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Original contribution

Is revision of cutoff values needed when using CD3 immunohistochemical staining in histopathologic diagnosis of lymphocytic colitis?☆

Anne-Marie Kanstrup Fiehn MD[a,*], Louise Nygaard Clausen MD, PhD[a], Ulla Engel MD[b], Signe Wildt MD, PhD[c], Lars Kristian Munck MD, MSc[c,d], Martin Kristensson MSc[e], Peter Johan Heiberg Engel MD[d,f]

[a]*Department of Pathology, University Hospital of Copenhagen, Rigshospitalet, 2100 Copenhagen, Denmark*
[b]*Department of Pathology, University Hospital of Copenhagen, Hvidovre, 2650 Hvidovre, Denmark*
[c]*Department of Medical Gastroenterology, Zealand University Hospital, 4600 Koege, Denmark*
[d]*Department of Clinical Medicine, University of Copenhagen, 2200 Copenhagen, Denmark*
[e]*Visiopharm, 2970 Hoersholm, Denmark*
[f]*Department of Pathology, Zealand University Hospital Roskilde, 4000 Roskilde, Denmark*

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Summary Lymphocytic colitis (LC) and LC incomplete (LCi) are common causes of chronic watery diarrhea. The diagnosis relies on clinical findings and histopathologic evaluation. The diagnostic criteria of LC are based on hematoxylin and eosin (HE) staining. However, supplementary immunohistochemical staining for highlighting the lymphocytes in borderline cases is now widely used. This change in diagnostics could lead to incorrectly diagnosing patients with LC and LCi if the present histologic criteria are used. The number of intraepithelial lymphocytes (IELs) was estimated and categorized in intervals based on HE- versus CD3-stained slides from patients with an HE diagnosis of normal colonic mucosa (n = 19), mucosa with nonspecific reactive changes (n = 24), LCi (n = 24), and LC (n = 40). The number of IELs was compared with clinical symptoms. Overall, the number of IELs was higher with CD3 stain compared with HE stain in 73% of cases, unchanged in 26% of cases, and lower in 1 case. The number of IELs detected was higher using the CD3 stain in 53%, 79%, 79%, and 75% of cases included as normal colonic mucosa, nonspecific reactive changes, LCi, and LC, respectively. Based on CD3 stain, 58% of the cases with nonspecific reactive changes fulfilled the HE criteria for LCi, and 79% of the cases with LCi fulfilled the HE criteria for LC. Automated image analysis of CD3-stained slides resulted in even higher numbers of IELs in all 4 diagnostic groups. Conclusively, our data support considering increased cutoff values for LCi and LC when assessed in CD3-stained specimens.

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* Corresponding author at: Rigshospitalet, Frederik V's Vej 11, 2100 Copenhagen, Denmark.
E-mail address: anne-marie.kanstrup.fiehn.01@regionh.dk (A. -M. K. Fiehn).

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1. Introduction

Microscopic colitis (MC) encompassing the 2 major subgroups of collagenous colitis (CC) and lymphocytic colitis (LC) is a common cause of chronic watery nonbloody diarrhea [1-3]. Until recently, a rapid increase in the incidence was reported especially in the Nordic countries and North America after the emergence of this entity in the 1980s [4-6]. The pathogenesis of LC remains unknown, although an association with smoking [7-9], autoimmune diseases [7,10], and certain medications including proton pump inhibitors (PPIs) and selective serotonin reuptake inhibitors (SSRIs) has been suggested [7,11-13]. Endoscopy reveals a macroscopic normal-appearing mucosa, although erythema, edema, and even mucosal tears can be seen [14,15]. Routine laboratory tests show normal results [8]. The diagnosis is based on the presence of both chronic watery diarrhea and characteristic histopathologic features. During the last decade, the entity of MC incomplete (MCi) has been accepted by some gastrointestinal pathologists. MCi includes cases of CC and LC that do not completely fulfill the histopathologic criteria, but the patients have similar clinical characteristics [16-19]. The MCi group seems to have an equal response to treatment as the MC group [19,20].

The accepted histologic features of LC are an increased number of intraepithelial lymphocytes (IELs) of at least 20 IELs/100 epithelial cells combined with an increased inflammatory infiltrate in lamina propria consisting of predominantly lymphocytes, plasma cells, and a smaller number of eosinophils and neutrophils. This is often accompanied by mucin depletion and flattening of the surface epithelium [15,17,18]. LC incomplete (LCi) does not completely fulfill these criteria.

The originally described histopathologic characteristics in 1989 were based on hematoxylin and eosin (HE)–stained slides [21]. Since then, immunohistochemical stains have become part of diagnostic routine and is used as a supplementary tool to refine the diagnosis [22].

In obvious cases of LC, the amount of lymphocytes is greatly increased and can easily be recognized in an HE-stained slide. The guidelines recommend that supplementary CD3 be applied in borderline cases [23]. A CD3 stain highlights the lymphocytes making it easier to estimate the true number. However, the use of CD3 differs between pathology departments and even among pathologists within the same department. This might be explained by economy, tradition, and individual preferences among pathologists [24]. In a previous study, we have shown that a supplementary CD3 staining resulted in higher interobserver agreement and reclassification of the diagnosis in one-third of the patients. In most of these cases, the CD3 changed a primary diagnosis of LCi to LC, simply because the supplementary stain revealed more IELs, thus fulfilling the existing criteria of LC [25].

Thus, the application of the histologic criteria based on HE stain could result in overdiagnosing LC if used on CD3-stained slides [24]. The present article elaborates on the possible consequences of assessing CD3-stained colonic biopsies. We examine the number of IELs based on HE versus CD3-stained slides in 4 diagnostic groups representing a spectrum of diagnostic entities with increasing number of IELs, as this might implicate changing cutoff values on CD3-stained slides.

2. Materials and methods

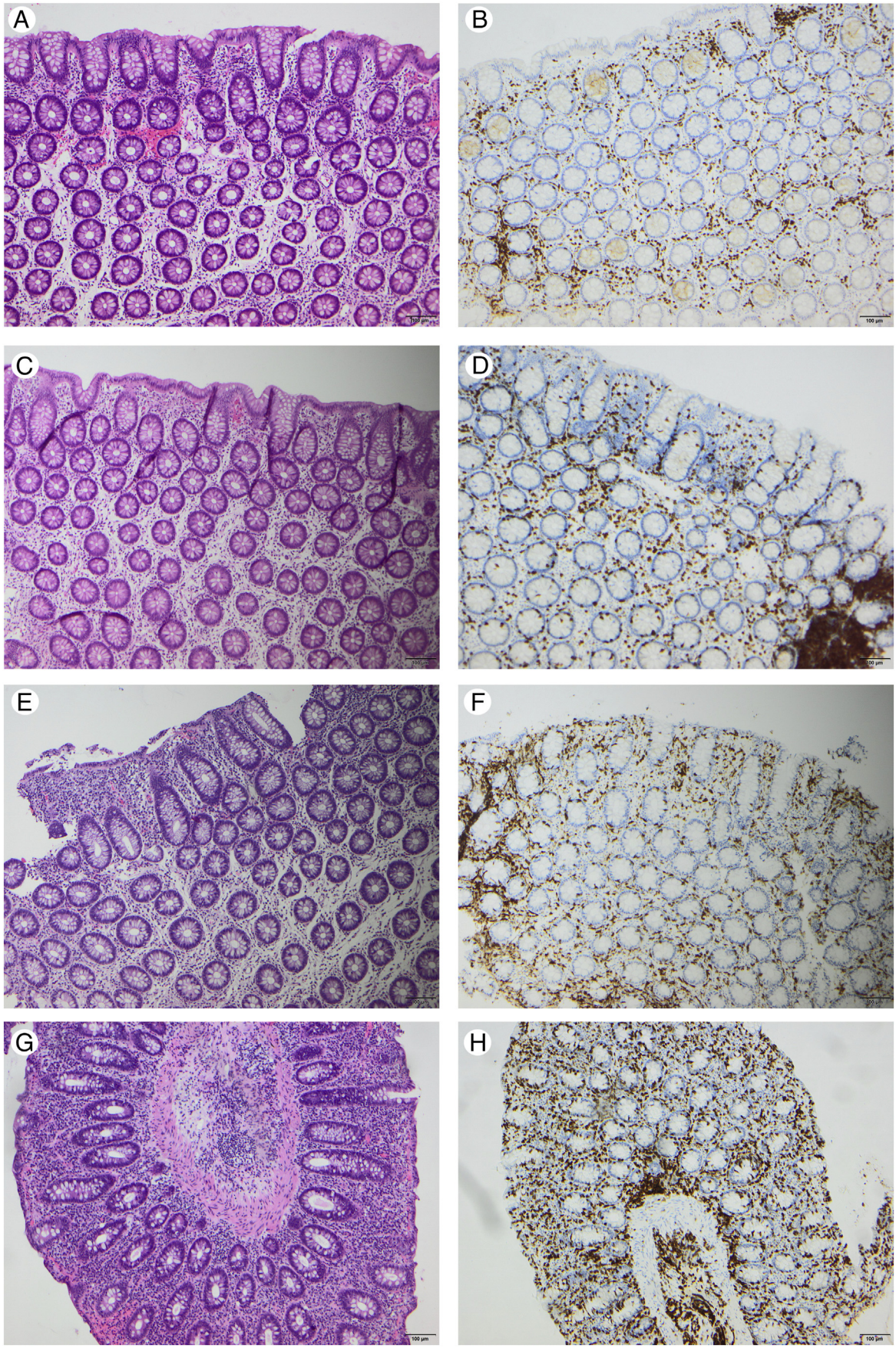
2.1. Patients and biopsy samples

The study included biopsies from 107 patients with normal colon mucosa (n = 19), colon mucosa with nonspecific reactive changes (n = 24), LCi (n = 24), and LC (n = 40). Patients were identified at the Department of Pathology, Region Zealand, Denmark, using the codes "colon mucosa" and "biopsy" combined with either "normal mucosa," "nonspecific reactive changes," "lymphocytic colitis incomplete," or "lymphocytic colitis." Histologic diagnoses were coded according to Systematized Nomenclature of Medicine (http://www.patobank.dk/). HE-stained slides were retrieved from the archives. Two pathologists specialized in gastrointestinal pathology reviewed the slides independently. Agreement between both pathologists was necessary for inclusion. In a few cases, the pathologists agreed on a diagnosis that diverged from the original diagnosis. These cases were included in the study with the reclassified study diagnosis. One slide representative of the diagnosis was selected for each case, and the corresponding formalin-fixed, paraffin-embedded tissue block was retrieved for further CD3 staining. The Department of Pathology, Region Zealand, Denmark, receives all pathology samples from the region including hospitals and private practices.

2.2. Histopathologic evaluation: HE- and CD3-stained slides

Histopathologic changes of LC and LCi had to be present in the surface epithelium of at least 100 epithelial cells. The number of surface IELs was estimated in areas with no spatial relation to dense lymphocytic aggregates in lamina propria. The histopathologic characteristics of each subgroup are at least 20 IELs, 10 to 19 IELs, 5 to 9 IELs, and less than 5 IELs for LC, LCi, nonspecific reactive changes, and normal colon mucosa. This must be accompanied by an inflammatory infiltrate in lamina propria for LC and LCi as well as some extent of surface epithelial damage. The group of nonspecific reactive changes is not well defined. It encompasses various subtle

Fig. 1. HE- and CD3-stained case of normal colon mucosa with only few scattered lymphocytes in the surface epithelium and no inflammatory infiltrate in lamina propria (A and B), mucosa with reactive changes (C and D), LCi with 10 to 19 IELs and a mixed inflammatory infiltrate in lamina propria (E and F), and LC with at least 20 IELs and a mixed infiltrate in lamina propria (G and H).


A
B
C
D
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F
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H

changes and close-to-normal morphology, and is commonly used by many pathologists. Fig. 1 shows 1 case belonging to each of the 4 diagnostic groups stained with HE and CD3, respectively.

All slides were stained using antihuman CD3 clone PS1 (catalog no. NCL-L-CD3-PS1; NovoCastra, Newcastle upon Tyne, United Kingdom) on the Dako Autostainer Link platform. Briefly, dewaxing and antigen retrieval were performed by immersing slides in EnVision FLEX Target Retrieval Solution, High pH (Dako; Denmark catalog no. K8004) and heated in the PT module at 97°C for 20 minutes. After pretreatment, slides were incubated with the primary antibody CD3 (1:50) for 30 minutes. The reactions were detected using EnVision FLEX+/HRP Detection Reagent and visualized with Envision DAB+ Substrate according to the manufacturer's instructions (Dako; catalog no. K8002). All sections were counterstained with hematoxylin and mounted with pertex. Negative controls were performed by omission of primary antibody. T lymphocytes in tonsillary tissue and appendix

Table 1 Clinical characteristics according to subgroups

	Normal colon mucosa (n = 19)	Nonspecific changes (n = 24)	LCi (n = 24)	LC (n = 40)
Sex, n (%)				
Male, n (%)	6 (32)	10 (42)	9 (37.5)	11 (27.5)
Female, n (%)	13 (68)	14 (58)	15 (62.5)	29 (72.5)
Age (y), mean (range)				
Male	56 (30-71)	51 (35-66)	59 (28-82)	70 (35-86)
Female	56 (22-76)	44 (19-74)	55 (26-83)	69 (33-89)
Localization of biopsy used in this study [a]				
Right	3	11	8	16
Left	13	7	9	16
Unknown	3	6	7	8
Diarrhea at time of referral for endoscopy, n (%)	6 (32)	13 (54)	23 (96)	40 (100)
Clinical activity at time of endoscopy, n (%)				
Yes	–	–	12 (50)	23 (57.5)
No			3 (12.5)	10 (25)
Unknown			9 (37.5)	7 (17.5)
Endoscopic findings, n (%)				
Normal	14 (74)	17 (71)	15 (62.5)	21 (52.5)
Subtle changes		3 (12.5)	1 (4) [b]	5 (12.5) [b,d]
Others			7 (29) [c]	9 (22.5) [c,d]
Unknown	5 (26)	4 (17)	1 (4)	6 (15)
Medication, n (%)				
NSAID	–	–	1 (4)	1 (2.5) [d]
SSRI			4 (17)	10 (25) [d]
PPI			0 (0)	5 (12.5)
Unknown			2 (8)	4 (10)
None of the above			17 (71)	21 (52.5)
Infection, n (%)				
No	–	–	23 (96)	35 (87.5)
Unknown			1 (4)	5 (12.5)
Autoimmune disease, n (%)				
Yes	–	–	4 (17)	4 (10)
No			17 (71)	31 (77.5)
Unknown			3 (12.5)	5 (12.5)
Treatment, n (%)				
Budesonide	–	–	10 (42)	20 (50)
Loperamide			1 (self-limiting; 4)	4 (2 self-limiting; 10)
Cholestyramine			2 (8)	–
Psylium			–	3 (7.5)
None			7 (5 self-limiting; 29)	7 (3 self-limiting; 17.5)
Unknown			4 (17)	6 (15)

[a] The left flexure was chosen as the dividing point between the left and right sides of the colon.
[b] Subtle changes include edema, erythema, and alteration in vascular pattern.
[c] Others include diverticula and adenomas.
[d] The total number exceed 100% because 1 patient is registered in more than 1 category.

were used as external positive controls, and tissue from the liver was used as an external negative control. The number of IELs was assessed independently by 2 pathologists and categorized in the intervals 0 to 4, 5 to 9, 10 to 19, 20 to 29, 30 to 39, 40 to 49, or greater than 50 per 100 epithelial cells. A single HE- or CD3-stained slide was available for assessment. In cases of disagreement, a consensus count was reached by reevaluation by a third pathologist using a multiheaded microscope.

2.3. Automated image analysis

All CD3-stained slides were digitized using a Nanozoomer HT 2.0 slide scanner from Hamamatsu Photonics (Hamamatsu, Honshu, Japan), and subsequently, the digital images were processed using Visiopharm Quantitative Digital Pathology software Version 2017.12 (Hoersholm, Denmark). For further details we refer to a previous study [26]. The automated image analysis (AIA) was further optimized to assess the number of CD3-stained lymphocytes in the surface epithelium. Separate counts in "hotspots" covering 100 epithelial cells and in the total area of surface epithelium in the biopsies were made. The 2 separate counts were made to simulate the pathologist's way of working with first an overall view followed by counting in selected hotspots.

2.4. Clinical information

Data concerning localization of the biopsy, indication for colonoscopy and endoscopic findings, and presence of diarrhea were derived from the original pathology report and patient charts. In LC and LCi cases, data on daily medication, stool samples for infectious courses, autoimmune diseases, disease activity at the first visit in outpatient clinic, treatment choice, and treatment response were recorded from patients' charts. Disease activity according to the Hjortswang criteria was recorded. Active disease was defined as a daily mean of 3 or more stools or 1 or more watery stools [27].

2.5. Data presentation and statistics

Data are presented as frequencies of IELs on HE and CD3 stain, and each of the 4 diagnostic groups is compared separately using a 2-sided Fisher exact test. *P* values of less than .05 was considered statistically significant. All statistical analyses were performed using GraphPad, QuickCalcs (https://www.graphpad.com/quickcalcs/contingency2/, accessed on June 14, 2018).

2.6. Ethics

The study was approved by the Local Committee on Health Research Ethics (record no. SJ-612) and the Danish Data Protection Agency (record no. REG-094-2017) according to the Declaration of Helsinki and Danish law.

3. Results

3.1. Clinical characteristics of study subjects

Clinical characteristics are presented in Table 1. The study population included 71 women and 36 men with a mean age of 60 and 59 years, respectively. Overall, 82 patients had diarrhea at the time of endoscopy. At the time of clinical assessment in the outpatient clinic, some patients had already received treatment, and active disease was present in 50% of the patients diagnosed as having LCi and 58% of patients with LC. No patients in the LCi or LC group had infectious disease. Autoimmune disease was reported in 17% and 10% of the patients belonging to the LCi and LC groups. Daily medication with nonsteroidal anti-inflammatory drug (NSAID), SSRI, or PPI was reported by 21% of the patients in the LCi group and 38% in the LC group. Endoscopy revealed normal-appearing mucosa in 74%, 71%, 63%, and 53% in patients with histopathology of normal mucosa, nonspecific reactive changes, LCi, and LC, respectively.

3.2. CD3 staining shows a higher number of IELs compared with HE-stained slides

Fig. 2 shows the number of IELs estimated by the pathologists on an HE versus CD3 stain and by AIA specified in intervals. In only 1 case (1%) of 107 cases was the number of estimated IELs on CD3 lower compared with IELs estimated on HE. This case belonged to the group of normal colonic mucosa. In 28 cases (26%), the number of IELs belonged to the same interval on HE and CD3 staining, whereas in 78 cases (73%), the number of IELs was estimated to a higher interval with a CD3 staining. The number of IELs was estimated to be higher in 53% of cases of normal colon mucosa, 79% of cases of nonspecific reactive changes, 79% of cases of LCi, and 75% of cases with LC ($P < .05$ for each of the 4 diagnostic groups; Table 2). Based on CD3 stain, 58% of the cases with nonspecific reactive changes histologically fulfilled the criteria of LCi, and 79% of the cases with LCi histologically fulfilled the criteria of LC. Fig. 2 also shows the count by AIA in hotspots. It seems that the number of IELs was higher using AIA when counted in hotspots compared with the pathologists in all 4 diagnostic groups. The hotspot count was always higher compared with the total surface area.

To examine if a diagnosis of LCi or LC had been made for any of these cases at a later time, the pathology registry was consulted. Of the 24 cases in the group of nonspecific reactive changes, only 3 patients were rebiopsied on a later occasion, and none were diagnosed as having neither LC or LCi. Likewise, only 3 patients of the 19 cases in the group of normal colonic biopsies were rebiopsied, and none of these were either diagnosed as having LC or LCi.

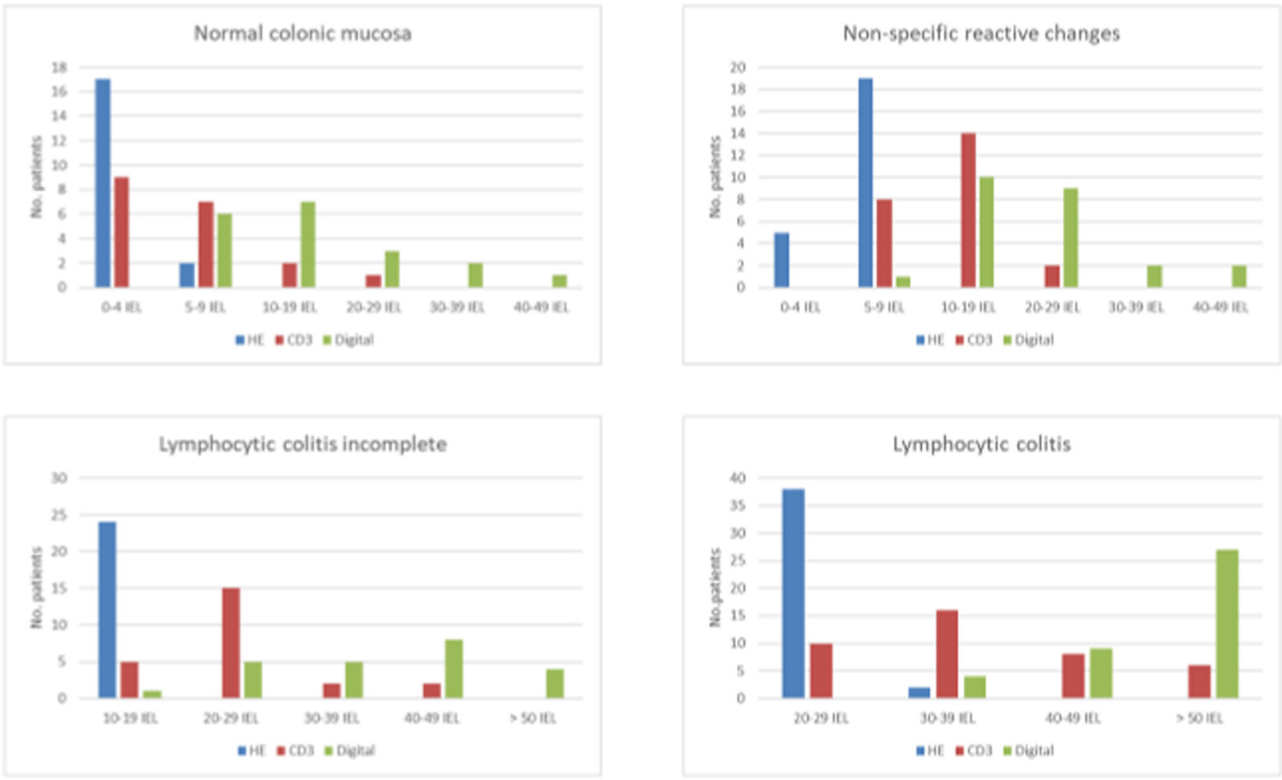

Fig. 2 The number of IELs according to histopathologic diagnosis assessed on HE- and CD3-stained slides by the pathologists and by digital analysis in hotspots.

3.3. Clinical symptoms do not differ between the LCi and the LC group

Diarrhea was present at the time of referral for colonoscopy in 23 of the patients included in the LCi group, whereas 1 patient had pain as primary complain. All patients in the LC group had diarrhea. The Hjortswang criteria for disease activity were met for 12 patients (50%) in the LCi group and 23 patients (58%) in the LC group. At the time of colonoscopy, 6 of the patients (32%) diagnosed as having normal colon mucosa had diarrhea. In the nonspecific reactive change group, 13 of the patients (54%) was reported to have diarrhea. No

Table 2 Number of IELs assessed on HE *vs* CD3 according to the histopathologic diagnosis and the associated movements between categories

HE	CD3							*P*
	0-4 IELs	5-9 IELs	10-19 IELs	20-29 IELs	30-39 IELs	40-49 IELs	>50 IELs	
	No. of patients (%)							
Normal colon mucosa								
0-4 IELs	8 (42)	7 (37)	1 (5)	1 (5)	–	–	–	.0128 [a]
5-9 IELs	1 (5)	–	1 (5)	–	–	–	–	
Nonspecific reactive changes								
0-4 IELs	–	3 (13)	2 (8)	–	–	–	–	<.0001 [b]
5-9 IELs	–	5 (21)	12 (50)	2 (8)	–	–	–	
LCi								
10-19 IELs	–	–	5 (21)	15 (63)	2 (8)	2 (8)	–	<.0001 [c]
LC								
20-29 IELs	–	–	–	10 (25)	16 (40)	8 (20)	4 (10)	<.0001 [d]
30-39 IELs	–	–	–	–	–	–	2 (5)	

[a] *P* value is calculated as ≤4 IELs compared with ≥5 IELs on HE *vs* CD3.
[b] *P* value is calculated as ≤9 IELs compared with ≥10 IELs on HE *vs* CD3.
[c] *P* value is calculated as ≤19 IELs compared with ≥20 IELs on HE *vs* CD3.
[d] *P* value is calculated as ≤29 IELs compared with ≥30 IELs on HE *vs* CD3.

information on activity was available for these 2 groups of patients. Six patients (25%) in the LCi group and 5 patients (12.5%) in the LC group experienced self-limiting disease.

4. Discussion

The present study confirms that the number of visualized IELs is higher in CD3-stained slides as compared with HE-stained slides. We further present a direct case-by-case comparison between these stains and digital imaging of the number of IELs in 4 groups of patients (normal, nonspecific reactive changes, LCi, and LC) with increasing lamina propria inflammation. All patients in the LC group had at least 20 IELs/100 epithelial cells based on HE stains, and the additional CD3 stain did not move patients to another diagnostic category. In all included cases with nonspecific reactive changes, we confirmed that the number of IELs was less than 10 of 100 epithelial cells on HE, whereas the number was higher for 79% on CD3-stained specimens and 58% of these fulfilled the current criteria for LCi. A large fraction (79%) of patients in the LCi group was categorized as LC using CD3-stained slides. We demonstrate that using the HE-derived criteria for LC and LCi in CD3-stained biopsies tends to change the diagnoses in comparison with HE-stained specimens from the same individuals.

The risk of miscategorizing patients is greatest in the diagnostic categories of nonspecific reactive changes. Only a few of the cases included had a later rebiopsy from their colon, and in none of these cases did LC or LCi evolve. This is in accordance with previous findings in our MC cohort [28]. Similarly, none of the 3 patients with normal histology rebiopsied developed LCi or LC, indicating that the likelihood of LC or LCi in patients included in the subgroups of normal mucosa and nonspecific reactive changes is low. Thus, a diagnosis of LCi or LC based on CD3 in patients with normal or near-normal histology on HE would probably not have been correct. By contrast, an additional CD3 stain moved 19 of 24 cases in the LCi group to a higher IEL interval, and these patients now fulfilled the histologic criteria of LC. As the indication for colonoscopy was diarrhea in 23 of 24 patients, it is difficult to make a true clinical distinction between patients having LCi and patients having LC. In this context, it is important to notice that the LCi group was younger and had more were men as compared with what is usually reported in LC [29]. Also, a higher number of cases with spontaneously resolution were seen in the LCi group compared with the LC group in the present study. However, ignoring the increased number of IELs identified by a CD3 stain in patients with persistent chronic diarrhea could withhold these patients a proper diagnosis and effective treatment [29].

Counting was made in hotspots, but it was noticed by the pathologists that when a higher number of IELs was usually seen, it was present in several biopsies and an area that was bigger than the minimum required. This corresponds well with the literature where LC is usually described to have an even distribution throughout the colon and to be only seldom patchy [14,28,30]. AIA revealed even higher counts in hotspots. This may be explained by the fact that the software easily and with certainty localizes and counts the hotspots, whereas localizing this is an individual assessment by the pathologist. Furthermore, the higher the number of IELs, the more difficult it is for the pathologist to discriminate the individual cells, making it more challenging to count an exact number. In AIA, the overall count seems best comparable with the count made by the pathologist.

The question of different cutoff levels, depending on the stains used, is a well-known issue in gastrointestinal pathology. The number of IELs has been investigated in several studies based on HE and CD3 stains in biopsies of duodenal mucosa, where coeliac disease is suspected. The upper limit of normal duodenal mucosa has been established to be 20 IELs/100 enterocytes on HE and 25 on CD3 [31]. In LC, it seems even more important to agree on a cutoff level for CD3-stained slides because confirmation of the diagnosis relies solely on histopathology.

It is important to discriminate LC and LCi from differential diagnoses including infectious colitis, inflammatory bowel disease, drug-induced colitis, autoimmune disease, and irritable bowel syndrome. There is an overlap in especially clinical symptoms, whereas the histopathology is usually different. Acute inflammation and crypt architectural distortion are not a prominent feature of LCi and LC. None of the patients in the LCi or LC group had bloody diarrhea or a positive stool test result for infectious disease. Many of the included patients would probably fulfill the criteria for irritable bowel syndrome. This diagnose is not relevant in the LCi and LC groups where histologic changes are seen. Unfortunately, this information is not available for the group of normal and nonspecific reactive changes. Not surprisingly, more patients in the group of LC had a daily consumption of medications previously reported to be associated with LC compared with the LCi group, suggesting a clinical difference between the 2 subgroups. In contrast to this, a higher percentage of autoimmune disease was seen in the LCi group, although the numbers are too small (4 patients in each group) to draw any conclusions. The endoscopy reported an increasingly lower number of normal-appearing mucosa over the histopathologic spectrum of normal mucosa through nonspecific reactive changes, LCi, and LC. Most of this difference was caused by a higher number of patients having diverticula and adenomas, and only a small number of patients exhibited slight macroscopic changes including edema, erythema, and alterations in vascular pattern. Macroscopic changes were not associated with a certain higher level of IELs.

Not all patients fulfilling the histopathologic criteria of LCi and LC had active disease according to the Hjortswang criteria, although they were referred to colonoscopy because of diarrhea. This index has only been validated for CC [27] but has been widely used in other studies dealing with health-related quality of life in patients with both CC and LC [32].

The high rate of spontaneous resolution of symptoms in all MC subgroups has recently been confirmed in a prospective study [13]. Another recent study furthermore suggests that the presence of nonclassical histopathologic features might possibly divide LC into several subgroups predicting response to therapy [33].

The use of supplementary immunohistochemical staining is associated with a further cost of the histopathologic analysis compared with HE alone. In some cases, it can be an aid for the pathologist to make a more precise diagnosis, and the need for further diagnostic examinations, possibly including rebiopsies and follow-up visits, will be reduced. Thus, the overall diagnostics would be cost-effective.

Conclusively, using identical cutoff values for IELs in CD3 as for HE-stained slides may result in some overdiagnosing of LCi and LC. On the other hand, some patients with LC will be misdiagnosed and withheld treatment if CD3 is not used. The present guidelines recommend using CD3 only in borderline cases and when in doubt [15,17]. If the original HE-based criteria for LC and LCi are maintained, we suggest combining this recommendation with a raised lower cutoff value for both LCi and LC when using CD3-stained slides. A cautious proposal would be a cutoff value of 15 IELs for LCi and 25 IELs for LC. An alternative conclusion could be that the original HE-based criteria for LC are too strict and the LCi group actually should be included in the LC group. In this case, the cutoff of 20 IELs on CD3 stain should be retained, and instead, the cutoff of 20 IELs on HE should be reduced. Further and prospective studies comparing histology and clinical course are needed to confirm our findings and in particular to validate the clinical interpretation of these. In case AIA is used for diagnosis, even higher cutoffs should be established. Finally, our study emphasizes that the diagnosis of LC cannot be based solely on increased IEL counts but requires close collaboration between clinician and pathologist.

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