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ORIGINAL ARTICLE

Food Allergy and Gastrointestinal Disease

Characterization of eosinophilic esophagitis variants by clinical, histological, and molecular analyses: A cross-sectional multi-center study

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Abbreviations: ALOX15, Arachidonate 15-lipoxygenase; C3, Complement component 3; CASP14, Caspase 14; CCDC80, Coiled-coil domain-containing protein 80; CCL26, CC-chemokine ligand 26 (eotaxin-3); CD3, Cluster of differentiation 3; CNGB3, Cyclic nucleotide gated channel subunit beta 3; CRTH2, Chemoattractant receptor-homologous molecule expressed on Th2 cells; CXCL10, C-X-C motif chemokine ligand 10; CXCL11, C-X-C motif chemokine ligand 11; DYNLL1P3, dynein light chain LC8-type 1 pseudogene 3; EoE, Eosinophilic esophagitis; EoE-HSS, EoE histology scoring system; Eos, Eosinophils; EPX, Eosinophil peroxidase; EREFS, EoE endoscopic reference score; FDR, false discovery rate; GALNT15, Polypeptide N-acetylgalactosaminyltransferase 15; GERD, Gastroesophageal reflux disease; H&E, Hematoxylin and eosin; Hpf, High power field; IgE, Immunoglobulin E; IL-1, Interleukin 1; IQR, Interquartile range; LEKTI, Lympho-epithelial Kazal-type-related inhibitor; MMP1, Matrix metalloproteinase-1; NF-kB, Nuclear factor kappa B; PI15, Peptidase Inhibitor 15; qPCR, Quantitative polymerase chain reaction; RNA, Ribonucleic acid; RNAseq, Next generation RNA sequencing; SD, Standard deviation; TGF-β1, Transforming growth factor beta 1; Th2, T helper 2; TNF, Tumor necrosis factor; TSLP, Thymic stromal lymphopoietin; ZEB2, Zinc finger E-box-binding homeobox 2; ZIC1, Zic family member 1.

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Abstract

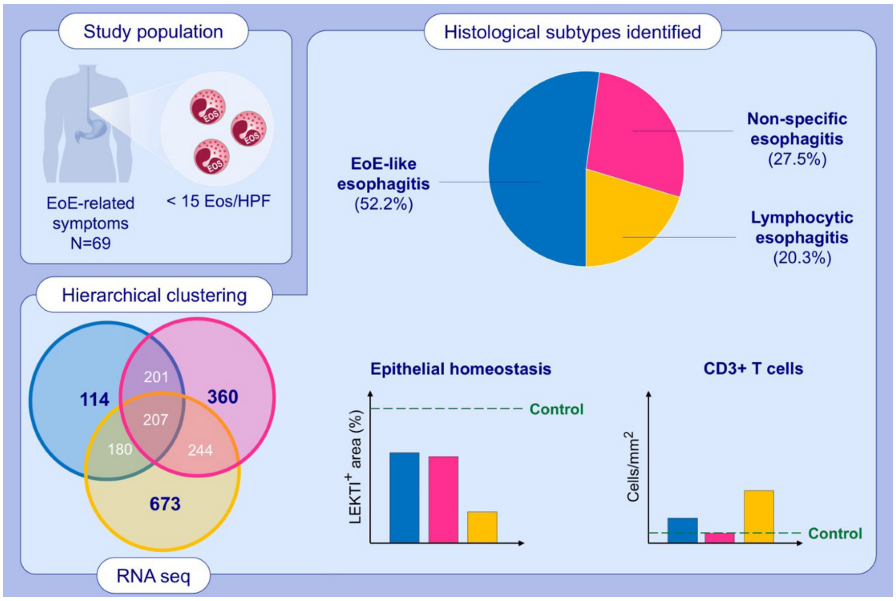
Objective: Physicians are increasingly confronted with patients presenting with symptoms of esophageal dysfunction resembling eosinophilic esophagitis (EoE), but absence of significant esophageal eosinophilia. The purpose of this study was to characterize and classify this group of EoE variants.

Design: Patients from six EoE-centers with symptoms of esophageal dysfunction, but peak eosinophil counts of $<60/mm^2$ (<15/hpf) in esophageal biopsies and absence of gastro-esophageal reflux disease (GERD) were included. Clinical, endoscopic, (immuno)-histological, and molecular features were determined and compared with EoE, GERD, and healthy controls.

Results: We included 69 patients with EoE variants. Endoscopic abnormalities were found in 53.6%. We identified three histological subtypes: *EoE-like esophagitis* (36/69, 52.2%), *lymphocytic esophagitis* (14/69, 20.3%), and *non-specific esophagitis* (19/69, 27.5%). Immunohistochemistry revealed—in contrast to EoE—no significant increase in inflammatory cell infiltrates compared with GERD and healthy controls, except for lymphocytes in lymphocytic esophagitis. EoE-typical Th2-response was absent in all EoE variants. However, considerable structural changes were detected based on histology and protein expression. Using next generation mRNA sequencing, we found the three EoE variants to have distinct molecular fingerprints partially sharing pronounced traits of EoE. Hierarchical sample clustering of RNA sequencing data confirmed the presence of an EoE-like (characterized by eotaxin-3 expression), non-specific, and lymphocytic variant cluster (characterized by CD3 cells and TSLP expression).

Conclusion: All EoE variants are clinically and histologically active conditions despite the absence of esophageal eosinophilia. EoE variants appear to be part of a disease spectrum, where classical EoE represents the most common and apparent phenotype.

KEYWORDS
dysphagia, eosinophilic esophagitis, esophageal eosinophilia, lymphocytic esophagitis, next generation rna sequencing



GRAPHICAL ABSTRACT

This study assesses clinical, (immuno)-histological, and molecular characteristics of 69 patients with EoE-related symptoms, but absence of significant esophageal eosinophilia. We identify three histological variants, EoE-like esophagitis, lymphocytic esophagitis, and non-specific esophagitis. All three variants show decreased LEKTI expression, while lymphocytic esophagitis also shows CD3-positive cell invasion. RNA sequencing reveals distinct molecular fingerprints in each variant and confirms the presence of an EoE-like, lymphocytic, and non-specific variant/cluster.

Abbreviations: CD3, cluster of differentiation 3; EoE, eosinophilic esophagitis; Eos, eosinophil; HPF, high-power field; LEKTI, lympho-epithelial Kazal-type-related inhibitor; RNA seq, RNA sequencing

1 | INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic inflammatory disorder of the esophagus that is defined clinically by symptoms of esophageal dysfunction and histologically by an eosinophil-predominant infiltration of the esophageal squamous epithelium with at least 15 eosinophils in at least one high power field [hpf].[1] Recently, several findings—such as the only modest correlation of eosinophil infiltration with symptom severity or the lack of efficacy of eosinophil-targeting medications—have questioned the role of eosinophils as the main driver of symptoms and inflammation, and therefore as the hallmark of EoE.[2–5] Moreover, a new EoE-like phenomenon has been described recently[6]: Five patients from four families with known EoE were evaluated for typical EoE symptoms, but without esophageal eosinophilia upon histological examination. These patients showed gene expression abnormalities similar to classical EoE, suggesting a uniform underlying pathogenesis[6]; thus, we hypothesized that EoE is only one phenotype of a broader disease spectrum.

Today, EoE centers around the world are increasingly confronted with patients reporting typical symptoms of esophageal dysfunction resembling EoE, but for whom finally the diagnosis cannot be established owing to the absence of any significant esophageal eosinophilia. It has yet to be determined whether these patients suffer from a distinct inflammatory entity, a variant of EoE or an early stage of EoE. Similarly, there are additional chronic inflammatory disorders of the esophagus, such as lymphocytic esophagitis, for which there is no singular etiology and could represent a histomorphological pattern that overlaps with EoE. Given these uncertainties, we performed a cross-sectional multi-center study with the following two aims: First, to determine clinical, endoscopic, and histological features of patients with EoE symptoms, but lacking a significant esophageal eosinophilia; and second, to immune-histologically and molecularly (based on whole genome mRNA profiles) scrutinize and classify these potential EoE variants in patients with lymphocytic and non-specific chronic esophagitis.

2 | METHODS

2.1 | Study design

In this multi-center study including patients from 6 EoE referral centers in Switzerland ($n = 1$) and the United States ($n = 5$), we analyzed clinical, endoscopic, histological, immunohistochemical, and molecular features of patients with EoE variants. All patients provided written informed consent prior to inclusion in the study. The study was approved by the local ethics committee of each of the participating centers (EKNZ 2015-388/CER-VD 148–15, COMIRB 07–0888, UNC IRB 14–1442, IRB-16–6910, STU00094108, ISMMS HS-10–00070).

2.2 | Patients and data collection

Patients were included in our study based on the following criteria: (i) presence of typical EoE symptoms (esophageal dysfunction: dysphagia up to bolus impactions in adults; failure to thrive, food

refusal, vomiting, abdominal, or chest pain in children); (ii) an available endoscopy report including images; (iii) availability of at least 6 esophageal biopsies following a structured biopsy protocol (3 from the distal and 3 from the proximal esophagus); and (iv) a peak eosinophil count of <15 eosinophils (eos) per hpf corresponding to <60 per mm^2 in esophageal epithelium regardless of presence or absence of other EoE-specific histologic features. Patients were excluded if they had other diseases associated with esophageal eosinophilia, particularly gastroesophageal reflux disease (GERD) and eosinophilic gastroenteritis, if they had congenital disorders of the esophagus (such as esophageal atresia) or underwent esophageal surgery in the past, if they had other non-IgE-mediated diseases such as lichen planus, Crohn's disease, celiac disease, or drug hypersensitivity, if they suffered from severe psychiatric comorbidities, or if they were on medical or dietary anti-eosinophil treatment. Patients on topical corticosteroids for other reasons (inhaled corticosteroids and nasal corticosteroids) were also excluded. GERD was excluded as previously described.[7] All esophageal biopsies were re-examined and re-classified (including re-calculation of peak eosinophil counts) by two EoE reference pathologists (MHC, CB). Patients whose peak eosinophil count was ≥15 eos per hpf on re-examination were excluded from further analyses. Endoscopic disease activity was graded using the EoE Endoscopic Reference Score (EREFS) grading and classification system based on the available endoscopic pictures.[8] Patients with classical EoE, erosive GERD, and esophageal healthy subjects served as controls. For details, see Supplementary Methods.

2.3 | Histological re-examination

All eight individual components of the validated EoE histological scoring system (EoE-HSS), in particular peak eosinophil count per mm^2, as well as lymphocytic infiltration and presence of acute inflammatory cells were assessed by two EoE reference pathologists.[9] Based on histology, patients were classified as the following three *a priori* defined EoE variants:

- EoE-like esophagitis: presence of 0–59 eos/mm^2 (<15 eos/hpf), but otherwise typical histological EoE features[6]
- Lymphocytic esophagitis: lymphocyte-predominant inflammation with high numbers of intraepithelial lymphocytes (≥30 per hpf), gathered mainly in peripapillary fields, peripapillary spongiosis (dilated intercellular spaces), and absence of intraepithelial granulocytes[10]
- Non-specific esophagitis: histological infiltration of lymphocytes or neutrophils not fulfilling the numerical and distributional criteria of lymphocytic esophagitis

2.4 | Immunohistochemical characterization

Formalin-fixed, paraffin-embedded esophageal biopsies were shipped at room temperature according to a material transfer agreement from each participating center to the Swiss EoE Clinic. The samples were sectioned, and slides were subsequently processed for immunohistochemical and immunofluorescent analyses as previously described.[6] For details on determined proteins and analyses, see Supplementary Methods.

2.5 | RNA Isolation and RNA sequencing studies

Esophageal biopsies from a subset of patients with classical EoE (10 samples), EoE-like esophagitis (13 samples), non-specific esophagitis (10 samples), lymphocytic esophagitis (5 samples), and esophagus-healthy individuals (7 samples) were processed for next generation RNA sequencing (RNA-seq), and RNA-seq libraries were prepared and analyzed as previously described.[11–15] For quality control, see Supplementary File S1. Samples were selected based on tissue availability and RNA quality. Differential gene expression analysis of experimental groups was performed using the Bioconductor package DESeq2.[16] For details, see Supplementary Methods. DESeq2 output tables can be found in Tables S2–S6 (excel files). Tertiary analyses were run with the R version 3.6.0 (for R-script see Supplementary File S2). To validate RNA-seq data, quantitative PCR (qPCR) was performed (Supplementary Methods and Table S1).

2.6 | Statistical analyses

For statistical analyses, GraphPad Prism software version 8.3.0 and R version 3.6.0 were used. Categorical data were compared using chi-squared test with post-test Bonferroni correction; one-way ANOVA with Bonferroni's multiple comparison test was used to analyze multiple groups (quantitative data) for statistical significance. For the purpose of this study, a Bonferroni-corrected *p*-value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Patient demographics

We identified 113 patients with typical EoE symptoms and peak eosinophil counts in esophageal biopsies of less than 60 eos/mm^2 (<15 eos/hpf) from 6 EoE referral centers. After histological re-examination, absence of significant esophageal eosinophilia was proven in 69 subjects (62 adults, 7 children aged 16y or younger). For patients included per study center, see Table S7. A study flow diagram is shown in Figure 1A. These patients had a mean age at diagnosis of 48.8 years (SD 24.0) with a median duration of symptoms of 28.1 months (IQR 12.4–74.4), 37 were females (53.2%) and 61 subjects were of Caucasian descent (88.4%). Atopic comorbidities were reported in 30 patients (43.5%), while family history for EoE was positive in 16 individuals (23.2%). For details, see Table 1.

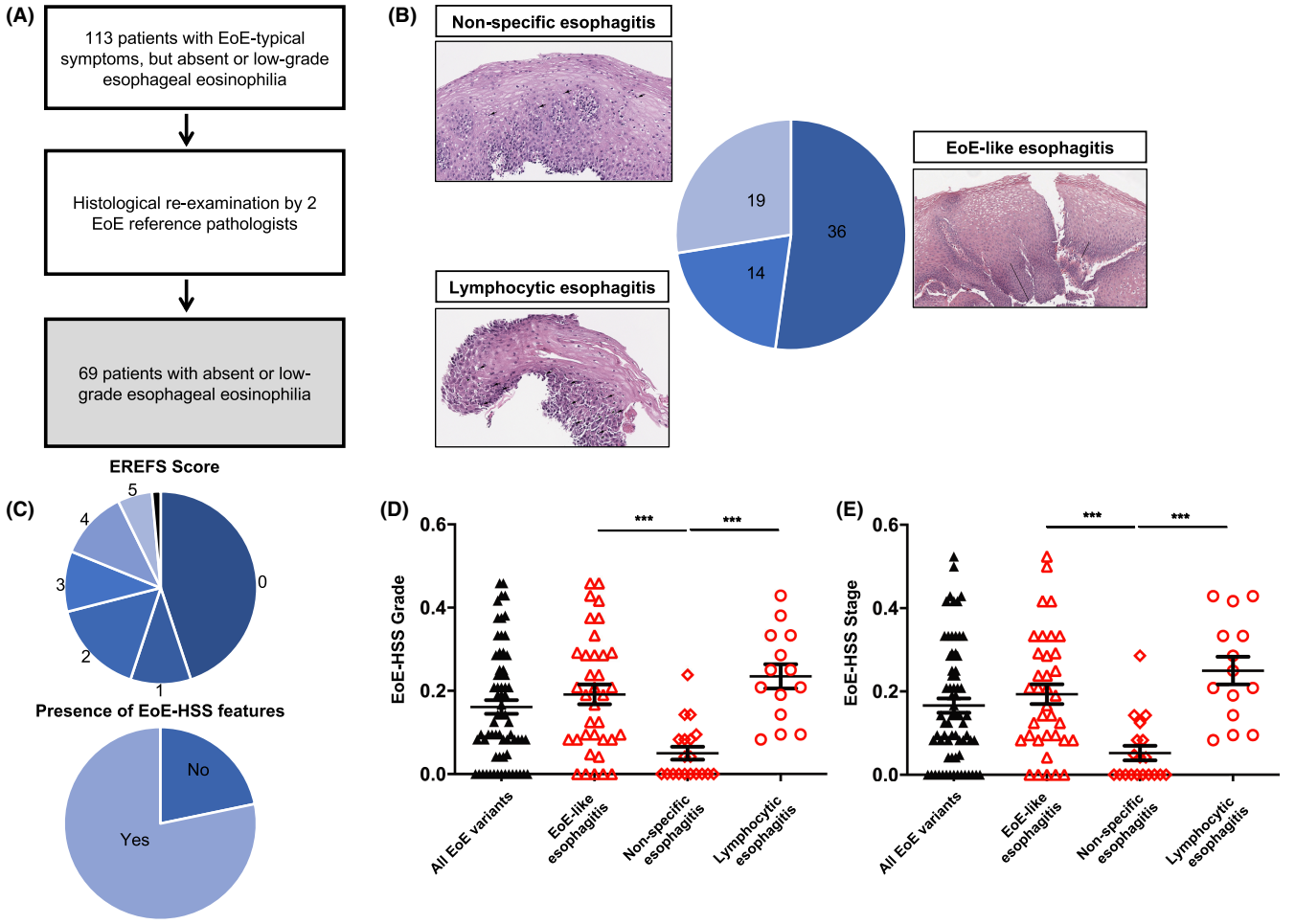


FIGURE 1 (A) Flow chart of study patients. (B) Proportion of patients classified into the EoE variants EoE-like esophagitis, lymphocytic esophagitis, and non-specific esophagitis with representative hematoxylin and eosin pictures. (C) Endoscopic (upper panel) and histological (lower panel) disease activity based on EREFS grading system (EREFS score) and EoE-HSS grading and staging system. (D) EoE-HSS grading for EoE variants (all patients) and each EoE variant separately. (E) EoE-HSS staging for EoE variants (all patients) and each variant separately. Bars indicate mean+/-SEM

3.2 | Histological classification

Based on hematoxylin and eosin staining, patients were classified into three EoE variants: 36 patients were classified as having EoE-like esophagitis (52.2%), 14 as having lymphocytic esophagitis (20.3%), and 19 patients as having non-specific esophagitis (27.5%). For details including representative pictures for each EoE variant, see Figure 1B. In 41 patients (59.4%), there was a complete absence of eosinophil infiltration in the esophageal mucosa (0 eos/mm^2). Patients with lymphocytic esophagitis were significantly older at disease onset compared with the two other subtypes (61.9y vs. 40.2 (EoE-like esophagitis) and 61.9y vs. 41.4 (non-specific esophagitis), $p < 0.05$). A considerable higher proportion of females had non-specific esophagitis and lymphocytic esophagitis. However, these differences did not reach statistical significance (Table 1). No difference with regard to patient demographics was seen when looking at EoE-like esophagitis without any eosinophils compared with EoE-like esophagitis with minor eosinophil infiltration. None of the patients with EoE-like esophagitis had a peak eosinophil count above 42 eos/mm^2, corresponding to <11 eos/hpf.

3.3 | Clinical, endoscopic, and histological disease activity at baseline

As per the inclusion criteria, all 69 patients had clinically active disease: 67 patients (97.1%) reported dysphagia and 49 patients (71.0%) experienced food impactions. The two patients without dysphagia were children reporting vomitus, abdominal pain, and failure to thrive. While chest pain occurred in 22 patients (31.9%), this clinical feature was significantly more common in EoE-like esophagitis (compared with lymphocytic esophagitis, 50.0% vs. 0%, $p < 0.01$). We identified 38 patients that were treated with swallowed topical steroids in the follow-up, which resulted in symptomatic improvement in 92%. Improvement rates were significantly lower in lymphocytic esophagitis (50%) compared with EoE-like esophagitis (100%, $p < 0.01$). Endoscopic activity was seen in 53.6% with mostly minor abnormalities (median EREFS score of 1 (IQR 0–3), Figure 1C). The leading endoscopic findings were rings (36.2%), strictures (36.2%), and edema (31.9%). No differences in terms of endoscopic inflammatory disease activity were observed when comparing the three

TABLE 1 Demographics, baseline disease characteristics

	EoE variants $n = 69$	EoE-like esophagitis $n = 36$	Non-specific esophagitis $n = 19$	Lymphocytic esophagitis $n = 14$
Demographics				
Sex (number of females, %)	37 (53.6%)	14 (38.9%)	13 (68.4%)	10 (71.4%)
Age at onset, years	43.8, 21.0	**40.2, 21.1**[a]	**41.4, 20.6**[b]	**61.9, 11.6**[a,b]
	45.6 (IQR 27.4-62.9)	**40.9, (IQR 20.7-62.3)**	**46.1, (IQR 25.2-55.5)**	**65.7, (IQR 52.1-70.7)**
Age at diagnosis, years	48.8, 20.4	46.1, 20.0	45.9, 20.9	62.6, 16
	52.5 (IQR 33.7-66.4)	46.7 (IQR 33.4-63.4)	48.0 (IQR 31.3-64.8)	66.4 (IQR 57.3-73.3)
Atopic comorbidities	30 (43.5%)	17 (47.2%)	7 (36.8%)	6 (42.9%)
Ethnics				
Caucasians	61 (88.4%)	31 (86.1%)	17 (89.5%)	13 (92.9%)
African Americans	5 (7.2%)	4 (11.1%)	0	1 (7.1%)
NA	3 (4.3%)	1 (2.8%)	2 (10.5%)	0 (0%)
Family history for EoE	16 (23.2%)	10 (27.8%)	6 (31.6%)	0 (0%)
Previous PPI	41 (59.4%)	20 (55.6%)	12 (63.2%)	9 (64.3%)
Steroids	0 (%)	0 (0%)	0 (0%)	0 (0%)
Diagnostic delay,	59.7, 72.9	56.6, 76.3	67.5, 73.7	54.9, 65.4
Months	28.1 (IQR 12.4-74.4)	24.7 (IQR 12.2-70.9)	39.3 (IQR 10.3-126.7)	24.7 (IQR 12.3-68.8)
Clinical activity				
Symptoms				
Dysphagia	67 (97.1%)	36 (100%)	18 (94.7%)	13 (92.9%)
Food impactions	49 (71.0%)	29 (80.6%)	12 (63.2%)	8 (57.1%)
Chest pain	22 (31.9%)	**18 (50.0%)**[c]	4 (21.1%)	**0 (0%)**[c]
Endoscopic bolus removal	3 (4.3%)	3 (8.3%)	0 (0%)	0 (0%)
Endoscopy				
EREFS Score	1.5, 1.7	1.6, 1.6	1.1, 1.6	2.0, 1.8
	1.0 (IQR 0-3)	1.0 (IQR 0.0-3.0	0.0 (IQR 0.0-2.0)	2.0 (IQR 0.0-3.0)
Stricture	25 (36.2%)	13 (36.1%)	4 (21.1%)[d]	8 (57.1%)[d]
Histology				
Peak eosinophil count, eos/mm^2	7.1, 12.2	**13.4, 14.2**[e,f]	**0.4, 1.6**[e]	**0, 0**[f]
	0 (IQR 0-10)	**9.0 (IQR 2.3-17.0)**	**0 (IQR 0-0)**	**0 (IQR 0-0)**
EoE-HSS Grade	16.1, 13.7	**19.1, 14.1**[g]	**5.0, 6.8**[g,h]	**23.5, 10.9**[h]
	12.5 (IQR 4.2-25.0)	**19.0 (IQR 8.3-28.7)**	**0 (IQR 0-8.3)**	**22.9 (IQR 15.5-32.1)**
EoE-HSS Stage	16.6, 14.3	**19.3, 14.3**[g]	**5.2, 7.7**[g,h]	**25.0, 12.4**[h]
	14.3 (IQR 4.2-28.6)	**17.9 (IQR 8.3-29.2)**	**0 (IQR 0-8.3)**	**22.9 (IQR 15.5-33.3)**
Subepithelial eosinophil count (available for 40), eos/mm^2	8.0, 13.0	9.3, 10.5	8.6, 18.9	2.0, 4.9
	0 (IQR 0-15)	4.0 (IQR 0-18.3)	0 (IQR 0-12.3)	0 (IQR 0-3.0)
Detetectable subepithelial eosinophilia (available for 40)	19/40 (47.5%)	14/22 (63.6%)	4/12 (33.3%)	1/6 (16.7%)
Subepithelial fibrosis (available for 42) - present	14 (33.3%)	**10 (45.5%)**[i]	**1 (7.1%)**[i]	3 (50.0%)

Note: Demographics and baseline disease characteristics in all EoE variants combined and stratified by each variant. Continuous data are shown as mean, standard deviation and median, interquartile range IQR. [a]$p < 0.05$; [b]$p < 0.05$; [c]$p < 0.01$; [d]$p < 0.05$; [e]$p < 0.001$; [f]$p < 0.001$; [g]$p < 0.001$; [h]$p < 0.001$; [i]$p < 0.05$.

EoE variants (Figure S1A). In contrast to inflammatory endoscopic abnormalities in only half of our patients with mainly minor findings, histological changes were considerable. Typical histological EoE features captured by the EoE-HSS were seen in 54 patients (78.3%). The leading findings were basal zone hyperplasia (59.4%) and dilated intercellular spaces (68.1%), see Figure 1C. EoE-HSS grading and staging scores were comparable in EoE-like esophagitis and lymphocytic esophagitis, but lower in non-specific esophagitis (Table 1,

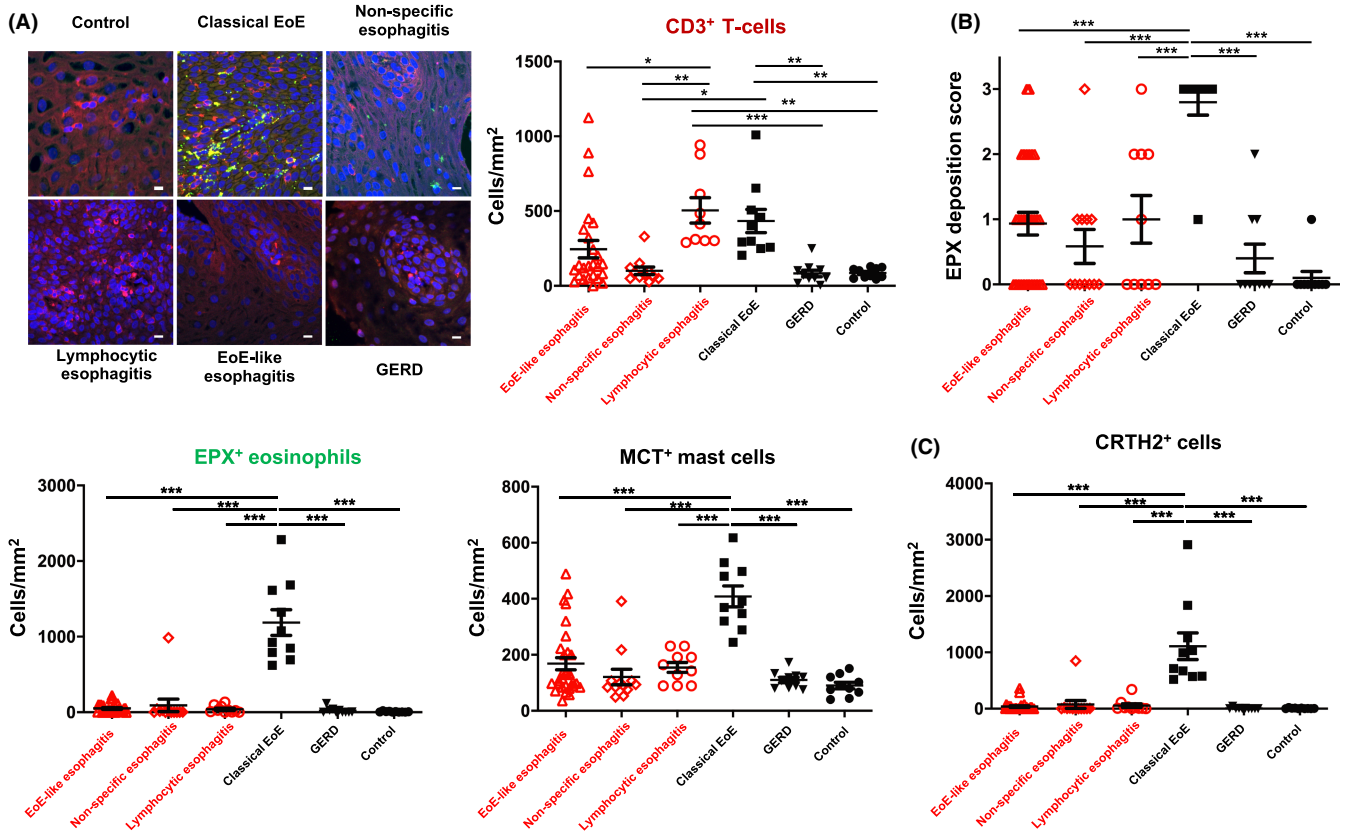


FIGURE 2 (A) Immunostaining for CD3 (red), EPX (green), and nuclear counterstaining (DAPI, blue) in healthy controls, classical EoE, EoE variants, and GERD (upper left panel). The three other panels show quantitative analyses. B) EPX deposition scores (ranging from 0 to 3) for classical EoE, EoE variants, GERD, and controls. (C) Number of CRTH2+ T-cells per mm^2 in classical EoE, EoE variants, GERD, and controls. Bars indicate mean+/-SEM

Figure 1D and E). Subepithelial eosinophilia was evaluated in 40 patients based on representative amount of lamina propria tissue available for analysis. In 19/40 individuals (47.5%), a mild subepithelial eosinophil infiltration was detected (mean eosinophil count 8.0 eos/mm^2, SD 13.0, IQR 0.0–15.0). Notably, only 1 patient showed a significant level of subepithelial eosinophilia (≥15 eos/hpf, ≥60 eos/mm^2). Although EoE-like esophagitis showed the highest percentage of positive subepithelial eosinophil levels, mild subepithelial eosinophil infiltration was found in every EoE variant. Subepithelial fibrosis was assessed in 42 patients. 14 of the 42 had at least mild fibrosis on histology (33.3%). Patients with EoE-like esophagitis more often had fibrosis compared with patients with non-specific esophagitis (45.5% vs. 7.1, $p < 0.05$). Based on these findings, we conclude that all three EoE variants are clinically *and* histologically active, while inflammatory endoscopic abnormalities are only subtle. Moreover, EoE variants are not simply a subepithelial form of EoE.

3.4 | Immunohistological characterization of inflammation

FFPE tissue was available from 52 patients with EoE variants. Immunohistochemistry confirmed—per inclusion criteria—a significantly lower number of EPX + cells (eosinophils) in patients with EoE variants compared with classical EoE (Figure 2A). Still, in some patients with EoE variants, degranulated and cytolytic eosinophils were detected (measured by the EPX degranulation score 0–3, Figure 2B); however, EPX degranulation scores were significantly lower than in classical EoE and not higher than in GERD and healthy controls. In addition, infiltration with mast cells (tryptase+) was significantly lower in EoE variants compared with EoE (Figure 2A). While some patients with EoE-like esophagitis and all patients with lymphocytic esophagitis exhibited high numbers of esophageal T-cells comparable with classical EoE (Figure 2A), no infiltration of CRTH2+ T-cells was seen (Figure 2C). Measurement of cytokine and chemokine expression revealed no increases in TNF-and eotaxin-3 levels. However, TSLP expression was significantly increased in lymphocytic esophagitis comparable with that of classical EoE (Figure 3A). No such increase was seen in the two other EoE variants. Expression of LEKTI, a protease inhibitor responsible for epithelial homeostasis, was decreased in lymphocytic esophagitis compared with controls as seen for classical EoE (Figure 3B), indicating an epithelial barrier dysfunction. Evidence for decreased LEKTI expression was also obtained in EoE-like and non-specific esophagitis. Based on these findings, we conclude that EoE variants exhibit structural and in some patients inflammatory changes, but lack an EoE-typical Th2-mediated inflammatory response.

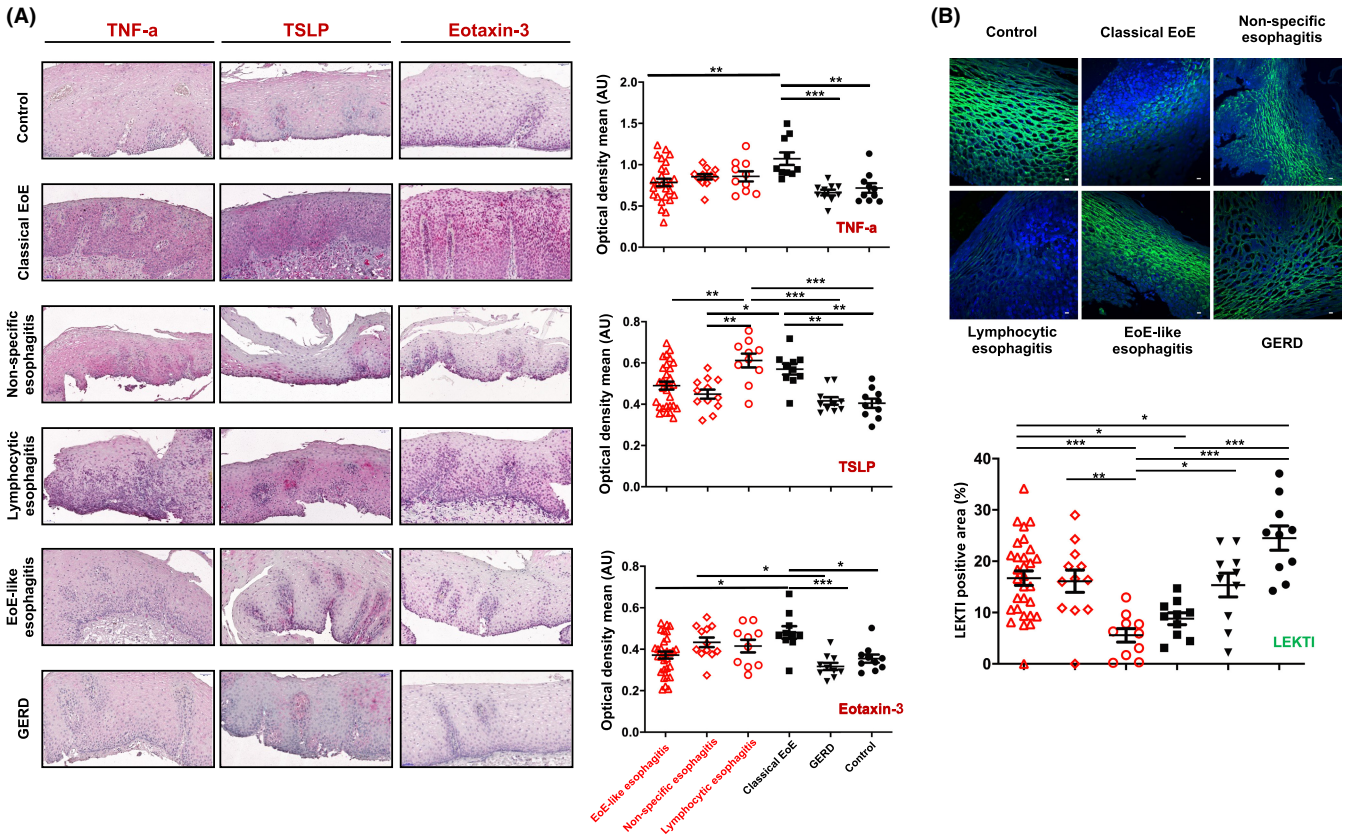


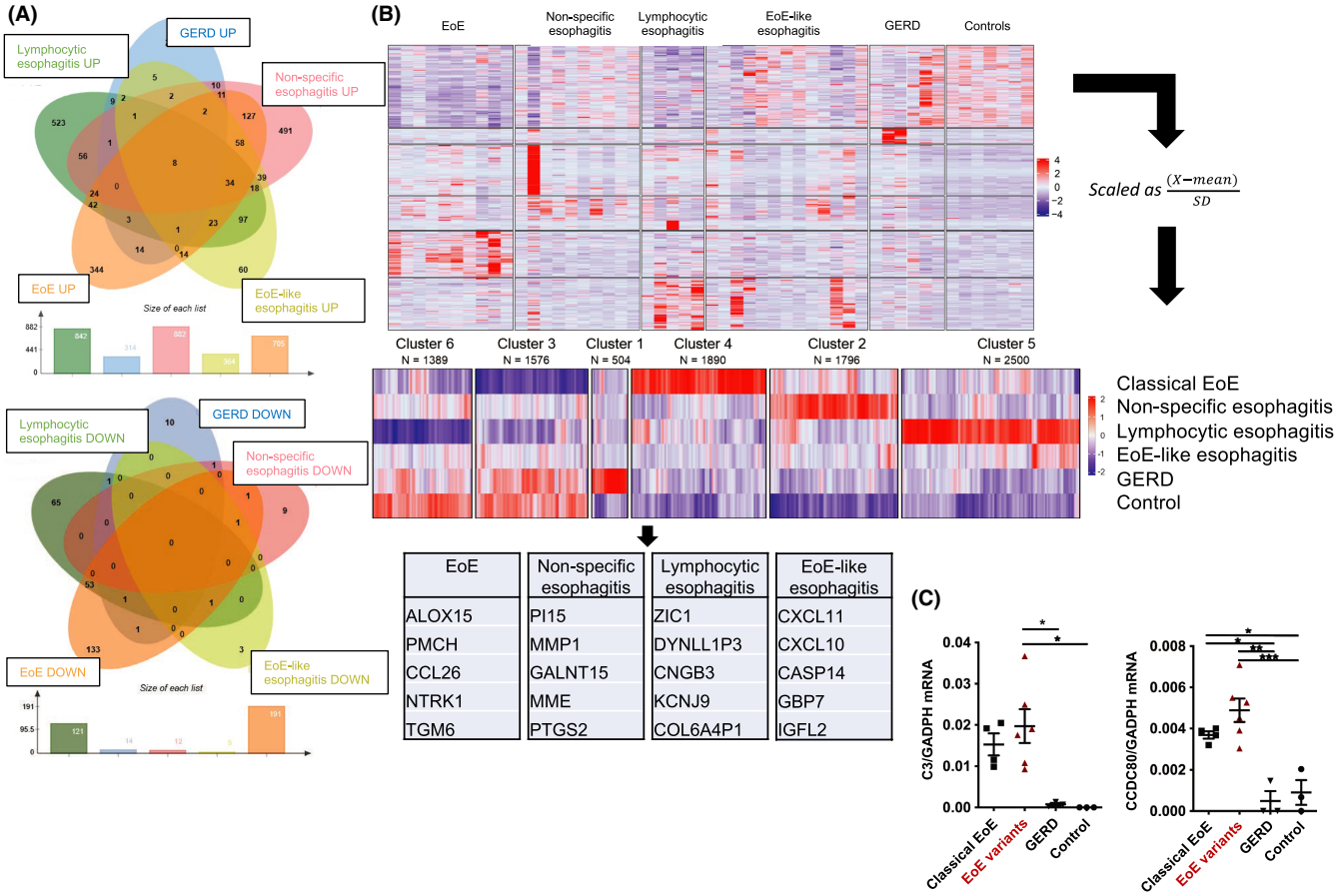
FIGURE 3 (A) Immunostaining for TNF-a, TSLP, and eotaxin-3 in healthy controls, classical EoE, EoE variants, and GERD (left panel). Right panels show quantitative analyses. B) Immunostaining for LEKTI (green) in healthy controls, classical EoE, EoE variants, and GERD. Lower panel shows quantitative analyses. Bars indicate mean+/-SEM

3.5 | Next generation RNA sequencing analyses

Esophageal RNA was available from 48 patients with EoE variants. For next generation RNA sequencing, we selected 28 patients with EoE variants (13 EoE-like esophagitis, 5 lymphocytic esophagitis, and 10 non-specific esophagitis), 10 subjects with classical EoE, 6 erosive GERD patients, and 7 esophagus-healthy controls. Of the 28 included subjects with EoE variants, 20 samples were from the Swiss EoE Clinics, while the remaining 8 samples were from the University of North Carolina. For principal component analyses, see Figure S2A. Heatmap for up- and down-regulated genes in each disease condition compared with healthy controls is shown in Figure S2B. Ingenuity pathway analyses revealed different top pathways in each EoE variant (Figure S3A). The two top hits in EoE-like esophagitis (agranulocyte adhesion/diapedesis and granulocyte adhesion/diapedesis) indicate the involvement of inflammatory cells and pro-inflammatory as well as pro-migratory cytokines. The top pathways in lymphocytic esophagitis indicate the involvement of T-cell activation and cell migration. Finally, non-specific esophagitis stands out with fibrogenesis as top pathway hit. Despite these differences among EoE variants, the top pathways in EoE-like esophagitis, lymphocytic esophagitis, and non-specific esophagitis are all among the most upregulated pathways of classical EoE (Figure S3B). For pathway-specific heatmaps (chemokines, interleukins, TNF superfamily, collagens, Th2 response, and kallikreins), see Figures S4, S5 and S6.

We next performed a comparison analysis between all RNA-seq datasets and sought to identify overlapping upstream regulators. We detected a considerable overlap between all EoE variants and classical EoE, but not erosive GERD with regard to upstream regulation through pro-inflammatory mediators such as IL-1, TNF-a, NF-kB, and through pro-fibrotic cytokines such as TGF-β1 (Figure S3C).

In a next step, we were interested in possible genes discriminating different EoE variants from each other. For this, we looked at all significantly up- and down-regulated genes (FDR<0.05, fold change ≥2) in EoE variants, classical EoE, and GERD compared with healthy controls (see Figure S7A). The lists of significantly changed genes in each condition were compared with each other, and the following gene sets were found to be disease-specific (upregulated): EoE-like esophagitis, 60 genes; lymphocytic esophagitis, 523 genes; non-specific esophagitis, 491 genes; classical EoE, 344 genes; and GERD, 245 genes (Figure 4A, upper panel). A total of 18 genes were specifically upregulated in all EoE variants, but neither classical EoE nor GERD. 34 upregulated genes were specific to EoE variants and classical EoE compared with GERD. The top discriminating genes can be found in Figure S7B and7C (specific genes for each condition) (specific genes for EoE and EoE variants *versus* others, and for EoE



EoE	Non-specific esophagitis	Lymphocytic esophagitis	EoE-like esophagitis
ALOX15	PI15	ZIC1	CXCL11
PMCH	MMP1	DYNLL1P3	CXCL10
CCL26	GALNT15	CNGB3	CASP14
NTRK1	MME	KCNJ9	GBP7
TGM6	PTGS2	COL6A4P1	IGFL2

FIGURE 4 (A) Venn diagram for significantly up- and down-regulated genes (fold change ≥2) compared with healthy controls in EoE variants, classical EoE, and GERD (upper panel with up-regulated genes, lower panel with down-regulated genes). (B) Cluster analysis of genes (in all patients, and with averaged values (z-scores)) that were significantly up- or down-regulated compared with healthy controls in at least one condition (classical EoE, EoE variants, and GERD). Lower panels show the most upregulated genes in cluster 4 (EoE), cluster 2 (non-specific esophagitis), and cluster 5 (lymphocytic esophagitis and EoE-like esophagitis). (C) qPCR for C3 and CCDC80 as possible biomarkers for EoE variants. C3 and CCDC80 have been identified as possible candidate genes in Supplementary Figure S7

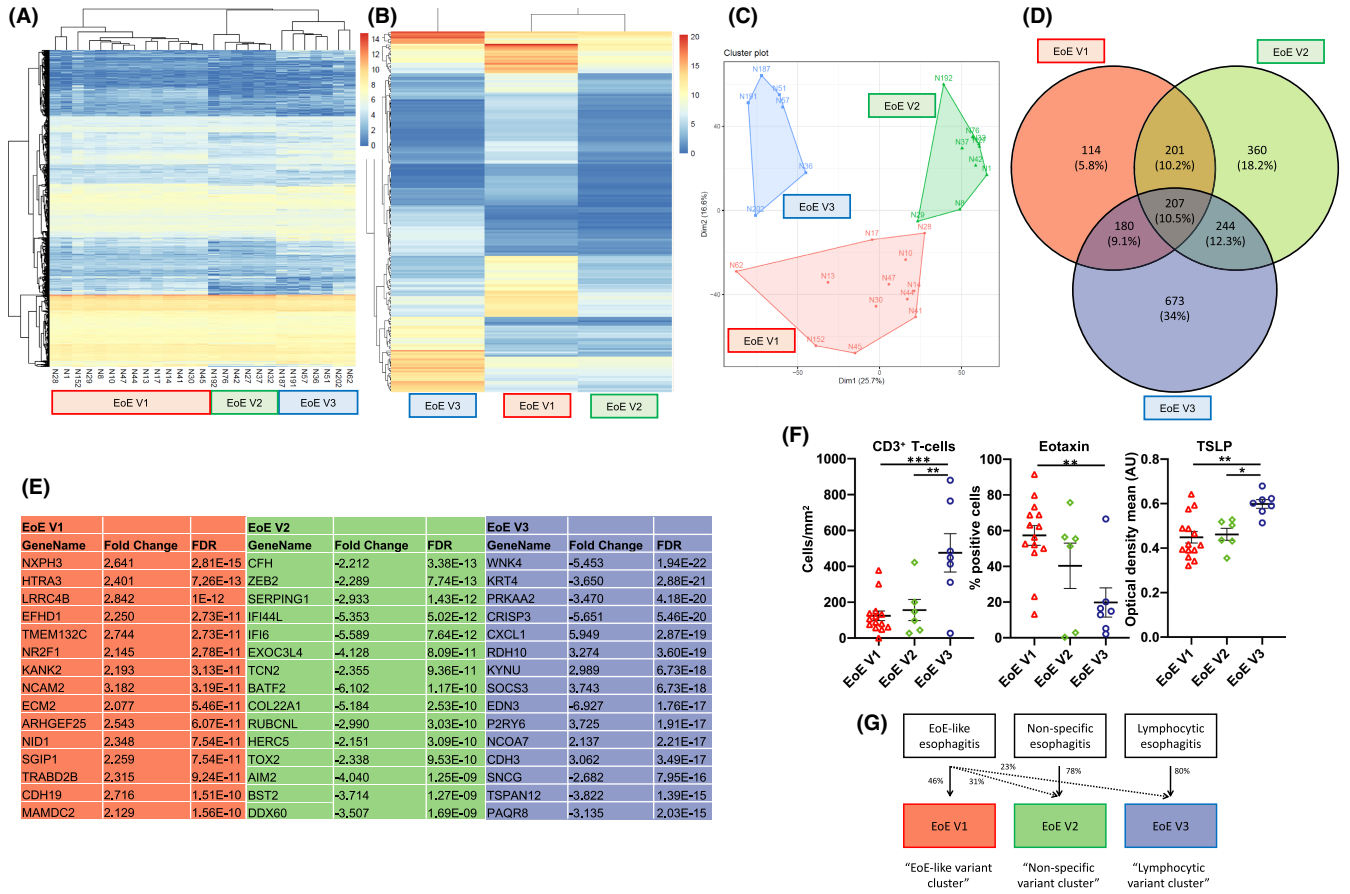
variants *versus* others). The three top genes specific for EoE variants *and* EoE were the complement protein C3, the zinc finger E-box-binding homeobox 2 (ZEB2) associated with TGF-β signaling, and the coiled-coil domain-containing protein 80 (CCDC80) involved in cell adhesion and matrix assembly. The following numbers of genes were uniquely downregulated in EoE variants: EoE-like esophagitis 3 genes, lymphocytic esophagitis 65 genes, non-specific esophagitis 9 genes (Figure 4A, lower panel).

In an alternative approach to identify possible biomarkers, we selected genes that were significantly up-/down-regulated compared with healthy controls in at least one condition ($p < 0.05$, Table S8). Condition specific expression patterns were then examined using cluster analysis (Figure 4B). We averaged the values in each group and plotted the scaled expression dataset based on each group's z-score. This scaled expression dataset revealed the existence of six differential clusters (Figure 4B) with specific genes for EoE (such as ALOX15 and CCL26), EoE-like esophagitis (CXCL11, CXCL10, and CASP14), non-specific esophagitis (PI15, MMP1, and GALNT15), and lymphocytic esophagitis (ZIC1, DYNLL1P3, and CNGB3). Variation of these genes are shown in Figure S8.

To assess relative differences in gene expression, we performed qPCR analysis for the most discriminating genes as mentioned above in high-quality mRNA samples. Indeed, CCDC80 and the complement protein C3 were able to identify EoE variant patients compared with GERD and healthy controls (Figure 4C). While no significant increase of C3 was seen in classical EoE, CCDC80 was able to distinguish EoE and EoE variants *versus* healthy controls and GERD patients.

3.6 | Hierarchical sample clustering

To assess the validity of hematoxylin and eosin-based classification of EoE variants, we performed hierarchical sample clustering (clustering based on correlation) of EoE variant patients (based on significantly up-/down-regulated genes in at least one condition, Table S8, Supplementary File S2). After exclusion of a considerable outlier—for PCA and cluster plot see Figure S9A,B—we identified 3 clusters (EoE variant clusters V1-3, Figure 5A and B). Cluster plots are shown in Figure 5C, respective gene lists can be found in Table S9–S11.



(E)

EoE V1			EoE V2			EoE V3		
GeneName	Fold Change	FDR	GeneName	Fold Change	FDR	GeneName	Fold Change	FDR
NXPH3	2.641	2.81E-15	CFH	-2.212	3.38E-13	WNK4	-5.453	1.94E-22
HTRA3	2.401	7.26E-13	ZEB2	-2.289	7.74E-13	KRT4	-3.650	2.88E-21
LRRC4B	2.842	1E-12	SERPING1	-2.933	1.43E-12	PRKAA2	-3.470	4.18E-20
EFHD1	2.250	2.73E-11	IFI44L	-5.353	5.02E-12	CRISP3	-5.651	5.46E-20
TMEM132C	2.744	2.73E-11	IFI6	-5.589	7.64E-12	CXCL1	5.949	2.87E-19
NR2F1	2.145	2.78E-11	EXOC3L4	-4.128	8.09E-11	RDH10	3.274	3.60E-19
KANK2	2.193	3.13E-11	TCN2	-2.355	9.36E-11	KYNU	2.989	6.73E-18
NCAM2	3.182	3.19E-11	BATF2	-6.102	1.17E-10	SOCS3	3.743	6.73E-18
ECM2	2.077	5.46E-11	COL22A1	-5.184	2.53E-10	EDN3	-6.927	1.76E-17
ARHGEF25	2.543	6.07E-11	RUBCNL	-2.990	3.03E-10	P2RY6	3.725	1.91E-17
NID1	2.348	7.54E-11	HERC5	-2.151	3.09E-10	NCOA7	2.137	2.21E-17
SGIP1	2.259	7.54E-11	TOX2	-2.338	9.53E-10	CDH3	3.062	3.49E-17
TRABD2B	2.315	9.24E-11	AIM2	-4.040	1.25E-09	SNCG	-2.682	7.95E-16
CDH19	2.716	1.51E-10	BST2	-3.714	1.27E-09	TSPAN12	-3.822	1.39E-15
MAMDC2	2.129	1.56E-10	DDX60	-3.507	1.69E-09	PAQR8	-3.135	2.03E-15

FIGURE 5 (A) Hierarchical sample clustering of EoE variants (heatmap); (B) Hierarchical sample clustering of EoE variants (averaged values, heatmap); (C) Cluster plot for EoE variant clusters detected by hierarchical sample clustering; (D) Venn diagram showing unique and overlapping genes in EoE variant clusters (significantly changed genes compared with healthy controls); (E) list of the most unique genes in each EoE variant cluster, ranked by false discovery rate (FDR) compared with healthy controls. (F) Immunostaining for CD3, eotaxin-3, and TSLP in EoE variant clusters (V1-3). (G) Re-classification of histologically defined EoE variants into variant clusters (V1-3). Bars indicate mean+/-SEM

A heatmap for genes involved in the cytokine-mediated signaling pathway (190/409 genes significantly changed in at least one condition compared to healthy controls) can be found in Figure S9C (with respective genes shown in Table S12). The three variant clusters showed specific gene sets with 114 genes uniquely changed in EoE variant cluster V1, 360 in EoE variant cluster V2 and 673 in EoE variant cluster V3 (compared with healthy controls, Figure 5D). Most unique genes are listed in Figure 5E (ordered by FDR compared with healthy controls). In a next step, we compared expression of these genes in one variant cluster with the other two variant clusters and identified the following number of genes significantly changed between variant clusters: 479 genes for EoE variant cluster V1 vs V2, 461 genes for EoE variant cluster V1 vs V3, and 409 genes for EoE variant cluster V2 vs V3 (Figure S10A). The following numbers of genes were significantly upregulated in only one variant cluster compared with the two others: EoE variant cluster V1 500 genes, EoE variant cluster V2 55 genes, and EoE variant cluster V3 267 genes (Figure S10B). The most discriminating upregulated genes in each EoE variant cluster V1-V3 can be found in Figure S10C.

When re-analyzing immunostaining with regard to the new EoE variant cluster V1-3 classification, we found EoE variant cluster V1 to have significantly increased eotaxin-3 expression, and EoE variant cluster V3 to have increased numbers of CD3 cells and increased TSLP expression, while EoE variant cluster V2 laid in between the two other clusters (Figure 5F). While non-specific esophagitis corresponded to EoE variant cluster V2 (78%) and lymphocytic esophagitis to EoE variant cluster V3 (80%), EoE-like esophagitis revealed to be heterogeneous with 46% of patients being classified into EoE variant cluster V1, 31% into EoE variant cluster V2 and 23% into EoE variant cluster V3 (Figure 5G). Thus, sample clustering analyses enabled re-classification of the heterogeneous EoE-like esophagitis group with otherwise consistent classification of hematoxylin and eosin-based groups (non-specific esophagitis and lymphocytic esophagitis). EoE variant cluster V1 showed an EoE-like phenotype (eotaxin-3), while EoE variant cluster V3 was consistent with a lymphocytic phenotype (CD3, TSLP). EoE variant cluster V2 laid in between (non-specific).

4 | DISCUSSION

Esophageal eosinophilia is the hallmark of and key diagnostic criterion for EoE. Observation of several patients with an EoE-like disease—defined by symptoms similar to EoE, but eosinophil-free biopsies—in EoE families has brought into question the role of eosinophils in EoE and whether similar conditions can overlap without eosinophils or with very few eosinophils.[6] However, detailed description of such conditions or potential EoE variants is lacking.

Here, we present clinical, endoscopic, (immuno)-histological, and molecular features of 69 patients from 6 large EoE referral centers exhibiting typical EoE symptoms, but less than 15 eosinophils/hpf in the esophageal epithelium. Our main findings are as follows: First, despite the absence of significant esophageal eosinophilia, a remarkable degree of histological disease activity can be observed allowing distinction of three EoE variants; second, decreased expression of the protease inhibitor LEKTI implies an epithelial barrier defect in the pathogenesis of these variants; third, while signs of (cellular) inflammation can be found in some patients, the EoE-typical Th2-weighted response is absent; and fourth, RNA-seq analyses revealed these esophagitis to have distinct molecular fingerprints partially sharing pronounced traits of EoE. Of note, the low subepithelial eosinophil counts in our 69 patients clearly highlight that EoE variants are not simply a subepithelial form of EoE.

The group of EoE variants can be classified into three subtypes based on conventional histology (hematoxylin and eosin staining). Degree and pattern of lymphocytic infiltration in combination with structural (=non-cellular) signs of chronic inflammation such as papillary elongation, spongiosis and basal zone hyperplasia, distinguish *among non-specific*, *EoE-like*, *and lymphocytic esophagitis*. Of note, histological examination by an experienced pathologist is needed to capture these minor histological changes. Additional CD3 staining is helpful to accurately assess lymphocytic infiltration. Hierarchical sample clustering of RNA sequencing data confirms the presence of three EoE variant clusters (V1-3) with considerable overlap of EoE variant cluster V2 with non-specific esophagitis and variant cluster V3 with lymphocytic esophagitis (CD3, TSLP). RNA sequencing further enabled re-classification of the heterogeneous EoE-like esophagitis group revealing a variant cluster 1 with a clear EoE-like phenotype (eotaxin-3). Thus, molecular analyses appear to help in the classification of EoE variants, but further studies are needed in order to check the applicability of mRNA markers for their diagnosis and potentially outcome prediction.

Our findings demonstrate that chronic esophagitis types that share features of EoE are clinically *and* histologically an active esophageal disease group. Clinical activity can be severe as more than 70% of our patients experienced food bolus impactions. As with EoE, patients with EoE variants are relatively young, show a considerable diagnostic delay until diagnosis is established and often report a positive family history for EoE. In contrast to EoE, no male preponderance was observed, except for the EoE-like esophagitis variant. This finding underscores potential differences between EoE variants with EoE-like esophagitis indeed exhibiting a more EoE-like phenotype than the two other variants. Future genetic studies of EoE variant patients might give insights into possible sex-specific differences. Despite an impressive clinical activity, endoscopic alterations are seen in only half of the patients and are mainly subtle; this represents a diagnostic challenge. Still, the presence of histological changes beyond esophageal eosinophilia is common, but requires expert evaluation.

In recent years, it has become apparent that pathogenesis of EoE goes beyond simple eosinophil infiltration of the esophageal mucosa. EoE is not a "single cell disease". Eosinophil-targeting treatments failed to induce clinical response despite impressively lowering eosinophil counts (although it cannot be ruled out that this was because not sufficiently lowering the number of esophageal eosinophils beyond 50%, ie not decreasing esophageal eosinophilia below 15 eos/hpf) and it is well known that symptom severity and degree of esophageal eosinophilia show at best a modest correlation.[2–5] Several other histological changes have been identified in EoE such as cellular infiltration with mast cells, basophils and CRTH2 cells, as well as structural alterations such as spongiosis and basal zone hyperplasia.[17–19] Our immunohistochemical and RNA sequencing analyses give further insights into pathogenesis. Although expression of TSLP, which initiates a Th2-inflammatory response in EoE,[20] is upregulated in one EoE variant (lymphocytic esophagitis), absence of CRTH2+ on T-cells, a marker of type 2 inflammatory diseases, and RNA sequencing analyses imply an attenuated Th2 signal in EoE variants. While absence of both significant eosinophil infiltration and extracellular EPX deposition was confirmed by immunohistochemistry, EPX deposition can still be observed in a subset of patients in each EoE variant. Presence of degranulated eosinophils highlights the potential overlap between these conditions and EoE.[21] Incongruence between EoE variant classification and immunohistochemical findings (with regard to EPX, but also CD3) can be explained by the fact that initial classification was purely based on conventional histology (hematoxylin and eosin). Downregulation of LEKTI implies an epithelial barrier dysfunction in EoE variants as it is well-known from classical EoE.[22–24] Defects in the epithelial barrier allow penetration of antigens through the epithelial layer into the mucosa, which then triggers an inflammatory response.[25] It has yet to be determined if this epithelial barrier defect plays a causative role[26] or whether it is a consequence of the inflammatory process in EoE and EoE variants and therefore just an epiphenomenon. Nevertheless, recent data—at least in EoE—suggest that an epithelial barrier defect leads to colonization with pathogens, subsequent sensitization, and possibly resulting in activation of eosinophils.[22,23] In addition, several environmental factors have been proposed to induce increased epithelial leakage, and a role of epithelial barrier defect has been suggested for previously considered functional gastrointestinal diseases.[27–31] Taken together, epithelial barrier disruption and TSLP upregulation—despite an otherwise attenuated Th2 signal and defects in eosinophil chemoattraction—appear to be key events in EoE variants clearly shifting away the focus from classical diagnostic approaches based on eosinophil-associated proteins.[32]

Our study has important clinical relevance: symptoms of esophageal dysfunction in combination with only subtle endoscopic alterations and lack of significant eosinophil infiltration is a diagnostic pitfall. Such patients are usually misdiagnosed as having "functional dysphagia" or "somatoform disorder" and may suffer over years, because neither drugs targeting motility disturbances nor psychiatric treatment modalities are effective. Although functional dysphagia cannot be completely ruled out in some patients, our findings are very robust showing histological changes with the presence of EoE-HSS features beyond eosinophilic infiltration, epithelial barrier dysfunction confirmed by LEKTI staining, and detection of inflammatory and/or fibrotic pathways by RNAseq in all EoE variants. Patients presenting with symptoms of esophageal dysfunction should therefore undergo structured esophageal biopsy sampling independently of endoscopic findings. Biopsies should be examined by an EoE-experienced pathologist with findings being reported using the EoE-HSS classification. Additional CD3 staining can help to assess degree and pattern of lymphocytic infiltration, while immunofluorescence for LEKTI can be considered to prove epithelial barrier defects. EoE variants could represent an early stage of EoE, particularly EoE-like esophagitis. The involvement of similar pathways makes progression to EoE or even from one variant to another likely. Longitudinal follow-up including repetitive biopsies will eventually show whether one or all of these variants can progress to EoE over time. Previous studies have demonstrated the usefulness of molecular fingerprinting by the EoE diagnostic panel for identification of EoE phenotypes.[33] In our present study, we expand this approach beyond EoE highlighting the presence of different phenotypes within a broader disease spectrum. In addition, the in-depth bioinformatic analyses give some pathophysiological clues and helped to identify novel gene products to be investigated in future, particularly mechanistic studies. Here, both discriminating genes (EoE variants versus each other) as well as overlapping genes (such as C3 and CCDC80) are of particular interest. Ongoing longitudinal data collection (including sequential RNAseq) will finally help to answer the question whether or not molecular profiles are changed over time (particularly in case of progression to classical EoE or transition from one subtype to another).

Our study also has some limitations. GERD has not been rigorously excluded by pH testing in all patients. However, GERD has been excluded similarly in many other EoE studies.[34,35] In addition, our inclusion criteria were rigorous requiring a typical history for EoE (and not for GERD). The presence of dysphagia and food impactions in all of our adult patients without any endoscopic signs of GERD (including peptic strictures) makes GERD as underlying disease in these patients very unlikely. Furthermore, RNA sequencing analyses reveal a completely different mRNA profile between EoE variants and GERD patients. EoE has been described as a patchy disease with the risk of missing esophageal eosinophilia. However, studies have shown a sensitivity of 100% for the diagnosis of EoE when taking five esophageal biopsies.[36] In our study, at dedicated EoE centers with >100 cases per year, availability of at least six biopsies (three from the distal and three from the proximal part) as part of a structured biopsy protocol was a prerequisite. In addition, none of our patients had a peak eosinophil count close to the cut-off value of 60 eos/mm^2 (15 eos/hpf), which might be interpreted as a near miss. The careful histological review by two EoE expert pathologists further limits such risk. Elimination diet[37] or antigen challenge of T cells[38] would serve as a proof-of-concept that EoE variants share a similar etiology to classical EoE, namely a non-IgE-mediated food hypersensitivity.[39] Analyses of food triggers would further help to unravel a possible association with another EoE feature, the recently described food-induced immediate response of the esophagus FIRE.[40,41] However, so far, none of our patients was treated with a dietary approach. Description and characterization of EoE variants is needed first to establish therapeutic outcome measures (in the absence of dense eosinophil infiltration). With more knowledge about EoE variants, dietary restriction as used in EoE[42,43] will be an appealing approach to further define and subtype this disease group. Longitudinal follow-up RNAseq before vs after treatment (either diet or topical steroid) will help to understand disease mechanisms and to find optimal treatment modalities for each variant. Finally, one must consider the difficult question of whether the overlapping features of esophagitis in these studies constitute a common etiopathogenesis or delineate common features of chronic esophagitis in general. Long-term follow-up will be essential to further answer this question.

In conclusion, EoE variants are clinically and histologically active conditions with variable overlap with EoE despite the absence of robust esophageal eosinophilia. Although similarities with EoE in terms of clinical presentation and (subtle) endoscopic changes exist, these variants show distinct immunohistological and molecular features. In particular, there is an absence of the EoE-typical Th2-inflammatory response. Nonetheless, there are also considerable pathogenic overlaps detected using whole transcriptome profiling between classical EoE and EoE variants. *EoE-like esophagitis*, *lymphocytic esophagitis*, and *non-specific esophagitis* appear to be part of a broader esophagitis spectrum that may share a common phenotype with classical EoE.

CONFLICT OF INTERESTS

TG has consulting contracts with Sanofi-Regeneron and Falk Pharma GmbH, received travel grants from Falk Pharma GmbH and Vifor, and an unrestricted research grant from Novartis. AS has consulting contracts with Actelion, Celgene-Receptos, Falk Pharma GmbH, Roche-Genentech, GSK, Novartis, Nutricia, and Sanofi-Regeneron. ES is a consultant for Celgene Corp., Regeneron Pharmaceuticals Inc., and Novartis. AMS is a consultant for Falk Pharma GmbH, Adare Pharmaceuticals Inc, Celgene-Receptos, and Sanofi-Regeneron. MHC is a consultant for Astra Zeneca, Allakos, Arena, Celgene, Esocap, GlaxoSmithKline, Regeneron and Shire, and has received research funds from Regeneron and Shire. LB has received consulting fees and/or speaker fees from Falk Pharma GmbH, Esocap AG, Sanofi-Aventis AG, and Calypso Biotech SA. GTF is a consultant for Takeda and Arena and has received research support from Holoclara. MC received research support from hire, Regeneron and Allakos, consulting fees from Shire, Regeneron, Allakos, Adare and Nutricia, and lecture honoraria from Nutricia, Medscape, and the Annenberg

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AUTHOR CONTRIBUTIONS

TG, AS, and HUS involved in study concept and design. TG, AS, MHC, CB, MC, ESD, GTF, NG, IH, FJM, ES, and AMS involved in acquisition of data. MHC and CB involved in histological examination. TG, AS, YFM, NG, AH, SY, DS, MHC, CB, and HUS involved in analyses and interpretation of data. TG, AS, and HUS involved in drafting of manuscript. YFM, NG, SY, DS, MHC, CB, MC, ESD, GTF, NG, IH, FJM, ES, and AMS involved in critical revision of the manuscript for intellectual content. TG, AS, and HUS involved in supervision. RNA-seq: RNA-seq was performed by the Next Generation Sequencing (GS) Platform of the University of Bern. RNA-seq data have been deposited in the Gene Expression Omnibus (GEO), accession number GSE148381.

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SUPPORTING INFORMATION

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