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Alimentary Tract

IgA anti-actin antibodies ELISA in coeliac disease: A multicentre study

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Abstract

Background. Previous studies have demonstrated that serum anti-actin antibodies are a reliable marker of intestinal damage severity in coeliac disease.

Aims. To validate in a multicentre study the clinical usefulness of serum IgA anti-actin antibody ELISA and its possible use in monitoring intestinal mucosa lesions during gluten-free diet.

Patients and methods. Four centres recruited 205 newly diagnosed coeliac disease patients with villous atrophy, 80 healthy controls and 81 "disease" controls. Twelve coeliac disease patients on gluten-free diet but with persistent symptoms underwent serum IgA anti-actin antibody assay and intestinal histology evaluation. IgA anti-actin antibody ELISA was performed with a commercial kit. All coeliac disease patients underwent intestinal histology study.

Results. IgA anti-actin antibodies showed a sensitivity of 80% and a specificity of 85% in the diagnosis of coeliac disease patients with villous atrophy. The area under the receiving operator curve for anti-actin antibodies was 0.873 [95% C.I. 0.805–0.899]. Serum anti-actin antibodies values were significantly higher in coeliac disease patients than in healthy or "disease" controls ($P < 0.0001$). Serum anti-actin antibodies were positive in 41 of the 60 coeliac disease patients with mild intestinal histology lesions (69%) and in 123 of the 145 with severe lesions (85.3%) ($P < 0.05$). There was a significant inverse correlation between anti-actin antibody values and the villi/crypts ratio ($r = -0.423$; $P < 0.0001$). In the 12 coeliac disease patients on gluten-free diet who underwent re-evaluation as they were persistently symptomatic, intestinal histology showed three cases with persistent villous atrophy: all of these were positive for serum anti-actin antibodies ELISA, whereas both serum anti-tTG and EmAs were negative. The other nine patients showed normal intestinal villi and were negative for serum anti-actin antibodies.

Conclusions. Anti-actin antibodies are a reliable marker of severe intestinal mucosa damage in coeliac disease patients and a simple ELISA technique offers an accurate method for their determination. These antibodies seem to be a very reliable marker of persistent intestinal damage in coeliac disease patients.

Keywords: Anti-actin antibodies; Coeliac disease; Intestinal histology

1. Introduction

Coeliac disease (CD) is an immune-mediated enteropathy triggered by gluten ingestion in genetically predisposed individuals. It is nowadays evident that CD is one of the

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most common life-long diseases, with a frequency around 1:100–1:200 in the general population [1–3]. The enormous increase in CD diagnoses in recent decades is in large part due to the availability of serum tests with excellent diagnostic accuracy, such the anti-endomysium and anti-transglutaminase assays [4–6]. However, these assays do not reflect the severity of the intestinal mucosa damage [7,8]. In this respect, the immunofluorescence (IF) assay of IgA anti-actin antibodies (AAA) has been suggested as a useful method [9,10] and we recently described an ELISA which demonstrated to be an accurate assay for their determination [11].

This multicentre study was designed to evaluate the clinical usefulness of serum IgA AAA ELISA in a greater number of CD patients and its possible use in monitoring intestinal mucosa lesions during gluten-free diet.

2. Patients and methods

CD patients were recruited in four centres (three for adults and one for paediatric CD) between January and December 2004. A total of 205 CD patients were enrolled. IgA AAAs were assayed on the stored sera of 155 adult CD patients (50 M, 105 F, median age 24 years, range 18–76 years) and 50 paediatric CD patients (22 M, 28 F, median age 16 months, range 1–14 years). The sera were collected at CD diagnosis, after overnight fasting, and frozen at −80 °C for a mean time of 14 months (range 12–24 months) before AAA determination. A previous study had shown that preservation at −80 °C did not significantly alter results (inter-assay coefficient of variation was 8.5%) [11]. The CD clinical presentation was typical (malabsorption syndrome) in 105 patients, atypical (absence of intestinal symptoms) in 85 and silent in 15 (patients identified through screening programs) [12].

In all patients, CD diagnosis was based on serum EmA and anti-tTG positivity associated with evidence of intestinal villi damage (villous height/crypt depth ratio <3), and in all cases on a subsequent gluten-free diet clinical symptoms disappeared and EmA and anti-tTGs became negative. As "healthy" controls we enrolled 80 subjects (54 adults evaluated for suspected hypercholesterolaemia: 25 M and 29 F, median age 29 years, range 18–59 years; and 26 children with recurrent pharyngo-tonsilitis, 10 M and 16 F, median age 2 years, range 1–11 years). None of the controls had symptoms or laboratory signs suggesting CD diagnosis and all were negative for EmA and anti-tTG assays. A further 81 adult subjects with autoimmune or gastrointestinal diseases other than CD were enrolled as "disease" controls for IgA AAA assay: type 1 autoimmune hepatitis (4 cases), systemic lupus erythematosus (5 cases), Sjogren's disease (4 cases), primitive biliary cirrhosis (2 case), active Crohn's disease (40 cases), active ulcerative colitis (20 cases), multiple food intolerance (6 cases). All these patients were negative for EmA and anti-tTG assays.

Finally, 12 CD patients (4 M, 8 F, median age 25 years, range 18–49 years) on gluten-free diet for a median time of 20 months (range 14–48 months), but with persistent symptoms, underwent serum IgA anti-tTG and AAA assays and gastroduodenoscopy with multiple duodenal biopsy.

Informed consent was obtained from the adult patients involved in the study and from the parents of the paediatric patients. The study was approved by the Ethics Committee of the University Hospital of Palermo.

2.1. Serology for CD diagnosis and IgA AAA indirect immunofluorescence assay

IgA EmAs and anti-tTGs were assayed with commercial kits as previously described [6].

IgA AAA enzyme immunoassay (ELISA) was performed in the laboratory of the "Buccheri La Ferla Hospital" of Palermo, using a commercial kit for anti-actin IgG determination (F-Actin Smooth Muscle, INOVA, San Diego, California, US, ref. 708785) and an anti-serum anti-human IgA conjugate (INOVA, ref. 508549). A 1:101 dilution of serum sample from each patient was prepared and 100 μL of the diluted serum was added to the wells and incubated for 30 min at room temperature. The wells were washed three times with buffer. Hundred microlitres of the IgA conjugate was added to each well and the plates were incubated for 30 min and washed again. Hundred microlitres of TMB chromogen was added to each well and incubated in the dark for 30 min at room temperature. Stopped reactions were read at 450 nm and 620 nm. The control wells (no serum) introduced in each plate showed an absorbance of <0.025. AAA results were expressed as arbitrary units (AU) in comparison with laboratory standards. The reference curve was obtained by pooling 20 sera from patients highly positive for AAAs (positive controls), evaluated by IF, whereas 20 sera negative for AAAs from healthy controls were mixed and used as negative controls. The threshold normal value was established at 5.8 AU, which was equal to the mean value +3 S.D. obtained in healthy subjects.

The examiners were unaware of the diagnoses and the other laboratory test results. Intra- and inter-assay coefficients of variation (CV) were calculated on 20 samples. The method showed a high reproducibility: intra-assay CV 3.4% and inter-assay CV 8.8%.

2.2. Intestinal histology

At least three biopsy specimens of the second part of the duodenum were obtained from each CD patient and orientated as previously described [6]. Specimens were embedded in paraffin. Slides were stained with haematoxylin and eosin and graded by conventional histology according to Corazza and Villanacci [13] as normal villi and crypts (Grade A), partial villous atrophy (Grade B), total villous atrophy (Grade C). Furthermore, the ratio between villous height and crypt depth was calculated for each sample. Histology

was described by an examiner unaware of the laboratory test results.

3. Statistical analysis

We followed the STARD checklist for studies on the diagnostic accuracy of tests [14].

The percentage (and 95% C.I.) of IgA AAA positive results was calculated in the CD patients, controls and in the CD patients with partial or total villous atrophy. The chi-square test was used for frequency analysis. The Mann–Whitney *U*-test was used to compare the serum IgA AAA ELISA results in CD patients and in the control subjects. Spearman's *r* correlation coefficient was used to evaluate the association of serum AAA values with severity of intestinal mucosa damage (villi/crypts ratio). In addition, receiving operator curves (ROCs) were plotted to show the discriminative ability of the tests. The model plots sensitivity (the proportion of CD patients attributed by the test to the CD group) versus 1-specificity (the proportion of control patients attributed by the tests to the CD group). The areas under the ROC curves and their 95% confidence intervals were also calculated using the non-parametric method described by Hanley and McNeil [15], developed as a statistical program.

4. Results

Fig. 1 shows the individual IgA AAA values in the study groups. Serum IgA AAA evaluated by ELISA were positive in 164 of the 205 (80%, 95% C.I. 72.2–87.8) untreated CD patients. Twenty-nine adults and 12 children with CD were negative. Among the 80 healthy controls, 1 subject was positive for serum AAAs, whereas in the group of "disease controls" 23 of the 81 patients were positive. On the basis of the above results, AAAs showed a sensitivity of 80% (95% C.I. 73–88.6%) and, considering as a single

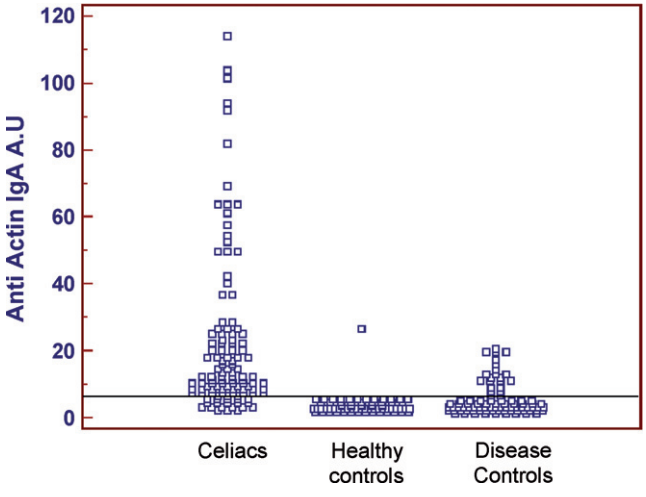

Fig. 1. Individual serum IgA AAA values in the three studied groups.

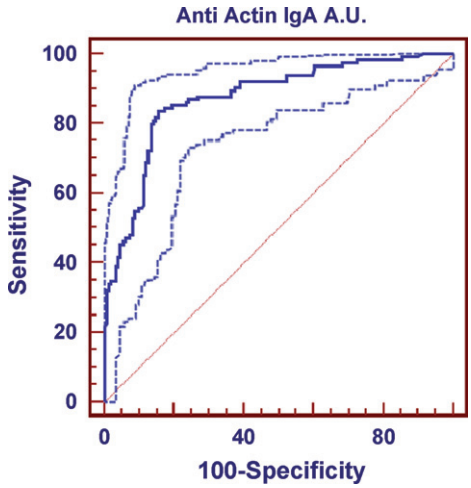

Fig. 2. Receiving operator curve (ROC) for serum IgA anti-actin performed after cumulative analysis of the data from CD patients and the whole group of control (healthy plus "disease" controls).

group both the healthy and "disease" controls, a specificity of 85% (95% C.I. 76–91%). The specificity went down to 72% when exclusively the "disease controls" were considered. After performing a cumulative analysis of the data from the patients and healthy plus "disease" controls, we plotted the ROC curve for AAAs: the area under curve was 0.873 [95% C.I. 0.805–0.899] (Fig. 2). However, when considering in the cumulative analysis the data from the CD patients and from the patients with non-CD autoimmune diseases or enteropathy ("disease" controls), the area under the curve was significantly lower: 0.836 (95% C.I. 0.778–0.885; $P = 0.05$).

Serum AAAs values were significantly higher in the CD patients (median value 17.7 AU, range 3–111) than in healthy controls (median value 2.4 AU, range 1.1–23.3) ($z = 13.34$, $P < 0.0001$) and "disease" controls (median value 3.8, range 1.1–21.6 AU) ($z = 9.32$, $P < 0.0001$).

Intestinal histology of the CD patients revealed that 60 had partial villous atrophy (Grade B) and 145 had total villous atrophy (Grade C). Serum AAAs were positive in 41 of the 60 CD patients with Grade B lesions (69%, 95% C.I. 59.9–78%) and in 123 of the 145 with Grade C lesions (85.3%, 95% C.I. 78.4–92.4) ($P < 0.01$). Table 1 resumed the percentage of positive IgA AAA in the study groups. Furthermore, there was a significant inverse correlation between AAA values and the villi/crypts ratio in each patient with $r = -0.423$ ($P < 0.0001$). Anti-tTG values and EmA titer showed a significant but lower inverse correlation with severity of intestinal mucosa damage (for anti-tTGs: $r = -0.381$; for EmAs: $r = -0.328$).

Table 2 summarizes the clinical characteristics, intestinal histology findings and serum assay results of the 12 CD patients on gluten-free diet who underwent re-evaluation as they were persistently symptomatic. Intestinal histology showed three cases with persistent villous atrophy (total in one patient and partial in two): all these were positive for serum AAAs ELISA, whereas both serum anti-tTG and EmAs were negative. These three patients declared they

Table 1
Number of cases and percentages of positive IgA anti-actin antibodies in the different study groups: coeliac patients (according to the grade of intestinal damage), healthy controls and "disease" controls

CD with partial villi atrophy	CD with total villi atrophy	Healthy controls	"Disease" controls
41/60 (69%)	123/145 (85.3%)	1/80 (1.3%)	23/81 (28%)

were strictly adhering to the gluten-free diet. The other nine patients showed normal intestinal villi, although most of them showed an elevated number of intra-epithelial lymphocytes. All these nine cases had negative serum AAAs, anti-tTG and EmAs.

5. Discussion

The best serological approach to diagnose CD is based on the EmA and anti-tTG assays, which have a very high diagnostic accuracy [4–6,16,17]. However, these serological tests do not correlate with histopathological features [8,18] and only recently another test – the AAA assay – has been proposed as a marker of the severity of intestinal mucosa damage in CD [9,10].

In the present multicentre study we assayed IgA AAAs with an ELISA which had demonstrated to be an accurate method for AAA determination [11]. Our results showed that in a group of CD patients, all positive for EmA and anti-tTG assays, serum IgA AAAs had 80% sensitivity and 82% specificity, thus we confirmed that they are less accurate than the traditional assays in CD diagnosis. Furthermore, it must be underlined that all the CD patients included in this study had various degree of villi atrophy and, as regards the test specificity, it is known that serum AAAs can be detected in patients with autoimmune diseases and in healthy subjects [19]. However, one study reported cases of CD patients negative for serum EmA and positive for IgA AAAs and suggested that this combination, EmA−/AAA+, could indicate complicated CD cases [20]. Others suggested that serum IgA AAA assay associated with other antibodies assays could substitute the EmA assay [21]. Our results, however, are in agreement with those reported by other Italian studies which showed that IgA AAA assay cannot replace EmA and anti-tTG in the diagnostic algorithm of CD [10,22]. Furthermore, we confirmed that serum AAA positivity is indicative of more severe intestinal histology damage. In fact, serum AAAs were found more frequently positive in the CD patients with total villous atrophy (85%) than in CD patients with partial villous atrophy (69%) and there was a significant inverse correlation between IgA AAA values and the villi/crypts ratio ($P < 0.0001$) with a correlation coefficient value higher than those shown by anti-tTG and EmA values.

Another important finding was IgA AAA positivity in the CD patients on gluten-free diet who showed persistent symptoms associated with intestinal mucosa damage. Interestingly, these patients declared they were adhering to the gluten-free diet and both anti-tTG and EmA assays were negative. Although we cannot explain the causes of the persistent histology damage at 14–20 months after commencement of the gluten-free diet, it is relevant that only IgA AAA assay correctly identified these patients and this assay was negative in all the other CD patients with persistent symptoms but contemporary evidence of complete intestinal villi recovery. This seems to confirm that in CD, serum IgA AAAs can be considered a direct expression of severity of intestinal damage and monitoring them could be useful to detect intestinal histology recovery or damage persistence.

IgA AAA ELISA, re-evaluated in the context of a multicentre study, confirmed to be a simple and reliable method. In fact, it was able to detect the antibodies which react against F-actin, the polymerized form of actin which *in vitro* study

Table 2
Clinical characteristics, intestinal histology findings and serum assay results of the 12 CD patients on gluten-free diet who underwent re-evaluation

Patient	GFD duration	Symptoms causing the re-evaluation	Intestinal histology	Serum anti-tTG	Serum EmA	Serum AAA
1	20	Anaemia, diffuse abdominal pain	Grade B	Negative	Absent	Positive
2	48	Dyspepsia	Grade A	Negative	Absent	Negative
3	20	Dyspepsia, anaemia	Grade A	Negative	Absent	Negative
4	14	Weight loss	Grade C	Negative	Absent	Positive
5	19	Anaemia	Grade A	Negative	Absent	Negative
6	17	Dyspepsia	Grade A	Negative	Absent	Negative
7	15	Diarrhoea, anaemia	Grade B	Negative	Absent	Positive
8	18	Diarrhoea, anaemia	Grade A	Negative	Absent	Negative
9	30	Dyspepsia	Grade A	Negative	Absent	Negative
10	32	Dyspepsia	Grade A	Negative	Absent	Negative
11	23	Anaemia	Grade A	Negative	Absent	Negative
12	24	Dyspepsia, anaemia	Grade A	Negative	Absent	Negative

Notes: (1) GFD: gluten-free diet; anti-tTG: anti-transglutaminase; EmA: anti-endomysium; AAA: anti-actin. (2) Duration of the GFD diet is given in months. (3) Severity of the intestinal histology damage is given as follows: normal villi and crypts (Grade A), partial villous atrophy (Grade B), total villous atrophy (Grade C) (see reference [13]).

[23] has shown to be induced in the intestinal epithelial cells of CD patients minutes after gliadin incubation.

In conclusion, we confirmed that IgA AAAs are a reliable marker of severe intestinal mucosa damage in CD patients and that a simple ELISA technique offers an accurate method for their determination. These antibodies seem to be a very reliable marker of persistent intestinal damage in CD patients.

Practice points

- IgA AAAs have been shown in a high percentage of CD patients with severe intestinal histology damage.
- A simple AAA ELISA showed high sensitivity in CD patients, comparable with that reported using the "classical" immunofluorescence technique.
- Using AAA ELISA the frequency of positive assays correlates with the severity of the intestinal damage.
- IgA AAA seems to be a reliable marker of persistent intestinal mucosa damage in CD patients on gluten-free diet.

Research agenda

- Prospective studies on a greater number of CD patients on gluten-free are needed to better understand the usefulness of AAA assay in monitoring the state of the intestinal mucosa.
- Further studies would be performed to evaluate whether the association of AAA assay with EmA and/or anti-tTG improves the diagnostic accuracy of serologic tests in CD diagnosis.

Conflict of interest statement

None declared.

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