2DM, 4 had celiac disease, 3 thyroids and one subject was suffering from vitiligo. Compared to the control group, patient group was having 15.58% TIDM, 12.98% T2DM, and one with both. A significant difference was observed between the two groups in terms of diabetes. Furthermore, when the other autoimmune diseases were compared between the two groups, no significant difference was observed.

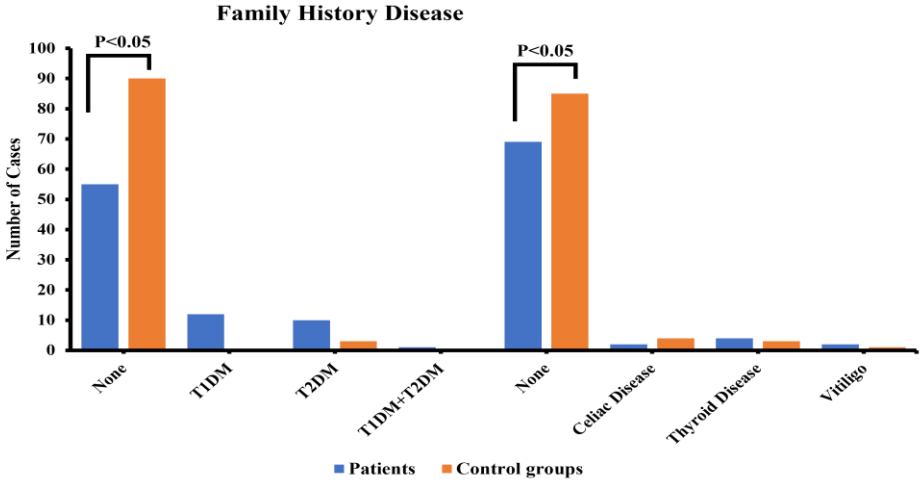

Figure 2: Family history of DM and autoimmune among patients and control groups.

Risk factors and Maternal status during pregnancy

Table 2 results revealed the maternal status during pregnancy and the medications used by the pregnant women, there was no significant difference between both groups.

Table 2: Risk factors and Maternal status during pregnancy.

Maternal diseases and drugs		Patients (77)	Control groups (93)	P value
		Number, percentage		
None		60 (77.9)	67 (72.05)	>0.05
Infection during pregnancy		15 (19.5)	23 (24.75)	
GDM		2 (2.6)	3 (3.2)	
Drugs	None	62 (80.5)	71 (76.34)	>0.05
	antihemorrhagic	1 (1.29)	0 (0)	
	Antibiotics	6 (7.8)	10 (10.75)	
	Analgesics	0 (0)	2 (2.15)	
	Antihypertensive	0 (0)	2 (2.15)	
	Antidiabetic	1 (1.29)	0 (0)	
	Others	7 (9.1)	8 (8.6)	
Maternal activity during pregnancy	Normal diet, no activity	52	84	
	Normal diet, high activity	4	8	
	Normal diet, reduced activity	1	0	
	Normal diet, Moderate activity	14	1	
	Keto diet	1	0	

Neonatal risk factors and diseases after delivery

Neonatal diseases and risk factors in children after delivery was studied (Figure 3), no significant difference was observed between both groups.

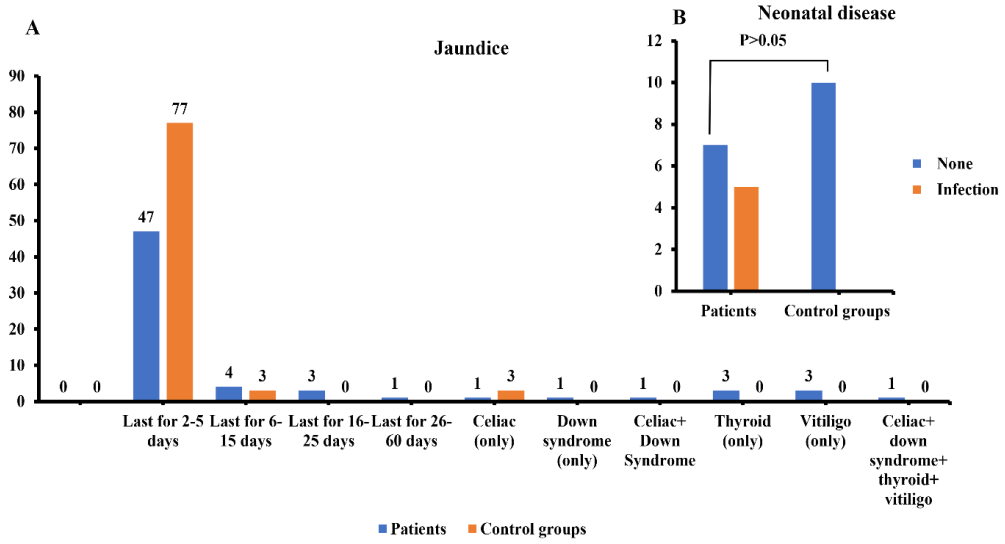

Figure 3: Neonatal risk factors and diseases after delivery. Number of the patients that has been infected by jaundice (A) and Neonatal disease (B).

Quantitative and qualitative measurement of the antibodies in both groups were performed using ELISA. The result revealed a significant difference when GAD65 was compared between the two groups. ICA antibody was positive in 50.4% of the patients while it was positive in 12% of the control groups IA2A was positive in 52.3% of the patients and it was 1.3% in the control group. On the other hand, the IAA antibody was positive in 32.8 % of the patients while none of the healthy subjects were having this antibody. The level of fasting C-

peptide in the patient group was 0.745±0.12 ng/ml while it was 2.12±0.48 ng/ml in healthy subjects. Furthermore, the HbA1C level in patients was 10.46±2.27% while it was 5.38±0.24 in healthy subjects. Figure 4 shows the prevalence of different antibodies in the patient group with both GAD65 and IA2A found in more than 59% of patients, while patients having both ICA and IA2A were less than 5%. Figure 4 is showing the level of different autoantibodies in the same patient and it is clear that GAD65 is shared by most patients.

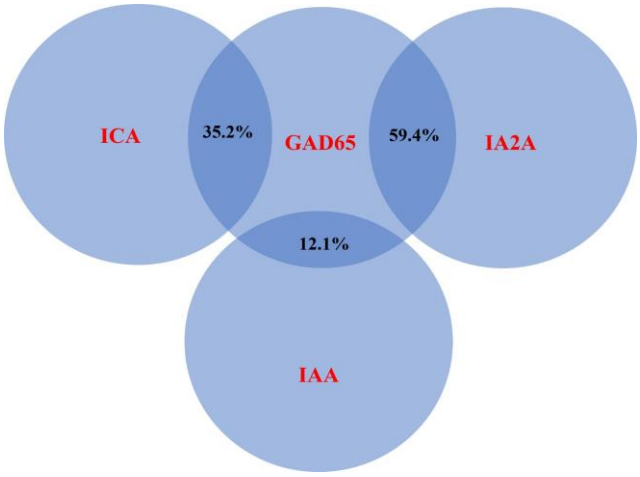

Figure 4: Prevalence of different antibodies using Venn diagram.

Discussion

In the last two decades, number of subjects suffering from diabetes was increasing in Kurdistan Region especially among infants and children. Globally, many risk factors have been studied to understand the etiology of TIDM including environmental, genetic and immunological factors that lead to the destruction of the beta cells of pancreas and a consequent TIDM. The incidence of TIDM continue to increase by a factor of 3% per year in Iraq **[36]**.

In the current study we examined the prevalence of several autoantibodies associated with T1DM and their associations with diabetes duration, family history of autoimmune disease, maternal status, and age of the onset. Simultaneously, the prevalence of additional autoantibodies affecting different organ systems was evaluated. We discovered that 92.1% of the individuals had at least one antibody. The most prevalent antibodies in our group were found to be anti-GAD antibodies (72.8%), followed by IA2A antibodies (52.3%), ICA antibodies (50.4%), and IAA antibodies (32.8%). According to data from worldwide literature, the prevalence of GAD autoantibodies, IA2A autoantibodies, ICA autoantibodies, and IAA, respectively, in children with new-onset T1DM ranges from 70% to 80%, 60% to 80%, 40% to 90%, and 30%-40% **[37]**.

Previous studies conducted in this earlier have revealed that 64.7% of people have GAD antibodies, 19.3% have IA2 antibodies, and 31.8% have ZnT8 antibodies. A high prevalence of GAD65 was available in-patient group while this antibody was observed in small number of control groups. About 75 % of the patients were having higher than normal of this antibody. The level of these antibodies is changing with time, and as most patients were diagnosed late so the level of the antibodies and the prevalence of some antibodies may be incorrect for that screening the patients that are at risk to get TIDM is necessary. Compared to the previous studies, the percentage of these autoantibodies is lower this could be due to late diagnosis of the disease and this may cause reduction of the antibody in the sera of these patients as the highest level of GAD65 was found in children aged ≤9 years old. According to the result of this study 59.4% of T1DM children had both GAD5 and IA2A especially in children aged less than 9 years old consequently they have the highest diagnostic value for T1DM. GAD65 and IA2A dynamics in T1DM are intricate. Autoantibodies can develop before a clinical diagnosis and last for years thereafter **[38]**, although they can also disappear at any time **[39]**.

In general, the prevalence decreases after the diagnosis. Intracellular antigens in β cells include glutamic acid decarboxylase (GAD) and insulinoma-associated protein 2 (IA2A). Intracellular autoantigens must be accessible for β cell autoantibodies to form. Intracellular antigens are released because of autoimmune injury that is cell mediated in β cells. As a result of the sequestered antigens being released, GADA and IA2A are created **[40]**. As the illness worsens, the number of cells reduces as a result of ongoing autoimmune damage, which causes the autoantigens to wane and subsequently the number of autoantibodies to decrease **[41]**. Studying these risk factors are crucial in encouraging decision-makers to begin the screening process for children who are most likely to acquire TIDM. The study compared the level of antibodies in male and female children and it was found that the prevalence of antibodies was higher in female compared to male patients, this result is in accordance with previous research that female tendency to autoimmunity have been observed in many autoimmune diseases **[42]**.

According to the result of this study, there is a significant difference between BMI in both groups, this shows that obesity is regarded as one of the risk factors for developing T1DM. previous studies examining how BMI affects the pathophysiology of T1DM have not always been reliable. However, our result showed a sustained increase in BMI may increase the probability of T1DM onset. The cause of this effect may be connected to insulin resistance when β cell activity is impaired **[43]**. This work suggests that an additional mechanism by which longitudinal elevation in BMI contributes to the preclinical stages of T1DM is obesity induced worsening of autoimmunity. Our data indicate that elevated BMI is associated with an acceleration in the progression of islet autoimmunity in some subgroups of people **[43]**.

We also studied the effect of family history on the development of T1DM. Results have shown that children who have a first-degree relative with T2DM were not at risk to get diabetes as there was no significant difference in a number of the first-degree relative suffering from T2DM and normal subjects **[44]**. Family history of other autoimmune diseases (AID) was studied in this research as well, and it was found that T1DM is more prevalent among children with a positive family history of AIDs such as celiac, thyroid and vitiligo. Additionally, we hypothesized that a significant humoral immune response to cell antigens would be brought on by a familial history of AIDs. An extensive activation of immune responses against T1DM-related autoantigens in the index child could result from an extensive activation of autoimmune reactions as seen by numerous distinct AIDs in the family. Maternal activity was studied during pregnancy and according to the results most pregnant women in both groups were having normal diet with low or no activity.

Another result obtained from this study showed the neonatal risk factors and diseases of the included patients after delivery was there were no statistically significant associations between autoimmune diseases such as thyroid, celiac, and vitiligo and the development of T1DM. Neonatal infection, celiac, down syndrome, thyroid, and vitiligo were not risk factors in developing T1DM, in contrary this autoimmune disease can be regarded as a risk factor for developing other autoimmune diseases such as thyroid, vitiligo, and celiac.

Conclusions

In conclusion, the prevalence of T1DM is increasing in the Kurdistan Region, especially in infants and children. Environmental, genetic, and immunological factors are the risk factors that contribute to the development of T1DM. The study has examined the prevalence of various autoantibodies related to T1DM and their associations with diabetes duration, family history of autoimmune diseases, maternal status, and age of onset. The most prevalent antibodies found were anti-GAD antibodies, IA2A antibodies, ICA antibodies, and IAA antibodies. The study has shown that obesity is one of the risk factors for T1DM, and a high BMI may increase the likelihood of T1DM onset. The family history of other autoimmune diseases such as celiac, thyroid, and vitiligo may increase the risk of T1DM, and a significant humoral immune response to cell antigens may be triggered by a family history of autoimmune diseases. Finally, maternal activity during pregnancy does not seem to be associated with T1DM in children. Studying these risk factors is important in identifying children who are at risk of developing T1DM and initiating early screening processes to diagnose the disease.

Conflict of interest

The authors confirm that they are not affiliated with or involved in any organization or entity with financial interests.

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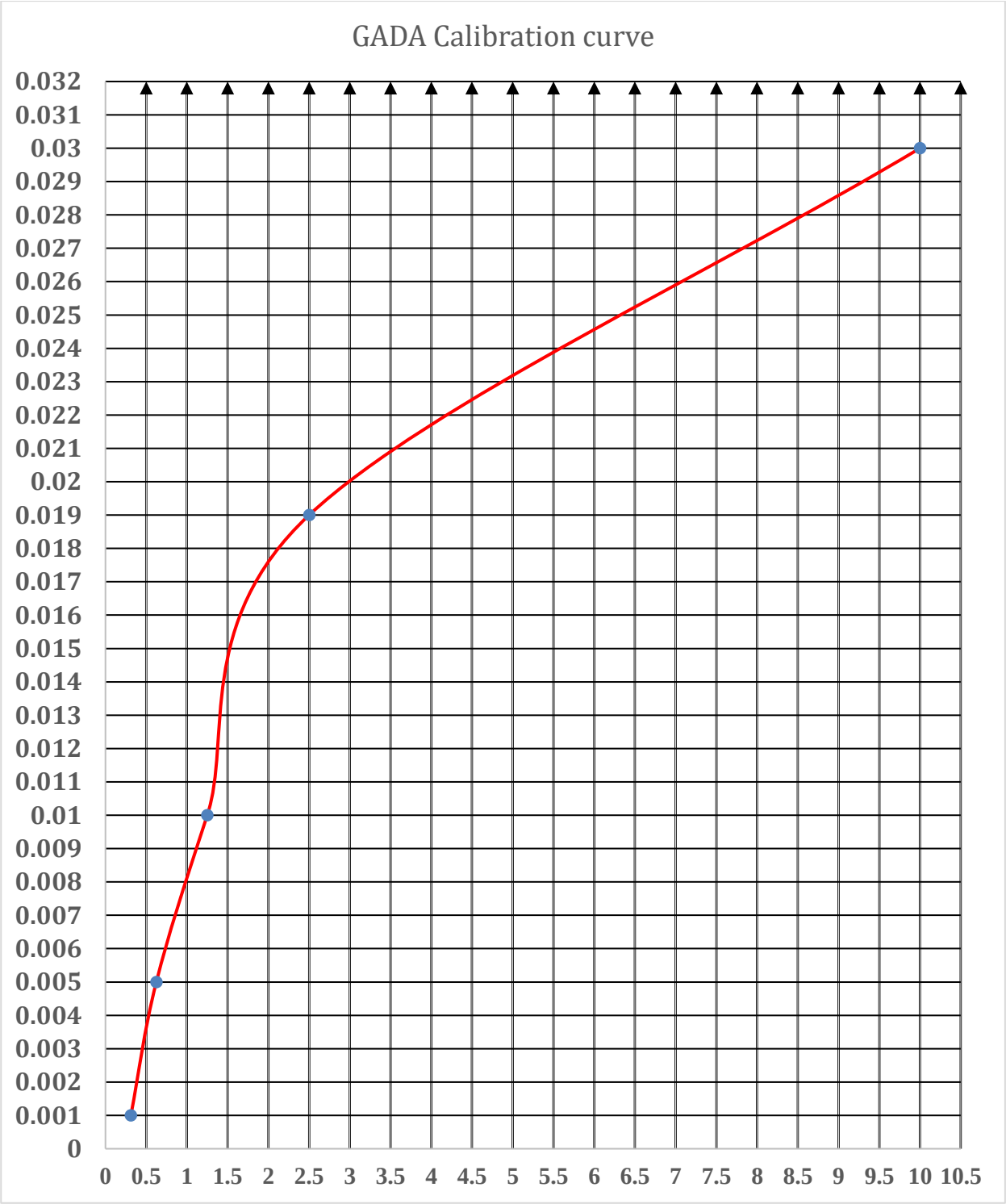
Appendices:

Appendix 1: The questionnaire employed in the sampling campaign.

1	Name
2	Age
3	Jender
4	Hight
5	Weight
6	BMI
7	Family history
8	Child number
9	Ethnic group
10	location, Environmental factors
11	Height and Mother weight during pregnancy
12	Mother have type 1 or type 2 DM?
13	Infection during pregnancy?
14	Gestational DM during pregnancy
15	Drug history during pregnancy
16	Mother life style and diet
17	Jaundice disease, for how long and severity?
18	Medical history
*	Thyroid disease
*	Addison disease
*	Celiac disease.
*	Vitiligo disease
19	Child's Activity Before DM
20	Family Education and Complication



Appendix 1: Calibration curve for the quantitative measurement of the GAD65 antibodies.