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REVIEW ARTICLE

The correct methodological approach to the diagnosis of celiac disease: the point of view of the pathologist

Vincenzo Villanacci[1], Rachele Del Sordo[2], Giovanni Casella[3], Gabriel Becheanu[4], Arianna Oberti[1], Baraa Belfekih[1], Gabrio Bassotti[5], Alberto Ravelli[6]

[1]Institute of Pathology, ASST Spedali Civili and University of Brescia, Brescia, Italy
[2]Department of Medicine and Surgery, Section of Anatomic Pathology and Histology, Medical School, University of Perugia, Perugia, Italy
[3]Medical Department, Desio Hospital, 20832 Desio, Italy
[4]Department of Pathology, Carol Davila University of Medicine & Pharmacy, 050474 Bucharest, Romania
[5]Gastroenterology and Hepatology Section, Department of Medicine and Surgery, University of Perugia, Perugia, Italy
[6]Gastroenterology and GI Endoscopy Unit, University Department of Pediatrics, Children's Hospital, ASST Spedali Civili, Brescia, Italy

ABSTRACT

The diagnosis of celiac disease relies on the assessment of serological data and the presence of histological alterations in the duodenal mucosa. The duodenal biopsy is pivotal in adults, and in some circumstances in children, to confirm the clinical suspicion of celiac disease. The correct interpretation of duodenal biopsies is influenced by numerous variables. The aim of this overview is to describe the correct methodological approach including the procedures of biopsy sampling, orientation, processing, staining and histopathological classification in order to avoid or minimize the errors and the variability in duodenal biopsy interpretation.
Multiple biopsies taken from different sites of the duodenum during endoscopy maximize the diagnostic yield of duodenal histological sampling. Proper orientation of the biopsy samples is of the utmost importance to assess histological features of pathological duodenal mucosa and to avoid artifacts that may lead even an experienced pathologist to a wrong histological interpretation with subsequent misdiagnosis of celiac disease. An immunohistochemical stain for CD3 can be invaluable to aid the pathologist in obtaining a more accurate intra-epithelial T lymphocytes count. A simplified histological classification facilitates the clinician's work and improves the communication between pathologist and clinician. An integrated clinical and pathological approach is required for a correct diagnosis of celiac disease since a relatively large number of conditions may cause duodenal damage with a histological appearance similar to that of celiac disease.

Keywords: Celiac disease, Duodenal biopsies, Biopsy handling, Biopsy orientation, Histopathological classification.

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Introduction

Celiac disease (CD) is a relatively frequent, immune-mediated disorder triggered by gluten ingestion in genetically predisposed individuals (1). CD is characterized by small intestinal enteropathy, clinical symptoms related to gastrointestinal (GI) involvement as well as extra-intestinal manifestations, frequent association with other autoimmune diseases (2), and serological positivity for antibodies to tissue transglutaminase 2 (tTG) and antiendomysial antibodies (EMA). The complex interplay of genetic and environmental factors underlies the wide spectrum of clinical presentations of CD ranging from asymptomatic to severe malabsorption (3). CD is one of the most common autoimmune disorders, with an overall prevalence of about 0.5–1% in the general population (4-8). The disease can present at any age

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Reprint or Correspondence: **Rachele Del Sordo,** *Department of Medicine and Surgery, Section of Anatomic Pathology and Histology, Medical School, University of Perugia, Perugia, Italy.*
E-mail: rachele.delsordo@unipg.it
ORCID ID: 0000-0003-3930-2957

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with two peaks of onset: one in the first 2 years of life after the introduction of gluten in the diet and the other in the second or third decade of life (9).

The diagnosis of CD requires a careful evaluation and integration of clinical, serological (tTG and EMA), genetic (HLA DQ2 –DQ8) and histological aspects. Apart from genetics, all these aspects can be reverted to normal by a gluten-free diet, and thus they must be evaluated while the patient is still on a diet containing gluten.

The duodenal biopsy with histological evaluation plays a pivotal role for diagnosing CD and still represents the gold standard for the diagnosis of CD in adults. In children, however, the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) guidelines state that if tTG-IgA level is ≥ 10 times the upper limit of normal (ULN), and EMA are positive, endoscopy with duodenal biopsy is not necessary for the diagnosis (10). The presence of symptoms and HLA testing are also not necessary in these cases.

Several studies have proposed a no-biopsy approach also for the diagnosis of CD in adults (11-14). IgA tTG titers ≥ 10 ULN have a strong predictive value for identifying adults with histopathological lesions associated with CD (15). However, this strategy is not yet recommended given the lack of multicenter validation and use of different serologic tests. Thus, duodenal biopsy remains mandatory for establishing a correct diagnosis in adults.

To obtain most representative biopsies and to minimize the errors and the variability in biopsy interpretation, a close collaboration is required between the endoscopist, the endoscopy-room nurse, the pathology laboratory technician and the pathologist (16-19).

Where to biopsy and how many biopsies are needed

In CD the histological lesions may not be uniformly distributed (i.e. they can be patchy) and villous atrophy may have a different grade even in the same sample (20, 21). Four or more biopsies reduce the chance of missing CD (22). To identify the patchy distribution of lesions, the biopsies should be taken from both the duodenal bulb and the second duodenal portion. In children, bulb duodenum biopsies are essential. In fact, several studies have reported that histological lesion can be restricted to the duodenal bulb in 2,5% to 13% of patients, especially in children (23-25). Two biopsies from the bulb and four biopsies from the second duodenal portion are therefore recommended for maximum diagnostic yield (22, 26, 27). In any case, since the decision on the tests to be carried out rests with the unquestionable judgment of the clinician, the only one who knows the patient's symptoms and laboratory data, establishing the exact diagnostic procedure and also for the biopsies the rule is to perform biopsies in the distal duodenum and in the bulb.

How to handle biopsies

The histological features of CD are represented by:

-Increased intra-epithelial T lymphocytes (IEL) count greater than 25 per 100 enterocytes;

-Crypt hyperplasia: more than one mitotic figure per crypt, usually accompanied by a decrease in mucosecretive activity and crypts elongation;

-Villous atrophy: decrease in villous height, together with crypt/villous ratio (normal 3:1) must be taken in consideration.

Proper biopsy orientation is pivotal to evaluate the above lesions of intestinal mucosa that are not pathognomonic for CD. In fact, such or similar lesions can be observed in other GI disorders (e.g. immune deficiency, GI infections, autoimmune enteropathy, collagenous sprue and tropical sprue) and can be related to certain drugs (e.g. angiotensin II receptor blockers and immune checkpoint inhibitors) (28, 29). The orientation of biopsies begins in endoscopy room. Each sample is placed by the endoscopist in a straight line on a strip of paper (we use and recommend a strip of acetate cellulose filter paper, see Figure 1) (30), with the luminal surface upwards. One end of the filter paper has a “clarinet beak-shaped cut”, identifying the beginning of the biopsy sequence so that the same strip can be used for all biopsy samples, from the distal duodenum or proximal jejunum to the duodenal bulb (Figure 1).

After fixation, the filter-biopsy combination is processed and then embedded. During this phase the technician rotates the filter-biopsy combination 90 degrees without detaching the samples, in order to place the samples in their normal, proximal to distal

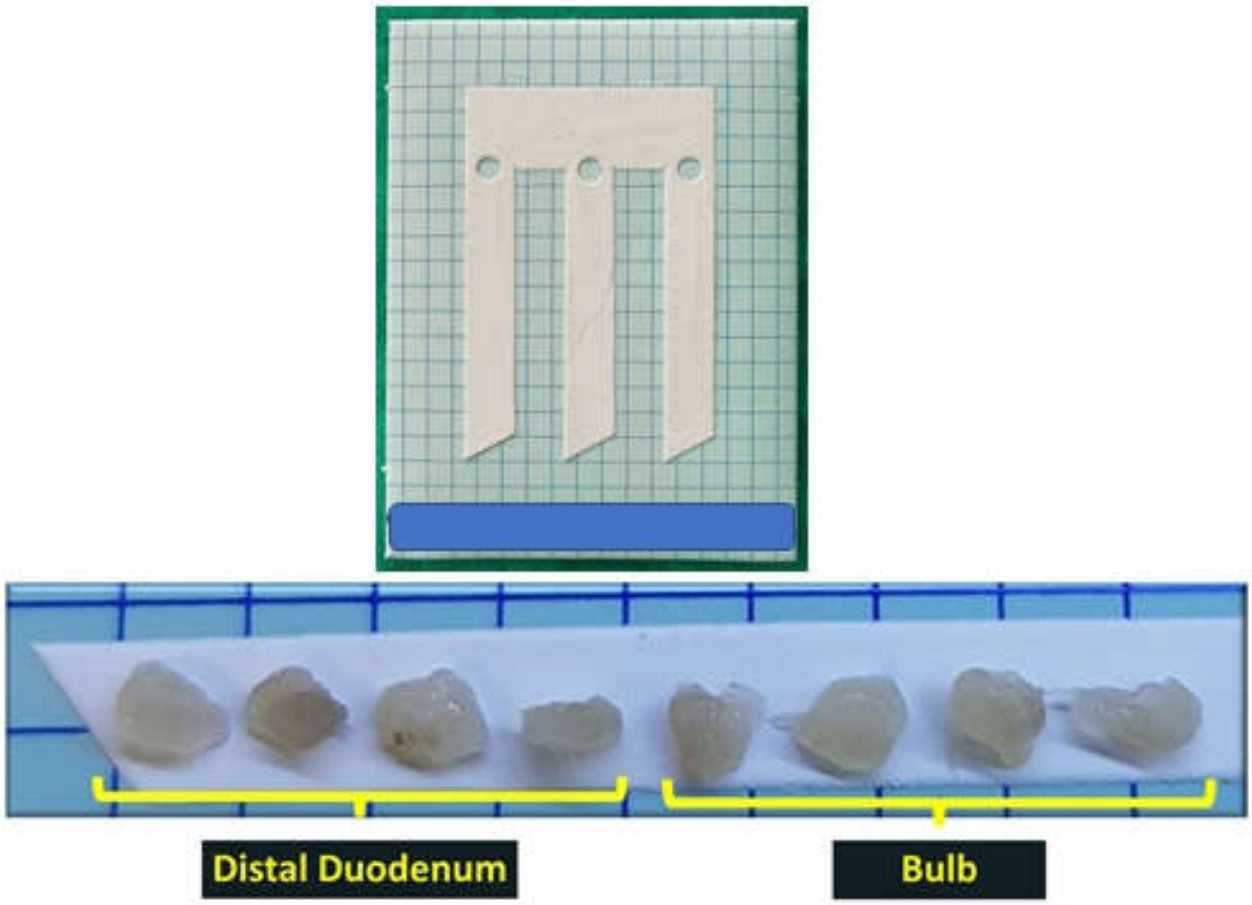

Figure 1. Acetate cellulose filter.

position. Placing the endoscopic biopsies in a straight line allows the pathology technician to cut sections that include all the biopsy samples (Figure 1). The cellulose acetate filters allow perfect adhesion of the biopsies (thus avoiding their dispersion in the fixation medium), do not react chemically with the fixatives and reagents used during processing of the biopsies, and do not offer resistance and do not fray during the cutting phase. This method also saves the technician's time by reducing the number of embeddings and the number of sections to be cut and stained. The pathologists, also, benefit from this method as it increases the diagnostic yield of biopsies (30). The one described is a method of undoubted advantage also from an economic point of view and in any case everyone can use the way of orientation they believe best.

Stains

One haematoxylin-eosin (H&E) stained section is enough to assess all the morphological features. Immunostaining with anti-CD3 can be useful for a correct count of IEL especially in initial forms of CD (30). CD8 immunostaining may be required in biopsies of elderly patients in order to exclude refractory CD, since the expression of CD8 may be reduced in this condition (31).

How not to handle biopsies

In many centers the biopsy specimens are placed by the endoscopist or nurse straight into the fixation medium. As a consequence, non-orientated biopsies are embedded in paraffin haphazard, and the orientation of samples on slide sections is left to chance. Duodenal biopsies that are not correctly orientated (Figure 2) may lead even an experienced pathologist to a wrong histological interpretation with subsequent misdiagnosis (usually false positive, rarely false negative) of CD.

The pathologist can fall into many pitfalls in the assessment of elementary lesions (17). An imperfect orientation of the biopsy may cause overlap of T lymphocyte nuclei and thus results in an inaccurate IEL

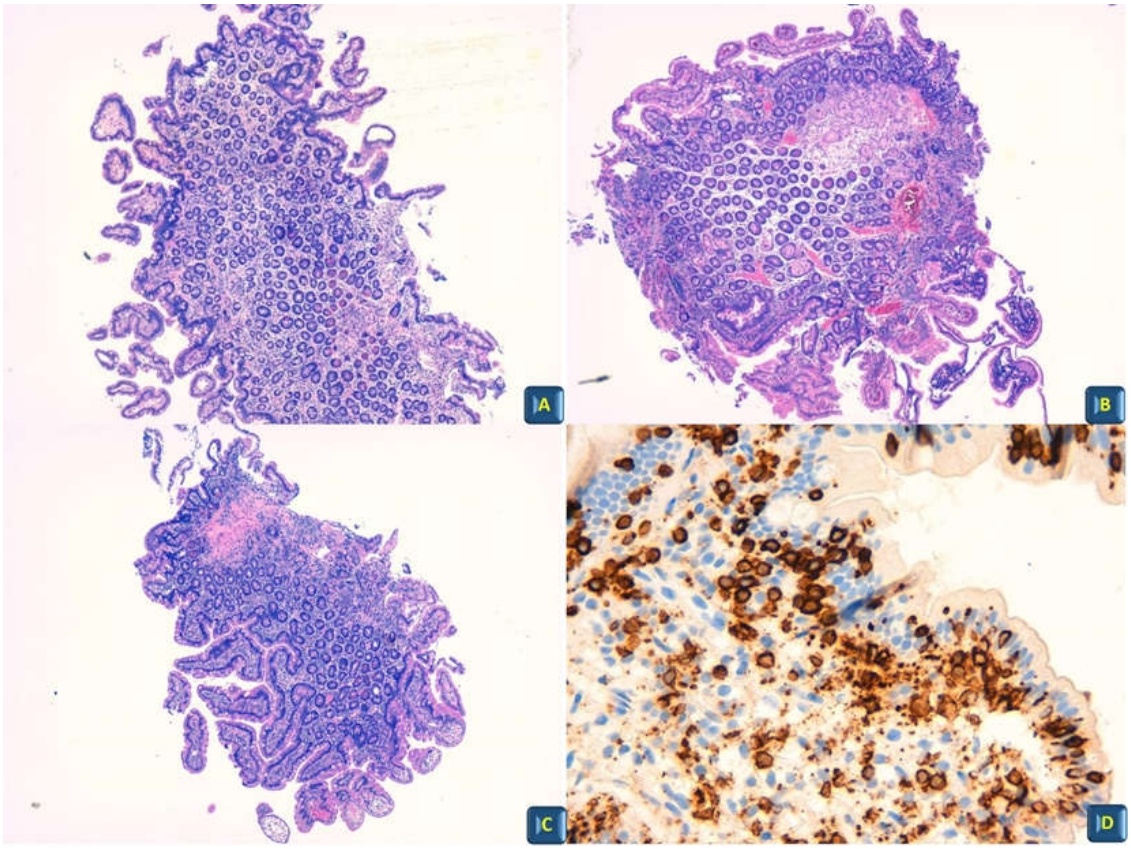

Figure 2. A-B-C-Biopsy non oriented: evaluation of villous height and the villous/crypt ratio is difficult (H&Ex4), D-An imperfect orientation causes overlap of enterocytes leading to overestimate intraepithelial T lymphocytes count (CD3 immunostain x20).

count (Figure 2) even on CD3 immunostaining sections, whereas the overlap of enterocytes leads to overestimate IEL count (Figure 2). In a poorly oriented biopsy it is also difficult to evaluate the villous height (Figure 2) and the villous/crypt ratio, as villi may appear shorter and crypts may look hyperplastic (Figure 2) with a false reduction of the villous/crypt ratio. Furthermore, it can be difficult to define the degree of histological lesions, since adjacent villi may appear partially fused or crushed, giving the wrong impression of partial villous atrophy and causing difficulties in IEL count.

The pathologist should attempt to clarify the histological artifacts by cutting other sections at deep levels and by repeating CD3 immunostaining on these sections.

Pitfalls in properly orientated biopsies

The thickness at which the histological section is cut can influence the IEL count, because it may contain more or fewer lymphocytes also in biopsies correctly orientated (19).

The IEL counts performed on CD3-stained sections will be higher than the corresponding H&E–stained slides due to the fact that the lymphocyte plasma membrane is indistinguishable from enterocytes, so a lymphocyte is counted on a H&E slide only when its nucleus is included in the section and stained. Immunohistochemistry (IHC), on the other hand, labels the membrane of CD3+ cells, so even only a small anucleate section of lymphocyte membrane will be stained. Thus, when a T lymphocyte is cut so that only its membrane is included in the slide, then it will be visible in CD3–IHC slides and invisible in H&E. Lymphocytes below the basement membrane, in the superficial lamina propria should not be counted as IELs. IHC-stained slides pose a particular challenge in this regard, because the detection reaction will make the stain extend slightly beyond the lymphocyte membrane, thus falsely appearing very close to enterocytes. The degree of the increase in IELs should also be considered: while virtually all CD patients will show more than 25 IELs per 100 enterocytes, the vast majority of them will show greatly increased counts

(i.e. >40/100). This is in contrast with most mimickers and borderline normal cases, where the IEL count will be borderline (20–30 IEL per 100 enterocytes) (Figure 3).

The location of lymphocytes within the villus is also important. Normally, IEL are more abundant along the sides of the villus and fewer at the tip. Especially in imperfectly-oriented biopsies, the pathologist should be aware of this fact and he/she should try to identify the villus tips.

Finally, especially in duodenal bulb biopsies, the epithelium overlying lymphoid tissue patches can normally show an increased IEL count, so the epithelium overlying lymphoid patches should not be used to assess IEL due to the risk of false positives.

Another example of histological artifact, not strictly related to inaccurate orientation of the biopsies is bigeminism or "twin villi" consistent of broad and duplicated villi (17). Its presence suggests partial villous atrophy. However, when further sections are cut along the vertical axis the villi show a normal appearance.

Histopathological classification

On the basis on the presence of one or more of the elementary lesions the histopathology of CD is subdivided into different category. The Marsh classification (32) is universally recognized and has been extensively validated. It classifies CD as follows:

- Type 1 or infiltrative lesion: only intraepithelial lymphocytosis;
- Type 2 or hyperplastic lesion: intraepithelial lymphocytosis and crypt hyperplasia;
- Type 3 or destructive lesion: intraepithelial lymphocytosis, crypt hyperplasia, and villous atrophy.

Oberhuber modified the Marsh classification (33) by splitting the type 3 lesion in three subgroups based on the degree of villous atrophy:

- Type 3a: mild villous atrophy;
- Type 3b: moderate villous atrophy;
- Type 3c: total villous atrophy.

Corazza and Villanacci (34) classify CD in only three categories:

- Type A (Non-Atrophic): intraepithelial lymphocytosis, with or without crypt hyperplasia, but without villous atrophy;
- Type B (Atrophic): intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy, further subdivided into
- Type B1: low-moderate villous atrophy (villi still recognizable, but villus/crypt ratio < 3:1);

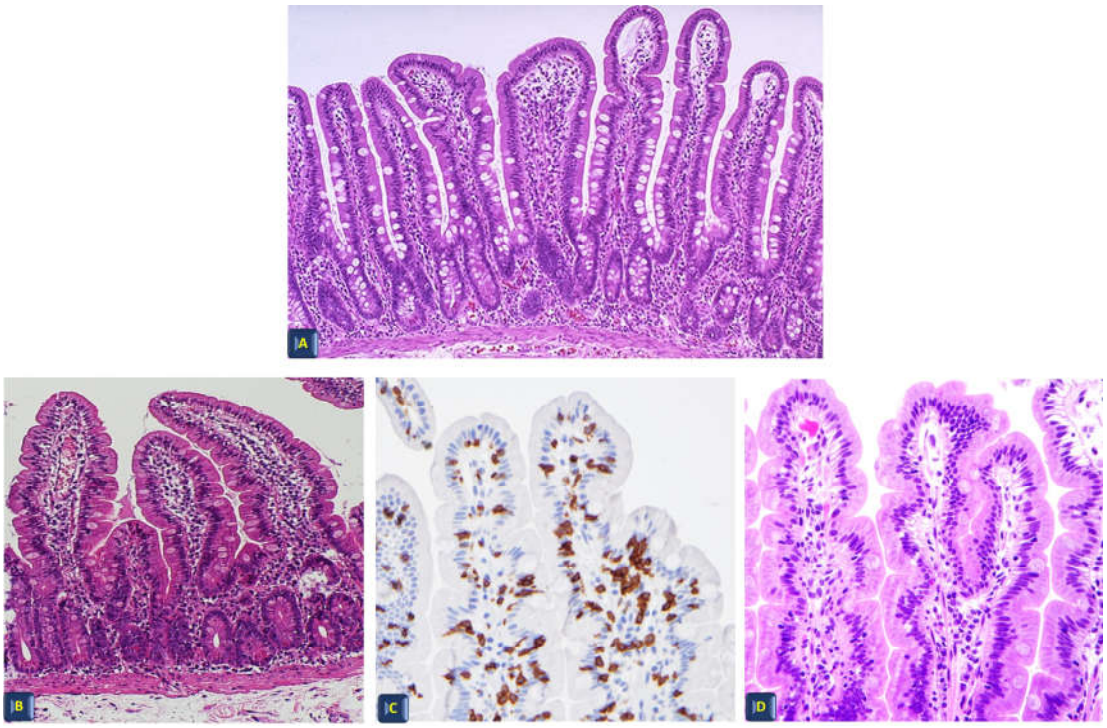

Figure 3. A-B-D: Normal duodenal mucosa: villus/crypt ratio over 3:1, C number of intraepithelial T lymphocytes < 25 per 100 epithelial cells (A H&E x4, B H&E x10, C H&E x20, C: CD3 immunostain x10).

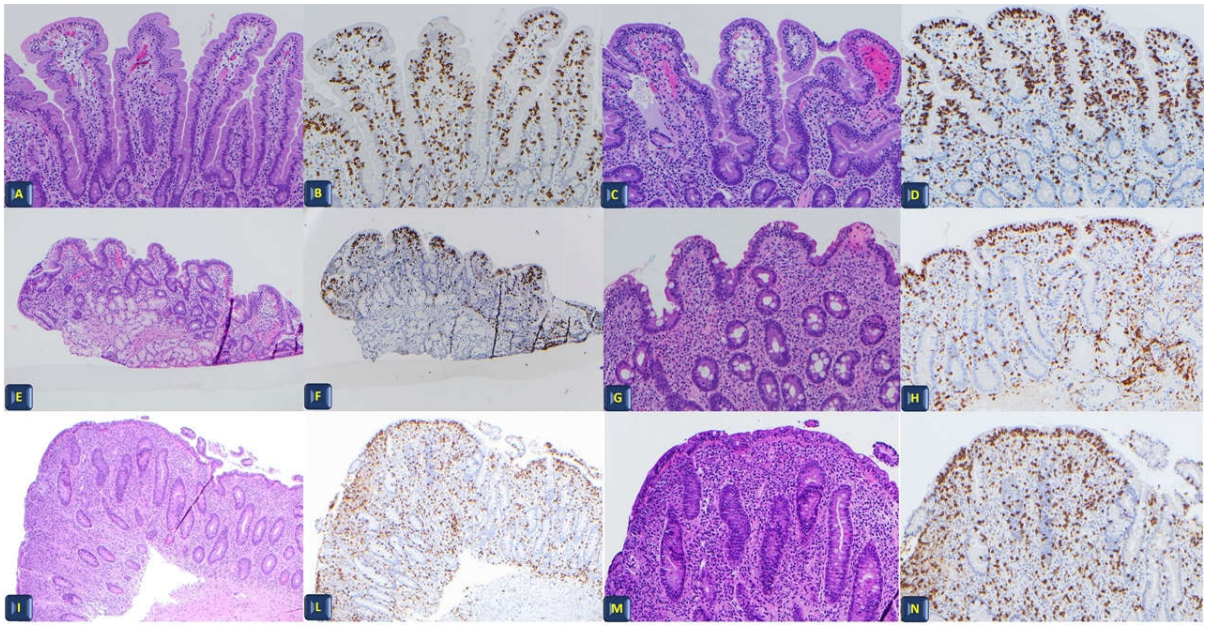

Figure 4. A-B-C-D: Type 1 – Grade A lesion; normal villi but with a pathological increase of intraepithelial T lymphocytes > 25 per 100 epithelial cells (A-C H&E x10, B-D CD3 immunostain x10). E-F-G-H: Type 3A-3B-Grade B1 lesion: mild to moderate villous atrophy with pathological increase of intraepithelial T lymphocytes (E-G H&E and CD3 immunostain x10, G-H H&E and CD3 immunostain x40). I-L-M-N: Type 3C - Grade B2 lesion; severe villous atrophy with pathological increase of intraepithelial T lymphocytes. I-L H&E and CD3 immunostain x10, M-N H&E and CD3 immunostain x20.

• Type B2: complete villous atrophy (villi no longer identifiable).

This latter classification aims at simplifying the diagnostic criteria and reduce the number of categories in order to increase the interobserver agreement, facilitate the clinician's work and improve the communication between pathologist and clinician (35).

Recently, an even more simplified classification with only two entities was proposed by Villanacci (36):

• Type A (Non-Atrophic): intraepithelial lymphocytosis, with or without crypt hyperplasia, but without villous atrophy;

• Type B (Atrophic): intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy, without further subdivisions (Figure 4).

Conclusion

The incidence of CD is increasing over time (37). CD can be diagnosed at any age, including elderly patients (38), and its clinical presentation is extremely variable: the symptoms vary from patient to patient, being sometimes more subtle or even absent (39). On the other hand, an increasing number of conditions may cause duodenal damage with a histological appearance similar to that of CD, so that the differential diagnosis can be challenging for pathologists (28).

Thus, an integrated clinical and pathological approach is required for a correct diagnosis of CD.

Conflict of interests

The authors declare that they have no conflict of interest.

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