New Para-Clinical Investigations in the Celiac Disease

Investigații paraclinice de actualitate în boala celiacă

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Abstract

Introduction: *Deficiencies of native antigliadin antibodies tests lead to the decrease of interest but, at the same time, to more serious studies of molecular biology in connection with this test. The discovery of specific B lymphocytes epitopes on certain deamidated gliadin molecules has led to a new serological test, the deamidated antigliadin antibodies. We aimed to evaluate these antibodies and to determine the possible connections with immunoglobulin A deficit in the child's celiac disease.* ***Method****: During 2008 we carried out an observational analytical study that determined both IgG and IgA immunoglobulin isotypes of the deamidated antigliadin antibodies in a group of 102 children from Cluj area, of which 31 children had celiac disease, under gluten-free diet, and 71 children, without diagnosis but with clinical signs of celiac disease.* ***Results****: After evaluating the qualities of the deamidated antigliadin antibodies, we obtained a sensitivity of 80% (95% CI 28-99) and a specificity of 88.4% (95% CI 74-96) for the 0-3 years age group (p=0,007). In older children, the test's sensitivity decreased but the specificity remained at close values, as the children got older. At the same time, in 4% of the children we observed a good correlation with the A immunoglobulin deficit.* ***Conclusions****: Assessment of the IgA+IgG deamidated antigliadin antibodies represents a useful test in the celiac disease screening, mainly for the 0-3 years age group. This test also helps detecting the A immunoglobulin deficit.*

Keywords*: Celiac disease, deamidated antigliadin antibodies, immunoglobulin A deficit*

Rezumat

Introducere: *Deficienţele anticorpilor antigliadină nativă au dus la scăderea interesului asupra lor, dar în acelaşi timp la aprofundarea studiilor de biologie moleculară legate de acest test. Descoperirea de epitopi ai limfocitelor B specifice pe anumite molecule de gliadină deamidată a dus la un nou test serologic, anticorpii antigliadină deamidată. Ne-am propus să evaluăm aceşti anticorpi şi să determinăm eventualele conexiuni cu defi-*

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*citul imunglobulinei A în boala celiacă la copil. **Metodă**: Pe parcursul anului 2008 am efectuat un studiu analitic observaţional, care a cuprins determinarea ambelor izotipuri imunglobulinice IgA şi IgG ale anticorpilor antigliadină deamidată, la un lot de 102 copii proveniţi din zona Clujului şi zonele învecinate din care 31 de copii cu diagnostic de boală celiacă, sub tratament fără gluten şi 71 de copii nediagnosticaţi dar cu semne clinice de boală celiacă. **Rezultate**: Evaluând calităţile anticorpilor antigliadină deamidată am obţinut o sensibilitate de 80% (95% CI 28-99) şi o specificitate de 88,4% (95% CI 74-96) la grupele de vârstă 0-3 ani (p=0,007). La copiii mai mari, pe măsura înaintării în vârstă, sensibilitatea testului scade, dar specificitatea se menţine la valori apropiate. De asemenea, la 4% dintre copii am observat o bună corelaţie cu deficitul imunglobulinei A. **Concluzii**: Determinarea anticorpilor IgA+IgG antigliadă deamidată reprezintă un test util în screeningul bolii celiace, în principal la grupa de vârstă 0-3 ani. Acest test favorizează de asemenea, detectarea deficitului imunglobulinei A.*

***Cuvinte cheie:** boala celiacă, anticorpii antigliadină deamidată, deficitul imunglobulinei A*

Introduction

Celiac disease is considered one of the most common chronic diseases. The fact that the prevalence of the disease increases and that it affects more and more systems and organs, determined the testing of children for this disease at younger ages.

At present, the celiac disease laboratory diagnosis can be established by determining the tissular anti-transglutaminase IgA antibodies (tTG IgA) and the Anti-endomysial IgA antibodies (EMA IgA) as screening tests, and by comparing their positive results with the intestinal biopsy as confirmatory test. The gold standard for diagnosing celiac disease is histology. Due to the fact that the serological methods are less invasive in children, in the last years researchers focused their attention on these methods. There are many data and studies about the tTG IgA evaluation through immunoenzymatic tests and EMA IgA evaluation through indirect immunofluorescence tests. The latest Setty [1]'s research demonstrated a good sensitivity and specificity in children, both for tTG IgA (98%, 96%) and for EMA IgA (90%, 100%). These tests' deficiencies are though recognized by many researchers. Basso [2] obtained best sensitivity (92,5%), specificity (97,6%), positive predictive value (98%) and negative predictive value (91,2%) using tTG IgA. Presence of IgA deficiencies (physiological or pathological) may affect the sensitivity of the IgA-based test, since severe IgA deficient children cannot produce IgA at detectable levels.

Molecular biology introduces us in a less studied domain. Skovbjerg [3] concludes that the tissular transglutaminase mediates the selective extra cellular deamidation of the gliadin peptides in the lamina propria. Korponay-Szabó [4] observes that the deamidated gliadin peptides diminish the linking of the specific monoclonal antibodies with the tissular transglutaminase, which shows that the celiac human serum contains additional antibodies oriented against certain gliadin epitopes, different from those of transglutaminase.

By using these deamidated gliadin peptides (DGP), loaded with specific B lymphocytes epitopes, the deficiencies of the lower specificity and sensitivity of the native antigliadin antibodies were eliminated from the immunoenzymatic tests. Studies brought to the specialists' attention the native antigliadin antibodies, focusing on the determination of both IgA and IgG immunoglobulin izotypes. We used this later approach in our study. Falini [5] states that DGP increase the immunoreactivity of the antigliadin antibodies from 25% to 50%. Rashtak [6] obtains, in the positive cases, a sensitivity and specificity of the DGP IgA (74%, 95%), DGP IgG (65%, 99%) and DGP IgA+IgG (75%, 94%) higher than that of the native antigliadin IgA antibodies (63%, 90%) and of the native antigliadin IgG antibodies (42%, 90%). Naiyer [7] compares the DGP sensitivity and specificity of the patients with active celiac disease with those of a witness

Table 1.Gender distribution of patients

Gender	Absolute frequency	Relative frequency(%)	Cumulative relative frequencies ascending(%)
Female	47	46.08	46.08
Male	55	53.92	100.00
Total	102	100	

Table 2.Age groups

Age	Absolute frequency	Relative frequency(%)	Cumulative relative frequencies ascending(%)
<=3	48	47.06	47.06
3-10	28	27.45	74.51
>10	26	25.49	100
Total	102	100	

group made of patients with Crohn disease and chronic hepatitis and notices that the sensitivity remains the same while the specificity decreases. Volta [8] compares the new test with other serologic markers of the celiac disease and finds the method's sensitivity lower than that of the tTG and EMA, but obtains a higher specificity than that of the tTG.

However, all the presented studies were carried out on adults, while in the case of children, according to the 2008 last agreement on celiac disease that took place under the care of the International Federations of the Pediatric Nutrition, Hepathology and Gastroenterology Societies [9], the new method still waits for wide confirmation.

Our study focused only on pediatric patients and had the following objectives:

1. Quantifying the association of the DGP IgA+IgG with the celiac disease;
2. The observational analytical evaluation of the test's qualities, with a focus on the small groups of age and on the immunoglobulin IgA deficit where there are deficiencies of the usual serologic tests, i.e. of tTG IgA and EMA IgA.

Material and method

Patients

Our group consisted of a representative population of 102 children of the Regional Management Center for Child Celiac Disease in Transylvania, organized within the Children Emergency Clinical Hospital, Cluj-Napoca, who were serologically tested for celiac disease. The gender distribution was: 54% boys and 46% girls (*Table 1*). The patients were divided into three age groups: 0-3 years, 3-10 years and 10-18 years old (*Table 2*). The testing established the serologic panel: tTG IgA, EMA IgA, DGP IgA+IgG and total immunoglobulin A.

Our group inclusion criteria comprised patients with classic signs of celiac disease: chronic diarrhea, weight loss, with influence on height growth, in serious cases malabsorption syndrome, anemia and patients with any primary or secondary intestinal disease [10, 11].

The sera were collected during 2008 from the patients coming for checkup with gastrointestinal symptoms, for diagnosis validation or invalidation and with celiac disease dia-

gnosis for monitoring the gluten-free therapy. All serum samples were stored at -20° C until testing.

The exclusion criteria included children with insufficient data regarding the tests' results. Serological testing of these children was performed on gastroenterologists' recommendation who did not request the dosage of all three serological markers, in all the cases; children without these markers have been excluded from our group. In persons tested more than once during the study, only the first test was taken into account.

The celiac disease was diagnosed based on the histopathologic examination of the intestinal biopsy in the patients discovered with positive tTG IgA and EMA IgA through serologic screening.

Methods

IgA EMA were determined through indirect immunofluorescence by using kits produced by INOVA Diagnostics Inc, San Diego, USA, which used as substratum already standardized (tissue sections of monkey esophagus mucosa). The analysis was performed with an Olympus CX31 fluorescence microscope. It is worth noting that the results obtained through this method were given as qualitative data: negative or positive.

tTG-IgA and DGP IgA+IgG were emphasized through ELISA by using in *vitro* diagnosis kits (IVD) produced by INOVA Diagnostics Inc., San Diego, USA. The samples were tested according to the producer's specifications, on the ELISA Chem Well 2910 Awareness Technology Inc automatic analyzer, and the obtained quantitative results were validated only after checking the internal quality control.

The total immunoglobulin A was determined through the turbidimetric method on the Konelab 30i analyzer, with reagents produced by Thermo Fisher Scientific, Waltham, USA.

Statistical Analysis

Statistical analysis was performed with the SPSS-PC+ software, version 13, and Microsoft Excel.

The relationship between specificity and sensitivity was expressed by drawing up the ROC curve. The quantification of the test's ability to differentiate between the diseased (EMA IgA positive) and healthy subjects (EMA IgA negative) was made by using the AUC indicator (the area under the curve), which varies between 0.5 and 1. The AUC 95% reliability interval was calculated, as well as the observed significance level (p) which tests the null hypothesis that the area under the ROC curve on the population sample is 0.5. If $p<0.05$ one can affirm that the area under the ROC curve is significantly higher than 0.5.

Results

I. Clinical and Demographical Characteristics of the Subjects

Serological testing comprised a group of 31 patients diagnosed with confirmed celiac disease, following a gluten-free diet, with an average age of 8.9 years and a 71 patients group with clinical signs and suspicion of celiac disease, with an average age of 5.5 years. The serologic tests were positive in 45% of the patients belonging to the first group and in 7.1% of the second group patients.

The clinical pathology of the celiac disease suspicious cases included chronic digestive disorders, growth disturbances and deficiency diseases, respiratory diseases, severe digestive diseases, metabolism and nutrition diseases, reno-urinary diseases.

The covered geographic area included patients from Cluj County and the counties in the Central and North-Western part of Transylvania; Maramureş, Alba, Sălaj, Bistriţa-Năsăud, Satu Mare, Suceava, Bihor, Hunedoara, Harghita, Mureş.

II. Evaluation of Test Qualities with* Anti-endomysial IgA Antibodies *as Gold Standard

General Evaluation of the Group

By drawing up the contingency table, we obtained the following distribution in our group: 8 disease carrying patients, 72 healthy patients, 11 patients with false negative results and

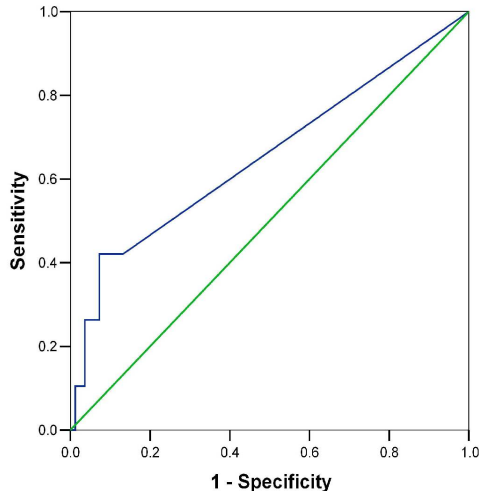

Figure 1. Test evaluation – the ROC curve

11 patients with false positive results. Analyzing the presented data by determining the test's performance indices we obtained a sensitivity of 42.1% (confidence interval CI 95%, 20.2-66.5) and a specificity of 86.7% (CI 95%, 77.5-93.1). The probability ratio showed small changes for the positive and negative value, 3.17 respectively 1.49. The Youden index was 2.88 (CI 95%, 0.54-5.22). The ROC curve area was 0.65 (CI 95%, 0.50-0.80), higher than 0.5 (p=0.037), which means that in almost 65% of all possible subjects' pairs where one has positive EMA IgA and one, negative EMA IgA, DGP IgA+IgG are highly probable to associate to the subject with positive EMA IgA (*Figure 1*).

We noticed from the contingency table a number of 11 patients with false positive results who in most cases correlated with the deficit in IgA, and 4 of those with IgA deficit cases correlated with the positive result of the intestinal biopsy *(Table 3).*

Evaluation on Age Groups

For the age group of 0-3 years, evaluating the test's performance indices, we found a sensitivity and specificity of 80% (CI 95%, 28.3-99.4) respectively 88.3% (CI 95%, 74.9-96.1) and the probability ratio showed moderate changes of the probability for the positive value (6.88) and small changes of the probability for the negative value (4.41). The Youden index had a value of 6.83% (CI 95%, 3.2-10.4). The analysis of the test's evaluation indices on location showed a value of 44.4% (CI 95%, 13.7-78.8) of the positive predictive value, but a negative predictive value of 81.8% (CI 95%, 86.5-99.9). The ROC curve area was of 0.870 (CI 95%, 0.65-1.00), so we can state that the AUC value was significantly higher (p=0.007) than 0.5 and even close to 1 (*Figure 3*).

Table 3. IgA values in patients with false positive AcDGP-IgA+IgG

Patients	Age (years)	EMA-IgA	DGP-IgA+IgG (U/ml)	IgA (mg/dl)
A.C.	4	Negative	41,5	31
F.S	3	Negative	129	13
N.S.	4,1	Negative	40	64
G.I.	4	Negative	41,3	162
R.G.	3	Negative	62,3	31
F.S.	4	Negative	115	12
P.L.	1	Negative	162	19
P.M.	4	Negative	38	36
S.C.	3	Negative	40,3	28
L.O.	4	Negative	99,2	52
S.E.	0,1	Negative	123	17

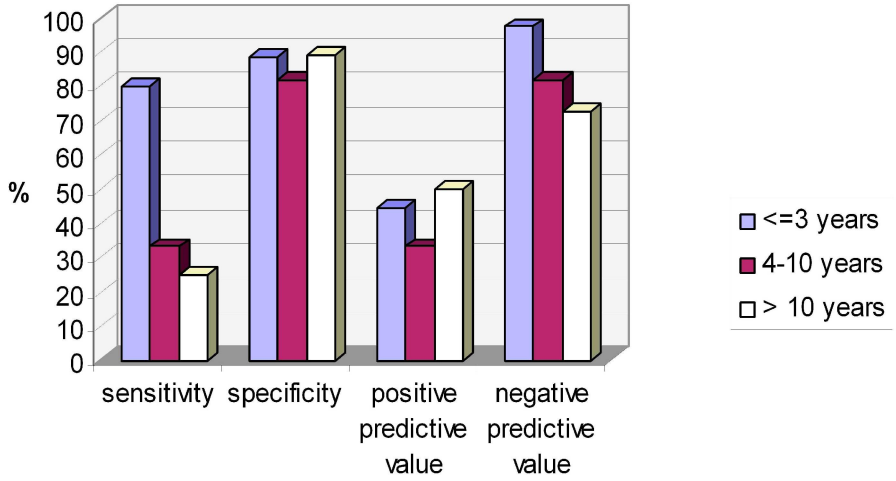

Figura 2. Evaluation test by age groups with IgA antiendomysium antibodies as gold standard

For the age groups between 3-10 and 10-18 years, we obtained analogies regarding the results, i.e.: sensitivity 33.3% (CI 95%, 4.3-77.7) respectively 25% (CI 95%, 3.1-65), specificity 81.8% (CI 95%, 59.7-94.8) respectively 88.8% (CI 95%, 65.2-98.6), positive probability ratio 1.83 respectively 2.25, negative probability ratio 1.22 respectively 1.18. The Youden index had close values in the case of the two age groups, of 1.51% (CI 95%, 2.58-5.61) respectively 1.38% (CI 95%, 1.94-4.72).

Evaluation According to the Disease's Presence or Absence

The sensitivity showed a value of 35.7% (CI 95%, 12.7-64.8) in the patient group with celiac disease, confirmed comparatively with 60% (CI 95%, 14.6-94.7) in the patient group with celiac disease suspicion, while the specificity showed close values in both groups: 76.4% (CI 95%, 50.1-93.1), respectively 89.3% (CI 95%, 79.3-95.6). In the case of the diseased patients' group, the probability ratio showed small changes for the positive value (1.51) and negative (1.18), compared to the patient group suspected of celiac disease where it showed moderate changes of the probability for the positive value (5.65), and small changes of the probability for the negative value (2.23). The Youden index was low 1.21 (CI 95%, 2.0-4.43) in the diseased patients' group compared to 4.93 (CI 95%, 0.58-9.29) in the group suspected of disease.

III. Quantifying the Association with Celiac Disease

To emphasize the association between EMA IgA and DGP IgA+IgG we used the exact Fisher test ($p<0.05$). To emphasize the association between tTG IgA and DGP IgA+IgG we used the chi-square test ($p<0.05$). The association with the celiac disease is also emphasized by the analysis of the standardized residues from which it results we have a significantly higher than expected number of patients with positive EMA IgA and tTG IgA, who also have positive DGP IgA+IgG *(Table 4)*.

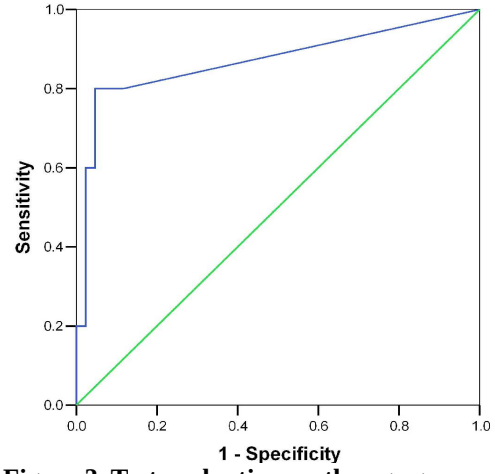

Figure 3. Test evaluation on the age group of 0-3 years - ROC curve

Table 4. Analysis of standardized residues

EMA-IgA		DGP-IgA+IgG		Total
		Negative	Positive	
Negative	Number of subjects	72	11	83
	% of total (column)	86.75	57.89	81.37
	Residues standardized	2.91	-2.91	
Positive	Number of subjects	11	8	19
	% of total (column)	13.25	42.11	18.63
	Residues standardized	-2.91	2.91	
Total	Number of subjects	83	19	102
	% of total (column)	100	100	100

tTG-IgA		DGP-IgA+IgG		Total
		Negative	Positive	
Negative	Number of subjects	17	10	27
	% of total (column)	20.48	52.63	26.47
	Residues standardized	-2.87	2.87	
Positive	Number of subjects	66	9	75
	% of total (column)	79.52	47.37	73.53
	Residues standardized	2.87	-2.87	
Total	Number of subjects	83	19	102
	% of total (column)	100	100	100

IV. Test Evaluation in Economic Terms of Cost-Efficiency

If we include both IgA and IgG immunoglobulin isotypes in one test, the economic costs of the serologic tests are reduced. This test's good results in the IgA deficit screening might also cover, in terms of cost-efficiency, the dosage of total immunoglobulin A.

Discussions

Test evaluation as a whole shows us a good value of the specificity and of the ROC curve area, statistically significant ($p<0.05$). At the same time, in the case of the patients with false positive results of DGP IgA+IgG, most of them of small ages, we can observe a good correlation with the immunoglobulin A deficit. The evaluation following age groups shows us good values of the statistic indices for the age group of 0-3 years (sensitivity and positive probability ratio) but with a low positive predictive value that might be explained by the immunoglobulin A deficit in these patients' case. The test's specificity had also a good value, with a good negative predictive value, same for the Youden index and ROC curve area statistically significant ($p<0.05$). For the other age groups, we note a decreased sensitivity but we found significant values only in the case of specificity and negative predictive value. The evaluation according to the disease presence or absence shows low values for the group of patients with celiac disease but good values of the sensitivity, specificity and Youden index for the control group.

The test evaluation in economic terms of cost-efficiency complies with the American Gastroenterological Association [12] recommendation that discourages the usage of more tests for the celiac disease diagnosis.

The Antiendomysial antibodies are considered to be the test with the highest sensitivity and specificity [1,13,14] in diagnosis of celiac disease. The method used in this study, employing standardized monkey esophagus sections, with commercially delivered buffer substances of well-known concentration and pH, eliminates the deficiencies that can appear when obtaining the sections with a microtome and preparing the buffer substances in the laboratory.

The qualities of the new test of DGP IgA+IgG proved higher precision in diagnosis compared to the native antigliadin antibodies and the anti-transglutaminase antibodies [15-17]. The population admitted in the present study as representative sample population was selected according to the clinical signs that confirm the presence or association of the celiac disease. This represents a difference, compared to the previous studies where the inclusion criterion was the presence of a positive serologic test [16, 17], criterion according to which the tests' evaluation was made. There is only one study that investigated the performance indices on consecutive patients, carried out by Niveloni [18], but he worked with separate immunoglobulin isotypes.

The good results of our study given by DGP IgA+IgG in the immunoglobulin A deficit screening, confirm the existent studies: Korponay-Szabo [4] stated that DGP IgA+IgG detected all 57 patients diagnosed with celiac disease and with immunoglobulin A deficit.

Celiac disease screening among the populations with high risk, such as family members of the patients with celiac disease or the adults with type I diabetes mellitus, remains an important issue. Because tTg and DGP have different target antigens, they can be used together to obtain the best sensitivity, without losing their specificity [19]. Generally, DGP test could be applied already from infancy as a celiac disease screening test to prevent late diagnosis and long term major complications, such as the risk of cancer development [20]. Consequently, further researches are necessary.

Conclusions

The ELISA immunoenzymatic testing of the deamidated antigliadin IgA+IgG antibodies opens new ways for celiac disease diagnosis, as it is a useful test as serological marker in the celiac disease screening and at the same time in the immunoglobulin A deficit for the 0-3 years age group, and a test with high precision diagnostic value in all child age groups.

We can conclude though from the obtained data that the test is not useful in monitoring the gluten exclusion therapy in the case of the patients with celiac disease.

High percentage (45%) of the positive serologic tests in the case of the patients with confirmed celiac disease demonstrates the necessity of extending the health education program in the sense of following the gluten-free diet among the adolescent population.

Abbreviations:

tTG - tissular anti-transglutaminase antibodies, EMA - Antiendomysial antibodies, DGP - deamidated gliadin peptides, IVD - in *vitro* diagnosis kits, CI - confidence interval

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