Hypersensitivity to acid is associated with impaired esophageal mucosal integrity in patients with gastroesophageal reflux disease with and without esophagitis

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Weijenborg PW, Smout AJ, Verheijen C, van Veen HA, Verheij J, de Jonge WJ, Bredenoord AJ. Hypersensitivity to acid is associated with impaired esophageal mucosal integrity in patients with gastroesophageal reflux disease with and without esophagitis. Am J Physiol Gastrointest Liver Physiol 307: G323–G329, 2014. First published June 12, 2014; doi:10.1152/ajpgi.00345.2013.—Increased esophageal sensitivity and impaired mucosal integrity have both been described in patients with gastroesophageal reflux disease, but the relationship between hypersensitivity and mucosal integrity is unclear. The aim of the present study was to investigate acid sensitivity in patients with erosive and nonerosive reflux disease and control subjects to determine the relation with functional esophageal mucosal integrity changes as well as to investigate cellular mechanisms of impaired mucosal integrity in these patients. In this prospective experimental study, 12 patients with nonerosive reflux disease, 12 patients with esophagitis grade A or B, and 11 healthy control subjects underwent an acid perfusion test and upper endoscopy. Mucosal integrity was measured during endoscopy by electrical tissue impedance spectroscopy and biopsy specimens were analyzed in Ussing chambers for transepithelial electrical resistance, transepithelial permeability and gene expression of tight junction proteins and filaggrin. Patients with nonerosive reflux disease and esophagitis were more sensitive to acid perfusion compared with control subjects, having a shorter time to perception of heartburn and higher perceived intensity of heartburn. In reflux patients, enhanced acid sensitivity was associated with impairment of in vivo and vitro esophageal mucosal integrity. Mucosal integrity was significantly impaired in patients with esophagitis, displaying higher transepithelial permeability and lower extracellular impedance. Although no significant differences in the expression of tight junction proteins were found in biopsies among patient groups, mucosal integrity parameters in reflux patients correlated negatively with the expression of filaggrin. In conclusion, sensitivity to acid is enhanced in patients with gastroesophageal reflux disease, irrespective of the presence of erosions, and is associated with impaired esophageal mucosal integrity. Mucosal integrity of the esophagus is associated with the expression of filaggrin.

esophageal sensitivity; gastroesophageal reflux; heartburn; mucosal integrity; nonerosive reflux disease

PATIENTS WITH GASTROESOPHAGEAL REFLUX DISEASE (GERD) have symptoms and/or esophageal mucosal damage due to the reflux of gastric content (25). Why these patients suffer from heartburn and/or regurgitation, whereas others with often similar reflux characteristics do not, is not entirely clear. It has been suggested that GERD patients have an altered esophageal sensitivity (23). In addition, although macroscopic erosions might be absent, subtle defects in esophageal mucosal integrity may lead to an enhanced perception of reflux (28). The exact relation between these parameters remains unclear, and better understanding of the complex mechanisms ultimately leading to reflux perception may lead to new therapeutic targets.

The permeation of noxious components of the refluxate into the mucosa is normally prevented by a tight barrier formed by the nonkeratinized stratified squamous epithelium (4, 18). In patients with esophagitis, the natural mucosal barrier is clearly breached at the site of erosions, and noxious stimuli can easily traverse the mucosa and subsequently activate the underlying nociceptors. However, the mucosa is macroscopically intact in patients with nonerosive reflux disease (NERD), and it is unclear how reflux can reach and activate nociceptors in these patients (3). A recent study (7) has demonstrated microscopic abnormalities in the esophageal epithelium of NERD patients, such as dilated intercellular spaces (DIS). DIS have been suggested to be a morphological feature of impaired mucosal integrity, facilitating the permeation of acid into the submucosa. Functionally, mucosal integrity can be assessed by mounting an esophageal tissue section or biopsy in Ussing chambers and measuring the transepithelial permeability of small molecules or transepithelial electrical resistance (TEER) (24, 27). Short exposure of the animal and human mucosa to components of refluxate can induce an increase in transepithelial permeability and a decrease in TEER (9).

A relationship between esophageal sensitivity to acid and mucosal integrity has only recently been proposed (28). Therefore, in the present study, we aimed to determine esophageal sensitivity and esophageal mucosal integrity of different subtypes of GERD patients and determine the relation between sensitivity to acid and mucosal integrity. We hypothesized that an impairment of mucosal barrier function leads to increased sensitivity to acid in patients with GERD and NERD.

METHODS

Subjects. We recruited 24 patients with GERD at the outpatient clinic of the Academic Medical Center. All patients presented with heartburn and/or acid regurgitation. Twelve patients were previously diagnosed with reflux esophagitis grade A or B according to Los Angeles classification (25). In the other 12 patients, diagnosis of

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GERD was established with 24-h pH-impedance monitoring, during which a positive relation between symptoms and reflux episodes (symptom association probability > 95%) was observed. In these patients, upper endoscopy showed no abnormalities, and they were therefore classified as NERD patients (25). The 24-h pH-impedance measurements were performed using a combined pH-impedance catheter assembly (Unisensor, Attikon, Switzerland), which was placed at 5 cm from the upper border of the manometrically localized lower esophageal sphincter (LES). Impedance and pH signals were stored on a digital datalogger (Ohmega, MMS, Enschede, The Netherlands). Each pH-impedance measurement was fully read by a gastroenterologist with expertise in gastrointestinal motility. We also recruited 11 healthy control subjects without a history of reflux symptoms or other abdominal complaints. Demographic data of all subjects are shown in Table 1. Each subject gave written informed consent, and the study protocol was approved by the Medical Ethics Committee of the Academic Medical Center.

Study protocol. All patients ceased the use of acid-suppressive or prokinetic drugs for a period of 3 wk. After 2 wk of pharmacological washout, an acid perfusion test was performed. Ensuring a 1-wk recovery period from the acid perfusion test, an upper endoscopy was performed. During the endoscopy, the presence or absence of episodes was confirmed, and mucosal integrity was assessed in vivo using an electrical tissue impedance spectroscopy (ETIS) probe (26). In addition, eight biopsies of the distal esophagus were obtained to assess the size of intercellular spaces by electron microscopy, to assess mucosal integrity in vitro in Ussing chambers, and to perform real-time PCR for genes related to barrier function.

Acid perfusion test. An acid perfusion test was performed using a conventional water-perfused manometry catheter with a sleeve sensor positioned at the location of the LES and an incorporated infusion channel 6 cm above the LES. Patients were seated in a semirecumbent position. After an accommodation period of 5 min, perfusion was carried out for 10 min with a neutral solution (0.9% NaCl buffered at pH 6.5) at a rate of 8 ml/min. Perfusion was then switched to an acidic solution (0.1 N HCl at pH 1.1) for 20 min. Subjects were blinded to the nature of the solution at all times and were not aware of when the acid perfusion was started. We noted the time to first perception of heartburn and time to discomfort. Furthermore, subjects were asked to score the intensity of their symptoms every 2 min on a horizontal 100-mm visual analog scale (VAS) with the extremes labeled “no heartburn and time to discomfort. Furthermore, subjects were asked to score the intensity of their symptoms every 2 min on a horizontal 100-mm visual analog scale (VAS) with the extremes labeled “no pain” and “worst possible pain.” By combining both parameters of acid perception, the perfusion sensitivity score was calculated as follows: [(total perfusion time − lag time to perception) × maximum VAS], similar to previously described method (10).

Mucosal integrity in vivo: ETIS. For the assessment of mucosal integrity in vivo, we used ETIS. A probe with four electrodes at the tip was advanced through the working channel of the endoscope and pressed against the mucosa. The probe was attached to a data-acquisition unit, the Mk 3.5 Tissue Impedance Meter (Medical Engineering Section, Royal Hallamshire Hospital, Sheffield, UK). An alternating current with a peak magnitude of 20 μA was created in a sweep of 30 frequencies ranging from 2 kHz to 1.6 MHz. Simultaneously, the potential difference was measured between the other two electrodes, and thereby an impedance spectrum was created. As previously described, the impedance of both the intracellular and extracellular compartments of the mucosa was calculated by fitting these impedance spectra to Cole’s equation (15). We (26) have previously demonstrated that extracellular impedance correlates well with DIS measured by electron microscopy and with TEER and permeability.

Mucosal integrity in vitro: Ussing chamber experiments. During upper endoscopy, four biopsies were obtained at 5 cm proximal to the Z-line. All biopsies were taken from macroscopically unaffected mucosa. Immediately after being harvested, biopsy specimens were immersed in ice-cold oxygenated Meyler buffer. Within 15 min, specimens were mounted in Ussing chambers using biopsy holders with an aperture diameter of 2 mm and a square area of 0.0314 cm². Biopsies were bathed in modified Meyler buffer composed of 105 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂-2H₂O, 1.0 mM MgCl₂-6H₂O, 20.0 mM NaHCO₃, 0.4 mM NaH₂PO₄-2H₂O, 0.3 mM Na₂HPO₄-2H₂O, and 10.0 mM HEPES at pH 7.4 and was continuously gassed with carbogen (95% O₂-5% CO₂). Biopsies were kept at 37°C using hot water jackets. After a 15-min acclimatization period, the luminal bathing solution was replaced with modified Meyler buffer containing fluorescein (376 Da) at a concentration of 0.5 mg/ml. The serosal bath was sampled at 0, 15, 30, 45, and 60 min, and the volume in the serosal chamber was kept constant with the initial modified Meyler buffer. The fluorescein concentration in the samples was measured with a fluorescence plate reader (BioTek Synergy, BioTek, Winooski, VT) using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Luminal to serosal flux was expressed as nanomoles per centimeter squared per hour. Furthermore, two sets of electrodes connected to a dual voltage clamp (World Precision Instruments, Berlin, Germany) were used to measure the voltage deflection induced by a bipolar constant current of 20 μA. TEER was calculated according to Ohm’s law. A baseline TEER value was measured 15 min after placement of the biopsy in the Ussing chamber. Baseline TEER values of all biopsies were averaged for each patient. TEER was measured every 15 min throughout the experimental protocol.

Transmission electron microscopy. Two biopsies were immediately immersed in a fixation solution containing 4% formaldehyde made from fresh paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Biopsies were stored in the same fixative at 4°C for several days. After fixation, biopsies were washed in distilled water, osmicated for 60 min in 1% OsO₄ in water, and washed again in distilled water. For contrast enhancement in the electron microscope, biopsies were block stained overnight in 1.5% aqueous uranyl acetate, dehydrated through a series of ethanol, and then embedded in LX-112 (Ladd). Ultrathin sections of 80 nm were cut with a diamond knife, collected on formvar-coated grids, and stained with uranyl acetate and lead citrate. Sections were examined with a FEI Tecnai-12 G2 Spirit Biotwin electron microscope.

An investigator blinded to subject status took 10 random photos of each biopsy at the basal layer (×4,600 magnification). Photos were analyzed using Leica QWin. The intercellular space ratio was calculated as previously described (8).

Quantitative real-time PCR and immunohistochemistry. One mucosal biopsy from each patient was stored in RNA stabilization reagent (RNAlater, Qiagen). Biopsies were homogenized, and total RNA was extracted using the RNeasy Micro Kit (Qiagen). The RNA concentration was assessed by a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA was synthesized using a reverse transcriptase reaction performed according to the MBI Fermentas cDNA synthesis kit (Fermentas, Vilnius, Lithuania) using both Oligo(dT)18 and D(N)6 primers. Quantitative real-time RT-PCR was performed on a LightCycler 480 (Roche Diagnostic, Almere, The Netherlands) using SYBR Green PCR Master Mix (Roche Diagnostic) and primers from Invitrogen. For quantitative real-time PCR, samples were normalized for the mean of the three most stable housekeeping genes (cyclophillin, GAPDH, and β-actin) as deter-

<table>
<thead>
<tr>
<th>Table 1. Patient characteristics</th>
<th>Erosive GERD Patients</th>
<th>Nonerosive GERD Patients</th>
<th>Healthy Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Age, mean (range)</td>
<td>52.2 (23–65)</td>
<td>47.8 (22–77)</td>
<td>38.5 (25–59)</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>5 (42)</td>
<td>8 (67)</td>
<td>4 (36)</td>
</tr>
<tr>
<td>Proton pump inhibitor use before inclusion, n (%)</td>
<td>12 (100)</td>
<td>12 (100)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

n, number of patients/subjects; GERD, gastroesophageal reflux disease.
mined by analysis with geNorm method software (http://medgen.ugent.be/~jvdesomp/genorm/). Transient levels of the tight junction proteins claudin-1, claudin-2, occludin, and zonula occludens (ZO)-1 and of the filament-associated protein filaggrin were determined in duplo.

One distal esophageal biopsy specimen was fixed in formaldehyde and embedded in paraffin. After deparaffinization, heat-induced antigen retrieval was performed using citrate buffer. Endogenous peroxidase was blocked with H2O2, and nonspecific IgG-binding sites were blocked with normal goat serum. Subsequently, sections were incubated with rabbit polyclonal anti-human filaggrin for 1 h (1:100, Sigma-Aldrich, St. Louis, MO). After being washed with PBS, sections were incubated with biotinylated goat anti-rabbit antibody (1: 200, Dako, Glostrup, Denmark) for 30 min followed by a 30-min incubation with labeled streptavidin-biotin-2 horseradish peroxidase (LSAB2, HRP kit, Dako). Filaggrin was visualized with 3,3′-diaminobenzidine (Dako), and sections were counterstained with Mayer’s hematoxylin before being mounted in glycercine-gelatin (Dako).

**Statistical analyses.** Data are expressed as means with SEs or medians with interquartile ranges (IQRs) when appropriate. The 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 ratios were compared using one-way ANOVA (Dunnett post hoc test). The multiple related measurements obtained for each individual subject with the ETIS probe and with the Ussing experiments were analyzed using a linear mixed-effects model. The model uses compound symmetry as a covariance matrix to address the correlation between separate measurements in each individual. Values of <0.05 were considered significant. All analyses were performed using Graph Pad Prism (version 5.01) and SPSS Statistics (version 19.01).

**RESULTS

**Esophageal sensitivity.** The acid perfusion test was successfully performed in all subjects. Seven of eleven healthy subjects (64%) did not experience heartburn during the entire infusion period; all healthy subjects tolerated and completed 20 min of acid perfusion. In contrast, in 9 of 12 esophagitis patients and in 8 of 12 NERD patients, acid perfusion was prematurely stopped due to pain. The mean lag time to symptom perception was significantly lower in both patients with esophagitis [mean: 3.5 min, 95% confidence interval (CI): 2.5–4.5 min] and NERD (mean: 4.3 min, 95% CI: 2.0–6.6 min) compared with control subjects (mean: 16.5 min, 95% CI: 13.7–19.3 min, P < 0.01 by log rank test; Fig. 1A). The maximum symptom intensity during the period of acid perfusion was significantly higher in patients with esophagitis (median VAS: 8.3, IQR: 5.1–8.5) and in NERD patients (mean VAS: 7.1, IQR: 3.6–9.1) compared with control subjects (mean VAS: 1.6, IQR: 0.4–1.2, P < 0.01; Fig. 1B). Therefore, the perfusion sensitivity score was also significantly higher in patients with esophagitis (median: 202, IQR: 141–232) and in NERD patients (median: 191, IQR: 101–220) compared with control subjects (median: 0, IQR: 0–92, P < 0.01; Fig. 1C).

**Mucosal integrity.** Extracellular impedance was significantly lower in patients with esophagitis compared with healthy control subjects (mean: 5,995 Ω·m, 95% CI: 4,411–7,586 Ω·m, and mean: 9,439 Ω·m, 95% CI: 7,788–11,090 Ω·m, respectively, P < 0.01), whereas the difference between NERD patients and control subjects tended to significance (mean: 7,232 Ω·m, 95% CI: 5,660–8,805 Ω·m, and mean: 9,439 Ω·m, 95% CI: 7,788–11,090 Ω·m, respectively, P = 0.06; Fig. 2A). Extracellular impedance was similar between patients with esophagitis and NERD patients.

Transepthelial permeability was significantly higher in esophagitis patients compared with control subjects (mean: 1,924 nmol·cm·2·h, 95% CI: 1,313–2,535 nmol·cm·2·h, and mean: 867 nmol·cm·2·h, 95% CI: 221–1,513 nmol·cm·2·h, respectively, P < 0.05). In NERD patients, transepithelial permeability did not differ significantly from control subjects (mean: 1,367 nmol·cm·2·h, 95% CI: 750–1,985 nmol·cm·2·h, and mean: 867 nmol·cm·2·h, 95% CI: 221–1,513 nmol·cm·2·h, respectively, P = 0.25; Fig. 2B). Baseline TEER was not significantly different between esophagitis patients, NERD patients, and control subjects, with only a trend toward a lower TEER in esophagitis patients (P = 0.06; Fig. 2C).

Dilation of intercellular spaces was successfully measured in 20 subjects. The intercellular space ratio was not significantly different between patients with esophagitis, patients with NERD, and control subjects (median: 0.22, IQR: 0.11–0.27; median: 0.21, IQR: 0.20–0.25; and median: 0.14, IQR: 0.11–0.22, respectively, P = 0.43; data not shown). Failure of intercellular space measurements occurred in some samples as the result of technical difficulties, mainly because the basal membrane was not always visible, hampering orientation.

![Fig. 1](http://ajpgi.physiology.org/)

**Fig. 1.** Parameters of acid perception in patients with esophagitis, patients with nonerosive reflux disease (NERD), and control subjects. A: lag time to initial heartburn perception. B: maximum symptom intensity. VAS, visual analog scale. C: perfusion sensitivity scores. *P < 0.05.
**Esophageal tight junction expression.** Transcript levels of the tight junction proteins claudin-1, claudin-2, occludin, and ZO-1 were not significantly different between patients with esophagitis, patients with NERD, and control subjects, although claudin-2 showed a trend toward a higher expression in GERD patients (Table 2). Filaggrin expression levels were also not significantly different between patient categories (Table 2).

Although no significant differences between patient categories were observed, expression levels of some investigated genes correlated with esophageal barrier function of GERD patients. The filaggrin expression level correlated with all functional parameters of barrier function: negatively with transepithelial fluorescein flux ($r = -0.69$, $P < 0.01$) and positively with TEER ($r = 0.74$, $P < 0.01$) and extracellular impedance in vivo ($r = 0.61$, $P < 0.01$; Table 3). The presence of filaggrin was confirmed immunohistochemically, as staining of esophageal biopsies demonstrated a cytoplasmatic granular pattern in the upper layers of the esophageal epithelium (Fig. 3).

In addition, ZO-1 expression levels correlated significantly with TEER ($r = 0.60$, $P < 0.01$) and extracellular impedance in vivo ($r = 0.55$, $P < 0.01$) but not with transepithelial fluorescein flux (Table 3).

**Relation between sensitivity and mucosal integrity.** There was a significant inverse correlation between perfusion sensitivity score and extracellular impedance as measured with ETIS ($r = -0.40$, $P < 0.05$; Fig. 4A). In concordance, there was a moderate positive correlation between perfusion sensitivity scores and transepithelial permeability ($r = 0.51$, $P < 0.01$; Fig. 4B) and a negative correlation with TEER ($r = -0.58$, $P < 0.01$; Fig. 4C). These correlations indicate that when the integrity of mucosa decreases in GERD patients, sensitivity to acid increases.

We subsequently defined all GERD patients (both erosive and nonerosive) having sensitivity scores within the 95th percentile of the sensitivity scores of our healthy subjects (<152) as normosensitive and defined all GERD patients having higher sensitivity scores as hypersensitive.

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**Table 2. Esophageal gene expression profile of barrier function-related proteins (relative to three housekeeping genes)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative Gene Expression, median (interquartile range)</th>
<th>Fold Change Versus Control Subjects</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occludin</td>
<td></td>
<td></td>
<td>0.93</td>
</tr>
<tr>
<td>Control subjects</td>
<td>$4.7 \times 10^{-3}$ (3.9–5.6 $\times 10^{-3}$)</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>NERD patients</td>
<td>$4.1 \times 10^{-3}$ (3.6–5.2 $\times 10^{-3}$)</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Esophagitis patients</td>
<td>$4.3 \times 10^{-3}$ (3.2–4.8 $\times 10^{-3}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZO-1</td>
<td></td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Control subjects</td>
<td>$4.0 \times 10^{-2}$ (2.6–4.2 $\times 10^{-3}$)</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>NERD patients</td>
<td>$4.6 \times 10^{-2}$ (3.0–5.1 $\times 10^{-3}$)</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>Esophagitis patients</td>
<td>$3.6 \times 10^{-2}$ (2.5–4.3 $\times 10^{-3}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Claudin-1</td>
<td></td>
<td></td>
<td>0.37</td>
</tr>
<tr>
<td>Control subjects</td>
<td>$6.9 \times 10^{-3}$ (6.0–8.3 $\times 10^{-3}$)</td>
<td>1.07</td>
<td></td>
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<tr>
<td>NERD patients</td>
<td>$7.4 \times 10^{-3}$ (6.1–9.0 $\times 10^{-3}$)</td>
<td>0.86</td>
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</tr>
<tr>
<td>Esophagitis patients</td>
<td>$5.9 \times 10^{-3}$ (5.1–9.0 $\times 10^{-3}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Claudin-2</td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>Control subjects</td>
<td>$0.0$ (0.0–0.0)</td>
<td>0/A</td>
<td></td>
</tr>
<tr>
<td>NERD patients</td>
<td>$0.0$ (0.0–2.0 $\times 10^{-3}$)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Esophagitis patients</td>
<td>$0.0$ (0.0–1.4 $\times 10^{-3}$)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Filaggrin</td>
<td></td>
<td></td>
<td>0.65</td>
</tr>
<tr>
<td>Control subjects</td>
<td>$1.4 \times 10^{-2}$ (8.6 $\times 10^{-3}$–5.3 $\times 10^{-2}$)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NERD patients</td>
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<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Esophagitis patients</td>
<td>$1.0 \times 10^{-2}$ (8.0 $\times 10^{-3}$–2.3 $\times 10^{-2}$)</td>
<td></td>
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</tbody>
</table>

Shown are esophageal gene expression levels of barrier function-related proteins in biopsies from patients with esophagitis, patients with nonerosive reflux disease (NERD), and control subjects. Expression is relative to the mean of the three housekeeping genes cyclophilin, GAPDH, and β-actin. ZO-1, zonula occludens-1; N/A, not applicable.
sitive GERD patients, transepithelial permeability was significantly higher than in normosensitive GERD patients (mean: 1,922, 95% CI: 1,179–2,664, and mean: 390, 95% CI: 38–743, respectively, P < 0.05; Fig. 5A). In addition, TEER was significantly lower in hypersensitive GERD patients compared with normosensitive GERD patients (mean: 99, 95% CI: 78–120, and mean: 148, 95% CI: 114–183, respectively, P < 0.05; Fig. 5B).

**DISCUSSION**

This study was designed to investigate the relation between impairment of the esophageal mucosal barrier and the perception of acid reflux in patients with GERD. Our study results indicate that 1) sensitivity to acid is enhanced to a similar degree in NERD patients and patients with esophagitis, 2) mucosal integrity is impaired in both esophagitis and NERD patients, 3) the observed esophageal hypersensitivity in GERD patients can be partly explained by an impairment in mucosal barrier function; and 4) esophageal barrier function in GERD patients correlates with the expression of filaggrin and changes in the expression of tight junction proteins seem of lesser importance.

In the normal situation, the esophageal nonkeratinized stratified squamous epithelium forms a tight barrier and prevents the diffusion of noxious substances into the deeper layers of the mucosa and submucosa, where acid-sensitive receptors are present (1, 5, 22). Exposure of the esophageal mucosa to acidic and weakly acidic solutions containing bile salts can give rise to an impairment of barrier function, i.e., the development of DIS and an increase in transepithelial permeability (8, 9). In addition, subtle defects in mucosal integrity are present in patients with NERD (7, 27).

Our study confirms the significant role of esophageal mucosal integrity in the perception of luminal acid, as when studied for each GERD patient individually, we observed a significant correlation between all functional mucosal integrity parameters and acid sensitivity. In addition, if patients were categorized as normosensitive or hypersensitive based on the sensitivity scores of our healthy subjects, in hypersensitive GERD patients transepithelial permeability was significantly higher and TEER was significantly lower compared with normosensitive GERD patients, who all demonstrated normal mucosal barrier function. This is in concordance with a previous study (28) demonstrating that acid-sensitive patients have lower intraluminal baseline impedance during 24-h pH-impedance testing, a recently proposed measure of mucosal integrity. These findings all support the hypothesis that impaired mucosal integrity facilitates the diffusion of noxious substances into the mucosa, leading to more pronounced activation of esophageal nociceptors.

In this study, we did observe overlap in all mucosal integrity parameters between our patients and healthy subjects and a significant interpatient variation, especially for the size of intercellular spaces on electron microscopy. We also observed DIS in some of our healthy subjects, a finding that has also previously been described in a study showing that up to 30% of symptom-free healthy subjects has DIS in esophageal biopsies (29).

The method of NERD diagnosis can be disputed. NERD refers to the condition in which reflux causes symptoms in the absence of erosions or Barrett’s esophagus. Evidence for a relation between symptoms and reflux is usually assessed using 24-h pH-impedance monitoring; however, neither in the Montreal definition nor in the Rome III criteria for functional disorders is it mentioned whether this relation between reflux and symptoms should be defined using esophageal acid exposure time or symptom association analysis (11, 25). In the present study, we hypothesized that reflux perception is enhanced due to a facilitated permeation of reflux into the mucosa, and therefore we decided to include patients in whom it seems that the sequence from luminal exposure to a trigger of a nociceptor occurs rapidly. We thus included patients in whom a direct temporal relationship between reflux episodes and symptoms was evident (symptom association probability >95%). Because we used symptom association as the main inclusion criterium for NERD, acid exposure time was variable in our NERD population. However, this had no effect on our outcome parameters. Transepithelial permeability and TEER did not significantly differ between NERD patients with pathological acid exposure time (pH < 4 during >6% of time) and NERD patients with physiological acid exposure time (pH < 4 during <6% of time; data not shown).

Esophageal sensitivity measured with the acid perfusion test was similar in our GERD patients, irrespective of the presence of esophagitis. It has been reported that NERD patients might be more sensitive to acid perfusion than esophagitis patients.
However, all of our patients were symptomatic during inclusion, and therefore our study more closely resembles a study by Hartono et al. (12), which also demonstrated similar esophageal sensitivity between symptomatic esophagitis patients and NERD patients.

As we conclude that esophageal hypersensitivity is only partly explained by impaired mucosal integrity, it remains to be answered what else contributes to esophageal hypersensitivity. An important known candidate is visceral sensitization, peripherally or centrally mediated (14). Peripheral sensitization is the reduction of the transduction threshold of peripheral acid-sensing receptors in response to the release of inflammatory mediators by excessively stimulated tissue. Central sensitization is the amplified response of spinal dorsal horn neurons to a stimulus after previous repetitive firing of peripheral nerves (21). In our study, minor defects in barrier function might already lead to an excessive stimulation of peripheral chemosensitive receptors, causing sensitization. This could explain why the difference in sensitivity between GERD patients and control subjects is more pronounced than can be expected on observed differences in barrier function alone.

Besides demonstration of the presence of mucosal barrier dysfunction in GERD patients, we also studied the molecular mechanism underlying these changes. We investigated the gene expression profile of biopsies to evaluate potential mechanisms of disturbed esophageal barrier function, and we focused on intercellular complexes. The apical epithelial intercellular complex consists of tight junctions, adherens junctions, and desmosomes. In particular, tight junctions seal the intercellular space and restrict paracellular diffusion (2). Therefore, we investigated the expression of several components of the tight junction complex that are known to be expressed in the esophageal epithelium (13, 16). In our study, we found no differences in the expression of the tight junction proteins ZO-1, occludin, claudin-1, and claudin-2. This is in contrast to a previous study that reported an upregulation of both claudin-1 and claudin-2 in patients with esophagitis (16). This might be explained by differences in patient population as patients with severe esophagitis grade C and D were not included in our study. Although expression was very low, we did find a trend toward higher expression of claudin-2 in GERD patients, and an upregulation of claudin-2 in GERD subjects would fit with the hypothesis that GERD is characterized by high transepithelial permeability, as claudin-2 is a pore-forming member of the claudin family and upregulation would result in increased permeability (20). However, no correlation between claudin-2 expression and transepithelial permeability was found in this study. We also investigated the expression of filaggrin, a keratin-binding protein important for skin barrier function in allergic disease. In atopic dermatitis, a loss of function mutation of filaggrin results in impaired skin barrier function (19), and it is known that polymorphisms in filaggrin expression are associated with psoriasis. In the context of esophagitis, the expression of filaggrin is decreased in patients with eosinophilic esophagitis, another esophageal disorder associated with disturbed epithelial barrier function (6). In our study, filaggrin is expressed in the upper epithelial layer in biopsy specimens in a cytoplasmatic granular pattern. We

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**Fig. 4. Correlation of perfusion sensitivity score and mucosal integrity in gastroesophageal reflux disease (GERD) patients with extracellular impedance measured by the ETIS probe (A; \( r = -0.40, P < 0.05 \)), transepithelial permeability (B; \( r = 0.51, P < 0.01 \)), and TEER (C; \( r = -0.58, P < 0.01 \)).**

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**Fig. 5. Mucosal integrity in GERD patients according to acid perfusion test results. Normosensitivity is defined as a perfusion sensitivity score within the 95th percentile of healthy control subjects. Hypersensitivity is defined as a perfusion sensitivity score higher than the 95th percentile of healthy control subjects. A: transepithelial permeability. B: TEER.**
observed no significant differences in filaggrin expression between GERD patients and control subjects due to large interpatient variability. However, filaggrin expression did correlate well with all functional barrier function parameters, suggesting that it might also be an important contributor to mucosal barrier function in GERD patients irrespective of classification. In the epidermis, filaggrin is produced by differentiating cells in the stratum granulosum and contributes to skin barrier function both by binding keratin and incorporating keratin filaments into the lipid envelope. The esophagus is a nonkeratinizing epithelium, and so it is not clear how filaggrin affects barrier function there but its localization is similar to the epidermis. Future work will have to determine the exact mechanism by which filaggrin contributes to barrier function in the esophagus.

In conclusion, this study demonstrates that sensitivity to acid is enhanced in patients with GERD, irrespective of the presence of erosions, and is associated with impaired esophageal mucosal integrity. Filaggrin potentially has a role in the observation of erosions, and is associated with impaired esophageal nonkeratinizing epithelium. Filaggrin is thought to have a role in the integrity of the lipid envelope. The esophagus is a nonkeratinizing epithelium and so it is not clear how filaggrin affects barrier function there but its localization is similar to the epidermis. Future work will have to determine the exact mechanism by which filaggrin contributes to barrier function in the esophagus.

**REFERENCES**


