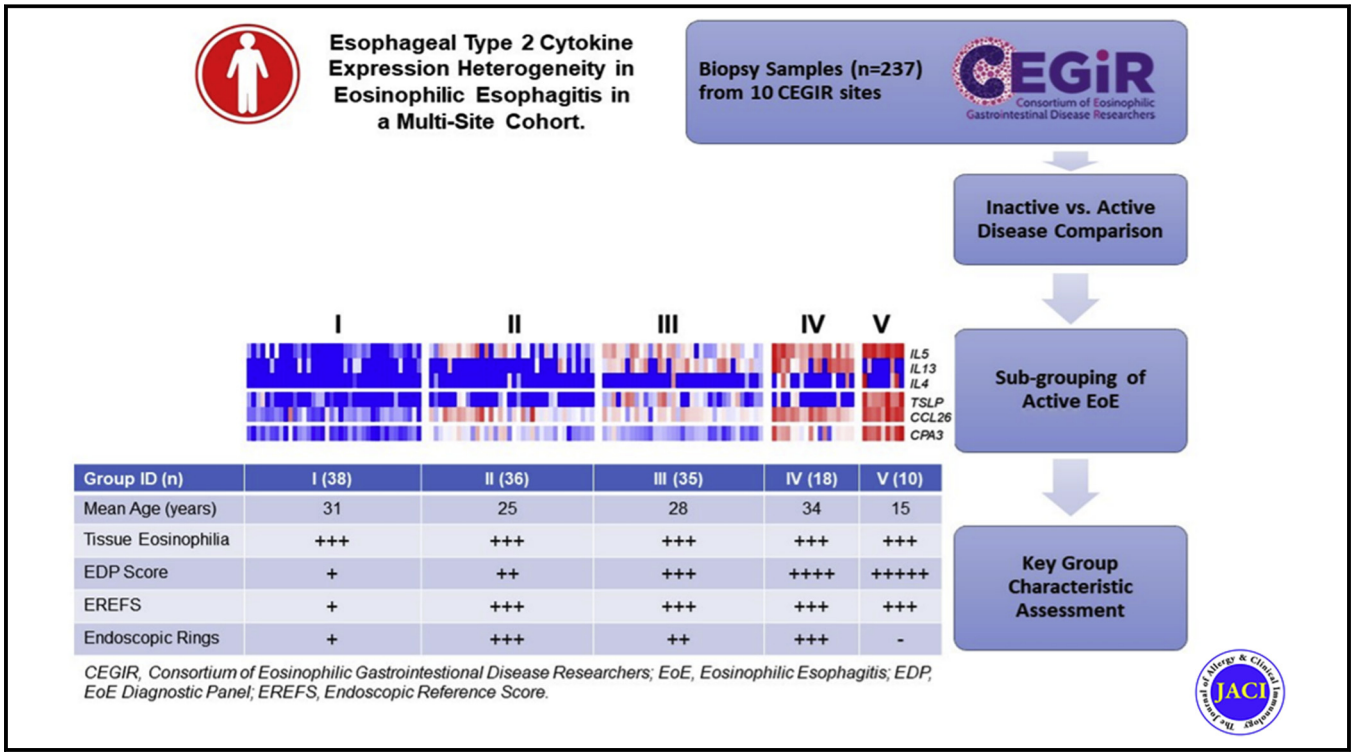
Esophageal type 2 cytokine expression heterogeneity in eosinophilic esophagitis in a multisite cohort

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GRAPHICAL ABSTRACT



Group ID (n)	I (38)	II (36)	III (35)	IV (18)	V (10)
Mean Age (years)	31	25	28	34	15
Tissue Eosinophilia	+++	+++	+++	+++	+++
EDP Score	+	++	+++	++++	+++++
EREFS	+	+++	+++	+++	+++
Endoscopic Rings	+	+++	++	+++	-

CEGIR, Consortium of Eosinophilic Gastrointestinal Disease Researchers; EoE, Eosinophilic Esophagitis; EDP, EoE Diagnostic Panel; EREFS, Endoscopic Reference Score.

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‡A list of participants in the CEGIR study is provided in this article's Online Repository (available at www.jacionline.org).

Background: There is strong evidence for a role of type 2 cytokines in the pathogenesis of eosinophilic esophagitis (EoE); however, heterogeneity in type 2 gene expression has not been examined.

Objective: We examined type 2 immunity–associated gene expression in esophageal biopsy specimens, aiming to determine the degree of cytokine heterogeneity and its potential clinical significance.

Methods: Patients (n = 312) were recruited from 10 sites associated with the Consortium of Eosinophilic Gastrointestinal Disease Researchers. In addition to histologic and endoscopic assessment, esophageal biopsy specimens were examined for expression of 96 genes within the EoE diagnostic panel.

Results: Five subgroups of patients with active EoE were identified by unsupervised clustering based on expression of *IL4*, *IL5*, *IL13*, C-C motif chemokine ligand 26 (*CCL26*), thymic stromal lymphopoietin (*TSLP*), Charcot-Leyden crystal (*CLC*), C-C motif chemokine receptor 3 (*CCR3*), and *CPA3*. These groups differed in age ($P < .02$) and EoE diagnostic panel score ($P < 1.08E–30$) but not in eosinophil levels. The group V patients had the highest expression of *IL5*, *TSLP*, and *CCL26* and genes associated with tissue remodeling, such as *COL8A1,* actin γ-2 (*ACTG2*), and tetraspanin 12 (*TSPAN12*). *IL5* and *IL13* were highly expressed in group IV; however, groups IV and V differed in age (34 vs 14 years [$P < .05$]). Groups II and III, which exhibited intermediate expression of *IL5* and *CPA3*, were differentiated by high *TSLP* and *IL13* in group III.

Conclusion: We observed heterogeneous type 2 gene expression among patients with active EoE. Type 2 gene overexpression was not directly proportional to disease features; this was especially true for tissue remodeling events. These findings highlight a clinical opportunity for leveraging molecular endotypes to implement personalized medicine in EoE. (J Allergy Clin Immunol 2020;145:1629-40.)

Key words: *Allergy, cytokine, eosinophil, fibrosis, IL-5, IL-13, inflammation, precision medicine, resolution, T_H2 cells*

Abbreviations used

ACTG2: Actin γ-2
ALOX15: Arachidonate 15-lipoxygenase
BZH: Basal zone hyperplasia
CCL26: C-C motif chemokine ligand 26
CCR3: C-C motif chemokine receptor 3
CEGIR: Consortium of Eosinophilic Gastrointestinal Disease Researchers
CLC: Charcot-Leyden crystal galectin
COL8A2: Collagen type 8 alpha 2-chain
Ct: Cycle threshold
ΔCt: Change in cycle threshold
DEC: Dyskeratotic epithelial cell
DIS: Dilated intercellular space
DSG1: Desmoglein-1
EDP: Eosinophilic esophagitis diagnostic panel
EI: Eosinophilic infiltration
EoE: Eosinophilic esophagitis
EoEe: Eosinophilic esophagitis endotype
EREFS: Endoscopic reference score
HSS: Histologic scoring system
LRRC31: Leucine-rich repeat containing 31
MMP12: Matrix metalloprotease 12
SLC26A4: Solute carrier family 26 member 4
SPINK7: Serine peptidase inhibitor, Kazal type 7
TGFB1: Transforming growth factor β-1
TNFAIP: TNF-α–induced protein
TSLP: Thymic stromal lymphopoietin
TSPAN12: Tetraspanin 12

Eosinophilic esophagitis (EoE) is a chronic inflammatory disease triggered by delayed immune hypersensitivity to foods and aeroallergens and resulting in the accumulation of excessive eosinophils in the esophagus.[1-3] Children and adult patients can present with vomiting, dysphagia, and food impaction.[4]

Substantial evidence is accumulating in support of the idea that EoE is driven by a type 2 immune response associated with IL-4, IL-5, and IL-13.[5-15] In particular, mRNA expression of *IL5* and *IL13* (and *IL4* to a lesser extent) is increased in the esophagus of patients with EoE compared with in controls.[6,16,17] IL-5 is required for induction of experimental EoE in mice, and overexpression leads to esophageal inflammation and remodeling.[10] IL-5 induces integrin ligation, activation, and survival of eosinophils, all of which can be inhibited in vivo in humans with a neutralizing antibody against IL-5, which is now approved for the treatment of eosinophilic asthma.[18,19] IL-13 appears to be particularly

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important in EoE, as overexpression in mice induces cardinal features of the disease, including induction of esophageal eotaxin expression, eosinophil accumulation, epithelial hyperplasia, angiogenesis, and fibrosis, processes that occur in the human disease.[15] In addition, IL-13 induces impaired barrier function of esophageal epithelial cells partially via upregulation of calpain-14 (*CAPN14*), which is encoded by a gene located at the site of one of the chief EoE susceptibility loci, 2p23.[12,15,20-22] Genetic studies have also identified genome-wide susceptibility at 5q22, the thymic stromal lymphopoietin (*TSLP*) locus. TSLP-activated dendritic cells cause T-cell polarization to T_H2 cells that serve as sources of IL-5 and IL-13 in EoE.[7,23,24] Antibodies against IL-5 and IL-13, including its receptor IL4Rα (also a receptor for IL-4), reduced esophageal eosinophils and improved endoscopic, histologic, and/or esophageal transcriptomic abnormalities in early clinical studies, but the effects have been variable.[5,11,13,14,25,26] Despite strong evidence for a role of type 2 cytokines in EoE pathogenesis, implementation of targeted therapeutics is hampered, in part, by the inability to predict responders and nonresponders and the heterogeneity of treatment response.

Levels of esophageal eosinophils correlate with the severity of endoscopic findings.[27] Yet, disease severity is associated more strongly with multiple histologic parameters than esophageal eosinophil counts alone.[28] Transcript expression profiling of esophageal biopsy specimens has identified unique disease-specific transcripts, notably rich in genes that encode type 2 cytokines or products induced by these cytokines (eg, IL-13–induced eotaxin-3).[6,17,29-31] An EoE diagnostic panel (EDP), composed of 96 genes involved in diverse functions, including inflammation, remodeling, and ion transport, effectively differentiates patients with active EoE from control individuals, including patients with EoE in remission.[17] Molecular profiling with the EDP has identified substantial molecular heterogeneity associated with distinct histologic and endoscopic features, which led to the identification of 3 distinct disease endotypes.[16] Herein, we examine biopsy specimens from 237 adult and pediatric patients with EoE and clinical features from 10 sites associated with the Consortium of Eosinophilic Gastrointestinal Disease Researchers (CEGIR).[32,33] Esophageal biopsy specimens were examined for the expression of type 2 immunity–associated gene products, aiming to test for the presence of patient heterogeneity based on type 2 immune responses, and its significance.

METHODS

Patient recruitment

This study was conducted within the context of CEGIR, a national collaborative network of 16 academic centers caring for adults and children with eosinophilic gastrointestinal disorders (see Table E1 in this article's Online Repository at www.jacionline.org). The CEGIR clinical trial, Outcomes Measures in Eosinophilic Gastrointestinal Disorders across the Ages (OMEGA), is a longitudinal cohort study aimed at understanding the natural history of EoE, eosinophilic gastritis, and eosinophilic colitis during routine clinical care.[16,32-34] With this aim, demographic, clinical, endoscopic, and histologic data, as well as gastrointestinal and blood samples, were prospectively collected from 2015 through 2018. A total of 10 sites provided the data on patients with EoE and samples for this study; samples without peak eosinophil counts were excluded from the current analysis (Fig 1 and see Table E2 in this article's Online Repository at www.jacionline.org). Clinical features of subjects were determined during standard of care evaluation with intake and follow-up forms. All subject data were stored at the Data Management and Coordinating Center at the University of South Florida in Tampa and were systematically extracted from the database. We defined patients with active EoE as having symptomatic esophageal dysfunction and a peak count of 15 or more eosinophils per hpf; samples with no documented eosinophil count at the time of biopsy were excluded from the current study. Specimens obtained from patients with confirmed EoE with fewer than 15 peak eosinophils per hpf were classified as inactive. Pediatric subjects were defined as those younger than 18 years of age. Atopy was defined on the basis of self-report of allergic rhinitis, dermatitis, asthma, or food allergy. The definition of the term *response to steroid* was determined by using a positive versus negative response to whether swallowed topical steroids had been effective on the basis of symptoms and pathology. These data were captured across 10 sites by the CEGIR questionnaire, as reported.[16,35] This study was approved by the institutional review boards of the participating institutions via a central institutional review board at Cincinnati Children's Hospital Medical Center. Participants provided written informed consent.

Biopsy specimen acquisition

We obtained distal esophageal biopsy specimens during endoscopy; 2 or 3 specimens were taken from the area 2 to 4 cm above the lower esophageal sphincter.[30] Gene expression in unfixed biopsy specimens was analyzed by quantitative PCR with the EDP as previously described.[16,17] The EoE transcriptome as measured by the EDP is enriched for epithelial genes as reported, although it also has embedded markers of T cells, mast cells, and eosinophils.[17] Changes in cycle thresholds (ΔCt) for all genes of interest were calculated by subtracting the cycle threshold (Ct) of glyceraldehyde-3-phosphate dehydrogenase. To control for the potential effect of variable glyceraldehyde-3-phosphate dehydrogenase detection on ΔCt values for samples with a Ct value equal to 40 (the lower limit of detection), ΔCt values associated with a Ct value equal to 40 were removed, the minimum expression of each gene was identified, and ΔCt values associated with a Ct value equal to 40 were replaced with (minimum – 1) values. The EDP score (previously described as EoE score) was calculated by summing the ΔCT values of the 76 highly dysregulated genes (ΣΔCT) in the panel, as described previously.[16,17] To reflect the disease-specific expression signature and disease severity, the CT sums were derived separately for the upregulated and downregulated genes, after which their 2 sums were combined. Of note, the EDP score is inversely correlated with disease severity. The assignment of the subject's EoE endotype on the basis of expression of 8 genes in the panel of genes was established by a method comprising linear discriminant analysis determining a probability distance by using Mahalanobis distance.[16] By using the same algorithm, probabilities and predicted EoE endotypes were calculated on the basis of the highest predicted probability.

Data analysis

Unsupervised clustering was used to identify groups of patients with active EoE based on expression of T_H2-associated genes previously shown to be upregulated in EoE, namely, *IL4*, *IL5*, *IL13*, *CXCL8*, C-C motif chemokine receptor 3 (*CCR3*), C-C motif chemokine ligand 26 (*CCL26*), and *TSLP*, as well as markers of eosinophils (*CLC*) and mast cells (*CPA3*). A value of k equal to 5 for patient clustering was selected on the basis of the results of a sensitivity analysis and also to obtain a sufficient fit and limit depletion of cluster size. Gene expression, EDP score, and age were compared between these groups by using 1-way ANOVA with a Bonferroni posttest, and significance with respect to patients with inactive disease was assessed with a Dunnett posttest. Values from the histologic scoring system (HSS),[28] endoscopic reference scoring system (EREFS),[27,36] and peak eosinophil count were compared by using a nonparametric Kruskal-Wallis test and Dunn posttest results. Features of the HSS were scored for grade (0-3) and stage (0-3), which were combined, and the sums were normalized to a range of 0 to 1 for the total scores. Lamina propria fibrosis could not be assessed in more than 30% of the samples; therefore, these measurements were excluded from the current analysis. Features of the EREFS were scored (0-2), and the features were added to obtain total scores in the distal and proximal esophagus; proximal and distal scores were in turn added to obtain a final score.

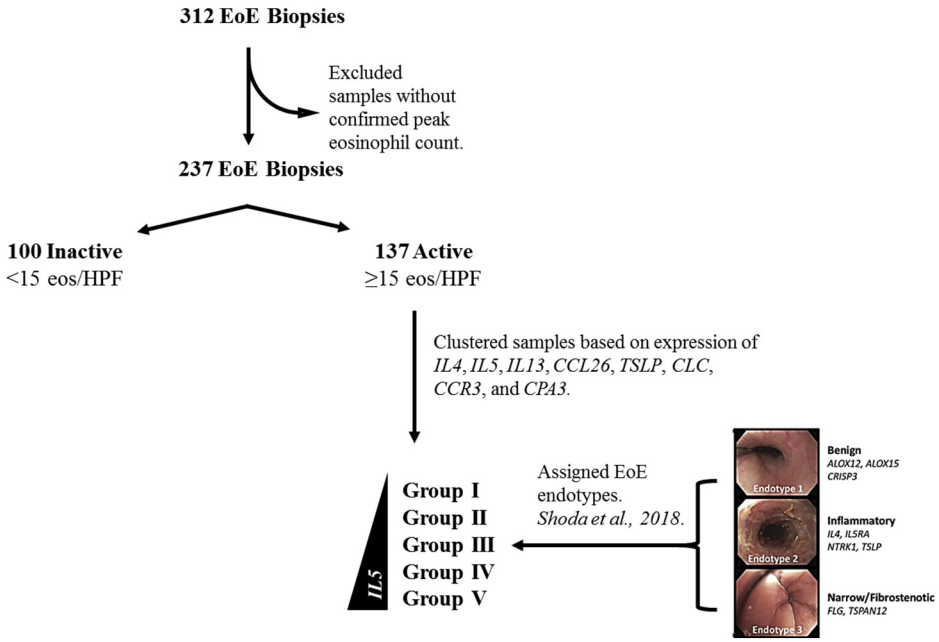


FIG 1. Workflow diagram depicting sample inclusion and categorization criteria. Biopsy specimens with confirmed active EoE were sorted into 5 groups by k-means clustering using the inflammatory genes *IL4, IL5, IL13, CCL26, TSLP, CLC, CCR3,* and *CPA3*. A published algorithm[16] was used to assign patients with active EoE to 1 of 3 EoE endotypes: benign (EoEe1), inflammatory (EoEe2), or fibrostenotic (EoEe3).

To characterize groups of patients with active EoE, we examined the distribution of previously described EoE endotypes. To assign endotypes, an algorithm with discriminant analysis was implemented on the basis of expression of 8 genes as previously described.[16] Data compilation, filtration, and k-means clustering were performed in R Studio (version 3.5.1), and statistical analyses were completed in GraphPad Prism (version 8.0.1); statistical significance was assigned at P values less than .01. Morpheus (https://software.broadinstitute.org/morpheus/) was used to generate heatmaps.

RESULTS

Characteristics of Patients with EoE

Patient age ranged from 4 to 71 years, with mean age of 26 years (Table I). A majority of the patients were male (67%) (Table I). A subset of patients were taking a proton-pump inhibitor, practicing dietary elimination, and receiving topical steroid therapy (Table I). Patients with active EoE had a mean value of 50 eosinophils per hpf, whereas patients with inactive EoE had a mean value of 3 eosinophils per hpf (Table I). The EDP total score differed in the active and inactive groups (123 vs 332 [$P < .0001$]) (Table I). Consistent with the increase in esophageal eosinophil levels, expression of esophageal *CLC* mRNA was increased in active EoE compared with inactive EoE ($\Delta\Delta$Ct = 5.45 [$P < .0001$]) (Fig 2). Levels of *CPA3*, a mast cell–specific transcript, were increased in biopsy specimens from patients with active EoE compared with inactive EoE ($\Delta\Delta$Ct = 2.84 [$P < .0001$]) (Fig 2).

Type 2 cytokine profiles in EoE

Of the upregulated genes in active versus inactive disease, we noted numerous type 2 immunity–associated genes, such as *IL5*, *TSLP*, and *IL13* (Fig 2). These included increases in several IL-13–inducible genes, including *CCL26* ($\Delta\Delta$Ct = 5.12 [$P < .0001$]), solute carrier family 26 member 4 (*SLC26A4*) ($\Delta\Delta$Ct = 4.88 [$P < .0001$]), matrix metalloprotease 12 (*MMP12*) ($\Delta\Delta$Ct = 4.32 [$P < .0001$]), leucine-rich repeat containing 31 (*LRRC31*) ($\Delta\Delta$Ct = 8.21 [$P < .0001$]) and arachidonate 15-lipoxygenase (*ALOX15*) ($\Delta\Delta$Ct = 7.47 [$P < .0001$]). Focusing on type 2–associated cytokines, we observed increased expression of *IL5* ($\Delta\Delta$Ct = 4.02 [$P < .0001$]) and *IL13* ($\Delta\Delta$Ct = 2.15 [$P < .0001$]). By contrast, we observed only a modest increase in expression of *TSLP* ($\Delta\Delta$Ct = 0.09 [$P < .05$]) and no significant change in transforming growth factor β 1 (*TGFB1*).

Expression of type 2 inflammatory genes distinguishes subgroups of active EoE

We set out to identify unique subgroups of patients with active EoE based on differential expression of type 2 cytokines (*IL4*, *IL5, TSLP,* and *IL13*) and the allergic inflammatory cell–specific markers *CLC* and *CPA3*. Using unsupervised clustering, we identified 5 groups of patients with low (group I), intermediate (groups II and III), and high (groups IV and V) expression of type 2 cytokine genes, which were ordered according to expression of *IL5* (Fig 3, *A*). *IL5* and *CPA3* were not significantly increased in group I with respect to patients with inactive EoE (Fig 3, *B*). Eosinophil levels were comparable among all 5 subgroups (Fig 3, *C*). Whereas expression of *IL13* was significantly increased in groups III to V, the increase in groups III and IV was most dramatic. *TSLP* expression was increased specifically in groups III and V. Of note, *IL4* mRNA was universally low; it was undetectable in 95 of 97 patients with inactive EoE, and in all group I patients; conversely, *IL4* expression was detectable in approximately 20% of *IL5*-high patients with active EoE (groups IV and V in Fig 3, *A*).

TABLE I. Patient demographics

Characteristic	Inactive (n = 100)	Active (n = 137)	*P* value
Age, y ± SD (range)	23 ± 17 (4-67)	28 ± 17 (5-71)	.07
Male (%)	64.0%	69.3%	
White (%)	95.0%	92.7%	
EDP score*	332 ± 83	123 ± 136	<.0001
Peak eosinophil count (eosinophils/hpf)	3 ± 4	50 ± 34	<.0001
Atopic status			
Allergic rhinitis (%)	44.0%	38.7%	
Asthma (%)	24.0%	25.5%	
Eczema (%)	26.0%	24.1%	
Food allergy (%)	42.0%	40.9%	
Ongoing diet therapy (%)	50.0%	51.1%	
Current PPI use (%)	34.0%	39.4%	
Current oral systemic steroids (%)	0%	0%	
Current swallowed topical steroids (%)	59.0%	47.4%	
History of steroid use (%)	68.0%	71.5%	
Response to steroid, symptom (% effective)	73.5%	63.3%	
Response to steroid, pathology (% effective)	64.7%	51.0%	

PPI, Proton-pump inhibitor.
*EDP score calculated as the sum of ΔCt values.

Groups I to V differed significantly in terms of total EDP score, both compared with one another and compared with patients with inactive EoE (Fig 3, *C*). The groups that did not significantly differ in EDP score were groups II and III (the *IL5*-intermediate groups) as well as groups IV and V (the *IL5*-high groups) (Fig 3, *C*). While there was no difference in age between patients with active or inactive EoE (Table I), age differed significantly among the groups of active patients ($P < .05$; Fig 3, *C*). Moreover, treatment status did not account for patient grouping, as chi-square analysis did not reveal significant enrichment of proton-pump inhibitor (PPI) use, steroid use, or ongoing diet therapy in any of the 5 patient groups (see Table E3 in this article's Online Repository at www.jacionline.org).

Cytokine heterogeneity is associated with histologic and endoscopic phenotypes, as well as with molecular endotypes

We aimed to determine whether the identified patient subgroups were associated with histologic and endoscopic features. In patients in group I with low *IL5* expression, we observed that HSS grade and stage scores were higher than in the inactive group, corroborating their assignment as having active EoE; however, we observed no significant increase in total endoscopic scores (EREFSs) with respect to inactive EoE (Fig 4, *A* and *B*). Peak eosinophil counts did not differ among the active groups; however, significant differences between patients with active disease and those with inactive disease and significant intergroup differences occurred among the individual features of the HSS (Fig 4, *C* and see Table E4 in this article's Online Repository at www.jacionline.org). The most frequent abnormalities were eosinophilic infiltration (EI), basal zone hyperplasia (BZH), and dilated intercellular spaces (DISs); EI and BZH were significantly increased in all groups compared with in individuals with inactive EoE. BZH was significantly increased in group V compared with in group I, and severity of DIS was significantly increased in groups II to V compared with in individuals with inactive EoE. Surface epithelial alteration was significantly increased in group V compared with in patients with inactive EoE. Eosinophil abscesses, eosinophil surface layering, and dyskeratotic epithelial cells (DECs) were less common, and significant increases were not found. Eosinophil surface layering was most common in group III, and DECs were most common in group V. Group V had the highest scores for EI, BZH, DISs, surface epithelial alteration, and DECs.

With regard to endoscopic features, the identified patient subgroups had unique associations. Group I did not have a significant increase in rings or edema, whereas the occurrence of edema was elevated across groups II to IV (Fig 4, *B*). The incidence of exudate was elevated across groups II to V. Although furrowing was observed in all patient groups with respect to inactive disease, the severity of furrowing in groups II, III, and V was significantly greater than group I. Of note, the incidence of rings (or trachealization) was highest in groups II, III and IV, and rings (trachealization) were absent in group V.

We aimed to understand the relationship between the identified cytokine heterogeneity and previously described EoE endotypes. Absence of rings in group V patients led us to question whether these biopsy specimens shared characteristics with the inflammatory EoE endotype 2 (EoEe2), which also exhibited a notable lack of rings.[16] Whereas group V overlapped entirely with EoEe2, group IV represented a combination of EoEe2 and the fibrostenotic endotype EoEe3 (Fig 4, *D*). Groups II and III were primarily represented by EoEe3, whereas the remaining patients had the normal-like endotype, EoEe1, or EoEe2. Finally, group I consisted primarily of patients assigned to the endotype EoEe1, whereas the remainder had the endotype EoEe3 aside from 1 individual with endotype EoEe2. Collectively, our data substantiate distinct EoE endotypes, independent of esophageal eosinophil levels in active biopsy specimens, based on cytokine expression.

Group-specific gene dysregulation corroborates endotype membership

We aimed to determine which genes and/or pathways were associated with the unique *IL5*-related endotypes. By enumerating the 10 genes that were the most highly upregulated in each group, we identified several genes that were upregulated in

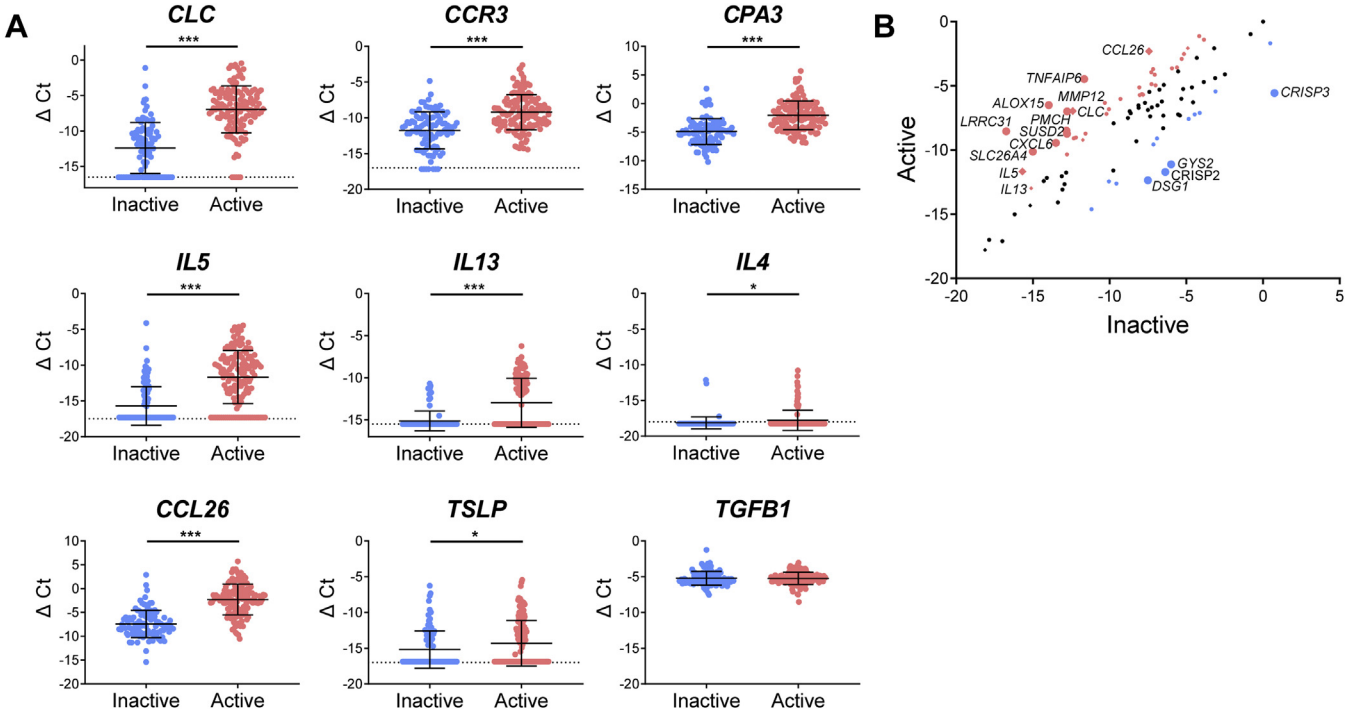


FIG 2. T_H2-associated gene expression in patients with active and inactive EoE. **A,** Expression of genes associated with eosinophils (*CLC* and *CCR3*) or mast cells (*CPA3*), T_H2 activation (*IL5, IL13, IL4,* and *TSLP*), and eosinophil chemotaxis (*CCL26*) was measured and normalized by subtracting the Ct of glyceraldehyde-3-phosphate dehydrogenase (ΔCt) (active EoE n = 137; inactive EoE n = 100). Significance was assessed by the Mann-Whitney test. Dotted lines indicate limit of detection. Data are presented as the mean ± SEM. **B,** Expression of genes in the EDP was averaged across patients with active and inactive EoE, and differentially expressed genes were identified and annotated. Genes with |ΔΔCt| greater than 2 are indicated in blue if their expression was higher in patients with inactive EoE or in red if their expression was higher in patients with active EoE. **P* < .05; *** *P* < .001. The data shown include the cumulative results of all samples performed over 78 Custom TaqMan gene expression array cards. The experiments each supported the trends shown.

all 5 groups, including *ALOX15, CCL26,* and *LRRC31*, which are induced by IL-13, as well as *CLC* and *PMCH* (Fig 5, *B*). *MMP12, SLC26A4,* and TNF-α–induced protein (*TNFAIP*) were among the most induced genes of groups II to V. In group III, there was notable upregulation of the structural remodeling genes *MMP12* and collagen type 8 alpha 2-chain (*COL8A2*) paired with a decrease in genes associated with epithelial integrity and differentiation such as *FLG*, desmoglein-1 (*DSG1*), and serine peptidase inhibitor, Kazal type 7 (*SPINK7*); this result corroborates the observation that group III is largely congruent with the fibrostenotic endotype (EoEe3 [Fig 5, *A*]).[16] Whereas *IL13* was preferentially expressed in group IV, several IL-13–dependent transcripts were preferentially expressed in group V, including *LRRC31* and *SLC26A4*. Similarly, we observed that both groups preferentially expressed specific genes associated with structural remodeling, specifically *COL8A2* in group IV and actin γ-2 (*ACTG2*), as well as *MMP12* in group V.

DISCUSSION

Herein, we have examined the relationship between type 2 gene expression and EoE-related disease features. In this study, we examined heterogeneous expression of the type 2 cytokines IL-5 and IL-13 because of their known roles in promoting eosinophil survival and activation through both direct and indirect mechanisms and the emerging set of therapies that target these cytokines and/or their receptors.[5,11,13,14,18] We have identified subgroups of patients with active EoE that have low, intermediate, and high expression of *IL5* and have furthermore demonstrated concomitant upregulation of genes associated with fibrotic tissue remodeling in *IL5*-intermediate patient groups (Fig 6, *A*). Treatment status did not account for the differential gene expression that determines patient grouping, as chi-square analysis did not reveal significant enrichment of proton-pump inhibitor use, steroid use, or ongoing diet therapy in any of the 5 patient groups. Importantly, of the 2 groups with high *IL5* expression, *IL13* expression was elevated in only 1 of them, suggesting the existence of a mechanism by which regulation of *IL5* and *IL13* gene expression is discordant either spatially or temporally, as the group IV patients were markedly older than the group V patients were. We have demonstrated a positive relationship between high *IL5* expression and increased disease severity as assessed by the total EDP score and endoscopic parameters. Specifically, we found that patients with active EoE but low expression of *IL5* did not exhibit a significant global increase in endoscopic disease severity scoring, although they did exhibit higher scores than did inactive patients via histologic scoring. These findings support the practice of obtaining biopsy specimens from patients suspected of having EoE even if the endoscopic appearance is normal.

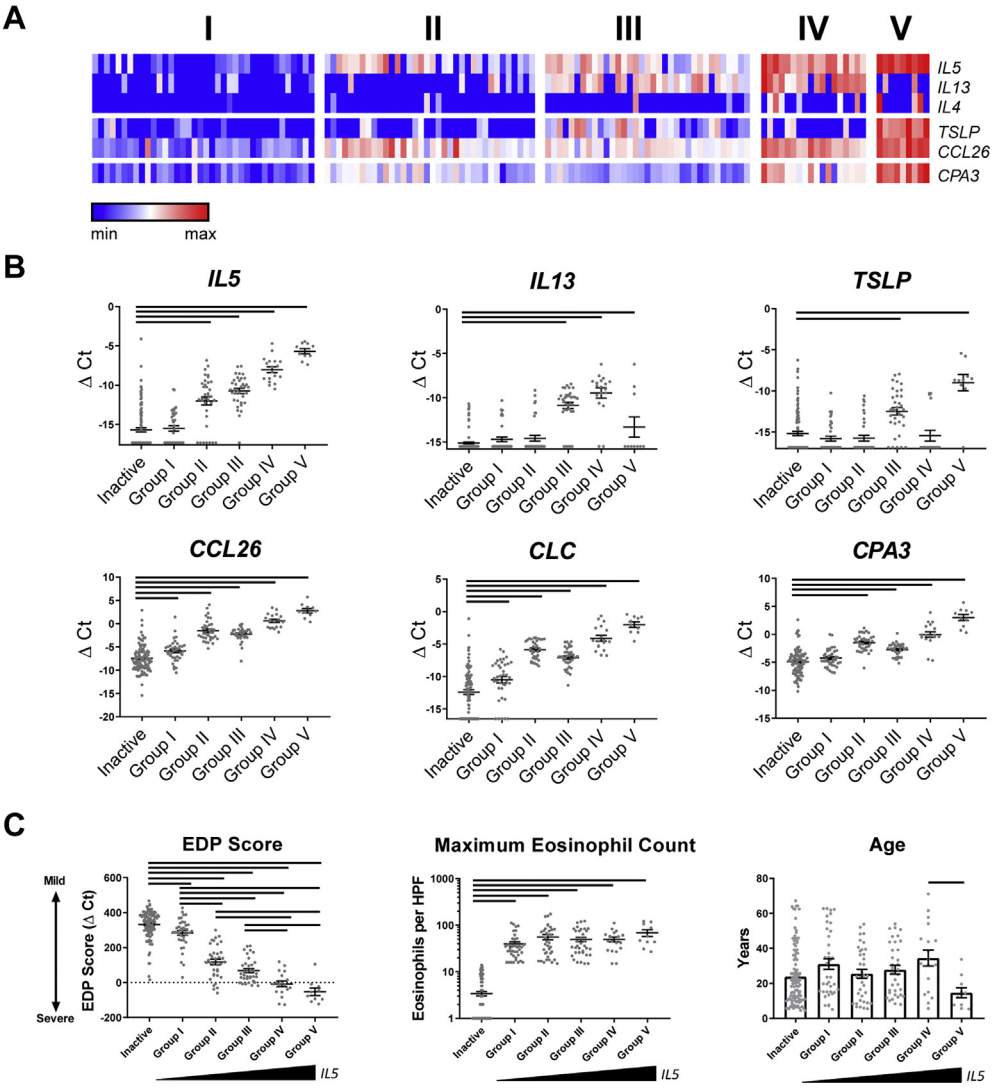


FIG 3. Expression of T_H2 genes in active EoE identifies distinct patient groups. **A,** Five subgroups of active EoE were identified by k-means clustering based on expression of *IL4, IL5, IL13, TSLP, CCR3, CCL26, CLC,* and *CPA3*. **B,** Normalized expression of T_H2 genes was compared in patients with inactive EoE and 5 groups of patients with active EoE. Bars indicate statistical differences ($P < .05$) between patients with inactive EoE and specified groups, assessed by 1-way ANOVA with a Dunnett posttest. Data are presented as mean ± SEM. **C,** EDP score and patient age were compared between patients with inactive EoE and patients with active EoE in groups I to V by1-way ANOVA with a Tukey posttest. Maximum eosinophil count was compared via a Kruskal-Wallis test with a Dunn posttest. Data are presented as the mean ± SEM. Bars indicate a *P* value less than .05. The data shown include the cumulative results of all samples performed over 78 Custom TaqMan gene expression array cards.

Previous studies have reported a correlation between eosinophil counts by histology and expression of inflammatory genes, including *IL5, IL13,* and *CCL26*.[6,17] In contrast, by focusing specifically on patients with more than 15 eosinophils per hpf, we have described differential gene expression in groups of patients with no difference in esophageal eosinophil counts. However, we observed discordance between *CLC* transcript and eosinophil levels. This result may be due to the presence of extracellular vesicles containing *CLC* mRNA, which may be indicative of eosinophil activation. Further investigation will be needed to establish a relationship between *CLC* transcript, intact eosinophils, and extracellular eosinophil–derived vesicles in biopsy tissue.

A recent single-cell RNA sequencing study of esophageal residing $CD3^+$ T cells[23] has identified the cellular source of IL-5 and IL-13 as an enriched population of activated effector memory T cells (designated the T8 cell population), which is consistent with prior reports.[37,38] The single-cell RNA sequencing article was limited to 17 subjects, whereas the current study examines more than 200 subjects, thus allowing a deeper analysis of patient heterogeneity and phenotypic associations. On a single-cell level, only a minority of pathogenic effector T_H2 cells produce only IL-5, whereas most produce IL-13. Collectively, our data substantiate patient-specific differences in the presence of $CD3^+$ T cells based on IL-5 and IL-13 expression; the data also demonstrate

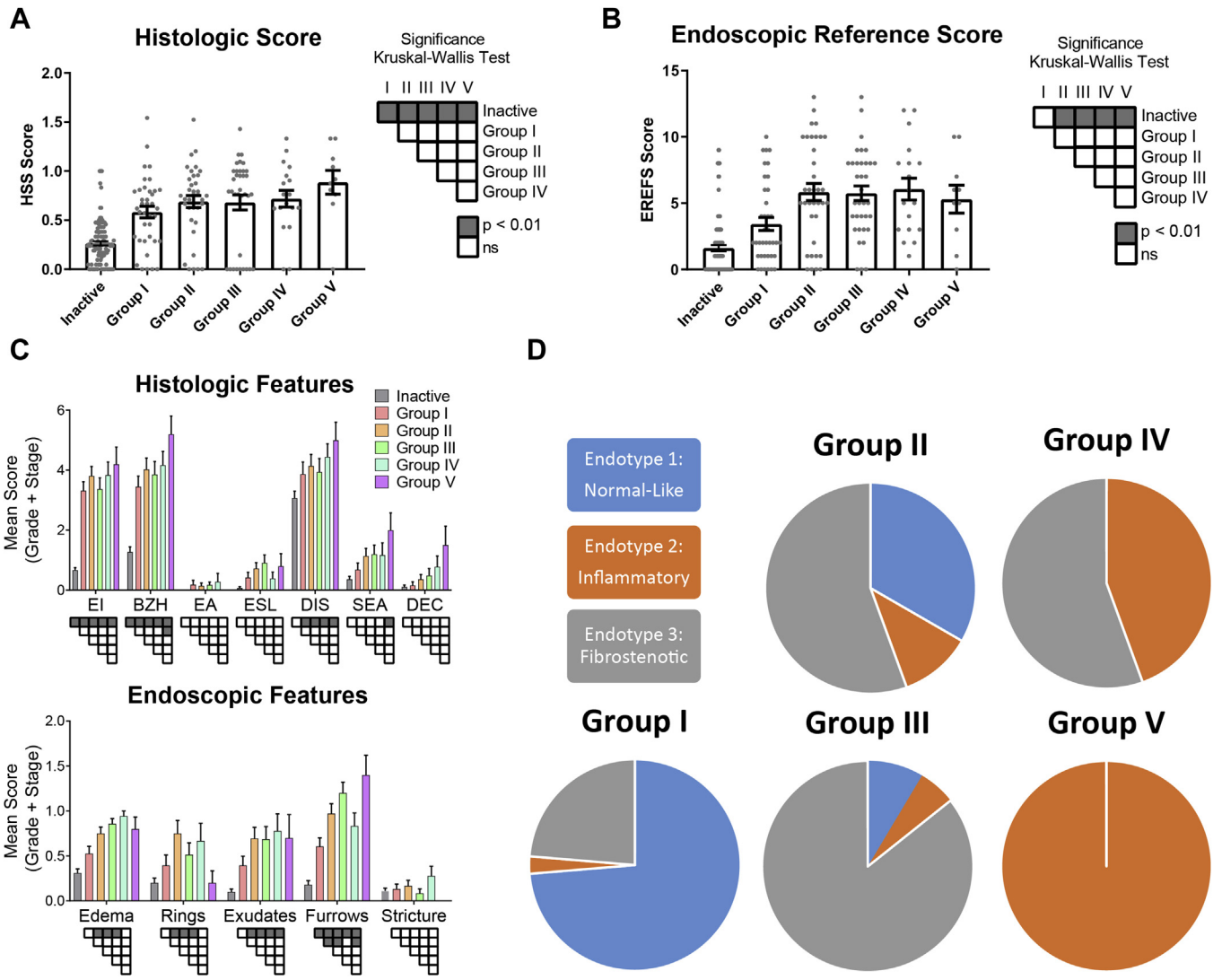


FIG 4. Inflammatory and fibrostenotic phenotypes among the *IL5*-high and *IL5*-intermediate groups. Histologic (**A**) and endoscopic (**B**) scoring of patients with inactive EoE or in groups I to V. Statistical significance determined by a Kruskal-Wallis posttest between individual groups is indicated as shading. For *A* and *B*, individual circles represent individual patients. **C,** Features of the EoE histology scoring system (HSS) and EoE endoscopic reference score (EREFS) were scored for grade (0-3) and stage (0-3) across groups I to V. Significance between patient subgroups with active EoE and those with inactive EoE was calculated with 2-way ANOVA and a Tukey posttest (shading; $P < .01$). For *A-C*, data are presented as mean ± SEM. **D,** EoE endotype assignment in groups I to V. Groups were found to be dependent on endotypes according to a chi-square exact test ($P < .0001$). Eosinophilic infiltration EI, BZH, eosinophilic abscess (EA), eosinophilic surface layering (ESL), dilated intercellular spaces (DISs), surface epithelial alteration (SEA), and DECs.

that this heterogeneity is associated with and is likely causative, at least in part, of distinct disease features.

Heterogeneity of disease presentation may be indicative of chronologic stages of disease or distinct disease mechanisms. Recently, we reported the identification of 3 molecular endotypes of active EoE with distinctive histologic and endoscopic features and presented evidence that patients transition from a normal-like endotype (EoEe1) to an inflammatory (EoEe2) endotype and finally a fibrostenotic (EoEe3) endotype.[16] Although the samples in this report represent a single time point, the data substantiate previous reports that EoE progression is not a simple linear pathway from low- to high-T_H2 cytokine expression.[39] By comparing the current findings with endotype assignments, these collective results support a disease mechanism that transitions from *IL5*-low/normal to *IL5*-high/inflammatory and finally to *IL5*-intermediate/fibrostenotic.

Whereas expression of the type 2 cytokines IL-13 and TSLP were detected at low levels in patients with low *IL5* expression or with inactive EoE, *IL4* transcript was undetectable in these patients and was expressed at only a low level in patients with active EoE and high *IL5* expression. It is possible that active protein production and signaling is not adequately accounted for by mRNA quantification. We have recently reported differential cytokine expression in T cells from patients with EoE and observed that IL-4 was readily detected by flow cytometry but not in RNA sequencing data, indicating that its regulation may occur

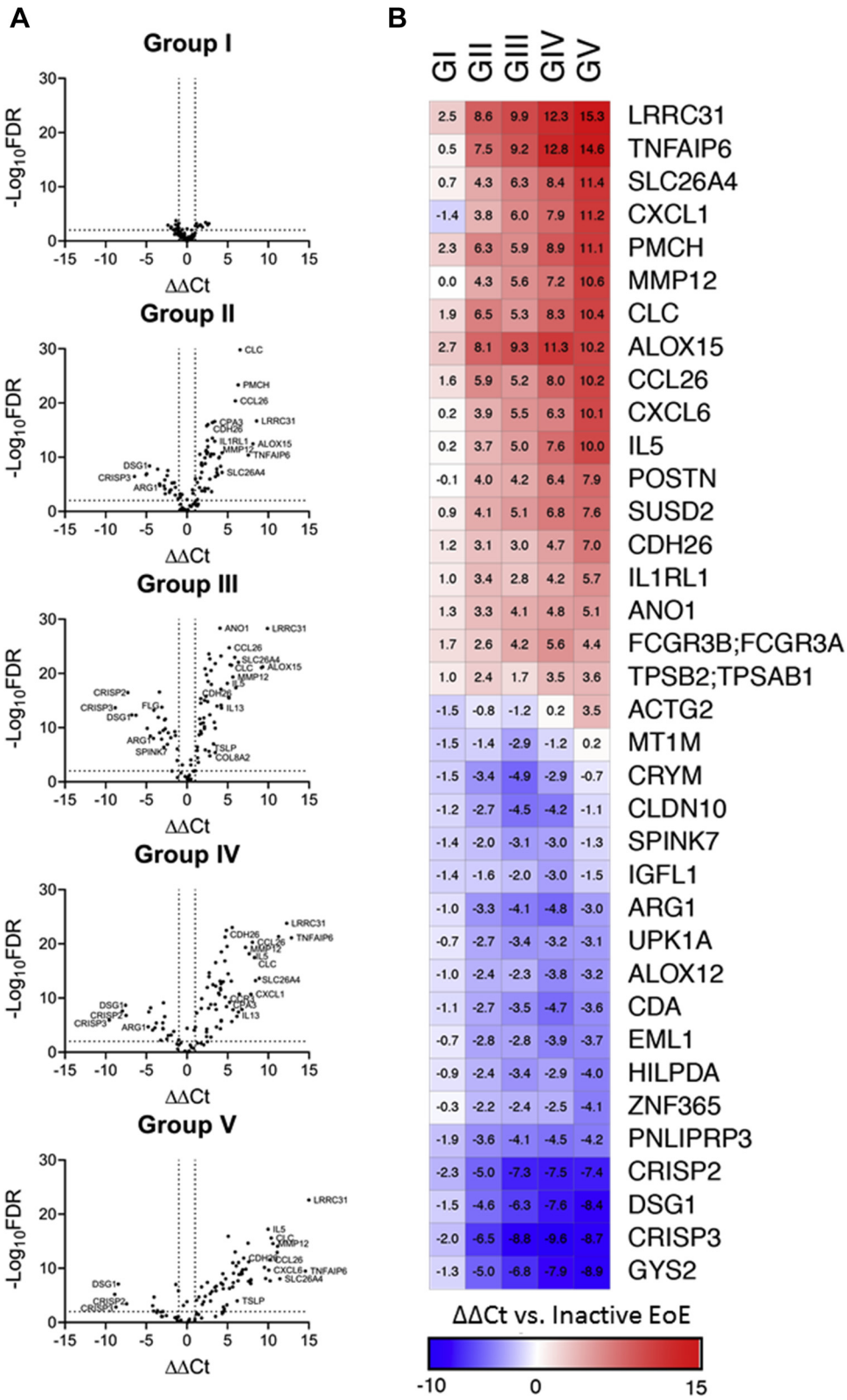


FIG 5. Differential gene expression in T_H2 subgroups. **A,** The ΔΔ Ct values ($\Delta Ct_{group} - \Delta Ct_{inactive}$) and accompanying statistical significance (–log10 false discovery rate [FDR]) were calculated for the entire EDP in each group with respect to patients with inactive EoE. Dotted lines indicate significance thresholds at a –log10 false discovery rate greater than 2 and |ΔΔ Ct| greater than 1. Genes of particular interest are annotated. **B,** ΔΔ Ct values (presented as numerical values in each square) of the highest upregulated and downregulated genes in each group with respect to inactive EoE.

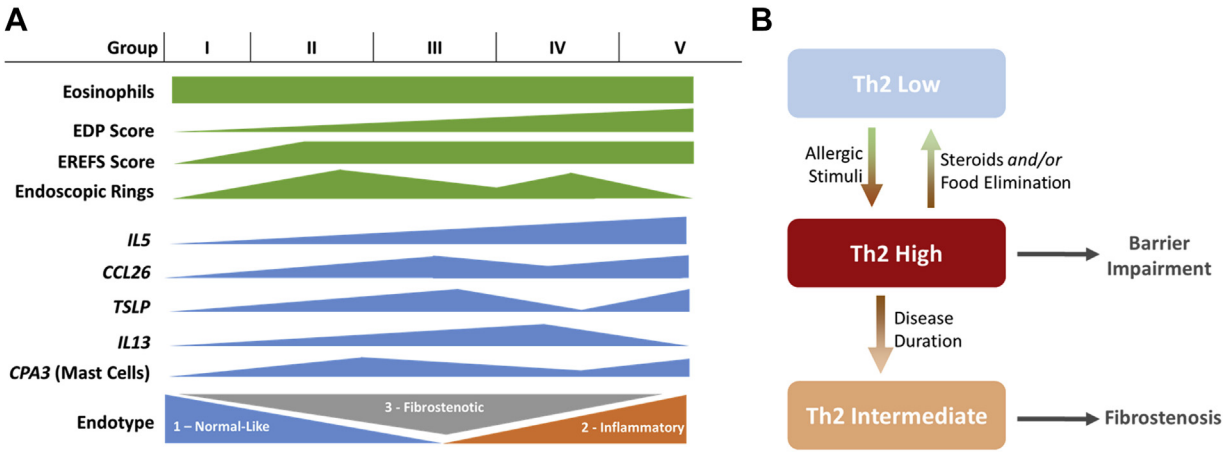


FIG 6. Summary of study results and proposed disease mechanism. **A,** Groups I to V as defined in this study were not statistically different on the basis of eosinophil count but exhibited increased disease severity as assessed by cumulative EDP scoring. Expression of cytokines and chemokines varied among the 5 groups, which also differed in terms of membership in EoE endotypes 1 to 3. **B,** Model depicting patient progression from a T_H2-low phenotype (congruent with EoEe1) to a T_H2-high phenotype (congruent with EoEe2) following allergic or inflammatory insult. With steroid treatment, food elimination, or biologic therapy, the T_H2 gene expression decreases, and patients either resolve inflammation by reverting to a T_H2-low phenotype or develop a fibrostenotic (EoEe3) signature.

posttranslationally and that biologically significant changes in IL-4 signaling may not be detected at the level of mRNA quantification.[23] Moreover, significant evidence exists that although IL-4 and IL-13 share receptor components owing to differential receptor subunit use, IL-4 exerts biologic activity at substantially lower concentrations than IL-13 does[40-42]; it is therefore possible that IL-4 signaling is a critical component of the signaling events in patients from groups IV and V, because low expression may be sufficient to skew the inflammatory environment. Furthermore, as cytokines largely exert their activity in local tissue microenvironments and immunologic synapses, mRNA quantification in bulk tissue may fail to detect bioactive quantities of IL-4 expression.

High-throughput EoE genetic studies used to identify common genetic susceptibility elements (single-nucleotide polymorphisms) and altered gene expression have implicated several genes associated with type 2 inflammation. TSLP, which acts on dendritic cells to promote T_H2 polarization, was identified by multiple genome-wide association studies as the site of multiple single-nucleotide polymorphisms that are associated with EoE.[7,43,44] We have shown that upregulation of TSLP expression in patients with EoE is affected by the presence of a risk allele in the 5q22 locus.[43] Whether patient genotype at the TSLP locus accounts for patients with high *IL5* expression but low *TSLP* expression, as in group IV, will be the subject of future study.

Improved understanding of the relationship between type 2 gene expression and clinical progression may enable selection of therapeutic interventions that target IL-13, IL-4Rα, and/or IL-5. Anti–IL-13 leads to improvement in clinical outcomes in EoE and suggests the presence of responders and nonresponders.[9,13] Trials using anti–IL-5 in EoE have shown significant improvement in some patients, as evidenced by decreased eosinophilia and tissue remodeling.[5,11,14,25,26] Anti–IL-5 (mepolizumab and reslizumab) has utility in asthma but only in the eosinophilic endotype[8]; as such, it is reasonable to expect that anti–IL-5 may be useful for EoE in patients with specific endotypes. Our data support the importance of studying anti–IL-5 therapeutics in patients from groups IV and V. Moreover, a recent report suggests that factors aside from IL-5 signaling may drive eosinophil survival in allergic disease[45]; therefore, cytokine expression alone may not predict eosinophil survival, and it would be beneficial to develop functional assays to assess eosinophil responsiveness to cytokines in inflammatory patients. It is interesting to speculate that anti–IL-4Rα– and anti–IL-13–based therapeutics may be particularly effective against subgroups III and IV, which harbor the highest levels of IL-13 mRNA.

Because of the nature of the CEGIR patient cohort, in this analysis we were able to retrospectively examine the relationship between inflammatory gene expression and patient characteristics in EoE; however, we are unable to make conclusive statements regarding the patient population as a whole or over time. We found that k-means clustering using 5 clusters resulted in reproducible groupings with biologically meaningful distinctions; however, the current study is not sufficiently powered to claim that these 5 groups are intrinsic to the EoE population or to propose diagnostic or prognostic value of these clusters. Future study will be necessary to evaluate patient outcomes in relation to type 2 gene expression. Moreover, this is 1 of several studies that suggest a progression from normal-like to inflammatory and finally to fibrostenotic phenotypes. A longitudinal analysis to examine changes in patient gene expression and phenotype over time will be an important future step for the field. Finally, whereas atopic status is reported for some patients in the CEGIR cohort, this information is not available for all patients, and it is therefore not feasible to observe associations between atopic status and inflammatory gene expression. It will be important for future studies to standardize the definition of atopic status and to ensure thorough collection of this information.

In conclusion, we have identified substantial heterogeneity of type 2 cytokine–associated gene expression in EoE, resulting in the identification of 5 groups of patients with active EoE, all of which do not differ significantly in esophageal eosinophil counts. Our findings support a nonlinear model to explain EoE

pathogenesis, in which cytokine expression is heterogeneous and associated with distinct endoscopic and histologic features. Our findings also demonstrate that elevated *IL5* is not directly proportional to the degree of histologic pathology and endoscopic abnormalities, esophageal eosinophilia, and tissue remodeling. Our data support a disease model in which a T_H2-low response transitions to a T_H2-high response associated with decreased barrier function, likely in response to allergic stimuli or food antigen exposure. Effective treatment reduces eosinophil numbers and is associated with decreased inflammatory gene expression, and we propose that under certain conditions, this decrease in inflammatory gene expression results in patients reverting to a relatively normal-like gene expression signature; over time, however, patients' gene expression adopts a fibrotic profile with intermediate inflammatory expression. Intermediate T_H2 responses are observed in older patients and are associated with fibrostenotic remodeling; interestingly, eosinophil levels remain relatively stable throughout these phases. Although this hypothesis needs to be validated by longitudinal studies, we present this pathogenic model for consideration (as summarized in Fig 6, *B*). Our model is consistent with the finding that the development of fibrostenosis is proportional to the length of time for which EoE is untreated[46]; however, we are not proposing that this is the only pathway to the fibrostenotic endotype. These findings provide a potential molecular basis for heterogeneity of patient responsiveness to therapeutic intervention and an emerging opportunity for personalized medicine approaches in the EoE field.

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Clinical implications: In addition to illuminating distinct disease mechanisms, the heterogeneity of *IL5*, *IL13,* and *TSLP* expression described herein is likely to affect patient response to biologic agents that target these cytokines.

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TABLE E1. CEGIR participants

Given name	Surname	Given name	Surname	Given name	Surname
J. Pablo	Abonia	Sandeep	Gupta	Robert	Newbury
Seema	Aceves	Frank	Hamilton	Quan	Nhu
Samuel	Almonte	Kirk	Harris	Oghenekpaobor (Joel)	Oyibo
Rachel	Andrews	Jennifer	Harris	Allisa	Paliana
Ashley	Arrington	Ikuo	Hirano	Zhaoxing	Pan
Nicoleta	Arva	Girish	Hiremath	Robbie	Pesek
Fred	Atkins	Nicole	Holland-Thomas	Kathryn	Peterson
Dominique	Bailey	Lea	Jacinto	Heidi	Poppendeck
Alexis	Berry	Amir	Kagalwalla	Philip	Putnam
Bridget	Besl	Timothy	Kaseta	Fabian	Rivera
Scott	Bolton	David	Katzka	Marc	Rothenberg
Peter	Bonis	Kaitlin	Keeley	Amanda	Rudman-Spergel
Wendy	Book	Emad	Khosh-Hemmat	Kathleen	Sable
Kimberly	Bray	Paneez	Khoury	Alain	Schoepfer
Teresa	Brown	Eileen	King	Melissa	Scott
Cassandra	Burger	Kara	Kliewer	Rachel	Sheridan
Deirdre	Burke	Amy	Klion	Selma	Sinanovic
Jonathon	Cahoon	Jennifer	Knowles	Jonathan	Spergel
Kelley	Capocelli	Kendra	Kocher	MaryJo	Strobel
Mirna	Chehade	Ellyn	Kodroff	Kiki	Sun
Margaret	Collins	Jeffrey	Krischer	Amy	Tasco
Carla	Davis	Shay	Kyle	Crystal	Tholen
Evan	Dellon	John	Leung	Katherine	Thompson
Maureen	DeMarshall	Meredith	Levy	Tiffany	Tomkinson
Lauren	DiTommaso	Chris	Liacouras	Daisy	Tran
Ranjan	Dohil	Denise	Mack	Alexandra	Tylicki
Michael	Eby	Lisa	Martin	Tiina	Urv
Gary	Falk	Ellen	Martin	Mei-Lun	Wang
David	Fleischer	Talaya	McCright-Gill	Joshua	Wechsler
Heather	Foote	Paul	Menard-Katcher	Barry	Wershil
Kelci	Foss	Calies	Menard-Katcher	Lisa	Wheatley
Joel	Friedlander	Gabriela	Mendoza	Leah	Wilkey
Patricia	Fulkerson	Melissa	Mingler	Guang-Yu	Yang
Glenn	Furuta	Mike	Minnicozzi	Angelika	Zalewski
Debra	Geno	Amanda	Muir	Amy	Zicarelli
Nirmala	Gonsalves	Vincent	Mukkada		
Thomas	Greuter	Cristin	Murray Petzold		

TABLE E2. Patient characteristics by CEGIR site

Institution	Sample count	Age (y) ± SD	Male (%)	EDP score ± SD	Peak eosinophil count per hpf ± SD
Ann and Robert H. Lurie Children's Hospital of Chicago	1	8.4	100%	219	9.00
Children's Hospital Colorado	45	10.9 ± 3.7	65%	217 ± 162	28.5 ± 33.6
Cincinnati Children's Hospital Medical Center	59	13.8 ± 5.1	85%	208 ± 159	35.6 ± 31.7
Rady Children's Hospital San Diego	9	8.4 ± 2.7	50%	241 ± 204	9.75 ± 12.5
The Children's Hospital of Philadelphia	4	10.9 ± 2.5	67%	101 ± 248	23.0 ± 27.9
Tufts University	3	47.6 ± 22.9	100%	306 ± 25.2	26.0 ± 36.8
University of California San Diego	1	67.1	100%	300	13.0
University of Colorado	27	39.5 ± 8.0	50%	139 ± 146	33.7 ± 25.9
University of North Carolina School of Medicine	53	41.2 ± 14.8	51%	195 ± 165	41.3 ± 48.2
University of Pennsylvania	35	38.6 ± 13.7	67%	186 ± 156	32.0 ± 29.8

TABLE E3. Patient treatment status and history by group

	Inactive		Group I		Group II		Group III		Group IV		Group V		Total
Ongoing diet therapy	50	50%	21	55%	17	47%	17	49%	10	56%	6	60%	121
Oral systemic steroids	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0
Proton-pump inhibitor	34	34%	17	45%	15	42%	13	37%	7	39%	1	10%	87
Swallowed topical steroids	59	59%	21	55%	19	53%	13	37%	7	39%	6	60%	125
History of steroid use	68	68%	28	74%	25	69%	22	63%	14	78%	10	100%	167
Response to steroid (symptom)	50	50%	19	50%	15	42%	13	37%	8	44%	8	80%	113
Response to steroid (pathology)	44	44%	14	37%	12	33%	12	34%	7	39%	6	60%	95
Mean disease duration, y ± SD	5.8 ± 3.6		7.3 ± 7.0		5.7 ± 3.0		6.4 ± 3.8		8.4 ± 5.8		6.3 ± 4.0		
Total samples	100		38		36		35		18		10		237

TABLE E4. Histologic and endoscopic scores by group

Score	Group I	Group II	Group III	Group IV	Group V
EREFS score					
Edema	0.53 ± 0.51	0.75 ± 0.44	0.86 ± 0.36	0.94 ± 0.24	0.80 ± 0.42
Rings	0.39 ± 0.72	0.75 ± 0.87	0.51 ± 0.78	0.67 ± 0.84	0.20 ± 0.42
Exudates	0.39 ± 0.64	0.69 ± 0.75	0.69 ± 0.83	0.78 ± 0.81	0.70 ± 0.82
Furrows	0.61 ± 0.59	0.97 ± 0.65	1.20 ± 0.72	0.83 ± 0.62	1.40 ± 0.70
Stricture	0.13 ± 0.34	0.17 ± 0.38	0.09 ± 0.28	0.28 ± 0.46	0.00 ± 0.00
HSS score					
Eosinophil inflammation	3.32 ± 1.85	3.81 ± 1.92	3.37 ± 2.22	3.83 ± 1.86	4.20 ± 1.81
Basal zone hyperplasia	3.45 ± 2.20	4.03 ± 2.25	3.86 ± 2.55	4.17 ± 1.95	5.20 ± 1.93
Eosinophil abscess	0.18 ± 0.87	0.14 ± 0.59	0.17 ± 0.57	0.28 ± 1.18	0.00 ± 0.00
Eosinophil surface layering	0.42 ± 1.08	0.72 ± 1.16	0.91 ± 1.56	0.39 ± 0.92	0.80 ± 1.32
Dilated intercellular spaces	3.87 ± 2.48	4.14 ± 2.33	3.94 ± 2.65	4.44 ± 1.85	5.00 ± 1.89
Surface epithelial alteration	0.68 ± 1.34	1.14 ± 1.53	1.20 ± 1.81	1.17 ± 1.76	2.00 ± 1.83
Dyskeratotic epithelial cells	0.16 ± 0.72	0.36 ± 0.96	0.49 ± 1.38	0.78 ± 1.52	1.50 ± 2.01

Values are shown as means ± SDs.