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ORIGINAL ARTICLE

## Usefulness of the organ culture system in the *in vitro* diagnosis of coeliac disease: A multicentre study

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### Abstract

**Objective.** Diagnosis of coeliac disease is based on the presence of villous atrophy which recovers following a gluten-free diet. The presence of circulating antiendomysial antibodies as well as their disappearance after a gluten-free diet supports the diagnosis. It has also been demonstrated that antiendomysial antibodies are detectable in supernatants of cultured intestinal biopsies from patients with coeliac disease. The objective of this study was to compare the histology and antiendomysial antibodies in culture supernatants of intestinal biopsies to validate the *in vitro* organ culture system as a future diagnostic tool for coeliac disease. **Material and methods.** Seventy-five antiendomysial serum-positive patients on a gluten-containing diet were evaluated. Patients underwent endoscopy with 5 biopsy fragments: 3 for histology, 1 cultured with and the other without gliadin-peptide activator. Antiendomysial antibodies were evaluated in all culture supernatants. **Results.** Sixty-eight patients had evidence of villous atrophy, while 73 out of 75 were positive to the organ culture system. The agreement rate between organ culture and histology results was 94%. **Conclusions.** As all the centres participating in the study obtained good agreement between organ culture and histology results, the new system could be considered a reliable tool for the diagnosis of coeliac disease. Nevertheless, it is possible to highlight cases with an organ culture-positive and -negative histology. This feature could be of considerable interest because, as the sensitivity of organ culture seems to be greater than the initial histology, the new system might be useful in uncertain cases where the risk of missing the diagnosis of coeliac disease is high.

**Key Words:** *Antiendomysial antibodies, coeliac disease, diagnosis, histology, organ culture*

### Introduction

The diagnosis of coeliac disease (CD) is currently based on a histological examination of the intestinal mucosa demonstrating villous atrophy which re-

covers following a gluten-free diet (GFD) [1]. The presence of circulating antiendomysial (EMA) and/or anti-tissue-transglutaminase (anti-tTG) antibodies and their disappearance after a GFD support the diagnosis [2,3].

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Today, positive serum EMA and/or anti-tTG alone are insufficient to diagnose CD. However, the widespread use of these screening tests, especially when they are used in a way that maximizes their sensitivity, contrasts with the limits that the histology involves, such as technical difficulties (i.e. correct orientation and sufficient size of the biopsy samples) and subjective data interpretation. Furthermore, at the routine histological examination, it is possible to highlight patchy features of intestinal damage (specially in randomness of the biopsy drawing condition) and, in the latent form of CD, absence of mucosal atrophy despite the appearance of villous flattening after a prolonged period of gluten ingestion [4,5].

On the other hand, it has previously been demonstrated that the production of EMA antibodies occurs in the intestinal mucosa and that these antibodies can be detected in the supernatants of cultured intestinal biopsies from untreated CD patients [6–8]. Subsequently, the use of other methods (e.g. phage display libraries) has confirmed that EMA antibodies are in fact produced in the small intestinal mucosa [9,10]. The presence of EMA antibodies can also be demonstrated in the supernatants of cultured intestinal biopsies from CD patients undergoing GFD after *in vitro* treatment with antigenic stimulus (*in vitro* gliadin challenge) [6]. The same results have been achieved by using a synthetic peptide corresponding to the 31–43 AA position of  $\alpha$ -gliadin [11]. Even though the *in vitro* gliadin challenge results reported in the literature tend to be variable [6–8], it is today known that the *in vitro* production of EMA antibodies is gluten dependent and highly specific for CD [12,13].

In this collaborative study, the overall aim was to compare histological findings and EMA detection in supernatants of intestinal biopsies cultured with or without gliadin to validate the *in vitro* organ culture system (EMA biopsy) as a future diagnostic tool for CD.

## Material and methods

### *Patients*

This multinational cooperative study involved six centres: five (Rome, Milan, Florence, Palermo – Italy; Buenos Aires – Argentina) dealt with adult and one with paediatric patients (Valencia – Spain). A total of 75 subjects (16 M, 59 F, median age 28 years, range 1–65 years) referred for diagnosis of CD were included in the study. The inclusion criteria consisted of symptoms suggestive of CD on a gluten-containing diet and positive serum IgA EMA.

In each centre, symptoms and signs were collected on specifically designed data sheets and sent to the coordination centre (Rome) for data processing. Fifty percent of the patients who took part in the study presented a body mass index of less than 20. The most frequent symptoms were: abdominal pain (37%); muscular cramps (36%); diarrhoea (34%); aphthosis (31%); defluvium, paresthesia and teeth enamel hypoplasia (27%); clubbing and diskarotosis (24%); spontaneous ecchymosis (21%). The most frequent abnormal blood data were: hypoferritinaemia (40%); iron-deficiency anaemia (35%); increase of alanine aminotransferase (ALAT) (32%) and aspartate aminotransferase (ASAT) (26%) transaminase; low serum levels of cholesterol (31%) and triglycerides (24%).

All patients in the study underwent oesophago-gastroduodenoscopy (OGDS) in order to collect 5 biopsy fragments from the II–III duodenal part (minimal weight required: 5 mg/fragment), 3 for routine histological examination and 2 for organ culture.

All procedures followed in this study, performed for diagnostic purposes, were in agreement with the ethics standards of the institutional committee responsible for human experimentation.

### *Histology*

Morphometric analysis was performed by means of the standard haematoxylin-eosin staining technique. The histological status was evaluated in agreement with both original and revised Marsh classification, and grade 3 lesions were considered diagnostic for CD [14,15].

### *EMA biopsy system*

*Organ culture.* Two duodenal specimens were cultured as previously reported [12]. Briefly: specimens were gently placed in a sterile tube containing 500  $\mu$ l of medium and then cultured for 48 h at 37°C, one along with and one without the activator (a synthetic peptide corresponding to the 31–43 AA position of  $\alpha$ -gliadin) (Antiendomysium Biopsy Kit – Eurospital, Trieste, Italy). Supernatants were collected and stored at –20°C, until in use.

*Antiendomysial antibody detection.* EMA antibodies were sought in culture supernatants by means of indirect fluorescence assay (IFA) on cryostat sections of monkey oesophagus (Eurospital). Undiluted culture supernatants were incubated for 45 min. Fluorescein isothiocyanate (FITC)-conjugated anti-human IgA, diluted 1:100, was incubated for 30 min. Positive results were identified by reticulon-like

staining of smooth-muscle bundles. For each centre participating in the study, the results were evaluated by an observer unaware of the clinical conditions of the studied subjects.

## Results

At the histological examination, 22 out of 24 Rome patients had evidence of villous atrophy (7 with IIIa, 4 with IIIb and 11 with grade IIIc of the Marsh classification) while, the remaining 2 patients, had no histological signs suggestive of CD (1 with I, and the other one with 0 grade of the Marsh classification). All 24 patients were positive to the EMA biopsy system, irrespective of the *in vitro* gliadin challenge.

### Florence

Sixteen out of 19 patients had evidence of villous atrophy (1 with IIIa and 15 with grade IIIb of the Marsh classification) while, the remaining 3 patients, had no histological signs suggestive of CD (1 with I and the other two with 0 grade of the Marsh classification). Seventeen patients (16 with III and 1 with grade 0 of the Marsh classification) were positive to the EMA biopsy system, irrespective of the *in vitro* gliadin challenge. Of the remaining two patients, one (grade 0 of the Marsh classification) was negative while the other patient (grade I of the Marsh classification) was positive only after *in vitro* gliadin challenge.

### Buenos Aires

All 10 patients had evidence of villous atrophy (1 with IIIa, 1 with IIIb and 8 with grade IIIc of the Marsh classification). Nine patients were positive to the EMA biopsy system, irrespective of the *in vitro* gliadin challenge. The remaining patient was positive after biopsy culture with medium alone and,

conversely, negative after biopsy culture in the presence of antigenic stimulus.

### Palermo

Seven out of 8 patients had evidence of villous atrophy (4 with IIIb and 3 with grade IIIc of the Marsh classification), while the remaining patient had no histological signs suggestive of CD (grade I of the Marsh classification). All 8 patients were positive to the EMA biopsy system, irrespective of the *in vitro* gliadin challenge.

### Milan

All 7 patients had evidence of villous atrophy (1 with IIIa, 4 with IIIb and 2 with grade IIIc of the Marsh classification). All 7 patients were positive to the EMA biopsy system, irrespective of the *in vitro* gliadin challenge.

### Valencia

Six out of 7 patients had evidence of villous atrophy (5 with IIIb and 1 with grade IIIc of the Marsh classification) while the remaining patient had no histological signs suggestive of CD (grade I of the Marsh classification). Five patients (all with mucosal atrophy) were positive to the EMA biopsy system, irrespective of the *in vitro* gliadin challenge. Of the remaining two patients, one (grade I of the Marsh classification) was negative while the other patient (grade IIIb of the Marsh classification) was positive only after *in vitro* gliadin challenge.

All data are summarized in Table I.

## Discussion

The diagnosis of CD is generally based on three parameters: clinical case identification, screening tests and confirmation tests. Over the past few years the clinical presentation of the disease has been better understood, added to which more sensitive

Table I. Centres participating in the study, the number of patients evaluated by each centre, the number of positive histologies (grades IIIa, IIIb and IIIc of the Marsh classification), the number of positive results of the EMA biopsy system (for both specimens cultured in medium alone and those cultured in medium plus gliadin) and the agreement rate between EMA biopsy and histology results.

Centres (no. of patients)	Positive histology	EMA biopsy positive results		Final results	Agreement rate
		Medium	Medium + gliadin		
Rome (24)	22	24	24	24	92%
Florence (19)	16	17	18	18	89%
Buenos Aires (10)	10	10	9	10	100%
Palermo (8)	7	8	8	8	88%
Milan (7)	7	7	7	7	100%
Valencia (7)	6	5	6	6	100%
Total patients (75)	68	71	72	73	94%

and specific diagnostic tools have been used. Notwithstanding, these parameters are currently being modified and upgraded.

In agreement with the last ESPGHAN criteria, if symptoms and signs (typical and/or atypical) plus screening tests (EMA and/or anti-tTG antibodies) are suggestive of CD, then intestinal mucosal samples with histological features as defined by the Marsh classification are sufficient to confirm the diagnosis. A favourable response to the GFD is mandatory, as well as disappearance of serum EMA and anti-tTG antibodies [2,3]. However total villous atrophy, identified as the gold standard for CD diagnosis, is considered the extreme condition of a modifying spectrum of tissue damage that can be revealed only during the acute phase of the disease [3]. For this reason, it is possible to highlight false-negative results, especially in the case of patchy atrophy (4) or in the absence of mucosal damage, a feature readily found in latent CD [5], and consequently the diagnosis could be missed. Together with the technical difficulty (correct orientation and sufficient size of the biopsy fragments), the subjective evaluation of the intestinal damage adds further limitations to the diagnosis of CD when the histological analysis is the only confirmation test performed. All these features indicate the need for considering other tools in the final step of CD diagnosis.

Given the evidence that EMA antibodies are produced by the intestinal mucosa and are then detectable in supernatants of biopsies from untreated CD patients cultured in the absence and/or presence of antigenic stimulus (gliadin) [6–8,12,13], we evaluated the reliability of the *in vitro* EMA biopsy in diagnosing CD, in comparison with the “classic” histology. The data highlight that all gastroenterology centres participating in the study obtained a high agreement rate between EMA biopsy and histology (from 88 to 100%, mean agreement rate 94%), suggesting that the new system could be useful in the diagnostic iter of CD. Although EMA determination in serum is easier than that in culture supernatant and, generally, immunofluorescence requires expertise, the EMA biopsy is an economical method that is easy to set up in each laboratory. Since to diagnose CD, an

endoscopy with biopsies is mandatory, reserving an intestinal fragment for EMA biopsy would offer an additional tool that could display the EMA antibodies locally where they are produced.

Moreover, in 70 out of 73 EMA biopsy positive cases, antibodies were detectable in supernatants irrespective of the *in vitro* gliadin challenge. In 2 of the remaining 3 cases, (1 in Florence and 1 in Valencia) EMA antibodies were detectable only after *in vitro* gliadin challenge. This feature suggests that the *in vitro* gliadin challenge should always be performed when the EMA biopsy system is used in the diagnostic protocol of CD. On the other hand, in the last EMA biopsy positive case (Buenos Aires), antibodies were detectable only after culture with medium alone. This was probably due to the low weight of the fragment cultured in the presence of gliadin peptide (1.2 mg versus 5 mg/fragment required), suggesting that particular attention should be paid to the size of the intestinal samples used for EMA biopsy tests.

In the two serum EMA-positive but EMA biopsy negative cases (1 in Florence and 1 in Valencia), the histology was also negative, even though both patients presented a clinical spectrum and a series of haematochemical data typical of CD. Furthermore, one of these patients presented a family history of CD and the HLA DQ2 type (Table II). A possible explanation for the EMA biopsy negative results could be that, in these cases, biopsy specimens were not adequate.

Finally, the histology was negative in 5 patients who showed EMA biopsy positive results (2 in Rome, 2 in Florence and 1 in Palermo). These patients presented positive serum EMA, HLA DQ2 type, family history, symptoms and signs suggestive of CD (Table III), but could not be given a diagnosis because villous atrophy was not present. This feature suggests a possible use of the EMA biopsy system to clarify the diagnosis in cases of gluten-sensitive enteropathy characterized by an infiltrative/hyperplastic histological pattern (I/II grade of the Marsh classification) or in cases of patchy condition of the villous atrophy.

As all the gastroenterology centres participating in the study obtained good agreement between EMA biopsy and histology results, the new system could

Table II. Clinical spectrum (family history, clinical presentation, HLA typing and haematochemical data) of the two patients with positive serum EMA but EMA biopsy and histology negative results.

Patients (centre)	Relatives with CD	Clinical presentation	HLA	Haematochemical suggestive data
Pts 1 (Florence)	Cousin	Typical	DQ2	↓ Haemoglobin; ↓ ferritin; ↓ cholesterol
Pts 2 (Valencia)	–	Typical	–	↓ Haemoglobin; ↓ iron; ↑ transaminase; ↓ cholesterol

Abbreviations: HLA = human lymphocyte antigen; EMA = antiendomysial; CD = coeliac disease; pts = patients.

Table III. Clinical spectrum (family history, clinical presentation, HLA typing and haematochemical data) of the five patients with serum and EMA biopsy positive results but negative histology.

Patients (centre)	Relatives with CD	Clinical presentation	HLA	Haematochemical suggestive data
Pts 1 (Rome)	Father; brother	Typical	DQ2	↓ Haemoglobin; ↓ iron; ↓ cholesterol
Pts 2 (Rome)	–	Typical	DQ2	↓ Haemoglobin; ↓ iron; ↑ transaminase
Pts 3 (Florence)	Father	Atypical	DQ2	↓ Ferritin; ↓ cholesterol; ↓ triglycerides
Pts 4 (Florence)	–	Atypical	DQ2	–
Pts 5 (Palermo)	Mother; sister	Typical	DQ2	↓ Iron

Abbreviations: HLA = human lymphocyte antigen; EMA = antiendomysial; CD = coeliac disease; pts = patients.

be considered a reliable tool for the diagnosis of CD. Nevertheless, it is possible to highlight cases, strongly suspected to have CD, with EMA biopsy positive results but negative histology. Consequently, correct values of sensitivity and specificity of the EMA biopsy system are not measurable if the choice of the reference gold standard is based on the histological evidence of villous atrophy. However, a prospective study to verify gluten dependency in patients with EMA biopsy positive results but negative histology is foreseeable. This study should be of considerable interest because, as the sensitivity of EMA biopsy seems to be greater than the initial histology, the new system could be useful for the uncertain cases (patchy atrophy or latent CD), where the risk of missing the diagnosis of CD is very high.

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