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ORIGINAL PAPER

ELISA Test for Analyzing of Incidence of Type 1 Diabetes Autoantibodies (GAD and IA2) in Children and Adolescents

Marina Delic-Sarac[1], Selma Mutevelic[2], Jasenko Karamehic[1], Djemo Subasic[1], Tomislav Jukic[2], Jozo Coric[3], Ognjen Ridjic[4], Mirsad Panjeta[5], Lejla Zunic[5]

[1]Department of Clinical Immunology, Clinical Center University of Sarajevo, Sarajevo, Bosnia and Herzegovina
[2]Department of Biomedicine and Health, Medical Faculty, Josip Juraj Štrosmajer University, Osijek, Croatia
[3]Department of Clinical Chemistry and Biochemistry, Clinical Center University of Sarajevo, Sarajevo, Bosnia and Herzegovina
[4]Sarajevo School of Science and Technology (SSST), Economics Department, International University of Sarajevo (IUS), Sarajevo, Bosnia and Herzegovina
[5]Faculty for Health Sciences, University of Zenica, Bosnia and Herzegovina

Corresponding author: Marina Delic-Sarac. Department of Clinical Immunology, Clinical Center University of Sarajevo, Sarajevo, Bosnia and Herzegovina. ORCID ID: orcid.org/0000-0002-3939-8197. E-mail:

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ABSTRACT

Introduction: Anti GAD (antibodies on glutamic acid decarboxylase) and anti-IA2 antibodies (against tyrosine phosphatase), today, have their place and importance in diagnosis and prognosis of Type 1 diabetes. Huge number of patients with diabetes mellitus type 1 have these antibodies. Insulin antibodies are of critical importance in diagnosis of diabetes mellitus type 1 for pediatric population. **Materials and methods:** During 2014, the samples of 80 patients from Clinical Center University Sarajevo (CCUS) Pediatrics clinic's, Endocrinology department were analyzed on anti-GAD and IA2 antibodies. The samples of serums of all patients were analyzed with ELISA tests using Anti GAD ELISA (IgG) kites from EUROIMMUN company. These are quantitative in vitro tests for human antibodies against decarboxylase of glutamine acid (GAD) and IA2, in serum or EDTA plasm. **Results:** During the period of one year, in CCUS's Organizational unit, Institute for Clinical Immunology, 80 samples of patients with anti GAD and IA2 antibodies were analyzed. Out of total number of samples, 41 were male patients, or 51% and 39 female, or 49%. The youngest patient was born in 2012, and the oldest in 1993. Age average was represented by the patients born in 2001. Share of positive results for IA2 antibodies and GAD antibodies was 37% for IA2 antibodies, and 63% for GAD antibodies. **Discussion:** During an autoimmune – mediated Diabetes mellitus type 1 leads to T-cell mediated destruction of beta cells of pancreatic islets, reduced production of insulin and glucose metabolism. Studies have shown that these bodies are the most intense single marker for identifying persons with increased risk for diabetes development.
Key words: anti GAD antibodies, anti IA2 antibodies, ELISA tests.

1. INTRODUCTION

Antibodies on Glutamic Acid Decarboxylase (GAD) are very important in research of Type 1 diabetes. GAD is made up of decarboxylase of glutamine acid and it is an enzyme located in the brain and pancreas, having few important roles. Its role is maintained in translation of amino acid's excitatory glutamate into inhibited neurotransmitter - GABA.

During the past 36 years the existence of the relationship between Type 1 diabetes and antibodies on pancreatic islets was known. The specific antigens were identified, against which specific antibodies are formed. Among them, are also IA-2 tyrosine phosphatase-bounded antigen, decarboxylase of glutamine acid 65, zinc transporter ZnT8 and insulin. These antibodies are detected for 96% of patients with Type 1 diabetes, detectable before appearance of clinical symptoms, as well as with patients with developed symptoms (1). Serologic tests on 50 patients with Type 1 diabetes and 50 control samples collected in 43 laboratories found the median sensitivity of 57% and 99% median of the specificities for IA-2 antibodies for Type 1 diabetes (2).

Prospective studies conducted on relatives with Type 1 diabetes patients have shown the appearance of one or more auto-antibodies for Type 1 diabetes (i.e. including IA-2 antibodies) which represent an early mark for Type 1 diabetes development (3). Detection of auto-antibodies, in patients who will develop Type 1 diabetes are usually detectable before they reach the age of three.

Within the study conducted on relatives who are seropositive on IA-2 antibodies, the risk for Type 1 diabetes development for 5 years is 65.3%. Some of

the patients with Type 1 diabetes are primary diagnosed, as Type 2 diabetes, because of the symptoms acquired in adulthood, social obesity and insulin resistency. These patients with „latent autoimmune adulthood diabetes" can be differentiated from Type 2 diabetes patients, by detecting one or more auto-antibodies connected with pancreatic islets' cells (including IA-2, as well) (4).

The best way for prevention and an early diagnosis of diabetes is currently based on combined testing of Type 1 diabetes antibodies. Insulin antibodies are of critical importance when identifying children with an increased risk for Type 1 diabetes. Their lower prevalence for older people means that they are less useful in prediction for adolescents and adults. Early appearance of these antibodies make them necessary for monitoring development of this island autoimmunity from birth.

Zanone et al. conducted researches by which they have confirmed that GAD (5) or IA2 antibodies are highly sensitive markers for Type 1 diabetes mellitus, in pediatric age and have identified a group of patients who lack ICA (e.g. antibodies on pancreatic islets cells) immunofluorescence. The persistence of antibodies of insular tyrosine phosphatase probably presents a mark of better glycemic control and lower needs for insulin, indicating residual beta cells' function, as evidenced by the clinical and prognostic relevance of these antibodies (5). In the study which included 1403 unselected school children in The Netherlands, all of the children were tested on GAD antibodies. Diabetes development was monitored within the period of 7 years. Five children (0.4%) were positive on GAD antibodies and one child (0.1%) was positive on IA2 antibodies. Two children have developed diabetes while being monitored. The first child was positive only on GAD antibodies, while the second child was positive on both GAD and IA2 antibodies (6).

IAA antibodies are of less importance in diagnosis of this disease when insulin therapy starts, because they can be masked by development of antibodies on exogenous insulin. Sensitivity and specificity of IAA depends on few factors, including population characteristics, as well as conduction of essay on its own (7). The intensity of IAA answer, reflected by the level of antibodies, changes the risk of progression, in diabetes. If there is a persistent positivity of IAA, in younger age, it makes a significant risk factor for progression of diabetes in Type 1 diabetes patients' brothers and sisters, while years in which IAA seroconversion comes and appearance of increased levels predicts the years of disease beginning for children with higher genetic risk.

School children with medium to high level of IAA (108-109 l/mol) have an increased risk to develop multiple diabetic antibodies, the same as Type 1 diabetes (8). It was found that a lower affinity IAA has a restriction of distribution of IgG subclasses and some have a dominant IgM responses that are not found in IAA high affinity. An early IAA answer is predominantly IgG1. Other IAA IgG subclasses are usually in the lower titer, but their presence together with IgG1 is connected with higher risk and faster progression in IAA positive family or children with increased genetic susceptibility. Number of detected IgG subclasses is also associated with IAA levels. Presence of IgG 4 IAA which is indicative on Th2 phenotype was not associated with protection from diabetes even when the answer was dominant. In the study, which has included 60 children with diabetes mellitus, results of two groups were compared: a group with detected GAD auto-antibodies and group without detected GAD antibodies. It was shown that there was no significant difference neither in the age, HbA1C and random checks of blood glucose level, nor in the fasting glucose level.

Contrary, a significant statistical difference ($P < 0.05$) was found between groups with a positive anti - GAD and groups with a negative anti - GAD antibodies, in terms of disease duration and Body Mass Index (BMI) (9). In a comprehensive study conducted in the USA (Diabetes Prevention Trial DPT-1), four auto-antibodies were analyzed (i.e. ICA, IAA, GAD65Ab, and IA-2Ab) in order to evaluate the risk of diabetes development. 98% of the first relatives who have developed Type 1 diabetes had one or more positive antibodies, and 80% had two or more positive antibodies. Individuals with two or more positive antibodies had 68% Five - year risk for Type 1 diabetes development, and those with all three antibodies 100% Five - year risk of diabetes development (10).

Even though IAAs have the highest diagnostical sensitivity (~50–60%) before age of ten, (11, 12), GAD 65 antibodies stay at 70 – 80% level, not depending on age (13).

2. MATERIAL AND METHODS

Samples of patients' suspected to Type 1 diabetes were sent from Endocrinology department of Pediatric clinic CCUS to Institute of Clinical Immunology CCUS, within the period of one year for analysis of GAD and IA2 antibodies.

ELISA kits EUROIMMUN Anti GAD ELISA (IgG) allow quantitative in vitro tests for human auto-antibodies against decarboxylase of glutamine acid (GAD) in serum or EDTA plasma. Within the first step of conducting ELISA method, patients' samples were being incubated, in wells. If they are positive, there comes a specific binding of antibodies on

Picture 1. Micro titer plates reader BioTek

GAD. Bonded antibodies form a bridging between GAD in wells and biotin - bonded GAD reagent, which is added in the next step. In order to detect a bonded biotin, a third incubation is conducted with enzyme bonded avidin which catalyzes a colored reaction. The intensity of coloration is proportional to the concentration of antibodies against GAD.

The method is carried out, such that 25µl of calibrators, negative and positive control and sample of patient's serum are loaded into individual micro titer wells, according to the pipetting protocol. Afterwards, the incubation lasts for an hour, at room temperature on shaker set to 50 rounds per minute (rpm). After incubation, the next step is washing on the automatic washer with micro titer plates three times with 450 µl, each, during which a wash buffer must be left on each well for 30 to 60 seconds, during each step of washing, and only after that wells are emptied. After washing, in every micro titer well, a 100 µl of biotin bonded anti GAD antibody is pipetted in. It is incubated for an hour at room temperature on shaker set to 500 rpm. After incubation, a previous step of washing is repeated. A 100 µl of enzyme conjugate peroxidase labeled avidin is pipetted into every well of micro titer plate. It is incubated for 20 minutes, at room temperature on shaker set to 500 rpm. After incubation, the same step of washing a micro titer plate is repeated. Then, a 100 µl of chromogen / substrate is pipetted in every well and incubated for 20 minutes at room temperature while being protected from direct sunlight. 100 µl of stop solution pipette in every well in the same order chromogen / substrate is applied.

A photometric measurement of color intensity is conducted at wave length of 450 nm and afterwards at 405 nm, using a micro titer plates' reader, within 5 minutes after adding stop solution. Before measuring, micro titer plates need to be softly shaken, in order to ensure a homogeneous distribution of solution. IA2 ELISA (IgG) is in vitro essay for human autoantibodies against tyrosine phosphatase (IA2) in serum or EDTA plasma. 50 µl of calibrator is added on micro titer plate, by utilizing a positive or negative control, as well as patient's serum sample, while additionally pouring 25 µl of sample buffer, in every well. The mixture is then covered and left for 5 seconds on shaker at 500 rpm. It is incubated for 16 – 20 hours at +4° do +8°C. After incubation, washing with wash buffer is performed three times with volumes of 450 µl on the automatic washer. 100 µl of IA2 (e.g. biotin labeled IA2) is pipetted in every well and incubated for an hour at +4° do +8°C. After incubation, the wells are emptied and previously described step of automatic washing is repeated. All other steps are the same, as with GAD ELISA essay.

3. METHODS

Within a time period of one year, at the Department of Clinical Immunology, Clinical Center of University of Sarajevo (CCUS), 80 samples of patients with anti GAD and IA2 antibodies were analyzed. Out of whole number of samples, 41 were male patients or 51% and 39 female, or 49%. The youngest patient was born in 2012, and the oldest in 1993. Age of birth median was comprised of these patients born in 2011. The mean value of IA2 results was 293.47 and mean value for GA results was 319.10. Median for all the results of GAD antibodies was 48.36, while median for IA2 antibodies was 179, 1. Mode for IA2 was 0, and for GAD was 7. 47. According to the

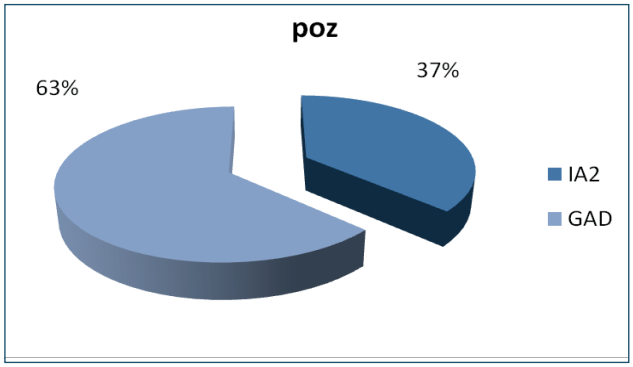

Diagram 1. Proportion of positive results for IA2 and GAD antibodies

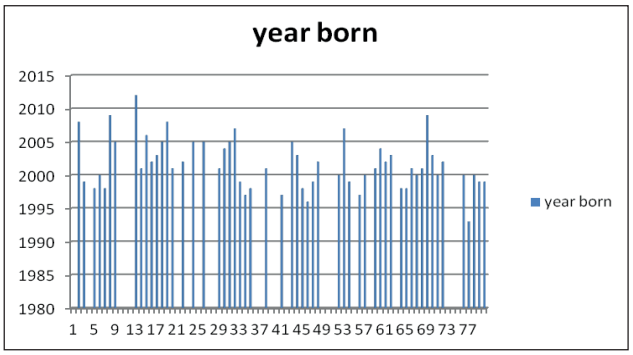

Diagram 2. Display of analyzed patients' year born

EUROIMMUNE protocol the suggested results' values are: <10 IU/ml are negative values of achieved results, and >10 IU/ml are positive values of achieved results.

According to that protocol, the proportion of positive results for IA2 antibodies and GAD antibodies was 37% for IA2 antibodies and 63% for GAD antibodies (Diagram 1).

The mean value for patients' date of birth for positive IA2 antibodies was 2002 and for negative IA2 antibodies was 2001. Mean value for patients' date of birth was comprised of those born in 2001, Anti-GAD antibodies, for both positive and negative values, which can further be explained by the focus group of patients monitored at the Pediatric Clinic, CCUS. In this group of 80 patients, for which anti - GAD and IA2 antibodies were analyzed, 15 patients were either, born in 2005, or were younger (Diagram 2).

According to the EUROIMMUNE protocol that we used, the lower anti IA2 ELISA (IgG) detection limit is defined as the mean extinction plus three times the standard deviation of an analyte free sample and is the smallest clearly detectable antibody titer. The detection limit of the ELISA was found to be 0.7 IU/ml. In our results we gained an additional, 0.7 IU/ml.

For anti-GAD ELISA the lower detection limit of the antibodies as recommended by EUROIMMUNE protocol was 0.2 IU/ml, while the lowest value in our GAD evaluation was also 0.2 IU/ml.

4. DISCUSSION

In Type 1 Diabetes mellitus is a result of T cell-mediated autoimmune disease during which the destruction of pancreatic isles beta cells occurs, followed by the lowered insulin production and glucose metabolism disorders, in genetically predisposed individuals (14). On various antigens of islet cells auto-antibodies begin to synthesize during an autoimmune

insulitis. Type 1 Diabetes is characterized by the presence of specific circulating auto-antibodies, including auto-antibodies on mean value for patients' date of birth (GAD), tyrosine phosphatase (IA2), insulin and auto-antibodies directed against cytoplasmic components of islet cells.

Measurement of auto-antibodies present in GAD and IA2 was shown, as highly important in diagnosis and prediction of Type 1 diabetes mellitus on patients' relatives, especially in the first-degree family ties (15, 16).

These antibodies can be detected in individuals' serum before disease starts. It is believed that they may show up in so-called prediabetes period. They may help in the risk assessment of whether a person will develop Type 1 diabetes mellitus (17). Anti GAD antibodies are detected in 70-90% of pre-diabetic patients and patients with Type 1 diabetes, and proved to be the most sensitive individual markers for identification of individuals with an increased risk for developing diabetes. The higher prevalence of anti-GAD antibodies occurs in older children and, consequently with late onset of Type 1 diabetes.

Latent autoimmune diabetes in adults (LADA) is a disorder with slow progression of autoimmune β-cell dysfunction. At first these patients does not require insulin (during first six month after initial diagnosis) so this slowly progressive form of autoimmune diabetes initially can be managed with diet and oral hypoglycemic agents (18). The following diagnostic criteria for LADA (Latent autoimmune diabetes of adult) are suggested: age between 25 and 65 years; absence of ketoacidosis or symptomatic hyperglycemia at diagnosis or immediately thereafter, without insulin requirement for 6-12 months; and presence of autoantibodies (especially GADA).

Autoimmunity and insulin resistance coexist in LADA and the contribution of these factors seems to be reflected in GADA titers. A subgroup, which is phenotypically and in terms of insulin requirement similar to type 2 diabetic patients, seems to be better identified based on the presence of low GADA titers, especially when these antibodies are present alone. On the other hand, subjects with high GADA titers and multiple antibodies show a phenotype close to that of classical DM 1 and are at a higher risk of premature beta-cell failure. Compared to GADA-negative diabetics, patients with LADA present a higher prevalence of other autoantibodies (anti-TPO, anti-21-hydroxylase and antibodies associated with celiac disease) and a higher frequency of genotypes and haplotypes indicating a risk for DM 1. Patients with high GADA titers may benefit from early insulin therapy and avoiding the use of sulfonylureas, delaying beta-cell failure. In contrast, patients with low GADA titers do not seem to have any disadvantage when managed as type 2 diabetic patients (GADA negative) (19).

One of recent studies suggested that children with autoimmune thyroiditis are at increased risk of developing Type 1 diabetes since the prevalence of Type 1 diabetes autoantibodies in patients with autoimmune thyroiditis was much higher than that observed in the general pediatric population (20). There were also other authors that evaluated presence of anti GAD antibodies and frequency of thyroid specific antibodies in adult patients with type 1 diabetes mellitus and concluded that there is much higher level of these antibodies in patients with diabetes mellitus type 1 comparing with anti GAD negative patients with type 2 diabetes mellitus (21). Some of the novel studies linked presence of elevated levels of anti GAD antibodies with specific neurological symptoms such as autoimmune epilepsy.

Authors suggested measurement of serum levels of anti GAD antibodies in patients with temporal lobe epilepsy (autoimmune epilepsy) developed at patients in middle age. There is a certain benefit of specific immune-modulating therapies with steroid pulse and intravenous immunoglobulin which could improve neurological complications, even in the chronic phase of the disease (22).

There are also suggestions that anti GAD antibodies, seemed to link chronic intestinal pseudoobstruction, autonomic neuropathy. That is significant of autoimmune origin of these patient's symptoms (23).

- Author's contribution: Marina Delic-Sarac was included in preparing of Introduction, Materials and methods, Results and Discussion; Selma Mutevelic - Introduction and Results; Jasenko Karamehic - Materials and methods and Results; Salih Saracevic - Results and Discussion; Djemo Subasic - Materials and methods; Tomislav Jukic - Materials and methods; Jozo Coric - Materials and methods; Ognjen Ridjic - Materials and methods; Mirsad Panjeta - Materials and methods. Lejla Zunic - Materials and methods, Critical review.
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