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Establishment of digital cutoff values for intraepithelial lymphocytes in biopsies from colonic mucosa with lymphocytic colitis

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ABSTRACT

Background/Introduction: Lymphocytic colitis (LC) and the incomplete form (LCi) are common causes of chronic watery diarrhea. Endoscopy is often inconspicuous, and the diagnosis relies on histopathological assessment of colonic biopsies. Digital Image Analysis (DIA) eliminates interobserver variation. The aim of this study was to establish digital cutoff values for LC and LCi on CD3 stained slides.

Material and methods: One hundred and six patients with a hematoxylin and eosin (HE) diagnosis of normal colonic mucosa (N = 19), non-specific reactive changes (N = 24), LCi (N = 23) and LC (N = 40) were eligible for analysis. The number of intraepithelial lymphocytes (IELs) reached by DIA in the total surface epithelium and in hot spots of the biopsies was compared with the diagnostic category assigned by the pathologists based on HE stained slides. The digitalized slides were analyzed for number of IELs using Visiopharm Quantitative Digital Pathology software. All digitalized slides were examined manually to identify differences in the approach to the evaluation of the biopsies by the pathologists and DIA.

Results: The median IEL counts and interquartile range in the total surface epithelium were 3.6 (3.2–4.3), 4.4 (3.4–5.3), 19.8 (16.6–30.0) and 41.3 (37.0–47.8) in normal colon mucosa, mucosa with non-specific reactive changes, LCi and LC, respectively. Discrimination between normal mucosa and non-specific reactive changes was not possible. Digital cutoff values with the best separation between non-LC, LCi and LC were > 13 IELs/100 epithelial cells for LCi and > 36 IELs/100 epithelial cells for LC. These cutoff values resulted in an agreement between the pathologist's and DIA that was very good with a kappa value of 0.90.

Conclusion: Despite differences among the approach of DIA and the pathologist's assessment of IELs in colonic mucosa DIA is able discriminate between the HE based diagnoses of the three subgroups non-LC, LCi and LC with high accuracy.

1. Background

Microscopic colitis (MC) is a frequently encountered diagnosis in patients with chronic watery diarrhea accompanied by a macroscopic normal or close to normal appearing mucosa. MC encompasses the two major subtypes lymphocytic colitis (LC) and collagenous colitis (CC) [1,2]. Differentiating between the subgroups is based solely on characteristic histological findings [1,3,4]. A consensus paper reports the mean frequency of MC in patients fulfilling the clinical criteria to be 12% [5]. Biopsies from patients with macroscopic inconspicuous mucosa that do not fulfill the histopathologic criteria of MC are often signed out by the pathologist as normal colonic mucosa or colonic mucosa with non-specific reactive changes.

The main histopathological criteria for LC are an increased number of intraepithelial lymphocytes (IELs) of ≥ 20 IELs/100 epithelial cells in the surface epithelium combined with a mixed inflammatory infiltrate in lamina propria predominantly consisting of lymphocytes, plasma cells, and sometimes including a smaller number of eosinophils

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and neutrophils. This is often accompanied by mucin depletion and flattening of the surface epithelium [1–3,6,7]. Lymphocytic colitis incomplete (LCi) comprises cases that do not completely fulfill these histopathological criteria but clinically seem to be similar [8–11]. In obvious cases the diagnosis can be made by hematoxylin and eosin (HE) stained slides [1,2]. In borderline cases observer variability has been reported [12]. A consensus statement of the European Microscopic Colitis Group recommends applying supplementary immunohistochemical staining (IHC) in borderline cases [13]. A CD3 stain highlights the T-lymphocytes, which makes counting easier and increases interobserver agreement [14]. At the same time CD3 staining represents a risk of over diagnosing since the diagnostic criteria was originally determined by HE stained slides and we recently suggested to raise the lower cutoff values for LCi and LC when using CD3 [15].

Digital image analysis (DIA) eliminates intra- and interobserver variance. Other advantages of digital pathology are education, teleconferences, frozen section, sharing slides and ergonomics which can be of value in research as well as in daily routine [16,17]. The reported concordance between the diagnoses achieved by digital microscopy and conventional light microscopy is high [18,19]. This is fundamental to apply DIA.

The aim of the present study was to establish digital cutoff values for biopsies from colonic mucosa with LCi and LC on CD3 stained slides and to identify and describe differences between the analysis made by DIA and the pathologist in counting IELs.

2. Material and methods

2.1. Patients and biopsy samples

The study included biopsies representing a spectrum of changes seen in colonic mucosa with chronic inflammation. Briefly, patients were identified at the Department of Pathology, Region Zealand, Denmark, and HE stained slides were retrieved from the archives. The slides were reviewed independently by two pathologists, and agreement on the diagnosis was required for inclusion. The HE based diagnose was defined as the “true” diagnose. One representative slide was selected including at least one biopsy with the crypts orientated perpendicular to the surface allowing for a correct manual assessment by the pathologist. The corresponding formalin fixed paraffin embedded tissue block was retrieved for further sectioning and staining with CD3. For more details of the study population we refer to previously published data [15].

2.2. Histopathological examination and immunohistochemical staining

Based on HE stained slides, in cases of LC and LCi the histopathologic changes had to be present in the surface epithelium of at least 100 coherent epithelial cells with no relation to dense lymphocytic aggregates in lamina propria. The histopathologic characteristics of each subgroup was defined as ≥ 20 IELs, 10–19 IELs, 5–9 IELs and < 5 IELs for LC, LCi, non-specific reactive changes and normal colon mucosa, respectively, accompanied by a mixed inflammatory infiltrate in lamina propria for the subgroups LC and LCi. Non-specific reactive changes comprised a group of cases with a variety of subtle changes.

All slides were stained using anti-human CD3 clone PS1 (cat.no. NCL-L-CD3-PS1, NovoCastra, United Kingdom) on a Dako Autostainer Link platform. Briefly, dewaxing and antigen retrieval were performed by immersing slides in EnVision™ FLEX Target Retrieval Solution, High pH (Dako, cat.no K8004) and heated in the PT-module at 97 °C for 20 min. After pre-treatment, slides were incubated with the primary antibody CD3 (1:50) for 30 min. The reactions were detected using EnVision™ FLEX+ /HRP Detection Reagent and visualized with Envision 3,3-diaminobenzidine-tetrahydrochloride (DAB) Substrate according to the manufacturer’s instructions (Dako, cat. no K8002). All sections were counterstained with haematoxylin and mounted with pertex. Negative controls were performed by omission of primary antibody. T-lymphocytes in tonsillary tissue and appendix were used as external positive controls, and tissue from liver was used as external negative control.

The number of IELs on the CD3 stained slides was assessed independently by two pathologists and categorized in the intervals 0–4, 5–9, 10–19 or ≥20 per 100 epithelial cells. In cases of disagreement, a consensus count was made at a multiheaded microscope with inclusion of a third pathologist.

2.3. Digital image analysis

All CD3 stained slides were digitalized using a Nanozoomer HT 2.0 slide scanner from Hamamatsu Photonics (Hamamatsu, Japan) and subsequently the digital images were processed using Visiopharm Quantitative Digital Pathology software (Hoersholm, Denmark). All slides were analyzed with an updated version (2017.12) of the previously developed software (APP no. 100085, Visiopharm, Denmark). The APP was built around a three-step approach 1) identifying the general tissue sample and outlining the edge of the tissue; 2) identifying subareas of the tissue edge, with discrimination between lamina propria and surface epithelium; 3) quantifying the number of IELs in the surface epithelium and calculating the percentage of positive IELs [20].

The initial detection of tissue was conducted automatically as a threshold on a low magnification (virtual 2x) version of the image, represented by features for DAB detection kit color-deconvolution, with Red/Green contrast and Green/Blue contrast. Post-processing step were used to remove small objects and filling unwanted holes. The final step created a rim around the tissue edge of approximately 45 µm. The detected edge served as the region of interest (ROI) in the following analysis as this area represents the surface epithelium.

The secondary detection was executed at virtual 10x magnification only within the previously detected edge, utilizing a Bayesian pixel classifier on features highlighting local linear object (Patent.no.: EP 2 327 040 B1), smoothness through a 11 × 11 pixel standard deviation filter.

The final analysis was conducted with a Bayesian pixel classifier at full magnification utilizing features highlighting round objects representing nuclei and discriminating was made between the positive CD3 stained nuclei and the negative nuclei of the epithelial cells. The fully analyzed images where used to create a heatmap, defined by the ratio of positive cells within an area following the contour of the heatmap including a minimum of 100 cells. Fig. 1a-1f illustrates the stepwise process of the digital analysis. (A more detailed protocol of DIA can be required by request).

Digital counts were made in the total area of surface epithelium and in hot spots of the biopsies. A hot spot was defined as an area covering 100 coherent epithelial cells reaching the highest IEL count. All digitized slides were manually assessed by two pathologists to identify differences in the evaluation of the biopsies between the pathologists and DIA. An attempt to optimize the software was performed (version 2018.12).

2.4. Data presentation and statistical analysis

The IEL count by DIA was compared with the IEL count by the pathologists on CD3 stained slides. Cohen’s kappa statistics (weighted values) was applied to evaluate the rate of agreement. Kappa values of < 0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80 and 0.81–1.00 reflect poor, fair, moderate, good and very good agreement. For Cohen’s kappa statistical analysis values were supplied both for the four separate groups and for only three groups when merging the group of normal mucosa and non-specific reactive changes into a non-LC group. Kappa statistical analyses were performed using GraphPad, QuickCals (https://www.graphpad.com/quickcalcs/, accessed on June 15, 2019). Next, comparison was made between the four diagnostic categories

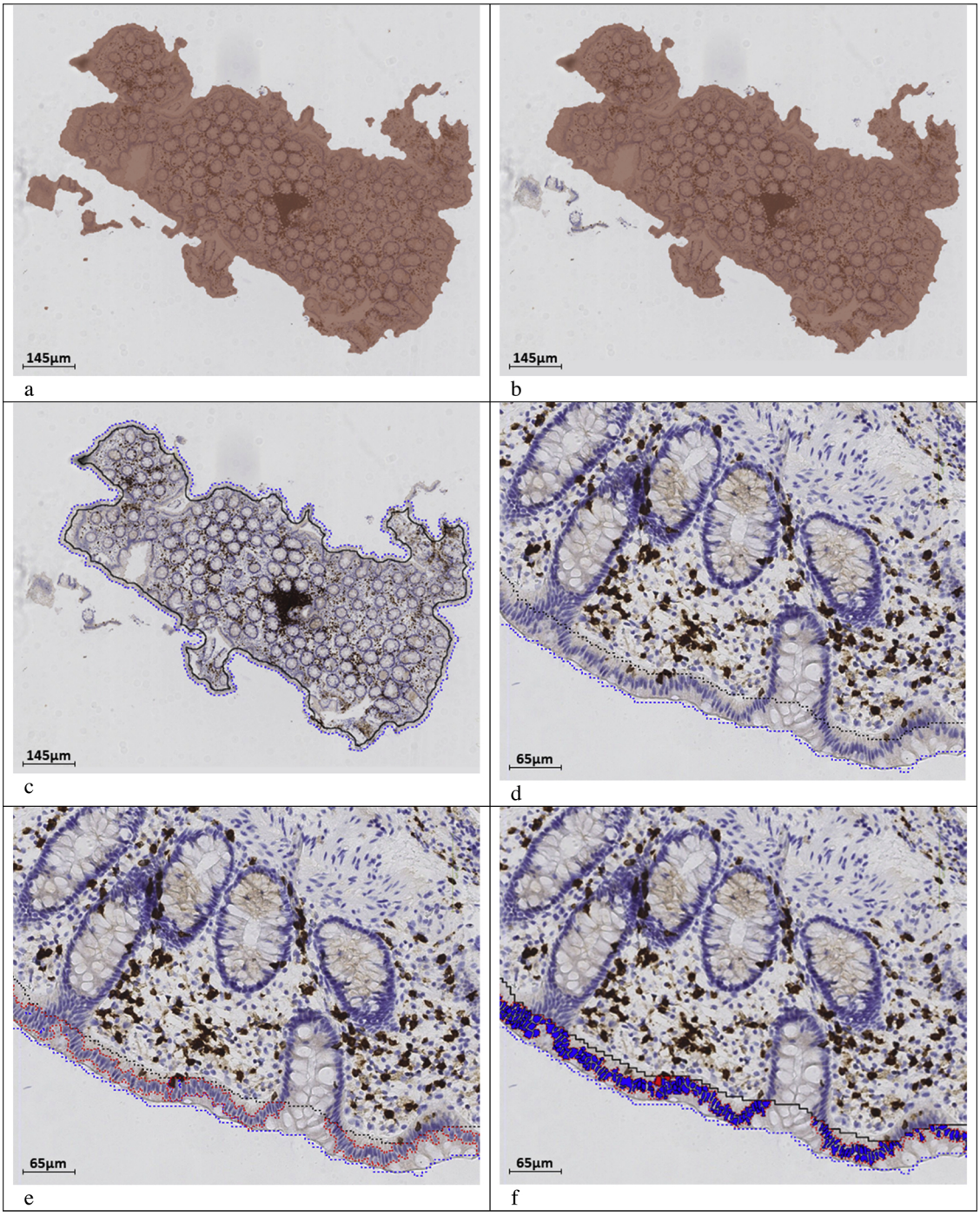

Fig. 1. 1a -1f 1a, initial detection of tissue (analyzed at 2x magnification, displayed at 5x magnification); 1b, small objects and holes are removed; 1c, detection of surface epithelium representing region of interest (ROI) localized between black and blue dotted lines; 1 d, 20x magnification close up of 1c; 1e, secondary detection (analyzed at 10x magnification, displayed at 20x magnification) within the ROI, with highlighting of local linear object localized between red dotted lines; 1f, highlighting round objects representing nuclei and discriminating between the positive nuclei representing lymphocytes (red) and negative nuclei representing epithelial cells (blue) within ROI (analyzed and displayed at 20x magnification).

assigned by the pathologists based on HE stained slides, and the IEL count by DIA. Median count and interquartile range (IQR) were supplied for the digital counts and shown according to the HE based diagnostic category. Suggested digital cutoff values reaching the highest concordance with the HE based diagnosis for the three groups non-LC, LCi and LC were calculated using Excel, version 1902 (Build 11,328.20222).

2.5. Ethics

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

Table 1
Clinical characteristics according to the four diagnostic subgroups.

	Normal colon mucosa, n = 19	Non-specific changes, n = 24	LCi, n = 23	LC, n = 40
Male, n (%)	6 (32)	10 (42)	9 (39.1)	11 (27.5)
Female, n (%)	13 (68)	14 (58)	14 (60.9)	29 (72.5)
Diarrhea at time of endoscopy, n (%)	6 (32)	13 (54)	22 (96)	40 (100)
Clinical activity at follow-up after endoscopy, n (%)	–	–	Yes 11 (47.8) No 3 (13) Unknown 9 (39.1)	Yes 23 (57.5) No 10 (25) Unknown 7 (17.5)
Endoscopic findings, n (%)	Normal 14 (74) Unknown 5 (26)	Normal 17 (71) Subtle changes[a] 3 (12.5) Unknown 4 (17)	Normal 14 (60.9) Subtle change[a] 1 (4.3) Others[b] 7 (30.4) Unknown 1 (4.3)	Normal 21 (52.5) Subtle change[a,c] 5 (12.5) Others[b,c] 9 (22.5) Unknown 6 (15)

LC; lymphocytic colitis, LCi; lymphocytic colitis incomplete.
[a] Subtle change include edema, erythema and alteration in vascular pattern.
[b] Others include diverticula and adenomas.
[c] The total number exceed 100% since one patients are registered in more than one category.

3. Results

3.1. Clinical characteristics of patients

The study included biopsies from 107 patients with normal colon mucosa (N = 19), colon mucosa with non-specific reactive changes (N = 24), LCi (N = 24) and LC (N = 40). One patient diagnosed as LCi was excluded due to insufficient quality of the scanned slides. The number of IELs detected by a CD3 compared with HE stained slides was higher in 53%, 79%, 79% and 75% of the cases included as normal colonic mucosa, non-specific reactive changes, LCi and LC, respectively. Clinical characteristics are presented in Table 1. Overall, diarrhea was present at the time of referral for endoscopy in 32%, 54%, 96% and 100% of the patients with normal mucosa, mucosa with non-specific reactive changes, LCi and LC, respectively. For more detailed description of the study cohort we refer to previously published data [15].

3.2. Comparison of pathologist CD3 count with digital CD3 count

When comparing the CD3 count by the pathologist with DIA the same or a lower number of IELs was identified in the total surface epithelium by DIA. In hot spots, IEL counts by DIA was only lower in one case and higher in 38 cases (35.8%). The kappa values were 0.61 in the total surface epithelium and 0.44 in hot spots reflecting good and moderate agreement, respectively when discriminating between four categories of IELs (Table 2a). Merging the groups of 0–4 and 5–9 IELs did not change the category of agreement but a minor increase of the kappa values to 0.72 and 0.49 in total surface epithelium and hot spots, respectively, was observed (Table 2b).

3.3. IEL counts by digital image analysis according to HE based diagnosis

Fig. 2a and b show the digital IEL counts in the total surface epithelium and in the hot spots according to the four diagnostic categories assigned by the pathologists on HE stained slides. The median IEL counts and IQR in the total surface epithelium were 3.6 (3.2–4.3), 4.4 (3.4–5.3), 19.8 (16.6–30.0) and 41.3 (37.0–47.8) in normal colon mucosa, mucosa with non-specific reactive changes, LCi and LC, respectively. The corresponding number for the hot spot counts were 15.4 (8.2–24.4), 25.4 (18.1–29.1), 41.7 (30.6–47.6) and 54.8 (45.0–60.3), respectively.

3.4. Digital cutoff values based on the histopathologic HE diagnosis

Based on the digital IEL counts in the total surface epithelium the cutoff values that gave the best separation between the groups non-LC, LCi and LC were < 13, 13–36, and > 36, respectively. Table 3 shows

Table 2a
Comparison of IEL counts on CD3 stained slides by the pathologists and the digital analysis in the total surface epithelium and the hot spots, respectively, divided into four IEL intervals (0–4, 5–9, 10–19 and ≥ 20). The number of cases with similar and divergent counts are shown in the table (total number of cases = 106).

		Pathologists count (IELs/100 EC)				Number of cases
		0-4	5-9	10-19	≥20	
Digital total surface epithelium count (IELs/100 EC)	0-4	9	15	10	1	35
	5-9	0	0	5	0	5
	10-19	0	0	6	11	17
	≥ 20	0	0	0	49	49
Number of cases		9	15	21	61	106
Kappa value					0.61	
Digital hot spot count (IELs/100 EC)	0-4	0	0	0	0	0
	5-9	5	2	0	0	7
	10-19	1	11	5	1	18
	≥ 20	3	2	16	60	81
Number of cases		9	15	21	61	106
Kappa value					0.44	

IELs; intraepithelial lymphocytes, EC; epithelial cells.

Table 2b
Comparison of IEL counts on CD3 stained slides by the pathologists and the digital analysis in the total surface epithelium and the hot spots, respectively, divided into three IEL intervals (0–9, 10–19 and ≥ 20). The number of cases with similar and divergent counts are shown in the table (total number of cases = 106).

		Pathologists count (IELs/100 EC)			Number of cases
		0-9	10-19	≥20	
Digital total surface epithelium count (IELs/100 EC)	0-9	24	15	1	40
	10-19	0	6	11	17
	≥ 20	0	0	49	49
Number of cases		24	21	61	106
Kappa value				0.72	
Digital hot spot count (IELs/100 EC)	0-9	7	0	0	7
	10-19	12	5	1	18
	≥ 20	5	16	60	81
Number of cases		24	21	61	106
Kappa value				0.49	

IELs; intraepithelial lymphocytes, EC; epithelial cells.

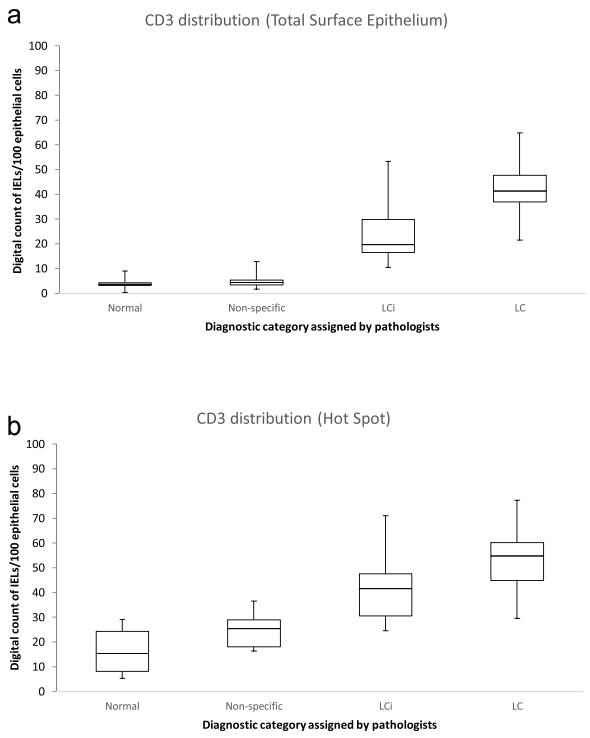

Fig. 2. 2a Exact number of IELs by digital image analysis in the total surface epithelium according to the HE based diagnostic category assigned by the pathologists.
2b Exact number of IELs by digital image analysis in hot spots according to the HE based diagnostic category assigned by the pathologists.
IELs; intraepithelial lymphocytes, Non-specific; non-specific reactive changes, LCi; lymphocytic colitis incomplete, LC; lymphocytic colitis.

Table 3
Comparison of original HE based histological diagnose (Non-LC, LCi and LC) and cutoff values for digital total surface epithelium resulting in the best separation between groups. The number of cases in each of the diagnostic categories are shown according to the established digital cutoff values (total number of cases = 106).

		Pathologists			Number of cases
		Non-LC	LCi	LC	
Digital counts	< 13	43	1	0	44
(IELs/100 EC)	13-36	0	19	6	25
	> 36	0	3	34	37
Number of cases		43	23	40	106

IELs; intraepithelial lymphocytes, EC; epithelial cells, Non-LC; non-lymphocytic colitis, LCi; lymphocytic colitis incomplete, LC; lymphocytic colitis.

the three groups based on these digital cutoff values compared with the diagnostic category assigned by the pathologist based on HE stained slides. The cutoff values resulted in an agreement of 90.6% and a kappa value of 0.90 corresponding to very good agreement.

3.5. Optimization of the digital image analysis

Manual examination of the digitally analyzed slides followed by a change in the settings of DIA (version 2018.12) resulted in only minor improvement in kappa values at the expense of a small decrease in separation between the diagnostic groups. We tried to optimize the DIA to better simulate the pathologist i.e. by including evaluation of denudated or displaced surface epithelium, counting in areas with big mucin vacuoles and areas with closer relation to lymphoid aggregates in the lamina propria which is otherwise avoided by DIA, as well as avoiding biopsies with tangential sectioning but this did not seem to be the right way to reach a significant improvement of DIA.

4. Discussion

CD3 visualizes a higher number of lymphocytes compared with HE stains [15]. This study shows that the IEL count by DIA is different compared with the count by the pathologist on CD3 stained slides. We establish digital cutoff values for the number of IELs in the surface epithelium distinguishing between non-LC, LCi and LC using DIA. The study was performed in a thoroughly described population of individuals with biopsies from colonic mucosa with histopathologic changes varying from normal colon mucosa to LC [15]. The IEL count provided by DIA is reproducible and furthermore gives an overall estimate across the whole surface epithelium while the pathologist counts only in hot spots. The digital cutoff values established in the total surface epithelium in this study results in a better separation between the diagnostic groups than do hot spot counts. Furthermore, we found that the total surface epithelium count is closer to the count performed by the pathologists compared with the hot spot count. This may be explained by the fact that especially in cases with a high number of IELs it can be difficult for the human eye to distinguish the individual cells. This may also reflect that the pathologist does assess the whole surface of the biopsy. In a previous study we have showed that the agreement between DIA and pathologists was comparable to the interobserver agreement among several pathologist's when counting in the total surface epithelium with a kappa value of 0.86 corresponding to very good agreement [20]. The kappa values that were reached in the present study when counting in the total surface area were comparable to the initial DIA study even though the present study included a higher number of normal biopsies, biopsies with non-specific reactive changes and a much higher number of LCi cases which are more difficult to classify. In the present study we refined DIA to count also in hot spots to simulate the pathologist's approach, but this approach was not preferable.

We have shown that there is marked overlap between the digital counts in the groups of normal colonic mucosa and non-specific reactive changes and therefore it is not reasonable to discriminate between these two groups; hence we merged them to a non-LC group. We observed significant differences in IEL counts between LC, LCi and the merged non-LC group, which were separated with very good agreement.

While we have thus identified digital cut-off values that are applicable in the clinical setting, our study has limitations. First, DIA may be challenged in identifying the surface epithelium of the biopsies. In two biopsies located in very close proximity, almost touching each other or cases with the surface epithelium being twisted and not lying on the surface of the biopsies the DIA was not able to identify this as surface epithelium. As a result, counting of these areas was not included. In addition, if the epithelium of the biopsy was detached the DIA occasionally counted in superficial areas of lamina propria instead, often resulting in a higher count of lymphocytes. Since denudation is rarely observed in cases with LC this was a limited problem. Second, the discrimination of individual cells is difficult in tangentially sectioned biopsies with a "star like" appearance and is avoided by the pathologist. The DIA included these areas. Third, the software application is programmed to avoid counting in crypt epithelium, where the epithelium appears lighter in the color due to many closely related mucin rich epithelial cells. Consequently, areas in the surface epithelium with big mucin vacuoles in the cytoplasm were not counted. Finally, the software is programmed to avoid surface epithelium in close relation to dense lymphoid aggregates in lamina propria and does this rigidly. As a result, it was observed that the DIA excluded areas where the pathologist might have counted. The observed differences resulted in higher or lower counts depending on the number of IELs in the concerned areas. Changing the settings of the DIA is complicated since avoiding counting in one area, might exclude another area otherwise acceptable for counting. We did try to refine the software which resulted in

minimally improved kappa values but decreased the separation between groups. Although the orientation of the biopsies is not as crucial in diagnosing LC as in CC it is still an essential parameter for manual assessment by the pathologist. In DIA the orientation of the biopsies was not as important and changing the settings to exclude tangentially sectioned areas (version 2018.12) did only lead to subtle differences. Still, the possibility of changing settings illustrates that using different software for DIA in different laboratories will probably lead to minor variation in the results.

Manual editing of each single case is a possibility, but this is very time consuming and the idea of minimizing inter- and intraobserver variability would be lost. A possible way for significant improvement of DIA would be double IHC staining of lymphocytes and epithelial cells which would probably eliminate the abovementioned limitations.

Of note, some basic requirement of the scanned slides, i.e. precisely mounted cover glass and clean slides with sparse fecal contents were mandatory for optimal DIA. Strengths of our study include a well described cohort; the analyses were performed blinded for diagnosis and the software was developed and tested in a previous study [20]. The study was not designed to answer how many biopsies that are needed to confirm or exclude the diagnoses LC, LCi or non-LC. Only one slide representative of the diagnose was selected for the study. Implementation of the method in daily routine would demand all available material analyzed given the variability of the condition throughout the colon.

Quantification of lymphocytes by DIA has been evaluated in an increasingly number of studies to obtain an exact estimate of tumor infiltrating lymphocytes in e.g. primary colorectal cancer, colorectal liver metastases [21,22] and breast cancer [23] as this might be of prognostic importance and in lung cancer also predictive of therapeutic response [24,25]. Using DIA in LC and a spectrum of cases with chronic inflammation in colonic mucosa faces other challenges. Localization of the ROI which as described above comprises only a narrow band of surface epithelium as well as calculating the ratio of lymphocytes to unstained normal surface epithelial cells is difficult and pose a significant pitfall in digital image analysis. Furthermore, LCi present borderline cases with interobserver variability by conventional light microscopy and the cutoff value for this group is somewhat arbitrary which is also reflected by DIA.

5. Conclusion

DIA is a supplementary tool now valuable especially in research, but the use in daily routine is limited by the expense of the equipment. We believe it will also be of importance in future diagnostic pathology with rapid technological development and decrease in costs. With the currently available software for counting the number of IELs in CD3 stained biopsies of colonic mucosa we suggest cutoff values of < 13 IELs, 13–36 IELs and > 36 IELs for normal/non-specific reactive changes, LCi and LC, respectively. To further assemble evidence that DIA is suitable for establishing a primary diagnosis of LC and LCi our findings require verification in additional studies.

Declaration of Competing Interest

Martin Kristensson is an employee of Visiopharm
No other authors have any disclosures.

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