

Best practices of handling, processing, and interpretation of small intestinal biopsies for the diagnosis and management of celiac disease: A joint consensus of Indian association of pathologists and microbiologists and Indian society of gastroenterology

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Access this article online

Website: www.ijpmonline.org

DOI: 10.4103/IJPM.IJPM_1405_20

Quick Response Code:



Submitted: 06-Dec-2020

Revised: 11-Dec-2020

Accepted: 07-Jan-2021

Published: 07-Jun-2021

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How to cite this article: Das P, Vaiphei K, Amarapurkar AD, Sakhija P, Nada R, Paulose RR, et al. Best practices of handling, processing, and interpretation of small intestinal biopsies for the diagnosis and management of celiac disease: A joint consensus of Indian Association of Pathologists and Microbiologists and Indian Society of Gastroenterology. Indian J Pathol Microbiol 2021;64(Special):S8-31.

ABSTRACT

The Indian Association of Pathologists and Microbiologists (IAPM) and Indian Society of Gastroenterology (ISG) decided to make a joint consensus recommendation for handling, processing, and interpretation of SI biopsies for the diagnosis and management of celiac disease (CD) recognizing the inhomogeneous practice of biopsy sampling, orientation, processing, and interpretation. A modified Delphi process was used to develop this consensus document containing a total of 42 statements and recommendations, which were generated by sharing the document draft, incorporating expert's opinion, followed by three cycles of electronic voting as well as a full-day face-to-face virtual ZOOM meeting and review of supporting literature. Of the 42 statements, 7 statements are on small intestinal (SI) biopsy in suspected patients of CD, site and the number of biopsies; 7 on handling, fixative, orientation, processing, and sectioning in pathology laboratories; 2 on histological orientation; 13 statements on histological interpretation and histological grading; 3 on the assessment of follow-up biopsies; 2 statements on gluten-free diet (GFD)-nonresponsive CD; 4 on challenges in the diagnosis of CD; 2 statements each on pathology reporting protocol and training and infrastructure in this area. The goal of this guideline document is to formulate a uniform protocol agreed upon both by the experienced pathologists and gastroenterologists to standardize the practice, improve the yield of small bowel biopsy interpretation, patients' compliance, overall management in CD, and generate unified data for patient care and research in the related field.

KEY WORDS: Celiac disease, guideline, mimickers, mucosal biopsies, orientation, pathology, processing, recommendations, reporting format, small intestine, training

INTRODUCTION

Celiac disease (CD) is a major public health problem with a pooled global seroprevalence of 1.4%, including 0.7% of biopsy-confirmed CD worldwide.^[1] Its prevalence in Asia and India are 0.5% and 0.67%, respectively.^[2] In India, its seroprevalence is highest in northern (1.23%) and north-Eastern states (0.87%) than in southern parts (0.10%).^[3] The Indian Council of Medical Research (ICMR) initiated and supported Indian Guidelines on CD, which recommends diagnosing CD based on a combination of positive celiac-specific serology and demonstration of villous abnormalities of modified Marsh grade 2 or more on small intestinal (SI) biopsies.^[4] Therefore, obtaining a duodenal mucosal biopsy is an integral part of the workup for patients with CD. However, the protocol of biopsy processing, orientation, and methods of interpretation varies widely leading to heterogeneity in reports. Due to the recognition of the high CD burden and its expanding extra-intestinal manifestations, pathologists from both Institutional and stand-alone laboratories are getting SI biopsies from suspected patients. Improper handling of biopsies will impart artifacts, impact interpretation, generate heterogeneous patient information, and make management difficult especially when a patient relocates to another city. Additionally, the high interobserver disagreement (up to 50%) reported in the histological interpretation of SI biopsies and grading is worrisome.^[5,6] A need was perceived for the standardization of the processing and interpretation protocols of proximal SI mucosal biopsy samples. The Indian Association of Pathologists and Microbiologists (IAPM) and the Indian Society of Gastroenterology (ISG) decided to make a joint consensus recommendation for handling, processing, and interpretation of SI biopsies for the diagnosis and management of CD. The authors believe that this joint consensus will not only refine and standardize the laboratory practices of the collection and processing of SI and further interpretation and bring uniformity.

METHODS

The areas covered in this guideline are based on the data collected from a Nationwide survey under the aegis of the IAPM and ISG. A set of 40 questionnaires were divided into 3 sections covering different aspects of biopsy handling, processing, and interpretation and circulated among 2000 registered members of the IAPM using the Survey Monkey platform. For most questions, the participants were asked if they strongly agree, agree, neither agree nor disagree, disagree or strongly disagree with the options provided and a total of 261 IAPM members completed the survey, whereas the others mentioned that they do not practice GI Pathology and others did not respond or given reasons for not participating. In summary, it was observed that only 40% of the biopsy fragments are filter paper mounted and separately labeled as per the site of biopsy sampling and about 50% of routine SI biopsy load is either not oriented or unfit for interpretation as stated by the participants. There were heterogeneity of site of proximal SI biopsies, number of biopsies sampled, clinical and endoscopic details received with samples, method of orientation, processing, sectioning, number of step sections examined, method of histopathological assessment of IELs, crypt depths (Cd), and Villous heights (Vh), histological grading of biopsies, and assessment of follow-up biopsies post-GFD. Unanimously 99.5% of the participants said that a uniform protocol agreed upon both by the pathologists and gastroenterologists is the need of the hour to standardize pathology practice, which will impact the patient compliance and management, as well as generate unified data for patient care and research in related filed.

Methods of formulating this guideline: A modified Delphi process was followed and this guideline document was made on the approval of the Executive Council and General Body of the IAPM and written approval from the Secretary-General of the ISG.^[7] Thereafter, under the chairmanship of the

current President and Past-President of the IAPM and the current Secretary-General ISG, a core committee was formed comprising of senior gastrointestinal pathologists and gastroenterologists with proven expertise in this field. A project coordinator was selected who, with the approval of the committee, formulated the questions and coordinated all activities related to the survey, formulated guideline draft, made revisions as per the members' suggestions, conducted online and final virtual voting for each recommendation mentioned in this document. A systematic review was performed on the relevant areas based on literature searched in the MEDLINE, EMBASE, and regional and International consensus statements and guidelines given on CD. The issues identified and discussed with the core committee members were detailed and uniform and acceptable terminologies to be used in this guideline document were formulated and finalized with the consent of all committee members [Table 1]. Voting was done anonymously.

The categorization of evidence, classification of recommendation, and voting scheme were according to the Canadian Task Force on the Periodic Health Examination [Table 2].^[8] A consensus statement was 'completely' accepted or 'accepted with some reservation' when voted favorably by ≥80% of members, and a statement was rejected when ≥80% of the members rejected a statement 'completely' or 'with some reservation'. Each statement was graded to indicate the level of evidence available and the

Table 1: Uniform Terminologies used in this document

CD - celiac disease
GFD - gluten-free diet
IEL - Intra-epithelial lymphocytes
tTG - tissue transglutaminase
IgA - Immunoglobulin A
EMA - Endomysial antibody
DGP - Deamidated gliadin peptide
ESPGHAN - European Society of Pediatric Gastroenterology and Nutrition
AGA - American Gastroenterology Association
NIH - National Institute of Health
DH - dermatitis herpetiformis
NCGS - non-celiac gluten sensitivity
RCD - refractory celiac disease
Vh - villous height
Cd - crypt depth
villous flattening - Cd:Vh ratio < 1:3
Crypt hyperplasia - Elongated tortuous crypts with cytoplasmic basophilia
GFD non-responsive patients - patients who did not show symptomatic or serological or histological response after one-year of GFD
D1 biopsy - duodenal bulb biopsy
D2/D3 biopsy - biopsies from second or third parts of the duodenum
PAS stain - Periodic acid Schiff stain
Q-histological - Quantitative histological
Mild villous flattening (modified Marsh grade 3a) - Cd:Vh ratio <1:3, but >1:1
Moderate villous flattening (modified Marsh grade 3b) - Cd:Vh ratio equal to 1:1
Severe villous flattening (modified Marsh grade 3c) - Cd:Vh ratio <1:1

strength of recommendation. There were three rounds of online voting by the core-group members and an online final face-to-face meeting to discuss each statement followed by real-time voting to finalize the document.

Target group to follow this guideline: This guideline is recommended to be followed and implemented by all centers and laboratories irrespective of their affiliations and size who deal with the SI biopsies from patients suspected of CD. In this guideline, a few recommendations have also been suggested for the Gastroenterologists/Endoscopist colleagues and it is expected that they will also follow these recommendations to help the pathologists to achieve a meaningful outcome of SI biopsy assessment. After the final approval of the draft guideline by the core-committee, the final guideline was published in Society Journals, displayed on the respective society's websites, and mailed to the participating members of the preceding Survey.

The Consensus Statements and Recommendations on handling, processing, and interpretation of SI biopsies for the diagnosis and management of celiac disease

1. Historical perspectives and SI biopsies in CD

- a) SI mucosal biopsy for demonstration of villous abnormalities is deemed essential in adult patients suspected to have CD.
Voting summary: A 88.05% B 10.78% C 0% D 1.17% E 0%
Quality of evidence: II-2
Type of recommendation: B
- b) A diagnosis of CD can be made even without SI biopsies in children if the anti-tTG antibody serotiter is 10 fold above upper limit of normal and the anti-endomysial antibody is positive in the second blood sample.
Voting summary: A 86.38% B 13.62% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B
- c) SI mucosal biopsies should be done in children suspected to have CD, if the serological criteria for the diagnosis is not met (anti-tTG Ab titer is less than 10 folds upper normal limit and/or a negative anti-endomysial antibody)
Voting summary: A 86.05% B 13.95% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B
- d) SI mucosal biopsies should always be done if small bowel diseases other than CD are also suspected.
Voting summary: A 91.05% B 6% C 0% D 2.95% E 0%
Quality of evidence: II-2
Type of recommendation: B

Table 2: Criteria for defining the quality of evidence, type of recommendations, and voting on recommendations

<i>Quality criteria & grading</i>	<i>Definitions</i>
Quality of evidence	I Evidence from at least 1 randomized control trial II-1 Evidence from well-designed control trials without randomization II-2 Evidence obtained from well-designed cohort or case-control study II-3 Evidence obtained from studies comparing time or place with or without intervention III Evidence derived from well-established authorities and based on expertise and experience of the core committee
Type of recommendations	A. There is good evidence to support the statement B. There is fair evidence to support the statement C. There is poor evidence to support the statement, but recommendations made on the ground D. There is fair evidence to refute the statement E. There is good evidence to refute the statement
Voting on recommendation	a. accept completely b. accept with some reservation c. accept with major reservation d. reject with reservation e. reject completely

Statement for which more than 80% of participants voted a and b are accepted

Over the past 70 years, the diagnostic method of CD has changed substantially. Being an enteropathy, demonstration of mucosal histological changes post-gluten intake was considered the mainstay of diagnosis in CD. Before the 1970s, whereas the 3-biopsy approach was the standard before and after the gluten intake, with the availability of celiac-specific serological tests as anti-gliadin antibody (AGA) and anti-reticulin antibody enzyme-linked immunosorbent assay (ELISA) kits, followed by anti-endomysium (anti-EMA) and anti-tissue transglutaminase (tTG) antibody kits, gradually the focus shifted towards less invasive and a combined clinical-serological-histology based diagnostic approach. Gradually, the European Society of Pediatric Gastroenterology and Nutrition (ESPGHAN) criteria published in 1990 with one biopsy approach became the accepted norm for diagnosis both in pediatrics and adults. In early 2000, anti-tTG screening became the standard of practice, although histological confirmation was required. In 2012, the revised ESPGHAN criteria recommended a non-biopsy approach of diagnosis, especially in symptomatic children based on the serum anti-tTG titer of >10 times upper normal limit, positive anti-EMA antibody test performed on a second blood sample in patients having HLA-DQ2/DQ8 alleles, with the consent of patient's first-degree-relatives to use the 'no-biopsy approach' for diagnosis in children consuming gluten before the biopsy. This approach was found to have a 99% positive predictive value (PPV) as assessed by an International prospective study group,^[9,10] and was later adopted by the British Society for Pediatric Gastroenterology, Hepatology, and Nutrition, with the modification of replacing the anti-EMA test with a second line anti-tTG test using an ELISA kit of different principle than that was used initially.^[11] The no-biopsy approach in children was favored as the endoscopic biopsies needed multiple passes for collecting >2 mucosal fragments under anesthesia, with inherent risks of invasive procedure and added high-cost of diagnosis.^[12] The American Gastroenterology Association (AGA) recommended intestinal biopsy in patients with strong clinical suspicion of CD even when the serology is negative, whereas the United States National Institute of Health (NIH) recommended a biopsy only when the serological

tests are doubtful.^[13] The ICMR also favored the evaluation of intestinal biopsies for mucosal changes along with other tests for establishing a diagnosis of CD in adults.^[4] Variability of the outcome of commercially available anti-tTG ELISA kits, lack of facilities for doing HLA haplotyping in India, and the question of affordability were the justifications for this recommendation. Besides, HLA-typing has a low PPV in the diagnosis of CD, and the HLA-DQ2/DQ8 haplotype/s is/are present in 13–30% of the Indians of which only $<1\%$ develop CD.^[4] In a study from the Central European region on the utility of intestinal biopsies in children, 20.6% of children with CD were diagnosed with 'no biopsy approach', and 51.9% of symptomatic children who underwent intestinal biopsy were also later assessed to be eligible for no-biopsy approach; however, the interval to diagnosis was significantly longer in children with 'no-biopsy approach' than in whom biopsy was done.^[14] HLA haplotyping in the 'no-biopsy approach' was responsible for this delayed diagnosis, and when it was excluded, the interval to diagnosis was similar for both the 'no-biopsy' and 'with-biopsy' approaches.^[15] However, intestinal biopsies in these patients can rule out a false positive anti-tTG ELISA test (in about 10%) and co-existing SI pathologies, not uncommon in South Asian countries.

Symptoms of malabsorption, diarrhea, weight loss, or anemia, are not specific to CD and may be seen in other disorders also. Although anti-tTG serology titer >5 -times upper normal limit (UNL) is mostly pathological, anti-tTG titers between 2 and 5 times UNL, and <2 times the UNL are dubious, and in such scenarios a SI biopsy is necessary.^[16] Kori M et al. identified mucosal pathological findings of CD in 17.4% of anemic children who underwent routine endoscopic biopsies for evaluation of anemia.^[17] Although it is possibly right that the no-biopsy approach works well in children aged <3 years old, in older children experts differ in their approach to diagnosis. Especially in India and Asia Pacific region, gastroenterologists prefer taking SI biopsies in suspected patients of CD when the age of children is >10 years in addition to serological assessment. Gluten challenge is not

an easy option in all centers and needs patients and their parents' cooperation.^[18] The problem using only serological tests for diagnosis is the development of transient immunity to gluten peptide or false- positivity in patients with other chronic autoimmune diseases having high serum proteins and an endoscopic biopsy can identify the etiology or rule out CD.^[19] Studies have shown that combining serological tests based on different principles can help in diagnosis in up to 78% of patients without a biopsy; however, an intestinal biopsy is indispensable in cases showing discordance among various serological tests and in patients with IgA deficiency.^[20] A SI biopsy can also help to assess mucosal response to GFD on follow-up intestinal biopsies. Although the symptomatic improvement is achieved within a few weeks of starting GFD, the mucosal healing can take months to a few years to become normal.^[21] SI biopsies also would be instrumental when the patients primarily present with dermatitis herpetiformis (DH), non-celiac gluten sensitivity (NCGS), extra-intestinal manifestations as in ataxia to rule out the possibility of CD and in children with IgA deficiency or patients with borderline high serology titer. However, the availability of IgG anti-deamidated gliadin peptide (anti-DGP) ELISA kit has high sensitivity and specificity for diagnosing CD especially in children who are IgA deficient. Overall, with expanding spectrum and recognition of extra-intestinal manifestations of CD even in the absence of primary GI symptoms, the number of patients undergoing diagnostic workup including SI biopsies is likely to increase in near future; hence, pathologists are expected to encounter more proximal SI biopsies for assessments.

2. Site of SI biopsies in patients suspected of CD

- a) SI biopsies should be obtained from both the second part of the duodenum (post-ampulla) and the duodenal bulb.
Voting summary: A 91.46% B 5.14% C 1.19% D 0.96% E 1.25%
Quality of evidence: II-2
Type of recommendation: B
- b) In suspected patients of CD in whom first biopsy is non-contributory, duodenal mucosal biopsies should be repeated and additional mucosal fragments should be obtained from distal duodenal (D3) or the jejunum.
Voting summary: A 63.65% B 32.66% C 3.69% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B

In patients with CD, the SI mucosa is said to be involved in a craniocaudal direction and the resolution of pathological changes occurs in the opposite direction.^[22,23] Most changes of CD have been documented in post ampullary parts of the duodenum (D2) and at duodenojejunal flexure. Duodenum is also a convenient site for taking endoscopic biopsies.^[24] But, in about 10% of cases, the duodenal bulb is the primary site of the disease, either isolated (often named as ultra-short

segment CD) or in combination with the involvement of the distal duodenum.^[25] Duodenal bulb biopsies particularly those from 9 o'clock and 12 o'clock regions, with 4 mucosal fragments from the distal duodenum (D2) show histological changes of CD in almost all cases.^[26-28] Mooney PD *et al.* found that the addition of a D1 biopsy fragment from any site was superior to a distal duodenal biopsy alone, increasing the diagnostic yield by 9.3%-10.8% ($P < 0.0001$).^[27] However, interpretation of duodenal bulb biopsies is challenging because of mucosal stretching by submucosal Brunner's glands [Figure 1a], or inflammatory changes inflicted by the gastric or pancreatic juice reflux.

Like duodenum, jejunal biopsies also show similar histological changes in CD. However, there are differences in the shape of villi and pathological cut-off of IELs between the duodenum and jejunum. Although the jejunal villi are finger-shaped, the duodenal villi are usually leaf-shaped. The proximal jejunal villi are taller and the distal jejunal villi are shorter than the duodenum [Figure 1b and c].^[29,30] Very rarely, histological changes of CD can be restricted to jejunum only (2.4%), whereas in the rest pathologies can be identified both in the duodenum and jejunum.^[31] The SI pathologies in CD can be patchy and evolving, hence, in 5-13.6% of patients with newly diagnosed CD, the diagnosis may be missed in the first endoscopic biopsy [Figure 1d].^[32] Ravelli *et al.* compared the mucosal pathological changes in the duodenal bulb, D1, D2, and D3 biopsies and found that the histological changes are more prominent in an aboral direction.^[24] Hence, in clinically suspected cases where the initial D2/bulb biopsies are noncontributory, repeat biopsies from the same sites, and additional biopsy fragments from the D3 or jejunum may reveal pathological changes.

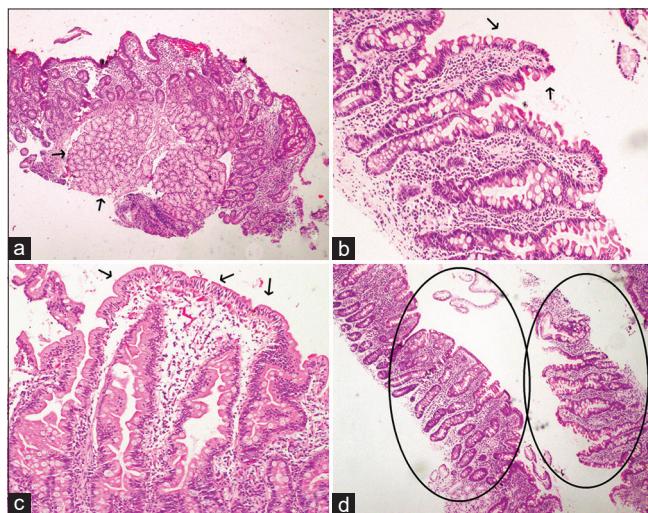


Figure 1: A duodenal bulb biopsy shows artifactual effacement of mucosa over Brunner's gland lobules [a x40]. Typical leafy villi in distal duodenum (arrows) [b x100], and villi with branching or fused tips (arrows) may lead to erroneous impression [c x100]. Figure D shows patchy pathological changes in patients with celiac disease, whereas the left circle shows changes of advanced celiac disease, the circle in the right shows relatively unremarkable villi [d x 40]

3. The ideal number of SI biopsy fragments

For optimum interpretation and reporting, at least 5-6 mucosal biopsies should be obtained including 4 (measuring approximately 3 mm in length each) biopsy fragments from the second part of the duodenum and one to two fragments from the duodenal bulb.

Voting summary: A 92.29% B 5.5% C 0% D 2.21% E 0%
Quality of evidence: II-2

Type of recommendation: B

The estimated probability of identifying pathological changes in CD in SI mucosal biopsies is 100% with 4-biopsy fragment protocol, 95% with 3-biopsy fragment protocol, and 90% with a 2-biopsy protocol.^[33,34] However, in a large pathology database-based study from the United States on 1,32,352 patients with malabsorption or suspected CD undergoing endoscopic SI biopsies adherence to 4-biopsy fragment protocol was observed only in 39% of patients, which improved the documentation of pathological changes in 1.8% cases versus 0.7% cases in those in whom <4 mucosal biopsy fragments were sampled ($P < 0.0001$).^[35] Bonamico, et al., in a study on 102 children with suspected CD, found duodenal bulb biopsy changes in 16 (15.6%) patients, whereas in 4.2% of biopsies the pathological changes were restricted to the duodenal bulb.^[22] Even with the 8-biopsy fragment protocol, the sensitivity of identifying pathological change reached 100% only when at least one biopsy fragment was obtained from the duodenal bulb, and the pathological findings were missed in 40–45% of cases when at least one biopsy fragment was not included from the duodenal bulb.^[24,27,36] Based on the above mentioned published data, it seems that obtaining least 5-6 duodenal biopsy fragments, including at least four fragments from the distal post-ampullary parts of the duodenum, measuring 3 mm each in length,^[3] as well as at one to two fragments from the 9 o'clock and 12 o'clock positions of

the duodenal bulb will yield the pathological changes of CD in suspected patients most efficiently.

4. Ideal fixative and duration of fixation for SI mucosal biopsies

SI mucosal biopsies should be fixed for 5-6 hours in 10% neutral buffered formalin (pH 6.8-7.4) in a ratio of 1: 10 tissue volume to fixative solution volume ratio for optimum results.

Voting summary: A 95.4% B 4.6% C 0% D 0% E 0%

Quality of evidence: II-3

Type of recommendation: B

10% neutral buffered formalin (pH 6.8–7.4) is an ideal fixative for optimum tissue sections and staining.^[37] The buffer used in 10% formalin preserves the efficacy of formalin for a longer time and prevents its degradation.^[38] For fixation of tissue and to produce minimal artifacts though alcohol-based or acid-alcohol-formalin-based fixatives are often preferred; for routine H and E staining and histological analysis neutral buffered formalin suffices. The use of picric acid-based fixatives has fallen short of routine use as it leads to tissue pigmentation and creates challenges in histological identification of the Paneth cell metaplasia. Formalin can also produce tissue pigmentation if the pH of the solution is 5.5 and hence a buffered formalin is recommended. In situations, where there is a clinical possibility of diseases such as Whipple's disease or microvillus inclusion disease (MVID), additional intestinal mucosal biopsy fragments should also be obtained and fixed in 2.5% glutaraldehyde solution for electron microscopy. In suspected abetalipoproteinemia, additional biopsy fragments should be sent fresh on foil paper on ice for frozen sectioning and fat staining. In all such special situations, pathology laboratories must be communicated in advance. Depending upon the clinical indications, special staining such as Periodic acid Schiff (PAS) with and without

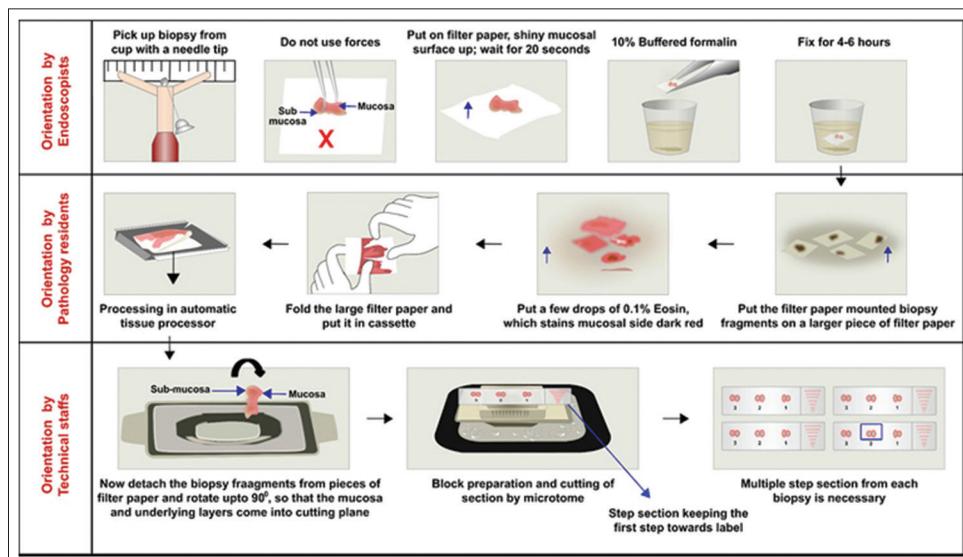


Figure 2: The diagram shows step by step method of handling, orientation, and processing of duodenal biopsy fragments. The three rows summarize the critical contribution of the endoscopy team, pathologist and pathology technicians

diastase digestion, Zeihl Nielsen stain, Auramine Rhodamine stain, Giemsa stain, Warthin starry silver stains can easily be performed on formalin-fixed tissues. Although not commonly used in SI biopsies, immunohistochemical (IHC) stains may be required to highlight organisms as cytomegalovirus inclusions, *H pylori*, or to establish the phenotypes of IELs in patients suspected to have a refractory CD or lymphoreticular lesions. The immunophenotyping can also be performed on FFPE tissue. One should avoid using undiluted 40% formalin as it produces a charring artifact, making subsequent interpretation difficult, or unnecessary heat to avoid producing smudgy or washed out nuclei appearance with inappreciable chromatin details.^[39]

An optimum fixation time for SI mucosal biopsies is about 5-6 hours. If biopsies are left in formalin for >36 hours (especially on weekends), the tissues become hard, brittle, and difficult to cut. Fixing the biopsies in formalin for a prolonged time also (>16 hours) leads to loss of immunoreactivity.^[38] However, not entirely the biopsies fixed for more than 6 hours become uninterpretable histologically, as can be unavoidable in centers that do not have in-house pathology laboratories. The ideal volume of formalin solution to the tissue volume should be between 25:1 and 10:1 to generate optimum osmotic driven forces and capillary driven forces for tissue penetration and should be adhered to.^[39]

5. Biopsy handling & transport to Pathology laboratory

- a) In the endoscopy room, fresh intestinal biopsy fragments ideally be oriented on filter paper by trained personnel keeping the mucosal surface up and stretching them optimally. In institutions where mounting of the biopsies over filter paper is not preferred, the pathology technical staff should orient free-floating biopsy fragments by looking at the contraction pattern of the fibers of muscularis mucosae at the bottom of the biopsy fragments. Voting summary: A 75.24% B 18.85% C 3.69% D 0% E 2.22%
Quality of evidence: II-3
Type of recommendation: C
- b) Biopsies from different sites of the SI should be sent in separately labeled tightly capped containers kept at room temperature.
Voting summary: A 93.62% B 4% C 2.38% D 0% E 0%
Quality of evidence: III
Type of recommendation: C
- c) The biopsy samples should be accompanied by an appropriately filled requisition form including relevant clinical information, serological data, site/s and number of samples (containers), endoscopic findings, previous pathology report if any and the clinical possibilities.
Voting summary: A 91.24% B 8.76% C 0% D 0% E 0%
Quality of evidence: III
Type of recommendation: C

6. Processing of biopsy samples in Pathology Laboratory

- a) Each paraffin block should not contain >4 biopsy fragments for the convenience of optimum processing.
Voting summary: A 90.92% B 9.08% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B
- b) At least 5 serial sections should be cut on each glass slide keeping the 'level-1-cut' towards the slide label to guide the reporting pathologist to understand the sequence of step sections and further step sectioning should be continued similarly until a properly oriented biopsy fragment is identified.
Voting summary: A 81.46% B 17.04% C 1.5% D 0% E 0%
Quality of evidence: III
Type of recommendation: C

All individual biopsy fragments should be separately mounted on a one cm² size rectangular piece of Whatman type I filter paper, white or colored, as per the preference of the pathologist. Cellulose acetate paper, gel foam, thick black paper, thin cards, or vegetable matrices such as thin slices of cucumber or potato can also be used for mounting the biopsy fragments. However, there are many centers, where pathologists do not prefer to mount biopsies on filter papers. The gastroenterologists/endoscopists play an important role in the biopsy orientation and mounting them on mediums mentioned above after gently separating the biopsy fragments from the endoscopic forceps cup by a needle tip without causing crushing artifact, keeping the shiny mucosal surface up. After separating, they may be gently flattened using clean sterile needles/pins, not over-stretched, so that the mucosal epithelium does not fall apart or edema like artifact due to overstretching is not produced [Figure 2].^[40] Biopsy fragments mounted on tissue paper face-down can cause artifactual mucosal flattening and best be avoided.^[41] Waiting for 10-20 seconds thereafter will result in adhesion of the biopsy fragments to mounting media by coagulation of plasma proteins. Then the biopsy fragments adhered to a filter paper should be dropped in 10% neutral buffered formalin keeping the tissue face-down so that the fragments do not float away [Figure 2]. It is essential to note that the biopsy fragments should not be pressed with forceps, fingers, or glass slides as they cause irretrievable damage. The use of a dissection microscope or the handheld lens may aid in proper tissue mounting on filter paper. The biopsy fragments obtained from different sites, as the duodenal bulb, D1, D2, D3, or jejunum should be put in separate labeled containers to enable pathologists to recognize the site of biopsies, apply appropriate criteria of interpretation, and generate site-wise biopsy reports. Within the containers, the biopsy fragments should be submerged with adequate fixative with a desirable fixative solution to biopsy volume ratio of at least 10:1 (ideal 25:1 to 10:1).^[38,42] It is best to transport the tightly capped sample containers as soon as possible to the pathology laboratory keeping them at room temperature. Inadvertent

storage in the freezing chamber of a refrigerator may result in the formation of ice-crystal artifacts in tissue sections. Heating in a microwave may produce smudgy nuclear artifacts. The fixation of biopsy fragments in fixative solution follows Fick's law. The diffusion coefficient depends on the concentration of the fixative solution, size of fragments, temperature, and potency of the fixative solution. Tissue fixated for a long time becomes brittle and difficult to cut, however, with experienced hands tissue fixed for a long time can be satisfactorily processed and interpreted under a light microscope. Tissues fixed for more than 24 hours lose antigenicity for further IHC based workup and become unsuitable for nuclei acid-based works.^[43]

The biopsies should always be accompanied by a properly filled requisition form. As the histological changes in most of the causes of malabsorption syndrome are similar, additional information will allow the pathologists to look for specific findings [Table 3]. Biopsy forms should at least include the following information^[40,44]:

- Patients name, age, sex, address, name of guardian/relative, hospital identification number
- Number of containers sent, number of biopsy fragments in each container, and site of biopsies
- Brief clinical history mentioning the clinical features, endoscopic indications, relevant investigation findings as celiac serology titer, stool examination findings, any relevant medical or surgical details, and clinical differential diagnoses. Mandatorily the HIV/Covid-19 or similar contiguous infective disease status should be mentioned to alters pathologists for taking extra precaution for processing.
- Mandatorily, a history of medications, especially non-steroidal anti-inflammatory drugs, angiotensin II receptor blockers, proton pump inhibitors, chemotherapeutic medications, anti-PD-L1 medications, etc., should be mentioned.

- If the patient is already on a gluten-free diet (GFD), the status of follow-up nutritional assessment, clinical symptoms, and serology titer should be mentioned.
- The contact number of the treating physician or resident must be made available to the pathologist so that issues can be easily discussed when needed. Changing the requisition form and converting it to a synoptic format including all these points will help to follow it on routine practice.

The biopsies can be stained with 0.01% Eosin solution during handling at the laboratory for easy identification of darkly stained already fixed mucosal pole. The filter paper mounted biopsy fragments should not be separated, and individually mounted biopsy fragments should be wrapped into a bigger piece of filter paper during grossing like an envelope, keeping the mucosal fragments up and kept in the cassette [Figure 2]. Only ≤4 biopsy tissue fragments should be put in one cassette to prevent overcrowding at a time depending on the size of the biopsy fragments. Just before embedding the biopsy fragments in paraffin blocks, the individual biopsy fragments should be carefully separated from filter paper mounts one by one by the technicians and rotated 90° to bring all layers of the biopsy fragment in the cutting plane of the FFPE tissue blocks [Figure 2].

Post-trimming, at least 5-step sections should be cut from each FFPE-tissue block keeping the level-1-cut towards the slide label for identifying the sequence of step sections during light microscopic interpretation [Figure 2].^[16] Examining each serial section helps one to choose the most appropriate section for interpretation. If needed, pathologists can ask for a second set of 5-step sections on another glass slide, and so on till an optimally oriented mucosal biopsy fragment is identified. Of all the step-sections, the most oriented step section should be marked for future reference. During review meetings or inter-departmental meetings, such marked

Table 3: Protocol of collection of samples from patients having malabsorption syndrome

Indications	How many biopsy fragment	Protocol	Indication of extra biopsy/serum samples
CD & Tropical sprue	4-6	10% neutral buffered formalin; fixative to tissue ratio- 10:1 H&E, IHC rarely for IgA anti TG2	Serum for IgA tTG titer or anti-EMA indirect immunofluorescence
Infectious etiology	5-6	biopsy containers should be kept inside a tightly sealed second plastic container to prevent any spillage or contamination H&E, IHC	1 biopsy fragments in a 2.5% glutaraldehyde solution at 4°C 1 biopsy fragment snap-frozen for PCR analysis for infectious agents
Whipple's disease	3-4	10% neutral buffered formalin; fixative to tissue ratio- 10:1 H&E, PAS-D stains	2 biopsy fragments in a 2.5% glutaraldehyde solution at 4°C
Abetalipoproteinemia	5-6	3-4 fragments in 10% neutral buffered formalin; fixative to tissue ratio- 10:1 H&E	1-2 biopsy fragments on aluminum foil kept on ice should be sent as soon as possible for frozen sectioning & Oil-red-O staining
Giardiasis	3-4	10% neutral buffered formalin; fixative to tissue ratio- 10:1 H&E, Giemsa, IHC	NA
IPSID	3-4	10% neutral buffered formalin; fixative to tissue ratio- 10:1 H&E, IHC	NA Serum sample for electrophoresis & immunofixation & serum Ig levels
CVID	3-4	10% neutral buffered formalin; fixative to tissue ratio- 10:1 H&E, Giemsa, IHC (as per indications)	Serum sample for Ig levels
Autoimmune enteropathy	3-4	10% neutral buffered formalin; fixative to tissue ratio- 10:1 H&E, IHC for parietal cells & enteroendocrine cells	Serum for anti-parietal cell or anti-goblet cell antibody

NA - not applicable, CD - celiac disease, IPSID - immune proliferative small intestinal disease, CVID - common variable immune deficiency

sections should be used to make a presentation, rather than going over all the sections. All sections should be 4 µm thick, as thick sections with cutting artifacts and nicks cause problems in microscopic evaluation, especially during IEL counting. Routinely, no special stain is needed for interpretation.

Related issues (collection of biopsies in a special situation)

Unmounted floating biopsy fragments

Some endoscopists do not prefer to mount the biopsy fragments on a mounting media and directly shake the biopsy forceps cups in the fixative solution and make their biopsy fragments freely float in the container. In an interesting study, Ruiz GC *et al.*, compared the occurrence of post-fixation mucosal contraction, crushing artifacts, and interpretability of the biopsies amongst those free-floating gastric and duodenal biopsies, biopsy fragments mounted on the cucumber slice, and also those mounted on the moistened synthetic foam-sponge. They observed that the contraction of the biopsy fragments was most observed most in free-floating samples. The orientation, absence of artifacts, and interpretability of the biopsies were appropriate on synthetic sponge mounted biopsies, followed by those mounted on the cucumber slices.^[42]

Post-fixation, identification of mucosal pole is difficult due to change of color of mucosa to tan-brown owing to the formation of acid formalin hematin and loss of mucosal shine. But, in most situations, the muscularis mucosae contract, leaving the mucosal surface on the concavity of the contracted biopsy fragments. Pediatric mucosal biopsies tend to be superficial and often the fibers of muscularis mucosae are not sampled, leading to further difficulty in orientation if they are not mounted. In an interesting study by Garg N *et al.* the authors compared orienting free-floating biopsies sent in containers over biopsies mounted on Whatman filter paper and lens papers, with and without application of albumin as an adhesive substance during grossing and found that with the use of lens paper and albumin, the % length orientation of the biopsy fragments was best, whereas those mounted on Whatman filter paper % orientation was the worst. The use of albumin to adhere to the biopsy fragments according to them also improved the orientation of biopsy fragments in Whatman filter paper.^[45] Such techniques should be standardized in laboratories when filter paper mounted biopsy fragments are not sent to the pathology laboratory.

When mounting media other than Whatman filter paper is used

When instead of laboratory-grade 1 Whatman filter paper cellulose acetate paper, gel foam, or vegetable matrices are used for mounting, the biopsy fragments can be embedded with the mount in-situ and cut through with microtome. In such cases, care should be taken during embedding so that the tissue is perpendicular to the cutting surface of the tissue block.^[16,46,47] If the biopsy fragments are not mounted, post-fixation, the contracted tissue fragments should be embedded on the edge. With experience, some technicians can straighten the curved tissue fragments in such a way that the sections provide a relatively straight orientation of the villi perpendicular to the muscularis mucosae. Many pathologists

use a hand-held lens to determine the post-fixation orientation, but its utility is not beyond doubt.

7. Section thickness for interpretation

The intestinal mucosal biopsies should be cut maintaining a section thickness at 4 µm for accurate counting of the intraepithelial lymphocytes.

Voting summary: A 93.5% B 6.5% C 0% D 0% E 0%

Quality of evidence: II-2

Type of recommendation: B

Cutting the processed tissue on glass slides of optimum thickness is one of the important steps for optimum microscopic interpretation. Sections cut at 4 µm thickness are most optimum for counting the IELs and sections thicker than this may pose difficulty due to overlapping of lymphocytes and epithelial cell nuclei. This may result in erroneous reporting of otherwise normal biopsies as Marsh 1 lesion. Some pieces of evidence tend to suggest that a section with higher thickness has a higher cut-off for IELs. Ferguson and Murray reported the upper normal limit of IELs as 40/100 ECs with 7 µm thick sections,^[48] Batman, *et al.*, reported 33/100 ECs as cut off with 5 µm thick sections,^[49] Hayat *et al.*, 25/100 ECs with 4 µm thick sections,^[50] and Mahadeva *et al.* 22 IELs/100 ECs with 3 µm thick sections.^[51] Later, 4 µm thick sections of duodenal biopsies were reported to uniformly define a pathological cut-off of IELs \geq 25/100 ECs across countries including North India.^[5,52] Hence, 4 µm thick sections of duodenal biopsies for counting the IELs seems optimum. Looking at the prevailing rate of gastrointestinal infection in India, setting a cut-off value of IELs to 30 IELs/100 epithelial cells at the tip of the villi appears ideal to avoid overdiagnosis of CD.^[52,53]

8. The orientation of mucosal biopsy fragments

a) Histological interpretation should only be made on oriented areas of mucosal biopsy fragments.

Voting summary: A 87.44% B 9.85% C 2.71% D 0% E0%

Quality of evidence: II-2

Type of recommendation: B

b) A biopsy is defined as properly oriented if 3-4 crypts, in a row, are arranged perpendicularly over the muscularis mucosae with overlying aligned villi.

Voting summary: A 93.37% B 5.13% C 1.5% D 0% E0%

Quality of evidence: II-2

Type of recommendation: B

One of the keys for appropriate interpretation and reporting of the intestinal mucosal biopsies is to have properly oriented mucosal biopsy fragments, characterized by 3-4 consecutive crypts perpendicularly arranged over the muscularis mucosae with properly aligned overlying villi visible from base to tips (as shown schematically in Figure 3a).^[40] The oriented crypts should reach the muscularis mucosae. The presence of only perpendicularly oriented

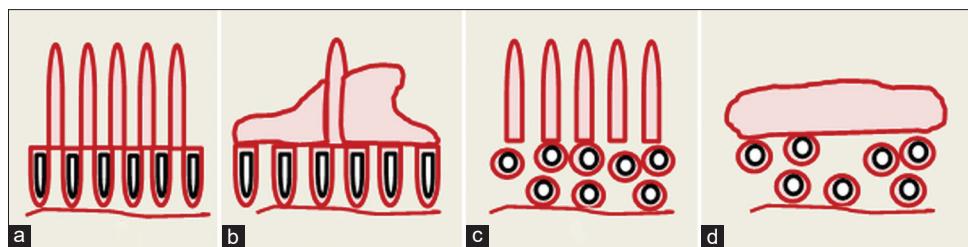


Figure 3: Schematic diagrams showing different situations a pathologist can encounter. (a) depicts an oriented mucosa; in (b) the villi are not aligned; in (c) the villi are aligned but the crypts are not oriented and in (d), neither the villi nor the crypts are properly aligned. The biopsy fragments mimicking (a) is most appropriate for interpretation

crypts may not be adequate to assess orientation as overlying villi may show slanting, fusion, denudation, or they may be artificially flattened when biopsies are taken over Brunner's glands or lymphoid follicles. The presence of even a single tall villus in a flattened mucosa should raise doubt on the quality of orientation and such biopsies should be interpreted with caution [Figures 3b and 4a-c]. Similarly, despite having aligned villi, the underlying crypts may be transversely cut round crypts [Figure 3c]. Interpretation in such areas should be avoided [Figures 3d and 4d]. Even when the biopsy fragments do not fulfill the criteria of orientation and the villi are cut transversely round, some pathologists still prefer to count IELs in them. Though this is not an ideal scenario the same should be internally validated and correlated with serological and clinical findings.

The orientation of each biopsy fragment should be assessed individually and there may be instances where only one of 5-6 biopsy fragments or none would be found oriented. Mounting the mucosal fragments on filter papers improves the orientation as discussed earlier. Identification of fibers of muscularis mucosae at the bottom of the mucosal fragments not only helps in the assessment of orientation but also prevents false flattening of the biopsy fragments.

Interpretation of histological parameters

9. IEL counting

- a) The IELs should be counted in the upper one-third of villi, in at least 5 oriented villi, starting from the villous tip and coming down on both sides. Their number should be expressed as the number of IELs per 100 enterocytes. Voting summary: A 92.42% B 7.58% C 0% D 0% E 0% Quality of evidence: II-2 Type of recommendation: B
- b) Alternatively, IELs can be counted in about 500 consecutive enterocytes restricted to the upper one-third of the oriented villi and averaged as the number of IELs per 100 enterocytes. Voting summary: A 87.8% B 9.75% C 2% D 0% E 0% Quality of evidence: III Type of recommendation: C
- c) The pathological cut off for IELs in India should be considered as $\geq 30/100$ epithelial cells. Anti-CD3 and/or

anti-CD8 immunostain for highlighting the IELs are not required in routine reporting of intestinal mucosal biopsies.
Voting summary: A 91.4% B 8.6% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B

10. Assessment of mucosal Cd and Vh

- a) For an appropriate assessment of Cd:Vh ratio, the presence of at least 4-5 oriented crypt to villous axes in a slide is optimal. However, Cd:Vh ratio can be interpreted even when a single-oriented crypt-villous axis is identified and that should be mentioned in the report.
Voting summary: A 71.25% B 27.25% C 1.5% D 0% E 0%
Quality of evidence: III
Type of recommendation: C
- b) Assessment of Cd:Vh ratio is best avoided over lymphoid follicles and Brunner's glands.
Voting summary: A 100% B 0% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B

In the normal SI, each villous is supported by 3-5 crypts, which are instrumental to produce new enterocytes and other differentiated mucosal cells when there is cell loss. The pathological changes in CD start with mucosal epithelial infiltration of IELS resulting in programmed cell loss. Initially (Marsh grades 1 & 2) the crypts can replenish the surface cell loss, however, when the disease is severe the crypt cannot keep up the supply of new enterocytes and the villi become flattened, which further is sub-graded into modified Marsh grades 3a, 3b, and 3c. These changes are sequential and follow the disease course. Not only does the surface mucosa lose enterocytes when there is increased IEL infiltration in patients with CD, but this is followed by crypt hyperplasia or varying degrees of villous flattening, the common histological features identified on light microscopy in patients with CD. However, none of these histological changes are specific and most infective, autoimmune, allergic, and drug-induced SI injuries will produce similar changes. Table 4 shows the causes of IELosis, villous flattening, and crypt hyperplasia in various diseases.

In some HLA DQ2/DQ2 positive symptomatic patients with increase serum anti-tTG titer, a duodenal biopsy may fail

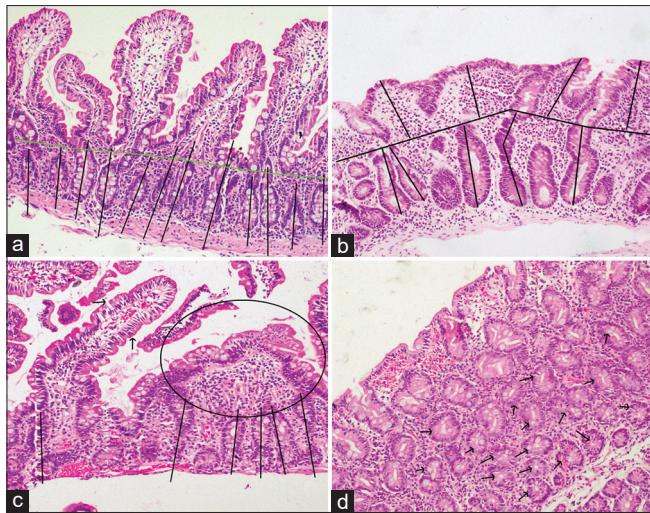


Figure 4: (a) shows a mucosal fragment, with crypts oriented perpendicularly over the muscularis mucosae (black lines) and villi with intact surface epithelium perfectly aligned [x100]. (b) shows villous abnormalities of modified Marsh grade 3c. The horizontal black line indicates the shoulder of the biopsy [x100]. (c) shows perpendicularly oriented crypts, with an aligned villous in the left (arrows), whereas the circled villous is inclined backward and uninterpretable [x100]. (d) shows all transversely cut, round crypts (arrows), not suitable for the interpretation [x100]

to show significant pathological changes (Marsh grades 0 & 1), called potential CD. On the other hand, patients having mucosal pathological changes may show serum titer within normal limits. In such condition's possibilities of false-negative and false-positive serum tests should be considered. Routinely, gastroenterologists advocate GFD to patients only fulfilling \geq Marsh grade 2 changes. However, it has been shown that symptomatic patients with early-enteropathy changes (Marsh 0 and 1) also respond to GFD with a reduction of epithelial programmed cell death.

Interpretation of IEL count

The orientation of the biopsy fragments and thickness of tissue sections can greatly impact the counting of IELs as discussed earlier. In duodenal biopsies from normal individuals, the IELs most often are concentrated towards the base of the villous. A villous cut transversely/obliquely through the base may appear flattened and IELs may be counted erroneously high [Figure 4]. The optimum site for IEL counting is the upper one-third of the villous,^[53,54] as the villous tips are commonly exposed to luminal gluten peptide, whereas the lower part of the villi and the crypts are not exposed to luminal contents unless the intestine is distended.^[55] The preferential infiltration of IELs at the villous tip is also known as the 'crescendo pattern', characteristically seen in CD. In certain clinical conditions such as that in tropical sprue (TS), IELs should be counted both at the upper part and the base of the villous. Predominant infiltration of the epithelium by the IELs covering the villous base is called a 'decrecendo pattern' [Table 5].

There are various methods for counting IELs. One of the methods is to start counting from the peak of the villus tip and continue both side slopes till 10 epithelial cells (nuclei) are counted on each side. Thus, one will get IELs per 20 epithelial cells at the villous tip [Figure 5a and b].^[53] The same procedure should be followed in 5 oriented villous tips to get IEL counts per 100 epithelial cells.^[53] The other method is to count at least 500 epithelial cells in a row and express the average IEL count per 100 epithelial cells.^[52] However, while counting the enterocytes in a row the high IEL count at the villous base, may result in a falsely high IEL count. Therefore, counting 500 epithelial cells only at the upper one-third of the villi is a preferred method. Demonstration of the crescendo pattern of IEL infiltration is one of the important histological pre-requisites in patients with CD.^[56] The pathological cutoff of IELs described in CD varied from time to time. A cut-off of \geq 40/100 ECs was described in the original Marsh classification, Oberhuber modified grading system and modified grading system by Corazza and Villanacci.^[6,57] Marsh MN, in an article in 2017 described 27/100 ECs as the cut-off as per his ROC curve analysis.^[58] In recent publications, a pathological cut-off of \geq 25/100 ECs has been described from various countries including India. However, to prevent an overdiagnosis it is better to stick to the cut-off of \geq 30 IELs/100 epithelial cells until further validation is done with other IEL cut-offs.

Although not recommended routinely, the IELs can be counted after immunohistochemical (IHC) staining with anti-CD3 (pan T cell marker) or CD8 antibodies.^[59] However, this is not routinely necessary and will add extra time and cost [Figure 5c and d]. IHC staining may be restricted to situations such as i) high serum anti-tTG antibody titer but microscopic Marsh 0 lesion or biopsies borderline high IEL count, ii) characterization of IEL phenotype in patients suspected to have either type I or type II refractory CD, or iii) when there is suspicion of the lymphoproliferative disease.^[51] Cooper R et al. compared the IEL counting in H and E stained slides with IEL counting on anti-CD3 stained sections and found that the number of IELs was higher in CD3 stained slides than the count on H and E stained slides. Hence, in CD3 stained slides, cases may be over-diagnosed as CD though interobserver agreements do not significantly differ between IEL counting on H and E stained slides and CD3 stained slides.^[60] To summarize, counting of IELs should ideally be done at the tips of at least 5 oriented villi on a 4 μ m thick section-glass slide. The routine use of IHC for IEL counting is not necessary.^[52]

Interpretation of crypt hyperplasia

Hyperplastic crypts appear visually as elongated and tortuous crypts with cytoplasmic basophilia [Figure 5e]. Crypt hyperplasia can be a result of the mucosal regenerative activity. Due to the nonspecific nature, it was not considered as a significant histological parameter in the grading systems proposed by Corazza & Villanacci or the Q-histological classification systems.^[5,6] But, in an appropriate clinical context with a positive celiac specific serological test, reporting of crypt hyperplasia along with IELs and with or without villous flattening is valuable [Table 4].

Table 4: Diseases where similar histological findings are in patients with celiac disease

<i>Increased IELs</i>	<i>Crypt Hyperplasia</i>	<i>Villous abnormalities</i>
Celiac disease	Chronic bacterial infections	Celiac disease
Bacterial infections	Chronic viral infections	Tropical sprue
Viral infections	Chronic parasitic infections	Crohn's disease
Parasitic infestations	Chronic fungal infections	Nutritional deficiencies
Fungal infections	Non-steroidal anti-inflammatory agents	Micro-villous inclusion disease
Non-steroidal anti-inflammatory agents	Drug-induced changes	Tufting enteropathy
H pylori gastritis and duodenitis	H pylori infection	Giardiasis
Giardiasis	Chronic Giardiasis	Autoimmune enteropathy
Autoimmune enteritis	Autoimmune enteritis	Graft versus host disease
Crohn's disease	Crohn's disease	HIV and AIDS
Common variable immunodeficiency	Common variable immunodeficiency	Common variable immunodeficiency
Tropical sprue	Celiac disease	Giardiasis
Intestinal lymphomas	Tropical sprue	Immune proliferative SI disease
Other medications, as Olmesartan, oral contraceptive pills, etc	Gastric juice induced chronic inflammation	Other SI lymphomas Other medications, as Olmesartan, oral contraceptive pills, MMF, Methotrexate, immune checkpoint inhibitors, Azathioprine, etc Spuriously in the duodenal bulb, on the top of the large lymphoid follicle, in poorly oriented mucosal fragments

Table 5: Histological differences of SI biopsies from patients with celiac disease, tropical sprue, Crohn's disease, and SI lymphoma/IPSID

<i>Histological features</i>	<i>Celiac disease</i>	<i>Tropical sprue</i>	<i>Crohn's disease</i>	<i>Giardiasis/H pylori-associated changes</i>	<i>CVID</i>	<i>Lymphoma, including IPSID</i>
Increase in IELs	Yes	Yes	Yes	Yes	Yes	Yes
The pattern of IEL infiltration	Crescendo, patchy	Crescendo or decrescendo, patchy	Throughout mucosal epithelium	Variable	Can be Crescendo or throughout the mucosal epithelium	Any epithelial cells can be targeted and destroyed
Flattening of villi	Yes	Yes	Yes	Yes	Yes	Yes
Server villous flattening (modified Marsh grade 3b & 3c)	Common	Uncommon	uncommon	Uncommon	May be seen	Uncommon
Crypt hyperplasia	Yes	Yes	Yes	Yes	Yes	Yes
Neutrophils in lamina propria	Only in severe disease	Usually Seen	When the disease is active	yes	Yes	No
Eosinophilic infiltrates	Seen (average count 5-6/HPF)	Seen (average count 13-14/HPF)	Seen (variable)	Seen (variable)	Yes	No (depends on lymphoma type)
Flattening of surface epithelial cells/thickening of subepithelial basement membrane)	Common	Uncommon	May be seen	Uncommon	Uncommon	Uncommon
Crypt destruction & apoptosis	Uncommon	Uncommon	Crypt loss may be seen	Uncommon	Uncommon	Common
Atypical lymphoid cells & lymphoepithelial lesions	Uncommon	Uncommon	Uncommon	Uncommon	No	yes
Mucosal or submucosal prominent lymphoid follicles	uncommon	No	No	May be seen	Present in 40-50%	May be seen
Plasma cells infiltration	May be seen	May be seen	May be seen	May be seen	May be seen	Sheets of plasma cells
Luminal organisms	No (unless superimposed infection)	No (unless has superadded infection)	No	Yes	Yes	Yes
Regional lymphadenopathy	Uncommon	Uncommon	Uncommon	Uncommon	Uncommon	May be seen depending on the stage of the disease
Serum Immunoglobulin	Normal, Except in patients with IgA deficiency	Normal	Normal	Normal	Deficiency of IgG, IgA, and IgM seen	High serum IgA in IPSID

Both the crypt hyperplasia and the villous flattening can alter the crypt depth to the villous height (Cd:Vh) ratio. In comparison

to Cd:Vh ratio of 1:5 to 1:3 in the European-Americans,^[13] the Cd:Vh ratio in healthy Indian adults is lower (1:2) as identified

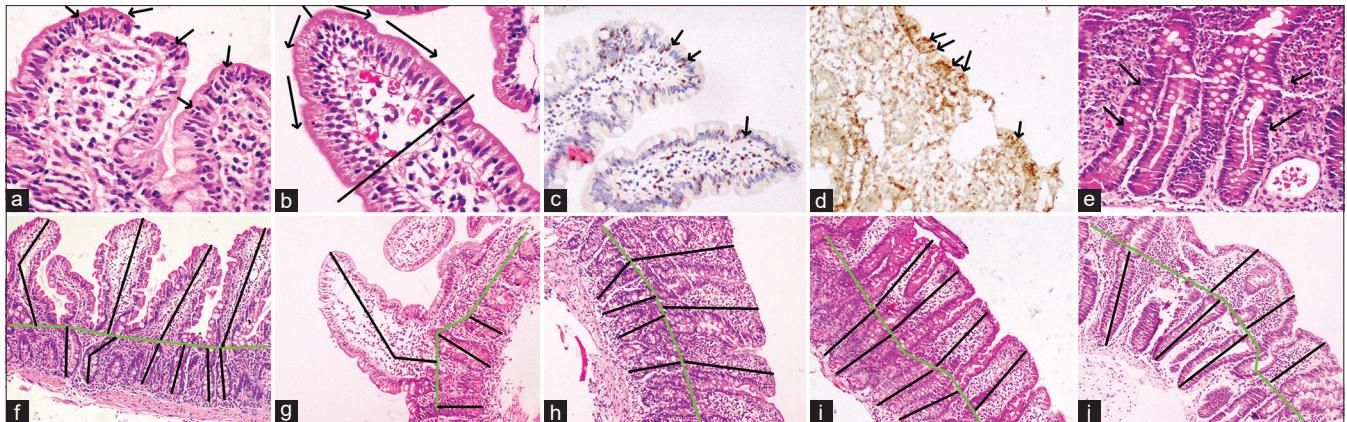


Figure 5: (a) shows the method of identifying the IELs (arrows), in contrast to the cigar-shaped elongated epithelial cell nuclei [a x 200]. (b) shows the method of counting of IELs in the upper 1/3rd of villi starting from the tip on both sides, covering at least 5 oriented villi [b x200]. Anti-CD3 stain highlighting the IELs in normal villi [c x100] and a biopsy showing modified Marsh 3c changes (arrows) [d x 100]. (e) shows elongated and tortuous hyperplastic crypts (arrows) [e 100]. Figures show Marsh 1 (f), Marsh 2 (g), modified Marsh 3a (h), 3b (i), and 3c changes (j). The shoulder of the biopsy fragments has been highlighted with green lines [f-j x 100]

in a study from the Northern part of India.^[5] Similar findings have also been reported both in adults and children by other investigators.^[50,61,62] It remains to be determined whether this morphological variation is due to the constitutively shorter villi in South-eastern Asian countries or because of mucosal damage following frequent gastrointestinal tract infections.

Villous height (Vh) interpretation

Flattening of the villi is the most characteristic and reliable histological feature among mucosal changes in CD [Figure 4]. Branching of villi is common in the duodenum and post-formalin fixation they can sway in any direction due to contraction of the mucosal fragments. It is important to identify the villous to crypt junction or the 'shoulder of the biopsy' correctly and in most cases, the shoulder is not a horizontal line. As the shape and Vh vary in the duodenum and the jejunum, labeling of mucosal biopsies sampled from different sites separately is essential. It is important to recognize that most available histological grading systems are based on qualitative criteria for sub-grading of the villous flattening, as 'mild', 'moderate' or 'severe' flattening which is subjective and thus results in high inter-observer disagreement.^[56,63] For better concordance, one can consider modified Marsh grade 3a change when the Vh is shorter than normal ($Cd:Vh < 1:2$) but still longer than the Cd, modified Marsh grade 3b changes when the Vh and the Cd are equal ($Cd:Vh$ ratio 1:1), and modified Marsh 3c change when the Cd is longer than the Vh ($Cd:Vh$ ratio $\geq 1:1$) [Figure 5f-j].^[64] It is still unclear whether the subclassification of villous abnormalities into modified Marsh grade 3a, 3b, or 3c has any clinical connotation. But it will be appropriate to use a histological classification system for uniform reporting and monitoring the mucosal changes post-GFD.^[64,65] Importantly, there can be regional variations of histological grades of severity of villous abnormalities in different fragments of the biopsies from the same patient, in such a situation, the highest-grade of severity should be reported, along with making a note about the heterogeneous nature of the histological changes.^[56]

It is also a relevant question that how many oriented crypt-villous axes are necessary at minimum for the interpretation of the Cd: Vh ratio? Although there is no defined standard, the finding of at least four to five oriented crypt-villous axes should suffice. However, keeping in mind the regional variations, as many oriented crypt-villous axes available should be assessed. Many pathologists also use the Cd:Vh and Vh:Cd ratio interchangeably, which may create confusion and misinterpretation not only by pathologists but also by the gastroenterologists.

Other histological findings in patients with CD

Additional histological findings in patients with CD are the presence of eosinophils in the lamina propria, shortening of the height of columnar enterocytes infiltrated with IELs, thickening of the subepithelial basement membrane, and mucosal neutrophilic infiltration, mostly in those having clinically severe CD. Some of these features help in making differentiation from the mimickers, but when identified isolated are not diagnostic [Table 5].^[64,65] These additional findings may be mentioned in the histopathology report for better interpretation.

11. Interpretation of SI biopsies, fallacies, and histological mimickers

- a) The diagnosis of CD is made on a combination of clinical, serological, and histological features.
Voting summary: A 91.28% B 8.72% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B
- b) Both pathologists and gastroenterologists should be aware of the limitations of the interpretation of the biopsies.
Voting summary: A 96.25% B 2.5% C 0% D 0% E 1.25%
Quality of evidence: II-2
Type of recommendation: B
- c) The changes that occur in the crypts and villi in patients with CD are non-diagnostic and similar changes occur in

a variety of other SI mucosal diseases.

Voting summary: A 96.31% B 3.69% C 0% D 0% E 0%

Quality of evidence: II-2

Type of recommendation: B

There can be the following fallacies that may impact histological interpretation:

- The changes can be patchy and may not be sampled at all
- The biopsy fragments are too few to represent the pathology
- The possibility of a bulb or D1-limited CD cannot be ruled out when not sampled
- Overdiagnosis in a case of misoriented biopsy fragments
- Overdiagnosis when the biopsy fragments have been taken only from D1
- Overdiagnosis of CD, when the pathologists do not know the serum antibody titer, clinical features, and clinical possibilities in case of malabsorption.
- Biased diagnosis in a case of false-high or false-low serology titer
- Overdiagnosis or under-diagnosis when the tissue is not properly fixed, cut with optimum thickness, optimally stained, and adequate efforts have been made to take step-sections to achieve an oriented fragment
- Use of improper pathological criteria to interpret the histological changes
- Overdiagnosis or underdiagnosis by pathologists who do not have enough experience to interpret the small bowel biopsies.

All have been discussed in detail. Not only the processing of SI biopsies needs expert technical hands, the pathologists interpreting the biopsies should make effort to gain experience to understand the nitty and gritty of their job. In discordant situations, always the case may be referred to an experienced pathologist for the second option and the issues may be discussed with the gastroenterology colleagues to effectively resolve them.

Histological mimickers of CD: The histological changes in patients with CD are not diagnostic and a diagnosis of CD is based on correlations of clinical symptoms, serology titer, and suggestive histological changes.^[66] Table 4 shows the diseases where similar histological changes.

The closest mimickers of CD in South-East Asia are the TS, parasitic infestations, and Crohn's disease, at least clinically and histologically. Though less commonly seen in North India, TS is quite prevalent in Southern India and some parts of Asia. In Table 5, the histological similarities and differences among these common entities have been highlighted. However, it is difficult to differentiate these conditions on histology alone, and attention should be given to the severity of clinical and endoscopic changes, serological titers, travel history, findings of stool examinations, etc. Most of the bacterial, viral, and parasitic

infections of the SI, Whipple's disease, food allergy, autoimmune enteropathy, drugs (NSAIDs, Olmesartan, immune checkpoint inhibitors), SI bacterial overgrowth, autoimmune enteropathy can result in mucosal pathology similar to that seen in CD.^[64,65] Hence, the availability of the relevant clinical details to the pathologists will help them to rule out the common mimickers and state if CD can be a possibility. If the villous abnormalities are of high grade and the anti-tTG antibodies are negative, one should repeat the serological tests from a different laboratory and an anti-EMA test should also be done. A short course of gluten challenge followed by a repeat biopsy, including those from distal SI and/jejunum may help to establish the pathological changes in such a scenario.^[67] A negative HLA DQ2/DQ8 haplotype will rule out CD.^[66,68-70] Programmed cell death of the enterocytes not only seen in CD, but are prominent in autoimmune enteropathy, graft versus host disease, and chronic intake of NSAIDs and Olmesartan. *Avoidance of NSAIDs at least 7 days before the biopsy procedure may help to exclude this change.* However, Olmesartan induced changes are mediated by IL-15 take 4-6 months to appear or regress; hence, short time discontinuation before biopsy may not help.^[71] Regardless of geographical locations, pathologists should not forget about parasitic infestations as giardiasis or *H pylori* infection especially in India and Southeast Asia, which may produce similar histology.^[72] The histological changes of combined variable immune deficiency (CVID) may also mimic that of CD. The absence of mature mucosal plasma cells, prominent lymphoid aggregates, and a low level of serum immunoglobulins should help in the identification [Table 5].

A diagnosis of lymphoma or immune proliferative SI disease (IPSID) should be considered when sheets of lymphoid cells or plasma cells are observed in the lamina propria along with destruction and/or loss of crypt [Table 5]. Levels of serum IgA, immunoblotting, or immunostaining with cryptic IgA, kappa, and lambda light chains may help in differentiation. The presence of prominent mucosal histiocytes should raise the considerations of Whipple's disease, histoplasmosis, or *Mycobacterium avium intracellulare* infection, particularly in immune-compromised patients. Prominent fat vacuoles in the enterocyte cytoplasm should raise the possibility of abetalipoproteinemia. In such a diagnosis, the biopsies should be repeated, and they should be collected in a specific manner as described in Table 5 for further workup. Although the presence of intracytoplasmic inclusions suggests a diagnosis of Cytomegalovirus infection, the presence of an intact glycocalyx layer on epithelial helps to rule out the possibility of MVID in infants with intractable diarrhea. Type of diarrhea, as histology of chronic protein-losing enteropathy, should raise the possibility of lymphangiectasia, or the presence of watery diarrhea should raise the possibility of microscopic enteritis. Also, sometimes pathological conditions may co-exist as giardiasis and CD, or CD and CVID, or IPSID in a background of CD, which needs multidisciplinary discussions and correlations.^[73]

12. Histological grading of SI biopsies in CD

- a) At present, the modified Marsh grading system is recommended for classifying the extent of the villous abnormalities and the diagnosis of CD is considered in the presence of villous abnormalities of modified Marsh grade 2 or more in an appropriate clinical and serological setting.
Voting summary: A 91.65% B 8.35% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B
- b) Patients having a positive celiac specific serological test but having villous abnormalities of modified Marsh grade 0 or 1 are classified as potential CD.
Voting summary: A 84.82% B 15.18% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B
- c) Using the semi-objective criteria for sub grading of villous abnormalities will reduce subjectivity in assessment, such as modified Marsh 3a- if the villous height is less than normal but more than the crypt depth (C:V ratio is <1:2), 3b- if Cd and Vh are equal (Cd:Vh- 1:1) and 3c changes- if crypt depth is more than the villous height.
Voting summary: A 83.56% B 16.44% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B
- d) Descriptive terminologies such as partial, sub-total, or total villous atrophy should be avoided.
Voting summary: A 70.93% B 25.74% C 3.33% D 0% E 0%
Quality of evidence: III
Type of recommendation: C
- e) Image analysis and Q-histological assessment techniques are objective and accurate tools to analyze the changes in mucosal dimensions, which improves the intra-observer and inter-observer agreements.
Voting summary: A 63.38% B 30.91% C 3.46% D 1.25% E 1%
Quality of evidence: II-2
Type of recommendation: B

Though a histological grading system described by Rubin *et al.* was already available,^[74] Marsh MN was the first to classify the histological changes as per the sequence of pathological changes,^[54] followed by the introduction of Oberhuber's modified Marsh classification system.^[57] The basic difference between the original Marsh and modified Marsh classification system was that the qualitative sub-grading of villous flattening was described in the latter, as mild, moderate, and severe villous flattening, leading to interobserver and intraobserver agreements in only about 50% of biopsies.^[5,56,72] This problem was recognized, followed by the introduction of subsequent modified Corazza/Villanacci classification,^[6,75] Ensari classification,^[53] which were also based

on qualitative criteria, and the off-late described Q-histological methods incorporating quantifiable criteria [Table 6].^[76,77]

Further sub grading of the villous flattening as mild, moderate, and severe villus flattening was questioned based on the findings of 3D image reconstruction studies, inconsistent correlation of mucosa infiltrating lymphocyte density, and grades of villous flattening, mathematical regression equation analysis, questionable clinical relevance.^[58] Clinically it is enough to know if seropositive patients have ≥Marsh 2 histological changes to advocate GFD.^[76] Seropositive patients with Marsh 0 or Marsh 1 changes are called potential CD, and their progression to higher modified Marsh grades has been documented in 30% of these patients after 12-months of follow-up.^[77]

Subjective criteria used for the sub grading results in low interobserver agreements. Werkstetter *et al.* highlighted the disagreement between the local and central pathologist in differentiating no CD (Marsh 0 and 1) and CD (Marsh 2 or 3) categories in up to 7% biopsies, and the disagreement rose to 58% when they were further asked for sub grading the villous atrophies into modified Marsh 3a-3c changes.^[10] For better concordance one can consider modified Marsh grade 3a change when the Vh is shorter than normal but still longer than the Cd, modified Marsh grade 3b changes when the Vh and the Cd are equal, (Cd:Vh ratio 1:1), and modified Marsh 3c change when the Cd is longer than the Vh (Cd:Vh ratio ≥1:1) [Figure 5f-j].^[64] But the most important justification for following the extended modified Oberhuber classification system is to assess the mucosal healing post-GFD, as symptomatic or serological improvements do not go hand in hand with the mucosal histological normalization.^[78] In a study by Lee SK, 12 months post-GFD, whereas 77% of patients had normal serology, only 21% of them showed histological reversal.^[79] Lanzini A, *et al.*, after 16 months post-GFD showed mucosal normalization only in 8% of patients. Interestingly, 83% of patients having baseline modified Marsh 3c changes showed a mismatch of histological and serological response after an average period of 16 months post-GFD.^[80] Among all serological tests available only EMA was found to be a fair indicator of mucosal healing, though not routinely performed during the initial screening and diagnosis, especially in India.^[81] In the presence of systemic autoimmune inflammation, both the anti-tTG titer and EMA can be falsely elevated/positive despite achieving mucosal healing.^[82] In the United States, persistent villous flattening was identified in up to 66% of patients on GFD after two-years of follow-up.^[83] In another study, after a year of post-GFD follow-up, only 50.5% of adult patients achieved mucosal recovery, whereas 81% of them showed mucosal healing after 8.1 years of follow-up.^[84] As the compliance to GFD in children is relatively better than in adults, increasing weight or height in children may be used as reliable clinical indicators of mucosal healing, besides the availability of biomarkers such as I-FABP (intestinal fatty acid-binding protein) or anti-DGP serology test.^[85,86] However, less than half of the adult patients with CD, especially those over 40 years of age, and who had modified Marsh 3c changes at baseline, the serological or

Table 6: Table showing a comparison of different histological classification systems available for SI biopsy interpretation from patients with celiac disease

Histological grading systems	Early enteropathy CD			Advanced enteropathy CD			The pathological cut-off for IELs
Marsh Classification ^[54]	Marsh 0	Marsh 1	Marsh 2	Marsh 3	Marsh 3	Marsh 3	≥ 40 IELs/100 ECs Later modified the IEL cut-off to about 27/100 ECs in 2017
Oberhuber Modification ^[57]	Marsh 0	Marsh 1	Marsh 2	Marsh 3a	Marsh 3b	Marsh 3c	≥ 40 IELs/100 ECs
Corazza & Villanacci classification (On jejunal biopsy) ^[6]	Grade 0	Grade A	Grade A	Grade B1	Grade B2		≥ 40 /100 ECs
Ensari classification ^[53]	Type 0	Type 1	Type 1	Type 2		Type 3	≥ 25 IELs/100 ECs
Q-histological classification ^[5]	Type 0	Type 1 (IEL count <25/100 ECs + Villous height fold change >0.7+Cd: Vh <0.5)	Type 1 (IEL count ≥ 25 /100 ECs + Villous height fold change >0.7 + Cd: Vh <0.5)	Type 2 (IEL count ≥ 25 /100 ECs + Villous height fold change ≤ 0.7 'OR' Cd: Vh ratio ≥ 0.5)	Type 3 (IEL count ≥ 25 /100 ECs + Villous height fold change ≤ 0.7 Cd: Vh ratio ≥ 0.5)		IEL count ≥ 25 /100 ECs

symptomatic changes are reliable indicators of mucosal healing, necessitating follow-up biopsies.^[84-89] Noninvasive biomarkers as serum I-FABP or Citrulline though have come up, large validation studies are needed before their clinical application in comparison to continuing with the gold standard SI biopsies.^[87] Keeping in mind the limitations of the light-microscopic histological classification systems, the Q-histological assessment based on quantifiable parameters improved intra-observer agreement by up to 85% and interobserver agreements up to 71%. This assessment also brought objectivity, is labor-intensive, time-consuming, and increases reporting time. Another advantage of the Q-histological grading system is that minor changes in villous dimensions can be measured, valuable in clinical trials. The application of artificial intelligence-based automation may simplify its use during routine reporting, and till that is available, its use does not seem practical for routine evaluation. Two simple approaches which can be adopted are (1) to train the pathologists who deal with SI biopsies through online webinars, hands-on-workshops, and (2) to improve routine assessment of biopsy dimensions is using eye-piece reticles in reporting microscopes to help assisted near-quantitative eyeballing of Vh and Cd, rather than directly measuring them as per the experience of the authors. However, this system needs further validation [Figure 6].

13. Assessment of healing and follow-up biopsies in patients with CD

- a) While routine follow up biopsies after GFD is not indicated at present, however demonstration of healing of mucosa on GFD is emerging as one of the treatment goals.
Voting summary: A 71.11% B 28.89% C 0% D 0% E 0%
Quality of evidence: III
Type of recommendation: C
- b) In patients with CD, while the resolution of symptoms and normalization of serology occur faster, the healing of mucosa often takes 6 months to one year or even more.
Voting summary: A 69.29% B 23.11% C 2% D 2% E 3.6%
Quality of evidence: III



Figure 6: The image shows the utility of using a microscope eye-piece reticle for quick quantitative eyeballing for assessment of Cd and Vh in a duodenal biopsy [x 40]

- c) While it appears appropriate to follow-up the patients with potential CD, the timepoints for reassessment is not yet defined.
Voting summary: A 79.89% B 18.51% C 1.6% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B

Follow-up SI biopsies help in identifying GFD-compliant patients. However, it needs to be recognized that there is no established guideline for follow-up intestinal biopsies in patients with CD and the protocol can vary depending on the patient's response and treating physician's choice. Literature review shows adult patients aged >40 years at diagnosis, males, and with baseline modified Marsh 3c changes would preferably need follow-up biopsies. Adult females on the other hand show relatively quick mucosal healing post-GFD.^[78,89] Unless sub grading of villous flattening into modified Marsh 3a, 3b, and 3c changes are done at baseline, triaging and follow-up assessment will be difficult.^[73] Follow-up

SI biopsies will help to work up for refractory celiac disease (RCD) in GFD-non compliant patients, or rule out a coexisting disease, in patients with high anti-tTG serology titer but normal histology; or in seropositive patients having histology corresponding to Marsh grade 0/1 changes prospectively after a period of 2-years while on a gluten-containing diet to establish the progression of mucosal pathology necessitating GFD, however, there is lack of established guideline regarding follow-up biopsies in potential CD patients.^[89-91] Generally, follow-up biopsies should be taken 12-months post-GFD as the histological changes of mucosal pathologies take time to reverse. The AGA recommends a second follow-up biopsy after a gap of 12-months if the first follow-up biopsy had Marsh 2 changes.^[86,92-95] There is also no established protocol on how to report the follow-up biopsies as the sequence of reversal of changes is exactly not known. Reporting of follow-up biopsies exactly in a similar manner as done for baseline biopsies with mentioning of current modified Marsh grade will help to compare existing mucosal changes with changes in baseline biopsies and assessing response to GFD. Applying Q-histological measurement in the follow-up biopsies also can provide accurate parameters for assessing response to GFD, but the use of this system seems is not yet easy to implement in routine practice and restricted to clinical trials.^[10]

14. GFD nonresponsive CD and Refractory CD

- a) All patients with GFD non-responsive CD should be evaluated appropriately including SI mucosal biopsies to rule out concomitant other SI diseases and refractory CD.
Voting summary: A 94.74% B 5.26% C 4.76% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B
- b) Almost 10-50% of patients with CD have incomplete healing of the mucosa and villous abnormalities of varying grade persist in them even after 1 to 7 years of initiation of GFD.
Voting summary: A 93.77% B 6.23% C 0% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B

GFD is the only recommended treatment for patients with CD. However, non-response to GFD has been reported in up to 10-50% of adults on follow-up.^[96] Non-response can be due to non-adherence to GFD, or mistaken diagnoses followed by GFD treatment or an associated disease which complicates the course of CD including the RCD type 1 and 2, followed by the risk of systemic complications of chronic malabsorption, lymphomas, and carcinomas reported especially in RCD type 2.^[3,97] On 12-months post-GFD follow-up, only about half of the patients with CD show mucosal healing. On the other hand, even after about 8 years of post-GFD follow-up up to 81% of patients showed mucosal healing.^[84] The diagnoses of GFD-nonresponse are not straight forward, need clinical and nutritional assessment, with a battery of investigations. Histologically

all GFD-unresponsive patients of CD show the persistent villous flattening and the validated histological features to predict mucosal response are not known. The annual incidence of RCD is about 0.83/10.000 CD patients,^[98] and immunophenotyping of the IELs by either IHC or flow cytometry are needed for diagnosis and typing, as follows: RCD type 1: CD3+CD103+CD8+IELs having polyclonal TCR γ chain gene, and RCD type 2: ≥50% by IHC or ≥25% of IELs by flowcytometry having CD3-CD103+/-CD8-/CD335+phenotype with clonal TCR γ chain genes.^[99] Hence, pathologists need to play an important role in the workup of patients with GFD-nonresponsive CD and to identify patients with RCD. A multidisciplinary approach and the collection of formalin-fixed biopsy fragments for IHC, and fresh biopsy fragments for isolation of IELs, followed by flow cytometry and clonality assessment are required.

15. Challenges in pathology interpretation of SI biopsies

- a) In patients on GFD without a confirmed diagnosis of CD, the diagnosis of CD can be confirmed with gluten challenge for 2-8 weeks.
Voting summary: A 60.7% B 34.79% C 2.81% D 0% E 0%
Quality of evidence: II-3
Type of recommendation: B
- b) In patients' having histological features of villous abnormalities but negative celiac specific serology, possibilities of seronegative CD and non-celiac enteropathies should be considered.
Voting summary: A 93.42% B 5.39% C 1.19% D 0% E 0%
Quality of evidence: II-2
Type of recommendation: B
- c) In case of diagnostic difficulty, it is preferable to get the biopsies reviewed by a Gastrointestinal pathologist/ or an experienced pathologist.
- d) In those, who have a high suspicion of a CD but negative celiac specific serology, IgA anti-tTG colocalized antibody deposits may provide a clue to the diagnosis.
Voting summary: A 74.14% B 23.24% C 1.5% D 0% E 1.3%
Quality of evidence: II-2
Type of recommendation: B

Scenario 1: Assessment of SI biopsies from those already on GFD (without a definitive diagnosis)

A perplexing clinical scenario is to confirm CD in patients who started GFD of their own without relevant investigations. In these cases, patients should be advised to undergo anti-tTG serology and a positive serology does not pose a difficulty in diagnosis. But seronegative patients should undergo HLA DQ2/DQ8 typing, which if comes negative rules out a CD. In seronegative patients having HLA DQ2/DQ8 haplotypes, a gluten challenge with 3-10 g of gluten (one to four slices of bread) every day for 2-8 weeks followed by duodenal biopsy would help to document gluten-induced mucosal pathologies. As the amount of gluten to use and duration needed to produce the mucosal changes vary according to different

studies the protocol needs to be individualized.^[67] However, gluten challenge should not be done in children <6 years old and during the pubertal spurt. It is strongly recommended to send such patients to tertiary care centers or refer them to experienced gastroenterologists before conducting gluten challenge because of the rare but fatal risk of developing celiac crisis.

Scenario 2: Negative serology in suspected patients

In patients having histological features like that in CD but negative IgA anti-tTG serology should be considered for IgA deficiency, unsolicited GFD, seronegative CD, non-celiac enteropathy as detailed in Table 3. In short, from an Indian perspective, possibilities of TS, Crohn's disease, parasitic infestation, food allergy, enzyme deficiencies, NASID induced changes, or CVID should not be forgotten and investigated accordingly.^[100] Before releasing the report, a second opinion on the biopsy and ruling out all the mentioned conditions would be advisable. Careful nutritional assessment, stool gluten Immunogenic Peptide (GIP) test, serum Ig levels estimation, IgG based serology, or HLA typing would be advisable.^[99]

Scenario 3: Positive serology and normal biopsy

Positive IgA anti-tTG serology can be seen in patients having normal duodenal histology. In up to 10% of cases, serology can be falsely positive, especially in patients having other autoimmune systemic diseases, chronic liver disease, congestive heart failure, or hypothyroidism. As discussed earlier, in such a scenario, the number of biopsy fragments included, their orientation should be checked, and a second opinion should be asked from an experienced GI-pathologist. Repeating the anti-tTG serology by an ELISA kit of a different make than the earlier used one, or a second-line anti-EMA test would also help. HLA DQ2/DQ8 typing would help to rule out CD in haplotype negative patients. A gluten challenge may be an option. If positive serology is confirmed or supported by other tests as detailed above in histologically normal patients, the patient should be categorized as a potential CD.^[67,101,102]

Applicability of IgA anti-TG2 immune deposit in tissues in a difficult diagnostic situation

In the above-mentioned situations where the diagnosis of CD is difficult, identification of IgA anti-TG2 immune deposits in the SI biopsies by dual-color IHC or dual-color confocal IF methods have shown high sensitivity and specificity.^[103,104] IgA anti-tTG2 deposits have been identified in CD, potential CD, seronegative

Quality of evidence: III Type of recommendation: C

CD, and dermatitis herpetiformis.^[104] Facilities for the same should be established in centers receiving a high load of celiac patients.

16. Reporting of SI biopsies

An ideal pathology report [Table 7] should describe the histological findings in a synoptic manner, followed by overall interpretation based on the clinical, serological, and histological findings and the modified Marsh grade based on the highest degree of pathological changes across all the biopsy fragments.^[3,16,44,105,106]

When serology titer is not high or not known, but histological features mimic CD, the pathology interpretation should be descriptive. E.g., when the biopsy shows 34 IELs/100 ECs with a C:V of 1:2 and crypt hyperplasia in a patient with anti-tTG serology titer of 6 (say the lab cut off is 4 IU/mL), the pathology report should be as 'Overall features are of mild villous abnormality with increased IELs and crypt hyperplasia. Although features may be consistent with CD, the patient should be further investigated to rule out other mimics.' In such cases modified Marsh grading should not be mentioned.

Section 16: Communication between two disciplines for optimal interpretation of SI biopsies

There is some basic but essential information that pathologists expect from the gastroenterology colleagues and the gastroenterologists expect from pathologists. Adherence to this checklist will help both to get the maximum yield of a SI biopsy procedure.

What Pathologists expect from gastroenterologists?

- Information on site of biopsies & biopsy fragment numbers
- Diagnostic biopsies or follow-up biopsies
- Biopsy fragments from different site labeled and sent in separate containers
- Information regarding the time of biopsy (to calculate the time of fixation)
- Biopsy fragments are oriented on mounting media or not
- Appropriate and relevant clinical information including serotiter
- Relevant investigation results
- Clinical differential diagnoses
- Any specific clinical question in mind

What is expected from the pathologists?

- Site wise descriptive report
- Reporting in a specified format
- Mention regarding the number of step cuts examined & biopsy orientation
- Final Interpretation
- Comments on limitations
- Suggestions
- Additional tests needed

- All SI biopsies should be reported in a well-defined and uniform format.
Voting summary: A 100% B 0% C 0% D 0% E 0%
Quality of evidence: III
Type of recommendation: C
- Both the pathologists and gastroenterologists should understand the expectations from each other and participate in multidisciplinary review meetings to resolve diagnostic dilemmas.
Voting summary: A 98.25% B 1.75% C 0% D 0% E 0%

Table 7: A model histology report should look like the following format

Name _____	Age _____	Sex M/F _____	Hospital ID: _____
OPD/WARD: _____	Contact number of Consultant/Resident _____		
Histopathology accession number _____			
Previous biopsy number/s _____			
Date of receiving the biopsy _____		Date of reporting _____	
Number of mucosal biopsy fragments obtained: D2 _____ D1 _____			
Are they collected and labeled separately: Yes/No.			
Biopsy fragment from duodenal bulb: Present/Absent.			
Comment on the orientation of biopsy fragments- Oriented/partially oriented/not oriented			
Number of step sections examined: _____			
Histological Findings:			
IELs: Increased/not increased (Normal: IELs < 25/100 ECs; Borderline: 25-29 IELs/100 ECs; Increased: IELs ≥ 30/100 ECs)			
Crypt hyperplasia: Present/Absent			
Cd:Vh ratio (mention ratio): _____			
Luminal parasites: Present/Absent. Describe (if yes) _____			
Lamina propria inflammation: mild/moderate/dense			
Inflammatory cell type: eosinophils (_____/HPF)/histiocytes/neutrophils/plasma cells			
Other Histological findings:			
Flattening of surface epithelium: Yes/No			
Loss of goblet cells in the surface mucosal epithelium: Yes/No			
Thickening of the subepithelial basement membrane: Yes/No			
Lamina propria fibrosis: Yes/No			
Significantly visible intracytoplasmic fat vacuoles in the surface epithelial cells: Yes/No			
Prominent dilated mucosal lymphatics: Yes/No			
Presence of prominent lymphoid aggregates & large Brunner's gland lobules in the mucosa or submucosa: Yes/No			
Presence of prominent plasma cells or atypical cells in the mucosa: Yes/No			
Intact glycocalyx layer on mucosal surface (in children): Yes/No.			
Presence of mucosal epithelial cell tufting (in children): Yes/No			
Final Interpretation:			
Modified histological Marsh grade-Comments if any: _____			
Signing Pathologist			

If biopsies have been taken from different sites, site wise report should be given

When serology titer is not high or not mentioned, but histological features mimic CD, the pathology interpretation should be descriptive. E.g. when the biopsy shows 34 IELs/ 100 ECs with a C: V of 1: 2 and crypt hyperplasia in a patient with anti-tTG serology titer of 6 (say the lab cut off is 4 IU/mL), the pathology report should be as 'Overall features are of mild villous abnormality with increased IELs and crypt hyperplasia. Although features may be consistent with celiac disease, the patient should be further investigated to rule out other mimics.' In such cases modified Marsh grading should

If the diagnostic issue is still not resolved, the following steps should be taken periodically:

- Joint conferences (physical, virtual, phone, What's App)
- Multidisciplinary review meetings.

17. Training and infrastructure

- | | |
|----|---|
| a) | Pathology technicians should put all efforts to process the SI biopsies as per suggested statements and recommendations described earlier with help of their supervising pathologists.
Voting summary: A 92.49% B 5.07% C 0% D 1.19% E 1.25%
Quality of evidence: III
Type of recommendation: C |
| b) | A due emphasis should be given in the training of postgraduate residents, fellows, and technical staffs in the handling, processing, orientation, and interpretation of SI mucosal biopsies in the prescribed format.
Voting summary: A 87.87% B 1.63% C 0% D 1.5% E 0%
Quality of evidence: III
Type of recommendation: C |

A joint and coordinated effort from all those involved in this process including the endoscopists, pathology technical staff,

and experienced pathologists is essential for optimum yield of this invasive procedure. In medical schools, during post-graduate training of doctors as well as nursing staff and paramedics training on intestinal biopsy handling is ignored. Arguelles-Grande C *et al.* evaluated agreement for primary diagnosis between pathologists working in community hospitals, commercial laboratories, smaller institutions, and University hospitals. The agreement was very good between this institution and university hospitals ($k = 0.888$) but moderate in community hospitals ($k = 0.465$) or commercial laboratories ($k = 0.419$). Within different Marsh score categories, the agreement was poor ($k < 0.03$) for Marsh 1 and 2 categories, both missed at other centers, and fair or moderate for scores 3a and 3b. Information regarding the degree of villous flattening and IELs was lacking in 26% and 86% of reports and non-quantifiable terminologies as 'blunting' or 'marked atrophy' were commonly used.^[107]

Trongwongsawat T *et al.* evaluated the outcome of training of the pathology technicians handling SI biopsies and endoscopy nurses who mount the biopsied fragments on filter paper.^[108] Without training, 46% of the biopsy fragments were interpretable, however, after training of technical staff, the interpretability rose to 60%. Furthermore, when endoscopy nurses and assistants were also trained regarding the handling and mounting techniques,

interpretability rose to 74% of all biopsies ($P=0.004$). Interestingly, when only the pathology technicians or the endoscopy nurses were trained, the improvement was not statistically significant.^[91] The authors also observed that the procedures followed by trained staff did not take extra time than the time taken by untrained staff.^[108] A focus on practical aspects during staff training, periodical evaluation of the quality of biopsy and interpretability, holding inter-disciplinary periodical meetings and internal quality assessments would help to improve the outcome. For smaller centers and non-academic establishments periodical, hands-on-workshop, and training courses both on biopsy handling and interpretation would help to generate a sense of awareness and uniformity in reporting. This guideline should be followed by all, irrespective of their nature of the practice, and implement to achieve the goal of this guideline. Pathologists should also take the lead to implement these SOPs in their respective laboratories and train their staff as per the guidelines. Institutions and pathology laboratories should strive to create a database of SI biopsies following the uniform reporting format to create a National database on SI biopsies in patients suspected of CD.

Knowledge gap and areas of priorities

Though the awareness regarding CD, and access to specialist gastroenterologists, and endoscopes have increased, still there is a heterogeneity of understanding, practice, interpretability, and yield of the SI biopsy procedure. The following are the areas of priorities that were identified before this guideline was made. However, this is only the first step and efforts should be made to work systematically on each aspect to improve the overall quality of practice:

1. Formulation of a detailed pathologist centered practice guideline on SI biopsy handling, processing, and interpretation, in consultation with the experienced gastroenterologists.
2. Improve the quality of practice and to bring uniformity by formulating a guideline acceptable to all.
3. Establishing a facility of centralized or regional training of pathologists and technical staff and endoscopy nurses with periodical assessment courses.
4. Working on the improved histological grading system of SI biopsies in patients with CD, including Q-histological classification and deep learning algorithm
5. Establishing facilities to workup nonresponsive CD and refractory CD
6. Establishing facilities to identify IgA anti TG2 deposits in biopsy tissues
7. Establishing laboratory diagnostic methods to confirm the extra-intestinal CD.

CONCLUSIONS

Small bowel biopsy still holds the position of the gold standard for evaluating the mucosal pathologies in patients with CD, especially in adults, and for evaluation of mucosal healing in follow-up biopsies. As the histological changes in CD are not specific, access to clinical, endoscopic findings and serological titer will improve the pathology reporting. Pathologists should make effort to rule out all mimickers of CD and follow the laid down reporting format for uniformity. Gaining

experience in the work by practice, undergoing training, and periodic assessment should be undertaken by pathologists, technical staff, and endoscopy nurses. A periodic quality assessment would give an insight into the nature of practice and difficult cases should be discussed in the multi-disciplinary meeting to understand the areas of deficiencies. Histological grading of the biopsies from patients with treatment naïve CD will help to identify patients who should be necessarily followed up and compare the findings of follow-up biopsies with baseline biopsies. To ensure the availability of clinical and serological detail, the requisition forms should be modified. Finally, pathologists should try to use digital image analysis software or a microscope eye-piece reticle for quick assisted semi-quantitative Cd and Vh assessment. This guideline intends to give a standard operating protocol formulated with the help of experienced pathologists and gastroenterologists and should be implemented by all for a uniform outcome.

Guideline Summary of Essential Recommendations

- Small intestinal biopsies are important to demonstrate mucosal pathologies in adults with CD, and older children having borderline serotiter.
- Small intestinal biopsies should be done from patients suspected of CD both from post-ampulla part of duodenum (4 fragments), and duodenal bulb (1-2 fragments); if not contributory in a case with strong clinical suspicion, repeat biopsies, including mucosal fragments from D3 or jejunum may further reveal pathological changes.
- Individual small intestinal biopsy fragments should be mounted on mounting media, as detailed in this document and should be sent to pathology laboratory in containers labeled site-wise, fixed in 10% neutral buffered formalin (pH 6.8- 7.4; ratio at least 1: 10) for 5-6 hours for optimum outcome.
- All small bowel biopsies should be accompanied with an appropriately filled requisition form mentioning relevant clinical information, serological data, site/s and number of samples (containers), endoscopic findings, previous pathology report if any and the clinical possibilities.
- In one block up to 4 mucosal biopsy fragments should be embedded and multiple step sections should be examined till an oriented mucosal fragment/s are identified for interpretation.
- Histologically, a biopsy fragment would be defined as oriented if 3-4 crypts, in a row, are arranged perpendicularly over the muscularis mucosae with overlying aligned villi.
- The IELs should be counted on 4 μm thick H&E stained sections in the upper third of the villous epithelium covering the tip of villi following the method/s specified in this document, and at present pathological cut off of ≥ 30 IELs/ 100 ECs should be considered.
- The assessment of Cd and Vh should be made only in oriented crypt-villous-axes.
- The pathological changes in small bowel are not specific, and histological mimickers of CD should always be

- excluded with diligent histological evaluation and clinical-endoscopic and serological correlations.
- Histological grading of small intestinal biopsies following the modified Marsh grading system helps to identify subjects suitable for gluten-free diet (GFD) [≥ 2 modified Marsh changes], potential CD, evaluate mucosal response post-GFD, identify coexistent other small bowel pathologies and patients of refractory CD, as clinico-serological response do not match with mucosal healing post-GFD.
 - Workup of difficult case-scenarios, including in patients on GFD without a confirmed diagnosis of CD, should be done as per the recommendations stated in this document, including consideration of gluten challenge and/or demonstration of IgA anti-tTG colocalized antibody deposits for diagnosis of CD.
 - The expectations of gastroenterologists from pathologists and expectation of pathologists from gastroenterologists have been stated in this document, should be kept in mind and report should be issued in the prescribed format for optimum compliance and uniform reporting.
 - Residents, Fellows and pathology technicians should be trained and evaluated by the pathologists so that the recommendations can be aptly implemented.
 - This joint statement document was made after identifying the heterogeneous nature of practice in India after a National Survey. All relevant areas were addressed by the individual members to achieve an acceptable and optimum document for improving the outcome of this invasive and costly procedure. Irrespective of affiliations or size of establishments, all pathologists are recommended to implement this guideline to bring uniformity of practice.

Acknowledgements

We acknowledge and deeply appreciate the office bearers of the IAPM and ISG to consider and approve this important guideline work under their aegis, all respected members of the IAPM who took part in the online National survey, and Ms. Yogyata Joshi for conducting and helping in technical aspects of face to face virtual meeting.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Singh P, Arora A, Strand TA, Leffler DA, Catassi C, Green PH, et al. Global prevalence of celiac disease: Systematic review and meta-analysis. *Clin Gasteroenterol Hepatol* 2018;16:823-36.
2. Singh P, Arora S, Singh A, Strand TA, Makaria GK. Prevalence of celiac disease in Asia: A systematic review and meta-analysis. *J Gasteroenterol Hepatol* 2016;31:1095-101.
3. Ramakrishna BS, Makaria GK, Chetri K, Dutta S, Mathur P, Ahuja V, et al. Prevalence of adult celiac disease in India: Regional variations and associations. *Am J Gastroenterol* 2016;111:115-23.
4. ICMR Guideline on Diagnosis and Management of Celiac Disease in India. Available from: <https://main.icmr.nic.in/sites/default/files/guidelines/ICMR%20-%20Diagnosis%20and%20Management.pdf>. [Last accessed on 2020 Oct 05].
5. Das P, Gahlot GP, Singh A, Baloda V, Rawat R, Verma AK, et al. Quantitative histology-based classification system for assessment of the intestinal mucosal histological changes in patients with celiac disease. *Intest Res* 2019;17:387-97.
6. Corazza GR, Villanacci V, Zambelli C, Milione M, Luinetti O, Vindigni C, et al. Comparison of the interobserver reproducibility with different histologic criteria used in celiac disease. *Clin Gasteroenterol Hepatol* 2007;5:838-43.
7. Linstone HA, Turoff M. Introduction to the Delphi method: Techniques and applications. *The Delphi method: Tech Appl* 1975;3:12.
8. Hill N, Frappier-Davignon L, Morrison B. The periodic health examination. *Can Med Assoc J* 1979;121:1193-254.
9. Husby S, Koletzko S, Korponay-Szabó IR, Mearin ML, Phillips A, Shamir R, et al. ESPGHAN Working Group on Coeliac Disease Diagnosis ESPGHAN Gastroenterology Committee European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *J Pediatr Gastroenterol Nutr* 2012;54:136-60.
10. Werkstetter KJ, Korponay-Szabó IR, Popp A, Villanacci V, Salemme M, Heilig G, et al. Accuracy in diagnosis of celiac disease without biopsies in clinical practice. *Gasteroenterol* 2017;153:924-35.
11. Murch S, Jenkins H, Auth M, Bremner R, Butt A, France S, et al. Joint BSPGHAN and Coeliac UK guidelines for the diagnosis and management of coeliac disease in children. *Arch Dis Child* 2013;98:806-11.
12. Burger JP, Meijer JW, Wahab PJ. Routine duodenal biopsy to screen for coeliac disease is not effective. *Neth J Med* 2013;71:308-12.
13. Rubio-Tapia A, Hill ID, Kelly CP, Calderwood AH, Murray JA. ACG clinical guidelines: Diagnosis and management of celiac disease. *Am J Gasteroenterol* 2013;108:656.
14. Riznik P, Balogh M, Bódi P, De Leo L, Dolinsek J, Guthy I, et al. The use of biopsy and "No-Biopsy" approach for diagnosing paediatric coeliac disease in the central European region. *Gasteroenterol Res Pract* 2019. doi: 10.1155/2019/9370397.
15. Wolf J, Petroff D, Richter T, Auth MK, Uhlig HH, Laass MW, et al. Validation of antibody-based strategies for diagnosis of pediatric celiac disease without biopsy. *Gasteroenterology* 2017;153:410-9.
16. Villanacci V, Ceppa P, Tavani E, Vindigni C, Volta U. Coeliac disease: The histology report. *Dig Liver Dis* 2011;43:S385-95.
17. Kori M, Gladish V, Ziv-Sokolovskaya N, Huszar M, Beer-Gabel M, Reifen R. The significance of routine duodenal biopsies in pediatric patients undergoing upper intestinal endoscopy. *J Clin Gasteroenterol* 2003;37:39-41.
18. Yachha SK, Poddar U. Celiac disease in India. *IJG* 2007;26:230-37.
19. Green PH. The role of endoscopy in the diagnosis of celiac disease. *Gasteroenterol Hepatol* 2014;10:522-4.
20. Bürgin-Wolff A, Mauro B, Faruk H. Intestinal biopsy is not always required to diagnose celiac disease: A retrospective analysis of combined antibody tests. *BMC Gasteroenterol* 2013;13:19.
21. Freeman HJ. Role of biopsy in diagnosis and treatment of adult celiac disease. *Gasteroenterol Bed Bench* 2018;11:191-6.
22. Bonamico M, Mariani P, Thanasi E, Ferri M, Nenna R, Tiberti C, et al. Patchy villous atrophy of the duodenum in childhood celiac disease. *J Pediatr Gasteroenterol Nutr* 2004;38:204-7.
23. Gonzalez S, Gupta A, Cheng J, Tennyson C, Lewis SK, Bhagat G, et al. Prospective study of the role of duodenal bulb biopsies in the diagnosis of celiac disease. *Gastointest Endosc* 2010;72:758-65.

24. Ravelli A, Villanacci V, Monfredini C, Martinazzi S, Grassi V, Manenti S. How patchy is patchy villous atrophy? Distribution pattern of histological lesions in the duodenum of children with celiac disease. *Am J Gastroenterol* 2010;105:2103-10.
25. Vogelsang H, Hänel S, Steiner B, Oberhuber G. Diagnostic duodenal bulb biopsy in celiac disease. *Endoscopy* 2001;33:336-40.
26. Kurien M, Evans KE, Hopper AD, Hale MF, Cross SS, Sanders DS. Duodenal bulb biopsies for diagnosing adult celiac disease: Is there an optimal biopsy site?. *Gastrointest Endosc* 2012;75:1190-6.
27. Mooney PD, Kurien M, Evans KE, Rosario E, Cross SS, Vergani P, et al. Clinical and immunologic features of ultra-short celiac disease. *Gastroenterology* 2016;150:1125-34.
28. McCarty TR, O'Brien CR, Gremida A, Ling C, Rustagi T. Efficacy of duodenal bulb biopsy for diagnosis of celiac disease: A systematic review and meta-analysis. *Endosc Int Open* 2018;6:E1369-78.
29. Carneiro Chaves FJ, Tavarela Veloso F, Cruz I, Gomes C, Domingues W, Marques da Silva E, et al. Subclinical tropical enteropathy in Angola: Peroraljejunal biopsies and absorption studies in asymptomatic healthy men. *Mt Sinai J Med* 1981;48:47-52.
30. Weir DC, Glickman JN, Roiff T, Valim C, Leichtner AM. Variability of histopathological changes in childhood celiac disease. *Am J Gastroenterol* 2010;105:207-12.
31. Valitutti F, Di Nardo G, Barbato M, Aloia M, Celletti I, Trovato CM, et al. Mapping histologic patchiness of celiac disease by push enteroscopy. *Gastrointest Endosc* 2014;79:95-100.
32. Lo W, Sano K, Lebwohl B, Diamond B, Green PH. Changing presentation of adult celiac disease. *Dig Dis Sci* 2003;48:395-8.
33. Pais WP, Duerksen DR, Pettigrew NM, Bernstein CN. How many duodenal biopsy specimens are required to make a diagnosis of celiac disease?. *Gastrointest Endosc* 2008;67:1082-7.
34. Rostom A, Murray JA, Kagnoff MF. American Gastroenterological Association (AGA) Institute technical review on the diagnosis and management of celiac disease. *Gastroenterology* 2006;131:1981-2002.
35. Lebwohl B, Kapel RC, Neugut AI, Green PH, Genta RM. Adherence to biopsy guidelines increases celiac disease diagnosis. *Gastrointest Endosc* 2011;74:103-9.
36. Hopper AD, Cross SS, Sanders DS. Patchy villous atrophy in adult patients with suspected gluten-sensitive enteropathy: Is a multiple duodenal biopsy strategy appropriate?. *Endoscopy* 2008;40:219-24.
37. Choi JH, Bennett AE, McDonald LG. Effects of fixation on gastrointestinal biopsies. *J Histotechnol* 2011;34:40-3.
38. Hewitt SM, Lewis FA, Cao Y, Conrad RC, Cronin M, Danenberg KD, et al. Tissue handling and specimen preparation in surgical pathology: Issues concerning the recovery of nucleic acids from formalin-fixed, paraffin-embedded tissue. *Arch Pathol Lab Med* 2008;132:1929-35.
39. Carson FL. Fixation and Processing. Available from: https://webapps.cap.org/apps/store/PUB123_Histologic_Sample.pdf. [Last accessed on 2020 May 27].
40. Serra S, Jani PA. An approach to duodenal biopsies. *J Clin Pathol* 2006;59:1133-50.
41. Asmussen L, Bernstein I, Matzen P, Holck S. Does the mounting of gastrointestinal biopsies on millipore filter contribute to an improved section quality?. *Ugeskr Laeger* 2009;171:2646-50.
42. Ruiz GC, Reyes-Gomez E, Hall EJ, Freiche V. Comparison of 3 handling techniques for endoscopically obtained gastric and duodenal biopsy specimens: A prospective study in dogs and cats. *J Vet Intern Med* 2016;30:1014-21.
43. Thavarajah R, Mudimbaimannar VK, Elizabeth J, Rao UK, Ranganathan K. Chemical and physical basics of routine formaldehyde fixation. *J Oral Maxillofac Pathol* 2012;16:400-5.
44. Robert ME, Crowe SE, Burgart L, Yantiss RK, Lebwohl B, Greenson JK, et al. Statement on best practices in the use of pathology as a diagnostic tool for celiac disease. *Am J Surg Pathol* 2018;42:e44-58.
45. Garg N, Majumdar K, Sakhija P. Orientation precedes interpretation: Comparison of different tissue handling techniques to attain well-oriented small-intestinal endoscopic biopsy. *J Clin Pathol* 2020;73:769-71.
46. Balasubramanian P, Badhe BA, Ganesh RN, Panicker LC, Mohan P. Comparison of filter paper and gelfoam as templates for orientation of endoscopic duodenal biopsies. *J Clin Diagn Res* 2019;13: p1-3.
47. Nada R, Duseja A, Sachdev A, Mohan H. Comparative analysis of duodenal biopsy tissue orientation supported on vegetable matrix versus filter paper. *Indian J Gastroenterol* 2002;21:110-2.
48. Ferguson A, Murray D. Quantitation of intraepithelial lymphocytes in human jejunum. *Gut* 1971;12:988-94.
49. Batman PA, Miller AR, Forster SM, Harris JR, Pinching AJ, Griffin GE. Jejunalenteropathy associated with human immunodeficiency virus infection: Quantitative histology. *J Clin Pathol* 1989;42:275-81.
50. Hayat M, Cairns A, Dixon MF, O'Mahony S. Quantitation of intraepithelial lymphocytes in human duodenum: What is normal? *J Clin Pathol* 2002;55:393-4.
51. Mahadeva S, Wyatt JL, Howdle PD. Is a raised intraepithelial lymphocyte count with normal duodenal villous architecture clinically relevant? *J Clin Pathol* 2002;55:424-8.
52. Rostami K, Marsh MN, Johnson MW, Mohaghegh H, Heal C, Holmes G, et al. ROC-king onwards: Intraepithelial lymphocyte counts, distribution & role in coeliac disease mucosal interpretation. *Gut* 2017;66:2080-6.
53. Ensari A. Gluten-sensitive enteropathy (celiac disease): Controversies in diagnosis and classification. *Arch Pathol Lab Med* 2010;134:826-36.
54. Marsh MN. Grains of truth: Evolutionary changes in SI mucosa in response to environmental antigen challenge. *Gut* 1990;31:111-4.
55. Fihm BM, Sjöqvist A, Jodal M. Permeability of the rat SI epithelium along the villus-crypt axis: Effects of glucose transport. *Gastroenterology* 2000;119:1029-36.
56. Dickson BC, Streutker CJ, Chetty R. Coeliac disease: An update for pathologists. *J Clin Pathol* 2006;59:1008-16.
57. Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: Time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999;11:1185-94.
58. Marsh MN, Heal CJ. Evolutionary developments in interpreting the gluten-induced mucosal celiac lesion: An archimedian heuristic. *Nutrients* 2017;9:213.
59. Järvinen TT, Collin P, Rasmussen M, Kyrönpalo S, Mäki M, Partanen J, et al. Villous tip intraepithelial lymphocytes as markers of early-stage coeliac disease. *Scand J Gastroenterol* 2004;39:428-33.
60. Cooper R, Papworth NJ, Harris C, Horne J, Lai J, Lai J, et al. Counting intraepithelial lymphocytes: A comparison between routine staining and CD3 immunohistochemistry. *Int J Surg Pathol* 2020;28:367-70.
61. Collin P, Wahab PJ, Murray JA. Intraepithelial lymphocytes and coeliac disease. *Best Pract Res Clin Gastroenterol* 2005;11:341-50.
62. Philips AD. The SI mucosa. In: Whitehead R, editor. *Gastrointestinal and Oesophageal Pathology*. New York: Churchill Livingstone; 1989. p. 29-39.
63. Oberhuber G. Histopathology of celiac disease. *Biomed Pharmacother* 2000;54:368-72.
64. Elli L, Branchi F, Sidhu R, Guandalini S, Assiri A, Rinawi F, et al. Small bowel villous atrophy: Celiac disease and beyond. *Expert Rev Gastroenterol Hepatol* 2017;11:125-38.
65. Sharma P, Baloda V, Gahlot GP, Singh A, Mehta R, Vishnubathla S, et al. Clinical, endoscopic, and histological differentiation between celiac disease and tropical sprue: A systematic review. *J Gastroenterol Hepatol* 2019;34:74-83.
66. Pallav K, Kabbani T, Tariq S, Vanga R, Kelly CP, Leffler DA. Clinical utility of celiac disease-associated HLA testing. *Dig Dis Sci* 2014;59:2199-206.

67. Leffler D, Schuppan D, Pallav K, Najarian R, Goldsmith JD, Hansen J, et al. Kinetics of the histological, serological and symptomatic responses to gluten challenge in adults with coeliac disease. *Gut* 2012;62:996–1004.
68. Ludvigsson JF, Bai JC, Biagi F, Card TR, Ciacci C, Ciclitira PJ, et al. Diagnosis and management of adult coeliac disease: Guidelines from the British Society of Gastroenterology. *Gut* 2014;63:1210–28.
69. Weeks CL, Batra A, Tighe MP. QUESTION 2: Is HLA typing for coeliac disease helpful in children with type 1 diabetes mellitus? *Arch Dis Child* 2016;101:590–1.
70. Martina S, Fabiola F, Federica G, Chiara B, Gioacchino L, Francesco DM, et al. Genetic susceptibility and celiac disease: What role do HLA haplotypes play? *Acta bio-med* 2018;89(Suppl 9):17–21.
71. Ianiro G, Bibbò S, Montalto M, Ricci R, Gasbarrini A, Cammarota G. Systematic Review: Sprue-like Enteropathy associated with Olmesartan. *Aliment Pharmacol Ther* 2014;40:16–23.
72. Dutta AK, Balekuduru A, Chacko A. Spectrum of malabsorption in India-tropical sprue is still the leader. *J Assoc Physicians India* 2011;59:420–2.
73. Elli L, Zini E, Tomba C, Bardella MT, Bosari S, Conte D, et al. Histological evaluation of duodenal biopsies from coeliac patients: The need for different grading criteria during follow-up. *BMC Gastroenterol* 2015;15:133–8.
74. Rubin CE, Brandborg LI, Phelps PC, Taylor HC. Studies of celiac disease. I. The apparent identical and specific nature of the duodenal and proximal jejunal lesion in celiac disease and idiopathic sprue. *Gastroenterology* 1960;38:28–49.
75. Villanacci V, Lorenzi L, Donato F, Auricchio R, Dziechciarz P, Gyimesi J, et al. Histopathological evaluation of duodenal biopsy in the Prevent CD project. An observational interobserver agreement study. *APMIS* 2018;126:208–14.
76. Das P, Gahlot GP, Mehta R, Makharia A, Verma AK, Sreenivas V, et al. Patients with mild enteropathy have apoptotic injury of enterocytes similar to that in advanced enteropathy in celiac disease. *Dig Liver Dis* 2016;48:1290–5.
77. Kondala R, Puri AS, Banka AK, Sachdeva S, Sahuja P. Short-term prognosis of potential celiac disease in Indian patients. *United European Gastroenterol J* 2016;4:275–80.
78. Sharkey LM, Corbett G, Currie E, Lee J, Sweeney N, Woodward JM. Optimising delivery of care in coeliac disease – comparison of the benefits of repeat biopsy and serological follow-up. *Aliment Pharmacol Ther* 2013;38:1278–91.
79. Lee SK, Lo W, Memeo L, Rotterdam H, Green PH. Duodenal histology in patients with celiac disease after treatment with a gluten-free diet. *Gastrointest Endosc* 2003;57:187–91.
80. Lanzini A, Lanzarotto F, Villanacci V, Mora A, Bertolazzi S, Turini D, et al. Complete recovery of intestinal mucosa occurs very rarely in adult coeliac patients despite adherence to gluten-free diet. *Aliment Pharmacol Ther* 2009;29:1299–308.
81. Vécsei E, Steinwendner S, Kogler H, Innerhofer A, Hammer K, Haas OA, et al. Follow-up of pediatric celiac disease: Value of antibodies in predicting mucosal healing, a prospective cohort study. *BMC Gastroenterol* 2014;14:28.
82. Pietzak MM. Follow-up of patients with celiac disease: Achieving compliance with treatment. *Gastroenterology* 2005;128:S135–41.
83. Rubio-Tapia A, Rahim MW, See JA, Lahr BD, Wu TT, Murray JA. Mucosal recovery and mortality in adults with celiac disease after treatment with a gluten-free diet. *Am J Gastroenterol* 2010;105:1412–20.
84. Hære P, Høie O, Schulz T, Schönhardt I, Rakki M, Lundin KE. Long-term mucosal recovery and healing in celiac disease is the rule—not the exception. *Scand J Gastroenterol* 2016;51:1439–46.
85. Adriaanse MP, Mubarak A, Riedl RG, Ten Kate FJ, Damoiseaux JG, Buurman WA, et al. Progress towards non-invasive diagnosis and follow-up of celiac disease in children; a prospective multicentre study to the usefulness of plasma I-FABP. *Sci Rep* 2017;7:8671.
86. Husby S, Murray JA, Katzka DA. AGA clinical practice update on diagnosis and monitoring of celiac disease—changing utility of serology and histologic measures: Expert review. *Gastroenterol* 2019;156:885–9.
87. Singh A, Pramanik A, Acharya P, Makharia GK. Non-invasive biomarkers for celiac disease. *J Clin Med* 2019;8:885.
88. Ciaccio EJ, Bhagat G, Naiyer AJ, Hernandez L, Green PH. Quantitative assessment of the degree of villous atrophy in patients with coeliac disease. *J Clin Pathol* 2008;61:1089–93.
89. Vahedi K, Mascart F, Mary JY, Laberenne JE, Bouhnik Y, Morin MC, et al. Reliability of antitransglutaminase antibodies as predictors of gluten-free diet compliance in adult celiac disease. *Am J Gastroenterol* 2003;98:1079–87.
90. Lebwohl B, Granath F, Ekbom A, Smedby KE, Murray JA, Neugut AI, et al. Mucosal healing and risk of lymphoproliferative malignancy in celiac disease. *Ann Intern Med* 2013;159:169–75.
91. Volta U, Caio G, Giancola F, Rhoden KJ, Ruggeri E, Boschetti E, et al. Features and progression of potential celiac disease in adults. *Clin Gastroenterol Hepatol* 2016;14:686–93.
92. Biagi F, Vattiatto C, Agazzi S, Balduzzi D, Schiepatti A, Gobbi P, et al. A second duodenal biopsy is necessary in the follow-up of adult coeliac patients. *Ann Med* 2014;46:430–3.
93. Sadeghi A, Rad N, Ashtari S, Rostami-Nejad M, Moradi A, Haghbin M, et al. The value of a biopsy in celiac disease follow-up: Assessment of the small bowel after 6- and 24-months treatment with a gluten free diet. *Rev Esp Enferm Dig* 2020;112:101–8.
94. Freeman HJ. Mucosal recovery and mucosal healing in biopsy-defined adult celiac disease. *Int J Celiac Dis* 2017;5:14–8.
95. Al-Toma A, Volta U, Auricchio R, Castillejo G, Sanders DS, Cellier C, et al. European Society for the Study of Coeliac Disease (ESSCD) guideline for coeliac disease and other gluten-related disorders. *United European Gastroenterol J* 2019;7:583–613.
96. Itzlinger A, Branchi F, Elli L, Schumann M. Gluten-free diet in celiac disease—forever and for all? *Nutrients* 2018;10:1–14.
97. Penny HA, Baggis EMR, Rej A, Snowden JA, Sanders DS. Non-responsive coeliac disease: A comprehensive review from the NHS England national centre for refractory coeliac disease. *Nutrients* 2020;12:216.
98. Van Wanrooij RLJ, Bouma G, Bontkes HJ, Neefjes-Borst A, Van Grieken NC, Von Blomberg BME, et al. Outcome of referrals for non-responsive celiac disease in a tertiary center: Low incidence of refractory celiac disease in the Netherlands. *Clin Transl Gastroenterol* 2017;8:e218.
99. Rowinski SA, Christensen E. Epidemiologic and therapeutic aspects of refractory coeliac disease – A systematic review. *Dan Med J* 2016;63:A5307.
100. Pallav K, Leffler DA, Tariq S, Kabbani T, Hansen J, Peer A, et al. Noncoeliac enteropathy: The differential diagnosis of villous atrophy in contemporary clinical practice. *Aliment Pharmacol Ther* 2012;35:380–90.
101. Kaswala DH, Veeraraghavan G, Kelly CP, Leffler DA. Celiac disease: Diagnostic standards and dilemmas. *Diseases* 2015;3:86–101.
102. Ludvigsson JF, Brandt L, Montgomery SM, Granath F, Ekbom A. Validation study of villous atrophy and SI inflammation in Swedish biopsy registers. *BMC Gastroenterol* 2009;9:19.
103. Borrelli M, Maglio M, Korponay-Szabó IR, Vass V, Mearin ML, Meijer C, et al. Intestinal anti-transglutaminase 2 immunoglobulin A deposits in children at risk for coeliac disease (CD): Data from the Prevent CD study. *J Clin Exp Immunol* 2018;191:311–7.
104. Gatti S, Rossi M, Alfonsi S, Mandolesi A, Cobellis G, Catassi C. Beyond the intestinal celiac mucosa: Diagnostic role of anti-TG2 deposits, a

- systematic review. *Front Med* 2014;1:9.
105. Bao F, Green PH, Bhagat G. An update on celiac disease histopathology and the road ahead. *Arch Pathol Lab Med* 2012;136:735-45.
106. Husby S, Koletzko S, Korponay-Szabó I, Kurppa K, Mearin ML, Ribes-Koninckx C, et al. European society paediatric gastroenterology, hepatology and nutrition guidelines for diagnosing coeliac disease 2020. *J Pediatr Gastroenterol Nutr* 2020;70:141-56.
107. Arguelles-Grande C, Tennyson CA, Lewis SK, Green PH, Bhagat G. Variability in small bowel histopathology reporting between different pathology practice settings: Impact on the diagnosis of coeliac disease. *J Clin Pathol* 2012;65:242-7.
108. Trongwongsat T, Tanboon J, Nimmannit A, Pongpaibul A. The specimen handling of GI mucosal biopsy: A simple and effective quality improvement initiative. *J Med Assoc Thai* 2013;96:1374-9.