Mucosal integrity and sensitivity to acid in the proximal esophagus in patients with gastroesophageal reflux disease

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Abbreviations: DIS, dilated intercellular spaces; ETIS, electrical tissue impedance spectroscopy; GERD, gastroesophageal reflux disease; LES, lower esophageal sphincter; PPI, proton pump inhibitor; PSS, perfusion sensitivity score; RDQ, reflux disease questionnaire; TEER, transepithelial electrical resistance; VAS, visual analogue scale.

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SUMMARY / ABSTRACT

**Background** Acid reflux episodes that extend to the proximal esophagus are more likely to be perceived. This suggests that the proximal esophagus is more sensitive to acid than the distal esophagus, which could be caused by impaired mucosal integrity in the proximal esophagus. Our aim was to explore sensitivity to acid and mucosal integrity in different segments of the esophagus.

**Methods** A prospective observational study, including 12 patients with gastroesophageal reflux disease. After stopping acid secretion-inhibiting medication, two procedures were performed: an acid perfusion test and an upper endoscopy with electrical tissue impedance spectroscopy and esophageal biopsies. Proximal and distal sensitivity to acid and tissue impedance were measured in vivo, and mucosal permeability and epithelial intercellular spaces at different esophageal levels were measured in vitro.

**Results** Mean lag time to heartburn perception was much shorter after proximal acid perfusion (0.8 minutes) than after distal acid perfusion (3.9 minutes); \( p = 0.02 \). Median in vivo tissue impedance was significantly lower in the distal esophagus (4563 \( \Omega \cdot m \)) compared to the proximal esophagus (8170 \( \Omega \cdot m \)); \( p = 0.002 \). Transepithelial permeability, as measured by the median fluorescein flux was significantly higher in the distal (2051 nmol/cm²/h) than in the proximal segment (368 nmol/cm²/h); \( p = 0.033 \). Intercellular space ratio and maximum heartburn intensity were not significantly different between the proximal and distal esophagus.

**Conclusion** In GERD patients off acid secretion-inhibiting medication, acid exposure in the proximal segment of the esophagus provokes symptoms earlier than acid exposure in the distal esophagus, whereas mucosal integrity is impaired more in the distal esophagus. These
findings indicate that the enhanced sensitivity to proximal reflux episodes is not explained by increased mucosal permeability.

**Keywords**  Acid sensitivity; Dilated intercellular spaces; Heartburn; Mucosal integrity; Mucosal barrier; Perception;
INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common chronic disorders, with a prevalence of 10-20% in the western world. (8) GERD is diagnosed when it is proven that reflux of gastric content is causing typical symptoms and/or esophageal mucosal damage. (24) Typical reflux symptoms are heartburn and regurgitation. (25)

In healthy subjects, gastroesophageal reflux occurs several times a day without causing symptoms. (4) Whereas a subset of GERD patients exhibit excessive esophageal acid exposure, most GERD patients do not have excessive reflux but are more sensitive to reflux than healthy subjects. They perceive reflux episodes more readily, even in the absence of esophageal erosions. (16) It is hypothesized that this enhanced esophageal sensitivity for reflux in GERD patients is caused by impaired mucosal integrity. (20) In a healthy esophagus, the squamous epithelium forms an effective barrier against gastric contents. In GERD patients however, an impaired mucosal barrier has been repetitively demonstrated, even in the absence of visible erosions. (22, 30) One of the hypotheses is that impaired mucosal integrity enables the refluxed material to reach the chemosensitive nociceptors easier and faster, resulting in reduced thresholds for pain elicitation. (20, 30)

GERD patients more often have reflux episodes reaching the proximal part of the esophagus than asymptomatic controls. (4, 6, 27) In both controls and GERD patients, proximal reflux generates symptoms more readily than reflux that only reaches the distal esophagus. (6, 9, 17) Even during proton pump inhibitor (PPI) treatment, which leads to reflux that is less acidic, proximal reflux episodes are more often associated with symptoms. (23, 31) Based on this evidence, the proximal segment of the esophagus seems to be more sensitive to exposure to gastric content than the distal segment. In the current study we aim to investigate the underlying mechanisms through which gastroesophageal reflux causes symptoms.
Our hypothesis was that the higher acid sensitivity of the proximal part of the esophagus of GERD patients, as compared to the distal esophagus, is due to a more pronounced impairment in mucosal integrity in the proximal part. If this would be the case, then future therapy could be directed at protection of the mucosa. For this reason, we evaluated the acid sensitivity and the mucosal integrity of the proximal and distal segments separately in patients with GERD.

**METHODS**

**Study subjects**

This prospective, observational study was conducted in the Academic Medical Center, Amsterdam, the Netherlands. We included patients > 18 years old, with heartburn lasting more than 12 months, and gastroesophageal reflux disease confirmed by a positive symptom association probability (SAP) > 95% between reflux-specific symptoms and acidic reflux episodes on ambulatory pH-impedance measurement.(28) Patients were recruited at the outpatient clinic of the Motility Center of our hospital. None of the patients had peptic ulcer disease, Barrett’s esophagus, history of gastrointestinal cancer or history of upper gastrointestinal tract surgery. All patients gave written informed consent and the study was approved by the Medical Ethics Committee of our hospital.

**Sample size**

We based our sample size on a previous pilot study by Niemantsverdriet et al. with a similar protocol in healthy volunteers.(17) In their study in 12 subjects they found significantly higher pain scores after proximal esophageal acid perfusion (mean 6.5) compared to distal esophageal perfusion (mean 3.6). When using the measured mean pain scores with the combined standard deviation of 3.1 and a paired 2-sided t-test with a significance level of 5%
and a power of 80%, the sample size required to measure a difference in our patient group was 11. To ensure enough power, we included 12 patients.

Study protocol

Patients on PPIs, H$_2$-receptor antagonists or prokinetic drugs underwent a 10-day pharmacological washout before upper endoscopy because these drugs can mask the effect of acid reflux on esophageal mucosa and can reverse the presence of dilated intercellular spaces (DIS) in the mucosa. For the reduction of severe symptoms, patients were allowed to take rescue medication in the form of antacids. After 7 days of pharmacological washout, patients were asked to fill out the reflux disease questionnaire (RDQ) before an acid perfusion test was performed. Three days later, an upper endoscopy was performed. The RDQ is a 12-item questionnaire assessing the current severity and frequency of 3 GERD-related symptom domains (heartburn, regurgitation and epigastric pain). Each domain is assessed by four questions, all rated on a 5-point Likert scale. The mean RDQ scores thus range from 0 to 5. This questionnaire was translated into Dutch and validated.

Acid perfusion test – Acid sensitivity

Patients underwent an acid perfusion test, according to a previously described protocol. A water-perfused manometry catheter was transnasally placed in the esophagus, with 2 infusion channels at 3 and 18 cm above the lower esophageal sphincter (LES). Through these channels, we perfused hydrochloric acid solution (0.1 N HCl, pH 1) or normal saline (0.9% NaCl, pH 6.5). After a 5-min adaptation period, each segment of the esophagus was perfused for 10 minutes with either a HCl or NaCl solution first and then with the other solution, at a rate of 2.5 ml/min. The sequence of the 4 perfusion periods was randomly assigned and patients were blinded to the nature of the infused solution. During HCl infusion via the proximal channel, bicarbonate (1.4% HCO$_3^-$) was infused through the distal channel, to
neutralize the acid in the distal segment. Simultaneous pH monitoring with a catheter with pH electrodes at the infusion sites, ensured that a pH < 4 was achieved at the HCl infusion site and a pH > 4 at the other segment.\(^{17}\)

During the test, subjects were asked to score the symptom intensity every 2 minutes on a horizontal 100-mm visual analogue scale (VAS) with the extremes labelled ‘no pain’ and ‘worst possible pain’. Furthermore, they were asked to report the first sensation of heartburn, discomfort and pain. When subjects experienced pain, acid infusion was discontinued immediately. The lag time from the start of acid infusion to initial discomfort and the maximum VAS score during acid perfusion were noted. Combining both parameters, the perfusion sensitivity score (PSS) was calculated as (total acid perfusion time - lag time to perception) \(\cdot\) maximum VAS.\(^{12}\)

*Upper endoscopy*

In each patient, after 10 days of pharmacological washout, an upper endoscopy was performed by one and the same gastroenterologist. After routine inspection of the esophagus, stomach and proximal duodenum, Electrical Tissue Impedance Spectroscopy (ETIS) measurements were performed at 3 cm and 18 cm proximal to the Z-line. Additionally, at each level 5 large mucosal biopsies were obtained with a jumbo biopsy forceps.\(^{14}\) These biopsies were used to investigate the mucosal permeability in Ussing chambers, and to measure dilation of intercellular spaces using transmission electron microscopy. All biopsies were taken from macroscopically unaffected mucosa.

*Electrical tissue impedance spectroscopy – In vivo mucosal integrity*

Electrical tissue impedance spectroscopy (ETIS) measurements were performed at two levels, in a 4-quadrant fashion, using a dedicated probe (diameter 2.3 mm).\(^{26}\) The probe with 4 electrodes on the tip (Medical Engineering Section, Royal Hallamshire Hospital, Sheffield,
UK) was passed through the working channel of the endoscope and pushed against the esophageal wall with a minimum angle of 30° and with a force that just caused a blanching of the mucosa. Two electrodes on the tip injected an alternating current with a peak magnitude of 20 µA, and the other 2 electrodes measured the impedance spectrum. The impedance was calculated as previously described.

_Ussing Chambers – In vitro mucosal integrity_

To evaluate the hypothesis that sensitivity is related to increased permeability to small molecules, transepithelial mucosal resistance and permeability was analyzed in Ussing chambers. Therefore, 4 mucosal biopsies from both the distal and proximal esophageal segments were immediately immersed in ice-cold oxygenated Meyler buffer. Within 15 minutes, the specimens were mounted in biopsy holders (aperture diameter 2 mm, square area 0.0314 cm²) in Ussing chambers (figure 1). Biopsies were kept at 37 °C, in Meyler buffer and continuously gassed with carbogen (95% O₂ - 5% CO₂) [figure 1].

After a 15-minute acclimatization period, the luminal bathing solution was replaced with a modified Meyler buffer containing fluorescein (376 Da, 0.5 mg/ml). The transepithelial flux of fluorescent molecules was measured by sampling the bath at the basolateral side of the biopsy after 0, 15, 30, 45 and 60 minutes. The volume in the basolateral bath was kept constant by adding Meyler buffer. The fluorescein concentration in the samples was measured with a fluorescence plate reader (BioTek Synergy, BioTek, Winooski, VT, USA). Luminal to basolateral fluorescein flux was expressed as nmol/cm²/h.

Furthermore, two sets of electrodes (World Precision Instruments, Berlin, Germany) were used to measure the voltage deflection induced by a bipolar constant current of 20 µA. The transepithelial electrical resistance (TEER) was measured after 0, 15, 30, 45 and 60 minutes throughout the measurement.
Transmission Electron Microscopy – In vitro mucosal integrity

Intercellular space between epithelial cells in the basal epithelial layers is considered a measure of mucosal integrity.(5, 22) Two mucosal biopsies, taken at 3 cm and 18 cm above the LES, were immediately immersed in Karnovsky fixative. After storage at 4° Celsius for several days, tissues were post-fixed, block-stained, dehydrated and embedded in epoxy resin. With a Philips CM10 transmission electron microscope (FEI Technai G2 Spirit), the laboratory technician, blinded to the status of the biopsy, took 10 random photographs of each biopsy at the basal layer (magnification 4600x), using a digital transmission EM camera. Dedicated software was used (Qwin, Leica Microsystems, Wetzlar, Germany) to calculate the intercellular space ratio by dividing the intercellular space surface by the total cell surface (figure 2).(26)

Statistical analyses

We performed all analyses using SPSS Statistics version 22.0 and Graph Pad Prism version 5.0. Normally distributed data are described as number and percentage or mean with range when appropriate. Not normally distributed data are described as median with interquartile range (IQR) or range when appropriate. Lag time to initial heartburn perception was analyzed using survival curves and the log rank (Mantel-Cox) test. Symptom intensity, perfusion sensitivity scores, and intercellular space ratio were compared using the Wilcoxon signed rank test (paired samples). The multiple related measurements obtained for each individual subject with the ETIS probe and with the Ussing experiments were analyzed by calculating a median of the multiple measurements, followed by the Wilcoxon signed rank test (paired samples). A p-value < 0.05 was considered significant.

RESULTS
Subject characteristics

We included 12 GERD patients (8 females), with a mean age of 49 years (range 28 - 66 years). The mean RDQ score was 3.4 out of 5. Seventy-five percent of patients reported that they had daily heartburn and regurgitation. Median (IQR) acid exposure time, as measured with 24-hour pH-impedance monitoring off PPI, was 9.7% (8.4 - 14.3%). Median number of reflux events was 134 (IQR 93 - 162). All patients had a symptom association probability (SAP) > 95% for acidic reflux episodes. All patients used PPIs and discontinued PPI use.

Esophageal acid sensitivity

In all patients, the esophageal acid sensitivity test was successfully performed [figure 3]. Two out of 12 patients (17%) did not experience heartburn during the test. The lag time to heartburn perception was shorter after proximal acid perfusion (mean 0.8 minutes, 95% CI 0.1 - 1.5) compared to distal acid perfusion (mean 3.9 minutes, 95% CI 2.4 - 5.4); log rank $p = 0.02$. Maximum heartburn intensity was similar during proximal (median VAS (IQR) 4.0 (1.3 - 7.1)) and distal (median VAS (IQR) 3.5 (1.8 - 6.9); $p = 0.638$) acid perfusion. There was a trend towards a higher perfusion sensitivity score in the proximal (median (IQR) 40 (11 - 59)) compared to the distal segment (median (IQR) 25 (5 - 41)), $p = 0.059$.

Esophageal mucosal integrity and intercellular spaces

Extracellular in vivo tissue impedance (ETIS) and in vitro fluorescein flux were significantly different between the proximal and distal esophagus [figure 4]. The tissue impedance was significantly lower in the distal esophagus (median (IQR) 4563 Ω·m (3640 - 5429)) compared to the proximal esophagus (8170 Ω·m (7353 - 10110); $p = 0.002$). Transepithelial permeability, measured by the fluorescein flux, was significantly higher in the distal than in the proximal segment (median (IQR) 2051 (1201 - 3708) nmol/cm²/h and 368 (0 - 1389)
nmol/cm²/h; \( p = 0.033 \). Both are signs of a more impaired mucosal integrity at the level of the distal esophagus.

Transepithelial electrical resistance (TEER) was comparable in the proximal and distal esophagus (median (IQR) 133 (92 - 149) Ω/cm² and 108 (83 - 146) Ω/cm² respectively; \( p = 0.11 \)).

Intercellular space ratio was not significantly different between the proximal (median (IQR) 0.17 (0.12 - 0.23) and distal esophagus 0.14 (0.10 - 0.26); \( p = 0.833 \)). In four samples the basal membrane could not be identified in the specimen, hampering orientation and therefore these biopsies were not used for assessment of intercellular space ratio.

**DISCUSSION**

In the present study we demonstrated that in GERD patients the threshold for induction of heartburn by intraesophageal acid perfusion was lower in the proximal than in the distal segment of the esophagus, while mucosal integrity was more impaired in the distal than in the proximal esophageal segment.

Several previous studies have shown that reflux reaching the proximal esophagus is more likely to provoke symptoms than distal reflux, in both GERD patients and controls.(6, 9, 11, 17) In addition, it has been found that GERD patients have a higher proximal esophageal acid exposure and longer duration of proximal reflux events than healthy controls(4, 6, 27) and that PPI-refractory GERD is associated with more proximal reflux episodes.(19, 23, 31) Understanding of the mechanisms that underlie the enhanced proximal sensitivity could lead to improvement of GERD treatment. The current study was the first combining the measurement of acid sensitivity and mucosal integrity in the proximal and distal segment.
separately, in an attempt to explain the underlying mechanism of enhanced sensitivity of proximal reflux.

We hypothesized that GERD patients would be more sensitive to proximal than distal acid perfusion due to more pronounced mucosal integrity changes in the proximal esophagus. Although we confirmed the presence of increased sensitivity to acid at the proximal segment, the mucosa was actually more impaired distally.

From our results, we conclude that the enhanced perception of proximal reflux that is frequently present in patients with GERD cannot be attributed to increased mucosal permeability in the proximal esophagus. Moreover, we think that the enhanced proximal sensitivity is not likely to be due to a larger reflux volume or a lower pH-drop, causing a stronger trigger for exposed nociceptors, as previously suggested.(11, 21) Our results demonstrate that infusion of small volumes of acid in the proximal esophagus lead to faster perception than infusion of similar volumes of acid in the distal esophagus. In our opinion, this makes it less likely that a larger reflux volume is underlying the faster perception of acid in the proximal esophagus. However, it should be noticed that in our study the proximal acid infusion area (3 to 18 centimeters above the LES) is larger than the distal infusion area (3 centimeters above the LES), even though we buffered the distal area during proximal acid infusion. This means that a larger exposed esophageal area or delayed acid clearance can still be a possible explanation for increased sensitivity in the proximal esophagus, but this is also the case in the esophagus after a reflux episode.(3) The results of a recent study suggest that the mucosal afferent nerves are located more superficially than in the distal esophagus. This feature also can be a possible explanation for the observed enhanced sensitivity to acid of the proximal esophagus.(29)
In the current study, we confirmed that, in patients with GERD, mucosal integrity in the distal esophagus is impaired. Only one previous study compared this to the mucosal integrity of the proximal esophagus, in healthy volunteers.\(^{(29)}\) We found that the mucosal integrity in the distal esophagus was lower than in the proximal esophagus, which is consistent with their results of a lower baseline impedance and a trend towards a lower TEER in the distal than the proximal esophagus.\(^{(29)}\) It is tempting to explain the isolated distal mucosal impairment by the fact that the distal segment is exposed to acid reflux more frequently and for longer periods of time than the proximal segment.\(^{(10, 18, 30)}\) However, Farré and co-workers observed that a 30-minute perfusion of acidic or weakly acidic solutions in healthy subjects provoked dilatation of the intercellular spaces not only in the distal but also in the proximal esophagus.\(^{(10)}\) Perfusion of a neutral solution did not provoke dilated intercellular spaces.\(^{(10)}\) In two other studies, in vitro acid exposure also provoked a significant decrease in transepithelial electrical resistance (TEER) in biopsies of both GERD patients and healthy subjects.\(^{(18, 30)}\) When biopsies were pretreated with alginate solution, the drop in TEER was no longer significant.\(^{(30)}\) Infusion with both acid and bile salts induced changes that were similar to those induced by acid alone. These findings support the hypothesis that enhanced mucosal permeability in the distal esophagus is provoked by reflux of stomach contents.

Our patients were allowed to take rescue medication in the form of antacids. It is possible that this may have reduced the abnormalities in mucosal permeability. However, no conclusive literature was found regarding the effect of antacids on esophageal mucosal healing. Moreover, prohibiting antacids could introduce selection bias and raise ethical concerns. Based on these methodological objections and the pharmacodynamics, we chose to allow antacid use.\(^{(7)}\)

In conclusion, the findings of this study show that, in GERD patients, acid exposure in the proximal esophagus provokes symptoms earlier than acid exposure in the distal esophagus,
whereas mucosal integrity is impaired more in the distal esophagus. These observations indicate that the enhanced sensitivity to proximal reflux episodes, characteristic of GERD, cannot be explained by increased permeability of the mucosa in the proximal esophagus.
REFERENCES


Figure 1. Ussing Chambers. The biopsy specimen is placed in between the two chambers filled with Meyler buffer. The luminal bathing solution contains fluorescein. The transepithelial flux of fluorescent molecules was measured by sampling the basolateral bath. Also, two sets of electrodes connected to a dual voltage clamp were used to measure the voltage deflection induced by a bipolar constant current of 20 µA, also called the transepithelial electrical resistance.

Figure 2. Two electron microscopy pictures of esophageal mucosal biopsies. In the left picture, dilated intercellular spaces (DIS) can be seen, in the right picture, normal intercellular spaces are shown. The basal layer is in both pictures visible on the right.

Figure 3. Parameters of acid perception in the proximal and distal esophageal segment of gastroesophageal reflux disease patients, A) Lag time to initial heartburn perception, B) Maximum symptom intensity and C) Perfusion sensitivity score.

VAS, visual analogue scale; PSS, perfusion sensitivity score.

Figure 4. Mucosal integrity represented by A) Extracellular impedance in vivo measured by the ETIS probe, B) Transepithelial fluorescein flux in vitro measured in Ussing chambers and C) Transepithelial electrical resistance in Ussing chambers.

ETIS, electrical tissue impedance spectroscopy; TEER, transepithelial electrical resistance
**A Time to perception**

- Log rank $p = 0.02$

**B Maximum intensity**

$p > 0.05$

**C Perfusion sensitivity score**

$p > 0.05$
**A Electrical tissue impedance (ETIS)**

- Proximal vs. Distal
- $\Omega \cdot m$

**B Fluorescein flux**

- Proximal vs. Distal
- nmol/cm²/h

**C Transepithelial electrical resistance (TEER)**

- Proximal vs. Distal
- $\Omega$ cm²

Statistical significance:
- $p = 0.002$
- $p = 0.033$
- $p > 0.05$
- $p = 0.002$