Pathology – Research and Practice 208 (2012) 197–202

Contents lists available at SciVerse ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.de/prp

Review

Tricks of the trade: How to avoid histological Pitfalls in celiac disease

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ARTICLE INFO

Article history:
Received 11 November 2011
Received in revised form
30 December 2011
Accepted 30 January 2012

Keywords:
Celiac disease
Gluten-sensitive enteropathy
Duodenal biopsy

ABSTRACT

Currently, the diagnosis of celiac disease (CD) is based upon the combination of raised serum anti-tissue transglutaminase or anti-endomysial antibodies and the presence of histological alterations of variable degree in the duodenal mucosa. Interpretation of duodenal biopsies is subjected to a number of variables, and the lack of standardization may cause diagnostic controversy or even misdiagnosis.

The aim of this overview is to solicit a standardization of the procedures of biopsy taking, orientation, processing, staining and interpretation in order to avoid or minimize misinterpretation of duodenal biopsies.

Based on a literature review and extensive personal experience, the appropriate methodology of duodenal biopsy taking, orientation, fixation, processing, staining and interpretation was thoroughly reviewed, and the most common and relevant errors and artifacts were identified.

To maximize the diagnostic yield of duodenal biopsy in CD, multiple specimens are best taken from different sites of the duodenum during endoscopy, and careful visual inspection of the duodenal mucosa may help identify abnormalities related to villous atrophy. Biopsy handling and orientation are of utmost importance to avoid artifacts that may impair the pathologist's ability to detect pathology and normality. Immunostaining with anti-CD3 monoclonal antibody should be carried out, and a simplified histological classification may help distinguish atrophic from non-atrophic stages of CD enteropathy.

Meticulous attention to biopsy orientation, handling and processing – together with the knowledge of the most common histological artifacts – is necessary to avoid a wrong histological interpretation which, in turn, may lead to misdiagnosis in CD.

Contents

Introduction

With a worldwide prevalence of up to 1:100, celiac disease (CD) is one of the commonest diseases in the world [10,9]. Nowadays, the routine diagnosis of celiac disease (CD) is based on the presence of typical histological alterations of the duodenal mucosa – mainly a degree of villous atrophy [16,19] – in a subject who has raised serum levels of anti-tissue transglutaminase (tTG) or anti-endomysial (EMA) IgA antibodies (except for patients with selective IgA deficiency). These antibodies have a sensitivity and specificity approaching 100%, and are used as the screening test in individuals suspected to have CD. Such individuals belong to four major categories: (1) patients with chronic gastrointestinal symptoms, (2) patients with a variety of extra-intestinal manifestations that may be due to CD (dermatitis herpetiformis being the most common), (3) individuals who suffer from a condition where the prevalence of CD is significantly higher than in the general population

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0344-0338/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
doi:10.1016/j.prp.2012.01.008

(e.g. another autoimmune disease, Down's syndrome, Turner's syndrome, selective IgA deficiency), and (4) individuals who have a first degree relative affected by CD [10,9]. In patients with positive tTG and/or EMA, an upper GI endoscopy with duodenal biopsies is recommended, and the finding of a T cell-mediated enteropathy leads to the final diagnosis of CD, which is commonly defined as "typical" or "classic" if the patient has GI symptoms, "atypical" if the patient has extra-intestinal symptoms, and "silent" or "subclinical" if no or minor GI symptoms are present [10,9].

Even in Western countries, physicians are not generally aware of the high prevalence of CD and of the many and diverse clinical manifestations that may be due to CD, so the pathway leading to the diagnosis of CD may not always be easy and straightforward, based on either a case-finding or an at-risk group screening strategy [10,9]. This knowledge, together with the high sensitivity and specificity of tTG, has led many leading adult and pediatric gastroenterologists – as well as the major gastroenterological societies – to question the need of small bowel biopsies in all cases of suspected CD [26,1]. For instance, everybody would probably agree that a patient with chronic diarrhea or a malabsorption syndrome in whom raised serum tTG and/or EMA are found is affected by CD unless proven otherwise. However, many would also agree that CD can be reasonably and reliably diagnosed without small bowel biopsy if raised TG or EMA are found in several other conditions, such as iron deficiency anemia, juvenile osteopenia, insulin-dependent diabetes mellitus, thyroiditis, and short stature (in children), especially if mild gastrointestinal symptoms are also present. Indeed, the presence of tTG > 100 units is almost always associated with a positive small bowel biopsy, i.e. a biopsy showing a lesion of histological grade ≥2 according to the Marsh–Oberhuber classification [1].

However, a proportion of patients in whom CD is suspected and serum tTG or EMA are raised do not have a "positive" biopsy but show a normal (grade 0) or nearly normal (grade 1) duodenal histology, regardless of the presence of clinical symptoms [22,13,17]. In subjects who have the HLA DQ2 or DQ8 haplotype predisposing to CD, these findings define the conditions known as "potential" or "latent" CD [30,21]. A grade 1 lesion is totally non-specific and can be found in a number of other inflammatory conditions of the GI tract, including infections, chronic inflammatory bowel disease, and food hypersensitivity other than CD [5]. However, genetically predisposed individuals with raised tTG/EMA who have a mild enteropathy may already have overt clinical symptoms and may eventually develop a full blown enteropathy with villous atrophy if a gluten-free diet is not commenced [14]. Therefore, the absence of duodenal villous atrophy cannot reliably rule out CD in these patients, who deserve a careful clinical, serological and histological follow-up.

Furthermore, antibody-negative CD may also occur [11], and, on the other hand, a gastrointestinal disease other than CD can cause the symptoms.

In the following paragraphs we provide some recommendations based on a review of the most recent literature and on our personal experience, aimed at minimizing the errors and the variability in biopsy interpretation, thus increasing the diagnostic yield of small bowel biopsy in CD.

Where and how to take biopsies

Histological lesions may not be uniformly distributed within the duodenum. In fact, "patchy" villous atrophy – i.e. villous atrophy localized only in some duodenal areas [27,31,2,4,3,32] or villous atrophy of different grade at different biopsy sites and/or within the same biopsy [23,25] – has been reported. These latter findings raised the question as to how many biopsies should be taken in order to reliably confirm or deny the diagnosis of CD. Whenever possible, biopsies should be targeted to areas showing the endoscopic features suggesting the presence of villous atrophy, such as a mosaic or cobblestone mucosa, flattening and scalloping of duodenal folds, visibility of the submucosal vascular pattern, and multiple erosions [24] (Fig. 1). However, the sensitivity and specificity of such endoscopic features are still controversial and highly dependent on the endoscopist's experience and expertise [15]. The visibility of mucosal changes, such as the mosaic appearance and scalloping of duodenal folds, can be increased by magnification and chromoendoscopy [15,28,18], but endoscopic magnification is not always available and chromoendoscopy substantially prolongs the duration of endoscopy. Furthermore, in order to use these procedures, the endoscopist should be aware of the clinical suspicion of CD, which is not always the case especially in busy open access endoscopy units. Therefore, at this time, the most realistic recommendation is probably to take 3–4 biopsies from different duodenal areas from the bulb to the distal duodenum or the duodenojejunal flexure [23,25,20]. In our experience, in infants and children, taking biopsies from the duodenal folds usually results in specimens of good size even when the smallest forceps (5 mm open cup diameter) are used, without increasing the risk of significant bleeding.

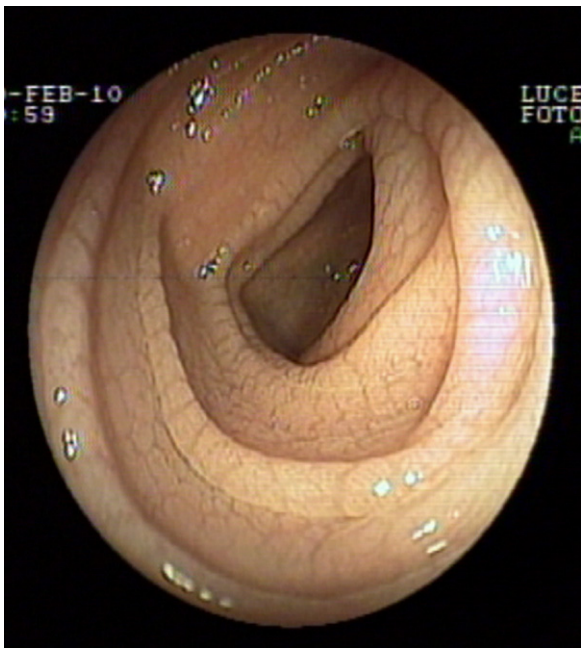

Fig. 1. Endoscopic view of the duodenum showing the most typical endoscopic features of celiac disease with total villous atrophy: cobblestone or "mosaic" mucosa and scalloping of duodenal folds.

Biopsy handling and orientation

Biopsy taking is only part of the job, however. Assuming the biopsy size is adequate, proper handling of the biopsy is equally important in that inaccurate orientation may result in misinterpretation and thus misdiagnosis. A few years ago, Green and Jabri observed that "a major pitfall in the diagnosis of CD is in pathological interpretation of intestinal biopsies" [10]. Indeed, biopsy handling can sometimes be inadequate even in referral centers, as shown by a multicenter European experience where the quality of biopsy specimens was reported as "unacceptable" in more than 10% of cases, and a reliable judgment could not be made mainly due to the poor orientation of the biopsy samples [6].

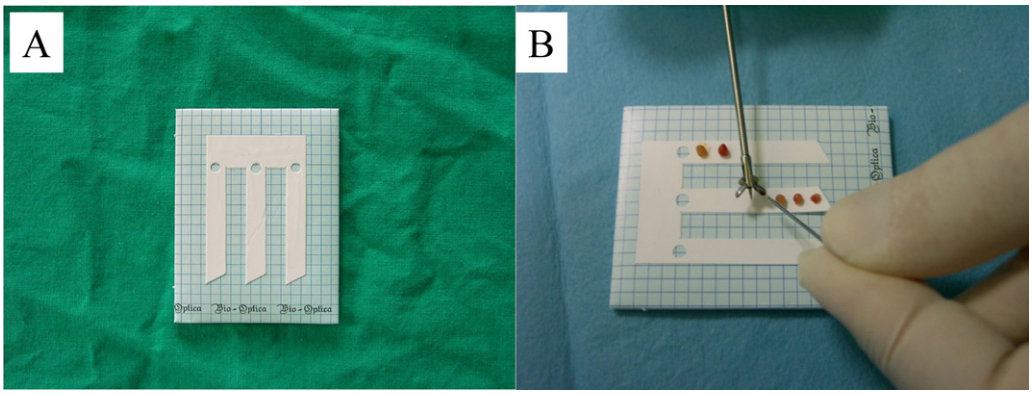

Fig. 2. (A) A three-strip cellulose acetate filter, with the oblique, clarinet mouthpiece-shaped end indicating the start of each biopsy sequence. (B) Positioning of duodenal biopsies from the biopsy forceps onto cellulose filters.

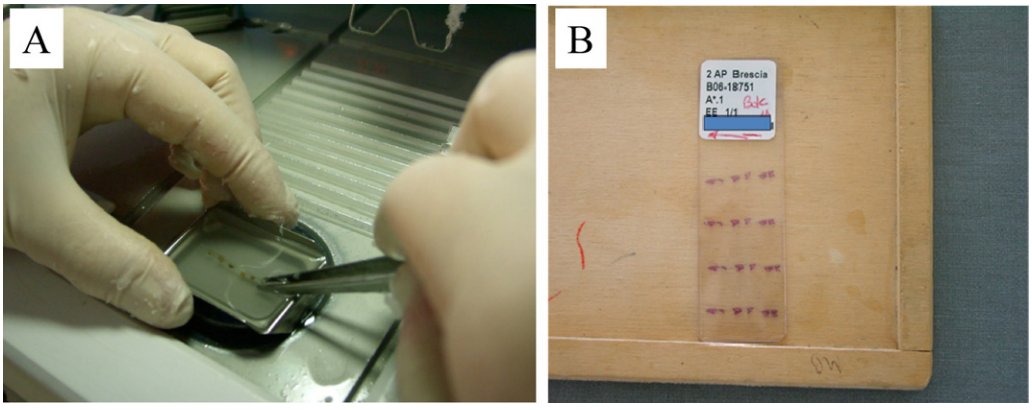

Fig. 3. (A) Inclusion/embedding of the biopsy/filter assembly and (B) a slide where a red arrow indicates the order of the biopsy sequence.

Guided by our personal experience and a careful review of the literature, we regularly apply a simple modification of the method based on the use of cellulose filters. In particular, after an initial experience with filters that needed to be cut with obvious waste of time and human resources, a comprehensive kit was developed on which three easily detachable filters with a beveled end shaped like a clarinet mouthpiece are already fixed (Fig. 2A). With a gentle clockwise or anti-clockwise rotation of the wrist, and the help of a needle if necessary, the endoscopist or endoscopy nurse gently transfers the biopsy from the forceps onto a filter, with the luminal side upwards (Fig. 2B). Handling the biopsies under the lens of a stereomicroscope may also help achieve an adequate orientation. Positioning of the biopsies on cellulose acetate filters is good for the laboratory staff, since with a simple 90° rotation it is possible to embed the whole biopsy-filter assembly (Fig. 3A). Even more importantly, this method provides adequate material for

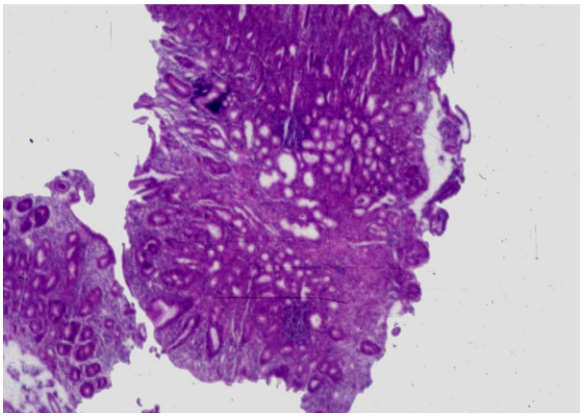

Fig. 4. Duodenal biopsy not correctly oriented, where it is impossible to define the degree of histological lesion (H&E, 20×).

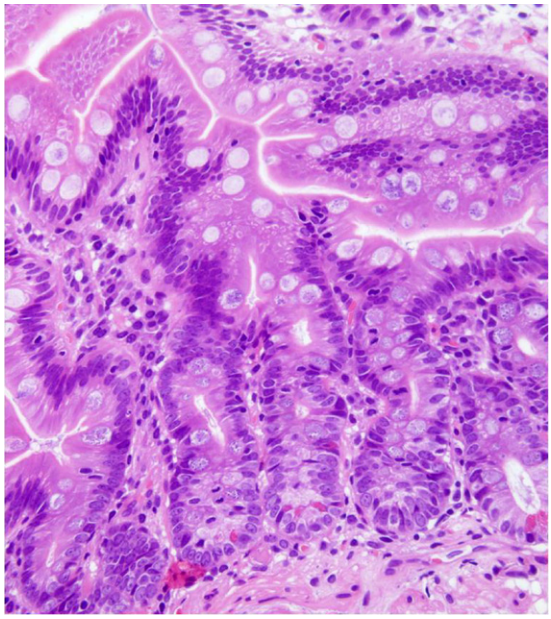

Fig. 5. A biopsy that has been cut tangentially due to bad orientation makes it extremely difficult if not impossible to correctly define the villous:crypt ratio (H&E, 40×).

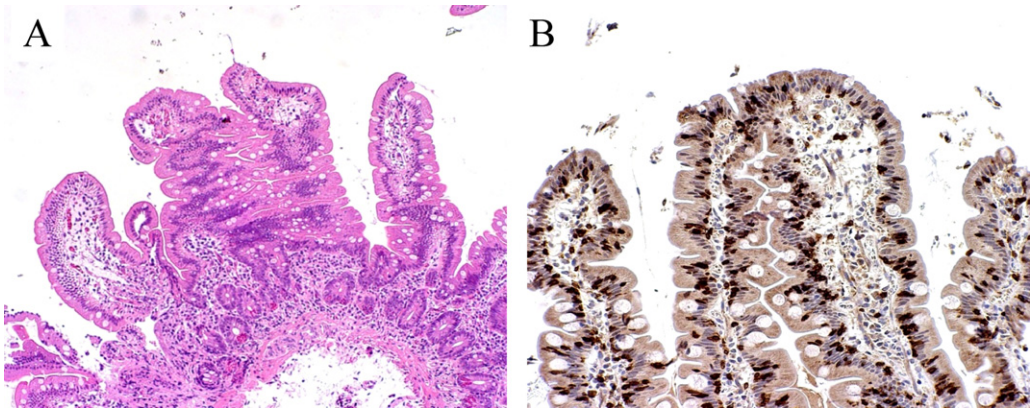

Fig. 6. (A) The crushing of villi may resemble partial villous atrophy (H&E, 10×), whereas (B) partial fusion of villi may prevent a correct count of intraepithelial lymphocytes despite CD3 immunostaining (CD3 immunostain, 20×).

a proper histological assessment by the pathologist (Fig. 3B). This method, based on the experience acquired at St Mark's Hospital in London [29], provides histological samples in which the mucosa and, if necessary, the submucosa of the removed tissue can be analyzed, respecting the normal anatomical relationship between the two different layers of the intestinal wall.

Fixation, embedding and processing of the biopsy

The use of cellulose acetate filters allows the perfect adhesion of the biopsies, avoiding their dispersion in the fixation medium. These filters also do not react chemically with the fixatives and reagents used during the processing of the sample. During the cutting phase, they do not offer resistance to the blade and, unlike tissue paper, they do not fray.

After the fixation stage, the filter-biopsy combination is processed and then embedded. During this last phase, the technician rotates the filter-biopsy combination 90° in order to place the samples in their natural position. After cutting, the biopsies are placed on a slide and, if necessary, the position of the beveled end is marked on the label to indicate the first biopsy.

This method, which can be applied on all segments of the gastrointestinal tract, can lead to considerable diagnostic and economic benefits by reducing the time, the number of embeddings, and consequently the number of sections to be cut and stained. When properly carried out, it is also of great benefit to the technician, who during the embedding phase does not have to search for the individual biopsies, which are sometimes fragmented and have no guiding landmark. And of course, this method is also beneficial to the pathologist, helping to avoid some artifacts due to compression of the specimen (see below).

Staining

Duodenal biopsies should always be subjected to an optimal hematoxylin and eosin (H&E) stain, whereas the periodic acid-Schiff (PAS) stain is generally not useful. Duodenal biopsies should be best immunostained with monoclonal anti-CD3 antibodies. This significantly increases the accuracy of IEL count, which is particularly important in the evaluation of biopsies with a normal villous/crypt ratio for the identification of grade 1 lesion, especially in patients with raised serum tTG [23,25]. It is currently agreed that IEL should be less that 25/100 epithelial cells in the normal duodenal mucosa [5,14,23,25]. A figure of 25–30/100 epithelial cells can be considered as borderline and is worth of further investigation, whereas an IEL count >30/100 epithelial cells is definitely abnormal and is usually found in CD [5,14,23,25].

Major histological artifacts

The pathologist's experience is certainly important and may often overcome some inaccuracies in biopsy handling and processing, but even the most skilled and experienced pathologist cannot overcome the challenge posed by a badly orientated or inadequately handled biopsy specimen (Fig. 4). A correct approach to the biopsies is particularly important for the evaluation of some pathological aspects such as the villous/crypt ratio and the T lymphocyte count. For instance, a poorly oriented biopsy, as shown by crypts cut tangentially (Fig. 5), will falsely increase the villous/crypt ratio, thereby leading to an underestimate of the severity of histological lesions. Inaccurate orientation can also result in crushing or partial fusion of adjacent villi, thereby giving the wrong impression of partial villous atrophy (Fig. 6A) or causing difficulties in IEL count

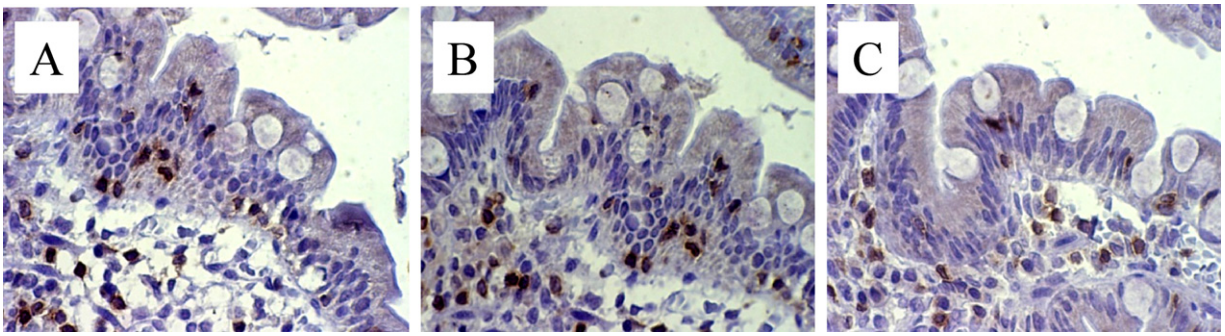

Fig. 7. An imperfect orientation of the biopsy specimen may also cause an overlap of T lymphocyte nuclei and thus result in an inaccurate intraepithelial lymphocyte count (A, B). Correct orientation of a nearby biopsy specimen is shown in C) (CD3 immunostain, 100×).

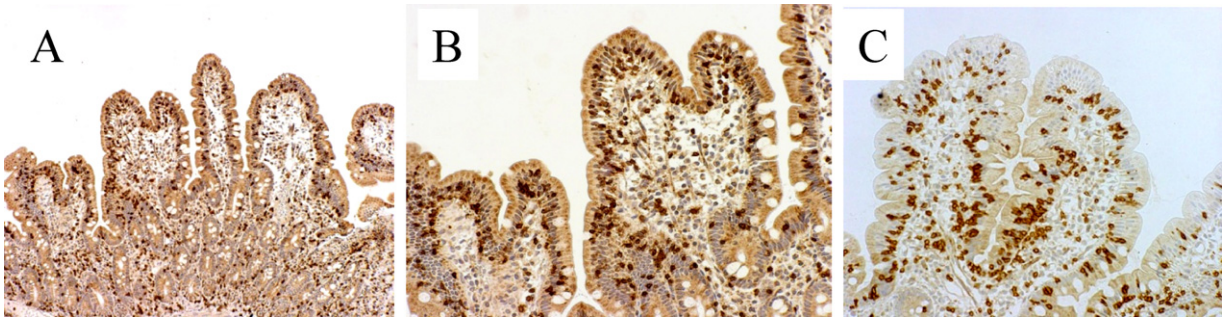

Fig. 8. Bigeminism or "twin villi" suggesting villous atrophy are present in (A) (CD3 immunostain, 10×), and (B) (CD3 immunostain, 20×). In (C) a further section cut along the vertical axis shows that villi are indeed regular or only mildly shortened (CD3 immunostain, 20×).

(Fig. 6B). Even if CD3 immunostaining is carried out, compression or imperfect orientation of the biopsy specimen may cause an overlap of T lymphocytes nuclei in some areas, which in turn may result in an underestimate of IEL count (Fig. 7). Another example of a histological artifact that may undermine the yield of small bowel biopsy is the so-called bigeminism (Fig. 8A). Although not strictly related to inaccurate orientation of the biopsy, the finding of broad and partially duplicated or "twin" villi is often considered a sign of partial villous atrophy. However, when further sections are cut along the vertical axis, normal-looking villi can be seen (Fig. 8B and C). In our opinion, the classification of histological lesions proposed by Corazza and Villanacci a few years ago could facilitate the correct interpretation of histological lesions and reduce the possibility of disagreement in gluten-sensitive enteropathy [7,8]. Briefly, this latter classification reduces the number of lesions from the five originally proposed by Oberhuber (who, in turn, modified the classification previously proposed by Marsh), to three [7] (Fig. 9). This reduction is based primarily on the observation that the recognition of Marsh–Oberhuber's lesion type 2 and the distinction between lesion type 3a and lesion type 3b – both indicating partial villous atrophy – are not essential for the diagnosis and follow-up of celiac disease [7,8]. The reduction is also based on the well known concept that the greater the number of diagnostic categories of a method, the lower its reproducibility. Indeed, the Oberhuber's classification bears a significantly high interobserver variability between pathologists (overall K value = 0.35) [7,8]. Using this classification, the level of agreement between pathologists was generally good for the normal mucosa (grade 0, $K = 0.53$) and the most severe lesion (grade 3C, $K = 0.73$), but was poor in case of intermediate lesions (e.g. grade 1, $K = 0.24$; grade 2, $K = 0.18$), even among experienced pathologists [7,8]. With the classification proposed by Corazza and Villanacci, on the other hand, the overall level of agreement rose from 0.35 to 0.55 among the same pathologists [7,8].

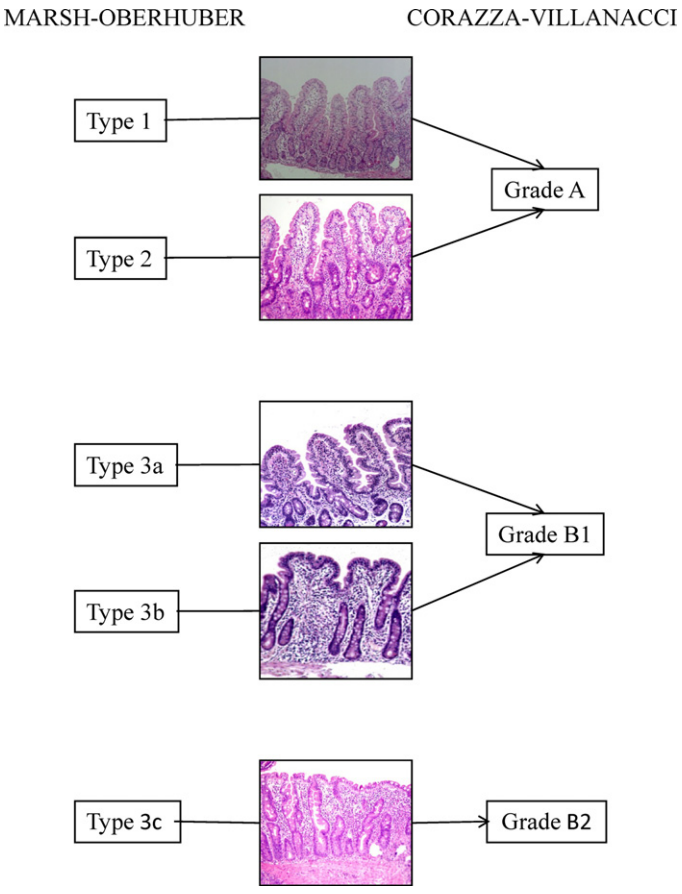

Fig. 9. Comparison between the histological classification of duodenal lesions in celiac disease according to Marsh–Oberhuber (left) and Corazza-Villanacci (right). The latter can be summarized as follows: lesion grade A incorporates both non-atrophic lesions described by Marsh–Oberhuber, i.e. type 1 (infiltrative lesions, characterized by increased intraepithelial lymphocyte count) and type 2 (hyperplastic lesion, characterized by increased intraepithelial lymphocyte count and crypt hyperplasia); lesion grade B1 includes all types of partial villous atrophy, i.e. type 3a (mild villous atrophy) and type 3b (moderate villous atrophy) as described by Oberhuber; lesions grade B2 include total villous atrophy only (i.e. lesion type 3c as described by Oberhuber). Thus the classification proposed by Corazza-Villanacci reduces the grades of duodenal lesions from 5 to 3.

Conclusions

The impact of small bowel biopsy in the diagnostic work-up of CD has been somewhat reduced by the widespread knowledge of the multiform clinical manifestations of CD and the availability of highly sensitive and specific serological tests, such as tTG and EMA. Small bowel biopsy can no longer be viewed as the gold standard for the diagnosis of CD, and the guidelines recently issued by the European Society of Pediatric Gastroenterology, Hepatology and Nutrition – likely to be endorsed by other gastroenterological societies worldwide – suggest that small bowel biopsy should be carried out only in selected cases of suspected CD [12]. On the other hand, for the same reasons illustrated above, the number of centers where CD is diagnosed is increasing, and early diagnosis is increasingly common. As more patients with silent or subclinical CD are identified in an early phase, mild histological lesions other than the classic "flat mucosa" or total villous atrophy are also found with increasing frequency [14,3,32,23]. These are the patients and lesions that more likely raise controversy, especially among less experienced clinicians and pathologists. This perspective further emphasizes the importance of a clinician–pathologist "cross-talk" and an accurate and standardized approach to each step, from biopsy taking to handling and processing of biopsy specimens, in patients with suspected CD.

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