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Microalterations of Esophagus in Patients With Non-Erosive Reflux Disease: *In-Vivo* Diagnosis by Confocal Laser Endomicroscopy and Its Relationship With Gastroesophageal Reflux

Chuan-Lian Chu, MD, PhD[1,2], Yan-Bo Zhen, MD, PhD[1,2], Guo-Ping Lv[1], Chang-Qing Li, MD, PhD[1], Zhen Li, MD, PhD[1], Qing-Qing Qi[1], Xiao-Meng Gu, MD, PhD[1], Tao Yu, MD, PhD[1], Ting-Guo Zhang, MD, PhD[3], Cheng-Jun Zhou, MD, PhD[4], Rui-Ji, MD, PhD[1] and Yan-Qing Li, MD, PhD[1]

OBJECTIVES: **Objectively diagnosing non-erosive reflux disease (NERD) is still a challenge. We aimed to evaluate the use of *in-vivo* confocal laser endomicroscopy (CLE) to examine the microalterations of the esophagus in patients with NERD and its relationship with reflux episodes monitored by multiple intraluminal impedance-pH (MII-pH).**

METHODS: **Patients with gastroesophageal reflux symptoms completed reflux disease questionnaires. NERD was determined by negative gastroscopy. Patients without reflux symptoms were recruited as controls. Pilot clinical study was followed by prospective controlled blinded study. All subjects were examined by white-light mode of the endoscopy followed by the standard CLE mode and then MII-pH monitoring. The microalterations seen on CLE images and the correlation between CLE features and reflux episodes were evaluated, the correlation between CLE and transmission electron microscope (TEM) data was also analyzed.**

RESULTS: **On CLE images, NERD patients had more intrapapillary capillary loops (IPCLs) per image than did controls (8.29±3.52 vs. 5.69±2.31, *P*=0.010), as well as the diameter of IPCLs (19.48±3.13 vs. 15.87±2.21 μm, *P*=0.041) and intercellular spaces of squamous cells (3.40±0.82 vs. 1.90±0.53 μm, *P*=0.042). The receiver operating characteristic analysis indicated that IPCLs number (optimal cutoff >6 per image, area under the curve (AUC) 0.722, 95% confidence interval (CI) 0.592–0.853, sensitivity 67.7%, specificity 71.6%), IPCLs diameter (optimal cutoff >17.2 μm, AUC 0.847, 95% CI 0.747–0.947, sensitivity 81%, specificity 76%), and the intercellular spaces of squamous cells (optimal cutoff >2.40 μm, AUC 0.935, 95% CI 0.875–0.995, sensitivity 85.7%, specificity 90.5%) diagnosed NERD with reasonable accuracy. Combined features of dilatation of intercellular space plus increased IPCLs provided 100% specificity in the diagnosis of NERD patients. The intercellular spaces of squamous cells observed on CLE were highly related to that on TEM findings (*r*=0.75, *P*<0.001). Multivariate progressive regression analysis showed that acidic reflux, especially in the supine position, was related to the increased number and dilation of IPCLs in the squamous epithelium (β=0.063, *t*=2.895, *P*=0.038 and β=0.156, *t*=1.023, *P*=0.04).**

CONCLUSIONS: **CLE represents a useful and potentially significant improvement over standard endoscopy to examine the microalterations of the esophagus *in vivo*. Acidic reflux is responsible for the microalterations in the esophagus of patients with NERD.**

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INTRODUCTION

Non-erosive reflux disease (NERD) is a chronic disease defined by the presence of troublesome reflux-associated symptoms and the absence of mucosal breaks seen by standard endoscopy (1); it can occur with or without abnormal esophageal acid exposure during 24-h ambulatory monitoring (2,3). NERD accounts for >50%

[1]Department of Gastroenterology, Qilu Hospital, Shandong University, Jinan, China; [2]Department of Gastroenterology, Jinan Central Hospital, Jinan, China; [3]Department of Pathology, Qilu Hospital, Shandong University, Jinan, China; [4]Department of Pathology, The Second Affiliated Hospital, Shandong University, Jinan, China. **Correspondence:** Yan-Qing Li, MD, PhD, Department of Gastroenterology, Qilu Hospital, Shandong University, 107 Wenhuaxi Road, 250012 Jinan, China. E-mail: liyanqing@sdu.edu.cn
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of cases of gastroesophageal reflux disease (GERD). In Asia, up to 70% of patients with GERD suffer from this disease. Unfortunately, the "gold standard" for the diagnosis of GERD is still lacking, and the sensitivity of 24-h pH monitoring for diagnosing NERD is unsatisfactory.

Multiple intraluminal impedance (MII) monitoring is a recently developed technique for examining gastroesophageal reflux content. Monitoring with MII combined with pH (MII-pH) allows for the detection of all reflux events in terms of acidity (acidic, weakly acidic, and weakly alkaline) and composition (liquid, gas, or mixed) (4–6). Study has shown that combined pH-impedance monitoring was more accurate than use of pH alone for the detection of both acidic and weakly acidic refluxes (7).

Currently, upper gastrointestinal endoscopy is the main clinical tool for visualizing esophageal lesions. However, microalterations in NERD may not be visualized by standard endoscopy. Advances in biomedical optics and diagnostic endoscopic tools have led to the improvement of conventional endoscopy for the detection of microalterations in the gastrointestinal tract. NERD patients have shown microscopic changes in the distal esophageal epithelium (8), which include elongation of the papillae, proliferation of basal cells, and dilated intercellular spaces within the squamous epithelium. The narrow band imaging endoscopy system, magnification and high-resolution endoscopy, chromoendoscopy, and newly developed i-scan endoscope provide numerous details of the esophageal surface in the diagnosis of squamous cell carcinoma, GERD, Barrett's esophagus, and adenocarcinoma of the esophagus (9–11). Sharma *et al.* (9) have reported detailed data on intrapapillary capillary loops (IPCLs) and microerosions of esophagus in GERD patients using narrow band imaging. Patients with GERD showed an increased number, tortuosity, and dilatation of IPCLs and were more likely to demonstrate microerosions and increased vascularity at the squamocolumnar junction. However, there is little *in-vivo* evidence of microalterations of the esophagus epithelium in patients with NERD.

Confocal laser endomicroscopy (CLE) is a newly developed endoscopic technique that allows the observation of living cells, tissue as well as vascular networks of the mucosal layer in the gastrointestinal tract during ongoing endoscopy (12,13). The highly magnified images (~1,000-fold) of the gastrointestinal tract mucosa can permit real-time histological analysis of the site during endoscopy. Therefore, CLE can provide precise assessment of the esophageal squamous epithelial cells and IPCLs without the need of biopsy. However, few studies have focused on the use of CLE to assess the microalterations of NERD *in vivo*.

We aimed to investigate the use of CLE for *in-vivo* evaluation the microalterations of the esophagus not observed by standard endoscopy in patients with NERD. In addition, we sought to evaluate the influence of different reflux episodes on the microalterations of NERD by CLE *in vivo*.

METHODS

Patients

From April 2008 to February 2010, outpatients with GERD symptoms were recruited from the endoscopy unit of Qilu Hospital. After giving written informed consent, all subjects completed a valid and reliable RDQ (reflux disease questionnaire, Chinese version). RDQ is a very simple questionnaire that contains 12 items to assess the frequency and severity of heartburn, regurgitation, and dyspeptic complaints in western countries (14,15). The Chinese version of RDQ (16,17), which has been reported validated by previous studies, contains heartburn, substernal chest pain, and regurgitation or food reflux. Symptoms were scored according to the severity and frequency (five scales). Subjects were enrolled if the score was ≥12. Erosive reflux disease was differentiated from NERD by endoscopic findings. Gastroscopy was used to diagnose NERD by no esophageal mucosal injury and erosive reflux disease by esophageal mucosal breaks. Patients without any reflux symptoms who were willing to participate in the study were recruited as controls. All patients were Chinese Hans. Eligible patients had not received proton pump inhibitors and/or H_2 receptor antagonists for at least 4 weeks before screening. The study protocol was approved by the Medical Ethics Committee of Qilu Hospital, Shandong University. The design of this study was followed the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines and checklist according to von Elm *et al.* (18).

Exclusion criteria were as follows: age <18 years, Barrett's esophagus, esophageal varices, evidence of cancer or mass lesion in the esophagus and gastric lesions (ulcer, cardiac polyp, and cancer), previous esophageal or gastric surgery, significant untreated medical conditions, history of alcohol or drug abuse, or severe uncontrolled coagulopathy.

Endoscopic and CLE procedure

All procedures in this study were performed using a confocal laser endoscope (Pentax EC-3870K; Pentax, Tokyo, Japan). The preparation of patients for endoscopic examination was similar to that for standard upper endoscopy. Patients received 20,000 units of a-chymotrypsin orally to remove adherent esophageal mucus. Then, 1 ml 2% fluorescein sodium was administered intravenously for allergy test. Patients were sedated intravenously with propofol and fentanyl and received cardiopulmonary monitoring during the examination. All patients were first examined by white-light mode of the endoscopy (WLE). Then, 5 ml fluorescein (10%) was applied intravenously for CLE imaging. During the examination, every quadrant of the distal 2 cm of the esophagus mucosa above the Z-line was placed at the lower left corner of the CLE window, and the confocal lens of the endoscope was placed in close contact with the mucosa; images from superficial to deep were obtained at these sites. The CLE procedures were performed by three experienced endoscopists (YQL, XMG, and TY), each of whom familiar with endoscopic diagnostic criteria for mucosal changes and had performed >300 CLE procedures before the commencement of this study. They obtained CLE images of all four quadrants of the distal esophagus in a standardized manner. After confocal images were obtained, biopsy specimens from every quadrant of the distal 2 cm esophagus were obtained from each individual. At least one sample block in every quadrant was included in the H&E stained specimen analysis. Half of the sample blocks were selected for transmission electron microscopy (TEM) analysis according to random number table. All biopsy specimens were routinely treated and evaluated by two pathologists (TGZ and CJZ) who were unaware of the endoscopic findings.

Evaluation of IPCL pattern in confocal images

Pilot study. Five patients with NERD and five controls were enrolled in a pilot study to evaluate and classify the CLE images. Based on the initial evaluation, the IPCL features (number of dilatations and irregular shape) seen on CLE were identified and agreed on by the investigator (YQL) of the prospective study.

(1) Number of IPCLs per image, counted manually.
(2) Diameter of IPCLs, measured in the clearest images of every examined site. In every image, three vessels with the largest diameter were selected, and the mean value was considered as the diameter of IPCLs at that site. Measurement involved an image processing software (Medcare, QingDao, China). Dilatation of IPCLs was accepted as a change that occurred in the pattern of the diameter or caliber of an individual vessel.
(3) Dilatation of intercellular spaces (DISs) in the squamous epithelium. The intercellular space was measured by using an image processing software (Medcare) in the clearest images of every examined site. At least 10 of the largest spaces were selected, and the mean value was considered as the mean intercellular space of the squamous epithelium.
(4) Presence or absence of irregularly shaped IPCLs, such as tortuous, pecilo-spiral shaped, and elongated IPCLs. Tortuosity was evidenced by the presence of corkscrewing or the twisted nature of an individual IPCL.
(5) Presence or absence of microcolumnar epithelial islands at the esophagogastric junction.

Prospective study. We obtained CLE images of four quadrant photographs of the distal esophagus *in vivo* for all patients and controls. Real-time analysis of IPCLs number and pattern and presence or absence of microcolumnar islands was performed *in vivo*. The IPCLs diameter and the DIS were measured on still images immediately after the procedure by an investigator blinded to the patient groups (QQQ). The IPCLs number was counted manually on still images again and the results were entered for the analysis. None of these images underwent any post-processing. The maximum and minimum numbers of IPCLs per representative field for each patient were manually counted. The presence or absence of microcolumnar islands and irregularly shaped IPCLs in the distal esophagus was recorded. The intercellular space of the squamous epithelium was measured and compared with that measured at transverse TEM images. The sample size in this prospective study was estimated by the previous pilot study. Based on the data obtained in pilot studies, we estimate that a sample size of at least 42 (21 NERD patients and 21 asymptomatic individuals) would give us a test power of ~80% and α value of 0.05.

MII-pH procedure

All patients and controls underwent ambulatory 24-h MII-pH monitoring 3–10 days (mean 4 days) after endoscopy. MII-pH monitoring involved use of a 2.1-mm diameter catheter (Medical Measurement Systems B.V., Enschede, Holland) with six impedance probes and one esophageal pH sensor. The pH sensor was calibrated by use of a buffered pH solution with pH 4.0 or 7.0 as specified by the manufacturer before the procedure. Then, the probe was intubated transnasally through the esophagus into the stomach, and the esophageal pH sensor was positioned 5 cm above the lower esophageal sphincter. In addition, the lower four impedance probes were positioned at the distal esophagus (3, 5, 7, and 9 cm above the lower esophageal sphincter) and the upper two measuring probes were positioned at the proximal esophagus (15 and 17 cm above the lower esophageal sphincter) to measure the impedance data. The catheter was connected to a data logger (Sleuth System; Sandhill Scientific, Highlands Ranch, CO), which stored data from the channels (six impedances and one pH). After the installation, the patients were allowed to leave the hospital with the apparatus on and could resume their routine activities.

According to the study design, all patients were required to keep a diary to record exact specification of meals, supine and upright phases of reflux measurement, as well as symptoms such as heartburn, regurgitation, and non-cardiac chest pain. Patients had no restrictions on meals except for foods with pH <4. After the monitoring, the patients returned to the hospital for removal of the probe.

Data of MII-pH monitoring

All data from 24-h MII-pH monitoring were analyzed by use of a dedicated software (BioView Analysis; Sandhill Scientific). Visual inspection on the tracing was performed before Autoscan function of the software to identify true retrograde events from artifact. The investigator (GPL), who was blinded to the clinical information of patients, had trained on visual detection and characterization of gastroesophageal reflux by impedance-pH monitoring. Meal periods were excluded from the analysis. The variables of the impedance signals included total number of reflux episodes, acid exposure time (% time with esophageal pH <4), number of reflux episodes in terms of composition (liquid, gas, and mixed reflux episodes), and pH (acidic, weakly acidic, and non-acidic).

Reflux episodes were classified as (i) liquid reflux, a sequential decrease in impedance to a minimum of 50% of the baseline value progressing from distal to proximal; (ii) gas reflux, an increase in only impedance (usually $>3{,}000$ Ohms) in at least two channels nearly simultaneous progressing from distal to proximal; and (iii) mixed reflux (gas–liquid or liquid–gas), a combination of liquid and gas impedance patterns. Reflux episodes detected by impedance combined with pH metrics were classified as (i) acid reflux, when the esophageal pH recorded decreases to 4 during the MII-detected reflux pattern or when the reflux occurred with the esophageal pH already <4; (ii) weakly acidic reflux, when the esophageal pH decreased <7 points but remained >7; and (iii) no acid reflux, reflux episodes during which the esophageal pH increased to 7. In addition, we separated all reflux patterns into upright and recumbent on the basis of patients' body position at the time of reflux episode.

Symptom index was defined as the percentage of reflux-related symptom episodes (19), expressed as a percentage. A symptom index ≥50% was considered as positive.

Symptom association probability was defined as the likelihood that patients' symptoms were related to reflux based on a statistical analysis (cross-tabulation) of a contingency table consisting of four possible combinations of reflux and symptoms (20). The symptom association probability calculation was provided in the software package of the MII-pH monitoring device. Based on the literature, symptom association probability values of ≥95% were considered as abnormal.

Esophageal acid exposure time (% time with esophageal pH <4) was defined as the total time at pH <4 divided by the time of monitoring. According to Zentilin *et al.* (21), <4.2% over 24h was considered as normal.

Histopathology and TEM

Biopsies were taken from subjects immediately after endomicroscopy. For histopathology, the specimens were fixed in 10% formalin, embedded in paraffin, and sectioned vertically and transversely to facilitate the comparison between histology and confocal images. The microscopic changes of the distal esophageal epithelium were routinely observed including elongation of the papillae, proliferation of basal cells, neutrophil/lymphocytes/eosinophil intraepithelial infiltration, and dilated intercellular spaces within the squamous cell epithelium according to Seefeld *et al* (22). For TEM, the specimens were prefixed in suspension with 2% glutaraldehyde in phosphate-buffered saline (pH=7.2), coded and treated for standard embedding in epoxy resin, then sectioned transversely to facilitate the comparison between TEM and confocal images. Ultrathin sections of each specimen (LKB-V, Uppsala, Sweden) were examined and photographed by use of an H-800 (Hitachi, Tokyo, Japan) transmission electron microscope. Photographs of at least 10 fields were magnified at ×10,000. Morphometric analysis of TEM microphotographs was performed by one investigator (QQQ) who was blinded to the clinical and endoscopic information of the patients. According to Tobey *et al.* (23), at least 10 randomly selected intercellular spaces were measured in each image, for at least 10 measurements in each case. Every transect was drawn at a distance of not closer than 1 µm.

Statistical analysis

Data are expressed as mean±s.d. Statistical analysis involved use of SPSS 13.0 for Windows (SPSS, Chicago, IL). Student's *t*-test was used for analysis of all continuous variables. χ^2 test was used for categorical variables. To determine factors predicting diagnosis, we used separate multivariate progressive regression analysis (with backward elimination). Differences were considered statistically significant when $P<0.05$. Sensitivity and specificity were calculated for the prediction of NERD by the characteristics of IPCLs (number and diameter) in confocal images. The receiver operating characteristic (ROC) curves were performed to find the optimal cutoff value capable of differentiating between NERD and controls. Bland–Altman plot (24) was used to evaluate the relationship of intracellular spaces in squamous epithelium by CLE and TEM and to examine for any systematic bias of two measurements (25). The abscissa (*x* axis) was drawn by assigning the mean of the two measurements, and at the limits of agreement, which are defined as the mean difference plus and minus 1.96 times the s.d. of the difference. The ordinate (*y* axis) was drawn according to the difference. To evaluate the agreement between the two methods, correlation was calculated using Pearson's correlation coefficient. The strength of rater agreement was as follows: 0–0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; and 0.81–1.00, almost perfect.

RESULTS

Patient characteristics

In total, 106 GERD patients underwent standard WLE; 34 patients were excluded because of mucosal breaks observed by WLE, and 20 patients did not meet the inclusion criteria (8 patients with Barrett's esophagus, 5 with gastroduodenal ulcer, 6 with gastric cardiac polyps, and 1 with subtotal gastrectomy). A total of 52 patients with NERD (25 males, 48%) underwent MII-pH monitoring, and 6 patients were excluded for failure of MII-pH tests. In the control group, one subject with Barrett's esophagus and four subjects with erosive esophageal mucosal breaks identified by WLE were excluded from the final analysis. In addition, five subjects were excluded from the final analysis because of incomplete data for MII-pH monitoring and poor-quality confocal images. Overall, data for 67 participants, including 46 with NERD and 21 controls, were eligible for the final analysis (**Figure 1**).

The mean age of the NERD patients was 48.9 years (s.d. 13.56; range 32–59 years).

Demographic data and clinical characteristics of patients and controls are shown in **Table 1**. The NERD patients and controls did not differ in mean age or gender. Under endoscopy, patients with NERD were more likely to show hiatus hernia as compared with controls (39.1 vs. 14.3%, $P=0.042$).

Evaluation of confocal images

A total of 6,100 CLE images from 46 patients and 21 controls were obtained. Regarding image quality, 2,440 (40%) confocal images were good, 2,745 (45%) confocal images were average, and the remainder images were poor and excluded from the final analysis. As a result, a total of 5,185 original CLE images were eligible for analysis. The mean numbers of CLE images for NERD and controls were 50±16 and 45±18, respectively. CLE imaging allowed for clear visualization of squamous epithelial cells and vasculature during ongoing endoscopy. The squamous epithelial cells had a rhombus appearance with clear borders and were arranged regularly (**Figure 2a,b**). IPCLs arise from the branch of the arborescent vessels and are directed toward the luminal surface through the epithelium. Patients with NERD had significantly more IPCLs per image than controls (8.29±3.52 vs. 5.69±2.31, $P=0.010$) (**Figure 2c**). The sensitivity and specificity of increased IPCLs were 67.7 and 71.6%, respectively. Subsequently, the ROC curve was plotted for the mean number of IPCLs in NERD patients and controls

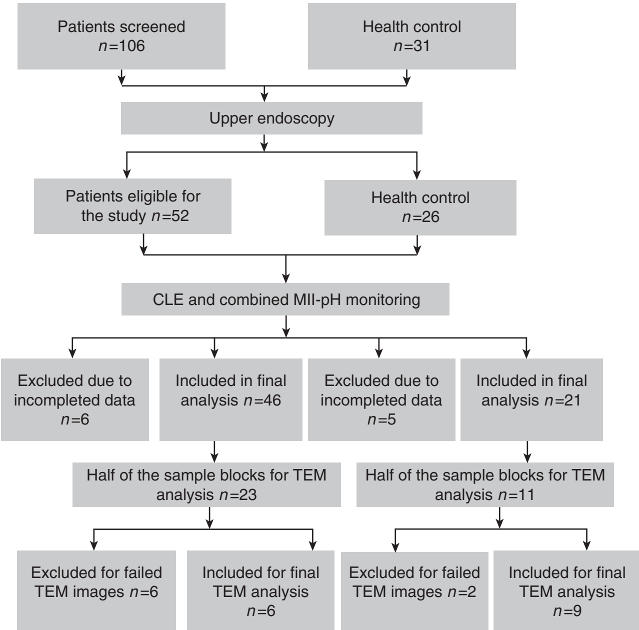

Figure 1. Study flow diagram. CLE, confocal laser endomicroscopy; MII-pH, multiple intraluminal impedance-pH; TEM, transmission electron microscopy.

Table 1. Characteristics of patients with non-erosive reflux disease (NERD) and controls

Group	NERD (*n*=46)	Controls (*n*=21)	*P* value
Mean age (range)	48.12	45.23	0.923
Males, *n* (%)	24 (52.1)	14 (66.7)	0.267
Patients with hiatus hernia, *n* (%)	18 (39.1)	3 (14.3)	0.042
DeMeester's score (mean±s.d.)	66±45	21±17	0.002
Positive symptom index (%)	24/46 (52)	2/21 (9.5)	0.001
Symptom association probability (SAP), (%)	31/46 (67.4)	3/21 (14.3)	0.000
Acid exposure time (% time with esophageal pH <4) (mean±s.d.)	7.82±2.91	4.09±2.93	0.001

to determine the cutoff value with the highest sensitivity and specificity (**Figure 3**). The optimal cutoff value between the two groups was evaluated as 6.0 per image (area under the curve (AUC) 0.722, 95% confidence interval (CI) 0.592–0.853). In all, 30 of the 46 patients (65.2%) in the NERD group had an IPCL number of >6 per image compared with 6 of the 21 control subjects (28.6%) ($P<0.001$).

As to the mean diameter of IPCLs, the mean value was 19.48 µm in NERD patients, whereas that of the controls was 15.87 µm. There was a significant difference between the two groups ($P=0.041$; **Figure 2c**). The optimal cutoff value between the two groups on the ROC curve was evaluated as 17.20 µm (AUC 0.847, 95% CI 0.747–0.947) (**Figure 3**). In all, 38 of the 46 patients (82.6%) in the NERD group had an IPCL diameter of >17.2 µm compared with 7 of the 21 control samples (33.3%) ($P<0.001$). The sensitivity and specificity were 81 and 76%, respectively.

Intercellular spaces of squamous cells of patients with NERD were significantly higher than that of controls (3.40±0.82 vs. 1.90±0.53 µm, $P=0.042$). The ROC curve showed AUC of 0.935 and 95% CI of 0.875–0.995. The optimal cutoff value between the two groups was evaluated as 2.40 µm (**Figure 3**). The sensitivity and specificity were 85.7 and 90.5%, respectively. In all, 38 of the 46 patients (82.6%) in the NERD group had DISs of >2.4 µm compared with 2 of the 21 control samples (9.5%) ($P<0.001$).

These findings and their sensitivity, specificity have been highlighted in **Table 2**.

However, NERD patients did not have larger proportion of irregularly shaped IPCLs (pecilo-spiral shaped, tortuous, and elongated) in the distal squamous epithelium (30.4 vs. 14.3%, $P=0.159$; **Figure 4**) and microcolumnar epithelial islands (**Figure 5**) at the esophagogastric junction (4 vs. 0%, $P=0.163$) than controls.

Sensitivity and specificity of combinations of IPCLs patterns and DISs of squamous cells on confocal images

Both IPCLs pattern and DIS were visible in most confocal images. Therefore, the combinations of IPCLs pattern with DIS were more significant and practical for the prediction of NERD than single characteristics. We investigated the sensitivity and specificity values for various combinations of endomicroscopic findings on CLE image *in vivo* for the diagnosis of NERD. The results showed that the combinations of increased IPCLs number and DIS had a specificity of 100% in the diagnosis of NERD patients (**Table 3**).

Reflux episodes

The number of reflux episodes (total, acid, weakly acid and non-acid, liquid, mixed, and gas) detected during the MII-pH monitoring are indicated in **Table 4**. NERD patients had a higher incidence of total mixed reflux episodes (69±33 vs. 28±9, $P=0.024$) and gas reflux episodes (66±90 vs. 10±4, $P=0.015$). With regard to the body position, similar results had been seen in the supine position (mixed reflux episodes: 14±13 vs. 6±2, $P=0.004$; gas reflux episodes: 20±35 vs. 3±4, $P=0.032$) as well as acidic (12±10 vs. 4±2, $P=0.001$) than did controls. The two groups did not differ in number of reflux events in the upright position or in weakly acidic, non-acidic, and liquid reflux episodes in the supine position (**Table 4**).

Symptom–reflux association and pH-metry

The DeMeester score was higher for NERD patients than for controls (66±45 vs. 21±17, $P=0.002$). Similar results of symptom association probability (67.4 vs. 14.3%, $P=0.000$) and positive symptom index (52 vs. 9.5, $P=0.001$) were found between the two groups. The acid exposure time (% time with esophageal pH <4) in patients with NERD and in control group was 7.82±2.91 and 4.09±2.93, respectively ($P=0.001$). The results have been shown in **Table 1**.

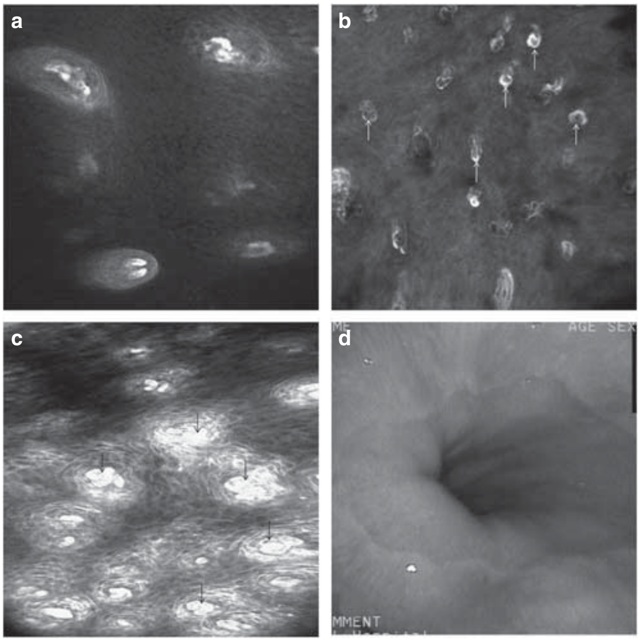

Figure 2. Confocal laser endomicroscopy (CLE) image of surface squamous epithelium in controls (**a**) and in patients with non-erosive reflux disease (NERD) (**b**), intrapapillary capillary loops (IPCLs) (arrow) were visible after fluorescein used intravenously. (**c**) NERD with dilated intercellular spaces and increased and dilated IPCLs (arrow). (**d**) Conventional white-light endoscopy view of an NERD patient showing absence of mucosal breaks.

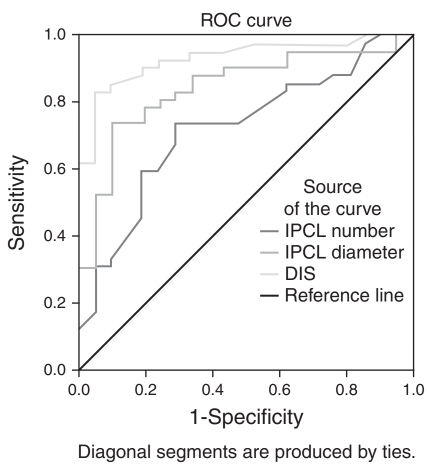

Figure 3. Receiver operating characteristic (ROC) curve analysis for intrapapillary capillary loops (IPCLs) number, IPCLs diameter, and dilatation of intercellular space (DIS) in squamous epithelium by confocal laser endomicroscopy (CLE) *in vivo* in non-erosive reflux disease (NERD) patients and controls. The blue, green, and yellow lines show the cutoff value, respectively. For colour figure see online.

Histopathology and TEM findings

For the routine histological examination of the esophagus, no eosinophilia and eosinophilic gastroenteritis were found in this study first. Assessment of the severity of microscopic changes was calculated by taking at least two of the four positive features. More microscopic changes in the distal esophageal epithelium can be observed in NERD patients than the controls, elongation of the papillae (15/46 (32.6%) vs. 3/21 (14.2%), $P=0.366$), proliferation of basal cells (16/46 (34.8%) vs. 3/21 (14.2%), $P=0.143$), neutrophil/lymphocytes/eosinophil intraepithelial infiltration (24/46 (52.2%) vs. 6/21 (28.6%), $P=0.111$), and dilated intercellular spaces (10/46 (21.7%) vs. 3/21 (14.3%), $P=0.74$). In total, 43.5% (20/46) of patients with NERD had microscopic esophagitis and 56.5% (26/46) were presented with normal histology findings, whereas the proportion of the subjects of controls with microscopic esophagitis and normal was 28.6% (6/21) and 71.4% (15/21), respectively. NERD patients were more likely to have histological evidence of esophagitis than controls, but the differences were not significant ($P=0.245$).

Of the 67 subjects who began the study, 34 were selected randomly for TEM according to the protocol. In all, 6 samples of the 23 NERD patients and 2 of the controls were failed for TEM images and were excluded from the final analysis. Finally, 26 sample blocks (17 NERD patients and 9 controls) entered the final TEM analysis. NERD patients and controls showed regular squamous epithelium cells. However, NERD patients showed obscure cellular borders and dilated intracellular spaces in the squamous epithelium, with decreased number of desmosomes. The mean esophageal intercellular spaces were higher for NERD patients than for controls (1.41 ± 0.46 vs. $0.90\pm0.20\,\mu m$, $P=0.005$). The maximal intercellular space for NERD patients and controls was 2.07 ± 0.23 and $1.48\pm0.19\,\mu m$, respectively, and the minimal intercellular spaces were 0.98 ± 0.10 and $0.55\pm0.06\,\mu m$, respectively (**Figure 6**).

Agreement of CLE and TEM in intracellular spaces in squamous epithelium

The mean intercellular spaces in the squamous epithelium by CLE and TEM for NERD patients were 3.40 ± 0.82 and $1.41\pm0.46\,\mu m$, respectively, and for controls were 1.90 ± 0.53 and $0.90\pm0.20\,\mu m$, respectively.

Figure 7 showed the Bland–Altman plot for the intracellular spaces observed by CLE and with TEM. The data points are scattered relatively closely around the x axis within the limits of ±1.96 s.d., indicating a relatively small difference between the two measurements. The mean differences did not approach zero, a proportional bias appears evident from **Figure 7**. Coefficient of repeatability was 3.95. However, Pearson's correlation coefficient showed that the intracellular spaces observed by CLE were highly correlated with TEM findings of the biopsy specimens from the same site ($r=0.75$, $P<0.001$).

Correlation of reflux episodes and CLE features

Multivariate analysis (with backward elimination) revealed that NERD patients were more likely to show an association between acidic reflux episodes and CLE features in squamous epithelium (increased number of IPCLs ($\beta=0.059$, $t=2.017$, $P=0.030$) and dilated IPCLs ($\beta=0.047$, $t=2.236$, $P=0.045$)). According to the body position, acidic reflux in the supine position was related to increased number of and dilated IPCLs ($\beta=0.063$, $t=2.895$,

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Table 2. Comparison of CLE findings in patients with NERD and controls

CLE findings	NERD (*n*=46)	Controls (*n*=21)	*P* value	Sensitivity (%)	Specificity (%)	95% CI
IPCL number (mean±s.d.)	8.29±3.52	5.69±2.31	0.01	—	—	
IPCL number >6 (per image) *n* (%)	30 (65.2)	6 (28.5)	<0.001	67.7	71.6	0.592–0.853
IPCL diameter (µm) (mean±s.d.)	19.48±3.13	15.87±2.21	0.041	—	—	
IPCL diameter >17.2 µm *n* (%)	38 (82.6)	7 (33.3)	<0.001	81	76	0.747–0.947
DIS in the squamous epithelium (µm) (mean±s.d.)	3.40±0.82	1.90±0.53	0.042	—	—	
DIS (>2.4 µm) *n* (%)	38 (82.6)	2 (9.5)	<0.001	85.7	90.5	0.875–0.995
Irregularly shaped IPCLs, *n* (%)	14 (30.4%)	3 (14.3%)	0.159	—	—	
Columnar islands, *n* (%)	4 (8.7)	0 (0)	0.163	—	—	

CLE, confocal laser endomicroscopy; DIS, dilation of intercellular spaces; IPCLs, intrapapillary capillary loops; NERD, non-erosive reflux disease.

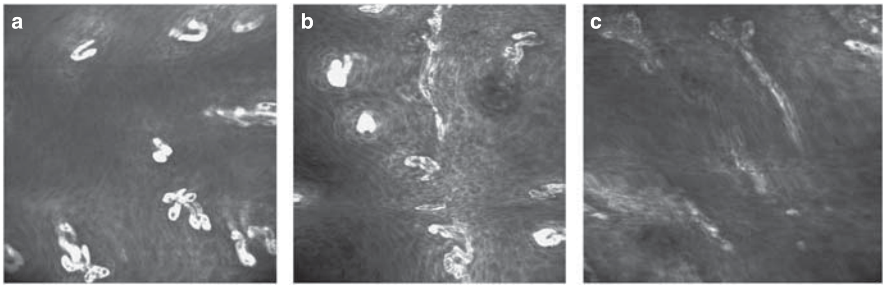

Figure 4. Confocal laser endomicroscopy (CLE) image of intrapapillary capillary loops (IPCLs) in the distal squamous epithelium: (**a**) pecilo-spiral-shaped IPCLs, (**b**) tortuous-shaped IPCLs, and (**c**) elongated IPCLs.

$P=0.038$ and $\beta=0.156$, $t=1.023$, $P=0.040$) in the squamous epithelium.

DISCUSSION

NERD is the most common phenotypic presentation in patients with GERD. Conventional WLE is not sufficient for diagnosing subtle changes of the esophageal mucosa in patients with NERD. Recently, CLE has allowed for better analysis of cellular and subcellular changes of the mucosal layer in the gastrointestinal tract (13,25) and can be used for *in-vivo* histology. Changes in the epithelium cells, vessel, and connective tissue can be classified during ongoing endoscopy.

In this study, we investigated IPCLs in the squamous epithelium of the distal esophagus of subjects with and without NERD using CLE *in vivo*. IPCLs are distinctive microvascular characteristics of esophageal epithelium. In previous studies, IPCLs could be observed by magnifying endoscopy or narrow band imaging to predict inflammatory or malignant lesions. Subtle changes of IPCLs such as dilation and elongation were suggestive of inflammatory changes in the esophagus (8,12,13,26). Sharma *et al.* (9) has reported increased number (odds ratio 12.6; 95% CI 3.7–42; $P<0.0001$) and dilatation (odds ratio 20; 95% CI 6.1–65.3; $P<0.0001$) of IPCLs were the best predictors for the diagnosis of GERD using narrow band imaging endoscopy. CLE allowed for clear visualization of IPCLs, which appears to be spiral shaped and bright vessels, which usually are spiral shaped and are much brighter than epithelial cells because of the flow of fluorescein in vessels. Elongated IPCLs are readily visible in NERD patients, along with the absence of vasculature within squamous cell epithelium *in vivo* at high resolution. Kiesslich *et al.* (26) provided preliminary data on the use of CLE in NERD. In 30 symptomatic GERD patients, the features of >5 capillary loops and dilated intercellular space >7 µm as defined by CLE had a sensitivity of 95% and a specificity of 85.4% as compared with conventional histological findings of GERD. In the present study, we evaluated the clinical utility of the CLE system *in vivo* in patients with NERD and controls. We found that the IPCLs of the distal esophageal mucosa were regular in normal squamous epithelium and the mean number of capillary loops per CLE image (475×475 µm) was 5.7 in healthy adults and 8.3 in patients with NERD. The ROC curve indicated that a mean IPCLs number of 6 is a cutoff score for diagnosing NERD with a sensitivity of 67.7% and a specificity of 71.6% and the diameter of 17.2 µm is a cutoff score for diagnosing NERD with a sensitivity of 81% and a specificity of 76%.

DISs of the esophageal epithelium are a sensitive marker of tissue damage in GERD patients and the most appropriate marker of damage evaluation in NERD (27,28). DIS diminishes transepithelial resistance of the esophageal epithelium and increases esophageal mucosal permeability. A recent esophageal perfusion study showed that the acidic and weakly acidic solutions can impair esophageal mucosal integrity (29). However, the specificity of DIS

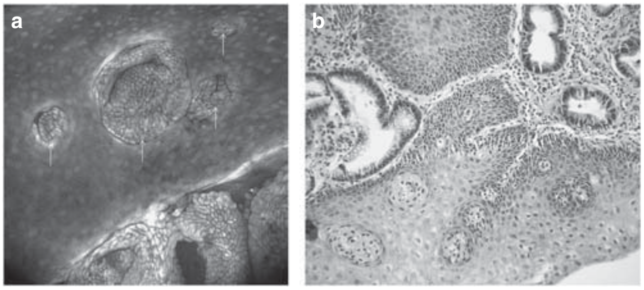

Figure 5. Confocal laser endomicroscopy (CLE) and histology images of columnar epithelial islands at the esophagogastric junction. (**a**) CLE images showing several columnar epithelial islands with typical gastric pits emerging surrounded by squamous epithelium cells after topical acriflavine. (**b**) Corresponding histology specimen of the columnar epithelial islands (hematoxylin and eosin staining; original magnification ×100).

Table 3. Sensitivity and specificity of various combinations for diagnosing NERD

Confocal findings	Sensitivity, %	Specificity, %
Increased IPCLs or dilated IPCLs or DIS	96.8	75
Increased IPCLs or dilated IPCLs	91	85.3
Increased IPCLs or DIS	88.6	89.8
Dilated IPCLs or DIS	92.3	90.4
Increased IPCLs and dilated IPCLs and DIS	42.5	100

DIS, dilation of intercellular spaces; IPCLs, intrapapillary capillary loops; NERD, non-erosive reflux disease.

in NERD patients is questionable (29,30), because it is present in up to 30% of asymptomatic healthy subjects and in patients with other esophageal disorders. In previous studies, DIS was measured and assessed quantitatively on TEM. The mean intercellular space diameter in NERD patients ranged from 1.31±0.08 to 1.5 µm *ex vivo* (28,31,32). A mean DIS of 0.74 µm is a cutoff score for esophageal intercellular damage (27) in GERD patients in western countries and 0.79 µm in China (31). Unfortunately, electron microscopy is not a practical technique for clinical application. CLE is a new endoscopic device enabling the visualization of mucosal microvascular architecture and cellular characteristics *in vivo*. In this study, we surveyed the spaces of intersquamous epithelial cells in the distal esophagus using CLE images and found intercellular space of > 2.40 µm is a predictive factor for diagnosing NERD. This study is an exploration on the intercellular space *in vivo*, how well do the cut points differentiate patients that were not included in the exploratory sample? Validity analysis for using the cut points in other study should be performed in future.

NERD patients in this study were more likely than controls to show microcolumnar epithelial islands at the distal esophagus. Normally, the esophagus is lined by stratified squamous epithelium and contains scattered mucous glands within the lamina propria of the mucosa; the stomach is lined by mucinous columnar epithelium and contains pure oxyntic glands or pure mucous glands in the deep glandular part of the mucosa of the corpus or antrum/pylorus, respectively. Columnar epithelial islands in Barrett's esophagus in white-light endoscopy have been considered to be the replacement of normal squamous epithelium by specialized intestinal metaplasia. Abundant histological and histochemical data support that the columnar epithelium located to the anatomic gastroesophageal junction represents a metaplastic epithelium that has developed as a result of GERD. Although Fass *et al.* (33) categorized GERD into three groups—NERD, erosive esophagitis, and Barrett's esophagus—evidence is lacking of columnar epithelial islands transforming directly into Barrett's esophagus. In the present study, the presence of microcolumnar epithelial islands in the squamous epithelium seen in NERD patients but not in controls may suggest that columnar epithelium metaplasia of Barrett's esophagus relates to NERD.

MII has been introduced as a new technique to detect all types of reflux episodes in terms of acidity and composition (4, 33, 34). Acid reflux is the major etiologic factor in the development of GERD and is the "gold standard" in diagnosing GERD. With the emergence of new diagnostic tools of diagnosis, such as Bilitec 2000, menomotry, and 24-h MII esophageal monitoring, other noxious contents of the refluxate besides acids, including bile acids, have been confirmed to be harmful in developing GERD. Animal experiments have shown that esophageal mucosa with a short exposure of esophageal mucosa to bile acids, both in acidic and in weakly acidic conditions, can impair mucosal integrity and provoke DIS (35). A group of leading esophageal specialists considered that MII-pH testing is the only available tool to monitor all types of reflux (4). Although abundant laboratory and clinical data support the relationship between reflux episodes and symptoms of GERD, little evidence exists of reflux patterns and IPCLs of the distal esophagus seen on CLE *in vivo*. We found higher numbers of acidic and mixed reflux events in the supine position in NERD patients than in controls. Considering the more instructive role of acid reflux in evaluating esophageal injury and predicting of healing, we analyzed acid exposure time between the two groups. The results showed that the acid exposure time (% time with esophageal pH < 4) in patients with NERD was higher than those in control group. The results are similar to those investigated by Savarino *et al* (36).

The Bland–Altman plot is useful to evaluate the relationship between the differences and the magnitude of two measurements (24). However, Bland–Altman plot in this study showed a proportional bias for CLE compared with TEM. There was a tendency with consistently larger DIS with CLE and increased bias with larger DIS and the mean differences were above zero. However, the coefficient of repeatability was 3.95. This may be mainly due to the tissue had been dehydrated with ethyl alcohol in the process of TEM, whereas the DIS in CLE image obtained *in vivo* without any dehydration. Moreover, the largest intercellular space in this study was 4.58 µm and strikingly larger than the maximal intercellular spaces under TEM. Pearson's correlation coefficient about DIS between the two measurements showed that the intracellular spaces observed by CLE were highly correlated with TEM findings of the biopsy specimens from the same site. CLE is a relatively new

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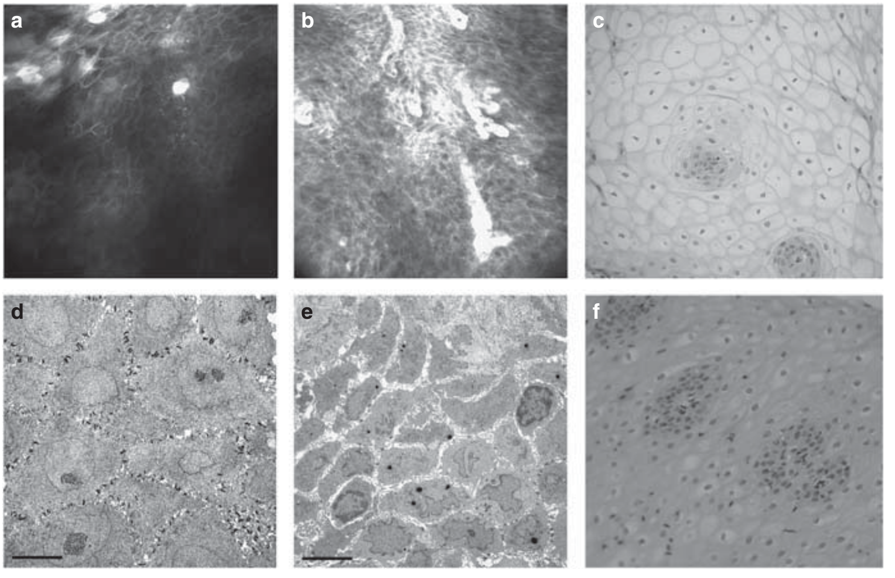

Figure 6. Comparison of confocal laser endomicroscopy (CLE) and transmission electron microscopy (TEM) images with histopathology of non-erosive reflux disease (NERD) patients and controls. (**a**) CLE image of surface squamous epithelium in controls with visible nuclei (arrow) after acriflavine staining showing squamous epithelium cells regular with clear borders. (**b**) TEM image of corresponding histology specimen (original magnification ×7,000, scale bar = 2 μm). (**c**) CLE image of surface squamous epithelium in a patient with NERD. (**d**) TEM image of corresponding histology specimen in a patient with NERD (original magnification ×7,000, scale bar = 2 μm). (**e** and **f**) Hematoxylin and eosin staining of corresponding transverse section of normal and NERD esophageal squamous epithelium.

Table 4. Number of reflux episodes for patients with NERD and controls according to composition (liquid, mixed, and gas) and pH (acidic, weakly acidic, and non-acidic) by position (upright and supine) during 24-h MII-pH measurement

Reflux episodes	Total		*P*	Upright		*P*	Supine		*P*
	NERD	Control		NERD	Control		NERD	Control	
Total number	95±44	55±28	0.094	68±34	40±28	0.340	35±23	18±9	0.276
Liquid	26±16	21±18	0.613	17±14	16±15	0.810	8±7	6±6	0.442
Mixed	69±33	28±9	0.024	55±32	22±8	0.054	14±13	6±2	0.004
Gas	66±90	10±4	0.015	46±73	10±1	0.339	20±35	3±4	0.032
Acid	48±36	29±12	0.297	36±31	21±9	0.328	12±10	4±2	0.001
Weakly acid	32±27	18±10	0.333	25±24	13±7	0.313	7±9	2±3	0.342
Non-acid	14±23	8±7	0.617	10±17	5±5	0.551	4±8	1±1	0.473

NERD, non-erosive reflux disease; MII, multiple intraluminal impedance.
Values expressed as means (±s.d.).

optical endoscopic imaging mode and being assessed for potential in enhanced endoscopic detection of gastrointestinal mucosal abnormalities.

Our study showed that CLE may improve the prediction of the histopathologic diagnosis *in vivo* and provide a potential new mucosal imaging standard of NERD, although it cannot completely replace conventional histopathologic examination currently. This evolving technique indeed needs validation and more sensitive and specific features of patients with NERD in larger studies in future.

The present study has certain limitations. First, there was a significant "drop-out" from original enrolled to final analyzable patient samples mainly because of mucosal abnormalities in patients with esophagitis and Barrett's esophagus, which often have reflux symptoms similarly to NERD. A small percentage of "drop-out" was due to invalid impedance tracing and TEM images, which should be paid more attention on in our future studies. Second, CLE is more costly than white-light high definition endoscopy and is not readily available; therefore, the use of CLE maybe restricted. Third, the surface area of the squamous epithelium to be examined by CLE is small, so the process is time consuming and tedious in examining the entire distal esophagus. For high rate of incidence of microscopic esophagitis in symptom and non-symptom NERD/GERD,

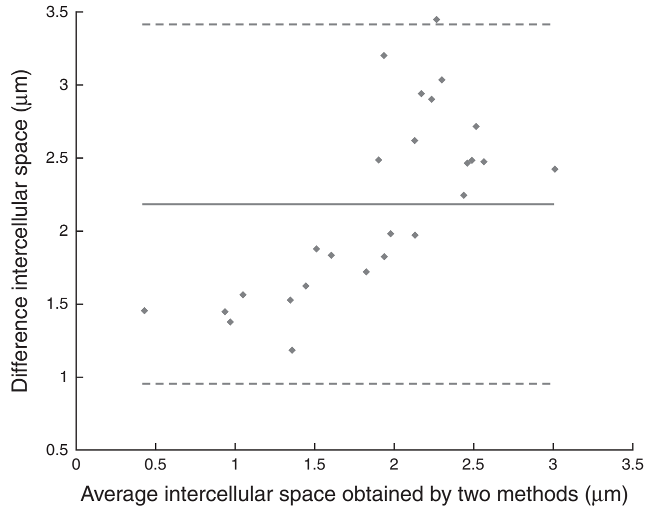

Figure 7. Bland–Altman plot analysis showing individual values of intracellular spaces in squamous epithelium by confocal laser endomicroscopy (CLE) *in-vivo* and transmission electron microscopy (TEM) images of biopsy samples for non-erosive reflux disease (NERD) patients and controls.

potential interobserver variability in counting or measuring CLE features and potential misclassification of control patients based on the GERD questionnaire may impact the decision, there are no known gold standard now. In addition, the cut points chosen from the ROC has not been validated in differentiating patients that were not included in the exploratory sample. The diagnostic yield performed by using the cut points in this study should be evaluated in future study. As well, we did not examine all NERD patients and controls by TEM. However, our objective software analysis may have circumvented this problem. Moreover, the diagnostic accuracy of CLE in NERD requires a larger sample and randomized trials in multicenters.

In conclusion, CLE could provide a histological diagnosis via "optical biopsy" and virtual histology. It should be useful for evaluating the microalterations of the esophagus *in vivo* and represent a significant improvement over standard endoscopy for the diagnosis of NERD. The described microalterations of the esophagus under CLE may be of crucial importance in clinical practice. In addition, we also found that acidic reflux is responsible for the microalterations in the esophagus of patients with NERD. However, these preliminary findings might not be sufficient to replace histological examination. The results need to be validated in future studies. Nevertheless, CLE, such newly developed method for studying microalterations *in vivo*, may provide objective assessment of histological observation of the mucosal layer. We believe that CLE will be used for the diagnosis of reflux disease and will have important clinical implications in the future.

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CONFLICT OF INTEREST

Guarantor of the article: Yan-Qing Li, MD, PhD.
Specific author contributions: Data collection: Chuan-Lian Chu, Yan-Bo Zhen, Guo-Ping Lv, Tao Yu, and Xiao-Meng Gu; data analysis: Chuan-Lian Chu, Qing-Qing Qi, Cheng-Jun Zhou, Ting-Guo Zhang, Zhen Li, and Chang-Qing Li; writing of the final version of the manuscript: Chuan-Lian Chu, Yan-Qing Li, Chang-Qing Li, Zhen Li, and Rui-Ji.
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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Non-erosive reflux disease (NERD) is the most frequent phenotypic pattern of gastroesophageal reflux disease (GERD) and has shown microscopic changes in the distal esophageal epithelium.
- ✓ Confocal laser endomicroscopy (CLE), highly magnified images (~1,000-fold) of the gastrointestinal tract mucosa, enables real-time histological analysis during endoscopy.
- ✓ Multiple intraluminal impedance-pH (MII-pH) monitoring allows for detection of all reflux events in terms of acidity (acidic, weakly acidic, and weakly alkaline) and composition (liquid, gas, or mixed).

WHAT IS NEW HERE

- ✓ Patients with non-erosive reflux disease (NERD) had significantly more number, larger diameter of intrapapillary capillary loops (IPCLs) and the intercellular spaces of squamous cells than controls.
- ✓ NERD patients had a higher incidence of mixed, gas, and acidic reflux episodes especially in the supine position.
- ✓ Confocal laser endomicroscopy (CLE) represents a useful and potentially significant improvement in examining the microalterations of the esophagus *in vivo*.

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