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Research paper

Diagnostic accuracy and applicability of intestinal auto-antibodies in the wide clinical spectrum of coeliac disease

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ABSTRACT

Background: Intestinal coeliac auto-antibodies are the marker of coeliac disease (CD). Since the determination of these antibodies is still not widely available, we used immunoassays to identify the most suitable technology for revealing intestinal auto-antibodies in the wide clinical spectrum of CD.
Methods: Intestinal auto-antibodies have been prospectively investigated in CD suspected children using two immunoassays: intestinal-deposits of IgA anti-tissue transglutaminase antibodies (anti-tTG) and biopsy-culture IgA anti-endomysium (AEA). Intestinal IgM antibodies have been determined in IgA-deficient subjects.
Findings: Two-hundred and twenty-one suspected CD patients were enrolled. Intestinal antibodies were tested positive for both assays in classical CD patients ($n = 178$) with villous atrophy and positive serum-CD antibodies, potential CD patients ($n = 16$) with normal intestinal mucosa and positive serum-CD antibodies, and pre-potential CD patients ($n = 14$) with normal intestinal mucosa and negative serum-CD antibodies. In 13/221 with normal intestinal mucosa, negative CD-serum antibodies and negative intestinal antibodies CD has been excluded. All classical, 14/16 potential and 11/14 pre-potential CD patients on gluten-free diet (GFD) improved their symptoms. In 9/11 pre-potential patients intestinal antibodies disappeared on GFD. Both assays were negative in 69/71 control subjects. The two assays showed high diagnostic sensitivity (100%) and specificity (99%).
Interpretation: Intestinal CD-antibodies make prompt diagnosis in the wide clinical spectrum of CD reducing the delay in diagnosis and treatment, especially in pre-potential CD patients. The easy handling biopsy culture assay is an effective diagnostic tool which should be carried out by any gastroenterology unit to recognize all CD clinical manifestations.
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1. Introduction

Coeliac disease (CD) is an intestinal auto-immune disorder induced by gluten ingestion in genetically susceptible individuals and characterized by small-bowel villous atrophy. Gluten induces a specific immune response characterized by the production of auto-antibodies against the tissue transglutaminase (anti-tTG) [1]. These auto-antibodies are produced by intestinal B-cells and bind to the tissue transglutaminase protein in the early phases of the disease, when the duodenal mucosa is still normal and the serum auto-antibodies are not detectable [2–5]. In symptomatic patients with positive-serum antibodies and villous atrophy, the CD diagnostic criteria are fulfilled and the diagnosis of classical CD is straightforward. However, thanks to greater awareness of CD, there is an increasing number of symptomatic patients with potential CD, who have positive-serum antibodies despite normal histological intestinal mucosa [6–8], and more patients with pre-potential CD, namely negative or fluctuating serum antibodies and normal intestinal mucosa [4,5,9]. In these two conditions, it has been observed that the presence of intestinal anti-tTG antibodies is the only mucosal immunological marker of CD. Significantly, these patients, who suffer from gastrointestinal and/or extra-intestinal symptoms (i.e. anaemia, chronic tiredness,

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Research in Context

Evidence before this study

Intestinal anti-tissue transglutaminase antibodies (anti-tTG) are a specific marker of coeliac disease (CD) to identify symptomatic patients without the CD-diagnostic criteria. These auto-antibodies are currently investigated by using the intestinal anti-tTG deposits immunoassay. This technique is limited to very few specialized centres because it requires frozen intestinal samples, special laboratory equipment and highly experienced operators.

Added value of this study

Intestinal CD-antibodies have been investigated in the wide clinical spectrum of CD by using both intestinal deposits and biopsy culture assays showing similar results in terms of sensitivity and specificity. These antibodies have been found not only in subjects with classical or potential CD but also in symptomatic pre-potential CD with normal intestinal mucosa and negative serum-CD antibodies. For the first time intestinal IgM antibodies have been investigated by using the biopsy culture method in IgA-deficient subjects suspected of CD.

Implications of all the available evidence

Biopsy culture is the easy handling assay which any gastroenterology unit can use to investigate the intestinal coeliac auto-antibodies in daily clinical practice in all the clinical manifestations of CD. These auto-antibodies make prompt diagnosis in symptomatic pre-potential patients who may benefit of a well-founded diagnosis reducing both unnecessary medical investigations and delay in diagnosis and treatment.

arthralgia) show great improvement on a gluten-free diet (GFD) with the disappearance of the intestinal mucosal anti-tTG [5,6,10]. Thus, it is very important to have a specific, user-friendly immunoassay for intestinal anti-tTG detection to supplement histology in diagnosing CD, especially in patients without villous atrophy. Currently, these auto-antibodies are detected as IgA deposits in distal duodenal biopsies by means of double immunofluorescence staining on intestinal cryosections [11]. Paediatric studies show that these IgA deposits had the best sensitivity values (100%) in detecting patients with classical CD, and gave scores ranging from 64% to 100% among patients with potential CD [11]. In non-coeliac patients the specificity of mucosal anti-tTG was between 82 and 100% [11,12].

Unfortunately, this methodology is not yet widely available because it is a time consuming and expensive analysis requiring frozen intestinal samples, special laboratory equipment and highly experienced operators. For this reason it has been suggested that in selected cases the frozen specimens should be sent to centres specialized in this immunoassay technique [13].

Another immunological test is to look for intestinal auto-antibodies to CD in the supernatants of cultured intestinal biopsies using the well-established IgA-anti-endomysium (AEA) assay already widely applied and standardised for serum IgA AEA determination [14]. This test is not too demanding because both the biopsy culture and the detection of the IgA AEA secreted in the supernatant can be performed by using commercial kits. In clinical settings, this test applied on the distal duodenum mucosa specimens produces very high values of sensitivity (98%) and specificity (100%) as well as diagnostic accuracy (98%) [12,15].

In this prospective pediatric study, we evaluated the reliability of these two immunoassays for detecting the intestinal IgA CD-antibodies in both duodenal bulb and distal duodenum mucosa of patients with classical, potential and pre-potential CD and we monitored the effects of GFD in these CD-clinical conditions. In addition, the two assays were used to search for intestinal IgM in subjects with total IgA deficiency, which are a group at risk of CD.

2. Patients and methods

2.1. Patients and study design

Pediatric patients were prospectively recruited at the Institute for Maternal and Child Health-IRCCS "Burlo Garofolo" in Trieste (Italy) from September 2016 to August 2018. Consecutive children undergoing clinically indicated upper gastro-intestinal (GI) endoscopy under deep sedation were enrolled. In this study have been included patients not fulfilling the new ESPGHAN guidelines for a serology based CD diagnosis without biopsy [16]. Patients suspected of CD, because of suggestive symptoms and serum CD antibodies positivity, were classified and diagnosed, after serological and intestinal immuno-histological evaluations, as classical and potential CD patients on the basis of international classifications [17,18] and as pre-potential CD patients on the basis of preliminary clinical experience [4,5,9,19]. These definitions are described in detail below:

Classical CD – symptomatic subjects positive for serum anti-tTG and/or AEA concentrations with intestinal villous atrophy;

Potential CD – symptomatic or asymptomatic subjects positive for serum anti-tTG and/or AEA concentrations with normal intestinal mucosa;

Pre-potential CD – symptomatic or asymptomatic subjects at risk of CD (e.g. first-degree relatives of CD patients, patients with autoimmune disorders) showing a previous serum CD auto-antibodies positivity converted to negativity at the time of GI endoscopy and with normal intestinal mucosa. Selected pre-potential CD patients testing positive for intestinal CD-antibodies and suffering from major symptoms (e.g. failure to thrive, anaemia, aphtous stomatitis, diarrhoea) were put on GFD and followed for at least one year. After one year of GFD, these patients were asked to undergo a second GI endoscopy to verify the disappearance of intestinal CD-antibodies and to confirm the CD diagnosis.

Children with other GI disorders (such as inflammatory bowel disease, gastritis, eosinophilic oesophagitis) at the diagnosis time or during the scheduled follow up were asked to take part in the study as a control group.

Patients with IgA deficiency were included in this study.

Following the ESPGHAN's recommendations (17), the parents of children testing positive for serum anti-tTG antibodies, were advised by their pediatrician not to carry out the gluten-free diet before the gastrointestinal endoscopy examination.

In all enrolled patients, intestinal CD-antibodies were investigated in bulb and distal duodenum specimens as anti-tTG deposits and AEA in biopsy culture supernatant. In patients in whom the intestinal CD-antibodies were detected by only one of the two immunoassays, a highly sensitive and specific phage-display antibody assay was used to verify the intestinal production of CD auto-antibodies. Written informed consent was obtained from the parents of the children enrolled, and the study was approved by the hospital's independent ethical committee (IRCCS "Burlo Garofolo" Comitato Indipendente per la Bioetica, Trieste; CE/V-131).

2.2. Serology tests

Serum IgA anti-tTG antibodies were measured using an ELISA assay (Eurospital Kit Eu-TG2, Trieste, Italy) following the manufacturer's instructions and values higher than 10 U/mL were considered positive.

Serum IgA AEA were investigated by an indirect immunofluorescence method following the manufacturer's instructions (Eurospital kit Antiendomysium 96, Trieste, Italy). Briefly, sera were tested diluted 1:5 on sections of monkey oesophagus as substrate and incubated for 30 min at room temperature. After sections were washed to remove serum and fluorescein isothiocyanate-labelled rabbit antibody against human IgA was incubated for 30 min. Serum samples were considered positive if a thin fluorescent network appeared around the smooth muscle fibres.

In patients with selective IgA deficiency (total IgA < 5 mg/dl) serum samples were investigated for IgG anti-tTG antibodies and IgG1 AEA.

2.3. HLA typing

Whole blood was used to test for the presence of the susceptibility alleles for CD using polymerase chain reaction with allele-specific primers that identify HLA DQ2 and DQ8, using the Eu-Gene_Risk kit (Eurospital, Trieste, Italy).

2.4. Intestinal deposits of anti-tTG antibodies

Intestinal IgA anti-tTG antibodies were investigated in 6 criosections obtained from both bulb and distal duodenum specimens by using a previously described immunoassay [3,10]. Briefly, unfixed frozen sections were incubated first with monoclonal mouse antibody against tTG (CUB7402; NeoMarkers Fremont, California, USA) followed by Alexa fluor 594-conjugated anti-mouse IgG secondary antibody (Thermo Fisher Scientific Waltham, Massachussetts, USA), and then with fluorescein isothiocyanate-labelled rabbit antibody against human IgA (Dako, Glostrup, Denmark). Multicolor analysis was performed using an Axioplan 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with the specific software AxioVision (Carl Zeiss, Oberkochen, Germany) to localize IgA anti-tTG deposits, which appear as yellow spot below the basement membrane, along the villous and the crypt, and around mucosal vessels.

In patients with selective IgA deficiency, IgM (Dako, glostrup, Denmark) anti-tTG deposits were investigated as previously described [20].

Samples were considered positive if at least 2/6 criosections from bulb or distal duodenum showed yellow spots.

In our experience started in 2010 [21], more than 2000 intestinal biopsies have been evaluated for both IgA and IgM intestinal anti-tTG deposits, and intraobserver and interobserver variations have both been 98% in the detection of the presence or the absence of intestinal deposits among two investigators.

2.5. Biopsy culture AEA assay

Two intestinal fragments, one from bulb and one from distal duodenum, were cultured for 72 h at 37 °C in the presence of peptic-tryptic digest of gliadin (PT-gliadin) following the manufacturer's instructions (Eurospital kit Antiendomysium biopsy, Trieste, Italy). The culture supernatants were collected and stored at −20 °C until analysis.

Intestinal IgA AEA secreted into culture supernatants were detected by an indirect immunofluorescence method following the manufacturer's instructions (Eurospital kit Antiendomysium 96, Trieste, Italy). Briefly, undiluted supernatants were tested on sections of monkey oesophagus as substrate and incubated for 30 min at room temperature. After sections were washed to remove supernatant and fluorescein isothiocyanate-labelled rabbit antibody against human IgA was incubated for 30 min. Supernatants were considered positive if a thin fluorescent network appeared around the smooth muscle fibres.

Intestinal IgM AEA were investigated in patients with selective IgA deficiency.

2.6. Phage display library

Phage-antibody libraries were constructed, as previously described 5, from the subjects' distal duodenum biopsy B-lymphocytes to search for CD-specific mucosal anti-tTG antibodies, which are primarily composed of the IGHV5-51 gene from the VH5 gene family. Selective IgA IGVH5-51 genes were amplified from the cDNA and assembled into single-chain fragment-variable (scFv) fragments by cloning into the phagemid vector pDAN5. ScFvs recognising human tTG were selected by affinity chromatography. After three cycles of selection, 45 clones were screened for reactivity to human tTG protein by ELISA.

All the serologic, genetic, immunologic and molecular analyses were evaluated by specialists blinded on the subjects' clinical data and not involved in clinical decisions.

2.7. Small bowel mucosal morphology

In each patient, 4 intestinal biopsy specimens were taken by endoscopy: 2 samples each from the bulb and distal duodenum. The histologic analysis was based on Marsh Oberhuber's and Corazza Villanacci Classifications [22,23]. An independent specialist (V.V.) evaluated the biopsy samples without prior knowledge of our subjects' clinical and laboratory data.

2.8. Statistics

Data are reported as mean±standard deviation for continuous variables and as proportion for categorical variables. The sequential serum samples were compared using the Wilcoxon signed rank test for paired data.

McNemar test and Cohen's kappa were used to evaluate the concordance between intestinal anti-tTG deposits and biopsy culture AEA. Landis and Koch propose Cohen's kappa as a method to describe the degree of concordance: 0.01−0.20, "weak"; 0.21−0.40, "fair"; 0.41−0.60, "moderate", 0.61−0.80, "substantial"; 0.81−1.00, "almost perfect" [24]. Sensitivity and specificity were reported with a confidence interval of 95%. A value of $P < 0.05$ was considered significant.

3. Results

Two hundred and ninety-two patients were enrolled, of whom 221 (76%) (86 boys and 135 girls; median age 7 years, range 1−18) had suspected CD. Seventy-one out of 292 (24%) (50 boys and 21 girls; median age 13 years, 1−18) had other GI disorders and were enrolled as the control group.

On the basis of serological data obtained at the time of GI endoscopy we found 194/221 (88%) suspected CD patients were still positive for serum IgA anti-tTG antibodies (104±164 U/ml) and/or AEA. The remaining 27/221 (12%), had seroconverted back to both IgA anti-tTG and AEA negativity, although they were on gluten-containing diet. In these last 27 children the positive serum anti-tTG concentrations were 18±6 U/ml measured 8 ± 2 months (mean value± standard deviation) before the GI endoscopy.

Among the 194 patients positive for serum CD antibodies on the basis of histological and immunological data we identified:

Classical CD − One hundred and seventy-eight symptomatic patients of 221 (80.5%) (Table 1) tested positive for HLA DQ2/8 haplotype and for serum IgA anti-tTG antibodies (114±172 U/ml) and/or AEA. Intestinal atrophy and high intraepithelial lymphocytes (IEL) density (102±49/100 epithelial cells) were observed in all patients, but in 19/178 (11%) only in the bulb duodenum. Both assays for intestinal IgA antibodies were positive in all patients (Table 2). In the two patients out of 178 (1%) who showed IgA deficiency and tested positive for

Table 1
Clinical findings of all the CD study groups and of the control group.

Clinical findings	Classical cd *n* = 178
Anaemia	11 (6%)
Diarrhoea	12 (7%)
Aphtous stomatitis	5 (3%)
Asthenia	20 (11%)
Failure to thrive	28 (16%)
Recurrent abdominal pain	61 (34%)
Family history of CD	42 (24%)
IgA deficiency	2 (1%)
Thyroiditis	5 (3%)
	potential cd *n* = 16
Diarrhoea	4 (25%)
Failure to thrive	10 (62.5%)
Recurrent abdominal pain	3 (19%)
Family history of CD	4 (25%)
Type 1 diabetes	1 (6%)
	pre-potential cd *n* = 14
Anaemia	1 (7%)
Diarrhoea	4 (25%)
Aphtous stomatitis	2 (14%)
Asthenia	4 (29%)
Failure to thrive	5 (36%)
Recurrent abdominal pain	8 (57%)
Family history of CD	2 (14%)
	not confirmed cd *n* = 13
Anaemia	1 (8%)
Diarrhoea	3 (23%)
Aphtous stomatitis	1 (8%)
Failure to thrive	3 (23%)
Recurrent abdominal pain	8 (61%)
Family history of CD	4 (31%)
IgA deficiency	3 (23%)
Thyroiditis	1 (8%)
Type 1 diabetes	1 (8%)
	Control group *n* = 71
Inflammatory bowel disease	29 (41%)
Eosinophilic oesophagitis	9 (13%)
Gastritis	17 (24%)
Reflux oesophagitis	11 (15%)
Others	5 (7%)

CD, coeliac disease.

both serum IgG anti-tTG antibodies (72±60 U/ml) and IgG1 AEA, intestinal IgM auto-antibodies were found. All 178 symptomatic patients were diagnosed as having CD and put on GFD.

Potential CD - Sixteen symptomatic patients (7%) (Table 1) tested positive for HLA DQ2/8 haplotype, tested positive for serum IgA anti-tTG antibodies values (14±17 U/ml) and/or AEA, showed normal both intestinal mucosa and IEL density (14±5/100 epithelial cells). Both assays for intestinal IgA antibodies gave positive results in all patients (Table 2). Fourteen out of 16 (87.5%) who had severe symptoms (failure to thrive, diarrhoea) and/or other autoimmune-associated disorders (diabetes type 1) were put on GFD.

Among the 27 patients transiently positive for serum CD antibodies and tested negative at the time of GI endoscopy, on the basis of histological and immunological data, we identified:

Pre-potential CD – fourteen symptomatic patients (6%) (Table 1) tested positive for HLA DQ2/8 haplotype, negative for both serum IgA anti-tTG antibodies (2 ± 1 U/ml) and AEA, and showed normal mucosa and IEL density (12±5/100 epithelial cells). Both assays for intestinal IgA antibodies were positive in all patients (Table 2). Eleven out of 14 (62.5%) suffering from major symptoms (anaemia, failure to thrive, aphtous stomatitis, diarrhoea) were put on GFD.

Not confirmed CD – thirteen patients (6%) (Table 1) tested positive for HLA DQ2/8 haplotype, negative for both serum IgA anti-tTG antibodies (1.9 ± 1 U/ml) and AEA, and showed normal intestinal mucosa and IEL density (10±5/100 epithelial cells). Intestinal auto-antibodies were negative in all patients (Table 2). Three/13 (23%) showed IgA deficiency. In these 3 patients, who were negative for both serum IgG anti-tTG (3 ± 2 U/ml) and IgG1 AEA, the intestinal IgM auto-antibodies were not found. All these patients remained on a gluten- containing diet (GCD).

Control group – the HLA DQ2 or DQ8 haplotype was positive in 19/71 subjects (27%) (Table 1), CD-serum markers were negative in all 71 and no CD-related histologic lesions were found. Both assays for intestinal IgA antibodies were negative in 69/71 patients (Table 2). The two control patients testing positive for intestinal CD-antibodies suffered from eosinophilic oesophagitis. One showed widespread duodenal anti-tTG deposits and AEA in the culture supernatant, while in the other who tested negative for HLA DQ2/8 haplotype, only the AEA assay was positive but limited to the duodenal bulb (Table 2). In the latter control subject, following our study design, the intestinal biopsy was then analysed using the phage-display antibody assay and no IGHV5-51 anti-tTG antibody clones were isolated: the subject was therefore not considered as being at risk of CD.

The sensitivity of the intestinal anti-tTG deposit immunoassay in the bulb and distal duodenum was 100% and 95% respectively in classical CD patients, and 100% and 94% in potential CD patients; specificity was 99% in both sites (Table 2). Sensitivity in the AEA biopsy culture immunoassay in the bulb and distal duodenum was 100% and 95% in classical CD patients and of 94% and 100% in potential CD patients; specificity was 98% in the bulb and 99% in the distal duodenum (Table 2).

Table 2
Sensitivity (Se) and Specificity (Sp) with 95% confidence interval for intestinal anti-tTG deposits and biopsy culture AEA. In this table are not reported the thirteen cases in which coeliac disease has been excluded.

	Intestinal anti-tTG deposits +				Biopsy culture AEA +			
	Bulb duodenum (n)	Diagnostic indicators	Distal duodenum (n)	Diagnostic indicators	Bulb duodenum (n)	Diagnostic indicators	Distal duodenum (n)	Diagnostic indicators
Classical CD n 178	178	Se 100% (97–100%)	169	Se 95% (91–98%)	178	Se 100% (97–100%)	169	Se 95% (91–98%)
Potential CD n 16	16	Se 100% (71–100%)	15	Se 94% (70–100%)	15	Se 94% (70–100%)	16	Se 100% (71–100%)
Pre-potential CD n 14	13	/	9*	/	14	/	9	/
Not confirmed CD n 13	0	/	0	/	0	/	0	/
Control group n 71	1	Sp 99% (92–100%)	1	Sp 99% (92–100%)	2	Sp 98% (90–100%)	1	Sp 99% (92–100%)

CD, coeliac disease; tTG, tissue transglutaminase; AEA, anti-endomysium antibodies; Se, sensibility; Sp, specificity.
* 1/4 was tested positive only in distal duodenum.

Table 3
Comparison between two diagnostic tests in bulb duodenum.

		Intestinal anti-tTG deposits		
		+	–	
Biopsy culture AEA	+	207	2	$P = 0.56$* $K = 0.97$ (0.95 – 1.00)
	–	1	82	

tTG, tissue transglutaminase; AEA, anti-endomysium antibodies.
* Mc-Nemar test; K=Cohen's Kappa with 95% confidence interval.

Combining the results obtained from intestinal anti-tTG deposits immunoassay with the AEA biopsy culture method, we obtained an overall concordance of 99.0% in bulb duodenum specimens (Table 3; $p = 0.56$, Mc-Nemar test) and of 99.7% in distal duodenum ones (Table 4; $p = 0.32$, Mc-Nemar test). K Cohen was calculated and gave a value of 0.97, 95% CI: 0.95–0.99 for bulb and of 0.99, 95% CI: 0.98–1.00 for distal duodenum.

3.1. Follow-up

After 12 months on GFD, all the patients with classical CD showed negative serum IgA AEA, a significant decrease in serum IgA anti-tTG concentrations (46±172 vs. 4 ± 2 U/ml, $P<0.0001$) and improvement in their clinical condition (Table 5). In the patients with IgA deficiency, serum values of IgG anti-tTG (72±60 vs. 4 ± 3 U/ml, $P<0.0001$) and IgG1 AEA were negative.

Fourteen/16 patients with potential CD were put on GFD: 12 months later, 13/14 (93%) showed negative serum AEA, a significant decrease in serum anti-tTG concentrations (14±17 vs. 2 ± 3 U/ml, $P<0.0001$] and improved symptoms (Table 5). The remaining continued to test positive for serum CD antibodies and underwent a second GI endoscopy. Histological analysis showed normal intestinal mucosa with an increased IEL density (35±2/100 epithelial cells) and intestinal auto-antibodies were detected throughout the duodenum. The two potential CD patients who were kept on a GCD but monitored, continued to test positive for serum IgA anti-tTG and AEA and had occasional abdominal pain. Both of them refused a second GI endoscopy to assess the intestinal condition.

After 12 months, the eleven pre-potential CD patients who were following a GFD all showed improvement in their symptoms (Table 5). They were asked to undergo a second GI endoscopy and 9/11 agreed. In all nine, normal histological findings were confirmed with the disappearance of the intestinal auto-antibodies (Fig. 1). The other three pre-potential CD patients who were being monitored on a GCD continued to test negative for both serum IgA anti-tTG and AEA with unchanged clinical condition (abdominal pain and asthenia).

The 13 patients, in which CD was not confirmed, continued to test negative for serum-CD antibodies on GCD with amelioration in their clinical condition. In particular, abdominal pain, diarrhoea and aphtous stomatitis disappeared, and anaemia (Hb 10.5 to 11 g/dl) and failure to thrive (from 15° to 25° centile) ameliorated.

The control subject suffering from eosinophilic oesophagitis who tested positive for intestinal auto-antibodies continued to test negative for serum-CD antibodies on GCD.

Table 4
Comparison between two diagnostic tests in distal duodenum.

		Intestinal anti-tTG deposits		
		+	–	
Biopsy culture AEA	+	194	1	$P = 0.32$* $K = 0.99$ (0.98 – 1.00)
	–	0	97	

tTG, tissue transglutaminase; AEA, anti-endomysium antibodies.
* Mc-Nemar test; K=Cohen's Kappa with 95% confidence interval.

Table 5
Clinical findings of the 3 CD study groups for the GCD and after 12 months of GFD.

	GCD	GFD
Active CD ($n = 178$)		
Anaemia	11 (Hb g/dl 9.5 ± 0.4)	11 improved (Hb g/dl 12.1 ± 0.6)
Diarrhoea	12	12 resolved
Aphtous stomatitis	5	5 resolved
Asthenia	20	20 disappeared
Failure to thrive	28	28 improved (from 5° – 10° to 25° centile)
Recurrent abdominal pain	61	61 improved/ disappeared
Potential CD ($n = 14$)		
Diarrhoea	4	4 resolved
Failure to thrive	10	10 improved (from 5° – 10° to 25° centile)
Recurrent abdominal pain	3	3 improved/disappeared
Pre-potential CD ($n = 11$)		
Anaemia	1 (Hb g/dl 8.5)	1 improved (Hb g/dl 12)
Diarrhoea	4	4 resolved
Aphtous stomatitis	2	2 resolved
Failure to thrive	5	5 improved (from 5° – 10° to 25° centile)

GCD, gluten-containing diet; GFD, gluten-free diet; CD, coeliac disease; Hb, hemoglobin.

4. Discussion

In this large pediatric prospective study, the assays for intestinal anti-tTG deposits and biopsy culture AEA both produced similar results in terms of sensitivity and specificity for the diagnosis of CD, even in those forms of CD in which the intestinal mucosa is normal.

Moreover, as regards detection of CD-specific intestinal auto-antibodies, the two immunoassays showed an overall concordance of 99.0% in bulb duodenum specimens and of 99.7% in distal duodenum ones. Interestingly, it was possible to determine the presence of intestinal IgM antibodies in patients with total IgA deficiency not only by means of the anti-tTG deposit assay [20], but also using the biopsy culture method. Therefore, the biopsy culture assay simplifies the search for CD-intestinal auto-antibodies in IgA-deficiency disorder characterized by a high CD prevalence (9%) [25]. In the daily laboratory practice, intestinal anti-tTG assay requires specialized personnel and specific equipment to prepare (cryostat) and analyse (specific software) the frozen biopsies. On the contrary the biopsy culture AEA is based on standardized kits, does not require specialized personnel and is less time-consuming making the investigation of intestinal CD-antibodies widely available. Considering materials, laboratory equipment and time cost of specialized personnel we estimate a cost of €40 per patient for the biopsy culture AEA assay and of €100 per patient for the intestinal anti-tTG deposits assay.

Intestinal CD-antibodies were detected in 2 sick control patients suffering from eosinophilic oesophagitis. Following our study design, one was considered a false positive, having tested negative for CD-related HLA DQ2/8 and for phage-display antibody assay. The other patient who was genetically predisposed to gluten intolerance was considered at risk of CD but remained negative to serum CD antibodies on GCD. This patient was deemed at risk of CD also given the high prevalence of CD among subjects with eosinophilic oesophagitis [26]. Of interest, none of the control subjects with chronic inflammatory bowel disease tested positive for intestinal CD-antibodies.

Importantly, by analyzing the duodenal bulb biopsies for both IgA anti-tTG deposits and culture AEA antibody, we obtained an increased detection rate of intestinal CD-antibodies. In particular, if duodenal bulb biopsies had not been performed, the intestinal CD-antibodies would have been missed in 14/208 (7%) of our diagnosed CD patients (178 classical CD, 16 potential CD and 14 pre-potential

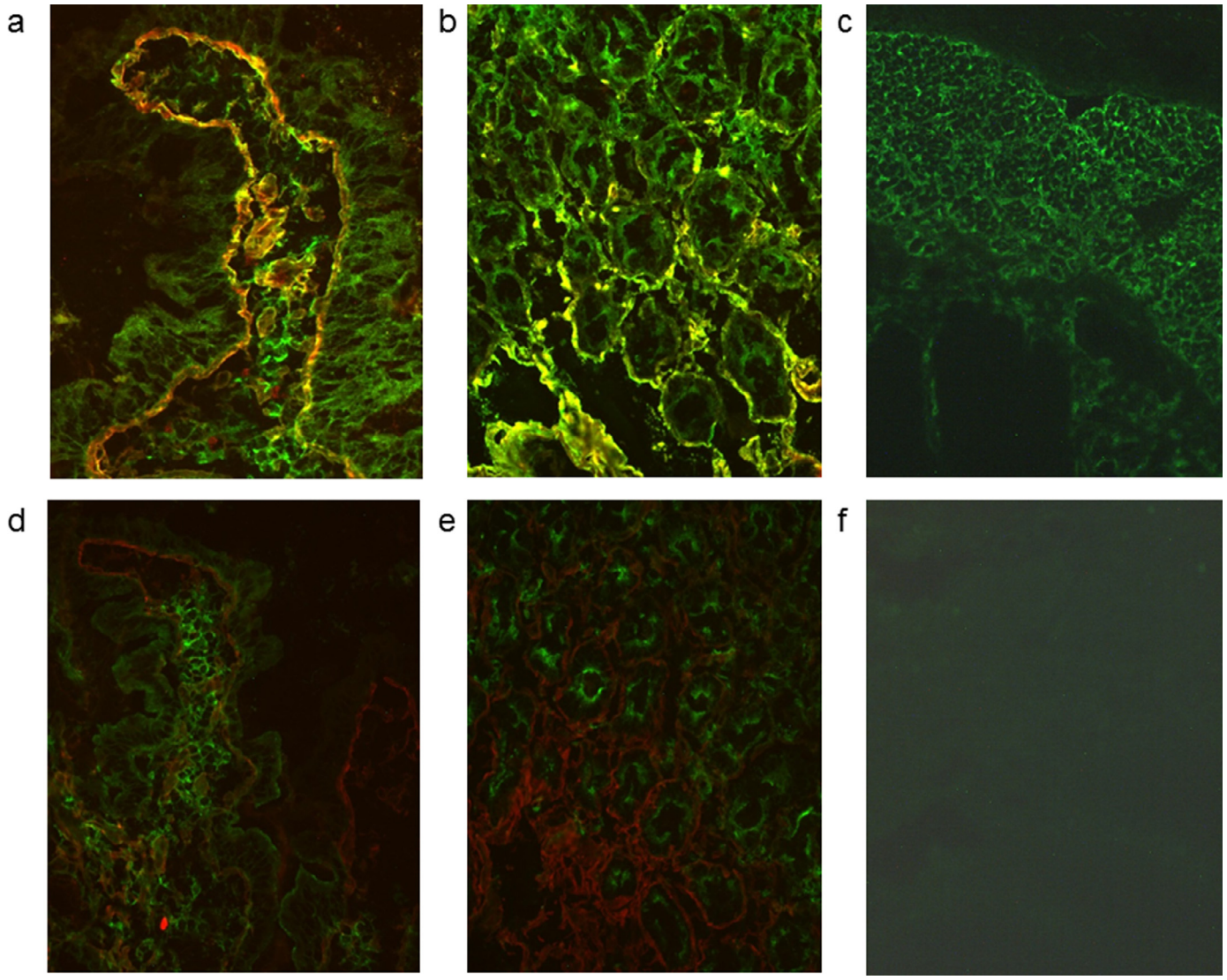

Fig. 1. Intestinal IgA anti-tTG deposits ((a) yellow spots in sub-epithelium, and (b) around crypts) and intestinal AEA in culture supernatant ((c) fluorescent network) at the diagnosis and disappearance of both specific fluorescent signals ((d),(e) intestinal IgA anti-tTG deposits, and (f): intestinal AEA in culture supernatant) after 12 months of gluten-free diet.

CD). This is in agreement with our recent observation [10] in which 10% of CD patients (particularly those positive for anti-tTG serum concentrations but with low values) showed intestinal antibodies only in the duodenal bulb while the distal duodenum was completely negative. In general, intestinal antibodies are measured in distal duodenal biopsies and for this reason, false negative results have been obtained in patients with both classical [27] and potential CD [7]. Moreover, the duodenal bulb is also the site that should be investigated for histological analysis, since at the time of diagnosis, from 2.4 to 10% of untreated CD patients had intestinal lesions confined to the duodenal bulb both in childhood [28–30] and adulthood [31]. In endoscopic practice, analysis of the duodenal bulb has positive implications for patients, allowing earlier commencement of GFD and reducing the number of unnecessary medical investigations associated with delayed diagnosis of CD [10,31].

More in general, testing for the presence of intestinal CD-antibodies not only made prompt diagnosis of CD possible, but when used together with histological analysis, enabled us to exclude CD in 6% of symptomatic genetically predisposed subjects negative for serum auto-antibodies, even though we are aware that a longer follow-up of these subjects is necessary.

The intestinal anti-tTG antibodies were found not only in subjects with classical or potential CD but also in symptomatic pre-potential coeliac disease. One concern about the diagnosis of potential and pre-potential CD is whether or not a strict GFD is required for these patients. There is evidence that CD-related clinical conditions (such as anaemia, diarrhoea and failure to thrive) are associated with gluten-dependent immunological inflammation at the intestinal level even in absence of intestinal atrophic lesions [6,32]. The clinical condition of our patients with potential or pre-potential CD improved on a GFD, in line with the improvement seen in patients with classical CD. Moreover, after GFD was commenced, there was a significant decrease in anti-tTG serum concentrations among the patients with potential CD and the disappearance of intestinal anti-tTG among patients with pre-potential CD, indicating that the gluten-dependent immune cascade had been turned off. These observations confirm that GFD cured CD-associated symptoms in patients testing positive for intestinal anti-tTG without intestinal lesions, supporting the idea that is not necessary (or wise) to wait for the onset of severe enteropathy before starting the most rational diet-therapy [5,6,9]. On the other hand [33,34] asymptomatic potential CD patients with intestinal anti-tTG deposits on GCD are more likely to develop intestinal

damage than those without. This reinforces the diagnostic value of this marker that should be investigated in routine diagnostics.

The prevalence of different CD-clinical types in children enrolled in our study was higher than expected for routine endoscopy practice. This may have resulted in an overestimate of the prevalence of potential, and in particular, pre-potential CD, which has been described here for the first time. This was a result of a referral bias to a centre with a specialist interest in CD recruiting patients from all the North Italian regions. However, these clinical conditions enabled us to test the reliability of the two immunological assays in presence of different versions of CD.

In conclusion, the current diagnostic criteria, based on conventional histology, are inadequate to identify the whole spectrum of CD that is much wider than described so far. The intestinal CD auto-antibodies should be investigated in all suspected CD patients who underwent GI endoscopy in order to identify potential and pre-potential CD patients. Regarding the pre-potential condition, multicentre and prospective studies are urgently needed to confirm the presence and the natural history of this new version of CD that we have identified by the intestinal CD auto-antibodies. Moreover, the biopsy culture AEA assay, that in our hands seems an effective diagnostic tool, should be applied in real field by any gastroenterology unit to further demonstrate the applicability in CD routine diagnostics.

Declaration of competing interest

All authors declare no competing interest.

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