Clinical and biological correlations in celiac disease in children: the prospective single experience of a romanian tertiary center

A case-control study (Strobe-Compliant study)

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Abstract

Celiac disease—a chronic inflammatory disease of the intestine—is triggered by gluten or associated protein consumption. The aim of our study was to assess the sensitivity, specificity of the combined anti-transglutaminase 2 (TG\textsubscript{2})/deamidated gliadin peptide antibodies (DGP), and antiendomisium antibodies (EMA), to determine the distribution of HLA-DQ\textsubscript{2}/DQ\textsubscript{8} for the 140 tested patients, and also to evaluate the clinical and laboratory characteristics of patients admitted with the suspicion of celiac disease (CD). Children included in the study were divided into: group 1, patients with confirmed CD; group 2, patients with "potential" CD; group 3, control group, patients without CD. We assessed the standard laboratory data, the level of TG\textsubscript{2}/DGP and EMA antibodies, as well as the distribution of HLA molecules in the selected patients. Histopathological examination was considered the criterion standard for diagnosis in most cases.

The sensitivity of TG\textsubscript{2}/DGP was 85% and the specificity 92%. EMA showed a sensitivity of 82% and a specificity of 98%. The vast majority of patients diagnosed with CD were either HLA-DQ\textsubscript{2,2,5} (encoded by DQA\textsubscript{1}*05 & DQB\textsubscript{1}*02) positive (87.5%) or HLA-DQ\textsubscript{8} (encoded by DQB\textsubscript{1}*05:02) positive (12.5%). One patient showed a positivity only for HLA-DQ\textsubscript{2,2}. Our study showed that the genetic risk for CD was present in more than one-third of the cases without a confirmed diagnosis of CD. Therefore, the awareness of genetic susceptibility for CD is essential because of the fact that these individuals can develop the disease at any point of their lives. The sensitivity of TG\textsubscript{2}/DGP and EMA were very similar, whereas EMA presented a higher specificity than that of TG\textsubscript{2}/DGP.

Abbreviations: ALT = alaninaminotransferase, AST = aspartataminotransferase, CD = celiac disease, DGP = deamidated gliadin peptides, EMA = anti-endomisium antibodies, Hgb = hemoglobin, HLA = human leucocyte antigen, Htc = hematocrit, LDH = lactate dehydrogenase, MCH = mean corpuscular hemoglobin, MCV = mean corpuscular volume, PLT = number of platelets, RBC = number of red blood cells, SD = standard deviation, TG\textsubscript{2} = tissue transglutaminase type 2.

Keywords: celiac disease, genetic testing, serology

1. Introduction

Celiac disease (CD) is a systemic immune-mediated disorder caused by gluten and other prolamins manifested in genetically susceptible individuals (HLA-DQ\textsubscript{2} or DQ\textsubscript{8} haplotypes). Immune reaction in the small intestine causes intraepithelial leukocyte infiltration, villous atrophy, and crypt hyperplasia.

Clinical manifestations vary from mild to severe, and the disease may be silent for many years. “Classic” symptoms include: chronic diarrhea, weight loss, malabsorption, and...
iron-deficiency anemia. In addition to gastrointestinal symptoms, the disease can have extraintestinal manifestations, such as osteoporosis, dermatitis herpetiformis, neurological problems, liver disorders, and arthritis.

The etiology of CD is multifactorial, but genetic factors have a major influence, as documented in twin studies and studies showing a strong dependence of HLA-DQ2 and HLA-DQ8 haplotypes. Ninety-five percent of patients with CD have the HLA-DQ2 heterodimer, which is encoded by alleles DQA1*05 and DQB1*02, either cis configuration or trans, and most of the remainders have the HLA-DQ8 heterodimer, encoded by the DQB1*0302 allele. The expression of these molecules, HLA-DQ2 and HLA-DQ8, is necessary but not sufficient to cause the disease because approximately 30% to 40% of the white population is HLA-DQ2-positive and only 1% develops CD. Individuals who lack heterodimers HLA-DQ2 or HLA-DQ8 can be ruled out from the group at risk to develop CD. The higher or lower risk of developing the disease depends on the presence or absence of these alleles, their combination, and the number of copies.

The DQ2 molecule presents gluten-derived peptides, modified by tissue transglutaminase, to CD4+ T cells. The DQ8 molecule is capable of binding to a different set of gluten-derived peptides with different affinities than the DQ2 molecule. This finding could explain the minor association between the DQ2 molecule and the development of CD.

The role of environmental factors and non-HLA alleles in CD development are well known. Study found an involvement of rotavirus in the development of CD, a finding that requires confirmation by further studies. The age at which gluten is introduced to the diet has been reported to affect the risk of CD autoimmunity in genetically predisposed children. Other studies have shown that the delayed introduction of gluten did not modify the risk of CD among at-risk infants.

The prevalence of CD in different populations is about 1%, but there are countries with a higher or lower prevalence. Although it is a relatively frequent disease, it remains underdiagnosed because of the great variation of symptoms and the diagnosis can be delayed for many years.

Very few data are available on the epidemiology of CD in Romania. A study published by Dobru et al. in 2003, conducted on a group of 2436 adult patients found a prevalence of 2.22% for CD. Popp et al. in a study on 148 asymptomatic adult family members of patients with CD, found a prevalence of 8.7%.

The diagnosis of CD is based on determining specific antibodies: anti-transglutaminase (TG2), anti-endomysium (EMA), and deamidated gliadin antibodies (DGP) as well as interpreting the histological changes in duodenal biopsies (villous atrophy, crypt hyperplasia). Anti-EMA antibodies are directed against the extracellular TG2. With the exception of anti-DGP, these antibodies are usually of the IgA class. In patients with IgA deficiency, the same type of antibodies, but of IgG class, can be detected.

TG2 appears in abundance in the gut and it functions by deamidation of proteins and peptides, including fragments of gliadin which increase the reactivity of T cells in patients with CD. Immunofluorescence assays, such as EMA, require microscopic evaluation and may be subject to interobserver variability. The specificity of the EMA test results is 98% to 100% in specialized laboratories. Positive titers of anti-TG2 and EMA are associated with a high probability of CD in children and adolescents.

Despite this fact, high levels of anti-TG2 have been described in a series of illnesses other than CD, such as hepatic disorders, psoriasis, autoimmune diseases, infections, tumors, and myocardial lesions. These diseases are not associated with positive EMA results, which explains the better reliability of EMA for the diagnosis of CD.

The aims of our study were to assess the genetic risk for CD in a pediatric population from Romania with suggestive clinical symptoms for CD, taking into account the clinical and laboratory parameters of the patients, and to establish the diagnostic value of the serological markers used for diagnosing CD.

2. Materials and method

A cross-sectional prospective study was performed on 173 pediatric patients with the clinical suspicion of CD hospitalized in the Pediatric Clinic of the Emergency County Hospital Targu Mures, Romania, Pediatric Gastroenterology Department, between 2007 and 2015. The parents of only 156 of the patients agreed to the inclusion of their children in our study and from them, only 140 patients remained after a selection according to sex and age to comply with the pair method. Clinical, demographic, laboratory, and histopathological data were obtained from the medical records of the patients.

Patients were divided into 3 groups: patients with confirmed CD—28 children; patients with genetic susceptibility for CD, which do not meet all the diagnostic criteria but they were proved to carry a genetic risk of CD (HLA DQ2 or DQ8 positive)—49 patients; and the control group consisting of patients without any proved criteria for the diagnosis of CD and without any genetic risk for CD (HLA-DQ2- and DQ8-negative)–63 patients.

We assessed the following data: demographic information (sex, age at diagnosis), symptoms, clinical data (weight, clinical examination), laboratory data (the level of anti-TG2 antibodies, anti-DGP antibodies, anti-EMA antibodies, HLA-DQ2/DQ8 testing, blood count, iron, liver enzymes, lactate dehydrogenase [LDH], albumins, total proteins), and histopathological data: the results of the duodenal biopsies. These data were assessed at the moment of diagnosis and at a subsequent reevaluation at 6 months, to appreciate the clinical and paraclinical changes determined by the gluten-free diet.

The diagnosis of CD was established according to the ESPGHAN criteria: a positive duodenal biopsy (Marsh 2/3) or a titer of TG2 >10 times the upper limit of normal, combined with positive EMA and genetic testing.

The inclusion criteria were: pediatric patients (1–18 years) admitted to the Pediatric Gastroenterology Department between 2009 and 2015 with the suspicion of CD based on clinical symptoms, such as: chronic, intermittent diarrhea or chronic constipation, recurrent abdominal pain, abdominal distension, recurrent or persistent vomiting, chronic fatigue, weight deficit, height deficit, and/or failure to thrive.

The exclusion criteria were: incomplete clinical or laboratory data, age <1 year and >18 years, associated autoimmune or chronic disorders, signs of acute infection at the moment of admission, previously introduced gluten-free diet, and patients whose parents refused to sign the informed consent.

HLA-DQ2/DQ8 testing was performed using a polymerase chain reaction amplification followed by hybridization technique: CeliacStrip-OPEGEN from OPERON (Zaragoza, Spain). We indicated as HLA-DQ2.5-positive the individuals who carried the DQA1*03 and DQB1*02 alleles. The individuals with DQB1*0302 and DQA1*03 alleles were indicated as DQ2-negative and those carrying the DQA1*02 alleles and DQB1*02 as DQ2-positive.
EMA IgA antibodies were measured by indirect immunofluorescence on a substrate of monkey esophagus (NOVA LITE Endomysial Antibody, INOVA Diagnostics, San Diego, CA). The EMA is the fine blade of connective tissue between the smooth muscle fibers of the muscular layers of the esophagus. If EMA antibody is present, it will bind to the connective tissue and will present a greenish fluorescence. An EMA positive-result was defined by the presence of a characteristic pattern of fluorescence at a dilution ≥1/5.

Serum TG2/DGP was measured using a determination kit via the enzyme-linked immunosorbent assay (ELISA) method: QUANTA Lite h-tTG/DGP Screen (INOVA Diagnostics, San Diego, CA). This kit allows a semiquantitative determination of IgA and IgG anti-TG2 and DGP. The antigens used were: human tissue transglutaminase and synthetic deamidated gliadin peptides. The patients’ sera were diluted at 1:101. We considered positive antibody titers >20U/mL, according to the manufacturer’s recommendations.

Duodenal biopsies were obtained by upper gastrointestinal endoscopy. We have taken 3 to 4 biopsies from different places of D1. The preparation of the biopsy fragments was performed using standard histopathological techniques. The fragments were examined after staining with hematoxylin-eosin, Pas-Alcian, and Giemsa. Intraepithelial lymphocytosis was evidenced by immunomarking with CD3.

For this study, we have obtained the consent of the ethics committee of the University of Medicine and Pharmacy in Targu Mures (approval no.13/18.07.2011). The parents of the pediatric patients signed an informed consent on agreeing to the processing of personal data, and an informed consent on agreeing to perform upper gastrointestinal endoscopy.

Statistical analysis was performed with the programs Excel 2007 and GraphPad Instat. To assess the normality of continuous variables, the Kolmogorov-Smirnov test was applied. Quantitative variables were compared using t test, Mann-Whitney U test, Wilcoxon test, analysis of variance test, or Kruskal-Wallis test, when appropriate. We interpreted all the tests against a P = .05 significance threshold, and statistical significance was considered for P values below the significance threshold.

3. Results

From the 28 patients with confirmed CD at which the TG2/DGP antibodies were determined, 4 patients had negative results, <20 U/mL (sensitivity = 85%). Seven of 28 patients with confirmed CD at which the anti-EMA antibodies were determined had negative results, for 2 of which the results were positive at reevaluation (sensitivity = 82%).

In the control group, 5 of 63 tested patients presented false-positive anti-TG2/anti DGP titers (specificity = 92%). The anti-EMA antibodies showed a slight positive result in 1 patient of 63 tested (specificity = 98%).

The positive predictive value for the TG2/DGP combined determination was 82.7%, whereas the negative predictive value was 93.5%. The positive predictive value for EMA determination was 95.8%, and the negative predictive value was 92.5%.

In the celiac group, 87.5% of the patients were HLA-DQ2.5 (DQA1*05 & DQB1*02)-positive and 12.5% HLA-DQ8 (DQB1*03:02)-positive. In group 2, of 49 tested patients, 69.4% were HLA-DQ2.5-positive and 44.9% HLA-DQ8-positive.

In the celiac patients group, the male/female ratio was approximately one-fourth and the mean age at diagnosis 9 years. The main signs and symptoms at the time of diagnosis as well as the secondary diagnoses of the patients in group 1 are illustrated in Figure 1.

For the celiac patients, duodenal biopsies were collected in 15 patients, and for the remaining patients, the diagnosis of CD was based on the TG2 values (>10× normal) coupled with a positive anti-EMA result and an increased genetic risk for CD. The biopsy results were: Marsh III in 14 cases (2 cases Marsh 3B, 3C Marsh 10 cases, unspecified in 2 cases) and Marsh II in 1 case (Fig. 2).

On comparing the values of TG2/DGP between groups, we obtained a statistically significant difference between patients with CD and controls (P < .0001) and between patients with CD and potential (P < .01) using the Kruskal-Wallis (Dunns) test; the values of TG2/DGP in the group with CD were higher (Table 1). Table 1 illustrates the data obtained by comparing the laboratory data of the patients from each group. We obtained statistically significant differences for hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, red blood cell number, iron, AST, and ALT. The differences between the laboratory data of celiac patients at the first admission and at reevaluation, respectively, are included in Table 2. We obtained statistically significant differences for hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, red blood cell number, iron, TG2/DGP, and ALT.

4. Discussions

The present study is one of the few to study the sensitivity and specificity of serological diagnostic methods and the distribution
of HLA molecules, in addition to clinical and laboratory characteristics of patients with CD on a population from Romania.

We found the sensitivity of TG2/DGP and EMA to be very close (85% vs. 82%), whereas EMA presented a higher specificity as that of TG2/DGP (98% vs. 92%). We also assessed the positive and negative predictive values of the 2 serological diagnostic methods. The positive predictive value for the TG2/DGP combined determination was 82.7%, much lower than that of EMA determination, which was 95.8%. The negative predictive values for the 2 methods were very close, 93.5% for TG2/DGP and 92.5% for EMA.

In a meta-analysis of 16 studies on the accuracy of serological tests for the diagnosis of CD, Giersiepen et al. achieved a sensitivity $\geq 90\%$ and a specificity of 98.2% for EMA. EMA test is based on an indirect immunofluorescence technique using a substrate which can be either monkey esophagus or human umbilical cord, the accuracy of the test is the same for each.

Figure 2. (A) Crypt hyperplasia, normal villi, Marsh II, hematoxylin and eosin (HE), 4×. (B) Marked villous atrophy and crypt hyperplasia, Marsh IIIB, HE, 4×. (C) Intraepithelial lymphocytes, Marsh IIIB, HE, 10×. (D) Complete villous atrophy and crypt hyperplasia, Marsh IIIC, HE, 4×.

### Table 1

Comparison between the laboratory tests of the patients in the 3 groups.

| Parameters          | Celiac group 1 (n = 28) | Potential group 2 (n = 49) | Control group 3 (n = 63) | P       |
|---------------------|-------------------------|----------------------------|--------------------------|---------|
| Hgb, g/dL (mean±SD) | 11.46±1.71              | 12.41±1.26                 | 12.44±1.92               | .02     |
| Htc (%) (mean±SD)   | 35.73±3.84              | 35.73±3.84                 | 36.53±4.71               | .61     |
| MCV, fl (mean±SD)   | 74.94±7.32              | 79.31±4.97                 | 78.65±4.61               | .003    |
| MCH, pg (mean±SD)   | 24.58±3.29              | 27.37±2.14                 | 27.05±2.77               | .004    |
| RBC, $\times 10^12$ cells/mm$^3$ (mean±SD) | 4.92±0.36 | 4.58±0.40 | 4.64±0.51 | .01    |
| PLT, $\times 10^12$ cells/mm$^3$ (mean±SD) | 336.2±126.2 | 329.4±113.2 | 347.6±127.4 | .10    |
| Iron, μmol/L (mean±SD) | 7.47±2.57 | 13.55±6.65 | 12.57±5.27 | .008    |
| AST, U/L, median (min–max) | 33.0 (12.6–309) | 27.9 (16.1–73.0) | 35.1 (16.1–137) | .02     |
| ALT, U/L, median (min–max) | 22.1 (8.1–356) | 16.4 (5.7–82.0) | 16.3 (8.8–236) | .03     |
| LDH, U/L, mean±SD    | 238.9±53.8              | 298.0±102.4                | 263.5±96.3               | .12     |
| Albumin, g/dL (mean±SD) | 4.16±0.57 | 4.62±0.43 | 4.54±0.40 | .24    |
| Proteins, g/dL (mean±SD) | 6.92±0.73 | 6.95±0.60 | 7.05±0.43 | .70    |
| TG2/DGP, median (min–max) | 133.00 (1.2–555) | 1.10 (0.40–1.80) | 1.99 (0.33–38.1) | .0001   |

Data were expressed mean±standard deviation (analysis of variance test) and median (min–max) (Kruskal-Wallis test).

ALT = alanin aminotransferase, AST = aspartat aminotransferase, DGP = deamidated gliadin peptides, Hgb = hemoglobin, Htc = hematocrit, LDH = lactate dehydrogenase, MCH = mean corpuscular hemoglobin, MCV = mean corpuscular volume, PLT = number of platelets, RBC = number of red blood cells, TG2 = transglutaminase.
The performance of anti-DGP antibodies is lower than that of TG2 and EMA. In a meta-analysis of studies comparing the performance of DGP antibodies and TG2, Lewis and Scott achieved a sensitivity of 87.8% versus 93% and a specificity of 94.1% versus 96.5%.\(^2\)

There have been few published studies on the combined determination of anti-TG2/DGP. Agardh et al conducted a study that included 119 children with CD, 57 children with other diseases, and 398 patients in the control group, yielding a sensitivity of 100% and a specificity of 89% in children with other diseases, and 97% in the group control.\(^3\)

### Table 2

| Parameters | Examination | Mean ± SD | P |
|------------|-------------|-----------|---|
| Hgb, g/dL  | Initial     | 11.46 ± 1.71 | .01\(^4\) |
|            | Reevaluation| 11.25 ± 1.28 | .96\(^5\) |
| Hct (%)    | Initial     | 35.73 ± 3.84 | .02\(^6\) |
|            | Reevaluation| 35.64 ± 8.95 | .07\(^7\) |
| MCV, fl    | Initial     | 74.94 ± 7.32 | .03\(^8\) |
|            | Reevaluation| 80.24 ± 6.40 | .09\(^9\) |
| MCH, pg    | Initial     | 24.58 ± 3.29 | .01\(^10\) |
| RBC, x10\(^6\) cells/mm\(^3\) | Initial | 4.92 ± 0.36 | .05\(^11\) |
| PLT, x10\(^9\) cells/mm\(^3\) | Initial | 395.2 ± 126.2 | .70\(^12\) |
| Iron, μg/L | Initial     | 7.47 ± 2.57 | .003\(^13\) |
| AST, IU/L  | Initial     | 33.0 (12.6–50.9) | .21\(^14\) |
| ALT, IU/L  | Initial     | 21.2 (8.1–35.6) | .03\(^15\) |
| LDH, IU/L  | Initial     | 15.4 (10.9–25.0) | .43\(^16\) |
| TG2/DGP, IU/mL | Initial | 238.95 ± 58.3 | .02\(^17\) |
| Reevaluation | 216.8 ± 62.90 | .01\(^18\) |
| Reevaluation | 133.0 (0.2–506.5) | .02\(^19\) |
| Reevaluation | 13.45 (1.0–201) | .05\(^20\) |

ALT = alanine aminotransferase, AST = aspartate aminotransferase, DGP = deamidated gliadin peptides, Hgb = hemoglobin, Hct = hematocrit, LDH = lactate dehydrogenase, MCH = mean corpuscular hemoglobin, MCV = mean corpuscular volume, PLT = number of platelets, RBC = number of red blood cells, TG2 = transglutaminase.

Student pair test.

Studies have demonstrated a sensitivity and specificity for IgA EMA of 95% and 99%.\(^21\) Despite the high specificity of the determination, there are certain aspects of this test that may limit its usefulness in clinical practice. It is semiquantitative, operator-dependent, expensive, and takes a long time for processing. IgA EMA testing can be useful if the anti-TG2 IgA test is negative, operator-dependent, expensive, and takes a long time for results.\(^22\) It is semiquantitative and specific but not possible to obtain overall performance estimates of sensitivity and specificity for the presence of CD in patients without bowel atrophic lesions.\(^23\)

In our study, we found that the majority (93.8%) of patients diagnosed with CD were either HLA-DQ\(_2\)-positive (DQA1\(^\ast\)05 and DQB1\(^\ast\)02)-positive (87.5%) or HLA-DQ\(_2\) (DQB1\(^\ast\)0302)-positive (12.5%). One patient had none of the antigens, instead showed a positivity for HLA-DQ\(_2\) (DQA1\(^\ast\)02 and B1\(^\ast\)02). Our results correspond to The European Genetics Cluster on Celiac Disease, which observed less DQ\(_2\) positive patients in Southern Europe than in Northern (84.3% vs. 90.2%).\(^24\)

Studies showed that 90% of patients with CD present the HLA-DQ\(_2\)-heterodimer, and most of the remainder are HLA-DQ\(_2\)-positive.\(^25\) The discovery that the predisposition for CD is virtually given by just these 2 heterodimers resulted in considering patients HLA-DQ\(_2\)-positive and HLA-DQ\(_2\)-negative without risk of CD. However, there are numerous studies that show a frequency of 3% to 5% of patients diagnosed with CD, which are HLA-DQ\(_2\)-and DQ\(_2\)-negative.\(^26\),\(^27\),\(^28\),\(^29\),\(^30\) Many of these patients were, as in our case, HLA-DQ\(_2\)-positive. Thus, to avoid misdiagnosis, it is important to determine HLA-DQ\(_2\) as well.

In our study, the main signs and symptoms of patients diagnosed with CD were diarrhea, weight-stature deficit, and lack of appetite (the same frequency, 31.8%), followed by a distended abdomen (27%), and abdominal pain (23%). The literature suggests a higher incidence of diarrhea (up to 50%) and abdominal pain (up to 90% of children in Canada\(^31\)) among the symptoms at the time of diagnosis.

The main diagnoses associated with CD were: anemia (44%), low weight (44%), and modified liver enzymes (27%). These consequences of CD are well known; studies showed a presence of anemia in 3% to 12% of patients, children and adults,\(^32\),\(^33\), low weight in 50% to 90% of children,\(^34\) and modified liver enzymes in 5% of patients.\(^35\) Two patients had Down syndrome, ...
and arthritis, respectively, which are described in the literature associated to CD in 0.3% and 1.5% of cases.\[^{[3,31]}\]

Comparing laboratory data from patients with CD at first evaluation and on reevaluation, we obtained statistically significant differences for hemoglobin, MCV, MCH and iron and RBC; in all cases, the average is higher on reevaluation. Secondary diagnosis and therapeutic intervention increase hemoglobin and red blood cell parameters, thus solving anemia. We observed a statistically significant decrease in ALT, a decrease in AST and LDH, increased protein and albumin, and an increase in body weight between diagnosis and reassessment, but without obtaining a statistically significant difference. We achieved a statistically significant difference between the values of TG at the moment of diagnosis and after a period of time after starting the gluten-free diet, which confirms the favorable role of dietary interventions on both the symptoms and laboratory parameters.

Certain limitations of this study should be mentioned. It included a small number of patients diagnosed with CD, which decreased the statistical power of the study. However, very few studies on CD, which include genetic testing have been published in Romania.\[^{[5,21]}\] It is an expensive determination, which is available in few hospitals, despite its great utility for the diagnosis. Some patients included in the study did not receive genetic testing due to parental refusal; however, the diagnosis was made certain using the histopathological examination. Nevertheless, to our best knowledge, this is the first study from Romania that aimed to assess the genetic risk for CD among pediatric patients together with the diagnostic values of serological markers used for diagnosing CD.

5. Conclusions

Our study showed that the genetic risk for CD was present in more than one-third of the cases without a confirmed diagnosis of CD. Therefore, the awareness of genetic susceptibility for CD is essential due to the fact that these individuals can develop the disease at any point of their lives. The sensitivity of TG2/DGP and EMA were very similar, whereas EMA presented a higher specificity as that of TG2/DGP. Laboratory parameters, such as hemoglobin, iron, and liver transaminases, shower to be significantly abnormal in patients diagnosed with CD, but their values improved at 6 months after the initiation of gluten-free diet.

Acknowledgements

This research was partially supported by the Collective Research Grants of the University of Medicine and Pharmacy Tirgu Mureș, Romania (“The role of mother’s genetic determinism in child’s obesity correlated with biompedance and anthropometric parameters” no.2.75/4/11.01.2017).

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