Chapter 7

Histological response to fluticasone in patients with eosinophilic oesophagitis is associated with improved functional oesophageal mucosal integrity

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Submitted
Abstract

**Objective:** The oesophageal mucosal integrity is impaired in patients with eosinophilic oesophagitis (EoO). We aimed to evaluate the effect of fluticasone propionate on functional and structural markers of oesophageal mucosal barrier integrity in adult patients with EoO.

**Design:** We included 15 (5 female) EoO patients (median age (IQR), 43 (30-45) years). Patients underwent upper endoscopy before and after 8 weeks of swallowed fluticasone propionate 500 μg BID. Several established parameters of oesophageal mucosal barrier integrity were evaluated: during endoscopy we measured the electrical tissue impedance of the oesophagus, and using oesophageal biopsy specimens we evaluated the transepithelial electrical resistance (TER) and transepithelial molecule flux in Ussing chambers, as well as intercellular spaces. Eosinophil & mast cells counts were scored in oesophageal biopsy specimens, and expression of inflammatory cytokines and barrier integrity proteins was investigated using qPCR and immunohistochemistry. Oesophageal signs and symptoms were also scored.

**Results:** Peak eosinophil and mast cell counts decreased significantly after fluticasone propionate treatment. The oesophageal mucosal integrity increased substantially after treatment, as shown by increased extracellular impedance and TER (both $p<0.01$). In line, the transepithelial molecule flux in Ussing chambers decreased after treatment ($p<0.05$). Whereas expression of genes encoding for inflammatory cytokines (IL-5, IL-13, eotaxin-3, periostin, TSLP) decreased after treatment, expression of genes encoding for barrier integrity proteins (filaggrin and desmoglein-1) increased.

**Conclusion:** Fluticasone propionate treatment decreases eosinophilic inflammation and improves the oesophageal mucosal barrier integrity in adult EoO patients. Normalization of gene expression of desmoglein-1 and filaggrin correlates with improvement of the mucosal barrier integrity.
Introduction

Eosinophilic oesophagitis (EoO) is a rapidly emerging disorder clinically characterized by dysphagia and food impaction.\textsuperscript{1,2} The pathophysiology of EoO is not completely known, although EoO is considered an atopic disease, and food allergens seem to play a pivotal role.\textsuperscript{2-4} EoO shows pathophysiological similarities with other atopic diseases such as atopic dermatitis (AD) and asthma. In AD and asthma, loss-of-function mutations in the filaggrin gene (FLG) are thought to contribute to an impaired epithelial barrier integrity, which is thought to cause increased allergen exposure.\textsuperscript{5,6} In patients with active EoO, gene expression of filaggrin is substantially reduced by IL-13, supporting the idea that impairment in barrier integrity may contribute to the development of EoO as well.\textsuperscript{7}

Very recently, impaired mucosal barrier integrity has emerged as an important factor in the pathophysiology of EoO. On the basis of a series of experiments, Sherrill et al. concluded that downregulation of DSG1 by IL-13 impairs the oesophageal mucosal barrier integrity and increases inflammation.\textsuperscript{8} Another study demonstrated that in EoO patients treated with fluticasone propionate, on a protein level, filaggrin expression was higher than in untreated patients.\textsuperscript{9} Using several established techniques, we have previously shown that the oesophageal mucosal barrier integrity is functionally and structurally impaired in patients with EoO compared with healthy controls.\textsuperscript{10} In patients with histological response to proton pump inhibitors, recently identified as having PPI-responsive oesophageal eosinophilia (PPI-ROE),\textsuperscript{11,12} improvement of the mucosal integrity paralleled the histological response to PPIs, whereas in patients that did not respond to PPIs the mucosal integrity remained impaired.\textsuperscript{10} Taken together, these data suggest that in order to achieve histological remission it might be necessary to restore the oesophageal mucosal integrity in patients with EoO. However, the effect of adequate treatment on the oesophageal mucosal barrier integrity has never been evaluated longitudinally in EoO patients.\textsuperscript{13}

We hypothesized that in EoO patients, a decrease in oesophageal eosinophilic inflammation would be associated with improvement of the oesophageal mucosal integrity. Using several techniques, we aimed to evaluate oesophageal mucosal inflammation and barrier integrity in adult patients with EoO before and after fluticasone propionate treatment.

Methods

Study subjects

In this prospective study, we included 15 adult patients with EoO (>15 eosinophils/high-power field (hpf), predominant symptoms of dysphagia and/or food impaction, endoscopic signs of EoO, and no response to PPI).\textsuperscript{2} Patients were consecutively recruited from the outpatient clinic of the Academic Medical Center. None of the study subjects had other oesophageal disease or had undergone surgery of the digestive tract. Each study subject gave written informed consent and the study protocol was approved by the Medical Ethics Committee of our institution.
Study protocol

After inclusion, patients were scheduled for upper endoscopy. Endoscopy was repeated after 8 weeks of treatment with swallowed fluticasone propionate 500 μg BID. Patients were not allowed to receive dietary, acid-suppressive or anti-inflammatory treatments from 8 weeks before the first endoscopy until the end of the study.

Upper endoscopy

After routine inspection of the duodenum and stomach, endoscopic pictures were taken of the oesophagus for assessment of endoscopic signs of EoO. Following this, electrical tissue impedance spectroscopy measurements were performed, and biopsy specimens were obtained for the evaluation of functional mucosal integrity in Ussing chambers, dilation of intercellular spaces, eosinophil and mast cell counts, and gene expression profiling.

To assess endoscopic signs of EoO, a physician blinded to the status of the patient scored the endoscopic images for presence of concentric rings, white exudates, linear furrows, strictures, oedema, and a crepe paper oesophagus. Total scores were compared before and after treatment.

Assessment of oesophageal mucosal integrity

In vivo and in vitro parameters of oesophageal mucosal integrity were measured as previously described. Briefly, electrical tissue impedance spectroscopy was performed at 5 cm proximal to the gastro-oesophageal junction. Four biopsy specimens taken at the same level with a large biopsy forceps (diameter 3.7 mm), were transferred immediately after endoscopy to Ussing chambers to assess the transepithelial electrical resistance (TER) of the mucosa and the transepithelial flux of molecules of different sizes (fluorescein, 0.3 kDa; rhodamine, 40 kDa) through the mucosa. As a structural marker of oesophageal mucosal integrity, the space between individual oesophageal epithelial cells was measured. The electron microscopy lab technician was blinded to the status of the biopsy specimen and selected 10 random photos of each specimen at the basal prickle layer (magnification 4600x). The surface of the intercellular space and total image surface were determined, and by dividing the intercellular space surface through the total image surface we calculated a detailed intercellular space proportion.

Histopathological analysis

For histopathological analysis, two biopsy specimens were taken at the distal (5 cm above the gastro-oesophageal junction), mid (10-15 cm above the gastro-oesophageal junction), and proximal oesophagus (5 cm below the upper oesophageal sphincter). Biopsy specimens were stained with H&E and tryptase for analysis of eosinophil and mast cell counts, respectively. An experienced gastrointestinal pathologist blinded to the biopsy location and treatment status of the patient analysed the specimens in random order, using an Olympus BX41 microscope. In each biopsy, the area of greatest eosinophil density was detected at
low-power view. Using a 400x magnification (1 hpf) the peak eosinophil count was determined. Mast cells were analysed accordingly. Several other histopathological features were also evaluated: the presence of eosinophilic microabscesses (defined as clusters of ≥4 eosinophils) at high power field; basal hyperplasia at low-power field (0 = absent; 1 = to lower third of total epithelial thickness (mild); 2 = to middle third (moderate); 3 = to upper third (severe); and spongiosis at low-power field (scored similar to basal hyperplasia).

Quantitative real-time PCR

During each endoscopy, one oesophageal mucosal biopsy specimen from each patient was collected and immersed in RNA stabilization reagent (RNAlater, Qiagen). Samples were left overnight at 4°C, and subsequently stored at -80°C until analysis. Biopsy specimens were homogenized and total RNA was extracted using RNeasy Micro Kit (Qiagen) according to the manufacturer’s recommendations. The concentration of RNA was assessed by means of the Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized by making use of a reverse transcriptase reaction which was performed according to the MBI Fermentas cDNA synthesis kit (Fermentas, Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers. Quantitative real time RTPCR was performed on the LightCycler 480 (Roche Diagnostic, Almere, The Netherlands) using SYBR Green PCR Master Mix (Roche Diagnostic) and primers from Invitrogen (Table I). For quantitative real-time PCR, samples were normalized for the mean of the three most stable housekeeping genes (GAPDH, β-actin en 36B4) as determined by analysis with geNorm method software (http://medgen.ugent.be/~jvdesomp/genorm/). Transcript levels of IL5, IL13, CCL26 (eotaxin-3), POSTN (periostin), TSLP (thymic stromal lymphopoietin), FLG (filaggrin), CASP14 (caspase-14), and DSG1 (desmoglein-1) were determined in duplicate.

Symptoms

Before each endoscopy, the frequency and severity of dysphagia for liquids and solids was assessed using a six-graded Likert scale, where 0 represents “no dysphagia” and 5 “daily”/“severe” dysphagia, analogous to the reflux disease questionnaire (RDQ). The RDQ was also filled out.

Statistical analysis

Statistical analysis was performed in SPSS Statistics version 22 (IBM Corporation, Armonk, NY, USA). Continuous data are expressed as median with interquartile range (IQR). Continuous data before and after treatment were compared using Wilcoxon’s signed rank test; proportions were compared using McNemar’s test. Correlations were calculated using the Spearman correlation coefficient. Significance was set at p<0.05.
Results

We included 15 patients (5 females) with EoO. Median age at first endoscopy was 43 (30-45) years, and the majority of patients (73%) was Caucasian. Atopic co-morbidity was reported by 60% of the patients: 40% food allergy, 40% allergic rhinitis, 13% asthma, and 13% atopic dermatitis.

Histopathological response to fluticasone propionate treatment

After treatment, pre-treatment peak eosinophil counts decreased to <15 eos/hpf in 10 of 15 patients (67%), and on a group level peak eosinophil counts in the distal, mid and proximal oesophagus were all significantly reduced (Figure 1). A similar effect was seen on peak mast cell counts. Before treatment, eosinophilic microabscesses were present in 10 patients (67%), whereas after treatment, eosinophilic microabscesses were seen in 4 patients (27%) (p=0.07). Thirteen patients (87%) had moderate to severe basal hyperplasia before treatment versus 2 patients (13%) after treatment (p<0.001). The number of patients with moderate to severe spongiosis also decreased after treatment, from 13 (87%) to 3 (20%) (p<0.01).

![Figure 1](Figure 1. Fluticasone propionate treatment reduced peak eosinophil (A, B, and C) and mast cell (D, E, and F) counts in the proximal, mid and distal oesophagus of EoO patients. Dotted lines indicate group medians. Shaded areas indicate remission (<15 eos/hpf).)
Oesophageal mucosal integrity

The extracellular impedance increased significantly from 2574 (1761-4285) Ω•m to 6618 (5040-9444) Ω•m (p<0.01) after treatment (Figure 2). The TER increased from 34.4 (28.7-43.3) to 64.3 (42.5-87.7) Ω•cm² (p<0.01). Accordingly, the mucosal permeability measured by the transepithelial flux of 0.3 kDa fluorescein molecules and 40 kDa rhodamine molecules decreased from 2768 (2091-3300) nmol/cm²/h to 693 (0-2589) nmol/cm²/h, and from 8 (1-21) nmol/cm²/h to 0 (0-2) nmol/cm²/h after treatment, respectively (both p<0.05). The median intercellular space proportion before treatment (0.40 (0.31-0.43) was not affected by treatment (0.35 (0.29-0.41), p=0.3).

Figure 2. Fluticasone propionate treatment significantly improved the oesophageal mucosal integrity. A) The extracellular impedance and B) transepithelial resistance of the oesophageal mucosa increased significantly, whereas the transepithelial flux of C) fluorescein molecules (size 0.3 kDa) and D) rhodamine molecules (size 40 kDa) through the oesophageal mucosa decreased significantly. Dotted lines indicate group medians.
Cytokine expression

The pre-treatment expression of inflammatory cytokines (IL5, IL13, CCL26, POSTN, and TSLP) decreased significantly after treatment, whereas the expression of barrier integrity proteins (FLG and DSG1) increased significantly (Figure 3). CASP14 was absent in the majority of EoO patients, and was detected in very low amounts in 4 patients.

Figure 3. Fluticasone propionate treatment significantly decreased the expression of genes encoding inflammatory cytokines A) IL5, B) IL13, C) CCL26 (eotaxin-3), D) POSTN (periostin), and E) TSLP (thymic stromal lymphopoietin). F) CASP14 (caspase-14) was not detectable in most EoO patients before and after treatment. The expression of genes encoding proteins important for maintaining mucosal barrier integrity G) DSG1 (desmoglein-1) and H) FLG (filaggrin) increased significantly after fluticasone propionate treatment. Dotted lines indicate group medians.
Filaggrin and desmoglein-1 expression correlate with markers of inflammation and barrier integrity

After treatment, FLG and DSG1 expression were significantly correlated with markers of inflammation: the peak eosinophil count (r=-0.57 and r=-0.54, respectively; both p<0.05), IL5 expression (r=-0.54 and r=-0.56, respectively; both p<0.05), CCL26 expression (r=-0.61 and r=-0.62, respectively; both p<0.05), and POSTN expression (r=-0.84 and r=-0.67, respectively; both p<0.05). FLG and DSG1 expression were also correlated with the transepithelial resistance of the oesophageal mucosa (r=0.60 and r=0.55, respectively; both p<0.05).

Symptoms and endoscopic signs

The frequency score of dysphagia decreased from 4 (0-10) to 0 (0-3) (p<0.05) after treatment, and the severity score decreased from 5 (0-7) to 1 (0-6) (p<0.05). RDQ total scores decreased (from 11 (4-16) to 3 (0-10), p<0.01). The total number of endoscopic signs decreased after treatment (from 4 (2-4) to 2 (1-4), p<0.05).

Discussion

In this study, we longitudinally evaluated the oesophageal mucosal barrier integrity in adult patients with EoO before and after treatment. We have previously demonstrated structural and functional impairments of the oesophageal mucosal barrier integrity in EoO patients. In the present study we demonstrated that in adult EoO patients, fluticasone propionate treatment improves the oesophageal mucosal barrier integrity and decreases the transepithelial passage of molecules with the size of common allergens. Improvement of the oesophageal mucosal barrier integrity paralleled the decrease in oesophageal eosinophilic inflammation in response to treatment. Furthermore, gene expression of inflammatory cytokines decreased significantly after treatment, whereas the expression of genes encoding for barrier integrity proteins (FLG and DSG1) increased significantly.

We have previously shown that the oesophageal mucosal barrier integrity is impaired in patients with EoO and in patients with PPI-ROE. In that study, we observed that in patients with PPI-ROE histological response to PPI treatment was associated with improvement of the oesophageal mucosal barrier integrity. In EoO patients, PPIs do not improve the impaired oesophageal mucosal integrity, which perhaps is not surprising since the magnitude of gastro-oesophageal reflux is not increased. However, similar to the effect of PPIs in patients with PPI-ROE, we now found that in adult EoO patients, improvement of the oesophageal mucosal barrier integrity in response to fluticasone propionate treatment paralleled the decrease of oesophageal inflammation. Therefore, it seems plausible that at least part of the barrier integrity impairments in EoO could be caused by intrinsic inflammatory cytokines and cytotoxic eosinophil and mast cell secretory products, similar to the mechanisms in asthma and atopic dermatitis.

Filaggrin expression is downregulated by IL-13 in active EoO, but its expression normalizes in adequately treated disease. Recent studies have identified filaggrin and desmoglein-1 as important regulators of mucosal barrier integrity in EoO. In the skin, loss of filaggrin causes
increased water loss. Genetic studies in atopic dermatitis strongly suggest that decreased filaggrin expression impairs skin barrier function and leads to increased percutaneous permeation of allergens. Downregulation of filagrin could play a similar role in the pathophysiology of EoO, since the oesophagus is frequently exposed to food allergens as well as swallowed environmental allergens. In active EoO, the strongly decreased gene expression of desmoglein-1 may also potentiate allergic inflammation. Desmoglein-1 is a component of desmosomes, which have a crucial role in maintaining epithelial barrier integrity. Desmoglein-1 downregulation has a central role in the pathophysiology of several skin diseases and SAM syndrome, in which patients suffer from severe skin dermatitis, multiple allergies and metabolic wasting.

In our study, gene expression of FLG and DSG1 increased significantly after fluticasone propionate treatment, while the expression of inflammatory cytokines decreased. Furthermore, FLG and DSG1 expression were negatively correlated with markers of oesophageal inflammation (peak eosinophil count, and expression of IL5, CCL26, POSTN) and were positively correlated with the transepithelial resistance of the oesophageal mucosa, which is a functional measure of the oesophageal mucosal barrier integrity. This suggests that normalization of gene expression is linked to normalization of functional barrier integrity. These results further corroborate the importance of filaggrin and desmoglein-1 in the pathophysiology of EoO.

We also measured the expression of CASP14 which encodes caspase-14, a protease that is required for filaggrin degradation to natural moisturizing factors in the skin. In patients with atopic dermatitis, active caspase-14 is downregulated in both laesional and non-laesional skin. Given the similarities between atopic dermatitis and EoO, we hypothesised that caspase-14 would also be downregulated in EoO and might be increased after treatment. We found that caspase-14 was not detectable in most EoO patients before and after fluticasone propionate treatment.

Our observations provide more evidence for the hypothesis that, in order to achieve histological remission in patients with EoO, restoration of the oesophageal mucosal integrity may be required. Given the limited treatment options for EoO, restoration of the oesophageal mucosal integrity may be an attractive novel therapeutic target. For instance, as in other oesophageal and atopic disease, application of topical agents on the oesophageal mucosa might theoretically protect or improve the mucosal barrier integrity in EoO.

In conclusion, fluticasone propionate treatment improves the impaired oesophageal mucosal barrier integrity in EoO patients and decreases the passage of molecules with the size of food and environmental allergens. In addition to previous research, our study endorses the role for impairment of the mucosal barrier integrity in the pathophysiology of EoO, and we confirm the importance of filaggrin and desmoglein-1 for maintaining oesophageal mucosal barrier integrity.
References