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Presence of Type 1 Diabetes-Related Autoantibodies in Pediatric Population in Bosnia and Herzegovina

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ABSTRACT

Background: Diabetes mellitus type 1 (T1D) is an autoimmune organ-specific disease with a wide range of clinical manifestations, in which the β cells of the pancreatic islets of Langerhans are destroyed by the action of autoreactive T lymphocytes and the formation of autoantibodies against β cell components. Among used serological markers of T1D, anti-glutamic acid decarboxylase antibodies (GAD65), anti-tyrosine phosphatase antibodies (IA2), islet cell antibodies (ICA), insulin autoantibodies (IAA) and anti-zinc transporter antibodies (Zn-T8) are of great significance. **Objective:** This study aimed to analyze presence of type 1 diabetes-related autoantibodies (GAD65, IA2, ICA, IAA and Zn-T8 and effects of age and gender on their occurrence in pediatric population. **Methods:** Sixty seven (N=67) T1D pediatric patients were included in the study. The levels of immunological parameters such as anti-glutamic acid decarboxylase antibodies (GAD-Ab), anti-tyrosine phosphatase antibodies (IA2-Ab), islet cell antibodies (ICA) and insulin autoantibodies (IAA) were determined by chemiluminescence immunoassay (CLIA) and anti-zinc transporter antibodies (Zn-T8-Ab) were determined by enzyme-linked immunosorbent assay (ELISA). For statistical analysis, we used SPSS statistical program. **Results:** Our study revealed that among 67 patients with T1D (40 male and 27 female), with an average age of 12,1±3,9 years. The average age of diabetes diagnosis was 6,15±3,29 years. 24 (35,8%) cases were positive for GAD65, 15 (22,4%) for ICA, 34 (50,7%) for IAA, 16 (23,9%) for IA2 and 36 (53,7%) for Zn-T8. The largest number of patients had single positive antibody, the most dominated among them was IAA dominated (40,9%), then Zn-T8 (31,8%). According to Spearman correlation test Zn-transporter shows a significant positive correlation with age of the participants (p=0.027) and disease duration (p=0.006). Anti IA2 shows significant negative correlation with HbA1c (p=0.043). Zn-transporter is associated with patients age and duration of T1D. **Conclusion:** In most cases, patients with T1D are positive for at least one of the specific autoantibodies. Zn-T8 is the most frequently detected and is an important serological marker of type 1 diabetes mellitus. Gender effects on autoantibodies seems to be insignificant, while age alongside disease duration shows important effects.

Keywords: Diabetes mellitus type 1, diabetes mellitus antibodies, immunological markers.

1. BACKGROUND

Type Type 1 diabetes (T1D) is an autoimmune disease caused by the selective destruction of pancreatic cells that produce insulin (1). This immune process has β cells as target of autoreactive B and T lymphocytes, which leads to their destruction and consequently to impaired insulin secretion. The autoimmune process usually begins months or even years before the manifestation of clinical symptoms (2), which manifest when 90% of pancreatic β cells are destroyed. The activation of the immune system and the immune response represent phases of T1D that precede the symptoms of the disease. The main immuno-

logical features of T1D are monocytic and lymphocytic infiltration of Langerhans islets, presence of antibodies to antigens on cells, HLA-DR expression, increased apoptosis of β cells (3).

T1D usually starts in childhood, or in the period of puberty between the ages of eleven and thirteen. The onset is usually acute and develops over several days or weeks. In over 95% of people with T1D, the disease develops before the age of 25, with equal frequency in both sexes (4).

Autoimmune destruction of β cells is confirmed by the success of immunosuppressive therapy which interrupts the progression of insulin dependence in a significant number of newly diagnosed type 1 diabetics. T lymphocytes are present in the islet cells of children who have just developed T1D, and autoantibodies to beta cell proteins are present in the serum even before the onset of T1D and sometime after the diagnosis (5).

The most widely used serological markers of T1D are anti-glutamic acid decarboxylase antibodies (GAD65), anti-tyrosine phosphatase antibodies (IA2), islet cell antibodies (ICA), insulin autoantibodies (IAA). Recently, a new high-ranking diabetes autoantigen was identified in zinc transporter protein 8 (ZnT8 or SCL30-A8). These biomarkers are evidence of an autoimmune process that precedes the clinical appearance of T1D and their combined determination can identify 80% or more of diabetic patients at disease onset or at risk for developing the disease (6).

The combined measurement of these autoantibodies raised the detection rate of diabetes autoimmunity to 98% at the onset of the disease, which resulted in the recognition of these markers in routine diagnostics. The presence of GAD and IA-2 are associated with older age, and GAD additionally with female gender (7). Autoantibodies can appear very early in life, and the order of appearance is related to genotype (8).

The appearance of antibodies to glutamic acid decarboxylase (GAD), islets of Langerhans (ICA) and endogenous insulin (IAA) are the earliest markers of β cell destruction. Not all people positive for autoantibodies will develop T1D, although the chances are higher with more than one autoantibody positive or a family history of T1D. After the onset of ICA, IAA or GAD, some patients have a long prodromal period before the complete destruction of β cells, increasing chances to be misdiagnosed as type 2 diabetes (9).

2. OBJECTIVE

This study aimed to analyze presence of type 1 diabetes-related autoantibodies (GAD65, IA2, ICA, IAA and Zn-T8 and effects of age and gender on their occurrence in pediatric population.

3. MATERIAL AND METHODS

The study included 67 patients, managed and selected by pediatricians at Pediatric Clinic- of the Clinical Center University of Sarajevo. The criteria of the International Diabetes Federation were used for the recruitment of patients with T1D. The study population consists of male and female patients aged 1-18 years who have been diagnosed with T1D for at least 6 months.

Samples for the immune analysis were obtained following standard laboratory procedures at the Department for Clinical Biochemistry and Immunology. The serum levels of GAD (anti-glutamic acid decarboxylase antibodies), IA-2 (anti-tyrosine phosphatase antibodies), ICA (islet cell antibodies) and IAA (insulin autoantibodies) were analyzed using MAGLUMI 800 Chemiluminescence Immunoassay (CLIA), while the serum level of Anti-zinc transporter antibodies (Zn-T) were determined by enzyme-linked immunosorbent assay (ELISA). The procedure were perforemd following recommended protocols by test manufacturer. The positive cutoff value for GAD, ICA, IAA, IA2, Zn-T was 17 IU/mL, 28 U/mL, 20 IU/mL, 28 IU/mL, 15U/mL, respectively.

Statistical analysis

Normality of data distribution was tested using the Kolmogorov-Smirnov and Shapiro-Wilk tests. The data did not fit normal distribution, so we analyzed the data using non-parametric tests. Difference between groups was analyzed with Mann-Whitney U test. The chi-square test was used in the analysis of differences in frequencies. Correlations between immunological parameters, patient demographics and clinical characteristics were determined by Spearman's correlation. The results of all tests were considered statistically significant at $p<0.05$. The analysis was carried out using IBM SPSS v 25.0.

4. RESULTS

The study included a total 67 pediatrics patients who had type 1 diabetes. 40,3% of patients were female and 59,7% male. The average age is 11.6 ± 3.9 for male patients and 12.7 ± 3.9 for female patients, there were not statistically significant differences in age (p=0,252). We divided the total sample into group 1 (1-12 years) and group 2 (13-18) based on the average age (12,1± 3,9) tab.1. The average disease duration was 6.14 ± 3.29 years. 58 patients (85,6%) had at least one positive autoantibody. As part of the routine laboratory treatment of patients, the values of HbA1C and c-peptide were measured, as

Gender distribution	
Female, N (%)	27 (40.3)
group 1-13 years	15 (55.6)
group 13-18 years	12 (44.4)
Male, N (%)	40 (59.7)
group 1-12 years	21 (52.5)
group 13-18 years	19 (47.5)
T1D duration (mean ±SD years)	6.14±3.29
HbA1c (mean ±SD %)	8.1±1.81
C-peptide (mean ±SD ng/mL)	102±176.9
Frequency of positive autoantibodies, N (%)	58 (85.6)
GAD	24 (35.8)
ICA	15 (22.4)
IAA	34 (50.7)
IA2	16 (23.9)
Zn-T8	36 (53.7)

Table 1: Demographics and clinical characteristics of diabetic patients (N=67). N=number of subjects; SD=standard deviation; T1D=Diabetes mellitus type 1

laboratory indicators in monitoring the treatment of insulin-dependent diabetes. The mean value of HbA1c was 8.1 ± 1.8%, and the mean value of C-peptide was 102 ± 176.9 ng/mL.

The largest number of patients had single positive antibody, the most dominated among them was IAA (40,9%), then ZnT8 (31,8%) (Figire 1).

According to the specified reference values of the examined parameters, we divided the findings into two groups (Reference - with reference parameter values, and Pathological - with parameter values higher or lower than the reference ones). In our total sample (n=67), IAA and ZnT8 parameters had more pathological than reference values (over 50%).

According to Mann- Whitney U test there is no significant difference between male and female patients in any of the observed autoantibodies and parameters.

Between two age groups there is a significant difference in values of GAD65 (p=0.044), ICA (p=0.038) i Zn-transporter (p=0.018). Differences in age and disease duration are expected (Table 3).

There is no significant difference in frequency of positive autoantibodies between male and female diabetic patients (Table 4).

There is a significant difference in frequency of positive GAD65 and Zn-transporter autoantibodies between age groups. Both, GAD65 and Zn-transporter have higher frequency of pathological values in older age group (p=0.047 and p=0.009, respectively) (Table 5).

According to Spearman correlation test Zn-transporter shows a significant positive correlation with age of the participants (p=0.027) and disease duration(p=0.006). Anti IA2 shows significant negative correlation with HbA1c (p=0.043). Zn-transporter is associated with patients age and duration of T1D (Table 6).

Parameter	Group	N	Median	IQR	Man Whitney U	p-value
GAD65	Male	40	9.75	6.89 - 34.55	492.500	0.544
	Female	27	8.20	6.73 - 28.90		
ICA	Male	40	6.33	2.00 - 27.22	473.500	0.391
	Female	27	5.39	2.00 - 14.00		
IAA	Male	40	20.45	11.92 - 58.65	522.000	0.818
	Female	27	17.10	12.40 - 67.10		
IA2	Male	40	5.27	2.50 - 29.05	445.000	0.209
	Female	27	2.76	2.50 - 14.60		
ZnT8	Male	40	31.25	7.30 - 86.80	465.000	0.337
	Female	27	9.80	7.10 - 61.50		
Age	Male	40	12.00	9.00 - 14.75	453.500	0.267
	Female	27	12.00	10.00 - 17.00		
Disease duration	Male	40	5.75	3.00 - 8.37	456.500	0.284
	Female	27	6.00	5.00 - 9.00		
Hemoglobin A1c	Male	40	7.80	6.72 - 8.50	508.500	0.687
	Female	27	8.00	7.30 - 9.30		
C-peptide	Male	40	15.25	3.40 - 109.00	499.000	0.599
	Female	27	14.30	5.10 - 193.00		

Table 2. Gender differences in values of observed parameters. N=number of subjects; IQR=Interquartile Range; GAD65=anti-glutamic acid decarboxylase antibodies, IA2=anti-tyrosine phosphatase antibodies, ICA= islet cell antibodies, IAA=insulin autoantibodies, ZnT8=anti-zinc transporter antibodies

Parameter	Group	N	Median	IQR	Man Whitney U	p-value
GAD65	1-13 y	36	7.75	6.49 - 25.35	398.000	0.044
	13-18 y	31	16.40	7.33 - 36.20		
ICA	1-13 y	36	3.89	2.00 - 19.62	394.500	0.038
	13-18 y	31	6.48	4.30 - 34.20		
IAA	1-13 y	36	27.20	12.32 - 95.65	423.000	0.090
	13-18 y	31	14.90	11.70 - 29.30		
IA2	1-13 y	36	3.62	2.50 - 26.4	549.000	0.907
	13-18 y	31	4.56	2.50 - 28.90		
ZnT8	1-13 y	36	7.90	6.30 - 51.22	369.500	0.018
	13-18 y	31	38.80	13.90 - 110.20		
Age	1-13 y	36	10.00	7.25 - 12.00	0.000	<0.001
	13-18 y	31	15.00	14.00 - 17.00		
Disease duration	1-13 y	36	5.00	2.12 - 6.00	189.500	<0.001
	13-18 y	31	9.00	6.00 - 10.00		
Hemoglobin A1c	1-13 y	36	7.80	6.62 - 8.95	543.000	0.850
	13-18 y	31	7.90	7.30 - 8.50		
C-peptide	1-13 y	36	10.19	3.40 - 121.75	496.500	0.438
	13-18 y	31	16.20	5.45 - 136.00		

Table 3. Age differences in values of observed parameters. N=number of subjects; IQR=Interquartile Range; GAD65=anti-glutamic acid decarboxylase antibodies, IA2=anti-tyrosine phosphatase antibodies, ICA= islet cell antibodies, IAA=insulin autoantibodies, ZnT8=anti-zinc transporter antibodies

5. DISCUSSION

T1D is the most common chronic disease in children and can appear at any age. We analyzed 67 pediatric patients, average age 12.1 +/- 3.9 years. No significant difference was observed in the age of the patient between the sexes (p=0.267). The occurrence of the disease between boys and girls was in favor of the male gender 59% vs 41%, which is in accordance with T1D being the only autoimmune disease that is not characterized by the predominance of the female gender (10).

Measurement of type 1 diabetes-related autoantibodies is a screening method for people with a genetic predisposition for developing the condition. Periodic testing for pancreatic protein autoantibodies help to assess the risk of diabetes in children with positive family history. According to The Environmental Determinants of Diabetes in the Young (TEDDY) cohort study, aimed at the prevention or delay of T1D, as an entry criterion for examining the prevalence, uses the presence of two or

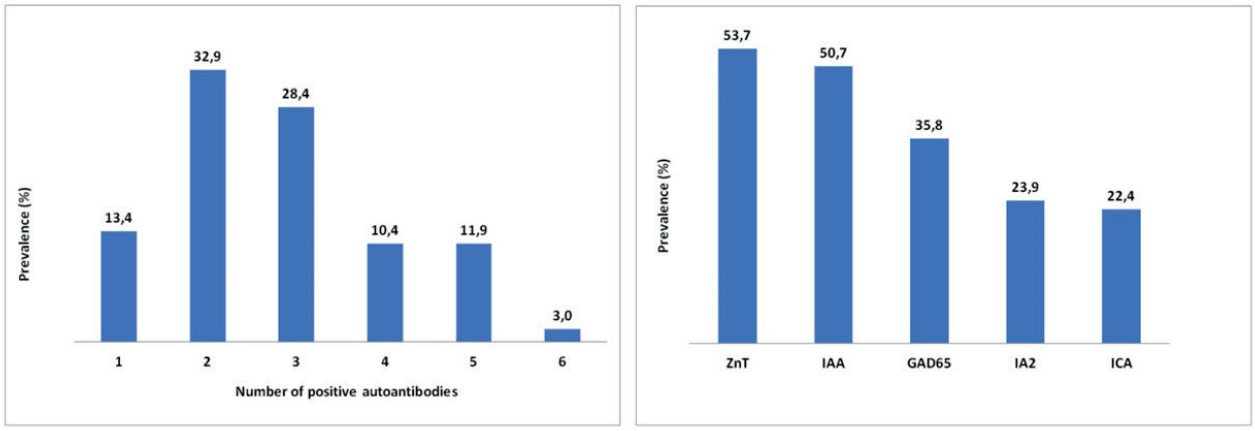

Figure 1. Frequency of positive autoantibody and prevalence of each of type 1 diabetes-related autoantibody

more antibodies. The presence of circulating autoantibodies GAD65, ICA, IAA, IA2, Znt8 suggests that a person is at risk of or has developed T1D. IAAs are primarily detected in children (11). GAD65 is the most common autoantibody detected in adults (12).

The greater the number of antibodies that can be detected as well as higher their titers, greater is the risk of developing T1D. Statistical analysis in our study showed that 58 patients (85.6%) had at least one positive autoantibody. According to literature, patients newly diagnosed with type 1 diabetes, 80% are positive for GAD or IA2 antibodies,1 whereas 20% are antibody negative at the time of diagnosis (13). Clinically defined type 1 diabetics without signs of autoimmunity exist in the Caucasian population. In our study 9 (14.4%) patients were not positive for any of the tested autoantibodies.

Parameter	Group	Pathologic values	Normal values	Total	Pearson Chi-Square	p-value
GAD65	Male	16 (40.0%)	24 (60.0%)	40 (100.0%)	1.416	0.234
	Female	7 (25.9%)	20 (74.1%)	27 (100.0%)		
ICA	Male	10 (25.0%)	30 (75.0%)	40 (100.0%)	0.390	0.532
	Female	5 (18.5%)	22 (81.5%)	27 (100.0%)		
IAA	Male	21 (52.5%)	19 (47.5%)	40 (100.0%)	0.419	0.518
	Female	12 (44.4%)	15 (55.6%)	27 (100.0%)		
IA2	Male	11 (27.5%)	29 (72.5%)	40 (100.0%)	0.715	0.398
	Female	5 (18.5%)	22 (81.5%)	27 (100.0%)		
ZnT8	Male	24 (60.0%)	16 (40.0%)	40 (100.0%)	1.569	0.210
	Female	12 (44.4%)	15 (55.6%)	27 (100.0%)		

Table 4. Gender differences in prevalence of positive autoantibodies GAD65=anti-glutamic acid decarboxylase antibodies, IA2=anti-tyrosine phosphatase antibodies, ICA= islet cell antibodies, IAA=insulin autoantibodies, ZnT8=anti-zinc transporter antibodies

Parameter	Group	Pathologic values	Normal values	Total	Pearson Chi-Square	p-value
GAD65	1-13 years	9 (25.0%)	27 (75.0%)	36 (100.0%)	3.963	0.047
	13-18 years	15 (48.4%)	16 (51.6%)	31 (100.0%)		
ICA	1-13 years	7 (19.4%)	29 (80.6%)	36 (100.0%)	0.388	0.533
	13-18 years	8 (25.8%)	23 (74.2%)	31 (100.0%)		
IAA	1-13 years	21 (58.3%)	15 (41.7%)	36 (100.0%)	1.792	0.181
	13-18 years	13 (41.9%)	18 (58.1%)	31 (100.0%)		
IA2	1-13 years	8 (22.2%)	28 (77.8%)	36 (100.0%)	0.118	0.732
	13-18 years	8 (25.8%)	23 (74.2%)	31 (100.0%)		
ZnT8	1-13 years	14 (38.9%)	22 (61.1%)	36 (100.0%)	6.895	0.009
	13-18 years	22 (71.0%)	9 (29.0%)	31 (100.0%)		

Table 5. Age differences in prevalence of positive autoantibodies. GAD65=anti-glutamic acid decarboxylase antibodies, IA2=anti-tyrosine phosphatase antibodies, ICA= islet cell antibodies, IAA=insulin autoantibodies, ZnT8=anti-zinc transporter antibodies

In Tiberti C. et all. two patients with insulin-dependent diabetes were negative for all antibodies as well as for high-risk HLA alleles clearly do not fall into any category established by the ADA the American Diabetes Association classification (14).

Further research will be needed for this non-1a non-1b diabetic subgroup of patients, trying to investigate their actual occurrence in different populations and, if necessary, should be considered for more appropriate division of the disease (15).

According to the specified reference values of the examined parameters, we divided the findings into two groups (Reference - with reference parameter values, and Pathological - with parameter values higher or lower than the reference ones). In our total sample (n=67), IAA and ZnT8 parameters had more pathological than reference values (over 50%).

Anti-islet autoantibodies serve as key markers in immune-mediated T1D and slowly progressive T1D, also known as latent autoimmune diabetes in adults (LADA). Autoantibodies to insulin IAA, GAD65, IA-2, and ZnT8 are currently used in the diagnosis, pathological analysis, and prediction of T1D. GAD65 can also be detected in nondiabetic patients with autoimmune diseases other than T1D and does not necessarily reflect insulitis. In contrast, IA-2 and ZnT8 antibodies serve as surrogate markers of pancreatic β-cell destruction. Combined

analysis of these four anti-islet autoantibodies in research of Kawasaki E. showed that 93-96% of acute-onset T1D and LADA cases were diagnosed as immune-mediated T1D, while the majority of fulminant T1D cases were negative for autoantibodies (6).

According to Mann- Whitney U test in our research, there is no significant difference between male and female patients in any of the observed autoantibodies and parameters.

Other studies have shown that T1DM is associated with a high prevalence of autoantibodies and rare occurrence of antibody negative T1DM. The association with other organ specific antibody (especially thyroid and adrenal glands) and celiac disease is substantial. Screening of other organ specific antibody e.g. anti TPO antibody, anti thyroglobulin antibody, anti tissue trans glutaminase antibody IgA (tTG IgA), and anti 21 hydroxylase antibody may also be useful in populations at a risk for other auto immune diseases (16).

	GAD65	ICA	IAA	IA2	ZnT8	
Age	rho	0.212	0.191	-0.240	0.007	0.270*
	p-value	0.085	0.121	0.051	0.953	0.027
Disease duration	rho	0.184	0.187	-0.178	0.111	0.332**
	p-value	0.137	0.129	0.150	0.371	0.006
Hemoglobin A1c	rho	-0.169	-0.080	-0.070	-0.247*	-0.045
	p-value	0.170	0.518	0.573	0.043	0.720
C-peptide	rho	0.053	0.137	-0.067	0.114	-0.026
	p-value	0.669	0.270	0.591	0.358	0.837

Table 6. Correlation between autoantibodies, demographics and biochemical parameters. GAD65=anti-glutamic acid decarboxylase antibodies, IA2=anti-tyrosine phosphatase antibodies, ICA= islet cell antibodies, IAA=insulin autoantibodies, ZnT8=anti-zinc transporter antibodies ; rho=Spearman Rank Correlation

We divided the total sample into group 1 (1-12 years) and group 2 (13-18) based on the average age (12,1± 3,9). Between two age groups there is a significant difference in values of GAD65 (p=0.044), ICA (p=0.038) i Zn-transporter (p=0.018). Differences in age and disease duration between age groups in our study are expected. There is no significant difference in frequency of positive autoantibodies between male and female diabetic patients. There is a significant difference in frequency of positive GAD65 and Zn-transporter autoantibodies between age groups. Both, GAD65 and Zn-transporter have higher frequency of pathological values in older age group (p=0.047 and p=0.009, respectively).

The combined presence of autoantibodies to the 65 kDa isoform of GAD65 and the ZnT8 in serum is the best predictive sign of loss of immune tolerance and clinical manifestations of autoimmune diabetes (17).

According to Spearman correlation test Zn-transporter in our research shows a significant positive correlation with age of the participants (p=0.027) and disease duration(p=0.006).

Salonen et all found that antibodies for ZnT8 are related to age and metabolic status at diagnosis but does not significantly improve the detection rate of β-cell autoimmunity in Finnish children and adolescents affected by T1D (18).

The Kawasaki e. et all. study showed that the rate of disappearance of anti-islet autoantibodies is faster in patients aged ≤10 years, and that although both proteins are localized in the insulin granule membrane, humoral autoimmunity to IA-2 and ZnT8 differs by age (19).

In addition, children aged 6–10 years had a higher prevalence of ZnT8A than other older French participants within 6 months of T1D onset in Garnier L. et all study (20). Taken together, it is speculated that age at diagnosis may play an important role in ZnT8A levels.

Anti IA2 in our results shows significant negative correlation with HbA1c (p=0.043).

Elevated HbA1c is a reliable time-predictive marker for the onset of type 1 diabetes. The increase of HbA1c values from the first to the third autoantibody and the decrease in HbA1c predicting the development of IA-2A are new findings that prove the link between HbA1c and the onset of autoantibodies (21).

6. CONCLUSION

In most cases, patients with T1DM are positive for at least one of the specific autoantibodies (85.6%). ZnT8 is the most frequently detected and is an important serological marker of type 1 diabetes mellitus, alongside other immunological markers (antibodies) for this disease. Gender effects on autoantibodies seems to be insignificant, while age alongside disease duration shows effects on autoantibody positivity (frequency and value). Age affects ZnT8 GAD65 and ICA values. ZnT8 is associated with patients age and duration of T1D. This protein seems to have a major role in the pathogenesis of type 1 diabetes.

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