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**Redefining the intraepithelial lymphocytes threshold to
diagnose gluten sensitivity in patients with architecturally
normal duodenal histology**

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3 Redefining **the** intraepithelial lymphocytes threshold to diagnose gluten sensitivity in
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5 patients with architecturally normal duodenal histology
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8 Short title: **mild enteropathy celiac disease**
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ABSTRACT

BACKGROUND: accuracy of intraepithelial lymphocytes (IELs) counts for diagnosing mild enteropathy celiac disease (MECD) in absence of villous atrophy can be limited by inappropriate controls included in the studies. **AIM:** to determine the diagnostic accuracy of IELs counts utilizing controls lacking HLA celiac disease-associated alleles

METHODS: IELs counting at villus tip and per 100 enterocytes were performed at Hematoxylin eosin (H&E) and CD3-stainings in: 29 cases (21 with potential celiac disease and 8 affected by latent celiac disease) representing the patient population and 14 non-celiac controls lacking HLA-DQ2/DQ8 alleles. **RESULTS:** Threshold (mean+2SD) of duodenal IELs at villus tip and per 100 enterocytes in non-celiac controls was respectively: 3.5 and 18 at H&E, 3.2 and 17 following CD3-staining. Considering the whole patient population, the sensitivity of tip IELs in detecting MECD was 89.6%(95%CI=72.6-97.8) both at H&E and CD3-stainings. The sensitivity of IELs per 100 enterocytes was 93.1%(95%CI=77.2-99.2) both at H&E and CD3-staining. Specificity of both IELs counts was 100%(95%CI=76.8-100). Using a threshold of 25 IELs per 100 enterocytes could miss 59% of cases at H&E and 48% following CD3-staining. **CONCLUSIONS:** IELs counts are diagnostic feasible tools to detect MEDC. Threshold of duodenal IELs may be lower than currently accepted.

INTRODUCTION

Celiac disease is a chronic inflammatory disease of the small intestine that is triggered by ingestion of gluten in genetically predisposed individuals¹. Virtually all patients with celiac disease have heterodimer HLA-DQ2 or HLA-DQ8 and the absence of these alleles provides a negative predictive value for the disease of close to 100%². Thus, celiac disease is virtually excluded if the individuals lack these disease-associated alleles. Although serologic testing for anti-tissue transglutaminase antibodies (anti-tTG) and anti-endomysium antibodies (AEA) is highly sensitive and specific for the diagnosis of celiac disease, duodenal biopsy demonstrating villous atrophy (Marsh III) is considered the diagnostic gold standard¹. However, according to the Marsh-Oberhuber classification, celiac disease progresses through various stages and villous atrophy (Marsh III C) represents only the end stage of mucosal lesions, while the early changes are characterized by intraepithelial lymphocytosis (Marsh I) and subsequently hypertrophy of the crypts (Marsh II)^{3,4}.

It is now recognized that even patients with mild enteropathy celiac disease in absence of villous atrophy, referred to as latent or potential celiac disease, are of sufficient clinical importance to be diagnosed and treated with a gluten-free diet as they may develop any of the manifestations encountered in celiac disease sufferers with villous atrophy. In fact, these patients may suffer from gastrointestinal symptoms and osteopenia or osteoporosis responding to gluten avoidance^{5,6}. Furthermore, they are at increased risk of autoimmune disease^{7,8} and mortality⁹.

A recent randomized, controlled clinical study showed that patients with positive AEA and mild enteropathy (Marsh I–II) only benefit from a gluten-free diet, making long delay in diagnosis unacceptable, highlighting the importance of early recognition of mild enteropathy celiac disease¹⁰.

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3 Increased intraepithelial lymphocytes (IELs) along superficial epithelium of villi in duodenal
4 mucosa has been considered a sensitive marker of the effect of gluten, but not specific,
5 since this lesion can be found in several other diseases^{11,12}. However, until now, what the
6 normal number of duodenal IELs should be has not been completely defined^{13,14}.

7
8 Accordingly, several studies have focused on other early mucosal findings suggesting
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10 **celiac disease**. Counting IELs present at the tips of the villi might suggest **mild enteropathy**
11
12 **celiac disease** in patients with an architecturally normal duodenal mucosal¹⁵⁻¹⁹.

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14 Furthermore, IELs distribution pattern along villi may be more sensitive than formal IELs
15
16 **counts** and it might also help in screening for **mild enteropathy celiac disease**¹⁵.

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18 Nevertheless, since both **counts** and pattern distributions of IELs have a high rate of false
19
20 positive cases, it has been suggested that they cannot be considered as a diagnostic
21
22 marker of **mild enteropathy celiac disease**^{11,13}.

23
24 However, only one¹⁷ of five studies¹⁵⁻¹⁹ established normal values of these parameters in
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26 patients in whom **celiac disease** was excluded with certainty because of the absence of
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28 genetic predisposition, namely HLA-DQ2 and DQ8 alleles.

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30 Latent **celiac disease** represents a condition in which patients have positive celiac serology
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32 and genetic predisposition but normal duodenal mucosa architecture while on gluten-
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34 containing diet; subsequently they develop duodenal villous atrophy compatible with **celiac**
35
36 **disease**. Therefore, latent **celiac disease** provides the opportunity to verify whether
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38 morphological features found in the absence of villous atrophy might predict the onset of
39
40 overt **celiac disease** subsequently.

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42 The aim of this study was to evaluate the accuracy of different IELs counts and
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44 distribution pattern to diagnose mild enteropathy celiac disease in patients with
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46 architecturally normal duodenal mucosa. A potential celiac disease group and a latent
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48 celiac disease group represented the targeted patient populations. A non-celiac group
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50 lacking HLA celiac disease-associated alleles represented the control population.
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MATERIALS and METHODS

Study Population

This was a retrospective study designed to fulfil the STARD (Standards for Reporting of Diagnostic Accuracy) recommendations²⁰. It was carried out at the Center for Celiac Disease of the University of Messina, which follows more than 2,500 patients and makes about 100 new diagnoses of **celiac disease** each year.

We reviewed our computerized files of patients attending the Center during the years 2002–2009 and selected all cases corresponding to the following criteria: (1) histology showing architecturally normal duodenal mucosa. Each duodenal biopsy had to be well oriented with villous height/crypt depth ratio of at least 3:1; (2) availability of celiac serological data; (3) availability of genomic HLA-DQ2 and HLA-DQ8 typing.

Based on these inclusion criteria and considering the predictive value of celiac serology and HLA to diagnose celiac disease, we selected 3 study groups. Table 1 summarizes demographic and clinical data in different groups.

The potential celiac disease group comprised 21 patients at risk of celiac disease with positive celiac serology and HLA-DQ2 or DQ8 alleles. To exclude false positive results of celiac serology, only patients with both anti-tTG and AEA positive, in at least two measurements at different times were included. Indication for upper endoscopy in these patients was the suspect of celiac disease on the basis of positive celiac serology. The mean follow-up of these patients was 2.4 years (range 1-8 years). Nine of 21 potential celiac disease patients included in the study underwent a gluten free diet and clinical and serological gluten dependence was demonstrated in all cases

The latent celiac disease group comprised 8 patients. One patient belonged initially to the potential celiac disease group, but during the study developed villous atrophy, and was then included in the celiac disease latent group. These patients were initially at the stage

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3 of potential celiac disease having both AEA and anti-tTG values positive and histology
4 showing a normal duodenal mucosa architecture. Subsequently they developed duodenal
5 villous atrophy compatible with celiac disease. This group of patients was used because
6 the values of IELs found at the time of normal duodenal mucosa undoubtedly indicate a
7 mild enteropathy celiac disease and then they can be used as a reference standard of mild
8 enteropathy celiac disease for comparing potential celiac disease and non-celiac group
9 values. Considering all latent celiac disease patients, the interval of latency between the
10 first intestinal biopsy showing normal mucosa architecture and the biopsy that revealed
11 intestinal villous atrophy was 13.9 months on average, range 5-27 months. All latent celiac
12 disease patients adopted a gluten-free diet and clinical and serologic recovery was evident
13 in all except one who had recently started on a gluten-free diet. Table 2 shows indicators
14 of mild enteropathy celiac disease in potential and latent celiac disease group.

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Finally, non-celiac disease control group comprised subjects with excluded celiac disease by negative celiac serology and lacking HLA DQ2 or DQ8 alleles. This group included 14 patients who had undergone upper endoscopy because of gastrointestinal symptoms (12 patients) or failure to thrive (2 patients).

Pathology Assessment

All duodenal biopsies taken from the first and second portion of the duodenum were correctly oriented on acetate cellulose filters (Bio Optica Milan Italy), fixed in 10% neutral buffered formalin for 8-12 hrs at room temperature and then included in paraffin at 56°C. Two serial 4 micron-thick sections were cut from each tissue block: one slide was stained with H&E and another was utilized for the IHC study. For this latter staining, sections were mounted on silane-coated slides and then subjected to antigen retrieval treatment by heating slides placed in 0.01 M citrate buffer pH 6.0 in a microwave oven for 3 cycles x 5 min each, before adding the primary antibody. The immunohistochemical procedure was

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3 performed in a moist chamber with the following steps: 1) 0.1% H₂O₂ in methanol to block
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5 intrinsic peroxidase activity; 2) normal sheep serum to prevent unspecific adherence of
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7 serum proteins; 3) with monoclonal primary antibody (mouse anti-human CD3,
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9 DakoCytomation, Denmark; working dilution 1:200, overnight at 4 °C); 4) anti-mouse
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11 immunoglobulin antiserum (Behring Institute; w.d. 1:25, 30 min at room temperature); 5)
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13 mouse anti-horseradish peroxidase-antiperoxidase complexes (DakoCytomation; w.d.
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15 1:25, 30 min at room temperature). For the demonstration of peroxidase activity, sections
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17 were incubated in darkness for 10 min with 3-3' diaminobenzidine tetrahydrochloride
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19 (Sigma Chemical Co., Stl Louis, MO, USA), 100 mg in 200 ml 0.03% hydrogen peroxide in
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21 phosphate-buffered saline. Nuclear counterstaining was performed by Mayer's haemalum.
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23 To test the specificity of CD3 immunostaining in order to exclude the possibility of a non-
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25 specific reaction, additional 4 micron thick serial sections of each specimen were tested by
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27 replacing the anti-mouse immunoglobulin antiserum by either phosphate buffered saline or
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29 normal rabbit serum: the results obtained were constantly negative. Moreover, the CD3
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31 immunoreactivity demonstrated in lymphocytes outside of intestinal epithelial cells, but
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33 present in the lamina propria of duodenal biopsies, was considered as positive control.
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35 Finally, in order to test the inter-run variability of CD3 staining, the same CD3-positive
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37 lymphnode tissue section was utilized in every run.
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41 All routinely and immunohistochemically stained slides were blindly examined by two
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43 experienced pathologists; neither the final diagnosis nor the relationship of the biopsy to
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45 the patient's diet was known at the time of assessment. Histomorphological analysis was
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47 done on light microscopy using a x20 and x40 objective lenses and x10 eyepiece. Only
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49 peri- or supranuclear lymphocytes above the basal membrane of villi were assumed as
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51 IEL. IELs counts were initially performed on H&E stained sections and after, on the
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53 corresponding CD3 immunostained slides. The relative IELs counts were separately
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55 recorded by determining the quantification of the following parameters: a) existence of a
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decrecendo-like pattern of sparse IEL infiltrate at H&E from the basal portion of villi to the middle and upper regions of villi, according to Goldstein²¹; *b*) villus tip IEL score calculated by counting the number of IELs per 20 enterocytes from 5 randomly chosen villus tips and then computing the mean value of the 5 villus tip IEL counts; *c*) the number of IELs was given as the mean/100 epithelial cells, after counting of at least 300 epithelial cells along both sides of 5 villous bodies.

Statistical Analysis

Statistical analysis was performed using Statistica release 7 (StatSoft) and GraphPad Prism 5. D'Agostino-Pearson normality test was used to assess Gaussian distribution data. Quantitative data were expressed as means, SD, ranges and 95% confidence intervals (CI). For categorical variables, Fisher's exact test was used. Comparison between multiple groups was carried out with one-way analysis of variance, followed by Bonferroni's Multiple comparison test; *P* value <0.001 was considered significant. The threshold of the IEL counts was defined as the mean + 2 standard deviations (SD) of non-celiac controls^{22,23}. For correlations between different IELs counts, the Pearson two-tailed t test was used.

RESULTS

The results of the IELs counts of the different stainings and study groups are summarized in Table 3.

IELs/20 enterocytes counts: the mean densities of tip IELs, both at H&E and CD3-stainings (Figure 1), in the potential celiac disease group were higher than the control group ($p < 0.0000001$), but not different from the latent group ($p = 0.12$ and $p = 1$ respectively). Comparing tip IELs counts of potential and latent celiac disease groups

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3 with the control group, there was no, or only slight, overlap following CD3-stainings, while
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5 there was slightly more overlap following H&E (Figure 2).
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8 The upper limit of the normal range of tip IELs (mean + 2 SD) in the control group
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10 following H&E was 3.5 IELs, and for CD3-stainings 3.2 IELs per 20 enterocytes,
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12 respectively. The upper limit of 95%CI was 2.4 IELs/20 enterocytes at H&E and 2.8
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14 IELs/20 enterocytes at IHC, respectively.
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17 **IELs/100 enterocytes counts**: with regard to IELs counts per 100 enterocytes, the mean
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19 densities of tip IELs, both at H&E and CD3-stainings, in the potential celiac disease group
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21 were higher than the control group ($p < 0.0000001$), but not different from the latent group
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23 ($p = 1$ and $p = 0.53$, respectively). Comparing IELs counts of the potential and the latent
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25 groups with the control group, there was no overlap following CD3-stainings, while there
26
27 was some overlap following H&E (Figure 3).
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31 The cut-off value (mean +2 SD) for IELs in the control group was set at 18 IELs/100
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33 enterocytes at H&E and 17 IELs/100 enterocytes at IHC. The upper limit of 95%CI was
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35 13.6 IELs/100 enterocytes at H&E and 13.3 IELs/20 enterocytes at IHC, respectively.
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38 **Diagnostic accuracy of IELs counts**: taking into account that there was no difference in
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40 IELs counts between the potential and the latent celiac disease group, the accuracy of
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42 IELs to diagnose **mild enteropathy celiac disease** was calculated by merging both groups
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44 in a single patient population.
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48 Considering the upper limit of the normal range of IELs (mean +2 SD) in control group,
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50 sensitivity and specificity of tip, and per 100 enterocytes counts following H&E and CD3-
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52 stainings, are summarized in Table 4.
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55 **Correlation between IELs counts and between stainings**: there was a **high** correlation
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57 between IEL counts per 20 and 100 enterocytes, both at H&E and CD3-staining ($r = 0.914$
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59 and $r = 0.929$; $p < 0.0000001$ respectively). Similarly, there was a **high** correlation between
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3 H&E and IHC staining considering IELs counts per 20 and 100 enterocytes ($r=0.761$ and
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5
6 $r=0.848$; $p<0.0000001$, respectively).

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8 **Considering the whole patient population**, sensitivity and specificity of IELs pattern along
9
10 villi at H&E are shown in Table 4. There was a difference comparing **the whole patient**
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12 **population** to control group regarding IELs pattern ($p=0.00001$).
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20 DISCUSSION

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22 Detecting early changes of celiac disease in patients with normal duodenal architecture
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24 has clinical relevance, but sometimes it is a challenge.
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27 An increased number of IELs is the earliest pathological sign of **mild enteropathy celiac**
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29 **disease**²⁴ and several morphologic criteria that raise the possibility of gluten sensitivity in
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31 the context of an architecturally normal duodenal mucosa have been proposed, as recently
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33 reviewed by Dickson BC et al¹³. Both sensitivity and specificity of a diagnostic test lie in
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35 the possibility of applying it to a control population explicitly not affected by the disease,
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37 and a study population known to be affected by the disease. Therefore, it is crucial, to
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39 avoid misleading results, to determine the upper normal value of duodenal IELs in control
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41 populations in which **celiac disease** has been excluded with certainty. In this setting, the
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43 ideal non-celiac disease control population should not only have negative serology, but,
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45 above all, the absence of any HLA allele associated with celiac disease, while the negative
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47 predictive value of HLA is virtually 100%². In our study, reference values for IELs were
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49 determined in a control population with low pre-test probability of **celiac disease**, negative
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51 celiac serology and a lack of HLA alleles associated with the disease, while, to our
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53 knowledge, only one study¹⁷ had a group lacking DQ2 or DQ8 haplotype as part of a non-
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55 celiac disease control group. Our control patients have an average age higher than the
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57 potential **celiac disease** group but it is noteworthy that intestinal density of IELs in healthy
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3 subjects increases with age²⁵. Therefore, we would have had an even lower IELs counts if
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5 we had enrolled younger controls
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8 As mentioned above, in evaluating a diagnostic test, it is important to have a group known
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10 to be affected by the disease, and there were several indicators that potential celiac
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12 disease patients in the study effectively had early-stage celiac disease. First, all patients
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14 have positive AEA and anti-tTG values, in at least two measurements at different times
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16 making false positive results highly improbable. Second, we included patients with latent
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18 celiac disease who initially presented positive celiac serology with a normal mucosa
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20 (potential celiac disease), but who later developed villous atrophy, to assess how the
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22 histological features of patients with potential celiac disease were comparable to those
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24 with latent celiac disease when they had a normal mucosa. In this regard, IELs count
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26 values of potential celiac disease group were comparable to those in latent patients, but
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28 different from the non-celiac disease group, meaning that changes in duodenal mucosa
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30 seen in our study group might predict future overt celiac disease or, in any case, indicate
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32 an abnormal response to ingested gluten. Third, HLA haplotypes associated with celiac
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34 disease were seen in all patients belonging to the potential celiac disease group, although
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36 3 out of 21 (14%) subjects had incomplete HLA-DQ2 heterodimer. However, 3 out of 8
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38 patients with latent celiac disease had only one celiac disease-associated HLA allele,
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40 namely DQ2-DR7, and therefore we speculate that the presence of incomplete HLA
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42 heterodimer may be one factor that delays the development of intestinal villous atrophy.
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44 Fourth, clinical and serological gluten dependence was present in all 9 patients of the
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46 potential celiac disease group who underwent a gluten-free diet.
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50 With regard to villus tip IELs counts, the mean and range calculated, both at H&E and
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52 CD3 stainings, in the non-celiac control group, were comparable to the study of Jarvinen
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54 and coworkers¹⁷, the only study that used a control group lacking HLA alleles associated
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56 with celiac disease. Accordingly, this method showed better diagnostic accuracy,
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3 especially regarding specificity, than previous studies^{15,18,19} including the study of Jarvinen
4 and coworkers¹⁷ who calculated diagnostic accuracy of tip IELs with a heterogeneous
5 control population in which the HLA-negative group only represented a part.
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10 With regard to villous IELs per 100 enterocytes counts, currently, the accepted normal
11 limits are 30 or 25 IELs depending on classification schemes^{13,23}. Based on the results
12 from our control group in which celiac disease was virtually excluded, the normal limits of
13 IELs, as 18 at H&E and 17 following CD3-stainings, resulted lower than currently
14 accepted with the mean values of duodenal IELs in accordance with previous studies
15 14,27,28. Mahadeva et al.²⁸ considered the upper limit of normal IELs at H&E as 20/100
16 enterocytes, but they reported higher SD. Veress et al.¹⁴ suggested 20 per 100
17 enterocytes at H&E and 25 per 100 enterocytes following CD3-stainings; however they
18 used the mean + 3 SD as IELs cut-off and reported higher SD following CD3-stainings.
19 Our control patients, unlike those in the above studies, are surely non-celiac due to lack of
20 HLA celiac disease-associated alleles, and this could explain the narrower range of IELs
21 values and the lower SD found in our study.
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38 According to our results, IELs per 100 enterocytes counts resulted in both high sensitivity
39 and specificity suggesting that and 20 IELs per 100 enterocytes can be reasonably
40 proposed as new threshold. Of interest, if the lowest current accepted cut-off of IELs had
41 been used, namely 25 per 100 enterocytes, we would have considered normal 17/29
42 (59%) at H&E and 14/29 (48%) following CD3-stainings of duodenal biopsies in the 29
43 subjects with mild enteropathy celiac disease. Importantly, latent celiac disease patient No.
44 6 (Table 2), with IELs countings below 25 per 100 enterocytes both at H&E and CD3-
45 stainings, developed insulin-dependent diabetes mellitus one and a half years after the
46 intestinal biopsy that revealed a normal mucosa in spite of positive celiac serology,
47 suggesting that the gluten-containing diet due to delayed diagnosis of mild enteropathy
48 celiac disease may have contributed to the development of diabetes in this patient and
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3 again highlighting the importance of recognizing **mild enteropathy celiac disease** early in
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5 patients with normal mucosa architecture.
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8 In this study, comparing tip IELs against per 100 enterocytes counts , both at H&E and
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10 CD3-staining, classical counting on complete villous was slightly superior in terms of
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12 sensitivity in detecting **mild enteropathy celiac disease** regardless of staining. Several
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14 studies suggested higher diagnostic sensitivity of tip IELs than IELs **counts** on complete
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16 **villus** to disclose early-stage **celiac disease**^{14,16-18}. However, 2 of these studies only^{16,18}
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18 reported diagnostic accuracy data of both IELs analyses, where tip IELs counts , namely
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20 IELs per 20 enterocytes, were compared with complete **villus counts** expressed as the
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22 total IELs per mm of **villus** epithelium, while we performed IELs **counts** per 100
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24 enterocytes.
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29 With regard to histological stainings, it was suggested that the value of the IELs counts is
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31 higher following immunohistochemistry with CD3 antibody than in H&E-stained specimens,
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33 although CD3-staining is not currently routine application¹³. In the present study overall
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35 diagnostic accuracy of IELs **counts** following CD3-staining and H&E were comparable, as
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37 demonstrated by the good correlation between H&E and CD3-staining, considering both
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39 villus tip and per 100 enterocytes **counts**, making IELs counting feasible following H&E
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41 where CD3-staining is not available in clinical practice.
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46 Finally, considering the distribution of IELs along the villi, the loss of normal pattern of
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48 IELs, following H&E-staining, showed **high sensitivity but unacceptable specificity in our**
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50 **study**. However the interpretation of this Gestalt approach is highly observer-dependent
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52 and this may explain, regarding the different stainings used in these studies, the reported
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54 diagnostic variability of this method **in previous studies**^{15,17}. In this regard, Mino et al.¹⁶,
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56 using a less observer-dependent method as such the IELs tip-to-base ratio, reported,
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58 following CD3-stainings, a sensitivity of 87.5%, and specificity 100% when **mild**
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3 enteropathy celiac disease patients with preserved villus architecture were compared to
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5 control subjects.
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8 In conclusion, the present study on diagnostic value of IELs findings was carried out using
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10 a control group certainly not affected by celiac disease and a population with potential
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12 celiac disease selected exclusively on the basis of multiple positivity of both AEA and anti-
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14 tTG.
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17 Our data indicate that IELs counts both at tip and along villi are diagnostic feasible tools,
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19 in terms of both sensitivity and specificity, for disclosing mild enteropathy celiac disease.
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22 The main result of our study is that the threshold of normal duodenal IELs may be lower
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24 than currently accepted and therefore, there is a need to update diagnostic criteria for
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26 celiac disease.
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29 The application of a lower duodenal IELs cut-off, as suggested by the present study, may
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31 allow other centers to re-evaluate pending diagnosis of mild enteropathy celiac disease,
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33 thus avoiding complications of disease due to a delayed diagnosis.
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Table 1: Demographic and clinical data in study groups

	Potential CD	Latent CD	Non-CD Controls
No. of patients	21	8	14
Female; <i>n</i> (%)	15/21 (71)	7/8 (87)	9/14 (64)
Age; mean (range), years	15.7 (3-48)	8.8 (2-29)	28.2 (3-68)
Symptoms before endoscopy; <i>n</i> (%)			
Anemia	2 (9)		
Failure to thrive	4(20)	3(37)	2 (15)
Abdominal pain	6(28)	4(50)	5 (35)
Constipation/diarrhea	5(23)	1(13)	2 (15)
Dyspepsia			5 (35)
Family history of CD; <i>n</i> (%)	4(20)		

CD=celiac disease

Table 2. Indicators of **mild enteropathy celiac disease** in potential and latent **celiac disease** groups with normal duodenal villous architecture

Patients	Sex/ Age ^a (years)	HLA ^b	Serology on GCD ^c		IELs ^d	GFD	Clinical/ Serological ^e gluten dependence	Associated disorders
			AEA	anti- tTG ^f				
Potential CD								
1	F/3	DQ2	+	4xN	+	+	+/+	IDDM
2	F/8	DQ2	+	14xN	+	+	+/+	-
3	M/9	DQ2/DQ8	+	4xN	+	+	+/+	-
4	F/12	DQ2	+	2.7xN	+	+	+/+	-
5	F/5	DQ2	+	2.2xN	-	+	+/+	Chronic Urticaria
6	M/15	DQ2	+	14xN	-	+	+/+	-
7	F/26	DQ2	+	14xN	-	+	+/+	-
8	F/6	DQ2	+	2.8xN	+	+	+/+	-
9	F/17	DQ2	+	5xN	+	+	+/+	Thyroiditis
10	F/40	DQ7-DR5	+	5xN	+	-	NA	Thyroiditis
11	F/3	DQ2	+	2xN	-	-	NA	-
12	F/10	DQ2	+	2.9xN	+	-	NA	-
13	F/3	DQ2	+	5.3xN	-	-	NA	-
14	M/13	DQ2	+	3.2xN	-	-	NA	-
15	M/38	DQ2-DR7	+	7.4xN	+	-	NA	-
16	F/9	DQ2	+	2.7xN	+	-	NA	-
17	F/17	DQ8	+	4.2xN	+	-	NA	Thyroiditis
18	M/4	DQ2	+	2xN	+	-	NA	-
19	F/20	DQ2-DR7	+	2.5xN	-	-	NA	-
20	F/25	DQ2	+	2xN	+	-	NA	-
21	M/48	DQ2	+	3xN	+	-	NA	-
Latent CD								
1	F/29	DQ2	+	1.3xN	+	+	+	Thyroiditis
2	F/3	DQ2-DR7	+	4.5xN	-	+	+	-
3	F/8	DQ2	+	1.9xN	+	+	+	Turner syndrome
4	F/2	DQ2	+	1.5xN	-	+	+	-
5	F/11	DQ2-DR7	+	1.5xN	+	+	+	-
6	F/3	DQ2	+	1.6xN	-	+	+	IDDM
7	F/13	DQ8	+	5.2xN	+	+	+	-
8	M/3	DQ2-DR7	+	5.7xN	-	+	NA ^g	-

HLA= human leukocyte antigen; GCG= gluten-containing diet ; GFD= gluten- free diet;

IELs=intraepithelial lymphocytes ; CD= celiac disease; IDDM= insulin-dependent diabetes mellitus

+ = positive/increased density; - = negative/normal density; N= normal value; NA= not available

^a Age at first observation

^b Serological equivalent HLA was reported

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3 ° Serology at the time of intestinal biopsy
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5 ° Considering both H&E and CD3-stainings and the current lowest limit accepted of
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8 25/100 enterocytes as normal limit of IELs
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10 ° Negative both AEA and ant-tTG
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12 ° Values expressed as multiple of normal limit (N)
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15 ° This patient started GFD recently because he belonged initially to potential CD group
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17 but during the study developed villous atrophy and was then included in CD latent
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20 group
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Table 3. Mean values, SD, 95% confidence intervals, number of abnormal values of IELs counts in small bowel mucosal histological findings in study groups

	Study groups		
	Potential CD	Latent CD	Non-CD Controls
IELs/20 enterocytes (H&E)			
- mean values (SD)	5.0 ^{b,c} (1.2)	5.9 (1.0)	2.5 (0.5)
- range	2.2-6.6	4.5-7.8	1.5-3.2
- 95%CI	4.5-6.6	5.0-6.8	2.2-3.8
IELs/20 enterocytes (CD3-staining)			
- mean values (SD)	5.7 ^{b,c} (1.6)	5.6 (2.2)	2.1(0.5)
- range	3.0-11	2.8-9.0	1.4-2.8
- 95%CI	5.0-6.4	3.8-7.5	1.9-2.4
IELs/100 enterocytes (H&E)			
- mean values (SD)	23.5 ^{b,c} (4.4)	24.8 (2.9)	12.2 (2.3)
- range	10.4-29.2	20.8-28.4	8.2-18.0
- 95%CI	21.5-25.5	22.3-27.2	10.9-13.6
- Abnormal values ^a ; <i>n</i> (%)	8/21(38)	4/8 (50)	0/14 (0)
IELs/100 enterocytes (CD3-staining)			
- mean values (SD)	25.4 ^{b,c} (4.1)	23.1(5.3)	11.9 (2.5)
- range	10.4-29.2	16.0-30.0	8.4-14.8
- 95%CI	21.5-25.5	18.8-27.6	10.4-13.3
- Abnormal values ^a ; <i>n</i> (%)	12/21(57)	3/8(38)	0/14 (0)

CD= celiac disease; IELs=intraepithelial lymphocytes; H&E= hematoxylin and eosin;

SD= standard deviation; CI= confidence Interval

^a Reference value considered was the current lowest limit accepted of 25/100 enterocytes

^b Statistically significant difference compared to controls ($p < 0.0000001$)

^c Statistically not significant difference compared to latent CD ($p > 0.05$)

^d Statistically significant difference compared to controls ($p = 0.00001$)

Table 4. Sensitivities and specificities with 95% confidence interval of IELs countings and distribution pattern in detecting mild enteropathy celiac disease in patient population ^a

	Sensitivity (%) (95%CI)		Specificity ^b (%) (95%CI)	
	H&E	CD3-staining	H&E	CD3-staining
IELs/20 enterocytes	89.6 (72.6-97.8)	89.6 (72.6-97.8)	100 (76.8-100)	100 (76.8-100)
IELs/100 enterocytes	93.1 (77.2-99.2)	93.1 (77.2-99.2)	100 (76.8-100)	100 (76.8-100)
IELs pattern ^c	96.5 (82.2-99.9)		78.5 (49.2-95.3)	

CI= confidence interval; H&E= hematoxylin and eosin; IELs= intraepithelial lymphocytes;

^a Potential CD group plus latent CD group

^b Non-CD patients without CD-associated HLA alleles served as controls to make comparisons and cut-off of IELs was defined as the mean + 2 standard deviations

^c Pattern of IELs along villi was evaluated only at H&E according to Goldstein (ref. 21).

Figure legends

Figure 1. **A,B,E,F**: normal villi (3/1 villous-crypt ratio) with normal number of intraepithelial lymphocytes. **A**, Hematoxylin and Eosin (H&E) x 20. **B**, CD3-staining x20. **E**, H&E x40; **F**, CD3-staining x40. **C,D,G,H**: normal villi (3/1 villous-crypt ratio) with pathological increase of intraepithelial lymphocytes. **C**, Hematoxylin and Eosin (H&E) x 20. **D**, CD3-staining x20, **G**, H&E x40; **H**, CD3-staining x40.

Figure 2. **Villus tip** intraepithelial lymphocytes (IELs) countings per 20 enterocytes at Hematoxylin and Eosin (H&E) and following CD3-staining in the different study groups. The solid lines represent the mean IELs value observed for each study group. The dotted lines are set at 3.2 and 2.8, which are the highest observed values in non-celiac disease controls at H&E and following CD3-staining, respectively.

Figure 3. **Villus tip** intraepithelial lymphocytes (IELs) countings per 100 enterocytes at Hematoxylin and Eosin (H&E) and following CD3-staining in the different study groups. The solid lines represent the mean IELs value observed for each study group. The dotted lines are set at 18.0 and 14.8, which are the highest observed values in non-celiac disease controls at H&E and following CD3-staining, respectively.

Specific author contributions:

All authors listed below approved the final draft submitted.

Salvatore Pellegrino: planning and conducting the study, collecting and interpreting data, statistical analysis, drafting the manuscript; Vincenzo Villanacci: planning the study, interpreting data, drafting the manuscript; Naire Sansotta: collecting and drafting the manuscript; Rosa Scarfì: collecting data; Gabrio Bassotti: interpreting data and critical revision of the manuscript for important intellectual content; Giuseppe Vieni: collecting and interpreting data; Alessandro Princiotta: collecting data; Concetta Sferlazzas: planning the study and interpreting data ; Giuseppe Magazzù: planning the study, interpreting data, drafting the manuscript, critical revision of the manuscript for important intellectual content; Giovanni Tuccari: conducting the study, interpreting data, drafting the manuscript, critical revision of the manuscript for important intellectual content.

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Potential competing interests: none

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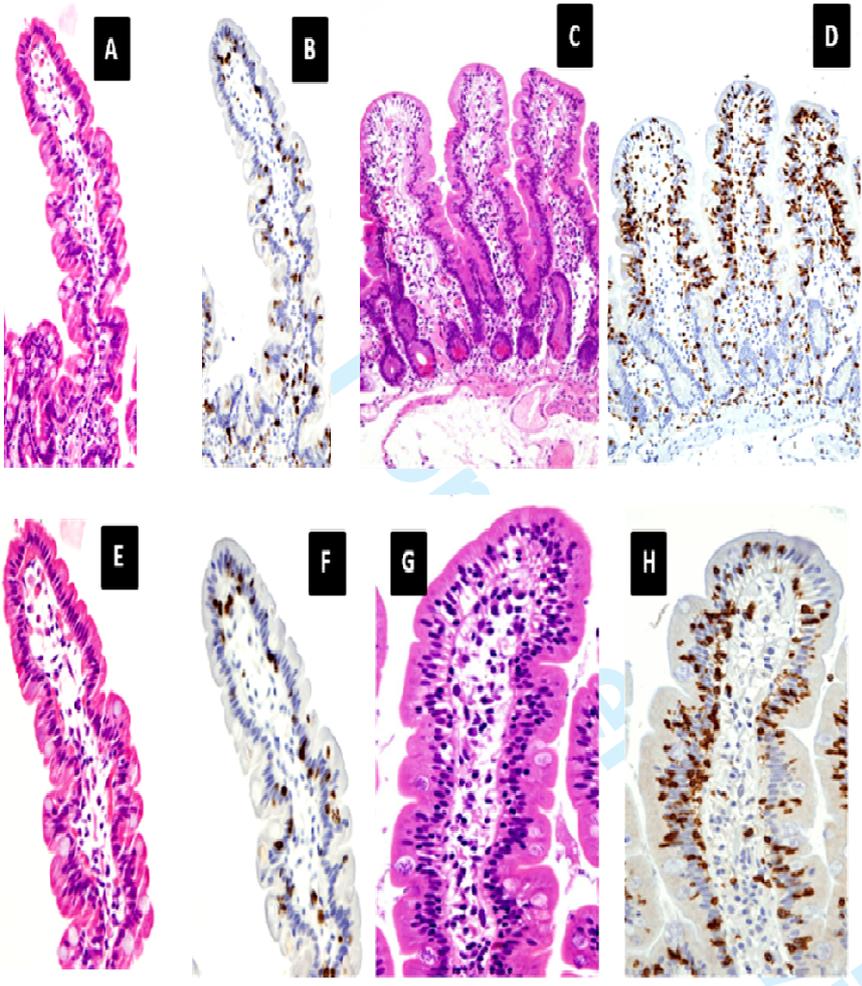


Figure 1.

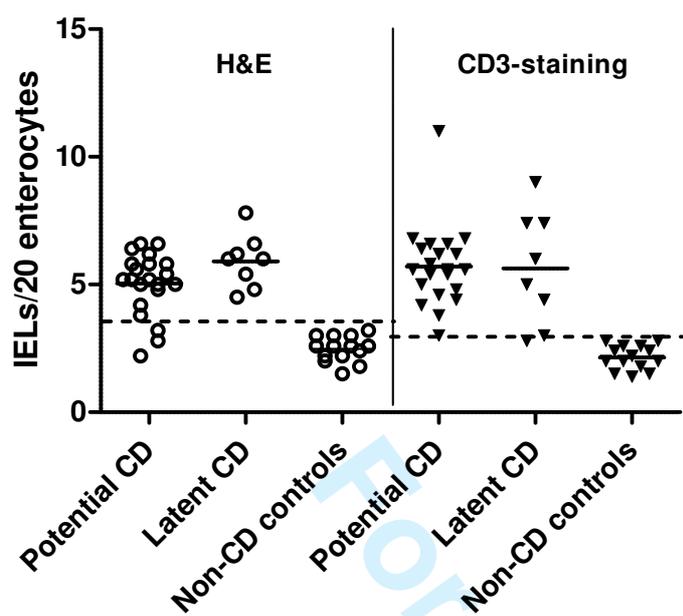


Figure 2.

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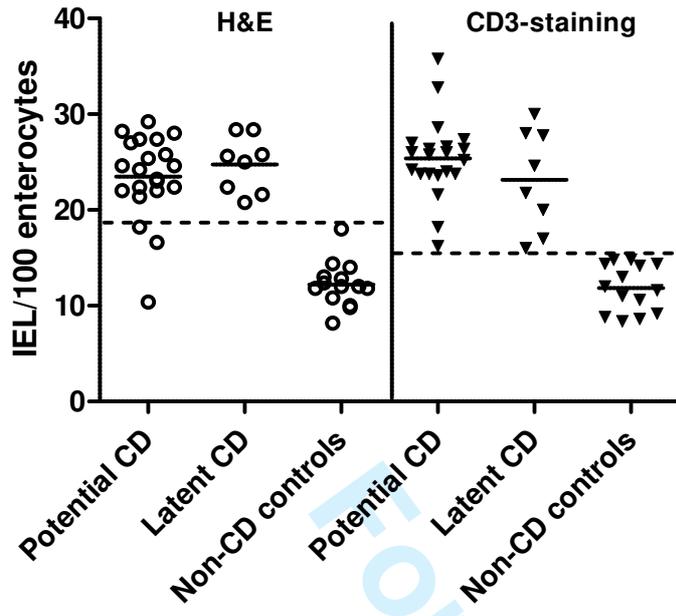


Figure 3.

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Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	1,2
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	4
METHODS			
<i>Participants</i>	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	5
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	5,6
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	5
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	5
<i>Test methods</i>	7	The reference standard and its rationale.	5, 6
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	6-8
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	7,8
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	7
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	7
<i>Statistical methods</i>	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	8
	13	Methods for calculating test reproducibility, if done.	Not done
RESULTS			
<i>Participants</i>	14	When study was performed, including beginning and end dates of recruitment.	5
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	5,6,19,20
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	Not applicable
<i>Test results</i>	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	5,6
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	Not applicable
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	Not done
	20	Any adverse events from performing the index tests or the reference standard.	Not applicable
<i>Estimates</i>	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	8-10,22,23
	22	How indeterminate results, missing data and outliers of the index tests were handled.	Not applicable
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	Not done
	24	Estimates of test reproducibility, if done.	Not done
DISCUSSION	25	Discuss the clinical applicability of the study findings.	10-14