The Potential Role for Impaired Mucosal Integrity in the Generation of Esophageal Pain Using Capsaicin in Humans: An Explorative Study

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INTRODUCTION: **Esophageal pain is mediated by sensory nerves, most importantly by the activation of the transient receptor potential vanilloid 1 (TRPV1) capsaicin receptor. TRPV1 is activated and sensitized by a broad range of pungent compounds, as well as inflammatory mediators and tissue irritants. Luminal stressors are suggested to impair the barrier function, which results in consequent activation of these sensory nerve terminals and pain. In this study, we investigated the effect of the perfusion of capsaicin, a TRPV1 agonist, on mucosal impedance and pain in asymptomatic volunteers.**

METHODS: **Thirteen asymptomatic volunteers completed a single-blind, saline-controlled, randomized crossover study. Capsaicin or saline was perfused for 30 minutes in the distal esophagus. Visual analog scale pain intensity scores and intraluminal impedance indicating mucosal integrity were determined. Distal and proximal biopsies were obtained 10 minutes later to measure TRPV1 messenger RNA and TRPV1 immunopositivity, as well as the intercellular space area.**

RESULTS: **Capsaicin perfusion resulted in significantly greater pain intensity ($P = 0.047$) and impaired recovery of the mucosal impedance compared with saline-treated controls ($P = 0.027$). Pain response was significantly associated with decreased mucosal impedance. Similar dynamics were seen in the proximal esophagus, but mucosal impedance recovered entirely to the preinfusion values there. There was a significant association between mucosal impedance and intercellular space width in the distal esophagus. TRPV1 transcription and expression were not significantly altered within this observation period.**

DISCUSSION: **Esophageal capsaicin perfusion results in pain, which is likely to be explained by impaired mucosal impedance and defective restoration capacity in the distal esophagus.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A795

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INTRODUCTION

Gastroesophageal reflux disease is a highly prevalent disorder characterized by reflux of gastric contents that cause pain symptoms (1). In patients with erosive esophagitis, macroscopic disruption of the mucosa is apparent. However, in a substantial proportion of patients with reflux symptoms, there is no evidence of macroscopic damage of the mucosa and symptoms can therefore be attributed to nonerosive gastroesophageal reflux disease (NERD) (2). Several studies have identified subtle mucosal injury at a microscopic level in these patients (3,4). It is hypothesized that this disruption of the esophageal mucosal barrier is an important factor in the generation of reflux symptoms in NERD by facilitating excitation of mucosal neural afferents responsible for pain signaling.

The question, however, arises whether such a mechanism is primarily related to impaired mucosal integrity or rather increased sensitivity of afferents in an otherwise intact mucosa. To understand these mechanisms, mucosal impedance has been

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introduced as a surrogate marker for esophageal mucosal integrity (5). Another tool to investigate the mucosal barrier is the evaluation of dilated intercellular spaces (DISs). The presence of DISs within the squamous epithelium is believed to be a marker for esophageal mucosal barrier damage (6).

For the investigation of sensory nerve function, previous studies have focused on molecules responsible for nociceptive signaling. Transient receptor potential cation channels, particularly the vanilloid 1 (TRPV1) capsaicin receptor, play an important role in nociceptive signaling of somatic and visceral pain. Capsaicin perfusion in the esophagus leads to the sensation of heartburn (7). TRPV1 expression was shown to be upregulated in the esophageal mucosa of patients with NERD (8,9). In a mouse NERD model, TRPV1 overexpression and impaired mucosal integrity were detected in comparison with control animals (10). In addition, proximity of sensory afferents to the lumen is also believed to contribute to increased hypersensitivity in NERD (11). These results suggest that an impairment of the mucosal barrier results in an increased exposure of the sensory nerve endings to the refluxate and consequently in pain generation through TRPV1 receptor activation.

Several studies have shown that the threshold for induction of symptoms in patients with gastroesophageal reflux disease and controls is lower in the proximal part compared with the distal part of the esophagus (12–15). Woodland et al. (16) observed that nerve endings in the proximal esophageal mucosa are closer to the lumen compared with the distal esophagus. The proximal to distal gradient in afferent sensitivity of the esophagus may reflect an important biological and homeostatic function.

There were 3 main aims of this study performed in asymptomatic volunteers: (i) to test the hypothesis that exposure of the esophageal mucosa to the TRPV1 agonist capsaicin results in an impairment in mucosal barrier function, measured by mucosal impedance and DIS in the "exposed" distal segment of the esophagus; (ii) to assess these effects in the "nonexposed" proximal segment of the esophagus; and (iii) to study the relation between symptom perception, mucosal integrity, and TRPV1 expression.

MATERIALS AND METHODS

This study was approved by the Medical Ethics Committee of Maastricht University Medical Center+ and was conducted in full accordance with the principles of the Declaration of Helsinki of 1975 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (1998). All volunteers gave written informed consent before participation. This trial was registered at www.clinicaltrials.gov as NCT02603783.

Participants

Participants between 18 and 65 years with a body mass index between 18 and 25 kg/m^2 were enrolled in this study. Participants were recruited by local advertisements. Participants were excluded if they (i) had any history of gastrointestinal diseases, including esophageal motility disorders, reflux disease, or any previous esophageal or gastric surgery; (ii) had an intake of more than 14 alcoholic units per week or smoking; (iii) regularly used capsaicin-containing foods (>1 per week); and (iv) has known allergy to capsaicin. Informed consent was obtained after an interval of at least 7 days.

Experimental design

Participants underwent esophageal perfusion of capsaicin and physiological saline (control) in a randomized order with a minimal wash-out period of 10 days in between the 2 test days. Only the participant was blinded for the type of solution perfused. Therefore, the researcher was able to stop the infusion of capsaicin when the participant experienced maximum pain scores. Randomization occurred using randomizer.org. Participants were requested to arrive at 9.00 AM after an overnight fast at the motility laboratory of the gastroenterology-hepatology department of the Maastricht University Medical Centre+. We performed esophageal manometry before insertion of the infusion catheter but with the intention to determine the position of the lower esophageal sphincter (LES). During this, no apparent motility disorders were observed; however, no formal motility assessment with 10 swallows as per the Chicago classification recommendation was performed. A single lumen nasoesophageal infusion catheter G-84300 (Medical Measurement Systems, Enschede, The Netherlands) was placed 12 cm proximal to the upper margin of the LES and connected to an infusion pump (Perfusor Space Infusion Pump System; B. Braun, Melsungen, Germany). Impedance measurements were performed using a combined pH-impedance catheter assembly that consisted of 6 impedance segments located at 3, 5, 7, 9, 15, and 17 cm above the upper border of the LES and 1 ISFET pH electrode (Unisensor AG, Attikon, Switzerland). The pH electrode was positioned 5 cm above the upper margin of the LES. Impedance and pH signals were stored on a digital data logger (Ohmega; Medical Measurement Systems), using a sampling frequency of 50 Hz. The position of the impedance catheter in the proximal esophagus was at 15 cm above the upper border of the LES (17).

After positioning of the catheters, the participants were instructed to stay in a semirecumbent position. Before the start of the perfusion, esophageal impedance was recorded for 20 minutes under basal conditions. Perfusions were performed at a rate of 2.5 mL/min for 30 minutes or until participants experience maximum tolerable discomfort (a single visual analog scale [VAS] score of 100 mm or 2 subsequent VAS scores of ≥ 80 mm). After esophageal perfusion was ended, recording of impedance was continued for 10 minutes. Directly after the removal of both catheters, a standard gastroscopy was performed by an experienced gastroenterologist (J.C.) to obtain 8 esophageal biopsy (4 distal at 3–5 cm above LES and 4 proximal 15–17 cm above LES) specimens. The time line of the test day is visualized in Figure 1.

Solutions

The total amount of capsaicin administered in this study was 1.5 mg in a 75 mL solution (capsaicin oleoresin 8.3% capsaicin; Tiofarma BV, Oud Beijerland, the Netherlands). This dose is equivalent to the allowed maximum daily intake according to the Scientific Committee on Food of the European Commission (18) and has been used by our group in previous studies for duodenal stimulation (19). In brief, first, 24 mg of capsaicin oleoresin was dissolved in 1 mL 96% ethanol (Brouwland bvba) and diluted with saline to 100 mL (Fresenius Kabi). Of this solution, 75 mL was infused. The pH value of the capsaicin solution was 5.5. A total of 75 mL of physiological saline (Braun Melsungen AG, Germany) (pH 6.2) was used as the control solution, similar to previous studies investigating esophageal mucosal integrity (5,17).

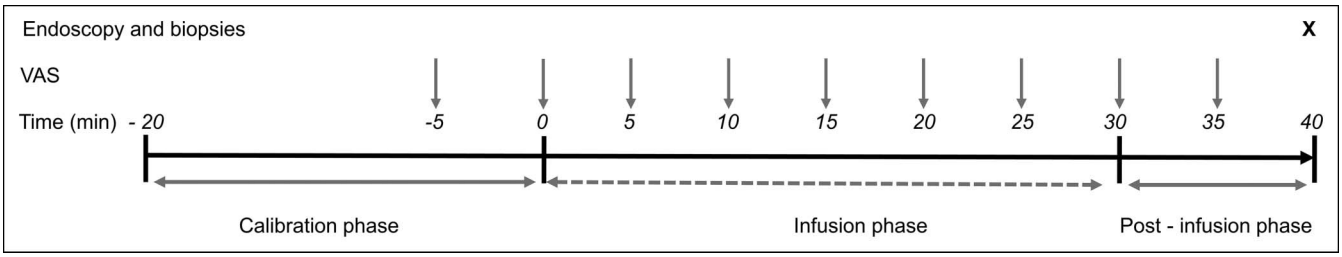

Figure 1. Timeline of the test day. Infusion of capsaicin solution or NaCl started at $t = 0$ minute after an overnight fast. 10 minutes after the end of the perfusion, an endoscopy was performed to obtain biopsies from the esophageal mucosa. VAS scores for retrosternal pain and heartburn were collected at regular intervals as indicated. Mucosal impedance was measured according to the following protocol: 20 minutes before perfusion (calibration phase), 30 minutes during perfusion, and 10 minutes after the end of the perfusion. VAS, visual analog scale.

Mucosal impedance measurements

Mucosal impedance was measured according to the following protocol: 20 minutes before perfusion (calibration phase), 30 minutes during perfusion, and 10 minutes after the end of the perfusion (Figure 1). After positioning the catheters, a 10-minute acclimatization period was allowed; thereafter, registration was started. We calculated the mean impedance value over a 10-minute period before the start of the perfusion. The impedance recovery after perfusion was calculated as follows: We included the mean impedance during the period between the third and fifth minutes after cessation of perfusion. The mucosal impedance in the first 2 min after the cessation of the perfusion was not included in the analysis to allow for complete capsaicin/saline bolus clearance from the distal esophagus. This 2-minute time for clearance of the infusion bolus was based on a study in which the total bolus transit time was measured in healthy volunteers (20).We excluded all reflux events and belching events from this calculation.

The analysis of mucosal impedance changes before, during, and after the perfusion was performed by one of the investigators (J.C.) with extensive experience in the interpretation of these impedance measurements who was blinded to the treatment order.

VAS scores for heartburn and retrosternal pain

All participants scored the intensity of heartburn and retrosternal pain, measured using VAS scores (0–100 mm) anchored at the low end with the most positive or lowest intensity feelings (extremely pleasant or not at all) and with the opposing terms at the high end (extremely unpleasant, very high, or extreme) (21). The VAS scores were collected at $t = -5, 0, 5, 10, 15, 20, 25, 30$, and 35 minutes.

TRPV1 immunohistochemistry and scoring

Four-micrometer sections from paraffin-embedded esophagus biopsy blocks were stained with a 1:100 dilution of guinea pig polyclonal anti-TRPV1 (GP14100; Neuromics, Edina, MN) primary antibodies. Slides were incubated with VECTASTAIN ABC-Peroxidase Kit, Guinea Pig IgG (PK-4007; BioMarker, Budapest, Hungary). The reaction was visualized by 0.01% hydrogen peroxide containing 3,3-diaminobenzidine tetrachloride, and histological counterstaining was performed with hematoxylin (22). TRPV1 immunopositivity was quantified by the individual assessment of 100 cells on each slide scored between 0 and 3 (0—negative, 1—minimal positivity, 2—moderate, and 3—strong positivity), performed by an experienced pathologist who was blinded to the treatment order (B.K.). A histological H-score was determined by the sum of the scores of the individual cells on each slide (ranging between 0 and 300). Incubating the esophageal mucosa with Tris-buffered saline instead of the primary antibodies served as the negative control, whereas sections of human dorsal root ganglia expressing-TRPV1 abundantly were used as positive controls. The antibody specificity has been validated by preabsorption of the respective blocking peptide (P14100 Neuromics, Edina, MN), as described previously (23). A representative image of TRPV1 immunohistochemistry is shown in Figure 2.

TRPV1 messenger RNA analysis

Sample homogenization was performed in 1 mL TRI Reagent (Molecular ResearchCentre, Cincinnati, OH), and total RNA was isolated with the Direct-zol RNA Miniprep isolation kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. Samples were then measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) to assess RNA quantity and purity. After treatment with deoxyribonuclease I enzyme (Zymo Research), total RNA (100 ng) was reverse transcribed with the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. To amplify transcripts, real-time quantitative Polymerase Chain Reaction (qPCR) was performed on a Stratagene Mx3000P qPCR System (Agilent Technologies, Santa Clara, CA) using Luminaris HiGreen Low ROX qPCR Master Mix (Thermo Fisher Scientific) (Bohonyi et al., 2017). The following primer pairs were used to amplify the genes of

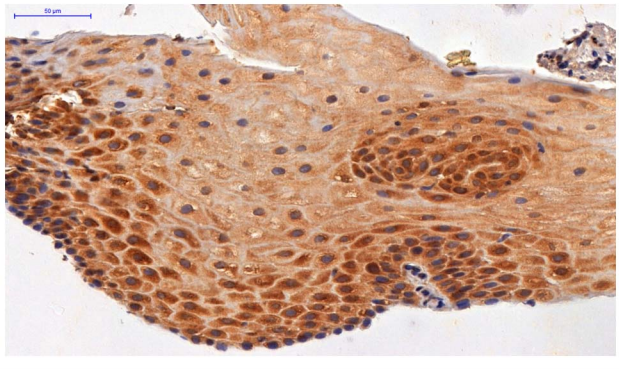

Figure 2. Representative image of TRPV1 immunohistochemistry: TRPV1 staining, approximately ×400 magnification. TRPV1, transient receptor potential vanilloid 1.

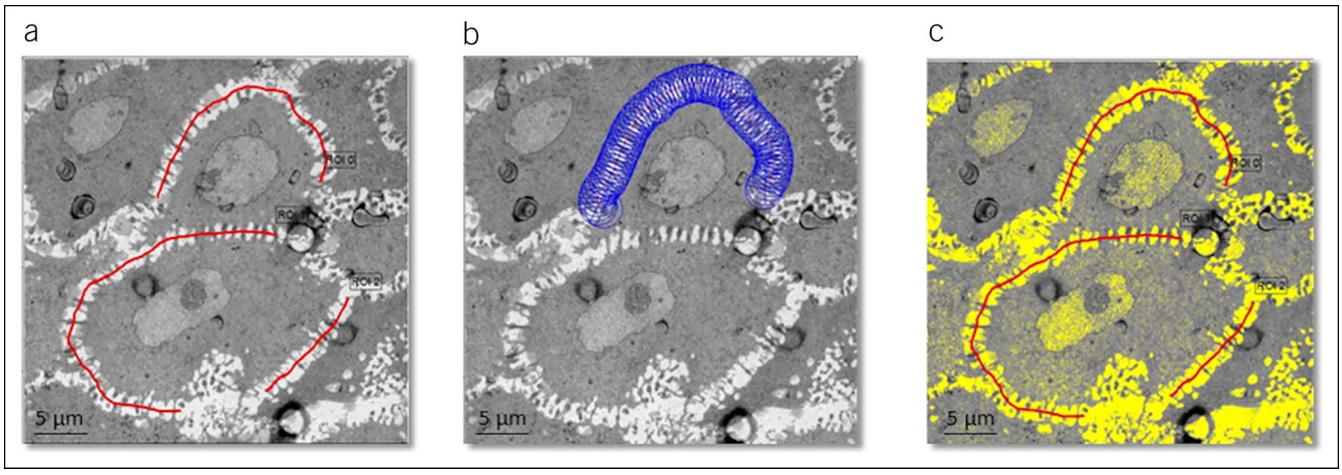

Figure 3. Illustrative image of the assessment of the intercellular space area (ISA). (**a**) Multiple lines (red) are drawn through the intercellular space. Crossings where intercellular spaces merge are not included. (**b**) A region of interest is marked around the lines to cover the selected intercellular space. (**c**) Based on the pixel threshold, the intercellular space area is then calculated and divided by its corresponding length.

interest: TRPV1 (NM_080706.3) (sense): 5′-CAGCTCAATTGCTGTGCAGGTTA-3′ and (antisense): 5′-TGCCAGTATGGATGGAGTGGAA-3′. All reactions were measured in triplicates, and the geometric mean of their Ct values was calculated, which was normalized to transcripts of the glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (NM_001289746.1) (sense): 5′-CCTGCACCACCAACTGCTTA-3′ and (antisense): 5′-TAGAGGCAGGGATGATGTTCTG-3′, used as reference gene. Primers with similar efficiencies were used, and melt curve analyses were performed to verify primer specificity. The determination of relative messenger RNA (mRNA) expression levels was performed according to the comparative Delta Cycle threshold (DCt) method. Analyses were performed by an investigator (K.C.) blinded to the treatment order.

Transmission electron microscopy

A single esophageal biopsy was directly immersed in 3% glutaraldehyde fixative buffered in 0.09 M KH2PO4 at pH 7.4 at room temperature. After a minimum of 24 hours of immersion, samples were then washed in 0.09 M KH2PO4 buffer with 7.5% sucrose and transferred to a 1% OsO4 + 1.5% ferrocyanide solution and buffered to pH 7.4 with 0.1 M veronal acetate for subsequent postfixation for 1 hour at 4 °C. After washing in phosphate veronal-acetate buffer containing 7% sucrose at pH 7.4, dehydration was performed rapidly in graded ethanol series followed by embedding in Epon. Ultrathin tissue sections were cut and examined with a Philips CM 100 electron microscope. Of each biopsy specimen, 3–5 TEM photographs were obtained. TEM photographs were obtained from the basal and suprabasal layers of the epithelium at ×4,000 magnification by an independent researcher, who was experienced in the recognition of the different layers of the epithelium and blinded for the status of the participant.

Intercellular space

The intercellular space area was evaluated in 3 representative TEM photographs per participant. For the assessment, based on the division of the area by the corresponding length (an estimation of the perimeter of the cell), first, a single line was drawn through the intercellular space around 2–3 cells in the microphotograph. The researcher was blinded for the treatment order. Multiple lines were drawn, which were optically the most suitable for calculation of the intercellular space. A region of interest (ROI) around this line was defined to include the intercellular space between the 2 cells, without inclusion of intracellular content. The software allowed us to customize an ROI of different µm in each case. Then, the pixel threshold was chosen to include only pixels from the intercellular space. Pixels considered for the analysis were visualized in yellow. Based on this threshold, the area around the line included in the ROI was automatically calculated and divided by the length of this line using custom-written image analysis software in IGOR Pro (WaveMetrics, OR). Per participant, the average of the area of different regions of interest from the 3 TEM photographs was used for the analysis (Figure 3).

Statistical analyses

Statistical analyses were performed with SPSS, version 25.0 (SPSS, Armonk NY). A linear mixed model was performed to analyze the mucosal impedance in the proximal and distal esophagus separately and VAS scores and the relation between these 2 variables. For the mucosal impedance and VAS scores for heartburn and retrosternal pain, a 3-way interaction between intervention (capsaicin or control), time (before, after, or −5, 0, 5, 10, 15, 20, 25, 30, and 35 minutes), and test day (1 and 2) with all lower terms were included in the model to check whether the intervention effect over time depends on the test day. In case the interaction was not significant, this interaction term was removed from the model, and the intervention effect at each time point was reported for both test days combined. For mucosal impedance, solely the time points preinfusion (between −10 and 0 minutes) and postinfusion (between +32 and +35 minutes) were included in the model to ignore the effect of the fluid perfusion and bolus clearance on mucosal impedance. A random intercept and/or slope (time) were included, and diffures (unstructured and variance components) were considered for these random effects, where the final model was chosen based on the Akaike information criterion.

For both locations separately, TRPV1 expression, transcription, and intercellular space area were analyzed with a linear mixed

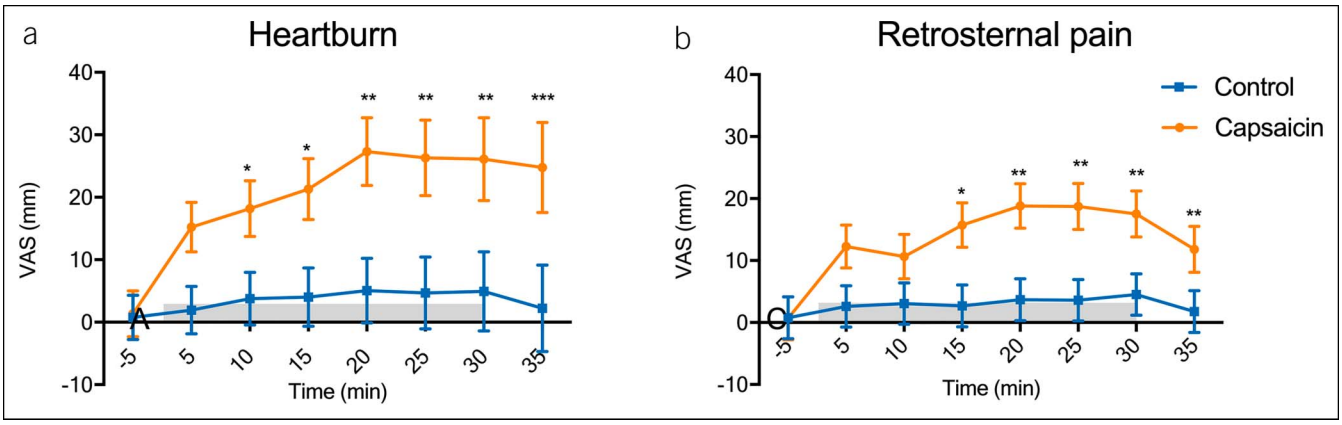

Figure 4. VAS scores for heartburn (**a**) and retrosternal pain (**b**) (estimated means ± SEM). Gray box 0–30 minutes: perfusion period. Overall *P* value heartburn: 0.004. Overall *P* value retrosternal pain: 0.047. **P* value<0.05, ***P* value<0.01, ****P* value<0.001. Data were analyzed using a linear mixed model. VAS, visual analog scale.

model which included a 2-way interaction between treatment and test day. In case the 2-way interaction was nonsignificant, the intervention effect was reported for the 2 test days combined.

Data are presented as means ± standard error of the mean (SEM) unless specified otherwise. *P* value ≤ 0.05 was considered as statistically significant.

Sample size calculation

Data for executing this sample size calculation were retrieved from Farré et al. (5) with the following parameters for mucosal impedance: alpha (α) = 0.05, power = 0.80, mean difference = 716 Ω (control vs esophageal acid perfusion = 2,960 vs 2,244 Ω), and SD (σ) = 690. According to this sample size calculation, we needed to include 14 asymptomatic volunteers to be able to reject the null hypothesis that the capsaicin infusion has a similar effect as the control solution on mucosal impedance with a probability (power) of 0.80. Regarding dropouts, we were able to include up to 15 asymptomatic volunteers.

RESULTS

Fifteen asymptomatic volunteers were included in this study. Two participants dropped out after screening and randomization; 1 volunteer was not able to schedule the test days, and 1 volunteer became ill due to reasons unrelated to the study and discontinued study participation. 13 volunteers were included in the study analysis (4 male volunteers; mean ± SD age: 27.6 ± 14.3 years, body mass index 22.5 ± 1.3 kg/m²) for analysis of mucosal impedance. The baseline characteristics and capsaicin tolerance are presented in the Supplementary file (see Table 1, http://links.lww.com/CTG/A795). This included 1 study participant who only completed 1 test day (saline condition) and dropped out before the second test day. As for the analysis of TRPV1 immunohistochemistry and TRPV1 mRNA expression, only samples from participants who completed both test days were included (n = 12). Similarly, for DIS analysis, each group contained 12 samples, except for the proximal biopsies in the saline group, which included 11 samples because of fixation artifacts.

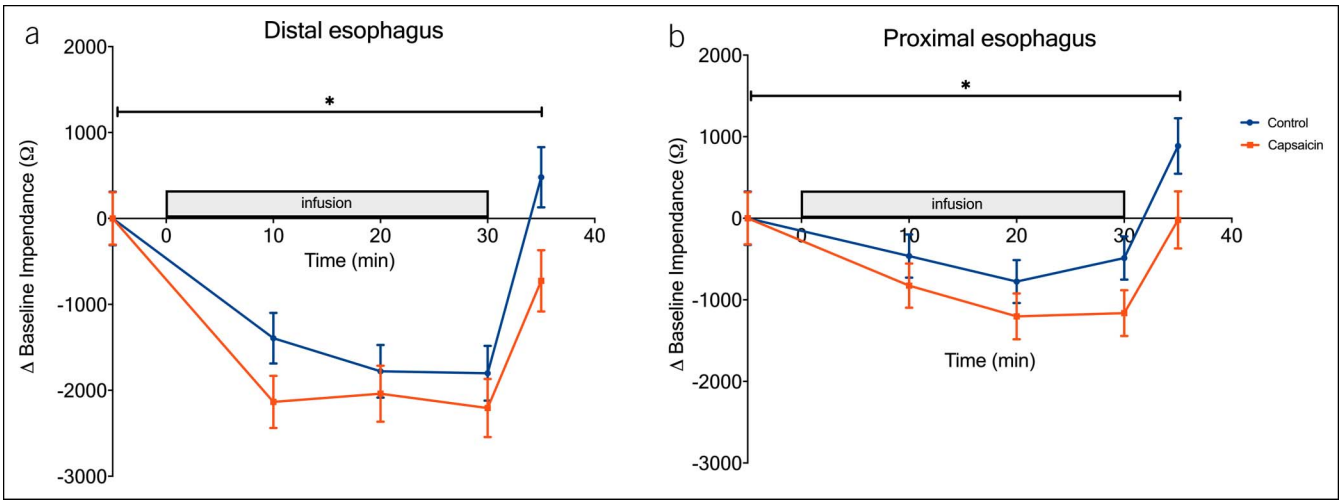

Figure 5. Δ mucosal impedance over time in the distal (**a**) and proximal (**b**) esophagus. Gray box: infusion period. *Significant difference between capsaicin and control perfusion, preperfusion vs postperfusion. Proximal: significant difference preperfusion and postperfusion between the 2 treatments, *P* value: 0.007. Distal: significant difference preperfusion and postperfusion between the 2 treatments, *P* value: 0.027. Data were analyzed using a linear mixed model.

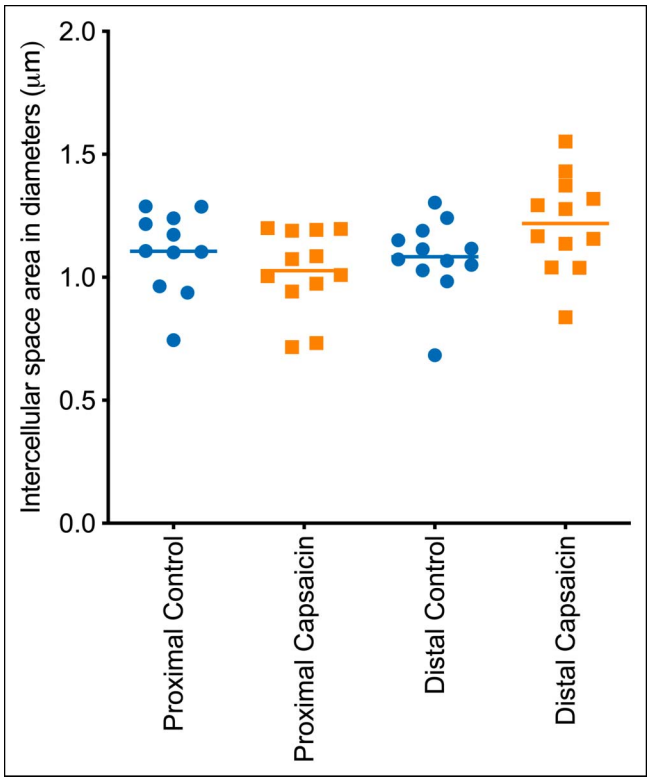

Figure 6. Mucosal intercellular space area in biopsies from the proximal and distal esophagus in asymptomatic volunteers after exposure to capsaicin and control. There was no significant difference for the treatment in both locations (*P* value proximal esophagus 0.330, *P* value distal esophagus 0.171). n = 12, proximal control n = 11. Data were analyzed with a linear mixed model, which included a 2-way interaction between treatment and test day.

Effects in the distal esophagus

Perfusion of capsaicin into the distal esophagus resulted in significantly higher scores for heartburn (overall *P* value 0.004) and retrosternal pain (overall *P* value 0.047) over time (Figure 4). Notably, both pain and retrosternal pain persisted after cessation of capsaicin during the timeframe of the experiment.

These effects were accompanied with changes in mucosal impedance in the distal esophagus (Figure 5a). Both capsaicin and saline perfusion resulted in decreased mucosal impedance. However, this decrease was of a greater magnitude after capsaicin perfusion compared with saline perfusion, albeit this difference was not significant. After discontinuation of capsaicin perfusion, mucosal impedance remained lower compared with the value before perfusion. After the perfusion of saline, mucosal impedance increased above preperfusion levels. We, therefore, observed a significant difference in recovery of 966 Ω (estimated mean [SEM] for capsaicin preperfusion and postperfusion: 3,468 [257] Ω and 3,313 [277] Ω vs saline preperfusion and postperfusion: 2,918 [261] Ω and 3,728 [266] Ω, *P* value for difference in change scores 0.027).

There was a significant inverse association between VAS scores for retrosternal pain and mucosal impedance for capsaicin and saline conditions combined (slope −0.006, *P* value: 0.031, 95% CI [−0.011 to −0.001]). For heartburn, there was a nonsignificant relation between mucosal impedance and heartburn in the distal esophagus (slope −0.007, *P* value: 0.080, 95% CI [−0.015 to 0.001]).

Mucosal impedance before the start of the perfusion on both test days was not significantly different (*P* value 0.369). The fluctuation of the mucosal impedance in an empty esophagus ranges between 2,000 and 4,000 Ω in normal adults (24).

Effects in the proximal esophagus

Distal perfusion of both solutions resulted in a decrease in mucosal impedance in the proximal esophagus over time (Figure 5b). Comparable with the distal esophagus, this decrease was also of a greater magnitude after capsaicin perfusion compared with saline perfusion in the proximal esophagus. However, the decrease was less pronounced compared with the distal esophagus for both conditions. After cessation of the perfusions, mucosal impedance recovered above preperfusion levels for saline. For capsaicin, mucosal impedance fully recovers to the value before perfusion, contrary to what was observed in the distal esophagus. This difference in recovery was 1,139 Ω (estimated mean [SEM] for capsaicin preperfusion and postperfusion: 3,017 [294] Ω and 3,047 [310] Ω vs saline preperfusion and postperfusion: 2,329 [294] Ω and 3,497 [301] Ω, *P* value for difference in change scores 0.007).

Intercellular space area

The intercellular space area was not significantly different between the type of solution in the proximal (*P* value 0.330) and distal esophagus (*P* value 0.171) (Figure 6). However, there was a significant negative association between intercellular space area and mucosal impedance just before obtaining the biopsy in the distal esophagus (slope −0.001, *P* value 0.002, 95% CI [−0.0001 to −0.00003], see Supplementary Figure 1, http://links.lww.com/CTG/A795).

TRPV1 immunohistochemistry and mRNA expression

There was no significant difference in H-scores for TRPV1 between the type of solution after correction for location (distal *P* value 0.970 and proximal *P* value 0.761) (Figure 7a). There was no significant difference in TRPV1 mRNA levels between the type of solution after correction for location (distal *P* value 0.829 and proximal *P* value 0.186) (Figure 7b).

DISCUSSION

The present results suggest a potential role for impaired mucosal integrity in the generation of esophageal pain induced by capsaicin infusion. Perfusion with the TRPV1 agonist mucosal stressor capsaicin in the distal esophagus resulted in an impaired capacity to restore mucosal impedance after perfusion. In addition, pain scores during the perfusion showed a significant association with the decreased mucosal impedance of the distal esophagus.

Besides the impaired mucosal impedance, pain generation can also be related to primary dysfunction of the afferent nerve endings (i.e., through sensitization). Capsaicin infusion is assumed to exert its effects by the activation of the TRPV1 receptor. It has been hypothesized that increased transcription of the TRPV1 receptor may contribute to the perception of heartburn in patients with NERD (8,9,25). Furthermore, the release of neuropeptides, such as substance P and calcitonin gene-related peptide, from TRPV1-expressing nerves may result in neurogenic inflammation and consequent activation of the inflammatory cascade in the esophageal mucosa, leading to mucosal afferent hypersensitivity, although these phenomena were not subject to analysis in this study. In addition, the inflammatory cascade can

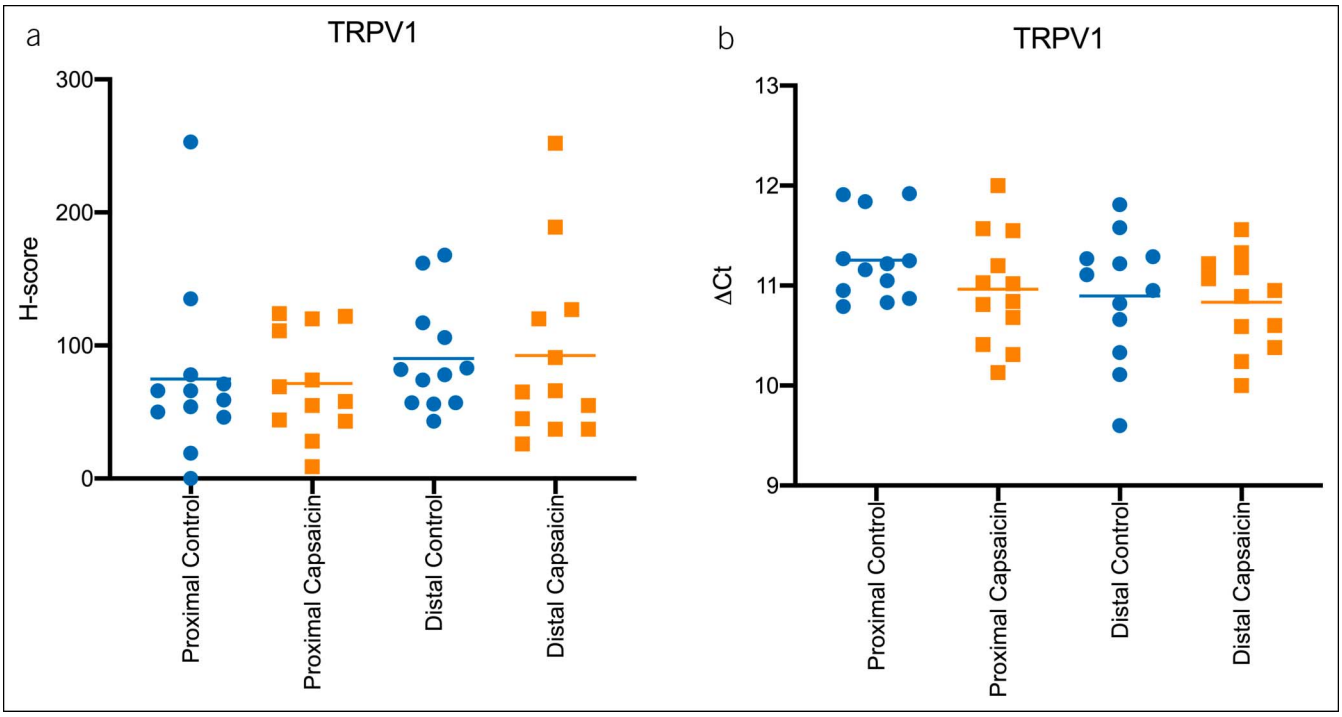

Figure 7.(**a**) TRPV1 immunohistochemistry: H-score for TRPV1. There was no significant difference between the treatments after correction for location (distal *P* value 0.970 and proximal *P* value 0.761). (**b**) TRPV1 mRNA expression: delta Ct value for TRPV1. There was no significant difference between the treatments after correction for location (distal *P* value 0.829 and proximal *P* value 0.186). n = 12. Data were analyzed with a linear mixed model which included a 2-way interaction between treatment and test day. TRPV1, transient receptor potential vanilloid 1.

also contribute to the impairment of the mucosal barrier (26). We did not observe significant alterations of TRPV1 expression either at mRNA or at protein levels, albeit prompt changes within this relatively short timeframe are unlikely to occur. In the esophageal mucosa, the TRPV1 receptor is expressed both on the sensory nerves and epithelial cells (27), but its mRNA is more likely to be derived only from the epithelial cells. The role of epithelial TPRV1 in relation to pain signaling remains to be established. On the other hand, it has been reported that peripheral inflammation induces axonal transport of TRPV1 mRNA from dorsal root ganglia to central and peripheral axon terminals (28), and such phenomena may also occur because of esophageal acid exposure (29).

Regardless of the localization within the esophageal mucosa, it seems that pain intensity is not related to the amount of the TRPV1 receptor. Indeed, pain sensation is a complex process, which is determined not only by mucosal mechanisms but also by central sensory and autonomic processing at the spinal cord and brain levels. Nevertheless, we speculate on the basis of the current findings that esophageal pain generation depends on the mucosal barrier resistance and consequent exposure of TRPV1-expressing sensory terminals to different stimuli, rather than TRPV1 upregulation per se. We provide an overview of the postulated mechanisms in Figure 8.

The mucosal impedance value as an indicator of the mucosal barrier function remained permanently reduced in the distal, capsaicin-perfused esophagus even after completing the stimulation. In the proximal esophagus, mucosal impedance fully recovered to the preperfusion control values after a moderate, transient decrease during the intervention, and the intercellular space area was not altered. However, it should be noted that mucosal impedance and the intercellular space width do not necessarily reflect the exact same pathophysiological mechanisms.

Interestingly, pain sensation persisted even after cessation of the capsaicin infusion. We speculate that the recovery of the mucosal impedance in both locations after the stimulation reflects the mucosal barrier restoring mechanisms to protect the mucosa from potentially noxious substances. The study design, however, did not allow to fully ascertain the dynamics of this recovery because the measurement of mucosal impedance had to be discontinued for biopsy retrieval. As far as the difference between the distal and proximal esophagus is concerned, this can be explained by the higher sensitivity of the proximal part to noxious stimuli as a defense mechanism to protect the airways (30) from the influx of potentially noxious substances. This is supported by results demonstrating that nerve endings in the proximal esophagus are closer to the lumen compared with the distal part (16).

In contrast to the present results demonstrating no intercellular space changes in the proximal biopsies after distal capsaicin perfusion, earlier studies with acid (both pH 2.0 and 5.5) perfusion in a similar way in 14 asymptomatic volunteers induced a significantly enlarged intercellular space area in both esophageal parts (17). Although protons also mainly activate the TRPV1 ion channel similar to the selective agonist capsaicin, the binding sites and the activation mechanisms are different (31). Furthermore, acid perfusion elicited almost no symptoms (17) in agreement with the observation that esophageal submucosal injection of capsaicin but not acid was able to induce pain (32). Therefore, we hypothesize that the reflectory reinforcement of the mucosal

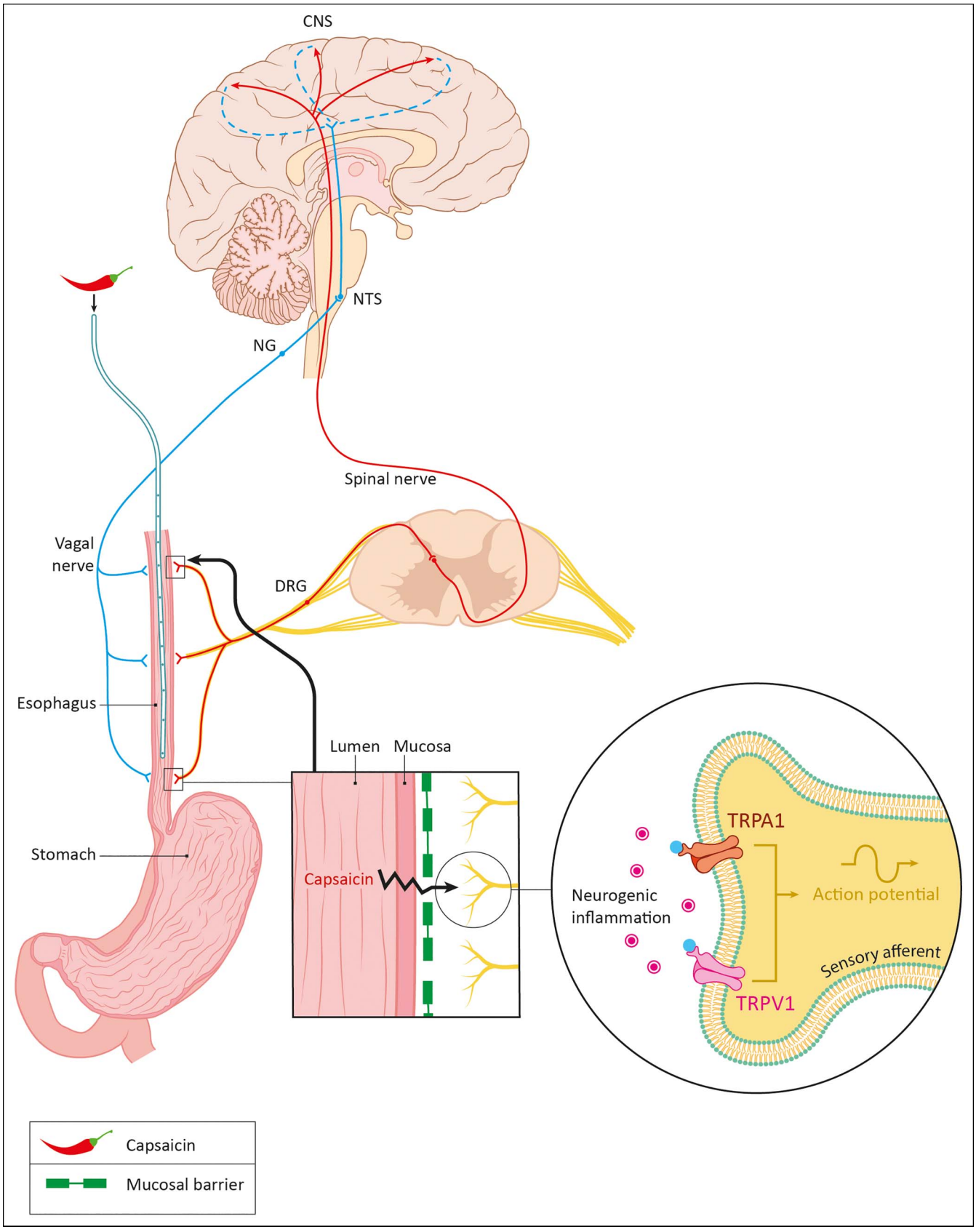

Figure 8. Schematic depiction of the hypothesized effects of esophageal perfusion with capsaicin. DRG, dorsal root ganglion; NTS, nucleus tractus solitarius; TRPV1, transient receptor vanilloid 1.

barrier, in particular in the proximal esophagus, is specifically induced when pain signals are generated.

An intriguing observation was that after 30 minutes saline perfusion, the mucosal impedance increased above the preperfusion values after the cessation of the stimulus in both the proximal and distal esophagus. This phenomenon was also observed in a previous study (5). The saline infused in our study had a pH value of 6.2, whereas the solution in the study by Farré had a pH value of 7.2 (5). The pH value of physiological saline can vary between 6.15 and 8.15 (33). In this sense, our control condition rather resembles the effect of a weak acid than that of a neutral solution and may therefore have resulted in the observed decrease of mucosal impedance. It seems that the same underlying mechanisms might be at operation during both conditions. Nevertheless, even when the direction of changes are comparable between capsaicin and saline, the effects of capsaicin infusion are significantly larger in magnitude, and this difference might be related to the degree of the mucosal barrier impairment and the pain response elicited.

Besides the important outcomes and novelties, there are limitations of this study, such as the relatively small sample size that might have resulted in a type II statistical error. This means that some results cannot be treated as firmly conclusive, in particular regarding the secondary end points, such as the effect on the intercellular cell area. In addition, the timing of the endoscopy and biopsy retrieval might have influenced the accuracy by which we were able to detect any findings on the intercellular space level. It may be possible that any alterations occurred were (partially) reversed at the moment of the biopsy retrieval. Regarding mucosal impedance measurements, technical factors, such as attachment of the catheter to the esophageal mucosa or fluid on the catheter, might have influenced measurements which can render values less accurate as surrogate markers for epithelial integrity. We cannot completely rule out that the impedance measurements in both the proximal and distal esophagus were possibly affected by retrograde flow of the infusion solution. However, retrograde flow of the infusion solution was highly unlikely because of careful positioning of the healthy volunteers (semirecumbent position). As this was a study performed in asymptomatic volunteers, it remains to be established what the exact role of the described phenomena is in patients with reflux/heartburn symptoms. Owing to the experimental design which was chosen to accommodate for biopsy retrieval as soon as possible after the infusion has stopped, we were unable to measure baseline impedance for a longer period. This would have been desirable to have a better understanding of the impact of the intervention on mucosal integrity. Another limitation is the different composition of the solutions infused; ethanol was added to the solution to dissolve capsaicin in saline. Ethanol was not added to the saline (placebo) solution. It is not known whether ethanol in the capsaicin solution might have affected mucosal impedance measurements. Another limitation is that we did not ascertain alternative markers for mucosal integrity (such as the expression of tight junction proteins), which would have added more clarity to the interpretation of the impedance data.

In summary, the most important novel findings of this study are that (i) capsaicin infusion in the distal esophagus resulted in the impaired recovery of mucosal impedance and (ii) pain intensity (sensory response) was related to the magnitude of this impairment but not to TRPV1 expression. Therefore, we speculate that pain sensation in the esophagus is likely a result of increased exposure of the sensory nerve terminals to stimulants through barrier impairment. Our results provide important novel insight into esophageal pain generation, which has clinical relevance for treating heartburn and related symptoms and for providing rationale for esophageal barrier protection as a therapeutic modality (34).

CONFLICTS OF INTEREST

Guarantor of the article: Annick M.E. Alleleyn, MD, PhD.

Specific author contributions: A.M.E.A.: conducting study, collecting materials, analyzing and interpreting data, and drafting and revising of the manuscript. D.K.: study concept and design, analyzing and interpreting data, and writing and revising of the manuscript. N.F.R.: study concept and design. K.C.: preparation of study materials and analyzing and interpreting data. B.K.: preparation of study materials. Z.H.: analyzing and interpreting data. B.W.: statistical analysis. A.A.M.M.: study concept and design and interpreting data. J.M.C.: principal investigator, study concept and design, and collecting data. All authors approved the final version of the manuscript.

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Potential competing interests: None to report.

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Study Highlights

WHAT IS KNOWN

- ✓ Patients with nonerosive reflux disease have typical reflux symptoms caused by the intraesophageal reflux of gastric contents without visible esophageal mucosal injury.
- ✓ The transient receptor potential vanilloid 1 capsaicin receptor plays an important role in nociceptive signaling of somatic and visceral pain.

WHAT IS NEW HERE

- ✓ Capsaicin infusion in the distal esophagus resulted in an impaired recovery of mucosal impedance.
- ✓ Pain intensity was related to the magnitude of this impairment.
- ✓ Pain is likely to be the result of increased exposure of the sensory nerve terminals to stimulants through barrier impairment.
- ✓ This study provides rationale for esophageal barrier protection as a therapeutic modality.

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