

Significance of smooth muscle/anti-actin autoantibodies in celiac disease

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Summary

Background/aim: Smooth muscle antibody (SMA) specific for the protein actin, a major component of the cytoskeleton of epithelial cells, is one of the most prevalent non-organ specific autoantibodies in the serum of celiac disease (CD) patients. Our aim was to explore the clinical relevance of the presence of IgA type anti-actin antibody (AAA) and SMA in a series of patients with CD. **Methods:** We evaluated frozen serum samples collected at diagnosis from 92 adult patients with CD and 52 control individuals in whom CD was excluded. Patients were re-evaluated a median time of 5 yr after treatment. IgA type AAA was detected using a modified commercial ELISA assay and IgA SMA was detected using indirect immunofluorescence on primate esophagus substrate. **Results:** At diagnosis, samples from CD patients had significantly higher AAA values than controls ($p < 0.00001$). While all active CD patients had serum AAA values over the cut-off for healthy controls, we observed a very significant reduction of these antibodies after treatment ($p > 0.0001$). AAA had a highly significant correlation with both, tissue, transglutaminase ($r = 0.62$) and antigliadin ($r = 0.60, p < 0.00001$) antibodies as well as the severity of the intestinal injury ($p < 0.05$). SMA was detected in sera of 35 consecutive CD patients. At diagnosis, SMA positive patients had significantly higher values of AAA ($p < 0.0002$), increased number

of autoimmune disorders ($p < 0.04$), delayed menarche ($p < 0.04$), lower hemoglobin levels ($p < 0.01$), increased fecal α -1 antitrypsin clearance ($p < 0.01$) and more severe diarrhea ($p < 0.06$). We also detected a trend to more severe complications at follow-up ($p = 0.059$).

Conclusions: Based on our findings we suggest that the presence of increased IgA AAA serum levels is a highly sensitive marker of the disturbed architecture of intestinal epithelial cells of CD patients with a potential relevance to diagnosis and follow-up. The presence of SMA seems to define a distinct subset of CD patients with a more severe clinical outcome.

Index (palabras claves): Celiac disease, autoimmunity, anti-actin antibodies, smooth muscle antibodies, gluten-free diet.

Resumen

Significado de los anticuerpos anti-músculo liso/anti-actina en la enfermedad celíaca

Introducción/objetivo: El anticuerpo anti-músculo liso (SMA) dirigido contra la proteína actina, un componente mayor del citoesqueleto de las células epiteliales, es el anticuerpo no-órgano específico más prevalente en enfermedad celíaca (EC). Nuestro objetivo fue explorar la importancia clínica de los anticuerpos anti-actina (AAA) y SMA en una serie de pacientes con EC. **Métodos:** Evaluamos 92 muestras serológicas de pacientes celíacos adultos recolectadas al momento del diagnóstico y la de 52 individuos controles no celíacos. Los pacientes fueron re-evaluados luego de un tiempo medio de 5 años en tratamiento. Evaluamos AAA tipo IgA mediante ELISA empleando un equipo comercial modificado y SMA IgA por inmunofluorescencia indirecta sobre sustrato de esófago de mono. **Resultados:** Al momento del diagnóstico, los pacientes celíacos tu-

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vieron valores de AAA significativamente más elevados que los controles ($p < 0.00001$). Todos los pacientes con EC activa presentaron niveles de AAA por encima del valor de corte determinado para el grupo control sano y se evidenció una reducción significativa de los niveles luego del tratamiento ($p > 0.0001$). Los AAA presentaron una correlación significativa con los anticuerpos anti-transglutaminasa tisular ($r = 0.62$) y anti-gliadina ($r = 0.60$) ($p < 0.00001$), de igual modo que con la severidad del daño intestinal ($p < 0.05$). Al momento del diagnóstico, se detectó SMA en el suero de 35 pacientes no controles. Los pacientes SMA positivos tuvieron valores significativamente mayores de AAA ($p < 0.002$), un incremento del número de enfermedades autoinmunes asociadas ($p < 0.04$), menarca tardía ($p < 0.04$), niveles bajos de hemoglobina ($p < 0.01$), incremento del clearance de α -1 antitripsina fecal ($p < 0.01$) y mayor severidad de la diarrea ($p < 0.06$). En ellos se evidenció una tendencia al desarrollo de complicaciones más severas durante el seguimiento ($p = 0.059$). **Conclusiones:** Sugerimos que la presencia de un valor sérico aumentado de AAA tipo IgA podría ser un marcador altamente sensible de las alteraciones histoarquitecturales asociado a la enteropatía celiaca con potencial relevancia en el diagnóstico y seguimiento. La presencia de SMA parece definir un subgrupo de pacientes celíacos con un pronóstico clínico más severo.

Introduction

Celiac disease (CD) is an enteropathy triggered by the ingestion of gluten by genetically susceptible individuals. The frequent association with other autoimmune disorders, the characteristic genetic background, and the immunopathologic features, among others factors, suggest that autoimmunity could have an important role in the pathogenesis of the disease.¹⁻³ Sera of the vast majority of CD patients have abnormal levels of circulating antibodies to foreign alimentary antigens, on the one hand, and against self-antigens, on the other hand.⁴ Some of these antibodies, especially anti-tissue transglutaminase and endomysial autoantibodies (a-tTG and EmA, respectively), are currently used for diagnostic screening, and follow-up purposes.⁵ Recently, Dieterich et al.⁶ identified the endogenous enzyme tissue transglutaminase (tTG) as the common autoantigen eliciting both a-tTG and EmA antibodies.

Several other autoantibodies recognizing different tissue antigens may be observed in serum of patients with CD.^{7,8} Among others, anti-thyroid microsomal, anti-gastric parietal cell, anti-mitochondrial, anti-B cells of the pancreatic islets, etc, have been detected with some frequency.⁹⁻¹² It has been hypothesized that these autoantibodies may indicate the presence of underlying or coexisting autoimmune disorders such as thyroiditis, primary biliary cirrhosis, and type I diabetes.⁹ Furthermore, non organ-specific autoantibodies can also be detected in serum of CD patients. In this context, anti-smooth muscle (SMA) and antinuclear antibodies are the most often detected autoantibodies which, up to now, have had a doubtful significance.^{13,14} Furthermore, it has also been observed that SMA may be apparent not only at the time of diagnosis but also while patients are on gluten-free diet.¹⁴ Interestingly, in some cases the detection of EmA antibody using indirect immunofluorescence on primate esophagus substrate is obscured by interfering fluorescence generated by SMA.¹³ Testing SMA positive sera at progressively higher serum dilutions has been used to unmask EmA that otherwise may escape detection. This procedure, however, is still not able to detect EmA in all cases.¹⁴

Antibodies to smooth muscle antigens (of the IgG isotype) are a common and non-specific finding often detected in several clinical disorders. They can be observed in 40% to 70% of patients with chronic active hepatitis and autoimmune hepatitis, in 50% of patients with primary biliary cirrhosis and in up to 28% of patients with cryptogenic cirrhosis.^{15,16} Furthermore, these antibodies can also be found in patients with several disorders and in approximately 2% of the normal population.¹⁶ The highest titers of SMA are found in autoimmune hepatitis cases and interestingly, in all these listed disorders the autoantibody is of IgG class and less frequently of the IgM class.¹⁷ A retrospective analysis of EmA tests in our lab allowed us to determine that 6.3% of serum samples from CD patients have a concomitant detection of SMA (unpublished observation). Furthermore, additional studies have demonstrated that in most SMA positive sera, the autoantibody is directed against the protein actin, a major component of the cytoskeleton of epithelial cells. Using immunofluorescence, Clemente et al¹⁸ have shown the presence of IgG and IgA anti-actin

antibodies (AAA) in serum of active CD patients. These AAA have been strongly linked with the severity of enteropathy and the persistence of the intestinal damage.¹⁸⁻²⁰

While it seems clear that AAA and SMA have little value for diagnosis of CD, they may represent nonspecific markers of disease that nevertheless may have a particular clinical or prognostic significance. Our objective in the present study was to analyze the presence of AAA in a series of consecutive CD patients at the time of diagnosis and after a period on a gluten-free diet and to elucidate the clinical relevance of AAA and SMA positive serology.

Material & methods

Subjects and controls

We evaluated clinical parameters and serum samples from 92 CD patients obtained at the time of diagnosis. All patients were clinically assessed at variable times after treatment (mean follow-up time: 5 yr, range: 1 to 9 yr). Serum samples from 32 of these patients were reevaluated when they were on treatment for a mean time of 5 yr (range: 4-8 yr). The patient population collected consisted in two different subgroups that were categorized according to the immunofluorescence evaluation of sera obtained at diagnosis. Group A consisted of 35 consecutively patients in whom a positive SMA was detected, and group B consisted of 57 consecutive patients evidencing EmA as the only CD-related autoantibody.

Diagnosis of CD was based on the combination of currently accepted clinical, histological and serological criteria.⁵ At diagnosis, all patients exhibited a characteristic celiac enteropathy (Marsh's classification).²¹ Diagnosis of CD in patients with a negative EmA test and normal anti-tTG values was only confirmed when a post-treatment (gluten-free diet) biopsy showed improvement of the mucosal architecture. Diagnosis of CD in patients with minor enteropathy (Marsh types 0 or I) was only established in the presence of dermatitis herpetiformis. Clinical data of patients at diagnosis and after treatment were obtained both from files and clinical interviews with all information collected on pre-designed specialized forms. Diagnosis of autoimmune disorders other than CD were performed by specialized physicians and based on clinical and bio-

chemical evidences and a concomitant specific serology. Intestinal biopsies (n>3 per procedure) were performed in the distal duodenum by upper videoendoscopy using endoscopic forceps. Duodenal histology was categorized according to Marsh's classification in types 0, I, II, IIIa, IIIb, IIIc and IV and an intraepithelial lymphocyte count (number of lymphocytes per 100 epithelial cells) was also reported.

We also evaluated serum samples from healthy control individuals (blood donors, n=35), patients with inflammatory bowel disease (n=10) and patients with chronic liver disease (primary biliary cirrhosis and autoimmune hepatitis, n=7).

Celiac disease-related serology

Antigliadin antibodies types IgA and IgG were determined using an enzyme-linked immunosorbent microassay (micro-ELISA) (INOVA Diagnostics Inc San Diego, CA, USA) as previously described.²² In our laboratory, the upper normal limit was 20 AU/mL for both AGA-IgA and AGA IgG. Endomysial antibody (EmA) was determined by the immunofluorescence method using monkey esophagus as substrate (INOVA Diagnostics Inc San Diego, CA, USA).²² While the presence of a thin fluorescence network around the smooth muscle fibers was considered EmA positive, SMA positive samples gave a homogeneous fluorescence of the whole myofibril. Serum samples were initially tested at 1:5 dilution with phosphate buffer. For the purpose of determining if the presence of SMA immunofluorescence was masking the underlying EmA fluorescence, we gradually increased serum dilutions (from the initial dilution of 1:5) until SMA immunofluorescence disappeared. IgA anti-transglutaminase antibodies were determined using a commercial native human red blood cell tTG ELISA assay (QUANTA Lite h-tTG IgA, INOVA Diagnostics Inc San Diego, CA, USA).

Other serological tests

IgA type AAA antibodies were determined using a modification of a commercial ELISA assay for IgG type anti-actin antibodies (QUANTA Lite Actin, INOVA Diagnostics Inc San Diego, CA, USA) and an anti-serum anti-human IgA conjugate (INOVA Diagnostics Inc San Diego, CA, USA). Serum samples were studied at 1:501 dilution. Serum samples

from a selected subset of CD patients (29 SMA positive patients and 20 SMA negative patients) were also tested for the following autoantibodies: anti-thyroglobulin, anti-thyroid microsomal, anti-gastric parietal cells, liver and kidney anti-microsomal, anti-mitochondrial, anti-nuclear, SMA, anti-neutrophilic (ANCA) and anti-b-cells of pancreas. Serum samples diluted 1:10 were incubated with cryostat-cut sections of human thyroid, human pancreas, rat liver, stomach and kidney and fixed preparations of cultivated human epithelial cells (Hep-2, Immunoconcepts, USA) and neutrophils (The Binding Site, UK) for immunofluorescence assays using FITC conjugate to human IgA.

Statistics

Data are presented as mean \pm SEM or 95% confidence interval or median and range as appropriate. The statistical analysis was performed using the Statistix 7 for Windows Analytical Software

(Tallahassee, FL, USA). Comparisons between groups were performed using the chi-square test or exact Fisher's test for qualitative variables and Mann-Whitney U test and Student t test for quantitative variables. Serum AAA values obtained at diagnosis and after treatment were compared using the Wilcoxon's matched pair test. Spearman's correlation coefficient was used to evaluate associations between AAA and either a-tTG or AGA tests. The actuarial percentage of patients with autoimmune disorders was estimated by the Kaplan-Meier curve, and differences were assessed by the Cox-Mantel log-rank test.

Results

Clinical features of patients

Table 1 shows epidemiological data, clinical characteristics and serology results of CD patients and control groups assessed at the time of diagnosis.

Table 1. Epidemiological data and clinical and serological characteristics of patients and controls. BMI: body mass index; EmA: endomysial antibody; a-tTG: native human red blood cells tissue transglutaminase antibodies; AAA: anti-actin antibodies.

	Celiac disease patients	Inflammatory bowel disease	Controls Chronic liver disease	Healthy individuals
n	93	10	7	35
Gender (F/M)	70/23	5/5	7/0	20/15
Age at entry the Study				
Median (range) yr	43 (22-80)	33 (20-64)	53 (42-62)	34 (19-52)
Age at diagnosis				
Median (range) yr	39 (14-75)			
BMI				
Mean \pm SEM. K/m ² .	19.2 \pm 0.3			
EmA				
N of +ve cases (%)	84 (90)	0/5	0/5	0/35
a-tTG				
N of +ve cases (%)	87 (94)	0	0	0
a-tTG serum values				
median (range)	170 (1-369)	0	0	0
AAA n of cases				
>110 AU/mL (%)	88 (95)	5/5	1/5	1/35
AAA serum values				
median (range)	744 (123-2671)	128 (52-197)	78 (34-221)	(5-140)
Hemoglobin				
Mean \pm SEM g%	11.9 \pm 0.2	-	-	-
Serum albumin				
Mean \pm SEM g%	3.45 \pm 0.07	-	-	-
Serum calcium				
Mean \pm SEM g%	8.3 \pm 0.1	-	-	-

Clinical features at presentation allowed the classification of 64 patients as classically symptomatic (mainly GI symptoms), 19 patients as atypical CD (no GI symptoms), and 9 as silent cases (no appreciable symptoms). At diagnosis, a concomitant CD-related serology confirmed diagnosis of CD in 83 patients (26 from the SMA positive group and all those from the EmA positive group). Positive serum values for a-tTG were determined in three of the nine EmA negative cases. Post-treatment biopsies for confirmation of CD were required in 6 patients. Microscopic examination of samples showed improved histology in all cases. At diagnosis, 83 of 92 (90.2%) patients of the overall population were EmA positive either at the initial dilution or after a progressive dilution of serum samples to eliminate concomitant SMA fluorescence. Positive a-tTG antibodies were detected in 86 of 92 (93.5%) serum samples obtained at diagnosis. Finally, IgA AGA was positive in 65 of 87 (75 %) patients assessed at diagnosis. While histological assessment of intestinal biopsies showed that minor abnormalities (Marsh's types I to IIIa) were detected in 10 patients, major mucosal damage was shown in 82 cases. Analysis of patient records revealed that four patients required use of steroids for failure to maintain clinically stable, six had severe complications, four developed malignancies, and six died.

Table 2 shows the comparative analysis of biochemical and serology parameters of a subset of 32

CD patients that were assessed at diagnosis and after a period on a gluten-free diet. Most parameters were significantly improved by treatment (p values between 0.04 and 0.0001). Serum albumin, hemoglobin concentration and serum calcium had a tendency to improve after treatment but this did not reach statistical significance.

Anti-actin antibodies

Healthy control individuals had very low levels of IgA AAA (median value: 43 AU/mL, range 5-140) with only one serum over 110 AU/mL. As shown in Table 1, disease controls had slightly greater, but non-significant median serum levels compared with healthy controls. All control patients with inflammatory bowel disease and one of five with chronic liver disease had mildly increased serum AAA values. Compared with the overall control population (median: 61 UA/mL, range: 5-221), serum samples from CD patients collected at diagnosis had highly significant greater mean values ($p < 0.00001$). Considering the upper cutoff for serum AAA levels in the healthy population ($X+2$ SD: 110 AU/mL), all patients (100%) assessed at diagnosis had abnormally increased values of the autoantibody. If we consider the upper end of the normal range (140 AU/mL) as the cutoff, 92.4 % (85 of 92) of patients had increased values of AAA. An unselected subset of patients ($n=32$) who were serologically reevaluated after treatment, showed a very significant

Table 2. Clinical, biochemical and serologic findings in a series of 32 patients assessed at diagnosis and after a mean time of 5 years on treatment.

Characteristic	At diagnosis	After Treatment	p
Age Median (range)	38 (14-63)	43 (22-68)	-
Sex F/M (n of cases)	22/10	22/10	-
BMI (mean \pm SEM)	19.1 \pm 0.5	21.4 \pm 0.2	0.002
EmA (n of +ve cases)	28	4	0.0001
AGA-A (n of +ve cases)	22	10	0.01
AGA-A Median (range) (AU/mL)	38 (4-240)	22 (1-70)	0.02
a-tTG (n of +ve cases)	30	8	0.0001
a-tTG Median (range) (AU/mL)	170 (1-369)	8 (1-200)	0.0001
AAA Median (range) (AU/mL)	988 (153-2671)	93 (7-1721)	0.0001
α -1AT CL Median (range) (mL/day)	44.3 (2-279)	11.6 (0.5-247)	0.0004
Hemoglobin Mean \pm SEM (gr %)	11.8 \pm 0.3	12.7 \pm 0.4	NS
Albumin Mean \pm SEM (gr %)	3.4 \pm 0.31	3.8 \pm 0.13	NS
Ca+ Mean \pm SEM (mg %)	8.2 \pm 0.2	8.6 \pm 0.1	NS
Prothrombin time. Median (range) (%)	71 (16-120)	97 (65-120)	0.04

decrease of serum levels of IgA AAA ($p < 0.0001$) (Figure 2). However, 14 samples (44%) still remained over the upper end for the healthy control group (110 AU/mL) at a mean time of 5 years after starting treatment (Table 2). Except for one patient, abnormally increased values in the post-treatment evaluation were very close to the cut-off for normal individuals. However, half of patients considered as

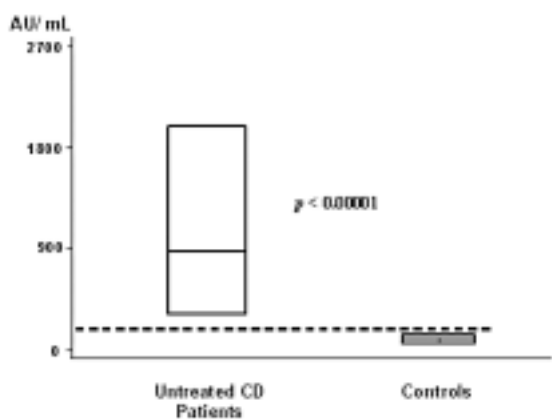


Figure 1. Distribution of data of serum AAA observed in patients and controls. Boxes represent to all data from samples included. The horizontal line into boxes represents the median value in the group. Horizontal dotted line represents the upper limit of normal AAA values (110 AU/mL).

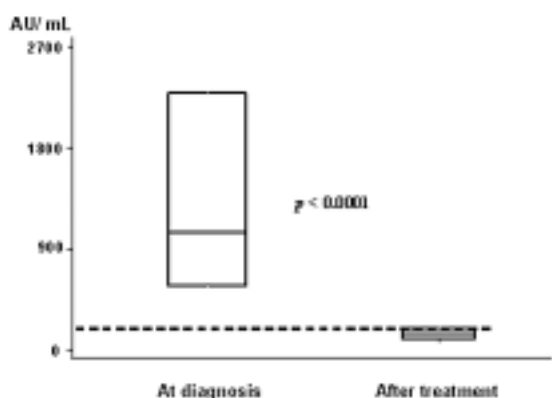


Figure 2. Distribution of data of serum AAA in a series of patients assessed at diagnosis and after a median time of 5 years after onset of treatment (range 1 to 9 yr). Boxes include data from samples. The horizontal line into boxes represents the median value in the group. Horizontal dotted line represents the upper limit of normal AAA values (110 AU/mL).

partially compliant or non-compliant with a gluten-free diet had increased serum values of AAA. According to the histological categorization (Marsh's classification) at diagnosis, patients with Marsh's type 0 to type IIIb ($n=8$) had significantly lower AAA serum levels (median 259 AU/mL; range: 123-825) compared to patients with types IIIb and IIIc ($n=84$) (831 Au/mL, range: 127-2671, $p < 0.05$) (Figure 3). A significant correlation was shown between AAA and a-tTG ($r: 0.62$, $p < 0.0001$), on one hand, and AGA type IgA ($r: 0.60$; $p < 0.0001$), on the other hand (Figures 4).

Smooth muscle antibody

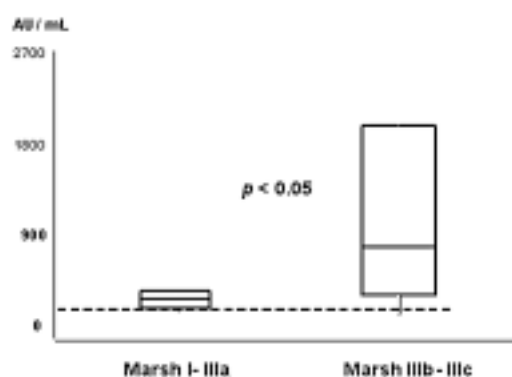


Figure 3. Distribution of AAA data of celiac disease patients assessed at diagnosis and grouped according to the severity of histological damage determined using Marsh's classification. The figure compares patients with enteropathy type I-IIIa vs. types IIIb and IIIc. Horizontal dotted line represents the upper limit of normal AAA values (110 AU/mL).

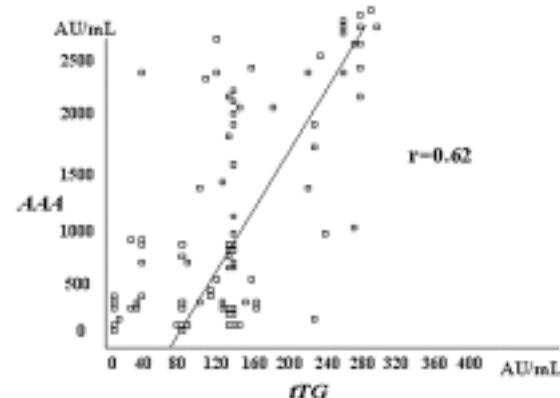


Figure 4. Correlation between serum AAA values and a-tTG.

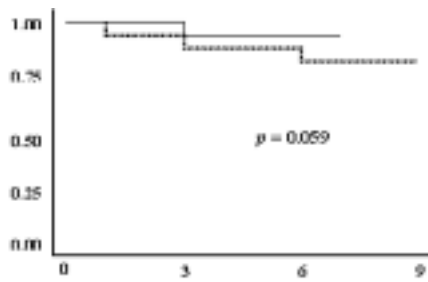


Figure 5. Kaplan-Meier estimates of the proportion of patients with complications in the follow-up (malignancies, refractory enteropathy and ulcerative jejunitis) of CD patients comparing SMA positive vs. SMA negative cases ($p=0.059$).

One of the study groups consisted of a series of consecutive patients in whom serum samples obtained at diagnosis of CD exhibited the presence of SMA. The antibody was also detected in two disease controls (one patient with IBD and another with chronic liver disease), but not in healthy controls. Interestingly, in this group of 35 patients SMA positive was heterogeneous in terms of presence of EmA. Thus, while in 9 patients EmA was not detected (and this negativity persisted despite increasing serum dilution), the presence of SMA was concurrently associated with EmA seropositivity in 26 samples. The epidemiological data and clinical characteristics of patients grouped according to whether SMA was present or were only EmA positives are shown in Table 3. An interesting finding was that compared with SMA positive patients, those presenting only EmA had a significantly greater number of first-degree relatives also affected with CD (odds ratio 5.9, 95% confidence interval 1.2 to 27.6, $p<0.03$). Furthermore, SMA positive patients had a significantly lower level of a-tTG ($p<0.02$), lower hemoglobin concentration ($p<0.02$), highly significantly increased serum AAA levels ($p<0.0001$), and increased fecal a1-antitrypsin clearance ($p<0.01$). While both subgroups had a similar number of patients with classical and atypical forms of the disease, a significantly lower numbers of silent cases ($p<0.03$) was observed among SMA positive patients. Fifteen patients presented autoimmune disorders at diagnosis with a signifi-

cantly greater prevalence in SMA positive cases (odds ratio 3.6, 95%CI 1.1 to 11.8, $p<0.04$). Four other cases developed autoimmune diseases during the course of the treatment of CD. While both groups had a similar number of cases with thyroid autoimmune disease or rheumatoid arthritis, SMA positive patients had an increased incidence of psoriasis (0.03) and autoimmune disorders ($p<0.03$) (Table 4). Despite the increased prevalence of autoimmune disorders in SMA positive patients at diagnosis, follow-up did not show any difference between groups (Cox-Mantel test $p=0.905$). Finally, histological characteristics of intestinal biopsies from both groups of patients did not show any difference in terms of Marsh's classification (data not shown). During follow-up, three SMA positive and one of cases only EmA positive required the use of corticosteroids because of progressive deterioration despite dietary measurements, and five from the SMA positive subgroup also received enteral and/or parenteral nutrition. Overall, six patients were complicated with lymphoma or refractory sprue (only one lymphoma case developed in a patient only EmA positive) (Cox-Mantel test: $p=0.059$) (Figure 5). All patients with lymphoma died.

Serum samples obtained at diagnosis from a random subset of 49 patients (29 SMA positives and 20 only EmA positive) were tested for several other autoantibodies. Using the Hep-2 cell substrate, we detected SMA-actin autoantibody fluorescence in 15 of 21 patients categorized as SMA positive (by using the monkey esophagus substrate), but also in 3 of 20 samples from only EmA positive cases. Other autoantibodies detected were: anti-thyroid, cANCA and pANCA and antinuclear.

Very interestingly, a subgroup of SMA positive CD patients ($n=9$) did not show any evidence of EmA seropositivity. As stated before, clinical and histological improvement after treatment with a gluten-free diet was required for a final CD diagnosed. Despite the fact that EmA was not detected after further dilution of sample, a-tTG serum levels were positive in three of the nine cases. While serum values of AAA were significantly greater than those of healthy controls ($p<0.000001$) and none of the patients had values below the cut-off (110 AU/mL), the mean value for this subgroup of patients was significantly lower than that of SMA positive/EmA

Table 3. Epidemiological, clinical, biochemical and serological parameters of celiac patients assessed at diagnosis comparing smooth muscle antibodies (SMA) positive (+ve) cases vs. those only EmA positive. AGA: antigliadin antibodies; a-tTG: anti-tissue transglutaminase; EmA: endomysial antibodies; AAA: anti actin antibodies.

	Only EmA	SMA +ve	p
Age Median (range) yr	43 (23-80)	44 (22-74)	NS
Age at diagnosis Median (range) yr	39 (8-75)	37 (4-68)	NS
Gender F/M (n of cases)	41/16	28/7	NS
BMI Mean \pm SEM	19.7 \pm 0.4	18.6 \pm 0.5	0.09
Clinical characteristics at diagnosis			
Classical (n of cases) (%)	36 (63%)	28 (80%)	NS
Subclinical (n of cases) (%)	12 (21%)	7 (20%)	NS
Silent (n of cases) (%)	9 (16%)	0	< 0.03
Menarche Median (range) yr	13 (10-16)	14 (9-16)	< 0.04
Abortions (n of cases)	24	17	NS
Autoimmunity (n of cases)	5	9	< 0.04*
Family members with CD (n of cases)	15	2	< 0.03*
Laboratory findings			
EmA (n of +ve cases)	57 (100%)	26 (74%)	< 0.0002
AGA-A (n of +ve cases)	38/53 (72%)	27/34 (79%)	NS
AGA-A Median (range) (AU/mL)	48 (4-106)	56 (2-224)	< 0.02
a-tTG (n of +ve cases)	55 (96%)	28 (80%)	< 0.03
a-tTG Median (range) (AU/mL)	166 (1-323)	175 (1-369)	< 0.06
AAA Median (range) (AU/mL)	417 (123-2437)	2067 (163-2671)	< 0.0000
Fecal volume Median (range) (g/day)	225 (23-792)	282 (40-1000)	< 0.06
á-1AT CI Median (range) (mL/day)	28 (1-219)	44 (3-280)	< 0.01
Hemoglobin Mean \pm SEM (g%)	12.2 \pm 0.3	11.4 \pm 0.2	< 0.01
Albumin Mean \pm SEM (g%)	3.5 \pm 0.1	3.4 \pm 0.1	NS
Ca+ Mean \pm SEM (mg%)	8.3 \pm 0.1	8.2 \pm 0.1	NS
Prothrombin time Median (range) (%)	88 (16-125)	73 (18-101)	NS
AST Median (range) (mg%)	28 (6-126)	40 (8-220)	NS
ALT Median (range) (mg%)	30 (7-125)	39 (4-146)	NS

* OR= 3.6 (95% CI 1.1-11.8)

** OR= 5.9 (95%CI 1.2-27.6)

Table 4. Prevalence of autoimmune disorders at diagnosis in celiac disease patients grouped according to the serological evidence of smooth muscle antibody (SMA) immunofluorescence on monkey esophagus substrate.

Disorder (Number of patients)	SMA negative (n=57)	SMA positive (n=35)	p
Thyroid disease	5	5	
Psoriasis	0	4	<0.03
Vitiligo	2	1	
Rheumatoid arthritis	-	2	
Glomerulonephitis	0	1	
Number of patients with autoimmune disorders	7	12	0.03
Total number of autoimmune disorders	7	13	0.02

positive cases (413 ± 211 AU/mL, $p < 0.00001$), but similar to those only EmA positives ($p = \text{NS}$). While one patient of this subgroup had a dermatitis herpetiformis with no enteropathy (Marsh's type 0), the eight remainder cases had a severe mucosal atrophy (Marsh's type IIIc). Compared with CD patients only EmA positive, this subgroup of patients had a tendency to present lower mean concentrations of hemoglobin, serum albumin and lower BMI (18.5 ± 1.9 K/m²). Two patients died due to intestinal lymphoma, one from a refractory course and one patient had an ulcerative jejunitis.

Discussion

The immunopathological evidence available strongly suggests that autoimmune phenomena participate in the pathogenesis of CD.²³ This is supported, among others features, by the presence of disease-specific autoantibodies (EmA and a-tTG) in serum of the large majority of patients and by the association of CD with other autoimmune disorders, most sharing a common genetic background. In addition, a variety of organ-specific and non organ-specific circulating autoantibodies occur with some frequency in the serum of patients with CD.⁶⁻⁸ However, their real clinical significance in the context of gluten sensitivity remains to be established. Thus, our aim in the present study was to determine if SMA and/or AAA, commonly detected in CD patients, might be considered clinically important for CD.

Using a novel ELISA assay to detect IgA AAA, our study showed that the autoantibody is the most frequent non-organ specific autoantibody present in serum of untreated CD patients. Based on the cut-off for healthy controls established using the mean + 2SD formula (110 AU/mL), seropositivity was evident in 100% of samples from patients assessed at the time of diagnosis. Interestingly, 93.5% of these patients had serum values greater than the highest end of the range for normal individuals (140 AU/mL). Furthermore, serum AAA values for active CD patients were significantly higher than those for disease controls. Our results also showed a highly significant reduction of serum AAA values induced by treatment with a gluten-free diet for a mean period of five years. Despite such clear

improvement, however, 44% of samples still remained above the upper limit for normal individuals. This observation is in agreement with the 37% of inadequate diet compliance assessed in the long-term analysis of the present study (data not shown). Our present data on the prevalence of AAA are different to those recently reported. While we detected increased values of AAA in all patients with active CD, Clemente et al¹⁸ found it in 71% of patients and Granito et al¹⁹ in 27% of the population. Discrepancies among studies could be due to the differences in the populations enrolled (e.g. children and adults seem to have different prevalence) and/or variations in the assays used for the detection of the autoantibody and the choice of cutoff. More recently, an Italian multicenter study detected IgA AAA in 82.5% of prospectively assessed patients and in 61.5% of samples retrospectively investigated.¹⁹ Similarly to former studies, the finding of a correlation between AAA and the degree of severity of the intestinal histological damage in active CD patients seems to be of major relevance. Taken together, the present and former observations suggest an important role for AAA as a marker of histological damage and, therefore, these antibodies could have potential implications in diagnosis and follow-up of patients.

A very recent study of Clemente et al²⁴ has shown that the presence of SMA/anti-actin antibodies is associated with a gluten-induced rapid disorganization of actin filaments in the cytoskeleton of enterocytes in the celiac enteropathy. Therefore, they could be viewed as markers of the chain of events involved in the histological damage of CD enteropathy.^{18-20,24} It has been hypothesized that at least two mechanisms may be involved in actin filament disorganization in the enterocyte. One of these mechanisms seems to be associated with the interplay of AAA, the quite specific a-tTG antibody and the presence of tTG in the epithelial membrane. This is supported by very recent study, still published in abstract form, where Italian researchers used an immunostaining method to show co-staining of membrane-associated type 2 transglutaminase and a-tTG antibody.²⁵ The authors suggested a functional role for the antigen-antibody association by producing a dose-dependent increased cytoskeletal rearrangement (actin depolymerization) and the

modulation of the cell cycle, both potentially resulting in the progression of the mucosal lesion in CD. On the other hand, Clemente et al²⁴ have shown that AAA could also be elicited by the zonulin-induced actin depolymerization in epithelial cells, which also provoke perturbation of the intestinal permeability.

A very interesting finding of our study not commonly reported by other serologic studies was the detection of a group of CD patients seropositive for IgA SMA using the EmA monkey esophagus assay. In most SMA positive sera, the antibody recognizes actin as the autoantigen. Furthermore, all SMA positive sera were found to have abnormally increased values of AAA (by ELISA). As it was previously mentioned, SMA immunofluorescence often hides underlying EmA staining. Although progressive dilution of serum has been useful in unmasking the EmA in most samples, a-tTG proves to be a more efficient method for detecting occult CD-specific seropositivity. Despite these tricks for unmasking the specific autoantibody, a small number of well-established CD cases still remain EmA and a-tTG negative. In general, SMA positive patients had significantly greater values of AAA than those cases found to be only EmA positive. In contrast, although increased AAA serum values was the rule, samples found to be only SMA positive had a significantly lower mean value of AAA than the subgroup positive for both SMA and EmA. Taking into consideration all this evidence, it seems very reasonable to consider that very high serum values of AAA are required for the expression of SMA seropositivity using the immunofluorescence technique. As suspected, seropositivity for SMA subsided in most patients as result of strict gluten avoidance.

The comparison of clinical features of patients grouped according to the presence or absence of IgA SMA allowed us to detect a series of novel and interesting findings. Our results suggested that SMA positive patients are a subgroup prone to more severe clinical compromise at diagnosis. We determined that the SMA positive CD subgroup included a significantly higher number of patients with classical and atypical symptoms, more severe diarrhea, lower hemoglobin concentration, increased fecal protein loss, delayed menarche and a greater number of autoimmune associated disorders. In

contrast, SMA positive patients had a significantly lower familial penetration and a lower BMI compared with those negatives, although this difference did not reach statistical significance. In addition, follow-up of patients suggests that those SMA positives may exhibit a trend to present more severe complications (lymphoma or refractory course including ulcerative jejunitis). A particular finding of our study is related to a subgroup of SMA positive CD patients who appeared EmA negative at diagnosis. This subset of patients had a more severe clinical compromise with significantly lower BMI, hemoglobin concentration and serum albumin. Furthermore, one third of patients of this subgroup presented complications or died. The association between the SMA seropositivity and the poor clinical behavior of patients seems to be an intrigue. We hypothesize that the presence of a high AAA serum value in SMA positive cases could be a marker of a more severe and extended histological damage and, therefore, the possibility for a more severe clinical course.

In conclusion, our present study showed that sera from patients with active CD have increased serum levels of IgA AAA. These antibodies correlate with the severity of histological damage of the intestinal mucosa and the level of antibody decrease in most patients after the introduction of a gluten-free diet. Highest serum values of AAA correlated with the presence of SMA by immunofluorescence on monkey esophagus substrate. Furthermore, we also showed that the presence of SMA seropositivity in a subset of CD cases seems to be clinically relevant for characterizing patients with a more severe clinical disease at diagnosis. SMA positive patients exhibit an increased prevalence of autoimmune disorders and a tendency to more severe outcome. We hypothesize that the former clinical observation might be related to the severity of the histological damage and probably, to a more extensive intestinal compromise. All studies agree that detection of IgA AAA seems to be a promising non-invasive test in the follow-up of the intestinal mucosal lesion of CD patients. Although the antibody results a very helpful adjuvant to more specific autoantibodies for diagnosing the CD enteropathy, prospective studies on unselected population are required to better define such role.

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