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A new eosinophilic esophagitis (EoE)-like disease without tissue eosinophilia found in EoE Families

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Abbreviations

CRTH2, chemoattractant receptor expressed on Th2 cells; EDP, EoE diagnostic panel; EoE, eosinophilic esophagitis; EPX, eosinophil peroxidase; EREFS, exudates-rings-edema-furrows-strictures classification; FFPE; formalin-fixed and paraffin-embedded; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GERD, gastro-esophageal reflux disease; GWA, genome-wide association; HPF, high-power field; IHC, immunohistochemistry; LEKTI, lympho-epithelial Kazal-type-related inhibitor; LyE, lymphocytic esophagitis; MCT, mast cell tryptase; NE, normal epithelium; PCR, polymerase chain reaction; PI, propidium iodide; TLDA, TaqMan low density arrays; TSLP, thymic stromal lymphopoietin.

Abstract

Background: Eosinophilic esophagitis (EoE) is a rapidly emerging, chronic inflammatory, genetically impacted disease of the esophagus, defined clinically by symptoms of esophageal dysfunction and, pathologically, by an eosinophil-predominant tissue infiltration. However, in four EoE-families, we have identified patients presenting with EoE-typical and corticosteroid-responsive symptoms, but without tissue eosinophilia. It was the aim of this study to clinically and immunologically characterize these patients with EoE-like disease.

Methods: Five patients suffering from an EoE-like disease were evaluated with endoscopic, histologic, functional and quantitative immunohistologic examinations, and mRNA expression determination.

Results: The frequency of first generation offspring of EoE-like disease patients affected by EoE or EoE-like disease was 40%. Immunofluorescence analysis confirmed an almost complete absence of eosinophils in the esophageal tissues of patients with EoE-like disease, but revealed a considerable T cell infiltration, comparable to EoE. In contrast to EoE, eotaxin-3 mRNA and

protein were markedly reduced in EoE-like disease ($P < 0.05$). The mRNA expression levels of three selected EoE genes (*eotaxin-3*, *MUC4* and *CDH26*) allowed to discriminate between EoE-like disease, EoE and normal epithelium.
Conclusions: Patients suffering from "EoE without eosinophilia" do not fulfill formally the diagnostic criteria for EoE. However, their clinical manifestation, immunohistology and gene-expression pattern, plus the fact that they bequeath EoE to their offspring, suggest a uniform underlying pathogenesis. Conventional EoE, with its prominent eosinophilia, therefore appears to be only one phenotype of a broader "inflammatory dysphagia syndrome" spectrum. In this light, the role of the eosinophils, the definition of EoE, and its diagnostic criteria must likely be reconsidered.

Eosinophilic esophagitis (EoE) is a recently recognized, rapidly emerging, chronic inflammatory disease of the esophagus, characterized clinically by symptoms of esophageal dysfunction and, histopathologically, by an eosinophil-predominant tissue infiltration (1). EoE has strong genetic and environmental components, with a risk of EoE for other siblings estimated at 2.4% and a sibling recurrence-risk ratio greater than 40 (2). Several genetic variants are associated with EoE (3), but one twin study has highlighted the preponderant role of a common environment in EoE susceptibility (2). Consequently, families with more than one affected member are frequently observed (4,5). Interestingly, familial and sporadic EoE have identical clinical manifestations, endoscopic features, histologic abnormalities, and even similar molecular background (4).

We report here on four EoE families with at least one member exhibiting confirmed EoE and at least one member suffering from an "EoE-like" disease, clinically resembling EoE with severe dysphagia up to bolus impaction and responding to topical corticosteroids, but without detectable esophageal eosinophilia. This observation raises several fundamental questions regarding EoE's pathogenesis, prompting us to investigate the underlying immunopathogenic and molecular mechanisms of this EoE-like disease, and to compare findings to conventional EoE patients and healthy individuals.

Materials and methods

Identification of EoE-families and patients with EoE-like disease

The Swiss EoE Clinic in Olten, Switzerland, established in the early 1990s, is a national tertiary referral center for suspected or confirmed EoE patients. The catchment area includes all of Switzerland (some 8 million inhabitants). Diagnostic and therapeutic procedures correspond to established standards (1).

The Swiss EoE Clinic currently attends 417 EoE patients, including 60 with at least one direct family member also having confirmed EoE. An additional 52 patients report a family member with clinically suspected, but not yet confirmed, EoE. These 112 patients originate from 46 families. In four of these families, we have identified five members suffering from severe dysphagia, but not fulfilling EoE's histopathologic criteria.

To enhance our understanding of this still-enigmatic disease, we evaluated these five intriguing patients clinically, endoscopically, histologically, immunohistologically, functionally (3/5), with gene expression analysis, and allergologically (3/5). We then compared findings to patients having active conventional EoE and to esophagus-healthy controls (normal epithelium, NE). The local Ethics Committee approved data collection into the Swiss EoE Database. All participants provided written informed consent.

Endoscopy, tissue sampling and histologic analyses

Upper endoscopies were performed, assessed and recorded in all five patients by board-certified gastroenterologists (AS and PH). Patients were sedated with intravenous propofol, starting with a dose of 0.5 mg/kg body weight. A standard video instrument

(Pentax EG-2940 K, Asahi Optical Co. Ltd., Tokyo, Japan) was used and all findings were documented. The global appearance of endoscopic abnormalities was assessed and, in addition, each sign associated with eosinophilic esophagitis (EoE) was recorded using the EREFS (Exudates-Rings-Edema-Furrows-Strictures) classification (6). In acknowledgement of the disease's patchy nature (7-9), at least four biopsy specimens were taken from the upper half and four from the lower half of the esophagus, using commercially-available endoscopic biopsy forceps (Boston Scientific Radial Jaw 4; Boston Scientific Corporation, Natick, MA, USA; and Olympus FB-11K-1; Olympus Medical Systems Corporation Tokyo, Japan). A standard video instrument (Pentax EG-2940 K, Asahi Optical Co. Ltd., Tokyo, Japan) recorded all findings that were then documented.

All esophageal biopsies were evaluated by an EoE expert pathologist (ChB); in particular, the eosinophils in the most densely infiltrated area were counted in five consecutive HPFs (high-power fields) (Zeiss Axiophot, Plan-Neofluar 40, ocular magnification 10x, area of microscopic field 0.3072 mm^2) and EoE-associated signs, such as spongiosis, papillary elongation and basal zone hyperplasia, as well as the amount of subepithelial fibrosis, were determined semi-quantitatively, as previously described (10,11).

Immunofluorescence and immunohistochemistry (IHC)

Immunofluorescence of formalin-fixed, paraffin-embedded (FFPE) biopsies from the five EoE-like patients (EoE-like) (m/f: 1/4; mean age: 60.2 yrs), eight conventional EoE patients (EoE) (m/f: 6/2; mean age: 36.1 yrs), and three NE controls (m/f: 1/2; mean age: 32.7 yrs) determined the expression of eosinophil peroxidase (EPX), CD3, mast cell tryptase (MCT), chemoattractant receptor-homologous molecule (CRTH2), thymic stromal lymphopoietin (TSLP) and lympho-epithelial Kazal-type-related inhibitor (LEKTI) (10-12). Primary antibodies used: anti-EPX (Lee Laboratory, Mayo Clinic, AZ); anti-CD3 (DakoCytomation, Glostrup, Denmark and Lab Vision/NeoMarker, Thermo Fisher Scientific, Fremont CA); anti-MCT (DakoCytomation); anti-CRTH2 and anti-TSLP (Santa Cruz Biotechnology Inc., Santa Cruz, CA); and anti-LEKTI (Novus Biologicals, Littleton, CO). A confocal laser scanning microscope (LSM 510, Carl Zeiss, Jena, Germany) analyzed the 20 fields of highest activity (1000x magnification, total area=0.34 mm^2), except for LEKTI analysis (450x magnification, total area=0.265 mm^2). Infiltrating T cell CRTH2 expression was obtained in a double staining of CRTH2 and CD3 (11). Extracellular EPX deposits and epithelial cell TSLP and LEKTI expression were evaluated semi-quantitatively using scores described previously (10-12).

For epithelial eotaxin-3 and TNF-α expression, samples were analyzed by indirect IHC, as previously described (13). Anti-TNF-α (Novus Biologicals) and anti-eotaxin-3 (R&D Systems, Minneapolis MN) served as primary antibodies. Evaluation was performed using a light microscope (Fluorescence Microscope Observer.Z1, Carl Zeiss, Germany) at 630x magnification (total area=0.44 mm^2) and we analyzed the 20 fields of highest activity. TNF-α positive cells were counted and semi-quantitative scores determined staining intensity of eotaxin-3 expression (10-12).

mRNA expression of EoE genes

Genome-wide association (GWA) analyses show that EoE has a distinct mRNA expression pattern, a so-called EoE transcriptome, consisting of approximately 500 genes (14). The EoE diagnostic panel (EDP), a representative set of 94 EoE genes with an approximate 96% sensitivity and 98% specificity, discriminates among EoE, gastroesophageal reflux disease (GERD) and NE (15). Complementing the established clinical and histologic approach, EoE can also be diagnosed at a molecular level by mRNA expression in FFPE biopsies (15,16). TaqMan Low Density Arrays (TLDA) from three EoE-like patients (m/f: 1/2, one of them at 2

different examinations, mean age: 57.33 yrs); four conventional EoE patients (m/f: 1/3 mean age: 30.25 yrs); and four NE individuals (m/f: 1/3, mean age: 42.75 yrs) analyzed a gene set of 94 transcripts, including genes dysregulated in treated and untreated EoE as well as control genes. Briefly, Aegis Sciences Corp (Nashville, TN) performed RNA extraction and TLDA PCR (polymerase chain reaction) on 4-5 10-µm FFPE biopsies, as described previously (15,17). Genes were analyzed only if the expression level was detectable in at least 4/13 biopsies. Raw data were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expression and the difference in gene expression calculated by the Delta-Delta-Ct method.

Radiological and functional examinations

In order to exclude morphological abnormalities, motility disorders and gastro-esophageal reflux, we performed a barium swallow, high resolution esophageal manometry with Manoscan 360 (Given Imaging Limited, Yoqneam, Israel) and 24-hour impedance pH-Monitoring with ZepHr® (Sandhill Scientific, Highlands Ranch, CO, USA).

Allergological work-up

After a comprehensive medical history, three patients with EoE-like disease underwent a skin prick test with a panel of environmental and food allergens applied on the forearm and read after 20 min. Histamine served as a positive, and sodium chloride as a negative, control (ALK Abello AG, Volketswil and Allergopharma AG, Therwil, Switzerland). The patch test provided an additional allergic challenge with application of aeroallergens (house dust mites, pollens, animal danders; Stallergenes AG, Dietikon, Switzerland), and native food (cow's milk, chicken eggs, rye and wheat flour, soy, carrots) on the back for 48 hours using Finn chambers, and evaluated at days 3 and 4. Peripheral blood levels of total immunoglobulin (Ig) E (normal value <100 kU/l), specific IgE to an environmental allergen mix (Sx1, normal value <0.35 kU/l) and allergen components (microarray with 112 allergens; ImmunoCAP ISAC, Thermo Fisher Scientific, Uppsala, Sweden) were measured, according to the manufacturer's instructions.

Statistical analysis

Data analysis was performed by HUS using the unpaired *t* test, and significance set at 0.05. Semi-quantitative, immunohistometric data were graded accordingly: 0 (absent, 0); + (mild, 1); ++ (moderate, 2); +++ (strong, 3); ++++ (very strong, 4). Biopsies from EoE-like patients were obtained and analyzed at different times during follow-up. Data represent means ± standard deviation of all available biopsies. For mRNA quantification, only data points above the detection limit are presented. Individual biopsies were considered as independent points; the median is shown. A non-parametric Kruskal-Wallis test was performed and multiple comparisons were corrected with Dunn's Multiple Comparison test.

Results

Pedigrees emphasize a strong inheritance

All 57 individuals from these four EoE-families were Caucasian. Four females and one male were identified as having the atypical, so-called “EoE-like” disease, but both male and female first generation offspring are afflicted with conventional EoE (Fig. 1). In family A, 1/2 (A.II.1) 1st generation children had confirmed, conventional EoE. In family B, 2/5 (B.II.7 and B.II.8) 1st generation had confirmed, conventional EoE, 1/5 suspected but unconfirmed (refused endoscopy) EoE-type pathology (B.II.5), and 1/5 with one EoE-like disease, B.II.2, mother to EoE patient, B.III.2, 1/2 children from the 2nd generation. In family C, 1/4 (C.II.7) 1st generation offspring has confirmed, conventional EoE as did another 1/10 (C.III.3) in the 2nd generation. Interestingly, neither parent of C.III.3 was affected with EoE or EoE-like disease. In family D, 1/4 (D.II.5) 1st generation offspring has conventional EoE and 1/4 (D.II.4) with EoE-like disease, and 1/4 (D.II.7) has suspected, but unconfirmed (refused endoscopy) EoE-type pathology, but 0/9 (as of this writing) in the subsequent 2nd generation. Therefore, 33% of the 1st generation offspring of EoE-like disease patients were affected by conventional EoE, and even 40% by EoE or EoE-like disease (only offspring of EoE-like disease patients are included in this calculation, Fig. 1). Taken together, these data suggest a strong and common inheritance of EoE and of EoE-like disease.

The clinical manifestation of EoE-like disease is similar to conventional EoE

As in conventional EoE, the leading symptom in all EoE-like syndrome patients was dysphagia for solids with food impactions (1,5,18), in one patient requiring even endoscopic food removal. Chest pain was present in 2/5 patients. Both dysphagia and chest pain persisted despite high-dose PPI treatment. In contrast, all these disturbances responded rapidly, in 4/5 patients even completely, to treatment with swallowed topical corticosteroids. Of note, symptoms relapsed in all patients shortly after medication cessation. Summarizing, the clinical manifestation of EoE-like disease is similar to conventional EoE (1,5,9,18). Table 1 summarizes demographic, clinical and disease-specific characteristics of the five EoE-like disease patients.

Lack of evident endoscopic abnormalities in EoE-like patients

Except for discreet mucosal irregularities, such as fine nodules and subtle rings, endoscopies in all EoE-like patients were unremarkable. Established EoE-associated signs (6), e.g., edema, furrows and white exudates, were not detectable, even on repeated examinations (Fig. 2A-C and Table 1).

Exclusion of distinct functional abnormalities and GERD in patients with EoE-like disease

The barium swallow performed in 4/5 patients was inconspicuous, showing a normal sized esophagus with a timely passage of the contrast agent without any narrowing or signs of dysmotility. High-resolution manometry and 24-hour impedance pH monitoring performed in 3/5 patients revealed neither pathologic gastro-esophageal reflux nor a specific dysmotility pattern.

Histological re-examination revealed no relevant tissue eosinophilia

Re-evaluation of all HE-stained esophageal biopsies from EoE-like patients revealed few lymphocytes and non-specific signs of chronic inflammation, e.g., basal zone hyperplasia and papillary elongation. Notably, no eosinophils were detected, except for two single eosinophils in 1/40 HPFs found in 1/8 biopsies from patient 5 (Fig. 2D-F and Table 1).

Quantitative immunohistological analyses confirm lack of eosinophilia, but reveal a strong T cell and moderate mast cell infiltration

In contrast to conventional histologic examination with HE staining which showed no tissue eosinophilia in EoE-like patients, double staining with EPX (eosinophil peroxidase) antibody and propidium iodide (PI) revealed a few eosinophils (Fig. 3A and Fig. S1). *Per definitionem* (1,18), we found a pronounced epithelial eosinophilic infiltration in patients with conventional EoE, but no eosinophils in the esophageal epithelium of healthy controls (NE) (Fig. 3A). Notably, we discerned no evidence of already degranulated and destroyed eosinophils (no extracellular EPX deposition) in EoE-like disease patients (Fig. 3A). Moreover, the almost complete absence of both tissue eosinophils and extracellular EPX deposits were constant findings throughout the follow-up (up to 6 years) (Fig. S1).

Compared to NE, most EoE-like disease patients had, as in conventional EoE (1,5,18), a significant increase in esophageal T cells that remained stable throughout follow-up (Fig. 3B and Fig. S1). Of note, the T cells were largely CRTH2-negative (Fig. 3B) and found primarily in a peripapillary location (Fig. 3B).

Tryptase staining revealed more mast cells in samples from EoE-like disease patients compared to NE, but still markedly fewer than in conventional EoE (Fig. 3C); this, too, remained relatively stable throughout follow-up (Fig. S1).

Slightly increased epithelial cytokine expression, but no barrier defect in EoE-like disease

Considering the lack of eosinophilic esophageal infiltration in EoE-like disease, measurements of chemokine and cytokine expression involved in eosinophilic recruitment (1,18) showed interesting results. Compared with conventional EoE, epithelial eotaxin-3 expression levels were significantly lower in EoE-like disease patients, being even within the healthy range (Fig. 4A). Increased expression of the cytokines, TSLP and TNF-α, was observed in some EoE-like disease patients, though conventional EoE patients tended to exhibit higher levels (Fig. 4B and 4C). Note that many healthy controls also showed TNF-α positive epithelial cells (Fig. 4C).

LEKTI, a protease inhibitor responsible for epithelial homeostasis, is reportedly reduced in active EoE, implying an epithelial barrier defect (12). EoE-like disease patients exhibited significantly higher LEKTI expression by epithelial cells as compared with conventional EoE, and no difference was observed relative to normal esophagus (Fig. 4D).

EoE-like disease and conventional EoE share similar gene expression abnormalities, but mRNA expression levels of three genes enable a discrimination between EoE-like disease, EoE and NE

Assessment of mRNA expression levels of 94 transcripts identified previously as being dysregulated in EoE (EDP) provided insight into the molecular pathogenesis of EoE-like disease (15). Using a low density array, quantitative mRNA level determination in FFPE tissue sections showed a significant up-regulation of *eotaxin-3* mRNA in conventional EoE, but was below the detection limit in EoE-like disease and NE (Fig. 5). We identified significant changes in the mRNA expression of several genes known to be dysregulated in IL-13-stimulated esophageal epithelial cells (15,16). In EoE-like disease, *CDH26, KCNJ2, UPKB* were strongly up-regulated, though not quite to conventional EoE levels, while *MUC4* was comparable in the two pathologies (Fig. 5). *DSG1* was significantly down-regulated in conventional EoE compared with controls (Fig. 5). Generally, in EoE-like disease, gene dysregulation levels were either comparable to conventional EoE or between conventional EoE and NE. Recapitulating, mRNA expression of *MUC4* and *CDH26* genes differentiated between EoE-like disease and healthy controls, whereas *eotaxin-3* mRNA

expression differed significantly between EoE-like disease and conventional EoE. These data suggest that EoE-like disease is a phenotypical variant of EoE, with some uniform underlying molecular pathogenesis.

Discussion

EoE is characterized clinically by symptoms of esophageal dysfunction and histopathologically by an eosinophil-predominant esophageal infiltration (1,9,18). We present here a series of patients, all with at least one family member having conventional EoE, suffering from PPI-refractory symptoms of severe esophageal dysfunction and thereby fulfilling formally the *clinical* EoE diagnostic criteria. Intriguingly, histological examination of serial biopsies failed to detect esophageal eosinophilia in these patients who, therefore, do *not* fulfill EoE's *histopathologic* requirement. Provocatively, one could designate the EoE-like disease as "EoE without eosinophilia". This so-far unrecognized disease has the potential to provide new insights into EoE's pathogenesis.

Similarities between conventional EoE and EoE-like disease include a common PPI-refractory cardinal symptom (dysphagia for solids, up to food impaction requiring endoscopic bolus removal), and rapid symptom improvement after swallowed topical corticosteroids which relapses after medication cessation (1,5,18).

Mechanisms leading to EoE's esophageal dysfunction are poorly understood (1), though cellular components, in particular eosinophils and mast cells with their preformed substance release, are suspect (1,18). Our novel results show clearly that EoE dysphagia is not necessarily linked to eosinophils or an eosinophil-related mediator in esophageal tissue, possibly elucidating the disappointing outcomes of eosinophil-targeted treatments, such as the anti-IL-5 antibodies, mepolizumab and reslizumab, where esophageal dysfunction persisted despite achieving a marked (>50%) reduction in tissue eosinophilia (10,19,20). Other clinical trials likewise report a poor correlation between symptoms and the density of tissue eosinophilia (21,22), suggesting a likely non-essential role of eosinophils in symptom provocation.

In contrast to their striking difficulty in swallowing, EoE-like disease patients had only minimal, or even no, endoscopic abnormalities. Almost all established inflammatory signs of EoE, such as white exudates, furrows and edema (6), were absent, suggesting two conclusions: Firstly, EoE's endoscopic pattern probably relies on the presence of eosinophils; and secondly, based on its inconspicuous endoscopy, EoE-like disease risks being undiagnosed or misinterpreted as functional dysphagia (23).

On average, 40% of the first generation offspring of EoE-like disease patients exhibited either confirmed, conventional EoE or EoE-like disease, suggesting that these two phenotypes have a common genetic and pathogenic background. In sporadic EoE, 2/3 of patients are male (1,5,18). One hallmark of multifactorial inheritance is that, when affected, the gender the least likely to have the disease is the most likely to produce affected offspring (14). Affected females are thus the most likely gender to produce affected offspring. Indeed, all female EoE-like disease patients had children with EoE, whereas neither child of the male patient was affected by the disease.

One could argue that EoE-like disease patients have a hidden eosinophilia due to: the patchy nature of the inflammation (7,8); eosinophils located in deeper, sub-epithelial wall layers; a previous degranulation; or to progression from inflammatory to fibrotic disease (24,25). In all patients, aggressive, structured epithelial and lamina propria tissue sampling from all esophageal segments revealed no relevant eosinophilic infiltration, either with HE or IHC EPX staining. The lack of fibrosis, endoscopically and histologically, provides a strong argument against non-inflammatory end-stage EoE. Ergo, the risk of missing a hidden eosinophilia in these patients is small and they likely suffer from a non-eosinophilic phenotype of immune-mediated esophagitis.

As blood eosinophil numbers are comparable to conventional EoE, but no eosinophils are present in the esophagus, we hypothesized that EoE-like disease patients might have a defect in chemoattraction for eosinophils, and determined therefore the

expression of chemokines and cytokines crucially involved in eosinophil recruitment. *Eotaxin-3* has been identified as the most highly induced gene in EoE patients, and the esophageal *eotaxin-3* mRNA level correlates strongly with tissue eosinophilia (26). Compared to healthy controls, EoE patients have increased epithelial expression of eotaxin-3 (27,28), while EoE-like disease patients showed levels between NE and EoE. TSLP and TNF-α expression showed similar, but less consistent, results. In conclusion, EoE-like disease patients might have a defect in the eosinophil recruitment pathway, leading ultimately to this non-eosinophilic inflammatory pattern.

Because the *eotaxin-3* gene is IL-13-inducible (26), one could speculate that IL-13 is not over-expressed in the tissue. We identified several genes, such as *CDH26* and *DSG1*, known to be dysregulated by IL-13 in esophageal epithelial cells and dysregulated in EoE, but only slightly dysregulated in EoE-like disease biopsies. These results, along with the histologic findings, suggest that EoE-like disease may present an attenuated Th2 signal.

Histology revealed no obvious inflammation in EoE-like disease patients, though IHC detected a considerable T cell infiltration of the mucosa, similar in intensity to conventional EoE. The rapid clinical response to anti-inflammatory medication further supports the key role of this common immunohistologically detectable infiltration in the pathogenesis of this disease. Of interest, in contrast to conventional EoE (11), the expression of the CRTH2 receptor on T cells was not significantly increased, signifying that patients with an EoE-like disease may have, as with conventional EoE, a chronic T cell inflammation, but unlike conventional EoE, no Th2-type inflammatory response.

Notably, T cells were located primarily in a peri- and intrapapillary site, suggesting an early stage of inflammation where T cells start to migrate into the epithelial layer. The pattern of a dominantly peripapillary T cell infiltration is reminiscent of lymphocytic esophagitis (LyE), a recently recognized, histo-pathologically defined disease of another chronic, immune-mediated esophagitis (29-32). As in conventional EoE and in EoE-like disease, dysphagia for solids is also the leading symptom of LyE but, unlike EoE, affects predominantly older women thereby resembling our EoE-like disease patients (29-32). Today, neither the cytokine expression nor the occurrence of chemotactic factors in LyE has been well characterized. However, it is tempting to speculate that all three types of chronic, immune-mediated esophagitis – EoE, LyE and EoE-like disease – have a common underlying pathogenic mechanism.

It is well established that, in actively inflamed EoE, mucosal integrity is disturbed (9,12). As such, dilated intercellular spaces, referred to as spongiosis, are a common histomorphological feature of active EoE (5). The widening of the gap between epithelial cells is considered to be a condition that allows antigens to penetrate into deeper layers of the mucosa, thereby initiating an inflammatory response (33,34). The intercellular epithelial connection is largely regulated through tight junctions and desmosomal proteins (34). Several studies have concordantly shown that spongiosis and reduced expression of tight junction and desmosomal proteins are associated with a reduced mucosal integrity, as assessed by functional tests (33,34). However, it is still an ongoing debate as to whether this barrier defect is an EoE-inherent feature, allowing allergens to invade the mucosa and to initiate an inflammatory response, or whether it is only a non-specific consequence of the inflammatory condition. This impelled us to search histologically for signs of spongiosis and to determine LEKTI, a marker shown to be responsible for epithelial homeostastis (12). In contrast to conventional EoE patients, patients with EoE-like disease exhibited no signs of spongiosis in the esophageal epithelium, and structural and immunohistological markers of mucosal integrity were not impaired. Expression of LEKTI was not reduced and appeared comparable to esophagus-healthy controls. This, together with the finding that, in conventional EoE, successful treatment of inflammation restores mucosal integrity, are strong indicators that barrier function defects in EoE are a consequence of active eosinophil inflammation and not a disease-inherent feature.

In conclusion, the comprehensive evaluation of five members of EoE families, presenting clinically with a syndrome similar to EoE, but without esophageal eosinophilia, reveals a substantial T cell and a minor mast cell infiltration clearly detectable by IHC. However, the symptomatic response of this EoE-like disease to corticosteroids and the inheritance of conventional EoE in offspring strongly suggests a uniform pathogenesis. Conventional EoE, with its predominant eosinophilia, is therefore likely only one phenotype of a broader dysphagia spectrum. Our observations have the potential to challenge some established paradigms, for instance, the pathogenic role of eosinophils, the currently used diagnostic criteria for EoE, and even the definition of "functional dysphagia", and beg reconsideration in light of this new, distinct, but difficult to diagnose, entity. However, these findings require confirmation in well-powered cohorts of patients suffering from sporadic EoE-like disease, including an analysis of the longitudinal development of this intriguing disease. Based on our preliminary data, we currently recommend that, when confronted with a patient suffering from solid food dysphagia, but not fulfilling the histologic criteria for EoE diagnosis, examinations be extended to include the determination of T cell infiltration; *eotaxin-3 (*allowing a differentiation between EoE-like disease and conventional EoE); and *MUC4* and *CDH26* mRNA expression analyses (allowing a discrimination between EoE-like disease and NE).

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Conflict of interest

AS has consultant contracts with Actelion, Falk, Novartis, Receptos, Regeneron and Roche-Genentech. ES received consulting fees from Aptalis Pharma and Novartis. AMS has consultant contracts with Falk, Novartis, Receptos, and Regeneron. CB is employed by Nestec and ChB by Viollier. The remaining authors have no potential conflict of interest.

Author contributions

Acquisition, analysis, or interpretation of data: AS, CB, SRH, ChB, PH, ES, DS, AMS, HUS.

Drafting of the manuscript: AS, CB, ES, AMS, HUS.

Critical revision of the manuscript for important intellectual content: AS, CB, SRH, ChB, PH, ES, DS, AMS, HUS.

Statistical analysis: CB, SRH, HUS.

Obtained funding: AS, CB, SRH, AMS, HUS.

Administrative, technical, or material support: AS, CB, SRH, ChB, PH, ES, DS, AMS, HUS.

Study supervision: AS, HUS.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Persistence of inflammatory cell infiltration in EoE-like disease over time. EoE-like disease patients were followed for up to 6 years. Inflammatory cells and extracellular EPX deposition were evaluated in biopsies from every endoscopy performed since clinical presentation. Single data points represent mean values at each time point.

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Table 1. Demographic, clinical and disease-specific characteristics of patients with EoE-like disease

Patient	Age	Gender	Age at symptom onset	Age at diagnosis	Duration of symptoms at diagnosis	Symptoms			History of allergies	BMI	Laboratory findings		Endoscopic features		Histology	Treatment response	
	years		years	years	years	Dysphagia for solids	Long-lasting food impaction requiring endoscopic removal	Spontaneously occuring chest pain	AA, ARC, OAS *	kg/m^2	Peripheral blood eosinophils eos/mm^3 (Norm <350 eos/mm^3)	Serum IgE kU/l (Norm <100 kU/l)	Description	EREFS Score **	HE staining	Symptom response to PPI	Symptom response to corticosteroids
1	60	f	52	59	7	yes	no	no	none	28.1	0	22	few plaques	0/0/0/0/0	no eosinophils, few lymphocytes	none	complete resolution
2	73	f	10	72	62	yes	yes	no	AA, ARC	18.4	200	99	few nodules	0/0/0/0/0	no eosinophils, few lymphocytes	none	complete resolution
3	53	f	10	52	42	yes	no	no	AA	23.2	100	40	few nodules	0/0/0/0/0	no eosinophils, few lymphocytes	none	complete resolution
4	71	f	65	70	5	yes	no	yes	none	24.1	100	15	unremarkable	0/0/0/0/0	no eosinophils, few lymphocytes	none	partial resolution
5	44	m	40	43	3	yes	no	yes	ARC, OAS	23.3	100	102	Subtle rings	0/1/0/0/0	2 eosinophils in 1/40 hpf, few lymphocytes	none	complete resolution
Average, ratio	60.2	m/f = 1/4	35.4	59.2	23.8	5/5	1/5	2/5	3/5	23.4						0/5	4/5
Range, percentage	44 - 73		10 - 65	43 - 72	3 - 62	100%	20%	40%	60%	18.4 - 28.1	0 - 200	15 - 102				0%	80%
SD	10.91		24.82	12.19	26.73					3.43							

* AA=allergic asthma, ARC= allergic rhinoconjunctivits, OAS= oral allergy syndrome

** EREFS-Score (Edema/Rings/Exudates/Furrows/Strictures): 0=absent 1=mild 2=moderate 3=severe

Figure legends

Figure 1. Pedigrees of the four EoE families identified with at least one relative suffering from EoE-like disease. The pedigrees of the four EoE families demonstrate that among EoE-like disease patients, 33% of the first generation offspring were affected by confirmed conventional EoE and, in total, 40% by EoE and EoE-like disease.

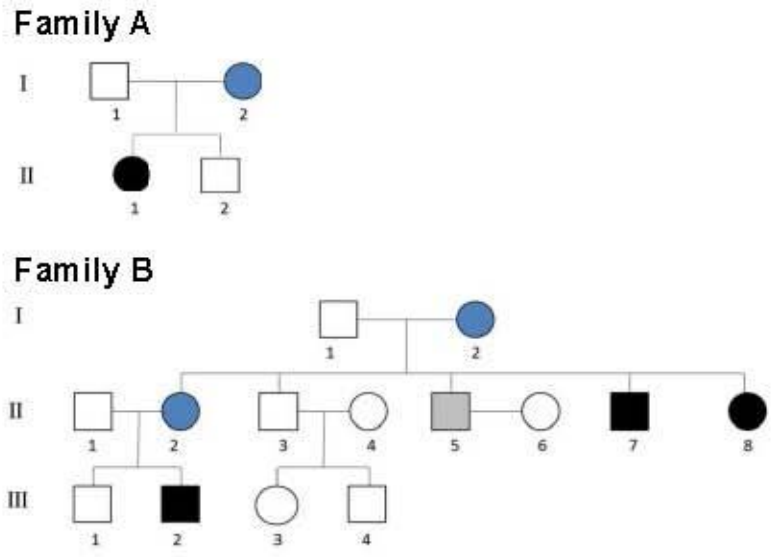
Figure 2. Endoscopic and histologic findings in patients with EoE-like disease. *Upper Panel:* Representative endoscopic pictures of three patients with EoE-like disease, showing an intact, non-inflamed mucosa with discrete abnormalities, such as some fine nodules (A and B), as well as subtle rings that disappear spontaneously or after insufflation of air (C). *Lower Panel:* Representative histologic pictures of three patients with EoE-like disease, showing a regularly-structured squamous epithelium with few lymphocytes (D and E) in the epithelium and lamina propria, mild basal zone hyperplasia (D), papillary elongation (D and F) and minimal sub-epithelial fibrosis (D and F), but no eosinophils. (Elastica van Gieson (D) and HE staining (E and F); original magnification x 200).

Figure 3. Inflammatory cell infiltration in EoE-like disease, EoE and healthy controls. Immunohistomorphometric determination of epithelial eosinophil, T cell and mast cell numbers in patients with EoE-like disease, conventional EoE and healthy controls (NE), demonstrating a lack of eosinophils or extra-cellular EPX granule protein deposits in patients with EoE-like disease (A); a significant T cell infiltration comparable with conventional EoE, but mainly with CRTH2 negative T cells (B); as well as a relatively mild infiltration with mast cells (C). *Right:* Representative images. Eosinophils, T cells, and mast cells were identified using the indicated lineage-specific antibodies. Nuclei were stained with propidium iodide (PI). In the images of panel B, the epithelial-subepithelial junction is highlighted (dashed line). Bars, 10 µm.

Figure 4. Cytokine expression in EoE-like disease, EoE and healthy controls. Immunohistomorphometric determination of esophageal cytokine expression in EoE-like disease, conventional EoE, and in healthy controls, revealing a significantly reduced expression of the eosinophil chemoattractant, eotaxin-3, as compared with conventional EoE (A), showing similar trends for TSLP (B) and for TNF-α (C). In contrast to conventional EoE, the expression of the mucosal integrity-associated marker, LEKTI, was not reduced (D). *Right:* Representative images. Eotaxin-3, TSLP, TNF-α, and LEKTI were identified using specific antibodies. Nuclei were stained with hematoxylin (panels A and C) and propidium iodide (PI) (panels B and D). Bars, 10 µm.

Figure 5. mRNA expression levels in formalin**-**fixed paraffin-embedded (FFPE) tissue biopsies from patients with EoE-like disease, EoE and healthy controls. Determination of mRNA expression with TaqMan Low Density Arrays (TLDA) for esophageal tissue sections from patients with EoE-like disease, conventional EoE and normal esophageal epithelium (NE) demonstrate that the *eotaxin-3* gene is significantly up-regulated in EoE, but not in EoE-like disease or healthy controls. In contrast, the epithelial-related gene, *MUC4,* is significantly up-regulated in EoE and in EoE-like disease as compared with controls. The EoE genes, *CDH26, KCNJ2, UPKB* and *DSG,* were found to be remarkably dysregulated in EoE and in EoE-like disease, but less so in the latter. Expression levels are shown as normalized to GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) expression. Each biopsy is presented as an individual point and genes were analyzed only if the expression level was detectable in at least 4/13 biopsies.

Eotaxin-3 mRNA levels in biopsies of patients with EoE-like disease and healthy controls were undetectable. Therefore, the lower detection limit of the assay was added in each of these cases (upper left panel).



	Conven-tional EoE		EoE + EoE-like disease		Diagnosed + suspected EoE	
Family	Ratio	%	Ratio	%	Ratio	%
A	1/2	50	1/2	50	1/2	50
B	2/5	40	3/5	60	4/5	80
	1/2	50	1/2	50	1/2	50
C	1/4	25	1/4	25	1/4	25
D	0/2	0	0/2	0	0/2	0
Total	**5/15**	**33%**	**6/15**	**40%**	**7/15**	**46%**

EoE-like disease

EoE diagnosis confirmed

EoE diagnosis suspected

Figure 1

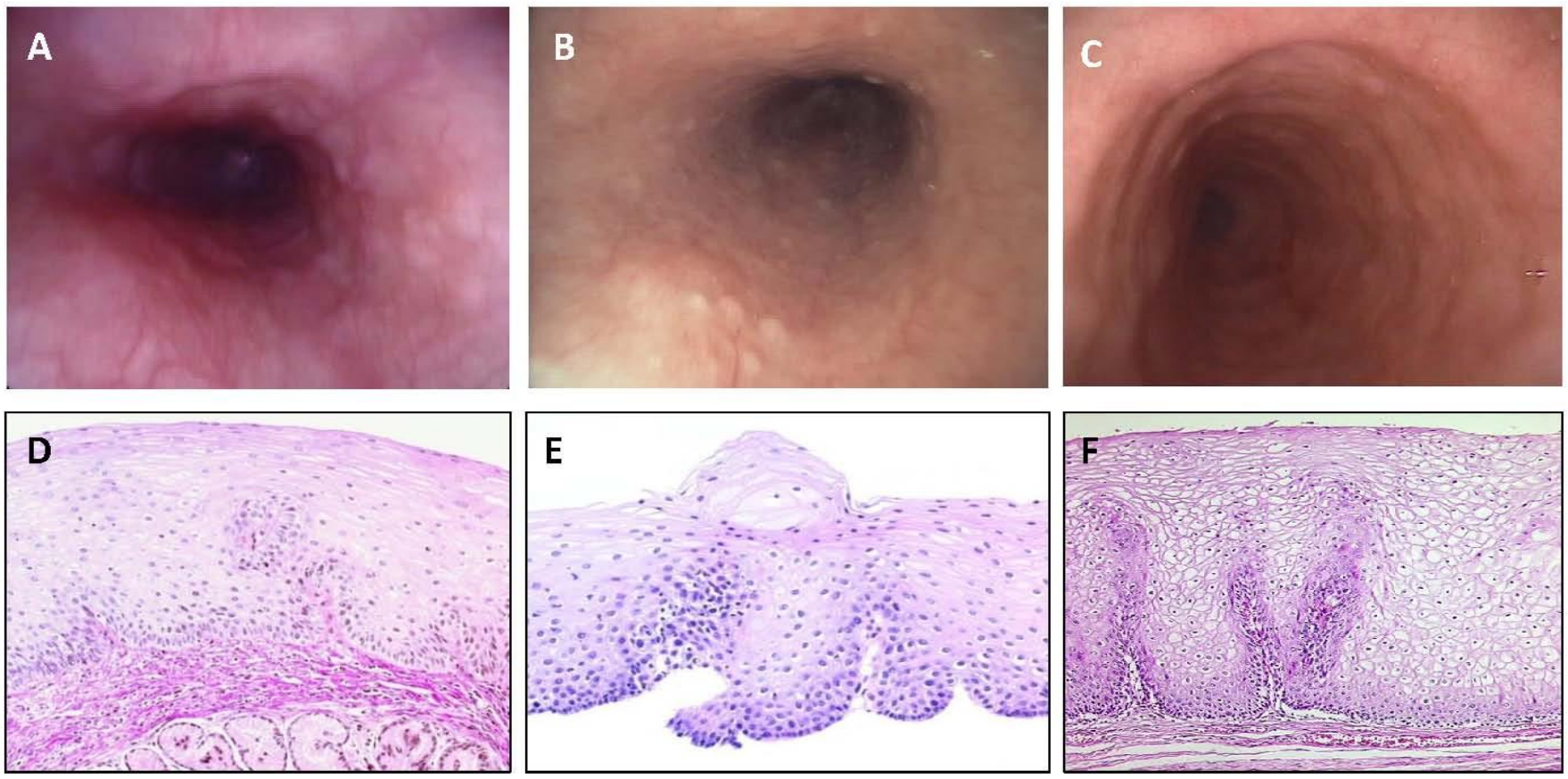


Figure 2

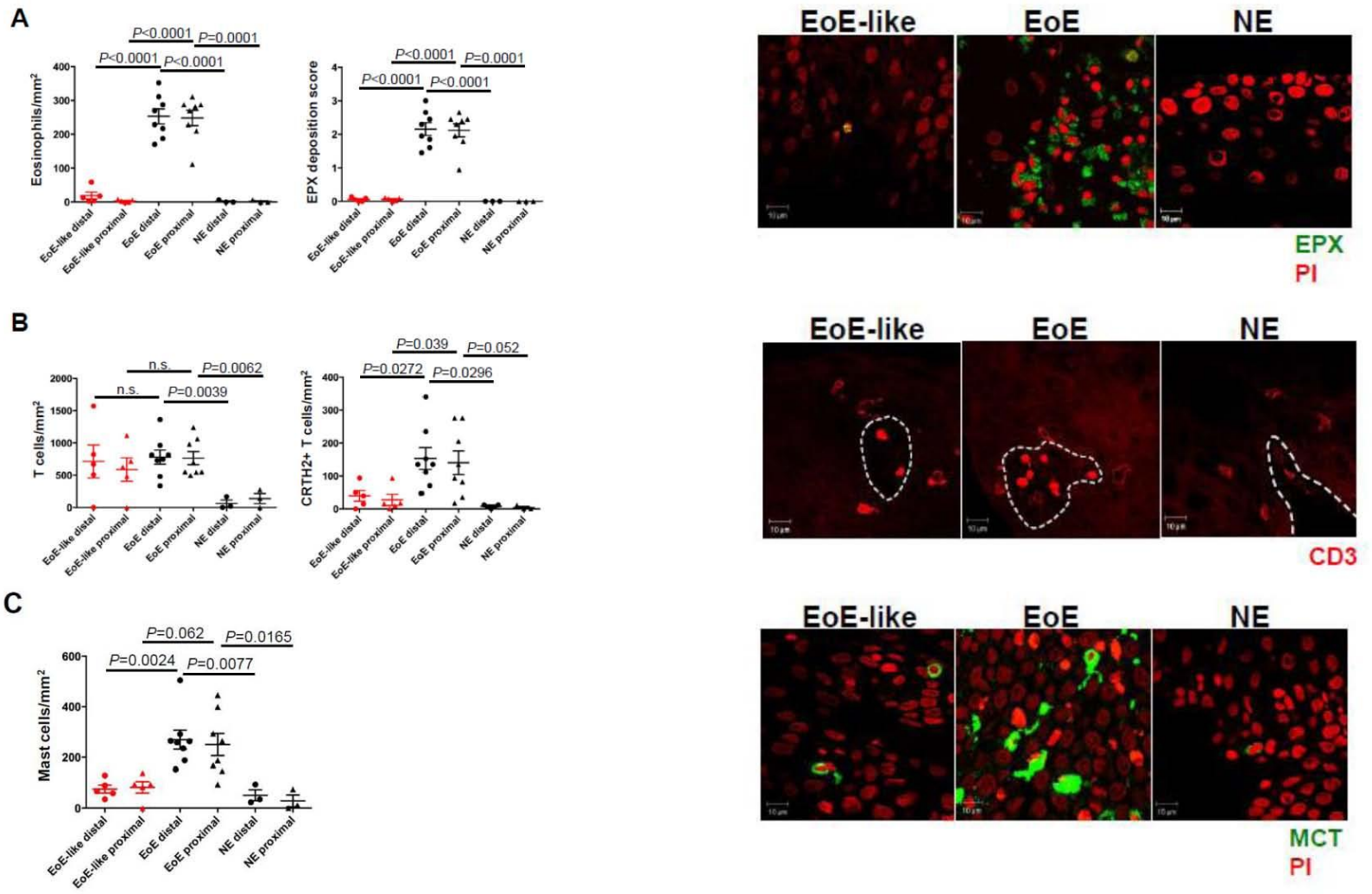


Figure 3

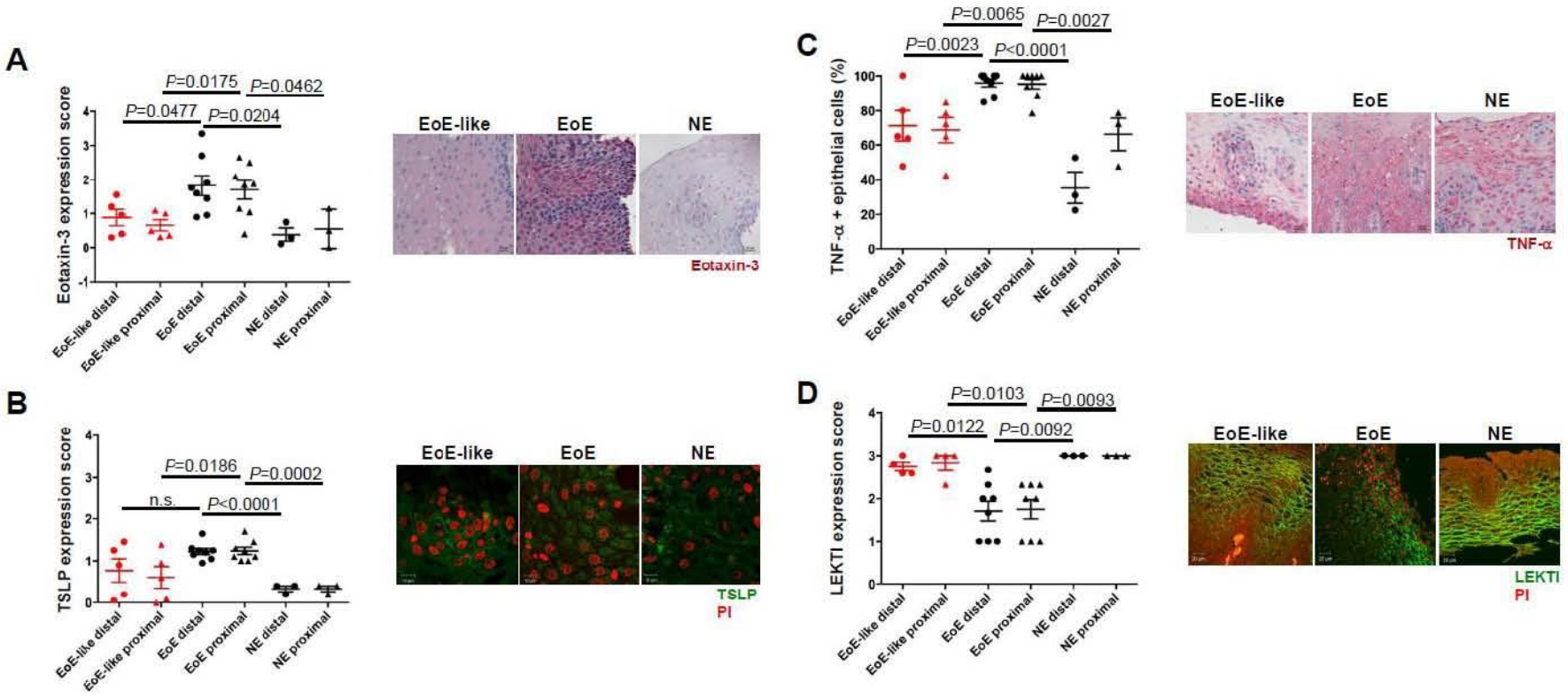


Figure 4

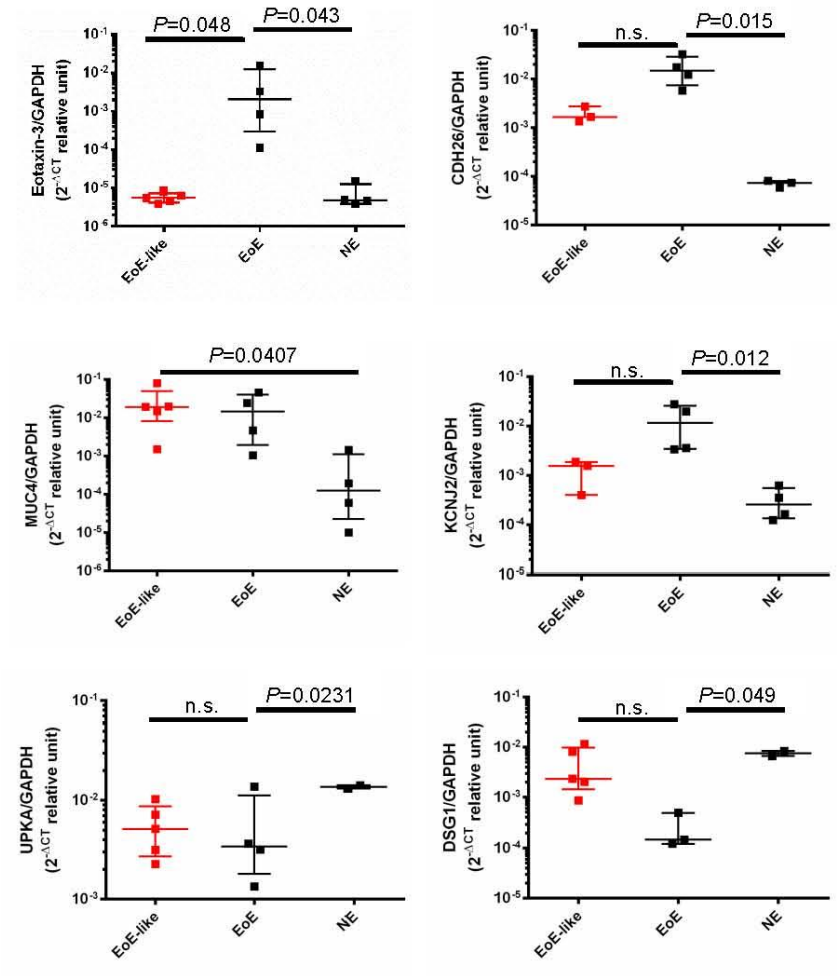


Figure 5