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Many Patients With Irritable Bowel Syndrome Have Atypical Food Allergies Not Associated With Immunoglobulin E

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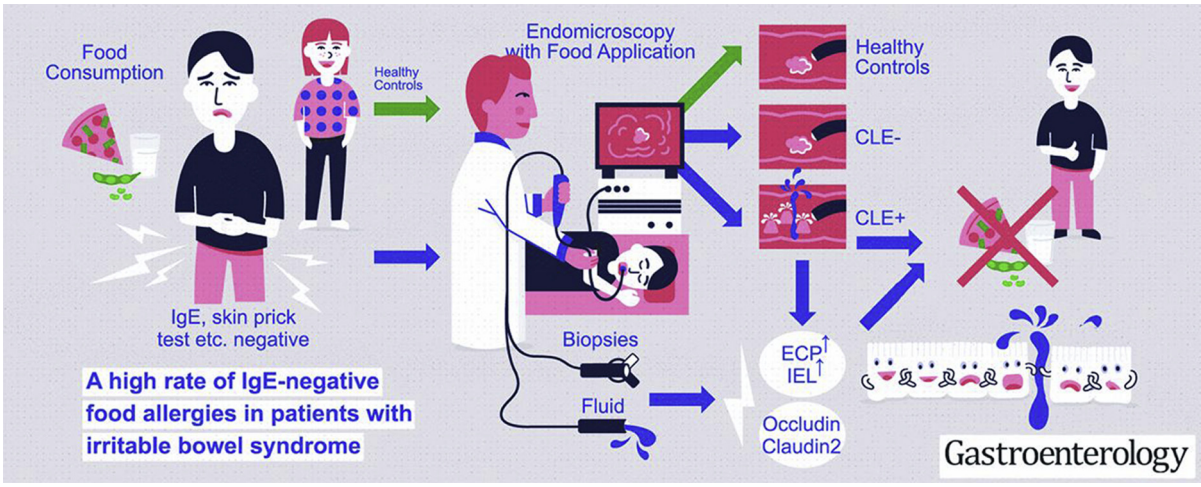

See editorial on page 15.

BACKGROUND & AIMS: Confocal laser endomicroscopy (CLE) is a technique that permits real-time detection and quantification of changes in intestinal tissues and cells, including increases in intraepithelial lymphocytes and fluid extravasation through epithelial leaks. Using CLE analysis of patients with irritable bowel syndrome (IBS), we found that more than half have responses to specific food components. Exclusion of the defined food led to long-term symptom relief. We used the results of CLE to detect reactions to food in a larger patient population and analyzed duodenal biopsy samples and fluid from patients to investigate mechanisms of these reactions. **METHODS:** In a prospective study, 155 patients with IBS received 4 challenges with each of 4 common food components via the endoscope, followed by CLE, at a tertiary medical center. Classical food allergies were excluded by negative results from immunoglobulin E serology analysis and skin tests for common food antigens. Duodenal biopsy samples and fluid were collected 2 weeks before and immediately after CLE and were analyzed by histology, immunohistochemistry, reverse transcription polymerase chain reaction, and immunoblots. Results from patients who had a response to food during CLE (CLE^+) were compared with results from patients who did not have a reaction during CLE (CLE^-) or healthy individuals (controls). **RESULTS:** Of the 108 patients who completed the study, 76 were CLE^+ (70%), and 46 of these (61%) reacted to wheat. CLE^+ patients had a 4-fold increase in prevalence of atopic disorders compared with controls ($P = .001$). Numbers of intraepithelial lymphocytes were significantly higher in duodenal biopsy samples from CLE^+ vs CLE^- patients or controls ($P = .001$). Expression of claudin-2 increased from crypt to villus tip ($P < .001$) and was up-regulated in CLE^+ patients compared with CLE^- patients or controls ($P = .023$). Levels of occludin were lower in duodenal biopsy samples from CLE^+ patients vs controls ($P = .022$) and were lowest in villus tips ($P < .001$). Levels of messenger RNAs encoding inflammatory cytokines were unchanged in duodenal tissues after CLE challenge, but eosinophil degranulation increased, and levels of eosinophilic cationic protein were higher in duodenal fluid from CLE^+ patients than controls ($P = .03$). **CONCLUSIONS:** In a CLE analysis of patients with IBS, we found that more than 50% of patients could have nonclassical food allergy, with immediate disruption of the intestinal barrier upon exposure to

food antigens. Duodenal tissues from patients with responses to food components during CLE had immediate increases in expression of claudin-2 and decreases in occludin. CLE$^+$ patients also had increased eosinophil degranulation, indicating an atypical food allergy characterized by eosinophil activation.

Keywords: Diet; Eosinophil; Food Allergy; Tight Junction.

Irritable bowel syndrome (IBS) is a common and global disorder with a major socioeconomic impact.[1–3] Food has long been suspected to cause symptoms in IBS patients, but evidence has remained scarce despite reports of the onset of symptoms after meals.[4] Dietary exclusion of fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (known as *FODMAPs*) can improve symptoms in some patients[4,5] but does not appear to be superior to standard IBS diets based on a balanced lifestyle; regular meals; and avoidance of coffee, spices, and fatty foods.[6] Exclusion of single foods such as wheat or gluten has effectively resolved IBS and intestinal inflammation in some patients.[7,8] However, dietary studies can neither reliably prove the pathogenic role of food nor allow insights into cellular or physiological changes that lead to the symptoms. Consequently, objective display and measurement of the pathology of the gastrointestinal response to defined foods is required to allow a valid and better definition of IBS patients who react to specific nutrients, both to understand the underlying pathophysiology and to identify the offending food for an evidence-based exclusion diet.

We recently showed that confocal laser endomicroscopy (CLE) can provide such an objective measure.[9] During CLE, when the specified food is applied to the duodenum via the endoscope channel, immediate changes can be seen and quantified at the cellular level in real time, including an increase in intraepithelial lymphocytes (IELs) and fluid extravasation through epithelial leaks. Of 36 patients with IBS who reported food-related abdominal symptoms, 22 reacted to 1 of 4 tested food antigen mixtures: wheat, milk, soy, or yeast (CLE$^+$). Symptoms improved dramatically within 3 months and over 1 year of follow-up when the reacting antigen was removed from the diet; no improvement was noted in 12 patients who did not react to any of the applied foods (CLE$^-$).[9] Although changes occurred at the duodenal mucosal level within 5 minutes of food exposure, clinical symptoms were delayed by several hours in some patients, making classical oral provocation studies problematic. CLE may thus serve as an objective criterion standard to define the presence of adverse, likely immune-mediated allergic reactions to specific food components, despite negative immunoglobulin (Ig) E and skin-prick testing.[9]

Our first feasibility study included only a small number of patients and did not attempt to investigate the underlying cellular and biochemical pathophysiology. Here, we use CLE as the criterion standard to prove the reaction to food in a larger patient population. Moreover, histologic and molecular methodologies were used to further analyze underlying mechanisms of the gastrointestinal reaction to food in this controversial and supposedly "functional" disorder.

WHAT YOU NEED TO KNOW

BACKGROUND

Many patients with irritable bowel syndrome (IBS) believe that their symptoms are related to the foods they eat. We used confocal laser endomicroscopy (CLE), combined with application of defined foods to the duodenum via the endoscope channel, to observe immediate changes in duodenal tissue and cells in real time in patients with IBS.

NEW FINDINGS

In patients with a reaction to defined food components – prominently wheat – during CLE, IBS symptoms were reduced when these food components were withdrawn from the diet. A mucosal permeability disorder and eosinophil activation were found in patients with responses to food allergens during CLE.

LIMITATIONS

The final analysis included 108 patients; larger studies in other populations are needed to confirm these findings.

IMPACT

More than 50% of patients with IBS could have an atypical food allergy, with negative results from skin tests and serologic analysis of immunoglobulin E.

Methods

Participants

Patients who believed that their symptoms were related to food ingestion[4]; fulfilled the IBS Rome III criteria[10]; and had moderate to severe daily symptoms for at least 1 year, as verified by Francis IBS symptoms score and visual analogue scale (VAS) well-being score questionnaires (see Supplementary Materials) were included in the study. Esophagogastroduodenoscopy/colonoscopy results were normal, and inflammation/infection markers in serum/stool, total IgE, IgE serum antibodies to common food antigens, and skin-prick test and celiac serology (IgA [IgG] anti-transglutaminase) results were all negative, including the celiac gene test for HLA-D2 and -DQ8 in those patients with increased IELs on histologic analysis. Genetic testing was performed only in patients with >30 IELs/100 epithelial cells and, when results were positive, led to patient exclusion. Patients were not tested for IgG4 to food antigens.

Exclusion Criteria. Patients with well-defined gastrointestinal diseases (IBS excluded), IgE positivity to foods, or a known allergy to methylene blue or fluorescein were excluded. Outpatients without IBS symptoms and negative IBS scoring

Abbreviations used in this paper: **CLE, confocal laser endomicroscopy; ECP, eosinophil cationic protein; HC, healthy control; IBS, irritable bowel syndrome; IEL, intraepithelial lymphocytes; Ig, immunoglobulin; IL, interleukin; MBP, major basic protein; PCR, polymerase chain reaction; Th, T helper; VAS, visual analogue scale.**

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results, undergoing CLE for Barrett's esophagus, served as healthy controls (HCs). The study was approved by the ethics committee of the Christian Albrechts University (Kiel, Germany). Patient data were recruited from unpublished patients and data from the initial study and second study, which was also registered at clinicaltrials.gov (NCT01139424). All authors had access to the study data and reviewed and approved the final manuscript. Patients could opt out of the study at any time. For all patients and HCs, a careful family history was taken for atopic disorders.

Study Design

This study is part of a more extensive double-blind, randomized, crossover dietary intervention study in IBS patients. It focuses on the cellular, immunologic, and biochemical pathophysiology of those patients with IBS who reacted to food antigens (CLE^+) vs those patients who did not react (CLE^-) or vs HCs. The other part of the study investigated the patients' somatization and well-being at baseline and after several diets, quantifying functional symptoms. It followed a complex design using 3 different diets, 4 established questionnaires, and a well-being score, and those results will be reported separately. The overall study flow chart is provided in Supplementary Figure 1.

We hypothesized that in patients with daily IBS symptoms who reported an improvement on food reduction or fasting, daily and regularly ingested food components could be responsible for their symptoms, which would then improve when the reacting food component was withdrawn from the diet. These reactions to food may cause pathophysiological changes of the gut mucosa, possibly due to an atypical allergy.

Two established scoring systems were used in the present report: (1) the Francis IBS symptom score and (2) the VAS for patient well-being for baseline[9] (see Supplementary Materials) to assess initial IBS symptoms. This allowed us to categorize patients as having moderate or severe disease before they were included in the CLE study and to confirm that the HC group of participants with Barrett's esophagus did not have IBS. These were also used to evaluate improvement of symptoms after dietary exclusion of the antigen that tested positive at CLE. Such a positive reaction at CLE was defined as postchallenge increase of epithelial leaks with secretion of fluorescein-labeled plasma into the lumen and the presence of fluorescent signal between enterocytes. Both dynamic changes would cause the intervillous space to widen, with a marked change of color from black to white. Symptoms needed to improve after the causative specific allergen defined by CLE was excluded from the diet, as we described previously.[9]

A minimum of 2 weeks after baseline esophagogastroduodenoscopy with 8 duodenal biopsies and fluid sampling (1–1.5 mL) with an endoscopic retrograde cholangiopancreatography catheter, CLE was performed with sequential application of 5 different main food antigens. Before each challenge, baseline CLE images were captured and stored, as was the reaction of the duodenal mucosa to the applied food antigens. The images of the CLE findings were interpreted by a separate team, not present at and blinded to the results of the CLE procedure. They were also blinded to the 2 patient groups: HC vs IBS. CLE images were evaluated randomly in batches up to 2 weeks after endoscopy. Patients were categorized as CLE^+ or CLE^- depending on whether the changes mentioned were present. Immediately after CLE, biopsy samples were taken for histologic analysis, and duodenal fluid was collected at the end of every CLE^+ procedure, in the CLE^- patients and HCs after all provocations had been completed and documented, approximately 10 minutes after the last provocation test.

Those patients whose symptoms improved according to the standard IBS Francis symptom score and the VAS score after the antigen/allergen exclusion diet were regarded as reactive to the food antigen that tested positive at CLE in the present study (Supplementary Figure 2). Baseline and postexposure biopsy samples and duodenal fluids were analyzed in CLE^+ and CLE– patients and HCs using histology/immunohistochemistry, polymerase chain reaction (PCR), and protein expression to support our hypothesis of a food antigen/allergen–induced mucosal inflammatory reaction and tight junctional impairment. An in-depth medical history was taken of the patients and their first-degree relatives for underlying atopic diseases, but no specific test results were requested.

Endomicroscopy and Food Challenges

At CLE, images were acquired and analyzed as previously described at baseline and immediately after food exposure.[9] The following CLE events were quantified at baseline and after food provocation[9]: (1) density of IELs; (2) epithelial breaks/leaks of fluorescein into the lumen (per 1000 epithelial cells/5 different locations/5 different images); (3) presence of fluorescent signal between enterocytes; and (4) widening of the intervillous space due to fluorescein leaking through the mucosa, changing the color on imaging from black to white. A macroscopically visible and quantifiable change of at least 2 of these 4 parameters within 5 minutes of provocation, always including leakage (2) as the prime parameter, was defined as CLE^+ (Figure 1 and Supplementary Video).

Patients received sequential food challenges with 20 mL of a standardized solution/suspension of wheat, milk, soy, yeast, or egg white and a control substance (water with polysilane, simethicone), as described in the Supplementary Materials. The endoscopist was blinded to the food components applied to the mucosa except for cow's milk, which could be identified by its color.

Endoscopy for Duodenal Biopsies and Fluid Collection

Upper endoscopy was performed 2 weeks before CLE for baseline biopsies. A second set of biopsies and fluid collection were performed immediately after all food exposures in the CLE^- patients and HCs and after a positive reaction in the CLE^+ patients, approximately 10 minutes after the last application of food antigen/allergen during CLE. Each time, 8 biopsy samples were taken from the second part of the duodenum; fixed in formalin or snap-frozen for pPCR; analyzed for mucosal structure, eosinophils, and IELs with H&E histology; and stained for T cells (CD3, 1:500), occludin (1:500), and claudin-2 (1:400) (all Sigma Aldrich, Darmstadt, Germany) by immunohistochemistry, as detailed in the Supplementary Materials. IEL level $>$ 25/100 epithelial cells was regarded as pathologic.[11–13] A total of 900 epithelial cells were examined for localization and quantification of occludin and claudin-2 along the crypt-villus axis in a series of at least 50 consecutive cells of the crypt, lower half of the villi, and villous tip.

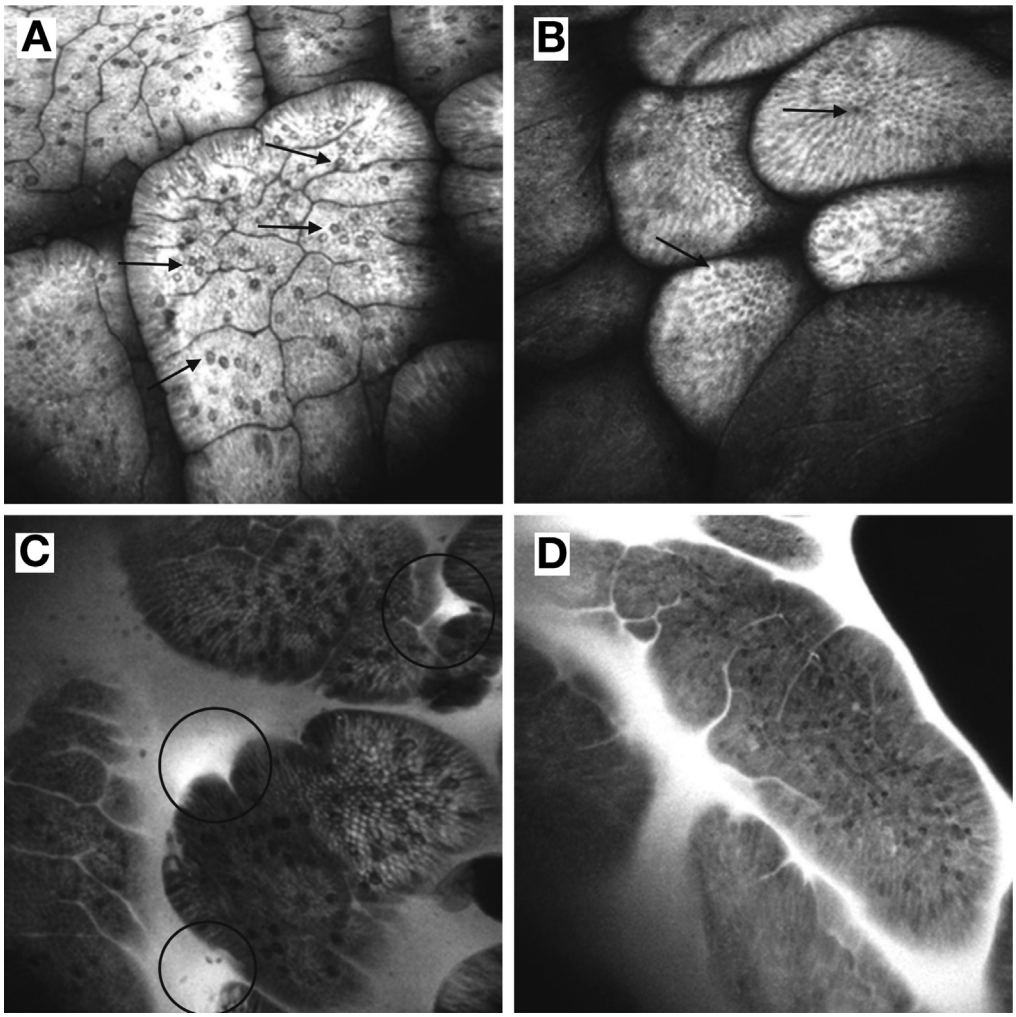

Figure 1. Still images of positive vs negative food reactions on CLE. (*A*) Baseline CLE image of a CLE^+ patient with multiple lymphocytes present (*arrows* point at IEL), compared with (*B*) low lymphocyte numbers in an HC (*arrows* point at IEL). (*C*) CLE image of a positive reaction to a food antigen with increased IELs, mucosal breaks/leaks, and intercellular extravasation of fluorescein-labeled plasma fluid into the widening intervillous space. The marked leakage of plasma changes the intervillous space images from black to white (*circles*). (*D*) End stage of a positive reaction.

RNA was extracted from snap-frozen biopsies, and complementary DNA was synthesized by using DNA polymerase (Eurogentec, Cologne, Germany). Gene expression levels of tight junction protein-1 (TJP-1), occludin, claudin-2 (Thermo Fisher Scientific, Foster City, CA), tumor necrosis factor-α, interleukin (IL) 4, IL-5, eosinophil cationic protein (ECP), and major basic protein (MBP) (Eurogentec, Cologne, Germany) were determined by quantitative reverse-transcription PCR by using the primers and probes listed in the Supplementary Materials. Results were normalized to expression levels of hypoxanthine-guanine phosphoribosyltransferase (HPRT) and expressed as fold increase.

ECP and α-tryptase were analyzed from the small amount of duodenal fluid (1.0–1.5 mL) that was recovered from the duodenum with an endoscopic retrograde cholangiopancreatography catheter and analyzed by fluorescence enzyme immunoassay in a Phadia 250 analyzer (Thermo Fisher Scientific), as detailed in the Supplementary Materials.

Statistics

No prestudy sample size calculation was performed, because insufficient prior data on effect strength were available. Results of immunohistochemistry are presented as mean and/or median and were analyzed with Wilcoxon rank sum test or Mann-Whitney test. All other data are presented as mean ± standard deviation and were analyzed with nonparametric (Wilcoxon signed rank test) or parametric methods (unpaired/paired *t* test), as appropriate. Analyses were performed using IBM (Armonk, NY) SPSS statistics software for Windows, version 21.0. A 2-sided *P* value $> .05$ was considered significant.

Results

A total of 170 IBS patients were enrolled into the study (95 female; age range, 18–76 years; mean age, 44.4 ± 15.4 years). Eleven patients had other diseases, such as mastocytosis or endometriosis, among others, or became pregnant; 4 had serious clotting disorders. Of the remaining 155 patients, 47 did not complete the full study because they regarded this as either too cumbersome or because symptoms improved with the first randomly assigned diet, resulting in refusal to continue with the randomized diet exposure. Overall, 108 IBS patients (52 with diarrhea-based IBS [IBS-D], 42 with mixed IBS [IBS-M], and 14 with constipation-based IBS [IBS-C]) completed the study: 76 patients were CLE^+ (an additional 6 patients reacted to pepsin-trypsin–digested gliadin,[14] which was tested outside

Table 1. Age Distribution of Patient Subgroups According to Identified Allergens

Group	Overall	CLE$^+$ (wheat)	CLE$^+$ (yeast)	CLE$^+$ (milk)	CLE$^+$ (soy)	CLE$^+$ (egg)
CLE$^+$						
n	76	46	15	7	5	3
Mean age (*y*) ± standard deviation	42.7 ± 14.9	40,6 ± 13,9	43,4 ± 18,6	42,2 ± 9,2	46,9 ± 16 ,5	41,2 ± 1 3,5
Range (*y*)	20–76	20–76	22–72	27–58	21–62	25–70
% of overall	100.0	60.5	19.7	9.2	6.6	3.9
CLE$^-$						
n	32					
Mean age (*y*) ± standard deviation	43.8 ± 15.8					
Range	22–75					
HC						
n	14					
Mean age (*y*) ± SD	49.8 ± 12.8					
Range	39–78					

of the study), and 32 patients were CLE$^-$. Typical positive reactions are illustrated in Figure 1 and the Supplementary Video. The characteristics of the 76 CLE$^+$ patients, their reactions to the different food antigens, the 32 CLE$^-$ patients, and HCs are shown in Table 1. Confirming our prior results in a smaller cohort of food-sensitive IBS-patients,[9] the majority of the CLE$^+$ patients reacted to wheat (n = 46, 60.5%), and the remainder reacted to yeast (n = 15, 20%), milk (n = 7, 9.2%), soy (n = 5, 6.6%), and egg white (n = 3, 4%). Nine patients reacted to 2 of the tested food antigens.

Patients' Clinical History

Patients' and/or their first-degree relatives' medical histories showed a highly increased prevalence of atopic disorders in CLE$^+$ patients, especially related to inhaled allergens, regardless of which food antigens they reacted to (68.9% vs 38.3 vs 15.4% in the CLE$^+$ patients vs CLE$^-$ patients vs HCs, respectively; $P = .001$) (Figure 2).

CLE findings

Baseline CLE findings before the administration of each food component were similar, irrespective of a more distal or proximal location in the duodenum to which the food was applied or whether it was the first or the last food challenge, as long as the reaction to food was negative. However, if a reaction to any of the food components took place, further food applications were discontinued, because such reaction would occur and be visualized throughout the accessible duodenum, invalidating further tests because of the absence of a baseline status. CLE was resumed at another time if another or an additional antigen/allergen was suspected.

Histology

Gross duodenal morphology results were normal after CLE and similar to earlier pre-CLE results,[9] but IELs were significantly higher in the CLE$^+$ (mean, 25.2 ± 8.4) than in CLE$^-$ patients (mean, 18.7 ± 11.8; $P = .03$) and HCs (mean, 14.4 ± 3.4; $P = .001$) (Supplementary Table 1). Compared with our prior exploratory study,[9] the now significant difference is likely due to the larger numbers of patients and to better selection of the Barrett's esophagus HC group that now had to yield normal results in the Francis score to exclude the presence of IBS. The IELs were found at the tips as well as in the upper two thirds of the lateral sites of the villi, and no obvious gradient was apparent between these sites. The comparable results of IEL quantification via histology vs via CLE are provided in Supplementary Table 1.

Immunohistochemistry and Quantitative PCR

In all biopsy samples, claudin-2 expression in enterocytes increased along the crypt-villus axis and was strongest in the upper third of the villus (crypt vs villus tip: HC, $P = .008$; CLE$^+$, $P < .001$) (Figures 3*A* and 4*A*), with no significant difference between CLE$^+$ and HC samples ($P = .721$). Although claudin-2 transcript levels before challenge were similar in CLE$^+$ and HCs (0.9 ± 0.51, $P = .16$), they were up-regulated after challenge in CLE$^+$ samples (before, 0.72 ± 0.54; after, 0.83 ± 0.39; $P = .023$). In contrast, occludin protein expression was reduced in CLE$^+$ vs HC ($P = .022$) (Figure 4*B*), was strongest in the crypts, and decreased steadily toward the villus tip (crypt vs villus tip: HC, $P = .006$; CLE$^+$, $P < .001$) (Figures 3*B* and *C* and 4*C*). In the villus tip, only occludin protein was significantly lower in CLE$^+$ patients compared with HCs ($P = .021$) (Figure 4*C*), whereas overall occludin transcripts remained unchanged

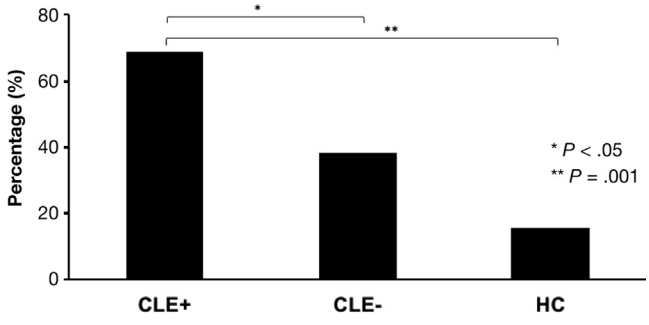

Figure 2. Prevalence of atopic disorders or first-degree family history of atopic disorders in CLE$^+$ patients, CLE$^-$ patients, and HCs.

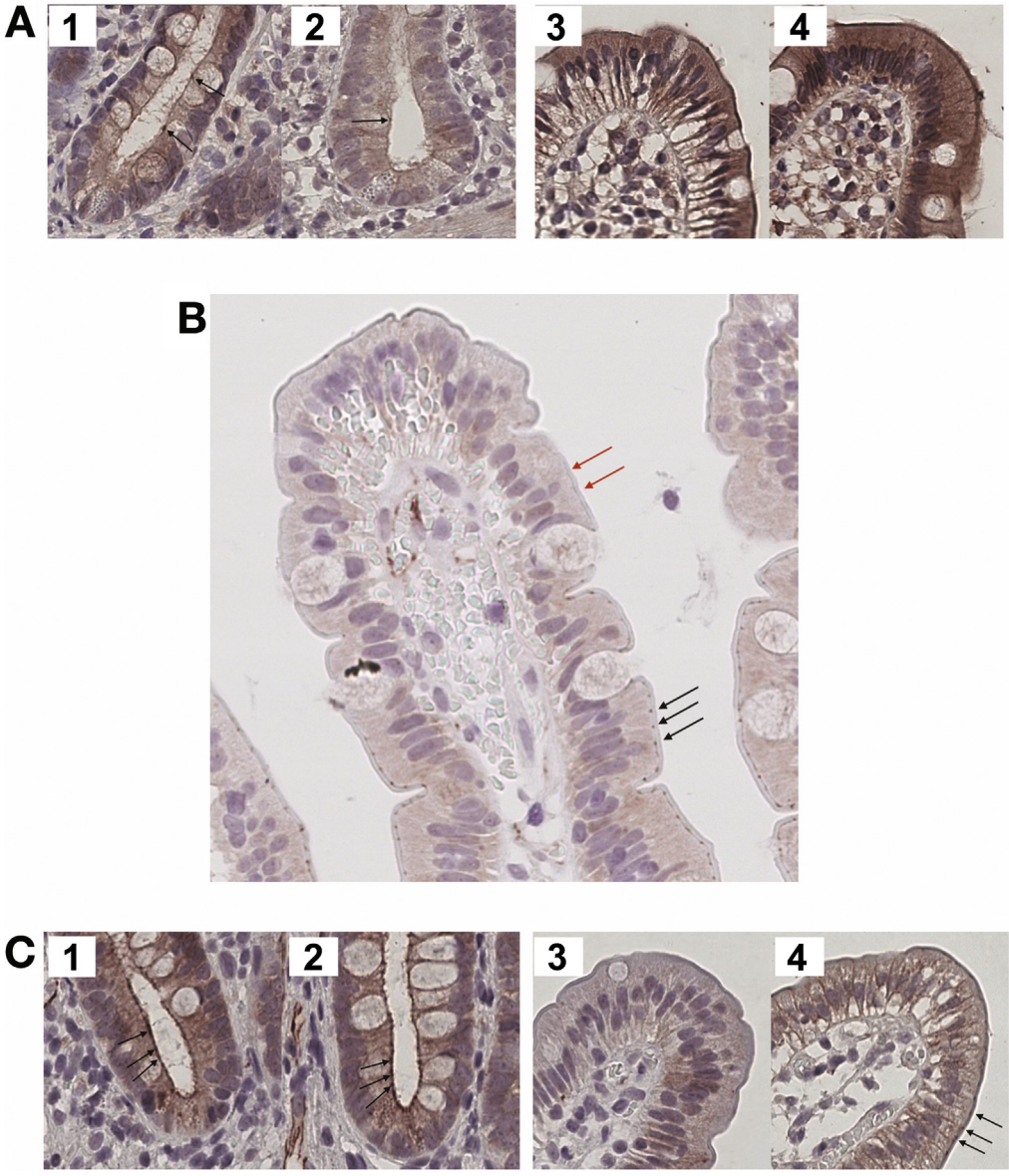

Figure 3. Pattern of claudin-2 and occludin protein expression. (*A*) Hardly any claudin-2 expression was noticeable in the crypts (*1*, *2*, *arrows*). Claudin-2 increases along the crypt-villous axis, with expression between almost every cell-cell contact in the villus tip (*3*, *4*) in both CLE^+ patients (*1*, *3*) and HCs (*2*, *4*). Original magnification ×40, taken with a Leica (Wetzlar, Germany) SCN 400 image viewer. (*B*) Overview of occludin expression in CLE^+, decreasing from crypt to villous tip. The black arrows show presence of occludin at every single cell-cell contact, which vanishes toward the villous tip (*red arrows*) when no occludin is present any more. (*C*) Occludin expression in the crypt between every cell-cell contact (*1*) in CLE^+ patient and (*2*) in an HC, decreasing steadily toward the villus tip in both CLE^+ and HC; (*3*) in the villus tip of the CLE^+ sample no occludin signal was seen, whereas (*4*) in the HC sample, occludin was reduced but still present. Original magnification ×40, taken with a Leica SCN 400 image viewer.

immediately after food challenge. There was no significant difference between patients reacting to wheat vs the other food antigens. There were also no significant changes in transcript levels for tight junctional protein (TJP-1) (P = .779 for baseline vs post-CLE challenge; P = .234 for CLE^+ vs HC) or the measured proinflammatory transcripts for tumor necrosis factor-α (P = .286 for baseline vs post-challenge; P = 1.00 for CLE^+ vs HC), IL-4 (P = .717 for CLE^+ vs HC), IL-5 (P = .673 for baseline vs postchallenge; P = .271 for CLE^+ vs HC), or ECP (P = .866 for baseline vs postchallenge; P = .297 for CLE^+ vs HC) or MBP (P = .917 for baseline vs postchallenge; P = .287 for CLE^+ vs HC).

Eosinophils, Secreted ECP, and Tryptase

Postchallenge ECP from duodenal fluid was significantly higher in CLE^+ patients compared with HCs (29.4 ± 51.2 vs 6.5 ± 7.9 μg/L; P = .03) but not vs CLE^- patients (15.5 ± 19 μg/L) (Figure 5). As already noted in our prior report,[9] the increased (baseline) test results in the CLE^- IBS patients suggest that some may suffer from a reaction to a different food antigen that we had not tested. Although regarded as CLE^-, some likely would have been CLE^+ if other, less-prevalent food antigens had been tested.

Prechallenge eosinophils in histologic and tryptase in duodenal fluid analyses were not different between CLE^+ or CLE^- patients and HCs, and they remained unchanged after antigen exposure (Figure 6). However, higher numbers of degranulating eosinophils were noted in the CLE^+ patients (mean, 5.32 ± 3.22) than in CLE^- patients (4.77 ± 3.10) and HCs (4.13 ± 3.33), but this was not significant, likely due to the short time of follow-up. In addition, in view of the degranulation, the cell counts in CLE^+ samples are likely an underestimate.

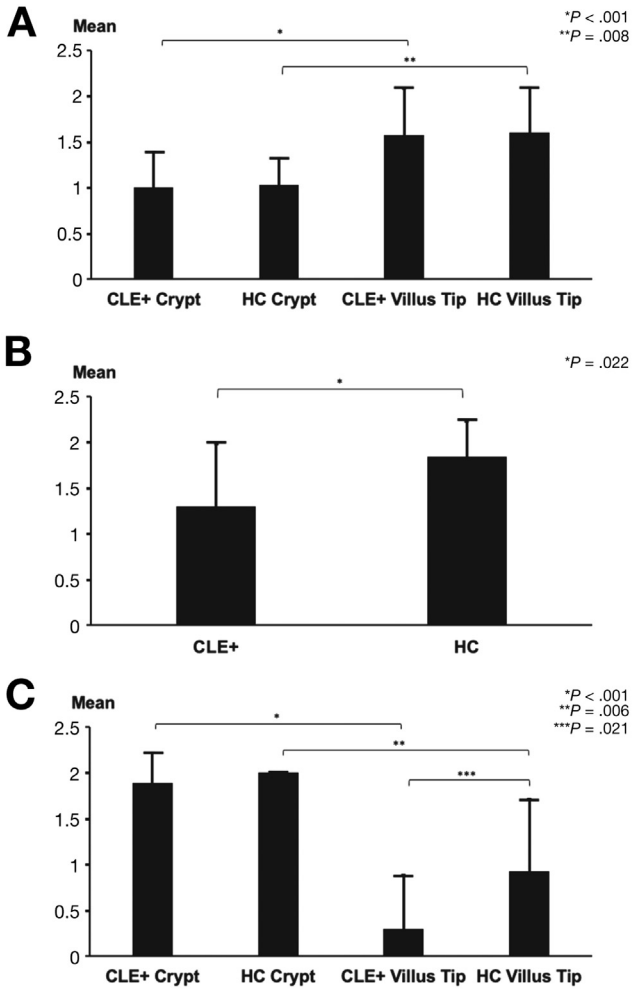

Figure 4. Quantification of claudin-2 and occludin expression. (*A*) Claudin-2 expression in crypts vs villus tips in CLE$^+$ vs HC samples did not show significant differences between groups; a significant difference was noted when claudin-2 expression was compared between crypts and villus tips. (*B*) Overall occludin expression was significantly lower in CLE$^+$ patients vs HCs ($P = .022$). (*C*) Occludin in crypts vs villus tips shows a reverse distribution compared with claudin-2 in Figure 3*A*. Other than for claudin-2 expression, which was not different between groups, occludin distribution was significantly lower in the villus tips of CLE$^+$ patients vs HCs ($P < .021$). *Error bars* = standard deviation.

Discussion

Although pathophysiological understanding of the spectrum of IBS remains incomplete, it is generally accepted that activation of the innate and acquired immune system is involved, finally leading to chronic intestinal low-grade inflammation.

This study confirms and expands our previous report[9] suggesting that at least 50%–60% of IBS patients may have a nonclassical food allergy. Notably, all patients displayed a negative response to food antigens on classical food allergy testing (skin-prick test and serum IgE to common food antigens), and clinical symptoms were delayed despite an immediate duodenal reaction on CLE. The delayed clinical symptoms in non–IgE-mediated food allergy in the gut are likely an amalgam of different pathophysiological processes, all with varying time frames involving changes in enteric secretion, permeability, motility, and sensation. As a consequence, clinical interpretation of oral provocation studies is problematic. Positive reactions usually take 2–6 hours to be clinically apparent, and a negative response can be uncertain for a number of days.[15] Patients diagnosed as CLE$^+$ with our methodology showed a highly significant, long-term response to exclusion of the identified food antigen/allergen up to the 12 months of follow-up.[9] Our current results further improved when the Francis symptoms and VAS scores were introduced to better define patients with IBS and to exclude HCs with hidden IBS symptoms.

CLE showed clear changes of increased fluid permeability of the duodenal mucosa immediately after contact with the particular food antigen/allergen in those patients with IBS who were sensitized. In contrast to IBD or other chronic intestinal diseases, for which no challenge can be or has been performed, we could observe an immediate response to food challenge, setting our study apart from prior studies on intestinal inflammatory diseases.[16,17] In the few patients with IBD whom we subjected to CLE food challenges outside of this study, no dynamic changes were seen to any of these food antigens, except for 1 IBD patient who proved allergic to cow's milk protein.

Osmolarity of the applied foods is unlikely to play a role in the mucosal changes that we observed, because (1) the reactions occurred only in challenged IBS patients and not in HCs, (2) exclusion of the identified food improved IBS symptoms significantly, and (3) the other normal daily foods that cover all ranges of osmolarity did not induce the symptoms. Moreover, 3 foods applied (wheat, soy, yeast) were suspended 1:10 in sterile water, yielding an osmolarity between 10 and 30 mOsm, except for milk, which was diluted 1:2, with an osmolarity between of 150 and 200 mOsm. Hypo-osmolar solutions, with pure water as the extreme, have not been reported to cause a disruption of the mucosal barrier.

In our previous CLE$^+$ patient cohort,[9] we already showed immediate disruption of the small intestinal barrier after provocation, with epithelial leaks forming within 5 minutes after food challenge. This was also the most prominent and constant finding of a positive reaction in the present study, with an accumulation of fluorescein between enterocytes followed by secretion of plasma into the intestinal lumen via paracellular pathways (Figure 1 and Supplementary Video). This phenomenon was never seen in any baseline CLE, CLE$^-$ patients, or HCs. The mechanism, here observed real time in vivo, is in accord with prior studies in IBS patients showing increased permeability to sugars,[18,19] tight junctional disruption on electron microscopy,[20] and changes in conductance in biopsy samples studied ex vivo in Ussing chambers.[21]

Experimental studies showed 2 distinctly regulated paracellular pathways across the duodenal epithelium through tight junctions: a claudin-dependent, charge- and size-restrictive (<0.4 nm) pore pathway[22,23] or a pathway though large-pore-size channels, regulated probably by

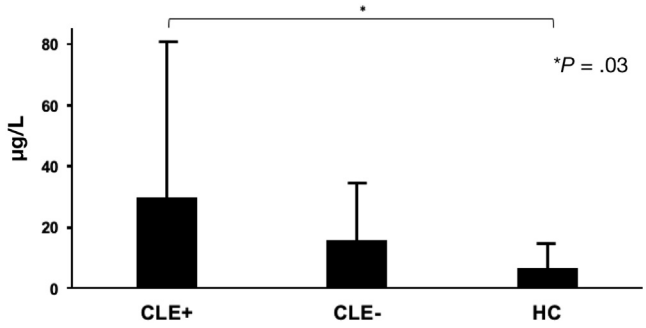

Figure 5. ECP in duodenal fluid of the CLE$^+$ patients compared with CLE$^-$ patients and HCs. Postchallenge ECP was significantly higher in CLE$^+$ patients vs HCs ($P = .03$) but not vs CLE– patients. *Error bars* = standard deviation.

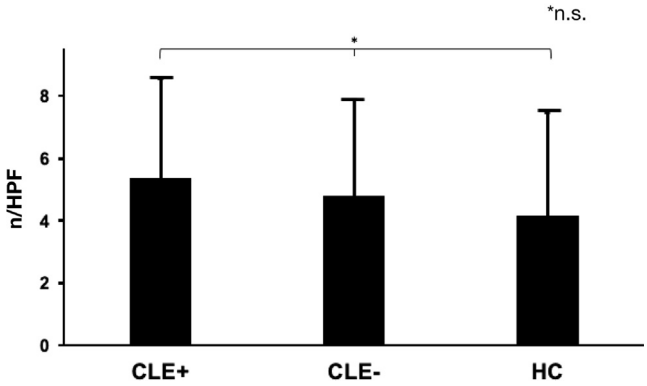

Figure 6. Eosinophil counts CLE$^+$ patients, CLE$^-$ patients, and HCs. There were no significant differences between groups. *Error bars* = standard deviation.

occludin, that is responsible for macromolecular permeability.[23] Claudins represent a family of molecules with divergent functional properties.[22] We focused on claudin-2 because of its high expression throughout the duodenal crypt-villous axis, its key role in salt/water paracellular movement, and prior data showing its significant up-regulation in IBS and IBD.[20,24–26] Thus, a significant increase of claudin-2 expression was previously seen in the jejunum of IBS patients[20] and in the colon of patients with Crohn's disease[25] using various methods. Although our analysis of claudin-2 expression did not show significant differences among IBS patients and HCs, claudin-2 transcript levels were up-regulated immediately after challenge in CLE$^+$ patients.

In contrast, overall occludin protein was significantly reduced in CLE$^+$ patients compared with HCs and even more so at the villus tip of CLE$^+$ vs HC. Although several other junctional and inflammatory transcripts and proteins remained unchanged, the unique baseline expression levels of claudin-2 and occludin, as well as their changes immediately after challenge, suggest significant constitutive and food antigen/allergen–triggered alterations in the structure and regulation of the tight junctions of CLE$^+$ patients. The observed decrease of occludin is in accord with findings of decreased expression in the duodenum of patients with active celiac disease[27] and in the colon of patients with Crohn's disease.[25] The decrease of occludin and increase of claudin-2 expression in the study of patients with Crohn's disease was related to epithelial dysfunction with decreased epithelial resistance, which the authors primarily attributed to the increase of claudin-2 expression.[25]

A variety of different functional pathways regulate intestinal permeability involving cellular and molecular changes of enterocytes, mast cells, T lymphocytes, eosinophils, and other immune cells.[20,24] In our CLE$^+$ patients, there was evidence of increased mucosal lymphocyte infiltration and eosinophil activation but not of mast cell activation, as assessed by immunohistochemistry and intestinal fluid analysis. Thus, duodenal fluid tryptase activity was not different from HCs. IELs likely increase permeability by activation of myosin light chain kinase and phosphorylation of myosin light chains[28,29] that are part of the contractile actomyosin ring of the tight junctions, in part by secretion of interferon gamma and by causing redistribution of occludin and claudins.[24] Eosinophils are multifunctional leukocytes involved in infections and allergic and inflammatory diseases.[15] They may act as antigen-presenting cells, expressing major histocompatibility complex class II and costimulatory molecules, thereby promoting T-cell proliferation and activation and their T helper (Th) type 1 or Th2 polarization, fueling antigen (allergen)-induced inflammation. In our study, we did not detect gross changes in Th1 or Th2 cytokines. However, biopsy samples were taken immediately after challenge which is too early to detect changes in associated cytokine or T-cell polarization patterns. In addition, eosinophils behave as direct effector cells by releasing their toxic granular proteins: MBP, ECP, eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and various lipid mediators.[15,30,31] Here, MBP, ECP, and eosinophil peroxidase have been shown to be toxic to the intestinal epithelium and to rapidly induce epithelial barrier dysfunction.[30–33]

Because we do not observe a histologic mast cell/basophil increase or activation and do not find increased mast-cell mediators (tryptase) in the duodenal fluid after positive challenge, we assume a nonclassical or atypical food allergy as the cause of the mucosal reaction observed by CLE. Apart from the tight junctional changes that we see in CLE and histology affecting claudin-2 and occludin, the only notable finding is modest eosinophil activation and a statistically significant ECP level in the duodenal fluid, whereas several other immune cell–related parameters, as determined by PCR, are unchanged. This could mean that the measured immune cell reactivities become statistically significant later than 10 minutes after positive challenge (ie, the time when samples were taken) or that other immune mechanisms, in addition to a modest eosinophil activation, are operative if not predominant later on. We are currently working with a mouse model of eosinophilic food allergy to get a better idea of the immune pathogenesis of these nonclassical food allergies.

Our results of increased duodenal eosinophils in CLE$^+$, increased IELs, and enhanced secretion of ECP into the duodenal fluid of CLE$^+$ patients after food challenge is in line with a role of eosinophils in this form of food

antigen–triggered food sensitivity or allergy. Other studies have shown that increased eosinophil density and, especially, eosinophil activation are the hallmark of atypical food allergies, including eosinophilic esophagitis and gastroenteropathy, and related pathologies.[33–38] Thus, our findings are remarkably reminiscent of an allergic reaction to food, despite a lack of IgE or skin test positivity to the incriminated allergen. In this regard, they are clinically reminiscent of food sensitivity in eosinophilic esophagitis or to airway hyperreactivity in patients with asthma and sputum eosinophilia,[37] in which hypersensitivity responses are delayed. Moreover, in these and our patients, the influx and activation of mucosal eosinophils are associated with a personal and family history of atopy. Interestingly, a similar mechanism has been postulated for some patients with ulcerative colitis and nonspecific colitis who are food intolerant.[39] We therefore hypothesize that our IBS patients have a form of non–IgE-mediated atypical food allergy involving enhanced eosinophil and intraepithelial lymphocyte activation. One may ask why wheat is the predominant allergen in these patients. An explanation could be that wheat not only contains a broad variety of potential gluten and, especially, non–gluten-related protein allergens, but also the nongluten amylase trypsin inhibitors that activate toll-like receptor 4 on intestinal mucosal myeloid cells.[40] As we could show, once activated by nutritional amylase trypsin inhibitors, the myeloid cells promote intestinal and airway allergies in allergen-sensitized mice, including mice with a humanized immune system.[39,41] More research needs to be done to answer the questions arising from our studies, especially further studies to elucidate the mechanisms by which changes in permeability are seen so quickly, although clinical symptoms are delayed.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2019.03.046.

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Acknowledgments
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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Methods

CLE Procedures

For CLE, the endoscope (EC-387CILK; Pentax, Tokyo, Japan) was guided into the deep duodenum with great care taken not to damage the epithelium, which would lead to lesions mimicking positive reactions to food. Food exposure was performed in sequence from the most distal point accessible toward the proximal D2. After intravenous injection of 5 mL 10% fluorescein (Fluorescein Alcon, Novartis, Nürnberg, Germany) and 40 mg methylscopolamine (Buscopan, CC-Pharma, Densborn, Germany), the laser was activated, and baseline CLE was performed if there were no signs of iatrogenic mucosal damage. Baseline/postchallenge images, including any real-time mucosal changes, were recorded, and images were analyzed in detail after the procedure by 2 different authors who were blinded to the antigens applied and to the CLE results, which were recorded by the endoscopist.

The following characteristics were quantified at baseline and immediately after food provocation (2–5 minutes after food provocation), as previously described[1]:

- Density of IELs were counted from 4 standardized 5 × 5-cm areas of a size of 250 × 250 pixels (edges, 500 μm; depth, 250 μm, 1 pixel equals 0.46 μm) at baseline and after food application. The numbers shown represent the mean of 2 examiners and areas.
- Epithelial breaks and leaks of fluorescein into the lumen, counted per 1000 epithelial cells in 5 different locations from 5 different images.
- Presence of fluorescent signal between enterocytes with leakage through tight junctions.
- Distance between villi (intervillous space), as measured per pixel of the image acquired in 4 different locations. Widening of the intervillous space indicated secretion of fluorescein into the lumen, with 1 pixel covering 0.46 mm^2 (475 mm^2/1024 pixels). A macroscopically visible change or reaction in at least 2 of these 4 parameters within 5 minutes, always including leakage as the prime parameter, was defined as a positive reaction (CLE^+) (Figure 1 and Supplementary Video).

IELs: CLE vs Histology

Results of IELs counted at CLE compared with histology are provided in Supplementary Table 1. Images of IELs in CLE are provided in Figure 1*A* for CLE^+ patients and Figure 1*B* for HCs (*black arrows* mark IELs).

In contrast to our earlier study,[1] in which the IELs in histologic analysis after food exposure were not statistically significant when compared with CLE^- patients and HCs, in this study there is a significance of $P < .001$. Although most data are similar to those of the earlier study, here the HCs provided much lower IEL count than. This is likely due to the larger overall number of patients, with a much higher range, but more so due to our improved selection for the HC group. Different from our previous study, in this study we used the Francis IBS symptom and VAS scores for baseline assessment of the severity of IBS in all participants, including the HCs, to avoid an earlier suspicion that there might be some patients with Barrett's esophagus who have IBS but denied the presence of IBS symptoms when asked. We believe that the use of the Francis IBS score for all participants led to the lower IEL count in the HC group and made the IEL histologic analyses between HC and CLE^+ samples significant (see Supplementary Table 1 and Figure 1*A* and *B*).

However, as already hypothesized in our prior report,[1] the increased baseline test results in many of the CLE^- IBS patients in our present study suggest that some may indeed have a reaction to a food antigen that was not included among our series and that these likely would have shown a CLE^+ reaction if other, less-prevalent food antigens had been tested.

Application and Concentration of Food Antigens

Patients received 5 sequential food challenges inserted through the endoscope channel with a syringe onto the duodenal mucosa as follows: 5 mL cow's milk from the shelf (Aldi Nord, Essen, Germany, 3.5% milk fat) mixed with 50% sterile water or suspensions in 20 mL sterile water of the following: wheat flour (type 405 [European standard]), 2 g; baking yeast (Aldi from the shelf), 1 g; and soy 2 g); 18 mL sterile water/2 mL simethicone (Sab Simplex, Kohlpharma, Merzig, Germany) served as a control substance.

Foods were applied in sequential and randomized order and blinded for the examiner, distributed from the distal to the proximal part of the duodenum. Exact placement localization points and extent of spread of the inserted food were not visible, given the necessity of placing the endoscope in direct contact with the duodenal wall for CLE imaging, which does not allow simultaneous endoscopic view without losing the endomicroscopic view. The only landmark visible was the starting point for the first food application in the deep duodenum beyond the papilla before the endoscope was placed/anchored to the wall. The exact locations at which to anchor the endoscope to the wall were chosen as found available for visualization with the laser endoscope and found undamaged, so that baseline examination would provide the necessary native mucosa. Exact definition of location was not necessary if the patient was CLE^-, because the food component would not cause changes, baseline images continued to be present, and any other area could be chosen for the next food exposure. It was also not necessary for the CLE^+ patients, because the reaction to food would cause the accessible duodenal mucosa to change within a very short time, forbidding further food provocation. The examination was stopped, and if necessary, the missing food components would be applied in a repeat CLE examination.

Because we did not use dye to mark the food, we are not able to define the exact size of the area in the duodenum covered by the food.

Diets

In this part of the study, we focused on biochemical mucosal changes and used 2 different diets to assess the success of excluding the antigen reacting at CLE.

Both diets were randomly chosen and performed in a double-blinded fashion. Diets were supervised, and for both diet plans, specially created bread was provided for each of the antigens used at CLE in case this antigen was positive; the most frequently used breads were yeast and wheat free. For the sham diet, normal bread was used with a reduced amount of salt, which gave a very different taste.

Diet A: Exclusion of the Antigen Found Reactive at CLE. This diet was designed to exclude the food component (food group) that had reacted on CLE and turned the normal duodenal mucosa into CLE^+. Either wheat, cow's milk, baking yeast, egg, or soy were eliminated from patients' diets for 2 weeks. Patients received professional dietary advice, a diet plan for 2 weeks, and a list of foods in the normal regional German diet that they would have to strictly avoid.

Diet B: Sham (Placebo) Diet. This diet did not exclude any food component or food group but only single items of such a food group. For instance, patients were not allowed cakes and cookies but were allowed the special bread and pasta, or patients were not allowed yogurt but were allowed other cow's milk–containing products. Patients received a diet plan for 2 weeks but also a list of foods in the normal regional German diet that they would have to strictly avoid, which were chosen at random.

Patients also received a customized bread, which had much lower salt than usual, so that it tasted very different and appeared to be special dietary bread, although all regular components were still present.

Customized Bread. Patients received 2 different types of bread that were especially produced for this purpose and were provided anonymously free of charge as a donation to our study by a local bakery (Firma Günther, Kiel, Germany). Breads were put in green and red covers so that patients could differentiate the color and associated diet: the green color indicated placebo-diet bread with lowered salt as the only change, and the red color indicated customized bread made without yeast, wheat, and soy.

Questionnaires

Francis IBS Severity Score. The Francis IBS Severity Scoring System was used to evaluate IBS symptoms at baseline, after food challenge, and after each of the exclusion diets. In the HC group, it was used to exclude any "hidden" IBS patients. This scoring system was described and initially validated in 1997 for the evaluation of the severity of IBS symptoms,[2] and in 2004, it was evaluated in a large patient cohort.[3] The Francis IBS Severity Scoring System measures 5 symptoms: intensity of abdominal pain, abdominal distension, stool habit, quality of life, and the number of days with pain within a time frame of 10 days, scored by the patient on a VAS of 1%–100%. Each item is recorded on VAS 1%–100% scale and transformed into a number; the number of days with symptoms is multiplied by 10. The sum of all 5 numbers provides an overall score of up to 500. The final number indicates the severity of the IBS symptoms ($<$75, in remission; 75–175, mild; 175–300, moderate; $>$300, severe).

We included only patients with at least moderate disease.

VAS

All patients were asked to classify their symptoms of abdominal pain, bloating, and/or diarrhea or constipation using a questionnaire that included the physical elements of the VAS-IBS[4] and a scoring system of 1–10. A symptom score of 10 used for each of the symptoms represented the baseline intensity of pain/bloating/diarrhea experienced immediately before/at inclusion in the study.

For the extended second part of the study, further questionnaires were used to understand the functional changes better, including the Patient Health Questionnaire 12 Somatic Symptom Scale, the Hospital Anxiety and Depression scale, and the Bristol stool chart. The results of these questionnaires were not used for this part of the study.

Results of the Antigen Exclusion Diet

After CLE, patients had randomly assigned 2-week test diets, including exclusion of the antigen that tested positive at CLE and a sham diet, with a 2-week washout phase in between. Baseline and potential changes of symptoms were assessed after each of the diets with questionnaires: (1) the standard IBS Francis symptom score and (2) a VAS. After 3 months, overall CLE^+ patients symptoms improved 70% when the allergen tested positive at CLE was excluded from the diet; it further improved to 73% after 6 months. Overall, 52 of 76 CLE^+ patients improved $>$80, 24 improved a mean of 61%, and 3 patients did not respond to the diet. The overall results are given in Supplementary Figure 2.

ECP and Tryptase Assay

ECP or tryptase was assayed by using the ImmunoCAP system in a Phadia 250 (Thermo Fisher Scientific) analyzer by fluoro-enzyme immunoassay. Duodenal fluid was centrifuged at 1200*g* for 10 minutes, then filtered. The filtrate was reacted with anti-ECP or tryptase mouse monoclonal IgG antibodies, as appropriate, covalently bound to a cellulose solid phase, followed by washing with a solution of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-2H-isothiazol-3-one. The bound ECP or tryptase antibody was then complexed with β-galactosidase–labeled anti-tryptase or anti-ECP at 3.5 μg/mL mouse monoclonal antibody. After incubation, the unbound antigen anti-tryptase or anti-ECP complex was developed with 0.01% 4-methylumbelliferyl-β-D-galactoside and 0.0010% 5-chloro-2-methyl-4-isothiazolin-3-one. The reaction was then stopped with a solution of 4% sodium carbonate, and the ECP or tryptase content was determined fluorometrically. A calibration curve was used to measure the final protein content.

Gene Expression Studies

RNA Extraction and Reverse Transcription. Extraction of total RNA from quick-frozen biopsy samples was performed with the NucleoSpin total RNA/Protein isolation kit (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. Total RNA was eluted in a volume of 60 μL H_2O. Genomic DNA was digested for 15 minutes with 1.5 U of DNAse I (Sigma-Aldrich, Darmstadt, Germany) at room temperature. Reverse transcription was carried out in a total volume of 30 μL containing 375 ng random hexamer primers (GE Healthcare, Freiburg, Germany), 0.5 mmol/L deoxynucleoside triphosphate (Promega, Mannheim, Germany), 0.01 mol/L dithiothreitol, 1$\times$ reaction buffer, and 150 U Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). The annealing step was carried out at 25°C for 10 minutes, elongation at 25°C for 10 minutes, and denaturation at 70°C for 15 minutes.

Real-Time Quantitative PCR. Gene expression was determined by real-time quantitative qPCR performed in 96-well plates in duplicate reactions. Each reaction (20 μL) contained 1 $\times$ qPCR Master Mix Plus (Eurogentec, Cologne, Germany), 900 nmol/L primers, 225 nmol/L hybridization probe, and 2 ìL of total complementary DNA. qPCR product accumulation was monitored by the ABI Prism 7700 Sequence Detection System (TaqMan, Applied Biosystems, Foster City, CA) over 45 cycles. Each cycle consisted of a denaturation phase (15 s at 95°C) and a hybridization/elongation phase (1 minute at 60°C). Primers for tight junction protein-1, occludin, and claudin-2 were obtained by Thermo Fisher Scientific primers for the inflammatory molecules tumor necrosis factor-α (forward: GAGGCCAAGCCCTGGTATGA, reverse: TCGAGATAGTCGGGCCGATT, probe: CCAGCTGGAGAAGGGTGACCGACT), IL-10 (forward: TGGGTTGCCAAGCCTTGTC, reverse: GGAGTTCACATGCGCCTTGA, probe: ACCTGGAGGAGGTGATGCCCCAAGC), IL-4 (forward: AAACGGCTCGACAGGAACCT, reverse: TTTAGCCTTTCCAAGAAGTTTTCCA, probe: CTGGCGGGCTTGAATTCCTGTCCTG), IL-5 (forward: AGCTCTTGGAGCTGCCTACGT, reverse: AGCAGAGTTCGATGAGTAGAAAGCA, probe: TGCCATCCCCACAGAAATTCCCACA), ECP (forward: CATGCCAGACCCCCACAGT, reverse: TTGTTAATTGCCCGCATTGC, probe: AGGGCTCAGTGGTTTGCCATCCAGC), and MBP (forward: CAACCTGGTTTCCATCCACAA, reverse: CTGTGATCCTGCCTCCAATCC, probe: TATCGAATCCAGTGTTCTGTCAGCG) by Eurogentec, Cologne, Germany. The data were normalized to expression levels of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) expressed as fold increase and presented as mean $\pm$ standard error of the mean.

Immunohistochemistry

For immunohistochemistry, 2-μm sections were cut with a microtome (Leica RM 2245, Leica Biosystems Nussloch, Nussloch, Germany) and placed on Leica BOND Plus Slides (Leica Biosystems, Richmond, IL). After drying for 12 hours at 54°C, paraffin wax was eluted by passing through baths of sequentially decreasing alcohol concentration (15 minutes xylol, 5 minutes 96% ethanol, 2 minutes 70% ethanol, 2 minutes 50% ethanol, 2 minutes distilled water, each 2 times). Sections were incubated in citrate buffer (pH 6.0) for 9 minutes and rinsed in Tris-buffered saline buffer (pH 7.4) for 5 minutes. Between each of the following steps, sections were washed 3 times with TBS. UltraVision Hydrogen Peroxide Block (Thermo Fisher Scientific) was incubated for 15 minutes at room temperature, followed by UltraVision Protein Block (Thermo Fisher Scientific) for 5 minutes. Next, the primary antibody was applied (occludin, 1:500, and claudin-2, 1:400; Sigma-Aldrich) diluted in antibody diluent (Zymed, Vienna, Austria) and incubated for 30 minutes at room temperature, followed by 4°C overnight. ImPRESS HRP Universal Anti-MOUSE/Rabbit Ig (Vector Laboratories, Burlingame, CA) incubated for 30 minutes at room temperature was followed by NovaRED substrate (Vector NovaRED Substrate Kit, Vector Laboratories) for 5 minutes. The sections were then rinsed with distilled water and counterstained with hemalum for 1 minute. They were dehydrated by passing through increasing alcohol concentrations and covered. Stained sections were analyzed by light microscopy (Leica DM 1000, Leica Biosystems). No protein expression was considered as 0, some expression as 1, and protein expression between every cell-cell contact as 2.

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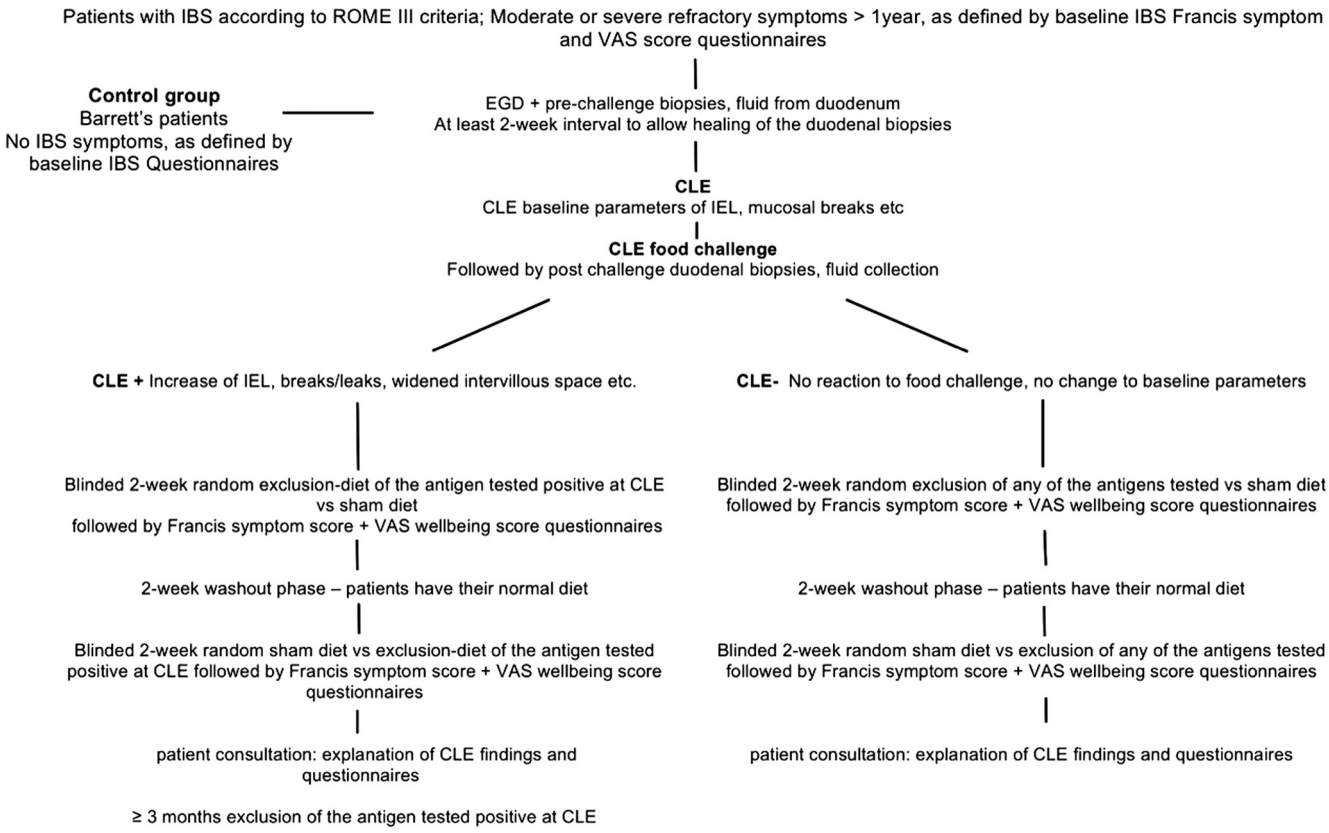
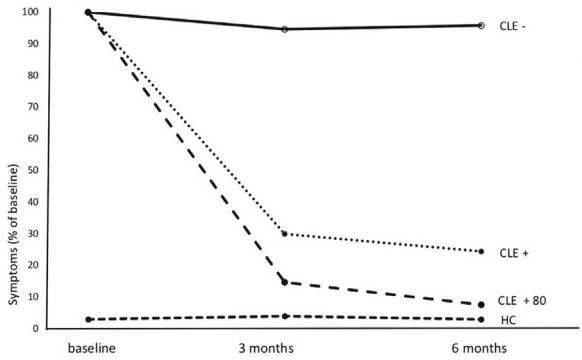


Supplementary Figure 1. Study flow chart.



Supplementary Figure 2. Improvement of symptoms in the CLE^{+} patients after food antigen exclusion. The graph shows mean values at baseline, after 3 months, and after 6 months of reactive food antigen/allergen exclusion in the CLE^{+} patients, differentiating between patients with and without a >80% improvement. Development of symptoms of CLE^{-} patients and HCs are also provided.

Supplementary Table 1. Overview of Changes and *P* Values for CLE+ vs CLE- Patients After Food Exposure Compared With Healthy Controls

	Healthy controls		CLE +		CLE -		
	Baseline	After exposure	Baseline	After exposure	Baseline	After exposure	*P* value
Patients, N	14		76		32		
Age, *y*	49,8 ± 12,8		42,7 ± 14,9		43,8 ± 15,8		NS
(*range*)	(39 - 78)		(20 - 76)		(22 - 75)		
IEL histology, IELs/100 cells		14,4 ± 3,40[a]	23,45 ± 12,03	25,2 ± 8,4[a]	20,27 ± 11,38	18,7 ± 11,8	<.001[a]
(*range*)		(5,0 - 19,0)	(3 - 50)	(8 - 53)	(5,0 - 45,0)	(2 - 40,5)	
IEL endomicroscopy, IELs/field	7,68 ± 0,9[b]	9,47 ± 2,71[a]	19,80 ± 4,7[b]	24,90 ± 4,7[a]	10,90 ± 1,90	12,10 ± 2,20	<.001[a] <.001[b]
(*range*)	(6,5 - 9,1)	(5,0 - 12,6)	(14 - 30)	(20 - 40)	(8 - 14)	(8 - 17)	

NOTE. Comparison of IEL in histologic analysis and at CLE in CLE^+ and CLE^- patients and HCs.
[a]Comparison of postexposure IELs in histology and in CLE images in CLE^+ vs CLE^- patients vs HCs.
[b]Comparison of baseline in CLE^+ patients and HCs.