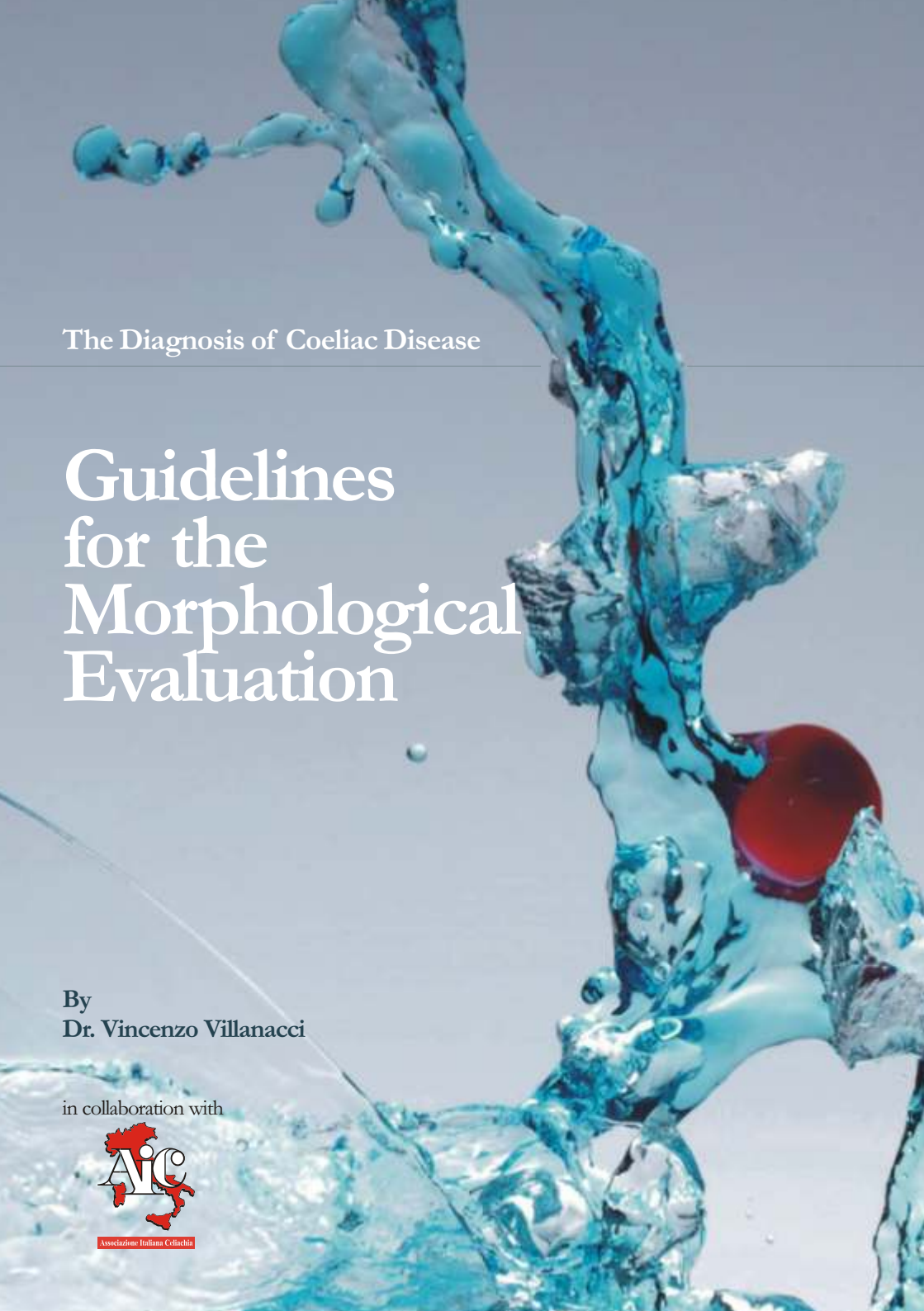

The Diagnosis of Coeliac Disease

Guidelines for the Morphological Evaluation

By
Dr. Vincenzo Villanacci

in collaboration with

Associazione Italiana Celiachia

2008 edition

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Foreword

The intestinal biopsy to this day is rightly considered the "gold standard" for the diagnosis of coeliac disease.

Those who work with this disease know that a critical phase in the diagnostic process is preparing the histological samples with the biopsy correctly oriented. They are also aware of the importance of an accurate and careful morphological evaluation that not only considers the degree of villus atrophy, but also the extent of intraepithelial lymphocyte infiltration and the presence of glandular crypt hyperplasia.

The pathologist's evaluation remains the element of primary importance that will then, together with serology, enable the clinician to formulate a correct diagnosis.

In ambulatories for coeliac disease, it is more and more common to come across patients whose diagnoses are wrong or anything but certain of coeliac disease precisely because of inaccurate interpretations of the intestinal biopsy.

As a way to remedy this problem, the Executive Council of the Italian Coeliac Association (AIC) asked the National Scientific Committee (CSN) of the AIC to choose an expert to write up the guidelines for the morphological evaluation of intestinal biopsy.

That choice fell on Dr. Vincenzo Villanacci, who was unanimously appointed the reference pathologist because of his undisputed experience and competence in this field.

The guidelines written by Dr. Villanacci are an extremely important document in which pathologists can find the answer to the many doubts and uncertainties that can sometimes make their reports unclear and not in keeping with the fundamental requisites for a correct diagnosis of coeliac disease.

We are sure that the use of these guidelines in evaluating intestinal biopsy, and in applying diagnostic and follow-up protocols (currently being approved at the ministerial level), will make the diagnosis of coeliac disease more reliable and certain.

Our particular thanks go to Dr. Villanacci for having collaborated with the AIC on writing up this very important scientific document.

Umberto Volta
AIC-CSN President

Adriano Pucci
AIC National President

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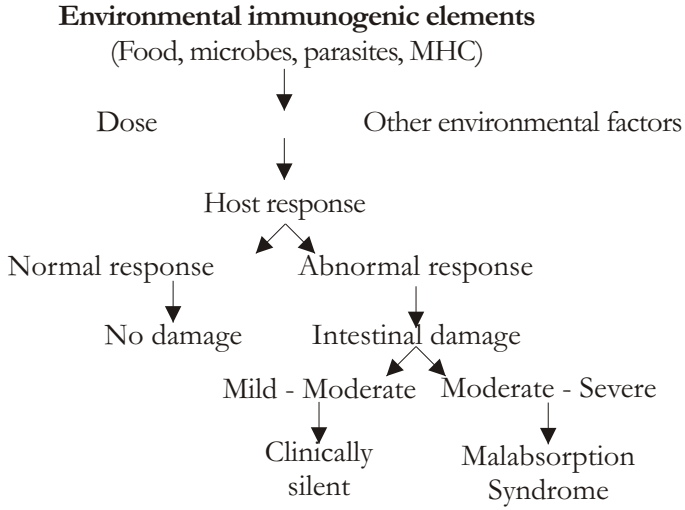
The morphological evaluation of the duodenal biopsy is the principal and irreplaceable diagnostic aid for coeliac disease (CD); and despite attempts to limit its role and efficacy, it still represents the "gold standard" [4 7 8 9 10 11 12 13 14 18 19 22].

Therefore, the pathologist has an important role to play in confirming whether the laboratory findings and clinical suspect of coeliac disease are valid.

The histopathological diagnosis, however, is just one step in the process leading to the definitive diagnosis of CD; a diagnosis that must nevertheless be made solely by the paediatric or adult gastroenterologist, who is the only one who knows the patient, who decides which tests to perform, and who receives the results.

In recent years, it has become clear that the intestinal lesion induced by gluten is not only the one that we have long been familiar with, or rather the one that is characterized by total villous atrophy. Today, in addition to the various clinical expressions of the disease, we also recognize a variability in the expression of the intestinal damage and thus of the damage morphologically evaluable. There is, in fact, a spectrum of gluten-dependent lesions that range from a simple increase in lymphocytes in an otherwise normal mucosa (latent and potential coeliac disease) to severe damage that is described as total mucosal atrophy.

See the Marsh[1] explanatory diagram below :



Methodological Introduction

The pathologist, working closely with other specialists such as paediatric or adult gastroenterologists, endoscopists and laboratory technicians, always needs to have the following clinical information:

- Reason for the biopsy: family history, history of previous diagnoses of coeliac disease, symptoms (these are particularly important);
- Clinical suspicion: serious, moderate or mild, depending on symptoms;
- Patient's diet (if possible): containing gluten, gluten-free, length of diet.

Laboratory tests

- Anti-Endomysial (EmA), Anti-Transglutaminase (anti-tTG) and Anti-Gliadin (AGA) antibodies;
- Serology with nearly absolute (EmA), elevated (anti-tTG) or low (AGA) predictability. Always specify the antibody class, the presence of IgA deficit and, if possible, the antibody ratio.
- Genetic (if performed and indicated): specify the HLA heterodimer pattern for coeliac disease.

What follows is a sample form containing the information and laboratory data to give to the pathologist.

The scheme was proposed by Prof Volta and is perfectly in keeping with what has already been said.

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Information sheet for the pathologist

Name _____

Surname _____

Birth date _____

Diet

Unrestricted □ Gluten-free □

Clinical suspicion

Serious □ Moderate □ Low □

Specify symptoms or associated pathologies that justify the exam

_____

_____

Serology:

Ema IgA _____

Anti-tTG IgA _____

AGA IgA anti-tTG IgG _____

(write the antibody ratio and normality values)
Specify if there is selective IgA deficit

Genetics (if available)

DQA1* 05 yes □ no □
DQB1* 02 yes □ no □
DQB1*0302 yes □ no □

Biopsy

Biopsy technique

For children under the age of two, the biopsy is performed with the Crosby-Watson capsule by mouth. However, today, the endoscopic examination is preferred for all ages because it makes it possible to explore other anatomical districts of the gastro-intestinal tract as well.

Biopsy location

The biopsy should always be performed in the first and second duodenal segments; and at least four biopsies are recommended, two for each segment. [26]

Orientation of the biopsy sample

This is essential for a correct histological evaluation.

It would be advisable, through close cooperation with endoscopists, to locate the biopsies on acetate cellulose filters (Fig 1 A-B).

This has obvious advantages:

A. for technicians of pathology labs because, with a simple 90° rotation, it is possible to include the filter and biopsies together (Fig 1 – C);

B. for the subsequent histological evaluation by the pathologist (Fig 1 – D Non-oriented biopsy Haematoxylin – Eosin x 20 and E Oriented biopsy Haematoxylin – Eosin x 10).

We consider this aspect so important in terms of the final diagnosis that we are adding an explanatory comment.

For 16 years now, mediated by the experience at St Mark's Hospital, a biopsy collection technique has been used in the II Pathology Lab of Brescia Hospital that makes it possible to orient gastro-intestinal biopsies correctly. This guarantees histological samples where it will be possible to analyze the mucosa and sub-mucosa of the collected tissue, thus respecting the normal anatomical relation among the various layers of the intestinal wall.

The orientation of the biopsies is no longer left to chance as in the past, but made possible thanks to collaboration with the endoscopist, who puts the bioptic samples on portions of cellulose filters pre-cut into rectangular shapes that vary in length according to need. The biopsies are then positioned in every little square, following a perfectly straight line. When needed to map an organ (e.g. colonic biopsies), we resort to a ploy by positioning the first biopsy near a bevelled end shaped like a clarinet mouthpiece which by convention indicates the starting point.

After the phase of fixation, the filter-biopsy is processed and then embedded. During this phase, the technician rotates the filter-biopsy 90° so that the collecting samples return to their natural position.

After cutting, the biopsy specimens are arranged on the slide and the position of the bevelled end showing the first biopsy indicated on the label if necessary.

If correctly applied, this method offers a great advantage for the pathologist as well as the

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technician, who during the embedding phase will not have to look for the single biopsies, at times fragmented and with no reference point for the orientation.

Using the filters allows the biopsies to adhere perfectly, without being dispersed in the fixation medium. Moreover, the filters do not chemically react with the fixatives and other reagents used during the sample processing; and when cut, they offer no resistance to the blade and do not become frayed.

This method applied on all segments of the gastro-intestinal tract has produced considerable benefits both in diagnostic and economic terms by reducing the time, the number of embedding samples and therefore the number of sections to cut and stain.

Despite years of using this method and the obvious advantages already mentioned, there are nevertheless a few problems:

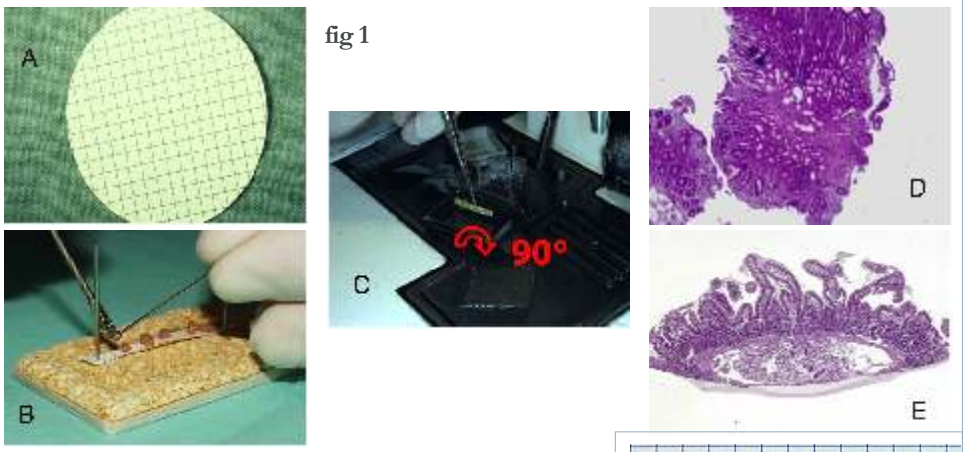


fig 1

A) Manual cutting of the filters;
B) Positioning the filters on the support;
C) Using one or more nurses to cut the filters;
D) Filters irregularly cut;
E) Biopsies not placed in a straight line;
F) Loss of time and human resources.

To obviate these problems, we have developed a kit complete with supports on which are placed three easily detachable pre-cut filters with a bevelled end.

Furnished with the support and the filters are vials, with adhesive labels, containing 10% neutral buffered formalin.

This kit, already tested in the endoscopic room, has the obvious advantages of saving time and human resources. Personnel are no longer needed to cut the filters. Moreover, the use of pre-cut filters, duly fixed on a support, ensures that the biopsies will be positioned correctly.

Therefore, the use of this kit is strongly recommended.

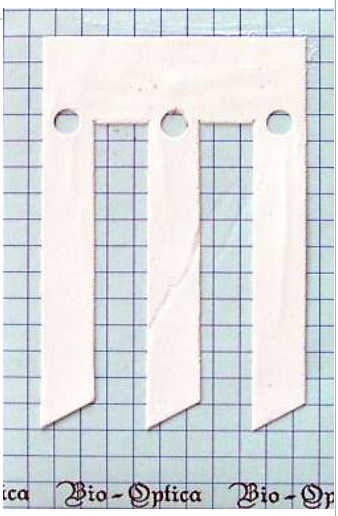


fig 2

Acetate cellulose filter

Haematoxylin–Eosin, or possibly a PAS stain, is sufficient to evaluate the morphologically essential elements (one or two sections are then set aside for the immunohistochemical evaluation).

MORPHOLOGICAL ASPECTS

Optical microscopy: First, we will consider the aspect of normal intestinal mucosa, and then the progressive histological pictures that can appear during coeliac disease.

NORMAL INTESTINAL MUCOSA

Villi: finger-like aspect with the villus to crypt height ratio always in favour of the villi (3:1 or more).

Enterocytes: normal height 29-34 microns, normal brush border.

Intra-epithelial lymphocyte infiltrate: A ratio of **25 lymphocytes for 100 epithelial cells**[6, 13, 21] is considered normal; the ratio of 40 lymphocytes for 100 epithelial cells is to be considered out of date and no longer valid.

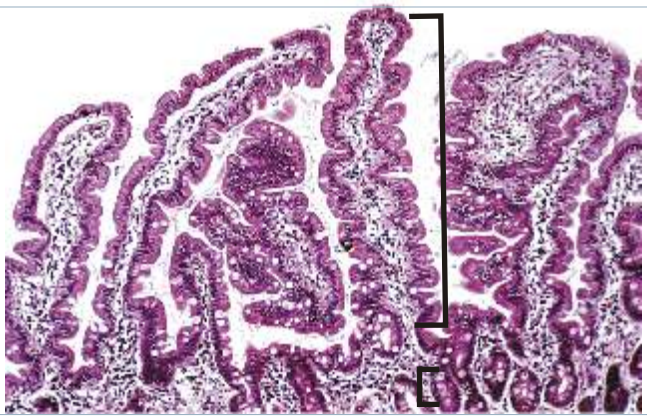
Glandular crypts: since crypts basically perform a regenerative function, cells in the phase of mitosis can be found there. Generally, the normal range is 1 mitosis for crypt.

Along with epithelial cells, there are also endocrine cells, Goblet cells and Paneth cells. However, these cells are of no value in diagnosing coeliac disease.

Lamina Propria: normally one finds plasmacells, eosinophils, mast cells, histiocytes and lymphocytes. Neutrophils are generally absent, except for cases of active duodenitis with possible gastric metaplasia closely associated with HP infection.

Plasmacells are the most numerous cellular component, as are lymphocytes, at times present as lymphoid aggregates and eosinophils, whose value should never be > 60 per 10 vision fields examined at x 40: (Fig. 3 Haematoxylin – Eosin x 10).

fig.3



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PATHOLOGICAL INTESTINAL MUCOSA (Marsh Classification) [1]

Type I or infiltrative lesion

1) Villous architecture within normal morphological limits (normal villous/crypt ratio = 3:1) (Fig. 4 A Haematoxylin – Eosin)
2) Increase in the number of intraepithelial lymphocytes (> 25 for every 100 epithelial cells) (Fig. 4 B immunohistochemical stain for CD3 x 40)

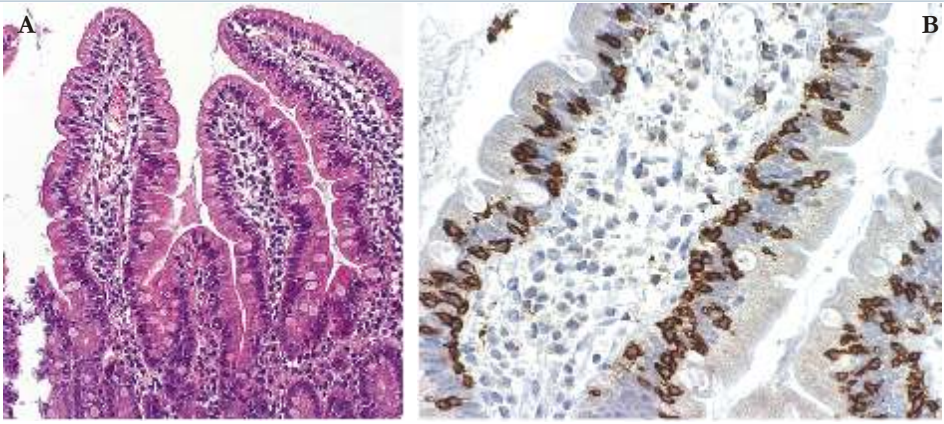


fig.4

Type II or hyperplastic lesion

1) Villous architecture within normal morphological limits (like type I)
2) Increase in the number of intraepithelial lymphocytes (> 25 for every 100 epithelial cells)
3) Hyperplasia of the glandular elements (regenerative aspect of glandular elements highlighted by the reduction of mucous activity and increase in mitosis) (Fig. 5A Haematoxylin – Eosin x 20 B x 40)

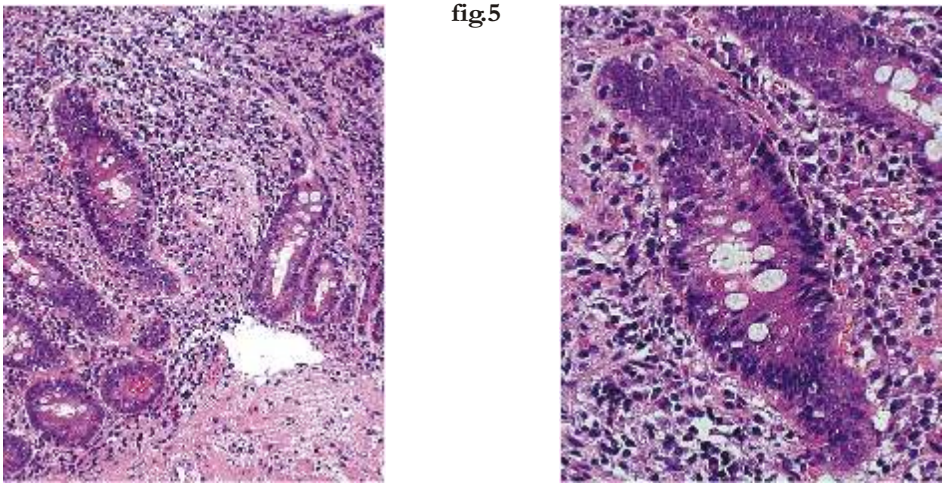


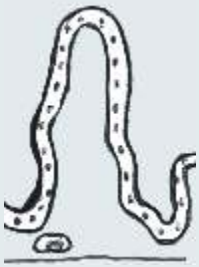
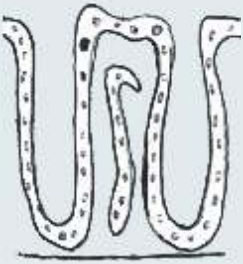
fig.5

Type III or destructive lesion

1) Villous atrophy of varying degrees associated with glandular crypt hyperplasia;
2) Superficial enterocytes with reduced height, brush-border irregularities and sometimes cytoplasmic vacuoles;
3) Increase in the number of intraepithelial lymphocytes (like type I and type II lesions).

The set of three factors described above is consistent with coeliac disease or gluten-sensitive enteropathy. Although schematic, the description represents the histological lesions observable in the course of coeliac disease. It is important to consider the lesions dynamic, progressive and not static, because they are function of the quantitative and temporal exposition to gluten.

The following diagram summarizes what we have explained (from Marsh GUT 1990)1

		
Infiltrative (Type I)	**Hyperplastic (Type II)**	**Destructive (Type III)**
Close relation coeliac patients;	Treated coeliac patients exposed to moderate gluten quantity;	Untreated coeliac disease;
Treated coeliac patients exposed to minimal gluten quantity;	Herpetic dermatitis without clinical enteropathy;	Treated coeliac patients exposed to elevated gluten quantity;
Herpetic dermatitis without clinical enteropathy;		Herpetic dermatitis without clinical enteropathy;
Tropical enteropathy		Tropical sprue;
Giardiasis		Giardiasis.

This is the universally recognized and amply valid classification for the diagnosis of celiac disease. The only weak point, if we want to call it that, is the fact that cases with mild, moderate or severe atrophy are all grouped together under a single category, i.e. the type III lesion.

For this reason, Oberhuber and colleagues[3] proposed a review of the classification by dividing Marsh's type III lesion into three subgroups:

3a) Mild villous atrophy and pathological increase of intraepithelial lymphocytes (Fig. 6 Haematoxylin – Eosin x 20).

3b) Moderate villous atrophy and pathological increase of intraepithelial lymphocytes (Fig. 7 Haematoxylin – Eosin x 20).

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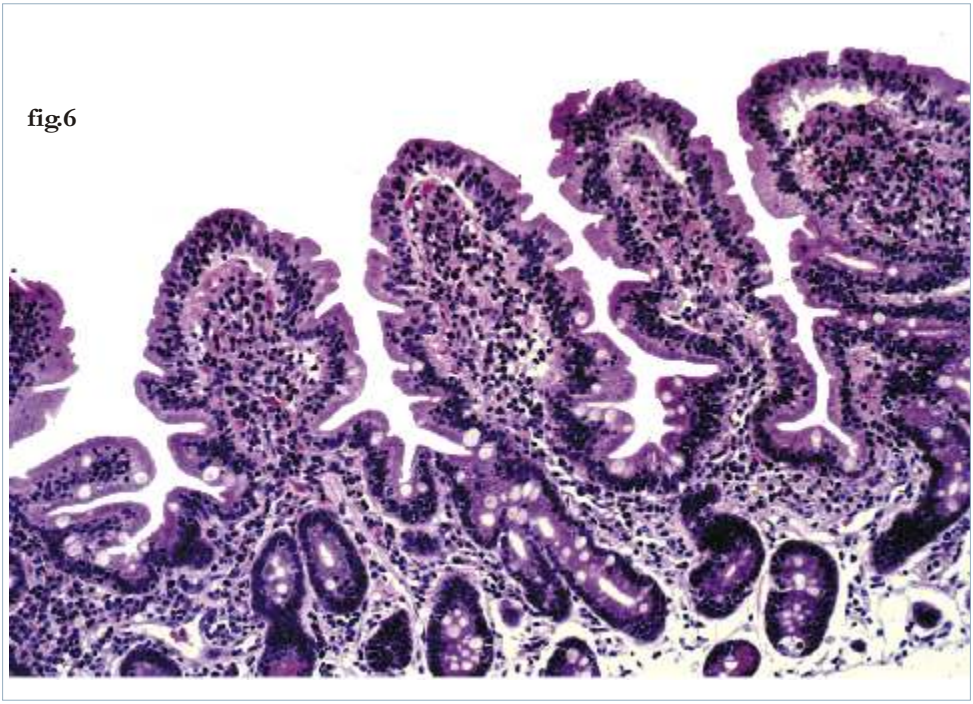


fig.6

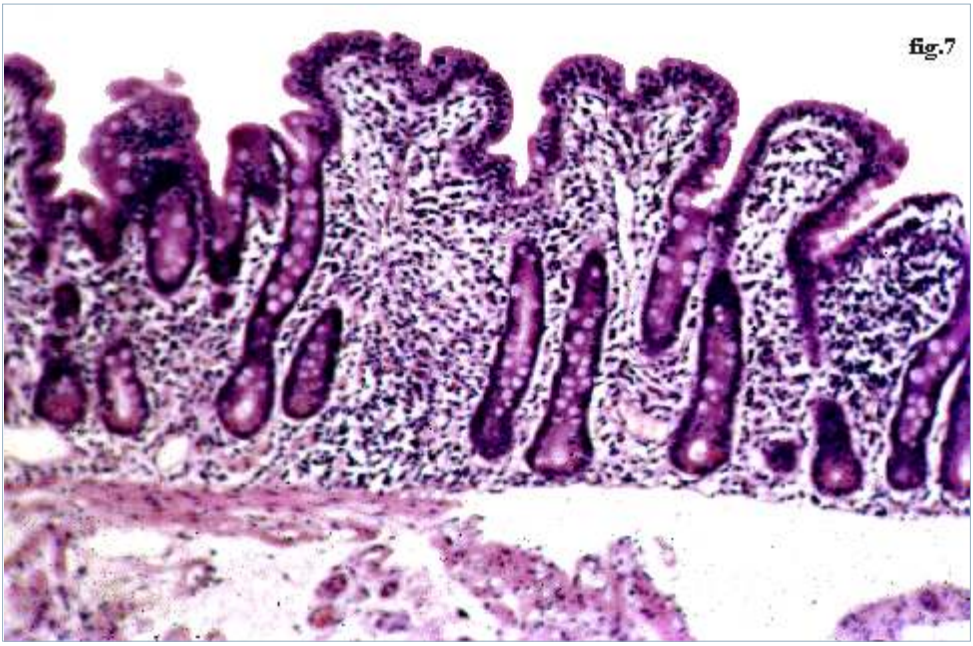
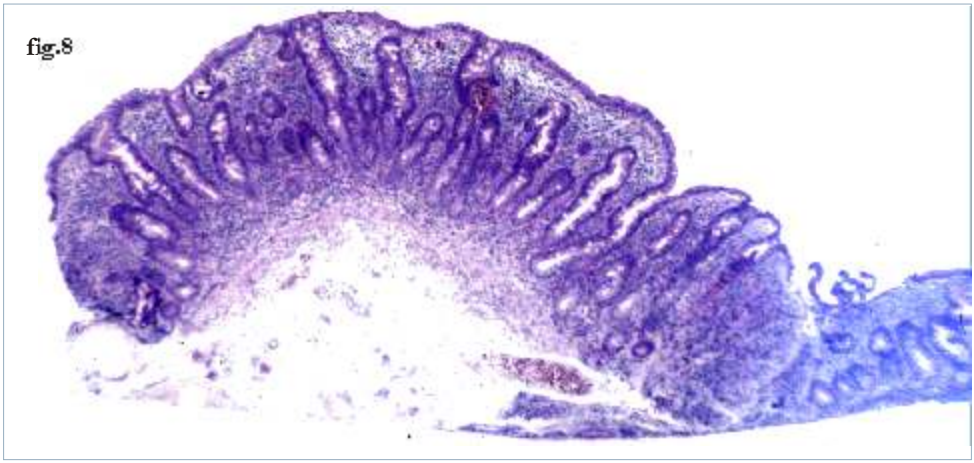


fig.7

3c) Total villous atrophy and pathological increase of intraepithelial lymphocytes (Fig. 8 Haematoxylin – Eosin x 20).



Notwithstanding all the other morphological criteria described, this last classification makes it possible to better describe the spectrum of lesions that may develop in coeliac disease, both in patients with an unrestricted diet and in those following one that is gluten free.

In keeping with this line, and in an attempt to simplify and standardize the work of pathologists and facilitate the rapport between pathologists and clinicians, a new version of histological classification was recently proposed by Corazza and Villanacci [15 23]. In particular, the lesions that characterize coeliac disease were divided into two categories:

Not-atrophic (Grade A) and Atrophic (Grade B)

The latter is divided again into

Grade B1

in which the villous/crypt ratio is < 3:1, with villi still identifiable, and

Grade B2

in which the villi are no longer identifiable.

The Grade A lesion is characterized by the pathological increase in the number of intraepithelial lymphocytes, better recognized by the use of immunohistochemical techniques.

Table 1 shows a comparison of the two classification systems

Classificazione di Marsh-Oberhuber	Classificazione di Corazza-Villanacci
Type 1	Grade A
Type 2	
Type 3a	Grade B1
Type 3b	
Type 3c	Grade B2

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IMMUNOHISTOCHEMICAL EVALUATION

As explained above, one of the essential points in the diagnosis of coeliac diseases is the intraepithelial lymphocyte count, which we recall are T lymphocytes, and in pathological conditions, their number must be greater than 25 lymphocytes for 100 epithelial cells.

This statistic is particularly important, especially today when we are witnessing a continuous diagnostic increase in the initial forms, type 1 and 2 lesions according to Marsh, or Grade A according to the Corazza-Villanacci classification.

The count can also be reasonably carried out using the normal and irreplaceable Haematoxylin – Eosin. However, particularly with the initial forms, we suggest always associating an immunohistochemical typization with monoclonal CD3 antibody that often allows having a more precise lymphocytes visualization. Another element could be the valuation of CD8 T lymphocytes, particularly useful in cases of old people in which it is possible to find refractory forms, considered pre-lymphomatous conditions and in which the CD8 expression could be negative if compared with the normal situation. If a frozen sample is available, an immunohistochemical typization with Gamma-Delta receptor of T lymphocytes could be achieved: this receptor in normal conditions is expressed by 2-3% of lymphocytes, whereas in the case of coeliac disease it could be reaching also 20-30%; this datum is particularly useful in the case of initial lesions (fig. 9).

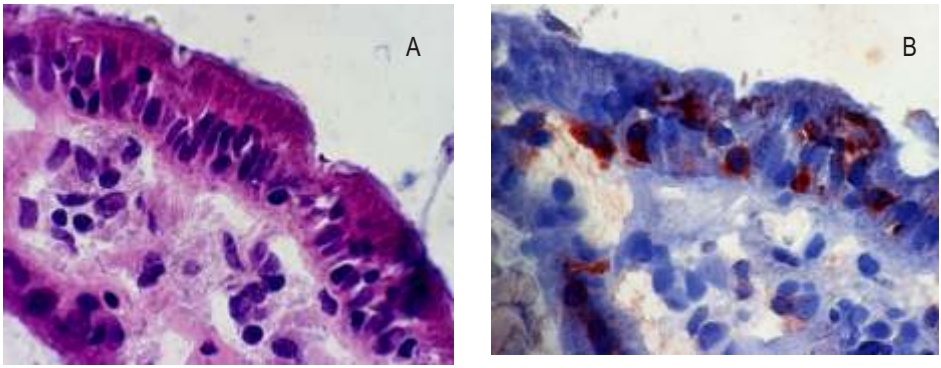

Fig. 9 Increase of gamma delta positive T lymphocytes in the superficial epithelial layer of the villi.

A Haematoxylin – Eosin x 40

B Immunohistochemical stain for Gamma – Delta receptors (x 100)

Practical Advice

In addition to what has been presented, we suggest eliminating the term sub-atrophy from the descriptive terminology because it is unclear and misleading. Instead, specify whether the villus is normal or atrophic, and if the latter is true, the degree of atrophy (from mild-moderate or severe); in the case of severe atrophy, the term total atrophy can be used. Do not use scores on singular morphological elements that are overly subjective and of no use in the final diagnosis.
Below a descriptive scheme is proposed, bearing in mind that it is also possible to relate the morphological picture with a simple description in which the same morphological elements described in the table are reported in sequence and concluding with compatibility or not with a diagnosis of coeliac disease.
In the final diagnosis, it is important not to use the term coeliac disease, but only to report the histological alterations encountered. The pathologist has the task of providing the image of one moment in a disease, whose final diagnosis is entirely up to the paediatric or adult gastroenterologist.
For this reason, we suggest:

- the completion of a form like the one proposed below

  or

- a precise description, possibly with a diagnostic orientation, and a diagnosis based only on the histological alterations encountered.

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Anatomical-Pathological Evaluation Form

Name _____

Surname_____

Sex M___ F___

Birth date _____/_____/_____

First biopsy _____ N°_____ Control_____ N°_____

N° Biopsies _____ Orientation correct _____ incorrect_____

Villi : normal_____ Atrophy ____ mild_____ moderate_____ serious_____

Villous/crypt ratio Normal [3/1] _____ Altered _____

Intraepithelial lymphocytes normal_____ increased_____

(< 25 lymphocytes / 100 epithelial cells) (> 25 lymphocytes / 100 epithelial cells)

Evaluation with CD3_____

Glands: normal _____ hyperplastic _____

Lamina propria _____

Diagnosis (in accordance with Marsh Oberhuber)

Type 0 _____

Type 1 _____

Type 2 _____

Type 3a _____

Type 3b _____

Type 3c _____

New proposed classification

Grade A _____

Grade B1 _____

Grade B2 _____

Histologically Evaluable Complications

There is a great deal of evidence that adult coeliac disease, unlike what happens in the child, is burdened by a mortality rate that is greater than in the general population. This is especially true if the disease is diagnosed late and even more so if not promptly treated with a rigorous gluten-free diet.

Removing gluten from the diet thus not only brings about an improvement in the bioptical and clinical situation, but also prevents the complications below, which must always be suspected if the adult patient continues to ail despite the diet.

Collagen Sprue

The patient does not respond to the diet and histologically we observe fibrous tissue in the superficial under-epithelial layer of the intestinal mucosa. This very rare pathology described in the literature is one that I have never personally encountered.

Ulcerative Jejunum Ileitis

Presence of extensive ulcerations in the intestinal mucosa.

Lymphoma

This is the most serious complication, and histologically must always be suspected when there is a prevalence of monomorphic atypical lymphocyte elements. In these cases, it is useful to make an immune-phenotype typification of the lymphoid population, generally composed of T lymphocytes.

DIFFERENTIAL DIAGNOSIS

What has been set forth above describes, in synthesis, all of the histological lesions that can be found in coeliac disease. Clearly, the pathologist has a crucial role to play in the diagnosis, if only to exclude the possibility that the clinically suspected malabsorption syndrome might be caused by other factors:

- parasitic (Giardia Lamblia, Cryptosporidium, Microsporidium);
- infective (Whipple disease);
- viral (Citomegalovirus, Herpes virus);
- idiopathic (Crohn's Disease);
- neoplastic.

Nevertheless, three conditions deserve particular mention:

• Forms of so-called "autoimmune enteritis" possible in children with immunological deficits (Common Variable Immunodeficiency, X–linked Agammaglobulinemia), in which the intestinal

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biopsy may be exactly like the one found in CD (Washington K.[2], Daniels J.A.[25]).

- Damage by drugs: there is increasing evidence in the literature that the use of non-steroidal anti-inflammatory drugs (NSAIDs) in particular can provoke morphological changes similar to those caused by coeliac disease. Keep this possibility in mind, especially when observing cases of elderly subjects who have a total absence of the serological markers.
- The possibility that a concomitant Helicobacter pylori infection in the stomach may determine a morphological picture like that of the initial lesions of coeliac disease, as recently reported by Memeo L et. al.[16] and confirmed by Villanacci et. al.[17]

At the end of this brief look at the anatomical-pathological aspects of coeliac disease and borrowing the definition that Arthur Hailey gives the pathologist in his novel "The Final Diagnosis" (1959), we can say that the pathologist is:

"the doctor the patient never sees, but who often has the task of dictating the final diagnosis that can save a life"

In other words, the pathologist is an important link in the diagnostic chain, allowing the gastroenterologist, paediatrician and general practitioner to make an accurate diagnosis.

Pathological aspects

What are the "certain" points in the diagnosis of coeliac disease?

From the anatomical-pathological perspective, an obvious premise to a certain diagnosis of coeliac disease is the observation and respect of a series of established criteria:

1. close collaboration between clinician, endoscopist and technician;
2. correct number of biopsies [26] (at least 4; 2 from distal and 2 from proximal jejunum);
3. correct orientation of the biopsies (use of nitrate cellulose pre-cut filters);
4. sufficient clinical information;
5. optimal sample quality.

• From what has been explained, it is clear that the diagnosis of coeliac disease is certain only when there is villous **atrophy** and a pathological increase in the number of intraepithelial lymphocytes **(> 25/100 epithelial cells).** In this situation, by applying the three classifications currently recognized and validated (Marsh, Marsh-Oberhuber and Corazza-Villanacci), there is no problem making the diagnosis and comparing the clinical and laboratory data.

• The atrophy must be certain and not pseudo-atrophy due to incorrect orientation and cutting of the filter. An useful suggestion in these cases is always to evaluate the number of intraepithelial lymphocytes, which 'must' always be pathological (> 25/100 epithelial cells). This is true both when they are evaluated with H&E stain and with CD3 immunohistochemical stain, which is always advisable.

• Pay attention to biopsies of the duodenal bulb, where the presence of Brunner glands may lead to a false diagnosis of atrophy. Always compare the bulb biopsies with biopsies of the distal portions, especially in the initial phases of the disease when there is a progression of the pathological process in a craniocaudal direction.

• If present, always describe the variable degrees of atrophy and do not limit yourself to the degree of major severity. Moreover, express a judgement of compatibility only in the description of the case, absolutely avoiding the word "coeliac disease" in the final diagnosis. In the diagnosis, only give to clinician a precise "photograph" of the state of the duodenal mucosa. Remember that the diagnosis of CD must be made only and exclusively by the paediatric or adult gastroenterologist.

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What are the "uncertain" points in the diagnosis of coeliac disease?

The "uncertain" points and areas for extreme caution for the pathologist in diagnosing CD are clearly represented by cases where one finds initial lesions

(Marsh 1-2 and Grade A according to the new proposed classification). In these cases:

1.carefully evaluate the orientation of the biopsies;

2.examine whether the villous/crypt ratio of at least 3:1 is maintained;

3.carefully count the number of lymphocytes in the superficial epithelial layer;

4.always associate the immunohistochemical evaluation with CD3;

5.compare clinical and laboratory data.

- The **two** essential elements to evaluate are always **the absence of atrophy and an increase in the number of intra-epithelial lymphocytes.** For this reason, always associate the immunohistochemical evaluation with CD3. Whether or not there is hyperplasia of glandular elements is irrelevant in terms of practical and therapeutic aims.
- Remember that the **"slide"** remains as evidence of the pathologist's evaluation and as such is subject to comparison and re-evaluation by other colleagues and specialists. Therefore, it is important to emphasize that the histological evaluation must only be made by pathologists and not by other "specialists".
- In the **"uncertain"** cases it is all the more important to express a **judgement** of possible compatibility only in the description of CD, limiting yourself to formulating the histological picture in the final diagnosis.
- If possible, exclude an attendant Helicobacter pylori infection (gastric biopsies should always be taken in this case), immune deficit, parasitic infections, allergies and other alimentary factors, and use of drugs.
- In these situations, it is useful to keep in mind the excellent review by Brown et al. [20] in which the final summary information can be conveniently applied to all the uncertain situations. As we have said, the pathologist must always be certain to be dealing with a pathological condition unequivocally demonstrated by an increasing number of intraepithelial T lymphocytes confirmed by evaluation with CD3. The final diagnosis will be based on the comparison of clinical and laboratory data.
- In paediatric patients in the first year of life, remember the possibility of intolerance to cow's milk proteins. In this case, an eosinophil count may be useful (pathological value > 60 per 10 vision field x 40)

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Appunti

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