In Vivo Confocal Endomicroscopy in the Diagnosis and Evaluation of Celiac Disease

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See CME quiz on page 2141.

Background & Aims: **Accurate histopathology of endoscopic duodenal biopsy specimens is critical in the diagnosis of celiac disease (CD) but sampling error and poor quality specimens may generate a false-negative result. Confocal endomicroscopy (CEM) is a novel technology allowing real-time in vivo microscopy of the mucosa that may diagnose CD and evaluate its severity and response to treatment more accurately than histopathology.** ***Methods:*** **Subjects with CD and controls prospectively underwent CEM. Features of villous atrophy and crypt hypertrophy were defined. A CEM score measuring CD severity was devised and validated against the diagnosis of CD and blinded histopathology. Receiver operator characteristics, sensitivity to change after treatment, and reliability of findings were assessed.** ***Results:*** **From 31 patients (6 untreated CD, 11 treated CD, and 14 controls), 7019 CEM images paired with 326 biopsy specimens were obtained. The accuracy of CEM in diagnosing CD was excellent (receiver operator characteristics area under the curve, 0.946; sensitivity, 94%, specificity, 92%) and correlated well with the Marsh grading (R-squared, 0.756). CEM differentiated CD from controls ($P < .0001$) and was sensitive to change after treatment with gluten-free diet (1787 optical biopsies; $P = .012$). The intraclass correlation of reliability was high (0.759–0.916). Of the 17 cases with diagnosed CD, 16 (94%) were diagnosed correctly using CEM but only 13 (76%) had detectable histopathology changes. The procedure was safe and well-tolerated.** ***Conclusions:*** **CEM effectively diagnoses and evaluates CD severity in vivo. This promising technique has the potential to improve endoscopy efficiency.**

With a prevalence of up to 1 in 67, celiac disease (CD), or gluten-sensitive enteropathy, is an underrecognized disease that may be complicated by nutritional deficiencies, infertility, malignancy, and osteoporosis.[1] Current macroscopic assessment by endoscopy alone is inadequate, and the diagnosis of CD relies on biopsy and histologic assessment.[2] The endoscopic features of nodular mosaic mucosa, scalloping and flattening of folds, correlate with severe villous atrophy, but overall sensitivity and positive predictive value in diagnosing CD is poor, even when zoom endoscopy is used.[3–5]

The diagnosis of CD, therefore, relies on histologic assessment of duodenal biopsies. Even with this approach, patchy CD changes or changes detected only in the jejunum may result in sampling error.[6] Nonrepresentative specimens, poor sampling quality, tangential sectioning, or failure to orientate tissue correctly after biopsies also may generate a false-negative result.[7,8] Rarely, forceps biopsies are complicated by bleeding or bowel perforation.[9,10] Histologic processing and interpretation is inefficient, may cause delays, and increases cost.

Confocal endomicroscopy (CEM) is a novel endoscopic technology that permits simultaneous macroscopic and real-time microscopic imaging of the gastrointestinal mucosa. A miniaturized laser confocal microscope is incorporated into the tip of a flexible endoscope. The 1000-fold magnified image of in situ living tissue histology has a sufficiently high resolution to distinguish cellular and subcellular structures.[11] CEM can rapidly acquire in vivo mucosal images at variable depths controlled by the user. Microscopic imaging is en face across a horizontal plane, does not require orientation of biopsied tissue, and avoids forceps trauma and crush artifacts. Therefore, CEM may immediately diagnose or exclude CD, assess severity, direct targeted 'smart' biopsies to improve the yield of biopsies, reduce the cost of histologic processing and interpretation, and avoid the complications of forceps-induced trauma and artifact. This study defined the CEM features of CD; evaluated its diagnostic sensitivity, specificity, and accuracy compared with forceps histopathology; and determined the CEM response

Abbreviations used in this paper: CCS, confocal celiac score; CD, celiac disease; CEM, confocal endomicroscopy; CH, crypt hypertrophy; D1 to D4, first to fourth parts of the duodenum; GFD, gluten-free diet; ROC, receiver operating characteristic; VA, villous atrophy.

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after treatment with a gluten-free diet. We found that CEM was highly accurate in identifying features of CD that correlated well with histopathology.

Methods

Subjects

This prospective study recruited subjects from a university hospital gastroenterology ambulatory clinic. Consecutive patients were recruited with known CD on treatment who required an endoscopy to evaluate mucosal recovery, or suspected CD for initial diagnosis. The latter included patients with nonspecific diarrhea or bloating, weight loss, iron deficiency, increased antigliadin antibody levels, and gluten-intolerance symptoms. Patients were classified as newly diagnosed untreated CD, CD treated with a gluten-free diet (GFD), or controls without CD, based on clinical and histology information revealed after data entry and data-lock. Controls by definition had never been labeled as having CD, had normal duodenal histology on a gluten-containing diet, negative tissue glutaminase serology in the absence of immunoglobulin A deficiency, and absent CD susceptibility HLA DQ2/DQ8 genotypes. The patients' clinical information was blinded to the endoscopist and remained blinded during data entry until data analysis. Pregnancy, lactation, and history of allergic reactions to fluorescein were exclusion criteria. Patients, or their guardians if they were minors, provided written informed consent for the study, which was approved by the Human Research Ethics Committee of Sydney South West Area Health Service, Sydney, Australia (registration code 05/093).

Equipment

Confocal gastroscopy (Pentax EC-3870FK, Pentax, Tokyo, Japan) was performed prospectively under conscious sedation. The lateral resolution of the confocal image was 0.7 μm and optical plane thickness was 7 μm. The depth of imaging can be controlled from the mucosal surface down to a depth of 250 μm. The fluorescent contrast agents used were intravenous fluorescein sodium (10%; Pharmalab, Lane Cove, New South Wales, Australia) and topical acriflavine hydrochloride (0.05%; Sigma Pharmaceuticals, Clayton, Victoria, Australia) given in aliquots to optimize imaging. Fluorescein labels vascular structures, cell cytoplasm, and the interstitial space, and is used commonly for ophthalmologic retinal vascular imaging and rarely induces allergic reactions. Acriflavine labels the superficial mucosal cells including the nuclei and has antiseptic properties. The staining of cell nuclei has the theoretic risk of mutagenicity, although there is little evidence of this occurring in human beings.[12]

Endoscopic Procedure

CEM was performed by 2 endoscopists (R.W.L.L., N.Q.N.) already experienced with the technology. For validation, CEM images and forceps biopsies of the same sites were taken sequentially at standardized locations at 5 small intestinal sites: (1) duodenal bulb, (2) D2 proximal to the ampulla of Vater, (3) D2 at the level of the ampulla of Vater, (4) D3 at the transverse portion of the duodenum, and (5) jejunum distal to the ligament of Treitz. Seven to 10 CEM images of different mucosal depths were collected from each site from the standardized locations for every forceps biopsy specimen and images were stored in a computer database for blinded assessment independent of the clinical and histology data after patient recruitment.

Histopathology

Small intestinal specimens were taken precisely matched to the CEM imaging sites. During stabilization of the endoscope for CEM imaging, the suction channel induces an erythematous patch adjacent to the CEM laser window. This CEM imaging site was targeted with forceps relative to this patch. Open-cup biopsies were oriented and processed using standard methodologies and were assessed by 2 experienced blinded histopathologists independently and subsequently reviewed during a single session for internal consistency. Grading was scored according to the Marsh classification as defined by Oberhuber et al.[13] Marsh 1 grade represented mucosal infiltration with intraepithelial lymphocytes in excess of 30 per 100 surface enterocytes; Marsh 2 grade represented crypt hyperplasia; Marsh 3 grade represented villous atrophy and was subclassified to A (partial villous atrophy), B (subtotal villous atrophy), and C (total villous atrophy); and Marsh 4 described a flat atrophic mucosa. Isolated intraepithelial lymphocytosis is not specific to CD.[14]

CEM Interobserver Agreement and Comparison With Histopathology

Before embarking on the definitive study, we standardized and defined the CEM changes of CD. The CEM features of 7 CD patients and 2 controls compared against known final histopathology was performed by an endoscopy focus group. A second cohort of patients then was recruited prospectively for the validation study. By using the CEM depth control, 10 progressive scans from the superficial surface down towards the lamina propria were obtained. Superficial CEM images showed the superficial surface villi and luminal spaces whereas deep images collected images of basal villi. The CEM changes chosen to represent villous atrophy (VA) and crypt hypertrophy (CH) had to be clearly recognizable, objectively defined, and feature prominently in cases but absent in controls.

Confocal Celiac Score, Reliability, and Sensitivity to Change

Increasing severity of CD was defined as more widespread disease and a higher histologic Marsh grade. The Confocal Celiac Score (CCS) is a ratio of images with

definite features of CD (VA or CH) against total images. This numeric value between 0 and 1 represented a scale of CD severity, with a higher score representing greater severity. The CCS could be calculated separately for VA by assessing only superficial scans, CH by assessing only deep scans, or combined by assessing all scans. The CCS allowed for disease severity to be validated against the parameters of construct validity (correlation with Marsh grading), discriminate ability (differentiation of treated CD, untreated CD, and controls), sensitivity to change (an individual's change in CCS after institution of treatment), and reliability (the constancy of CCS along the bowel segments in CD and controls). Treatment-naive CD subjects were invited for a follow-up procedure after at least 6 months of a GFD to determine the improvement of CCS on treatment. Statistical reporting was assessed on a per-patient basis.

Statistics

SPSS 15.0 (SPSS Inc, Chicago, IL) was used for statistical calculations. The chi-square test was used for comparison of categoric variables. The kappa score of interobserver agreement was calculated for the recognition of VA, CH, and the overall diagnosis of CD. The per-patient receiver operating characteristic (ROC) curve including 95% confidence intervals of sensitivity vs 1-specificity (or false-positive rate) was generated for various cut-off definitions of a positive diagnosis of VA, CH, and overall CD. The Pearson correlation and *R*-squared were used for comparing the continuous scale of the CCS with the Marsh grading transformed into a linear scale for the purpose of statistical analysis. Discriminate ability was calculated with the Kruskal–Wallis test and the Wilcoxon signed-rank test measured the sensitivity of CCS to change on GFD. Reliability and consistency of findings across different small-bowel segments were analyzed per segment by using the intraclass correlation.

Results

A total of 31 patients (9 males, 22 females; median age, 41 y; range, 14–83 y) were recruited prospectively; 17 with CD (6 pretreatment, 11 on a GFD) and 14 controls. This yielded 7019 optical biopsies and 326 paired forceps biopsies. Four treatment-naive CD patients had a follow-up CEM after a mean of 362 days (range, 147–427 days) of treatment with a GFD.

Definition of CD on CEM and Interobserver Agreement

The CEM features of VA and CH, showing similarities in comparison with scanning electron microscopy, are shown in Figure 1. Normal villi (Figure 1*A*) are shown in contrast to VA, which was defined by the presence of 5 or fewer blunt-shaped villi seen on superficial scans (Figure 1*B*). Normal deep imaging (Figure 1*C*) contrasts CH, which was defined as 1 or more crypts on small-bowel deep CEM imaging (Figure 1*D*). Crypts appeared as round structures with radiating enterocytes surrounding the slit-like central openings and almost invariably multiple crypts were found. Scanning electron microscopy images of normal small-bowel villi (Figure 1*E*) and crypt hypertrophy (Figure 1*F*) are included to aid orientation and for illustrative and comparative purposes only and were not part of the study protocol. By using these CEM features, the kappa scores of interobserver agreement were 0.67, 1.00, and 1.00 in the diagnosis of VA, CH, and in combination, respectively, producing an overall diagnosis of CD. These defined CEM features were used for the prospective study in a new cohort of patients.

ROCs, Sensitivity, and Specificity

The ROC curves of CEM were graphed to illustrate the sensitivity thresholds relative to specificity. First, CEM ROC was compared with a true diagnosis of CD (irrespective of Marsh grading) to determine whether CEM could diagnose mild CD that did not have histologic changes. Second, CEM ROC was compared with histologic findings of CD (irrespective of actual diagnosis) as a direct comparison of optical biopsies with standard histopathology. There was excellent accuracy with the ROC areas under the curve of 0.946 (95% confidence interval, 0.860–1.031) in comparison with a true diagnosis of CD, and 0.959 (95% confidence interval, 0.897–1.021) in comparison with histologic features of CD (Figure 2). CCS thresholds were as follows: a CCS of 0.06 or higher accurately diagnosed CD and a CCS of 0.22 or higher reflected histopathologic features of CD. A CCS of 0.06 or higher had a sensitivity of 94.1% and a specificity of 92.3% in making a true diagnosis of CD. A CCS of 0.22 or higher had a sensitivity of 84.6% and a specificity of 94.1% in detecting histopathologic features of CD (Figure 2). Four subjects with treated CD had normal histopathology. Of these 4, CEM correctly diagnosed 3 (75%) as having CD based on a CCS of 0.06 or higher. Only 1 of the 17 patients with CD had a CCS of less than 0.06.

Construct Validity and Discriminate Ability

Histopathology according to Marsh grades correlated well and linearly with the CCS (*R*-squared, 0.756; $P < .0001$; Figure 3). CEM accurately differentiated controls from patients with CD, and the median CCS of controls, treated CD patients, and untreated CD patients were 0.02, 0.26, and 0.54, respectively ($P < .0001$). CH had better discriminating characteristics than VA in the differentiation of the 3 patient populations as shown in Figure 4. The median CCS and interquartile ranges of controls, treated CD patients, and untreated CD patients in the findings of VA, CH, or combined parameters ($P = .003$, $P < .0001$, $P < .0001$), respectively, are disclosed in the table within Figure 4.

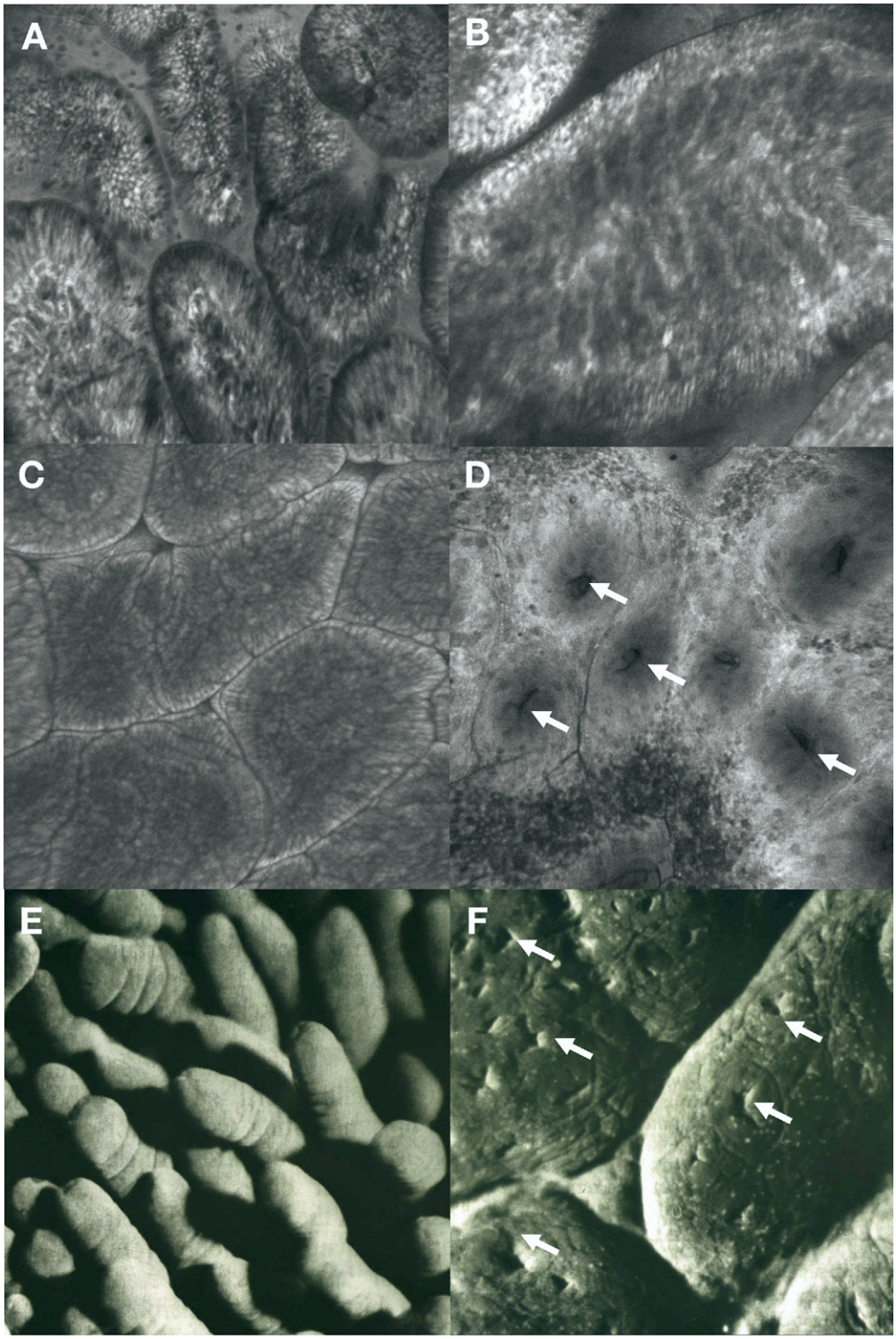
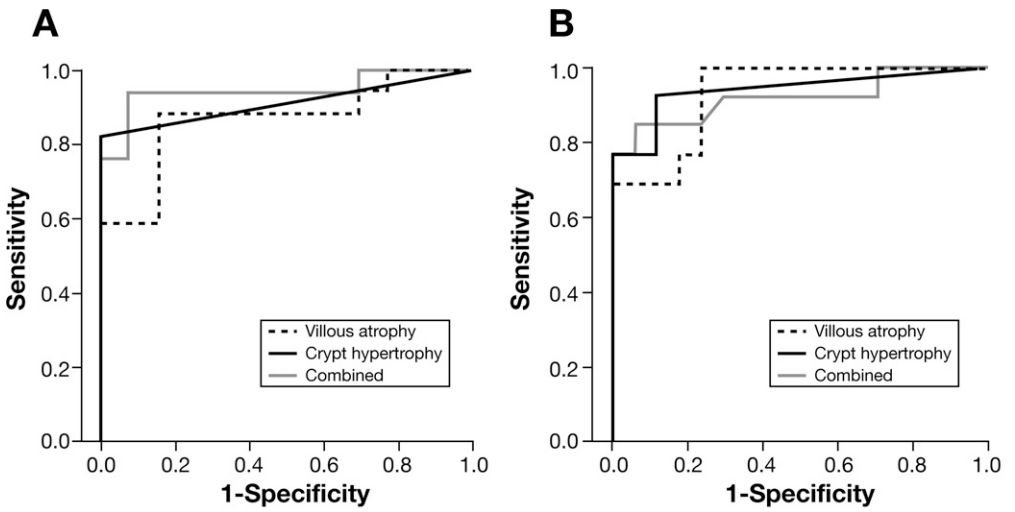


Figure 1. Superficial CEM imaging showing (*A*) normal small intestinal villi, (*B*) villous atrophy, (*C*) normal deep imaging, and (*D*) crypt hypertrophy. For illustrative purposes (*E*) normal villi and (*F*) crypt hypertrophy on scanning electron microscopy are shown. *White arrows* denote crypt openings. All CEM images are shown at 1000-fold magnification.

Sensitivity to Change and Reliability

Four treatment-naive patients (1787 CEM images) had a follow-up CEM at a mean of 362 days (range, 147–427 days) after the initial procedure. All had been assessed and educated by an accredited dietician, symptomatically improved after commencement on a GFD, or their CD serology had normalized. The median pre-GFD CCS of VA, CH, and combined parameters were 0.64, 0.64, and 0.64, respectively. After treatment, the median post-GFD CCS for VA, CH, and combined parameters were 0.15, 0.12, and 0.14, respectively ($P = .012$).



True Diagnosis	AUC	95% CI	*P**
Villous atrophy	0.869	0.736 - 1.001	.0001
Crypt hypertrophy	0.912	0.799 - 1.025	<.0001
Combined	0.946	0.860 - 1.031	<.0001

Histological Features	AUC	95% CI	*P**
Villous atrophy	0.894	0.771 - 1.016	<.0001
Crypt hypertrophy	0.939	0.841 - 1.036	<.0001
Combined	0.959	0.897 - 1.021	<.0001

*Null hypothesis that true area = 0.5

True Diagnosis	Sensitivity	Specificity
Villous atrophy (CCS>0.12)	88.2%	70.6%
Crypt hypertrophy (CCS>0.06)	82.4%	100%
Combined (CCS>0.06)	94.1%	92.3%

Histological Features	Sensitivity	Specificity
Villous atrophy (CCS>0.31)	69.2%	100%
Crypt hypertrophy (CCS>0.06)	92.3%	88.2%
Combined (CCS>0.22)	84.6%	94.1%

Figure 2. ROC curves, area under curve (AUC) with 95% confidence intervals, sensitivity and specificity of CEM in the diagnosis of VA, CH, and combined parameters in comparison with (*A*) a true diagnosis of CD and (*B*) histologic features of CD.

Reliability measured the similarity and consistency of the CEM findings at all 5 standardized small-bowel locations using intraclass correlation for each of the 3 patient groups. The intraclass correlation for controls was 0.916 ($P < .0001$), for treated CD patients was 0.875 ($P < .0001$), and for untreated CD patients was 0.759 ($P = .010$). This indicated very good CEM reliability throughout the small bowel in all 3 patient groups.

CCS and Optical Biopsy Yield

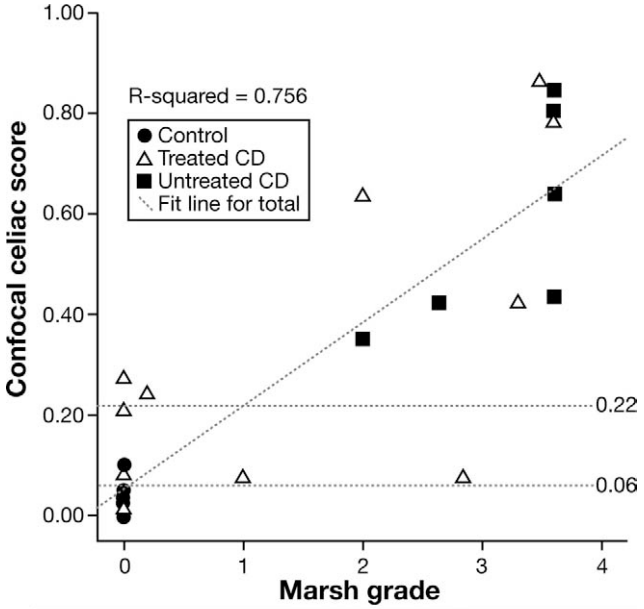
The yield of CEM in detecting CD in different sites of the small bowel was assessed using the CCS. The highest yield of CEM changes as indicated by the highest median CCS was in the duodenal cap (D1). In those with treated CD, the median CCS of the 5 standardized small intestinal sites from proximal to distal were 0.53, 0.15, 0.24, 0.17, and 0.06. In patients with untreated CD, the median CCS were 0.62, 0.54, 0.55, 0.25, and 0.30. These changes, however, were not statistically significant.

Complications

A mean of 4 mL of intravenous fluorescein and 10 mL of topical acriflavine were used per patient. CEM was safe and well tolerated without any serious adverse events. All patients had transient mild yellow discoloration of urine caused by the excretion of fluorescein. CEM increased the procedure time by a mean of 15 minutes because of the protocol requiring an extensive number of optical biopsies with matched forceps biopsies. In fact, a diagnosis of CD could be established on initial CEM imaging in some patients as soon as CH was found, given its high specificity. A survey of patients after the procedure uniformly showed a preference for immediate feedback rather than waiting for a formal biopsy report. No patients refused a follow-up procedure if deemed necessary.

Discussion

CEM correlated well with the histology of conventional forceps and our preliminary findings suggest that it also may detect minor abnormalities in treated CD that were not detected by histology. The high accuracy of CEM has potential clinical utility in diagnosing or excluding CD in vivo and minimize, if not replace, the need for biopsies. This may result in improvement in endoscopy workflow, reduced histopathology dependence, and greater efficiency and patient satisfaction, but further studies are required to support this. The ROC curves indicated high sensitivity and specificity not only when compared with established histopathologic features, but also in detecting diagnosed CD. This indicated that despite seemingly optimal treatment in established CD, CEM still could detect mild microscopic small-bowel changes. The sensitivity of CEM in detecting CD changes



Marsh grade	Controls (n=14)	Treated celiac disease (n=11)	Untreated celiac disease (n=6)
Normal	14	4	0
Grade 1	0	2	0
Grade 2	0	2	1
Grade 3A	0	1	1
Grade 3B	0	2	0
Grade 3C	0	0	4
Grade 4	0	0	0

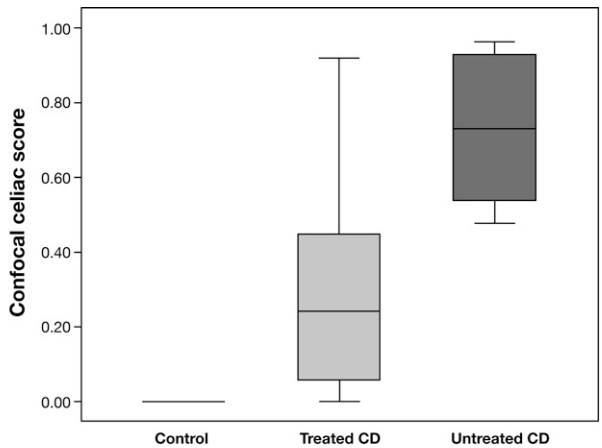
Figure 3. Correlation of the confocal celiac score with the Marsh grading of severity of CD (*R*-squared, 0.756). A CCS of 0.06 or higher indicates a true diagnosis of CD whereas the threshold of detectable histopathologic features of CD is a CCS of 0.22 or higher.

was therefore 94% in comparison with 76% by conventional histopathology. The CCS thresholds of 0.06 or higher and CCS of 0.22 or higher were predicted for a true diagnosis of CD and histopathologic changes, respectively. We hypothesized that the advantages of in situ CEM imaging may be through the avoidance of forceps-induced tissue crush artifact, misalignment of specimens, or poor microtome preparation of paraffin-embedded specimens. Diagnosing minor CD activity may be clinically important because inadequate treatment with ongoing immunologic damage may lead to long-term adverse sequelae.

CEM is not only safe and accurate in the immediate diagnosis of CD, but can grade disease severity. As a ratio of abnormal-to-normal images, CCS was independent from the total number of images acquired and reflected overall mucosal damage. The CCS is an easy-to-use quantitative score that correlated well and linearly with histologic assessment, with excellent validity, discriminate ability, sensitivity to change, and reliability. In contrast, the Marsh classification of histologic biopsies is categoric rather than a progressive scale, and intragrade changes may not be observable. The safety of acriflavine and similar compounds is poorly understood in human beings. Animal and cell culture data suggest it causes DNA damage and has the potential for mutagenicity.[12] The use of acriflavine in human beings for the purpose of confocal staining should be considered experimental and further study on safety is needed at this time. Administration of intravenous fluorescein alone may be sufficient for the evaluation of CD because detection of CH and VA does not rely on nuclear staining provided by acriflavine.

CEM alone cannot diagnose intraepithelial lymphocytosis. Specific immunofluorescent CD4 labeling of gamma-delta lymphocytes, however, may help define CEM intraepithelial lymphocyte characteristics. Longitudinal studies have shown that intraepithelial lymphocytosis in the absence of Marsh grades 2 and 3 changes was a poor predictor of CD on follow-up evaluation.[15] In this study, CEM was able to diagnose CD independently from intraepithelial lymphocytosis, and mild VA and CH on CEM is likely to support a diagnosis of CD with or without intraepithelial lymphocytosis. Other case reports also have shown the ability of CEM to detect CD changes, but they did not perform any prospective large-scale systematic validation or evaluate severity.[16,17]

Endoscopy often takes on a secondary role to biopsies in the evaluation of gastrointestinal diseases. However, this study suggests that with current advances in technology, in vivo living tissue histopathologic evaluation is possible during endoscopy. CEM already has shown its



Confocal celiac score	Control (n=3,013 images)	Treated CD (n=1,935 images)	Untreated CD (n=2,071 images)	*P*
Villous atrophy - median - 25-75% QR	0.04 0.02 - 0.10	0.38 0.11 - 0.77	0.46 0.16 - 0.69	.003
Crypt hypertrophy - median - 25-75% QR	0.00 0.00 - 0.00	0.26 0.09 - 0.57	0.73 0.53 - 0.94	<.0001
Combined parameters - median - 25-75% QR	0.02 0.00 - 0.05	0.26 0.08 - 0.67	0.54 0.40 - 0.82	<.0001

Figure 4. The discriminate ability of the median CCS, their interquartile ranges (IQR) in differentiating controls from treated CD patients and untreated CD patients in identifying crypt hypertrophy and the Kruskal–Wallis test *P* values.

clinical utility in a variety of gastrointestinal illnesses including the diagnosis of colorectal neoplasia,[18] *Helicobacter pylori*,[19] gastric cancer,[20] Barrett's esophagus,[21] collagenous colitis,[22] and ulcerative colitis surveillance[23] among other conditions.[24] This study systematically evaluated the CEM characteristics of treated and untreated CD, defined a potential role for CEM in this disease, and included longitudinal CEM follow-up evaluation to determine CEM accuracy and reversibility of CEM changes after treatment. CD is a common and underrecognized condition that is evaluated commonly by endoscopists and real-time diagnosis is advantageous to both patients and endoscopy units. The high specificity and ease in identifying crypt hypertrophy allowed CD to be diagnosed rapidly in this study. Furthermore, CEM may increase the yield of biopsies through targeting of lesions in patients with patchy CD, or who have a coagulopathy or platelet dysfunction to minimize biopsy-induced bleeding. In our experience, CEM can be learned easily and should not be limited to highly specialized centers. In vivo diagnosis is possible after training and highly specific features allow for instantaneous real-time diagnosis of CD. The long-term cost effectiveness of the instrument needs to be determined. In conclusion, this study showed that CEM was accurate compared with histopathology in identifying and evaluating CD severity. Further studies are encouraged to define its clinical utility in the management of patients or under evaluation for CD.

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