DETERMINATION OF THE RATE OF AUTOANTIBODY CARRIER STATE IN PATIENTS WITH CELIAC DISEASE BY MONO- AND MULTIPLEX IMMUNOASSAY

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The search for concomitant autoimmune disorders (ADs) in patients with celiac disease is a pressing issue. The study aimed to determine the rate of the carrier state for antibodies (Abs) being the markers of AD development in patients with celiac disease using various immunological approaches. Enzyme-linked immunoassay and hydrogel microarray-based multiplex immunoassay (MI) were used to determine Abs against thyroid peroxidase (TPO), thyroglobulin (TG), glutamate decarboxylase (GAD), pancreatic islet cells (ICA), tyrosine phosphatase (IA2), 21-hydroxylase (P450c21), Castle's intrinsic factor, tissue transglutaminase (TGM2) in blood serum of patients with celiac disease (group 1, $n = 27$) and healthy individuals (group 2, $n = 16$). The microarray also enables testing of Abs against interferons (IFN) alpha and omega, interleukin 22. In group 1, Abs against IA2 (30%), TPO (22%), TG (19%), GAD (19%) were detected by the enzyme-linked immunoassay, and in group 2 Abs against IA2 (38%), TPO (19%), GAD (19%) were detected. In group 1, Abs against TPO (11%), TG (11%), P450c21 (4%), IFN-alpha (4%), ICA (4%) were detected using the microarray, and in group 2 Abs against TPO (13%), ICA (13%), TG (6%), IFN-alpha (6%) were identified. No significant differences in the rate of elevated Abs in the groups were revealed ($p > 0.05$). Patients, in whom the Ab carrier state was established using microarrays, with negative results enzyme-linked immunoassay can develop the delayed ADs, which suggests prognostic value of MI. The lack of significant differences in the rate of elevated Abs in patients with celiac disease and healthy individuals can result from small size of the studied groups and can suggest high prevalence of potential AD forms in these cohorts.

Keywords: celiac disease, autoimmune diseases, screening, multiplex immunoassay, hydrogel microarray

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ОПРЕДЕЛЕНИЕ ЧАСТОТЫ НОСИТЕЛЬСТВА АУТОАНТИТЕЛ У ПАЦИЕНТОВ С ЦЕЛИАКИЕЙ МЕТОДАМИ МОНО- И МУЛЬТИПЛЕКСНОГО ИММУНОАНАЛИЗА

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Поиск сопутствующих аутоиммунных заболеваний (АИЗ) у пациентов с целиакией является актуальной задачей. Целью исследования было определить частоту носительства антител (АТ) — маркеров развития АИЗ у пациентов с целиакией с помощью различных иммунологических подходов. У пациентов с целиакией (группа 1, $n = 27$) и здоровых лиц (группа 2, $n = 16$) в сыворотке крови с использованием ИФА и метода мультиплексного иммуноанализа (МИ) на гидрогелевом биочипе определены АТ к тиреоидной пероксидазе (ТПО), тиреоглобулину (ТГ), глутаматдекарбоксилазе (GAD), островковым клеткам поджелудочной железы (ICA), тирозинфосфатазе (IA2), 21-гидроксилазе (P450c21), внутреннему фактору Кастла, тканевой трансглутаминазе (TGM2). Биочип также позволяет проводить исследование АТ к интерферонам (ИФН) альфа и омега, интерлейкину 22. Методом ИФА в группе 1 выявлены АТ к IA2 (30%), ТПО (22%), ТГ (19%), GAD (19%), в группе 2 — к IA2 (38%), ТПО (19%), GAD (19%). В группе 1 с использованием биочипа обнаружены АТ к ТПО (11%), ТГ (11%), P450c21 (4%), ИФН-альфа (4%), ICA (4%), в группе 2 — к ТПО (13%), ICA (13%), ТГ (6%), ИФН-альфа (6%). Значимых различий в частоте повышения АТ в группах не выявлено ($p > 0,05$). У пациентов с носительством АТ, выявленных на биочипах, при отрицательном результате ИФА не исключается развитие АИЗ в отсроченном периоде, что позволяет предположить прогностическую значимость МИ. Отсутствие значимых различий в частоте повышения АТ среди пациентов с целиакией и здоровых лиц может быть обусловлено ограниченной численностью групп наблюдения и свидетельствовать о высокой распространенности потенциальных форм АИЗ в данных когортах.

Ключевые слова: целиакия, аутоиммунные заболевания, скрининг, мультиплексный иммуноанализ, гидрогелевый биочип

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Celiac disease is an autoimmune disorder (AD) resulting from gluten intolerance in genetically predisposed individuals and characterized by damage to the small intestinal mucosa. The celiac disease clinical manifestations include diarrhea, weight loss, as well as delayed growth and development in children. It should be noted that classic gastrointestinal symptoms (abdominal pain, nausea, flatulence) associated with celiac disease are currently less prevalent due to the disease morphogenesis alteration in the last 30 years. Celiac disease often has atypical course and manifests with skin lesions, reproductive disorders, and neurological symptoms [1]. It is well known that celiac disease is often associated with other ADs, such as type 1 diabetes mellitus (T1D), Hashimoto's thyroiditis (HT), autoimmune hepatitis, dermatitis herpetiformis [2–4], as well as Sjogren's syndrome, selective IgA deficiency, juvenile chronic arthritis, autoimmune myocarditis [5]. The data have been published on the celiac disease comorbidity with primary biliary cirrhosis, primary sclerosing cholangitis, Addison's disease, vitiligo, alopecia areata, dermatomyositis, peripheral neuropathy, rheumatoid arthritis, and other ADs [6]. Moreover, some of the above ADs are considered to be clinical "masks" of celiac disease, for example alopecia areata [1]. Thus, patients with ADs represent the group at risk of developing celiac disease. To ensure timely diagnosis, patients with ADs require screening for celiac disease. At the same time, individuals with the established diagnosis of celiac disease should be recommended assessment aimed to rule out possible concomitant ADs. In particular, in the published studies, the analysis of antibodies (Abs) being the markers of T1D, autoimmune thyroiditis, Sjogren's syndrome, anti-nuclear, anti-mitochondrial Abs, Abs against DNA, to the Smith antigen, neutrophils, smooth muscles, microsomes, stomach parietal cells to the Smith antigen, is conducted [7]. The authors have not revealed higher rate of the carrier state for Abs specific for ADs in patients with celiac disease compared to healthy subjects. The researchers believe that such results are due to small number of patients and their adherence to gluten-free diet. However, the paper reports higher rate of concomitant ADs in patients with celiac disease relative to healthy individuals. Thus, the authors draw a conclusion about the need for both clinical and laboratory testing of patients with celiac disease in case of suspected concomitant AD and recommend to go beyond blood testing for antibodies.

Considering the large number of potential ADs the patient can develop, it can be very difficult to conduct regular screening by ELISA due to high cost and duration of testing, as well as the need to collect a large amount of biomaterial. In this regard, it is feasible to consider the possibility of using the multiplex immunoassay allowing one to obtain information about the presence/absence of a large number of Abs specific for various ADs in a small volume blood sample (5 μL) in a short time in order to optimize the assessment algorithm for patients with celiac disease. The hydrogel microarray-based multiplex immunoassay is used for the diagnosis and screening of celiac disease. Currently, the diagnostic kits are produced allowing one to simultaneously detect Abs against gliadin and tissue transglutaminase (TGM2) in a patient [8, 9], along with Abs against the endomysium [10]. The results obtained using the multiplex immunoassay match the data yielded by monoplex methods [10] and are characterized by high sensitivity and specificity [9]. It is assumed that due to preanalytical, analytical and cost advantages, as multiplex immunoassays are implemented, the need for biopsy to confirm the diagnosis will be significantly reduced [8].

Considering high risk of developing several ADs by one patient, the multiplex immunoassay-based diagnostic kits have been designed allowing one to assess Abs specific not only for celiac disease, but also for other ADs. The multiplex electrochemiluminescence analysis method has been proposed for detection of Abs against insulin, glutamate decarboxylase (GAD), tyrosine phosphatase (IA2), tissue transglutaminase, thyroid peroxidase (TPO), thyroglobulin (TG), IFN-alpha [11]. At the same time, there are no published studies focused on assessing the rate of the Abs carrier state, including Abs specific for endocrine ADs, in patients with celiac disease by multiplex immunoassay.

The study aimed to determine the rate of the carrier state for antibodies being the markers of autoimmune disorders in patients with celiac disease by ELISA and multiplex immunoassay.

METHODS

Study site and period

The study was conducted at the Loginov Moscow Clinical Scientific Centre of Moscow Healthcare Department, Endocrinology Research Centre of the Ministry of Health of the Russian Federation, and Engelhardt Institute of Molecular Biology of the Russian Academy of Sciences.

Material was collected in March–June 2023, laboratory testing was performed in March–December 2023; analysis of the results was conducted in January–June 2024.

Studied cohorts

Cohort of patients with celiac disease (group 1)

Inclusion criteria: male or female sex; age 18 years and over; the diagnosis of celiac disease verified based on the clinical, immunological, and instrumental testing data (in accordance with the data of medical records provided). Exclusion criteria: pregnancy, lactation; acute infection; exacerbation of chronic disorder; severe life-threatening conditions (decompensated chronic heart failure, chronic kidney disease (stage 3b and above), pulmonary failure and liver failure; immune system disorder (including congenital and acquired immunodeficiency; hypersensitivity reactions occurring within the period of participation in the study); use of drugs affecting the immune system function (interleukins, interferons, immunoglobulins, immunosuppressants, cytostatics) within a month before inclusion in the study; vaccination/revaccination within a month before inclusion in the study (in accordance with the data of medical records provided).

Cohort of conditionally healthy participants (group 2)

Inclusion criteria: male or female sex; age 18 years and over, no subclinical/overt autoimmune disorder (in accordance with the data of medical records provided). Exclusion criteria: symptoms of celiac disease (diarrhea, anemia, weight loss), pregnancy, lactation; acute infection; exacerbation of chronic disorder; severe life-threatening conditions (decompensated chronic heart failure, chronic kidney disease (stage 3b and above), pulmonary failure and liver failure; immune system disorder (including congenital and acquired immunodeficiency; hypersensitivity reactions occurring within the period of participation in the study); use of drugs affecting the immune system function (interleukins, interferons, immunoglobulins,

immunosuppressants, cytostatics) within a month before inclusion in the study; vaccination/revaccination within a month before inclusion in the study.

A total of 27 patients aged 29–51 years (median age 36 years) were included in group 1, among them 17 (63%) were females. Group 2 included 16 participants aged 30–52 years (median age 41 years), among them 12 (75%) were females. In patients of group 1, the disease duration at the time of inclusion in the study was 7 [3; 21], (1, 36) years. In group 1, concomitant ADs were diagnosed in three patients (11%), including Hashimoto's thyroiditis (HT) in two patients (7%) and hypoparathyroidism in one patient (4%).

Sampling method for several studied cohorts

A continuous sampling method was used.

Study design

Multicenter, interventional, cross-sectional, two-sample comparative study.

Methods

Criteria to establish the diagnosis of celiac disease

The diagnosis of celiac disease was established based on comprehensive assessment of the patient considering clinical features of the disease, elevated serological markers (levels of antibodies against tissue transglutaminase (IgA and IgG) above the reference values), as well as morphological features of the small intestinal mucosa (signs of hyperregenerative type small intestinal mucosal atrophy based on the Marsh–Oberhuber classification) in accordance with the guidelines of the all-Russian consensus on the diagnosis and treatment of celiac disease in adults and children [4].

All the individuals enrolled underwent determination of Abs markers of ADs by ELISA methods, chemiluminescence analysis, and hydrogel microarray-based multiplex immunoassay.

Enzyme-linked immunoassay

All the patients enrolled underwent assessment of the levels of Abs being the markers of autoimmune thyroiditis (against TPO, TG), type 1 diabetes mellitus (against GAD, pancreatic islet cells (ICA), IA2), autoimmune adrenal insufficiency (against 21-hydroxylase (P450c21)), autoimmune gastritis (Castle's intrinsic factor), celiac disease (levels of IgA against tissue TGM2) within the framework of screening for concomitant ADs.

Blood was collected from the cubital vein into vacuum test tubes with inert gel in the morning (08:00–10:00) in the fasting state (fasting for at least 8 h and no more than 14 h before blood collection). The resulting samples were centrifuged within 15 min after blood collection using the Eppendorf 5810R centrifuge (Eppendorf, USA) at a temperature of 4 °C and 3000 rpm for 15 min, and then processed. The levels of Abs against TPO, TG were determined on the day of blood collection. Serum samples for further determination of the levels of Abs against P450c21, GAD, IA2, ICA, Castle's intrinsic factor, and tissue TGM2 were temporarily frozen in micro test tubes at a temperature of –80 °C. Abs against TG were determined using the Cobas 6000 electrochemiluminescence analyzer (Roche, Germany); Abs against TPO were determined by chemiluminescence immunoassay using the Architect i2000 automated analyzer (Abbott, USA). Abs against P450c21, IA-2, GAD, ICA, Castle's intrinsic factor, tissue TGM2 were determined by ELISA using the commercially available kits (BioVendor, Czech Republic (Abs against P450c21); Medipan, Germany (Abs against IA-2); Biomerica, USA (Abs against GAD, ICA); Orgentec Diagnostika, Germany (Abs against Castle's intrinsic factor); Xema, Russia (Abs against tissue TGM2, total IgA)). The reference ranges of blood immunological indicators were as follows: Abs against P450c21 — < 0.4 U/mL, TPO — 0–5.6 IU/mL, TG — 0–115 IU/mL, GAD — < 1 U/mL (1–1.05 — "gray zone", > 1.05 — positive test), IA2 — < 8 U/mL (8–10 — "gray zone", ≥ 10 — positive test), ICA — < 0.95 U/mL (0.95–1.05 — "gray zone", > 1.05 — positive test), Castle's intrinsic factor — < 6 U/mL, IgA Ab against tissue TGM2 — ≤ 20 U/mL, total IgA — 0.9–5.0 g/L. The values within the"gray zone" were considered as elevated levels.

Hydrogel microarray-based multiplex immunoassay

Hydrogel microarrays were produced by the copolymerization immobilization method based on the hydrogel microarray technique developed at the Engelhardt Institute of Molecular Biology RAS. The earlier designed and tested microarray [12] allowing of the one to identify both organ-specific Abs (against P450c21, GAD, IA-2, ICA, TG, and TPO) and Abs against cytokines (against IFN-omega, IFN-alpha, and interleukin 22) was modified for identification of the IgA Abs against tissue TGM2. For that the microarray was supplemented with the hydrogel elements containing tissue TGM2 (R&D Systems, USA). To ensure simultaneous detection of the G and A class antibodies, the mixture of antibodies against human IgG labeled with the Cy5.5 fluorescence dye and antibodies against human IgA labeled with the Cy3 fluorescence dye was used. Conjugates of the F(ab')2-fragments of the goat antibody against human immunoglobulin G (Invitrogen, USA) and F(ab')2- fragments of the goat antibody against human immunoglobulin A (Invitrogen, USA) were produced in accordance by the method developed by the manufacturer of fluorescence dyes Cy5.5 and Cy3, respectively (Lumiprobe RUS, Russia). Fluorescent microarray images recording and fluorescent signal calculation were accomplished using the analyzer and software (Engelhardt Institute of Molecular Biology RAS, Russia). Interpretation of the microarray-based assay of the results and determination of the Abs presence/absence in blood serum was performed as previously reported [12].

Statistical analysis

Statistical processing of the results was performed by standard methods using the STATISTICA 13 (StatSoft, USA, 2017) and MedCalc (MedCalc Software Ltd, Belgium, 2020) software packages. The median and the interquartile range were specified for quantitative traits. Nonparametric tests were used, since the trait distribution was non-normal. To compare quantitative data of two independent samples, the Mann–Whitney *U*-test was used; qualitative traits were compared using the Chi-squared test and Yates's Chi-squared test. When testing statistical hypotheses, the critical significance level was considered to be equal to 0.05. Bonferroni correction was applied to counteract the multiple comparison problem. After applying correction, p-values within the range between the calculated value and 0.05 were interpreted as a statistical trend.

Multiparametric analysis of the signals reported based on the results of the microarray-based blood serum sample testing, which corresponded to autoantibody levels, considering the

Table 1. Levels of antibodies assessed by enzyme-linked immunoassay and the rate of elevated Abs in groups 1 and 2

Antibody level***				Rate of elevated antibodies, *n* (%)			
Antibodies	Group 1 *n* = 27	Group 2 *n* = 16	*p**	Antibodies	Group 1 *n* = 27	Group 2 *n* = 16	*p***
against TPO, IU/mL	0.8 [0.5; 4.1]	0.5 [0.3; 2.0]	0.315	against TPO	6 (22)	3 (19)	0.907
against TG, IU/mL	16.4 [13.6; 83.1]	14.0 [12.2; 18.2]	0.149	against TG	5 (19)	2 (13)	0.929
against GAD, U/mL	0.5 [0.5; 0.8]	0.5 [0.4; 0.8]	0.734	against GAD	5 (19)	3 (19)	0.699
against IA2, U/mL	3.6 [1.0; 8.4]	1.0 [1.0; 9.5]	0.792	against IA2	8 (30)	6 (38)	0.595
ICA, U/mL	0.4 [0.3; 0.5]	0.3 [0.3; 0.5]	0.49	ICA	2 (7)	0	0.715
against P450c21, U/mL	0.1 [0.1; 0.1]	0.1 [0; 0.1]	0.866	against P450c21	0	0	–
against Castle's intrinsic factor, U/mL	3.2 [0; 4.6]	1.7 [1.1; 2.6]	0.253	against Castle's intrinsic factor	4 (15)	–	–
against TGM2, U/mL	1.2 [0.7; 3.5]	0.9 [0.6; 1.5]	0.125	against TGM2	0	0	–
				At least one Ab	20 (74)	10 (63)	0.424

Note: * — Mann–Whitney *U*-test. Threshold p_0 = 0.004 (after applying Bonferroni correction: 14 comparisons). ** — Chi-squared test and Yates's Chi-squared test. Threshold p_0 = 0.004 (after applying Bonferroni correction: 14 comparisons). *** — Median value, [Q_1; Q_3] Note: TPO — thyroid peroxidase; TG — thyroglobulin; GAD — glutamate decarboxylase; ICA — pancreatic islet cell antibodies; IA2 — tyrosine phosphatase; P450c21 — 21-hydroxylase, TGM2 — tissue transglutaminase.

presence or absence of the diagnosis of celiac disease was conducted by the decision tree method for construction of a single tree in accordance with the classification and regression algorithm (CART). The resulting division into classes based on the signal value ranges was assessed using the Fischer's exact test to test the homogeneity hypothesis. Then the Fischer's exact test for pairwise comparison and Bonferroni correction were used to conduct post-hoc analysis. Calculations and construction of figures were accomplish in R using the rpart v. 4.1.24, rpart.plot v. 3.1.2, ggplot2 v. 3.5.1, rstatix v. 0.7.2 software packages.

RESULTS

No significant intergroup age (p = 0.372) and sex (p = 0.633) differences were revealed. The levels of Abs assessed by ELISA and the rate of detecting elevated levels are provided in Table 1. Elevated IgA against tissue TGM2 was detected in none of the study participants. In group 1, no elevated Abs were detected in seven patients (26%), one elevated Ab was reported in 13 patients (48%), two elevated Abs — in five (19%), three elevated Abs — in one (4%), four elevated Abs — in one patient (4%). In group 2, no elevated Abs were detected in six assessed individuals (38%), one elevated Ab was reported in eight individuals (50%), two elevated Abs — in one (6%), four elevated Abs — in one individual (6%). The median total IgA level determined by ELISA in patients with celiac disease was 1.1 g/L [95% CI: 0.9; 1.5], while in the group of healthy individuals it was 1.1 g/L [95% CI: 1.0; 1.4]. In one patient of group 1, the total IgA level was below the detection limit (< 0.06 g/L).

Values of signals of the microarray elements corresponding to the test Abs levels, that were obtained by multiplex immunoassay, and the rate of detecting elevated levels are provided in Table 2. Antibodies against TGM2 (TGM2-IgA) were also found in none of the patients. In group 1, no Abs were detected in 18 patients (66%), one Ab was reported in eight patients (30%), two Abs — in one patient (4%). In group 2, no Abs were detected in 12 assessed individuals (75%), one Ab was found in three (19%), three Abs — in one individual (6%).

In the whole studied cohort (n = 43) there were eight Abs carriers (18.6%), in whom more than one Ab against the target

Table 2. Values of the microarray element signals corresponding to the levels of studied Abs obtained by multiplex immunoassay and the rate of elevated Abs in groups 1 and 2

Values of microarray element signals, relative units***				Rate of elevated Abs, *n* (%)			
Antibodies	Group 1 *n* = 27	Group 2 *n* = 16	*p**	Antibodies	Group 1 *n* = 27	Group 2 *n* = 16	*p***
against TPO	1.1 [0.9; 1.4]	1.4 [1.0; 1.8]	0.247	against TPO	3 (11)	2 (13)	0.383
against TG	0.8 [0.7; 1.0]	0.95 [0.7; 1.2]	0.496	against TG	3 (11)	1 (6)	0.446
against GAD	1.0 [0.9; 1.1]	0.9 [0.8; 1.0]	0.126	against GAD	0	0	–
against IA2	1.0 [0.9; 1.0]	0.9 [0.8; 1.1]	0.724	against IA2	0	0	–
ICA	1.0 [0.9; 1.3]	1.4 [0.8; 2.1]	0.358	ICA	1 (4)	2 (13)	0.37
against P450c21	0.9 [0.8; 1.0]	1.1 [0.8; 1.9]	0.097	against P450c21	1 (4)	0	0.297
against IFN-omega	1.0 [0.7; 1.1]	0.85 [0.5; 1.0]	0.529	against IFN-omega	0	0	–
against IFN-alpha	0.9 [0.8; 1.2]	1.2 [0.9; 1.7]	0.346	against IFN-alpha	1 (4)	1 (6)	0.31
against IL-22	1.0 [1.0; 1.3]	0.9 [0.8; 1.1]	0.449	against IL-22	0	0	–
against TGM-2 (IgA)	1.1 [0.9; 1.1]	0.8 [0.3; 1.2]	0.065	against TGM-2 (IgA)	0	0	–
				At least one Ab	8 (30)	3 (19)	0.668

Note: * — Mann–Whitney *U*-test. Threshold p_0 = 0.003 (after applying Bonferroni correction: 16 comparisons). ** — Chi-squared test and Yates's Chi-squared test. Threshold p_0 = 0.003 (after applying Bonferroni correction: 16 comparisons). *** — Median value, [Q_1; Q_3] Note: TPO — thyroid peroxidase; TG — thyroglobulin; GAD — glutamate decarboxylase; ICA — pancreatic islet cell antibodies; IA2 — tyrosine phosphatase; P450c21 — 21-hydroxylase; IFN — interferon; IL-22 — interleukin 22, TGM2 — tissue transglutaminase

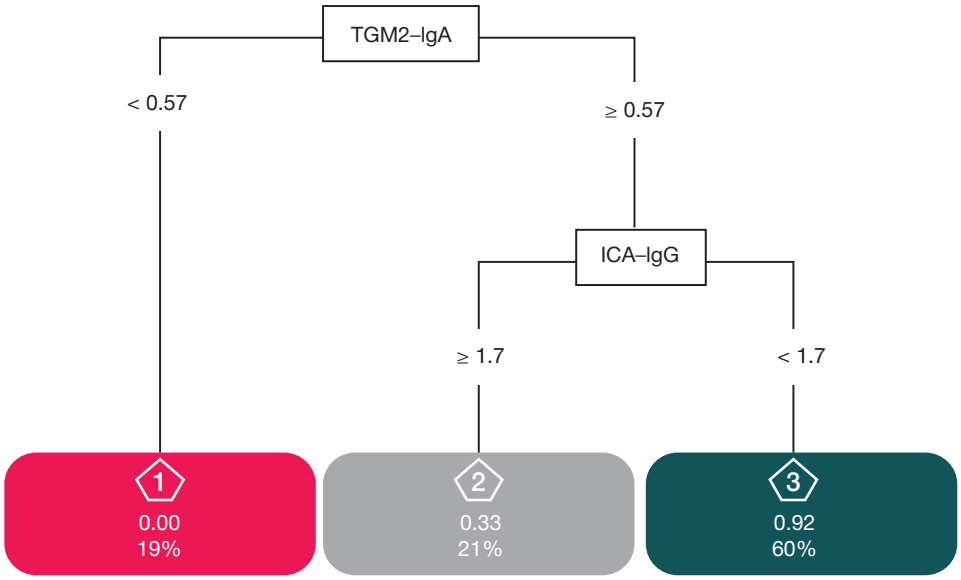

Fig. 1. Classification and regression tree (CART). Each terminal node of the tree (leaf) contains the name of the identified class (here: 1, 2, 3), likelihood of being diagnosed with celiac disease (here: 0.00, 0.33, 0.92), share of patients in the class relative to the entire sample (here: 19%, 21%, 60%)

group of proteins were found and/or the Abs identified were confirmed by two methods: two carriers of Abs against protein marker of diabetes mellitus — a patient with celiac disease and a healthy individual; six carriers of anti-thyroid Abs — five patients with celiac disease and no established diagnosis of autoimmune thyroiditis and one conditionally healthy patient with no established diagnosis of autoimmune thyroiditis. It was determined using multiplex assay that two patients were carriers of the Abs against interferon alpha (a conditionally healthy patient with MEN-1 and a patient with celiac disease). In four patients with celiac disease, the Abs against Castle's intrinsic factor associated with autoimmune gastritis were detected by enzyme-linked immunoassay.

Due to the fact that none of the methods detected elevated levels of the IgA Abs against tissue TGM2 in patients with celiac disease, we conducted multiparametric analysis of the signals of microarray elements based on the results of testing blood serum samples of the studied groups. A classification and regression tree (CART) was constructed based on the input array of signals obtained using microarrays corresponding to various levels of ten studied Abs (Fig. 1).

The resulting tree demonstrates the probability of being diagnosed with celiac disease in a patient with the accuracy of 0.92, if two criteria are met: TGM2-IgA 0.57, ICA–IgG < 1.7. Distribution of patients across the classes of the tree constructed based on the diagnosis of celiac disease (Fig. 2, Table 3) was assessed using the Fisher's exact test. The *p*–value obtained was < 0.0001.

The results of post-hoc analysis involving the use of the Fisher's exact test and Bonferroni correction (Table 4) suggest the differences at the significance level of $p < 0.005$ in classes 2 and 3, classes 1 and 3. The difference between classes 1 and 2 is negligible.

DISCUSSION

The sample of patients with celiac disease can be considered representative based on sex (predominance of females is reported) and age of the disease onset.

This study has revealed elevated Abs against tissue TGM2 in none of the samples. Among possible causes of false-negative

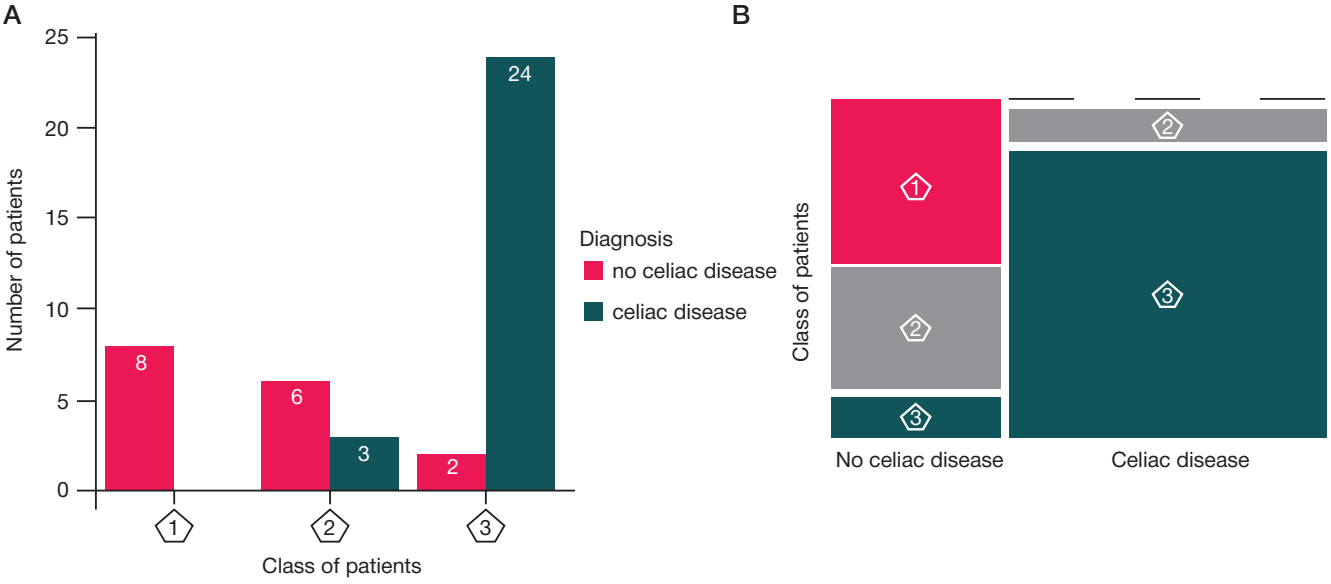

Fig. 2. Distribution of patients across the classes based on the fact of being diagnosed with celiac disease in the form of the column a) and mosaic b) charts. The corresponding classes are designated by numbers in pentagons

Table 3. Distribution of patients across the classes depending on the signals acquired using microarrays and the fact of being diagnosed with celiac disease

Diagnosis of celiac disease	Class 1	Class 2	Class 3
Yes	0	3	24
No	8	6	2

serological testing results in celiac disease, selective IgA deficiency, gluten intake reduction or gluten-free diet, use of corticosteroids or immunosuppressants are distinguished [13].

To rule out IgA deficiency in patients and healthy donors by ELISA, serum total IgA levels were determined. IgA deficiency was found by ELISA in one patient of group 1 (male, 33 years): the total IgA level was below the detection limit. Other patients of group 1 had total IgA levels within normal range, the same was reported for group 2. Celiac disease is an autoimmune disorder associated with IgA deficiency. However, the data on the prevalence of IgA deficiency among patients with celiac disease in different populations vary within wide limits: between 0.55 and 16.67% [16]. The results of the patient sample assessed are consistent with the earlier published data (IgA deficiency was found in one patient out of 27; 3.7%). Thus, since 26 patients out of 27 have no immunoglobulin A deficiency, negative results of testing for IgA against tissue TGM2 result from the long celiac disease duration and adherence to gluten-free diet in the majority of patients.

In patients with celiac disease, HT at the time of enrollment was found only in 7% of cases (n = 2). In one of these patients, ELISA revealed both elevated Abs against TG and elevated Abs against TPO, while multiplex immunoassay revealed none of these Abs. In the second patient, only elevated Abs against TG was detected by both methods. According to the results reported by other authors, the prevalence of HT among patients with celiac disease is 5.7%–18.9% [15, 16]. Considering the fact that the patients are young and the rate of elevated Abs against TPO and TG is high (the rate of elevated Abs against TPO and TG determined by ELISA is 22% and 19%, respectively), it is highly likely that the rate of HT in this group will increase with time.

Hypoparathyroidism was diagnosed in one patient. Sporadic cases of the combination of celiac disease and hypoparathyroidism are reported in the literature. It is assumed that hypoparathyroidism is not a primary disorder, but results from cross reactivity between Abs against endomysium and parathyroid antigens [17]. It is important to note that this patient was also diagnosed with diabetes mellitus against the background of decreased C-peptide levels and normal levels of Abs against insulin, GAD, IA2, zinc transporter 8 (ZnT8), ICA (testing of the expanded panel of T1D Abs markers was conducted before inclusion in the study). Thus, the autoimmune genesis of the disease has not been confirmed. Previous genetic testing revealed a pathogenic mutation in the gene *GCK*, thereby confirming maturity-onset diabetes of the young (MODY). No increase in Abs against Castle's intrinsic factor, 21-hydroxylase, IFN-alpha, and IFN-omega was found in this patient. As for Abs against thyroid tissue, ambiguous results were obtained: the levels of Abs against TG measured by two methods were within normal range, the same as the levels of Abs against TPO determined by ELISA, while multiplex immunoassay determined that the levels of Abs against TPO were within the “gray zone”. The patient’s history is notable for the development of subacute thyroiditis requiring prescription of prednisolone. However, treatment had been terminated before blood collection (euthyroidism was confirmed).

It should be noted that the rate of concomitant ADs determined in our study (11%) is lower compared to the previously reported data [13]: 33.7% of patients had at least one AD, among them 8.1% had multiple autoimmune disorders; the most prevalent was HT (18.9%), T1D (13.5%) and other disorders were less frequent. This is probably due to small number of study participants.

We have found no significant differences in the rate of elevated Ab markers of endocrine and non-endocrine ADs using both ELISA and multiplex assay when comparing the groups. Similar results were reported in the literature, when the rate of carrier state was assessed for Abs against TPO, TG, GAD, ICA in patients with celiac disease and healthy subjects [7]. At the same time, there are reports of the higher rate of Abs against GAD among patients with celiac disease [18]. It should be noted that the rate of elevated Abs against TPO in patients with celiac disease varies considerably; according to the data provided by different authors, it is 3.9–30.5% [7, 13, 19, 20–22], which is likely to result from the differences in the subjects’ age and the diagnostic kit sensitivity. According to the literature data, the rate of elevated Abs against TG in celiac disease is 11.2–11.7% [20, 23], against GAD — 0–13.5% [7, 13, 18, 19, 24], against IA2 — 1–1.25% [18, 19], against stomach parietal cells — 10.9% [19], against P450c21 — 2.2% [19].

In the assessed cohort of patients with celiac disease, the rate of elevated Abs was significantly higher compared to the data obtained in other studies: 13 assessed patients out of 74 (17.6%) were carriers of one Ab, 9 (12.2%) were carriers of two or more Abs [15]. However, it should be noted that the authors analyzed Abs against GAD, TPO and the anti-nuclear Abs only. In the study conducted by other authors, the carrier state for at least one Ab was reported in 31.5% of 92 surveyed patients (the study was focused on determining Abs against insulin, GAD, IA2, ZnT8, TPO, stomach parietal cells, P450c21) [19]. In contrast to our study, in this paper the carrier state for at least one Abs was significantly more frequent in individuals with celiac disease, than in the cohort of healthy individuals (a total of 237 individuals were assessed). The authors note that the rate of the Ab carrier state increases with age: thus, in the group aged 34–50 years elevated Abs levels are found in 34.8% of cases, while in patients aged 18–34 years these are found

Table 4. Results of pairwise comparison of classes

Classes	p	p-adjusted*
1 and 2	0.206	0.618
1 and 3	0.00000248	0.00000744
2 and 3	0.0012	0.0036

Note: * — Bonferroni adjusted p-value.

in 28.3% of cases. In our cohort, the multiplex immunoassay revealed Abs against P450c21 (2.8 relative units) in one patient of group 1, which can be also associated with the patient's age (76 years).

We used multiplex immunoassay to assess Abs against type 1 interferons (IFN-alpha and IFN-omega) and interleukin 22, along with organ-specific Abs. Abs against interleukin 22 associated with mucocutaneous candidiasis and Abs against IFN-omega were found in none of the patients of groups 1 and 2. High titers of Abs against type 1 interferons showing high specificity for type 1 autoimmune polyglandular syndrome can be also detected in patients with some other disorders, including systemic lupus erythematosus, myasthenia gravis, thymic tumor [25]. A rather low titer of Abs against IFN-alpha (2.5 relative units) was found in one patient with celiac disease, in whom other antibodies were within reference ranges and for whom no data on concomitant ADs were obtained. Abs against IFN-alpha (5.6 relative units) were also found in one patient of group 2 having type 1 multiple endocrine neoplasia (MEN-1), including hyperparathyroidism and insulinoma. It is noteworthy that our previously published paper [12] also reported the patient, who was a carrier of Abs against IFN-alpha having the MEN-1 syndrome, the components of which also included insulinoma and hyperparathyroidism. These data need the accumulations followed by a thorough analysis. Also noteworthy is one feature typical for the IFN-alpha Ab carrier state: as shown by the previously reported study, this is associated with the risk of severe COVID-19 course [26].

The lack of significant differences in the rate of elevated Abs in groups 1 and 2 can result from small size of the studied groups and suggest high prevalence of potential AD forms in the surveyed cohorts; is also confirms conditional nature of the term "healthy".

In group 1, markers of T1D and HT were most often detected by both ELISA and multiplex immunoassay, which is consistent with the literature data on the high risk of these disorders in individuals with celiac disease [6]. It is important to note that the Abs carrier state was more often determined using ELISA that is currently considered to be a "gold standard" in clinical practice, than using multiplex immunoassay in both group 1 and group 2. However, in three cases multiplex immunoassay allowed us to reveal the Ab carrier state (Ab against P450c21 in one patient of group 1 and ICA Abs in two subjects of group 2), while ELISA yielded a negative result. No signs of hypocorticism and T1D were found when assessing hormonal profiles of patients with elevated Abs, but we continued follow-up of these subjects (since delayed development of ADs was quite possible) in order to correctly estimate prognostic value of the results obtained.

Thus, since the results of determining this or that indicator by different immunological methods can differ, detection of one Ab in a patient having no clinical signs of the disease during screening requires clarification testing.

Due to the complexity of multidimensional data analysis and the non-obviousness of the conclusions drawn, it is necessary to use mathematical methods to interpret such data arrays. The decision tree methods, specifically the classification and regression tree (CART), are used for analysis of medical data due to the capability of discovering complex interactions between variables and providing visual representation of the results interpreted [27]. In the study conducted we analyzed ten-dimensional data arrays in the form of microarray signal values for 43 patients. It has been shown that when conducting the microarray-based multiplex assay, it is possible to classify patients into one of three classes, in which the third is significantly ($p < 0.005$) different from the first and second in the rate of detecting subjects with celiac disease. Furthermore, class 1 included only healthy subjects, class 2 included both healthy individuals and patients with celiac disease, whose test results were within the conditional "gray zone", and class 3 was constituted by 89% of patients with celiac disease and two (12.5%) subjects of group 2. It should be noted that one conditionally healthy patient classified into class 3 was diagnosed with idiopathic hypoparathyroidism and Fahr's syndrome. The second conditionally healthy individual is a carrier of Abs against gliadin. However, elevation of these Abs is nonspecific and does not constitute grounds for the diagnosis of celiac disease.

Introduction of new approaches to ruling out celiac disease in individuals adherent to gluten-free diet is currently a pressing issue, to resolve which special methods are being developed [28]. The decrease in the levels of various autoantibodies in patients with celiac disease adherent to gluten-free diet has been reported earlier [29, 30]. In particular, in patients with celiac disease, the gluten-free diet is associated with reduction or extinction of the islet-specific autoantibodies, including ICA-IgG [30]. The decrease in the values of ICA-IgG and P450c21-IgG (data not shown) in patients with celiac disease relative to the group of healthy individuals was reported for our sample. Inclusion of the ICA-IgG values together with the TGM2-IgA values in the CART algorithm ensured the best division of patients into classes based on the fact of being diagnosed with celiac disease with the accuracy of 0.92.

CONCLUSIONS

The lack of marker Abs against tissue TGM2 in patients with celiac disease can result from the disease duration and adherence to gluten-free diet. The rate of elevated Ab markers of endocrine ADs in patients with celiac disease is not significantly different from that in healthy individuals. The lack of significant differences in the rate of elevated Abs in the groups of patients with celiac disease and healthy individuals can be due to small size of the studied groups and can suggest high prevalence of potential ADs forms in these cohorts; it also confirms the conditional nature of the term "healthy". Patients, who are Abs carriers based on the multiplex immunoassay data, and have negative ELISA results can develop delayed ADs, which suggests prognostic value of the multiplex immunoassay method. Multiparametric analysis of autoimmune disease markers determined using microarrays has demonstrated the possibility of diagnosing celiac disease in patients on a gluten-free diet when the levels of antibodies against tissue TGM2 are within normal range. Thus, it has been shown that the multiplex immunoassay method can be used in the phase of the celiac disease diagnosis verification and for screening of organ-specific Abs in blood of patients with ADs.

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