

A Novel Algorithm for the Diagnosis of Celiac Disease and a Comprehensive Review of Celiac Disease Diagnostics

Orit Rozenberg · Aaron Lerner · Avi Pacht ·
Maya Grinberg · Dina Reginashvili · Clara Henig ·
Mira Barak

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Abstract There is an urgent clinical need for a better laboratory celiac disease diagnosis with both less false positive results and minimal underdetection. The aim of the present study was to evaluate the performance and diagnostic accuracy of different assays in an outpatient population setting for the diagnosis for celiac disease (CD) in order to design an optimal algorithm. We used 15 different ELISA assays to assess 47 blood samples of newly diagnosed children (positive biopsy results) and 52 samples from age- and sex-matched children with negative biopsy results for CD. Scoring criteria were established for grading the assays performance and characteristics. The combined gliadin and tTG assays exhibited the best sensitivity (100%). The addition of other assays to the CeliCheck neo-epitopes assay improved specificity so that the final algorithm had 100% sensitivity, 96.2% specificity, and 98.1% accuracy. The clinical demand for both maximal sensitivity and maximal specificity cannot be achieved with a single test. Using a combination of a sensitive assay

together with specific assays improved celiac disease detection rates, with an acceptable number of false positive results. This model, however, needs to be confirmed prospectively in both children and adults.

Keywords Celiac disease diagnosis · Tissue transglutaminase · Deamidated gliadin · Intestinal histopathology · Antibodies

Introduction

Celiac disease (CD) is an autoimmune-mediated enteropathy caused by permanent gluten intolerance in susceptible individuals [1, 2]. According to the known etiology of CD, T cell clones that are activated by gliadin peptides (the alcohol-soluble prolamins in wheat) presented in the context of HLA-DQ2 or HLA-DQ8 haplotypes (found in almost 100% of celiac patients) [3]. CD is almost unique in the context of autoimmune diseases since both the signal (the gliadin peptide) and the responsible autoantigen tissue transglutaminase (tTG) enzyme could be identified [4]. At high concentrations of calcium, as in the extracellular matrix, tTG catalyses the transfer of a glutamine residue of one protein to a lysine residue of another protein. The glutamine-rich gliadins are excellent glutamine donors for tTG-catalysed cross-linking reactions and can be cross-linked to tTG itself and cause parallel glutamine deamidation. Therefore, antigenic neopeptides created by the cross-link formation could trigger the autoantibody production against tTG. Such deamidation renders the gliadin peptides to fit much better into the binding groove of the celiac disease-predisposing HLA-DQ2 or HLA-DQ8 than their non-deamidated counterparts, causing a better antigen presentation and a more vigorous T cell response [5].

Orit Rozenberg, Aaron Lerner and Mira Barak contributed equally to the study.

O. Rozenberg · M. Grinberg · D. Reginashvili · C. Henig ·
M. Barak (✉)
Central Laboratory of Haifa and Western Galilee,
Clalit Health Services,
47 Ha'taasia Street,
Nesher, Israel 20300, POB 428
e-mail: miraba@clalit.org.il

A. Lerner · A. Pacht
Pediatric Gastroenterology and Nutrition Unit,
Carmel Medical Center,
Haifa, Israel

A. Lerner
Technion-Israel Institute of Technology,
Haifa, Israel

The prevalence of CD in the western world has been increasing steadily, and it is now recognized as a common disorder [6]. The overall CD prevalence in Europe was recently shown to be 1.0% (95% CI 0.9–1.1), with large unexplained differences in adult age across different European countries, 2.4% in Finland (2.0–2.8), 0.3% in Germany (0.1–0.4), and 0.7% in Italy (0.4–1.0). Only 68% of the antibody-positive individuals showed small-bowel mucosal changes typical for CD (Marsh II/III lesion) [7]. A possible explanation for differences in prevalence is the serological test used for screening [8]. It has been also suggested that using one serologic marker is insufficient for establishing the true prevalence of CD [9]. A systematic review of published papers revealed that the overall prevalence of celiac disease in the general population appears to be around 1/160 (6.2‰), but this figure varies widely according to the diagnostic criteria used in the original papers. However, even though the rate of diagnosis has increased in recent years, the majority of affected individuals are still undiagnosed, according to the accepted iceberg concept [10]. CD incidence has increased due to recognition of atypical CD forms on one hand [11] and identification of silent cases [12] on the other hand, based on the availability of serological tests with ongoing improved performance. The classical manifestation of CD in children: diarrhea, weight loss, constipation, abdominal pain, anemia, vomiting, and failure to thrive may include also other manifestations such as extraintestinal symptoms: irritability, anorexia, short stature [12], and autoimmune [13] and endocrine disorders [14]. In adults, the symptoms may be osteoporosis, hypothyroidism, low body mass index, low levels of cholesterol, ferritin or folic acid [15, 16], IBS [17], infertility [18], hemolytic anemia [19], neurological disorders [20], and gastroesophageal reflux disease [21].

According to the latest consensus report on CD, small bowel biopsies are considered the gold standard and are used for diagnosis confirmation [22]. However, the use of the histopathology for CD diagnosis has some drawbacks [23]. The presence of such a lesion is not entirely specific for celiac disease, as it also occurs in conjunction with other enteropathies such as giardiasis and gastroenteritis [24]. The diagnosis of celiac disease is rendered even more complicated in cases where villous shortening is patchy, being evident only in restricted areas of the small-bowel mucosa [25, 26]. Furthermore, the gluten-induced small-bowel mucosal lesion develops gradually from mucosal inflammation to crypt hyperplasia and finally to villous atrophy [27]. The interpretation of borderline findings is often difficult and celiac disease might thus be easily overlooked. Histologic assessment should invariably be made from well-oriented high-quality biopsy specimens, but sampling, processing of biopsies, and

interpretation are often compromised for technical reasons (i.e., incorrect orientation and insufficient size of samples) [28]. Further diagnostic difficulties may arise if patients have reduced their gluten intake on their own account, this possibly leading to partial recovery of the mucosal lesion [29].

In 1969, the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) proposed diagnostic criteria, based on three serial small intestinal biopsies dependent on gluten elimination and provocation [30]. Due to great progress in the field of serological diagnostic tests for CD, new ESPGAN recommendations were established in 1989 [31]. The diagnosis of celiac disease was based on the appearance of flat small intestinal mucosa with the histological features of hyperplastic villous atrophy (with no need for further gluten challenging) and on unequivocal and full clinical remission after withdrawal of gluten from the diet. The finding of circulating antibodies (IgA gliadin, antireticulin, and antiendomysium) at time of diagnosis and their disappearance when the patient was on a gluten-free diet add weight to the diagnosis. The first immunofluorescent test for detection of antibodies against gliadin (AGA) was suggested as a screening test for celiac disease in 1976 [32]. In this study, antibodies against gliadin were detected in serum samples of 19 from 23 children (83%) with celiac disease, while in a control group of 71 children with clinical diagnosis of another malabsorptive disorder, gliadin-IF-antibodies were found in only three patients (4%). In 1983, it has been first suggested that an ELISA test for AGA seem to be a good marker of the immune reaction in the jejunum triggered by gluten, when tested in 31 sera samples of young patients (less than 2 years) versus 36 controls [33]. However, the presence of these antibodies in other gastrointestinal and liver disorders and even in normal healthy controls casted a shadow on the diagnostic significance of gliadin antibodies [34]. Forty-eight of 97 (49.5%) nonceliac patients were positive for AGA, and diagnosis-specific incidences ranged as high as 75% in patients with small bowel disease. In addition, the levels of AGA were dependent upon age as their presence increased from 12% in children with a mean age of 10, to 35–40% in controls within the 60–70 age group. The lack of celiac disease specificity of AGA [35, 36] was not due to either the source of gliadin nor to the sensitivities of the test methods. Both the ELISA and the immunofluorescence methods gave comparable results [34]. In 1984, it has been described that IgA anti-endomysial antibodies (IgA-EmA) were detected on monkey esophagus by immunofluorescence. EmA which are directed against the intermyofibril substance of the smooth muscle occurred in about 70% of 28 patients with celiac disease [37]. The IgA-EmA could not be detected in 122 control sera from patients with other gut or skin diseases.

The shortages of the immunofluorescent assay were overcome in 1998 when an ELISA assay to determine IgA anti-tTG titers in serum samples was established [38]. The performance of this test was 98.1% sensitivity and 94.7% specificity, when tested in 106 celiac patients with partial or subtotal villous atrophy, 43 celiac patients on a gluten-free diet, and 114 diseased and healthy controls [38]. However, the prevalence of the tissue transglutaminase antibodies was markedly higher than that of the positive histological diagnosis, which may lead to an over-estimated prevalence of CD by using these antibodies [39]. The first ELISA assay detecting deamidated gliadin peptides (DGP) antibodies, using two synthetic peptides, was reported in 2001 [40]. An enhanced response against deamidated peptides was observed in 4 (13%, IgA) and 22 (71%, IgG) of 31 untreated CD patients for peptide-1 and in 25 (81%, IgA) and 29 (94%, IgG) patients for peptide-2. Since glutamine-rich gliadins can be cross-linked to tTG itself [3], the gliadin can be modified by deamidation or transamidation [41]. In vitro studies have demonstrated that transamidation is the major reaction (75% vs. 25% for deamidation) [41]. In order to create a serological test with high correlation to the physiological conditions of the disease, the purified cross-linked complex which is formed under physiological in vivo conditions was designed to detect antibodies to tTG, modified gliadin peptides, as well as the newly formed cross-linked complex (neo-epitope) [42]. The neo-epitope-based ELISA assay demonstrated a sensitivity of 92.31% and a specificity of 82.89% when testing 254 sera samples of patients suspected for CD, 26 of them with positive biopsy results [43]. The zonulin protein is involved in the modulation of intestinal permeability by interaction with intercellular tight junctions. Its expression is upregulated in several autoimmune diseases, including celiac disease. Presence of IgA anti-zonulin antibody was found to correlate with the acute phase of celiac disease as reported back in 2000 [44]. Indeed, serum zonulin levels correlated with intestinal permeability in adult patients previously diagnosed with celiac disease and on a gluten-free diet for more than 1 year [45].

Some of the available serological tests for CD diagnosis were proved to be effective for monitoring the disease. The levels of tTG antibodies were found to correlate with Marsh type in children as well as adults and was an independent predictor for Marsh type 3 lesions [46]. Up to 95% of children and 53% of adults would be correctly diagnosed without biopsy. However, despite gluten-free diet (GFD) and decreased tTG antibody levels, 25% of the adults did not recover from villous atrophy during the second year after diagnosis. Thus, strongly positive tTG antibody titers might be sufficient for CD monitoring in children, but duodenal biopsy cannot be avoided in adults because disease presentation and monitoring are different [46].

Another study showed that celiac disease-related antibodies tested in sera of 82 adult celiac disease patients at baseline and every 3 months thereafter for 1 year, may be useful for monitoring dietary compliance [47]. Strictly adherents had significantly lower concentrations of antibodies and smaller proportion of positive samples for IgA endomysial, IgA antiactin antibodies (suggested to correlate with intestinal mucosal damage [48]), and IgA antigliadin (15.6%, 17.4%, and 23.9%, respectively) than partially compliant. At 1 year, IgA endomysial, IgA antiactin antibodies, and anti-tissue transglutaminase predicted the degree of compliance. Antibodies to deamidated gliadin have been also proved suitable for monitoring the disease [49, 62]. There are no studies on the monitoring capabilities of the neo-epitope antibodies.

The nowadays diagnosis of CD, or more precise, of gluten-sensitive enteropathy (GSE) is based on: history and clinical presentation; serological screening of antibodies for tissue transglutaminase, endomysium, and DGP; histological findings in proximal small-intestinal biopsy; and clinical and serological response to a gluten-free diet [22, 50].

The algorithm previously used in our laboratory included screening with tTG IgA and confirming with EMA. The negative samples were further tested for total IgA, due to the high prevalence of IgA deficiency among celiac patients [51]. Samples with low total IgA were tested for Deamidated Gliadin Peptide (DGP IgA+IgG). According to the local physicians' practice, this algorithm was criticized for being neither sensitive nor specific enough.

In the following study, we analyzed 15 available different serological assays in order to find the optimal algorithm for CD diagnosis, based on biopsy-proved samples obtained without gluten intake restrictions.

Material and Methods

Study Population

Patients who had undergone intestinal biopsy and serological tests for CD during the years of 2008–2009 were retrospectively identified by the gastroenterologist physician.

Our cohort study included 99 children as detailed in Table 1. All patients were on gluten-containing diet. Blood samples were drawn on the day of biopsy. Diagnosis of celiac disease was proved by intestinal histology. The performance of all 15 assays for celiac disease diagnosis was determined in 72 sera, but the results of the five best performing assays was available, for a total of 99 serum samples. There was no significant difference regarding age, gender, biopsy Marsh level, and total IgA levels between the tested group and subgroup (data not shown).

Table 1 Study population characteristics

	No.	Age				Gender		Marsh		Total IgA (mg/dL)			
		AVG	SD	Range	Median	Males	Females	I/II	III/IV	AVG	SD	Range	Median
All	99	8	5	0–19	6	51	48			151	91	38–608	140
Controls	52	8	6	0–18	8	26	26			161	127	38–608 ^a	140
Celiac patients	47	7	5	1–19	6 ^b	25	22	11	36	144	52	50–268 ^c	142 ^d

AVG average, SD standard deviation

^a 3 IgA-deficient controls

^b No significant difference between controls and patients, $p < 0.7$

^c 1 IgA-deficient patient

^d No significant difference between controls and patients, $p < 0.4$

EMA Indirect Immunofluorescence Assay

Thirty-one samples were available for endomysial antibodies (EMA) testing.

IgA-EMA was determined by indirect immunofluorescence microscopy using fixed cryostat sections of monkey esophagus (The Binding Site, Birmingham, UK) as the antigen substrate, as previously described [52]. All sera were diluted at 1:2.5, and positive samples were further diluted to the highest dilution yielding fluorescence.

ELISA Assays

Celiac diagnosis assays were routinely tested on the *ETI-MAX 3000* analyzer (DiaSorin Saluggia, Italy). NEQAS is routinely used as the external quality control program. Assay characteristics of all 15 kits are detailed in Table 2.

The AESKU assay was performed on the TRITURUS analyzer (GRIFOLS SA, Barcelona Spain), the DiaSorin assay on the Liaison (DiaSorin Saluggia, Italy), and the DPC assay on the Immulite (Siemens Healthcare Diagnostics Inc. Deerfield, IL, USA). All others were performed on the *ETI-MAX 3000* analyzer.

Endoscopy and Intestinal Histology

All patients underwent esophago-gastro-duodenoscopy using GIF-xp 20 endoscope (pentax, Tokyo, Japan). At least five biopsies were obtained: four from the second part of the duodenum for the diagnosis or exclusion of CD and one from the antrum.

The biopsies were immediately fixed in buffered formalin and embedded on edge in paraffin. Sections were stained with hematoxylin–eosin and Giemsa, analyzed by the pathologist, and graded according to Marsh criteria, as previously described [52]. The local ethical committee approved the study.

Statistical Analysis

Pearson correlation coefficients, ROC analysis (including ROC cut-off), Area under the curve (AUC), as well as difference between areas, p significance levels, 95% confidence intervals, and sensitivities and specificities were calculated with the MedCalc statistical software (Maria-kerke, Belgium). A p value less or equal to 0.05 was considered significant. Our goal was to find an assay or combination of assays with 100% sensitivity and maximal specificity for celiac disease diagnosis. In order to equally evaluate the various examined assays, we established an arbitrary scoring taking into account various criteria and gave an appropriate score.

Results

Analytical CV% (intra-run imprecision) was assessed by testing 20 aliquots of each of the kit controls (low-level and high-level controls) in one run. The analytical CV% did not exceed 10% in the various assays except for the low- and high-level kit controls of the IMMCO tTG IgA assay (CV=16%, 26% respectively), the low-level kit control of the AESKU kit (CV=25%), and the low-level kit control of the EUROIMMUN GAF-3X kit (CV=11%). The IMMCO under-development assays as well as the tTG IgG assays (ORGENTEC and IMMCO) were not included in the analytical CV analysis.

Inter-run CVs imprecision could not be assessed due to limited number of runs.

Diagnostic sensitivity and specificity of the different assays were calculated with the recommended manufacturer cutoffs according to biopsy results (Table 3). Borderline results were regarded as positive results. Sensitivities ranged from 67% for the IMMCO new tTG IgG assay to 100% for the AESKU neo-epitope screen

Table 2 Assay characteristics of the evaluated kits

Kit name, antigen	Short name	Manufacturer	Antibodies	Manufacturer cut-off (borderline range)
Aeskulisa CeliCheck, neo-epitopes (human recombinant tTG cross-linked with gliadin specific peptides)	AESKU	Aesku.Diagnostics, Wendelshiem, Germany	IgA, IgG	16–24 U/mL
Celikey, human recombinant tTG	PHADIA	Phadia GmbH, Freiburg	IgA	5–8 U/mL
Quanta Lite celiac DGP screen, deamidated gliadin peptide (DGP)	INOVA DGP screen	Inova Diagnostics, Inc, CA, USA	IgA, IgG	20–30 U
Quanta Lite h-tTG/DGP screen, deamidated gliadin peptide and human tTG	INOVA tTG-DGP screen	Inova Diagnostics, Inc, CA, USA	IgA, IgG	20 U
Anti-gliadin (GAF-3X) ELISA	EUROIMMUN GAF-3X	Euroimmun AG, Lubeck, Germany	IgG	25 RU/mL
Liaison tTG IgA	DiaSorin (Liaison)	DiaSorin SpA, Saluggia, Italy (Liaison)	IgA	7.2–8.8 AU/mL
Anti-human tTG antibody IgA ELISA, human tTG	IMMCO tTG IgA	IMMCO Diagnostics Inc, NY, USA	IgA	20–25 EU/mL
Celiac tTG, recombinant human tTG ^a	IMMCO new tTG IgA	IMMCO Diagnostics Inc, NY, USA	IgA	20–25 EU/mL
Celiac tTG, recombinant human tTG ^a	IMMCO new tTG IgG	IMMCO Diagnostics Inc, NY, USA	IgG	20–25 EU/mL
Celiac G+, deamidated gliadin peptide ^a	IMMCO new G+IgA	IMMCO Diagnostics Inc, NY, USA	IgA	20–25 EU/mL
Celiac G+, deamidated gliadin peptide ^a	IMMCO new G+IgG	IMMCO Diagnostics Inc, NY, USA	IgG	20–25 EU/mL
Anti-tTG IgA, human recombinant tTG	ORGENTEC tTG IgA	ORGENTEC Diagnostika GmbH, Mainz, Germany	IgA	10 PGL U/mL
Anti-tTG IgG, Human recombinant tTG	ORGENTEC tTG IgG	ORGENTEC Diagnostika GmbH, Mainz, Germany	IgG	10 PGL U/mL
Anti-tTG screen, human recombinant tTG	ORGENTEC tTG screen	ORGENTEC Diagnostika GmbH, Mainz, Germany	IgA, IgG	15 PGL U/mL
Anti- tTG IgA	DPC (IMMULITE)	Siemens Healthcare Diagnostics Inc. Deerfield, IL, USA (Immulate)	IgA	4 U/mL

tTG tissue transglutaminase, U units

^a Assay under development

assay and for the INOVA tTG-DGP screen assay. Specificities ranged from 80% for the IMMCO new G+IgA and 100% for the Phadia tTG IgA assay. Thirty-one sera were also tested for EMA antibodies. Comparing to histology results, this test showed four false positive results, giving a specificity of only 87%.

AUC in all assays was above 0.9 (Table 3), with no statistically significant differences among assays (data not shown). ROC cutoffs were compared to the recommended manufacturer cutoffs (Table 3). If the manufacturer cutoffs included a borderline range, the lowest borderline level was compared to the calculated ROC cutoff. The assays of AESKU, INOVA tTG-DGP screen, IMMCO tTG IgA, IMMCO new tTG IgA, IMMCO new G+IgG, DCP (Immulate), and ORGENTEC tTG screen all exhibited a change in the cutoff less than 25% between ROC cutoffs and manufacturer cutoffs.

Sensitivity and specificity were also calculated according to the ROC cutoff (Table 3). Sensitivity at 95% specificity above 95% was found in the following assays: AESKU,

PHADIA, INOVA tTG-DGP screen, DiaSorin (Liaison), ORGENTEC tTG IgA, ORGENTEC tTG screen. Sensitivity of 90–95% at 95% specificity was found in the following assays: INOVA DGP screen, DPC (Immulate), and IMMCO new tTG IgA. The other assays showed sensitivity lower than 90% at 95% specificity.

Using Pearson correlation, we assessed linear correlation among all the 15 different assays. The correlation coefficients (*r*) were depicted separately according to the type of the tested antibodies: tTG IgA assays, Gliadin-derivatives assays, tTG IgG assays, and tTG-DGP screen assays. Among the tTG IgA assays (Table 4), the lowest correlation coefficients were observed when comparing with DPC (Immulate). The correlations ranged between 0.63 and 0.91. Very good correlations were observed (range 0.83–0.97) between all the other tTG IgA assays,

Among the Gliadin-derivatives assays (Table 5), the lowest correlation coefficients were observed when comparing with IMMCO G+IgA. Very good correlations were observed (range 0.89–0.93) for all the other comparisons

Table 3 Calculated sensitivities, specificities and ROC curves

Manufacturer	Manufacturer cut-off or borderline range	Sensitivity	Specificity	AUC	ROC cut-Off	ROC sensitivity	Sensitivity (95% specificity)	ROC specificity	Percent difference between cut-offs (%)
AESKU	16–24 U/ml	1.00	0.95	1.00	<i>14.2–23.3</i>	1.00	1.00	0.95	0
PHADIA	5–8 U/ml	0.85	1.00	0.99	2.4	0.97	0.97	0.95	51
INOVA DGP screen	20–30 U	0.95	0.93	0.99	11.9	1.00	0.95	0.93	41
INOVA tTG-DGP screen	20 U	1.00	0.90	1.00	22.9	1.00	1.00	0.95	15
EUROIMMUN GAF-3X	25 RU/mL	0.87	0.95	0.98	4.7	1.00	0.90	0.88	81
DiaSorin (Liaison)	7.2–8.8 AU/mL	0.95	0.98	1.00	4.8	1.00	1.00	0.95	34
IMMCO tTG IgA	20–25 EU/mL	0.87	0.93	0.97	16.3	0.95	0.82	0.90	19
ORGENTEC tTG IgA	10 PGL U/mL	0.97	0.95	0.99	7.4	1.00	0.97	0.93	26
ORGENTEC tTG screen	15 PGL U/mL	0.97	0.95	0.97	<i>12.0–15.1</i>	0.97	0.97	0.95	0
DPC (IMMULITE)	4 U/mL	0.97	0.92	0.98	<i>2.7–5.4</i>	0.97	0.94	0.95	0
IMMCO new tTG IgA	20–25 EU/mL	0.92	0.90	0.99	<i>24.9–27.8</i>	0.92	0.92	0.98	0
IMMCO new tTG IgG	20–25 EU/mL	0.67	0.93	0.93	6.6	0.95	0.62	0.83	67
IMMCO new G+IgA	20–25 EU/mL	0.82	0.80	0.91	1.3	1.00	0.31	0.73	94
IMMCO new G+IgG	20–25 EU/mL	0.95	0.90	0.97	<i>19.4–20.2</i>	0.95	0.85	0.90	0
ORGENTEC tTG IgG	10 PGL U/mL	0.79	0.98	0.98	6.2	0.90	0.90	0.98	38

The statistical parameters were calculated with the MedCalc statistical software. In cases where the ROC CO (italicized numbers) was the closest to the recommended manufacturer CO, the next point in the ROC curve was also listed in the table. Manufacturer recommended lowest borderline value was used as cutoff

CO cut-off, ROC receiver operating characteristics, AUC area under the curve

among the Gliadin-derivatives assays. Low correlation was found when comparing the two tTG IgG assays of IMMCO and of ORGENTEC, 0.67. Best correlation was found with all 99 samples when comparing the two tTG-DGP screen assays of AESKU and INOVA, 0.97.

Four samples from four different patients with negative biopsy results were found to be positive in at least eight of the serological assays. These samples were referred to a second biopsy. One patient was found to be a celiac patient and to have a positive biopsy (Marsh 4), the second patient was found to have Marsh 1 with more than 30 intra-epithelial lymphocytes (IEL)/100 enterocytes. The third patient's biopsy was negative; however, he has type I diabetes mellitus. The fourth had positive serological results but without classical histological changes on biopsies. The overall analysis was based on the results of the second biopsies.

In order to evaluate the performance of all assays in a uniform manner, we established an arbitrary scoring for the various evaluated assays (Table 6). This scoring took into account the importance of the various criteria to our lab and gave an appropriate score. The various criteria and the particular score for each criterion are listed in Table 6. The accumulated score showed that the

best performing assays are (Table 6): AESKU neo-epitopes screen, INOVA tTG-DGP screen, DiaSorin (Liaison) tTG IgA, ORGENTEC tTG IgA, ORGENTEC tTG screen.

In order to assess the performance of the best-performing assays, we added another set of biopsy-proven samples. Sensitivities of all five assays were above 95%, with AESKU screen and INOVA tTG-DGP screen with 100% sensitivity. Specificities were between 87% to 96%, with INOVA tTG-DGP screen performing the lowest specificity and DiaSorin (Liaison) tTG IgA performing the highest specificity. Thus, the assay with best overall performance in all the tested samples was the AESKU screen assay.

Another assay with high specificity and with no overlap between the false negative results was the ORGENTEC screen assay (data not shown).

According to all these findings, we set a new algorithm (illustrated in Fig. 1) starting with AESKU screen as a screening test. A negative result with the screening test is a final negative result; however, a positive result with the screening test is further confirmed with DiaSorin (Liaison) tTG IgA. Samples positive for both tests are regarded as positive samples for celiac disease diagnosis. Samples positive in the screening test and negative in the DiaSorin

Table 4 Pearson correlations

tTG IgA assays						
Manufacturer	PHADIA	IMMCO tTG IgA	DiaSorin (Liaison)	ORGENTEC tTG IgA	DPC (IMMULITE)	IMMCO new tTG IgA
PHADIA	1.00					
IMMCO tTG IgA	0.90	1.00				
DiaSorin (Liaison)	0.95	0.85	1.00			
ORGENTEC tTG IgA	0.95	0.97	0.91	1.00		
DPC (IMMULITE)	0.63	0.91	0.66	0.84	1.00	
IMMCO new tTG IgA	0.90	0.94	0.83	0.97	0.79	1.00

(Liaison) tTG IgA test are further confirmed with the ORGENTEC tTG screen assay. A negative result in the last confirming test (thus positivity in the screening test only) renders a final negative result. Samples positive for both tests, the screening and the ORGENTEC tTG screen assay, are regarded as serological positive samples for celiac disease. This algorithm enabled our lab to detect CD with 100% sensitivity, 96.2% specificity, and 98.1% accuracy according to the evaluated blood samples.

Discussion

The present study enabled us to evaluate new available assays and set a new biopsy-proven algorithm for CD diagnosis. This new algorithm combines high sensitivity screening with the AESKU screen assay and high specificity by confirmation with DiaSorin (Liaison) tTG IgA and ORGENTEC screen assays. In our previous algorithm, we have used IMMCO tTG IgA as our screening test. Here, we show that this assay lacks both sensitivity and specificity (87% and 93%, respectively). EMA assay and total IgA antibodies were not included at all in the new celiac algorithm. In the past, EMA was found to have good diagnostic reliability compared to the old generation serological markers of CD. Compared to the high specificity of the presently chosen screening assay (AESKU neo-epitopes screen), the use of EMA as a confirming test was no longer reasonable. Total IgA

antibodies assay was added to celiac algorithms in order to compensate for CD IgA deficient patients. However, as the screening test as well as one of the confirming tests (ORGENTEC screen), both include IgG antibodies, the determination of total IgA in the new celiac algorithm was unnecessary.

In the present study 100% sensitivity was found with two assays, both including tTG and DGP as antigens, and IgA and IgG as antibodies. The enhanced sensitivity of INOVA tTG-DGP screen assay was previously shown in anti-tTG-seronegative patients with CD-like enteropathy [53, 54].

It has been previously shown [55] that tissue transglutaminase (tTG) can both deamidate gliadin peptides as well as transamidate them, with transamidation being the major reaction (75% vs. 25% for deamidation). Such cross-linking also occurs outside the active site of tTG and results in permanently and covalently linked deamidated gliadin peptide/tTG complexes. The AESKU screen assay detects antibodies to tTG, to DGP, and to the neo-epitope and showed 100% sensitivity. This high sensitivity was suggested to provide an early detection, compared to other tTG ELISA kits [42, 43]. However, such cases might be interpreted as false positive cases on the first line screen. Applying the second step screen, with high specificity, can eliminate the false positivity. Taking into account the iceberg model, where diagnosed:undiagnosed CD ratio is 1:9, the present screening algorithm is reasonable. Monitoring these cases will be a reasonable approach.

Table 5 Pearson correlations

Gliadin derivatives assays				
Manufacturer	INOVA DGP screen	EUROIMMUN GAF-3X	IMMCO G+IgG	IMMCO G+IgA
INOVA DGP screen	1.00			
EUROIMMUN GAF-3X	0.89	1.00		
IMMCO G+IgG	0.93	0.90	1.00	
IMMCO G+IgA	0.78	0.60	0.64	1.00

Table 6 Arbitrary scoring of the evaluated assays

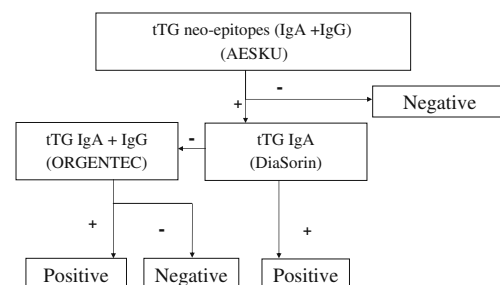
Assay	FN (40%)	FP (25%)	Sens (95% spec) (10%)	Delta between cut-offs (10%)	External QC-participation (5%)	Analytical CV (10%)	Score
INOVA tTG-DGP screen	40	12.5	10	10	5	10	87.5
AESKU	40	12.5	10	10	5	5	82.5
ORGENTEC tTG screen	20	12.5	10	10	5	10	67.5
ORGENTEC tTG IgA	20	12.5	10	5	5	10	62.5
DiaSorin (Liaison)	20	12.5	10	5	5	10	62.5
DPC (IMMULITE)	20	12.5	0	10	0	10	52.5
INOVA DGP screen	20	12.5	0	5	5	10	52.5
PHADIA	0	25	10	0	5	10	50
IMMCO new tTG IgA	20	12.5	0	10	5		47.5
IMMCO new G+ IgG	20	12.5	0	10	5		47.5
IMMCO tTG IgA	0	12.5	0	10	5	5	32.5
ORGENTEC tTG IgG	0	12.5	0	5	5	10	32.5
EUROIMMUN GAF-3X	0	12.5	0	0	5	5	22.5
IMMCO new tTG IgG	0	12.5	0	0	5		17.5
IMMCO new G+ IgA	0	0	0	0	5		5

False negative results (40%), 0=40 points; 1–4=20 points; >4=0 points. False positive results (25%), 0=25 points; 1–4=12.5 points; >4=0 points. Sensitivity at 95% specificity (10%), >97%=10 points; 95–97%=5 points; <95%=0 points. The percentage of difference between ROC CO and manufacturer CO (10%), <25%=10 points; 25–50%=5 points; >50%=0 points. Participation in external control programs (5%), participation=10 points; no participation=0 points. Analytical CV% (10%), <10%=10 points; >10%=0 points

In spite of the very good performance of the screening test, using one assay only for screening could lead to false positive results. Indeed, it has been previously suggested that screening of CD in children cannot be based on one test only [9, 56]. Thus, we added two assays with superior specificities for confirmation of the positive results. These two specific assays showed no overlapping between their false negative missed cases. The DiaSorin (Liaison) tTG IgA assay was indeed previously demonstrated to perform high specificity and sensitivity [57]. However, even if the two specific assays would give a negative result, and the screening test was positive, the physician has to weight all this diagnostic information in the view of the clinical data and decide whether to retest for serology in a few months, to refer for endoscopy, or to suggest GFD.

There are many studies describing the performance of the various assays for CD serological diagnosis. A systemic review [58] calculated the weighted mean of the sensitivity and specificity along with 95% confidence intervals and summarized receiver operating characteristic (ROC) curves including all the studies appearing in MEDLINE (1966 to October 2003) and EMBASE (1974 to December 2003) databases. The pooled specificity of EMA-monkey esoph-

agus was close to 100% in adults and children. IgA-EMA demonstrated sensitivities of 96% and 97% in children and adults, respectively. The pooled specificity of transglutaminase antibody was between 95% and 99%. The pooled sensitivity of tTG in adults and children was 98% and 96%, respectively. The performance of anti gliadin antibody was inferior to that of EMA and tTG. A recently published meta-analysis [59] summarized evidence on the performance of diagnostic serological tests for identifying celiac disease in adults presenting with abdominal symptoms in primary care. Sixteen studies were included in the review. Pooled estimates for IgA antiendomysial antibodies (eight

**Fig. 1** The new celiac diagnosis algorithm

studies) were 0.90 for sensitivity and 0.99 for specificity. Pooled estimates for IgA antitissue transglutaminase antibodies (seven studies) were 0.89 for sensitivity and 0.98 for specificity. The IgA and IgG antigliadin antibodies showed variable results, especially for sensitivity (range, 0.46–0.87 and range, 0.25–0.93, respectively). This meta-analysis suggested that IgA antitissue transglutaminase antibodies and IgA antiendomysial antibodies have high sensitivity and specificity for diagnosing celiac disease [59]. However, the EMA assay (fluorescence endomysial antibodies, usually also IgA) is very laborious and interobserver-dependent; it is used mainly as a confirmatory test. Another recent publication [60] reviewed the available tests for CD, including antibodies against gliadin, endomysium, tissue transglutaminase, and deamidated gliadin, and the evidence for preferential use of specific tests in different settings. It concluded that despite several novel developments, standardized ELISA-based assays for IgA autoantibodies against tissue transglutaminase remain the test of choice for most of the population. Kurppa et al. suggested [61] that the sensitivity of deamidated gliadin peptides antibodies was superior to tissue transglutaminase antibodies and comparable to endomysial antibodies in celiac patients having early-stage celiac disease with normal villous morphology. Superior specificity of the deamidated gliadin peptides antibody test have raised the suggestion that patients who test positive with this assay require a thorough follow-up, even if the tissue transglutaminase antibodies and endomysial autoantibodies assays are negative [62]. In a study conducted on 144 consecutive patients with gastrointestinal and extraintestinal signs suggestive for CD, the combination of IgA tTG and IgG DGP provided the best diagnostic accuracy for CD due to high sensitivity performance of the first assay and high specificity performance of the second test [63]. Such combination was suggested to be an excellent screening algorithm for suspected cases of celiac disease [64], and our algorithm indeed proves very good diagnostic performance for such a combination of antibodies.

Small bowel biopsies are considered the gold standard for diagnosis of CD [22]; however, biopsy results could be sometimes misleading. Duodenal histology is frequently patchy and may show variable severity even within a single biopsy fragment [65]. It has been suggested that the duodenal bulb mucosa might be the only area showing histological changes; thus, the biopsies should not only include distal duodenum but also bulb as biopsy sites in order to improve the diagnostic yield [66]. Moreover, measurement of ≥ 25 IEL /100 enterocytes correlates with serologic indicators of celiac disease and a higher intraepithelial lymphocytes threshold could miss 50% of cases [67]. In view of the most recent trend to change the diagnostic criteria for CD, such as to skip the routine

intestinal histology and replace it by reliable serologic and genetic markers [68, 69] and to surrogate the workup according to different algorithms in symptomatic and asymptomatic subjects (ESPGHAN meeting, Istanbul, June 2010, not published yet), the present screening algorithm is a step forward. The interplay between serology and intestinal histology in the CD diagnostic process is demonstrated by the following examples. In our study, four cases had positive serology and negative histology, and thus second biopsies were performed. Two of them revealed positive biopsy results (Marsh 4 and Marsh 1 with >30 IEL/100 enterocytes), the third suffered from type I diabetes mellitus and as for the fourth case, another biopsy was ordered by his physician. Based on the first biopsy results, the performance of our algorithm would render 100% sensitivity, 92.6% specificity, and 96.3% accuracy. Due to the alert raised by the positive serology results by most of the evaluated assays, the performance of the second biopsies improved the algorithm specificity and accuracy by 3.6% and 1.8%, respectively, rendering 96.2% specificity and 98.1% accuracy. It is well known that there are shared genetic risk factors for type 1 diabetes and celiac disease [70], and around 10% of the diabetic type I patients will develop CD after a lag period [71]. Children with celiac disease develop celiac disease-associated antibodies mostly at a younger age or the same age at which they develop diabetes-associated autoantibodies [72]. Thus, as further follow-up of the type I diabetic patient suggestion of GFD is warranted for possible improvement in the diabetes symptoms. It seems that CD is in the verge of changing in several aspects: pathophysiology, knowledge, diagnostic criteria, and therapeutic strategies [73]. The present study describes a new algorithm for CD diagnosis. This algorithm identified all patients with CD with few false positive results. Prospective studies are needed to confirm our findings and to determine if extrapolation to adults is warranted.

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