from CSL Behring; the institution has also received grants from Grifols for other works; she has grants with CSL Behring and has received payment for the development of educational presentations from CSL Behring. R. A. Rachid's institution has received a grant from the University of South Florida for the work under consideration and grants from Aimmune Therapeutics and CSL Behring for other works; she has received consultancy fees from Access Therapeutics and Aimmune Therapeutics. C. M. Duff has received consultancy fees and grants from CSL Behring. F. A. Bonilla's institution has received a grant from CSL Behring for other works; he has received consultancy fees from CSL Behring, Baxalta, The Cowen Group, Gerson-Lehrman, Grand Rounds Health, Grifols, Harvard Pilgrim Health Care, Immune Deficiency Foundation, and Advance Medical Consultation; is employed by Blood Product Advisory Committee (US Food and Drug Administration); has received payment for lectures from Pediatric Update; received royalties from UpToDate; and support for travel from Immune Deficiency Foundation. M. E. M. Younger has received consultancy fees from CSL Behring and RSM Products and is on the advisory board for Shire. T. M. Burns has received consultancy fees from CSL Behring. The rest of the authors declare that they have no relevant conflicts of interest.

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Eosinophilic esophagitis versus proton pump inhibitor–responsive esophageal eosinophilia: Transcriptome analysis

To the Editor:

The presence of esophageal symptoms and findings of dense eosinophilia in the esophageal epithelium are suggestive of eosinophilic esophagitis (EoE).[1] According to the current clinical consensus and guidelines, histological observation of more than 15 eosinophils/hpf should be a confirming factor for EoE diagnosis by itself.[1,2] Furthermore, based on the response to high-dose proton pump inhibitor (PPI) treatment, esophageal eosinophilia has been classified into 2 entities, known as EoE (PPI-resistant) and PPI-responsive esophageal eosinophilia (PPI-REE), each showing a frequency of about 50%. Therefore, being able to distinguish EoE from PPI-REE before PPI treatment would be clinically important for improving a patient's quality of life and initiating early, effective treatment. However, all the methods tested to date, including clinical characteristics, endoscopic/histologic findings, pH monitoring, and tissue/genetic markers, have failed to distinguish EoE from PPI-REE.[3,4] That suggests that EoE and PPI-REE may be variations of a single disease entity.

Previously, Wen et al[5] used an EoE diagnostic panel and demonstrated that untreated PPI-REE shares a largely similar molecular transcriptome with EoE. Because the study was restricted to a set of 94 esophageal transcripts, there is a possibility that the remaining, unexamined transcripts contain differences between the 2 types of disease. The whole-genome mRNA expression profiles of esophageal biopsy specimens might provide an unbiased insight into the local inflammatory mechanisms at the molecular level. Therefore, we used a genome-wide approach to investigate whether EoE and PPI-REE are the same disease entity.

Detailed information about the methods is described in this article's Online Repository at www.jacionline.org. Briefly, esophageal biopsy specimens derived from patients with EoE (n = 4) and PPI-REE (n = 6) before PPI treatment and control specimens (n = 4) were subjected to genome-wide transcriptome analysis. Consequently, we confirmed the absence of major differences between EoE and PPI-REE by several analytical methods. For instance, unsupervised principal-component analysis of EoE, PPI-REE, and control specimens showed no separation between EoE and PPI-REE specimens (Fig 1, *A*). As shown in Fig 1, *B*, we identified 1263 (red circle) and 1947 (blue circle) differentially dysregulated transcripts in the patients with EoE and PPI-REE, respectively, compared with the controls on the basis of the following criteria: more than 2-fold change between the 2 groups and a *P* value of less than .05 with a false-discovery rate (Fig 1, *B*). Moreover, cluster analysis showed no separation between EoE and PPI-REE specimens (Fig 1, *C*). We show a list of 2097 dysregulated genes at our Web site: http://ee.shimane-u-internal2.jp/25.html. Gene ontology analysis of each EoE and PPI-REE transcriptome also revealed significant overlap of gene ontology terms (15 of 21 in EoE; 15 of 22 in PPI-REE) and a similar tendency for the *P* value, suggesting shared basic biological functions, processes, and components between EoE (red bars) and PPI-REE (blue bars) (Fig 1, *D*). To further examine these similarities, scatter plots comparing EoE to PPI-REE transcriptomes are shown for all genes (Fig 2, *A*), all transcription factor gene expression levels (Fig 2, *B*), and the 2097 dysregulated genes (Fig 2, *C*). When we also directly compared EoE and PPI-REE, we found 35 significant genes ($P < .05$, >2-fold change, with Westfall-Young false-discovery rate correction) whose raw signals were relatively low (see Table E2 in this article's Online Repository at www.jacionline.org).

Here, our comprehensive microarray data obtained from patients with PPI-REE substantially overlapped with the data from patients with EoE, strongly suggesting that they are the same or at least variations of a single disease. In good agreement with Wen et al's report,[5] our microarray data also showed similar increases in inflammatory markers, including eotaxin-3 (*CCL26*), cadherin 26 (*CDH26*), periostin (*POSTN*), carboxypeptidase A (*CPA3*), and desmoglein-1 (*DSG1*) (Fig 2, *C*, and gene

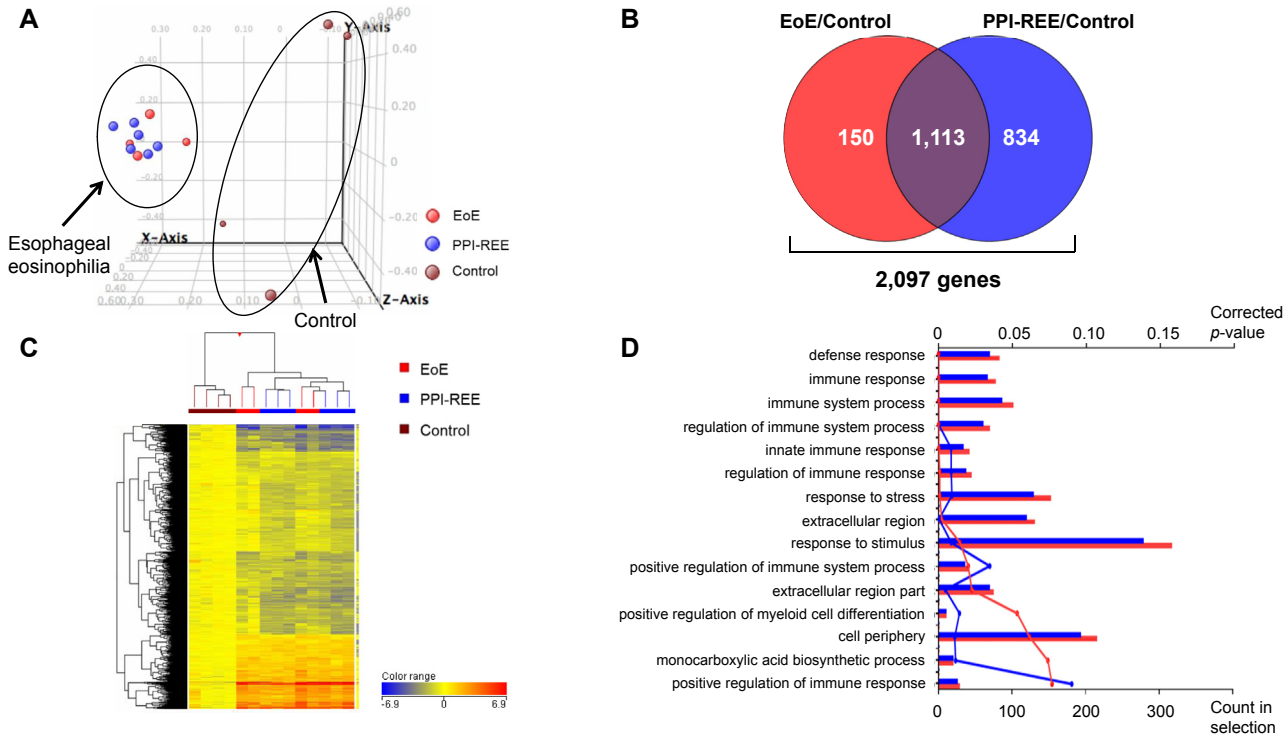

FIG 1. Expression profiles of esophageal biopsies and microarray analysis. **A,** Unsupervised principal-component analysis of esophageal eosinophilia (EoE and PPI-REE) and the controls. **B,** Venn diagrams comparing the number of genes identified as dysregulated in both EoE and PPI-REE, each primarily compared with control. **C,** Heatmap of 2097 differentially dysregulated genes' expression profiles ($P < .05$, >2-fold). Clustering analysis within each group; each column represents an individual patient or control. **D,** Gene ontology analysis of each EoE and PPI-REE transcriptome.

list at http://ee.shimane-u-internal2.jp/25.html). In addition, our analysis demonstrates that none of the transcription factor genes showed significant differences between the 2 groups (Fig 2, *B*). These observations further support our conclusion that EoE and PPI-REE should be considered to be the same disease entity.

We speculate several reasons for why only half of the patients with esophageal eosinophilia respond to PPI. First, PPI-REE might comprise less severe cases of the same disease that are mild enough to respond to PPI treatment. Indeed, in patients with PPI-REE, the 2097 significant expression profiles were reversed by PPI therapy (see gene list at http://ee.shimane-u-internal2.jp/25.html and Fig E1, *A*, in this article's Online Repository at www.jacionline.org). In particular, the reduction in eosinophils/hpf, as well as *CCL26* levels, has been reported by multiple groups on different continents.[4,5] Moreover, commonly dysregulated genes showed no major differences, but were slightly shifted to EoE, indicating minor differential molecular reactions (Fig 2, *C*). Recent studies also revealed that patients with more severe histological findings showed a lower response rate to PPI treatment.[6,7] Importantly, our data also showed that both the *CCL26* level (Fig 2, *D*) and eosinophil count (Fig 2, *E*) were higher in patients with EoE than in patients with PPI-REE. Furthermore, the *CCL26* level and eosinophil count (of each individual) were positively and significantly correlated ($r = 0.91$; $P < .0001$) (Fig E1, *B*).

Second, esophageal eosinophilia might be heterogeneous due to the time passed since disease onset. Because the primary clinical symptoms are mild to moderate in most patients, it is difficult to investigate the development of acute esophageal eosinophilia. However, a long-term follow-up study of patients with PPI-REE reported that typical features of EoE were observed in 86% of patients at some time during the course.[8] Thus, successful PPI treatment at an earlier clinical visit by these patients might have been reflected in a shorter disease duration.

Third, the composition of immune cells other than eosinophils might be slightly different. For instance, the number of group 2 innate lymphocytes in the esophageal epithelium was reported to be higher in patients with EoE than in patients with PPI-REE.[9] Such lymphocytes might be responsible for PPI-resistant inflammation, resulting in PPI-unresponsiveness.

Compared with Wen et al's study,[5] we observed minor differences, probably due in part to our smaller sample size, genetic and environmental factors, and the use of different array platforms between the studies. For instance, *KCNJ2* expression—a candidate gene for distinguishing EoE from PPI-REE—showed a tendency to be highly expressed in EoE compared with PPI-REE (Fig 2, *F*), but the difference was not significant in our study. Although smaller in scale, a strength of our study is that we performed genome-wide transcriptome analysis, providing an unbiased insight.

In conclusion, the gene expression profiles of mucosal biopsy specimens showed no major differences between patients with EoE and patients with PPI-REE before PPI treatment. Accordingly, our data suggest a single syndrome, which calls

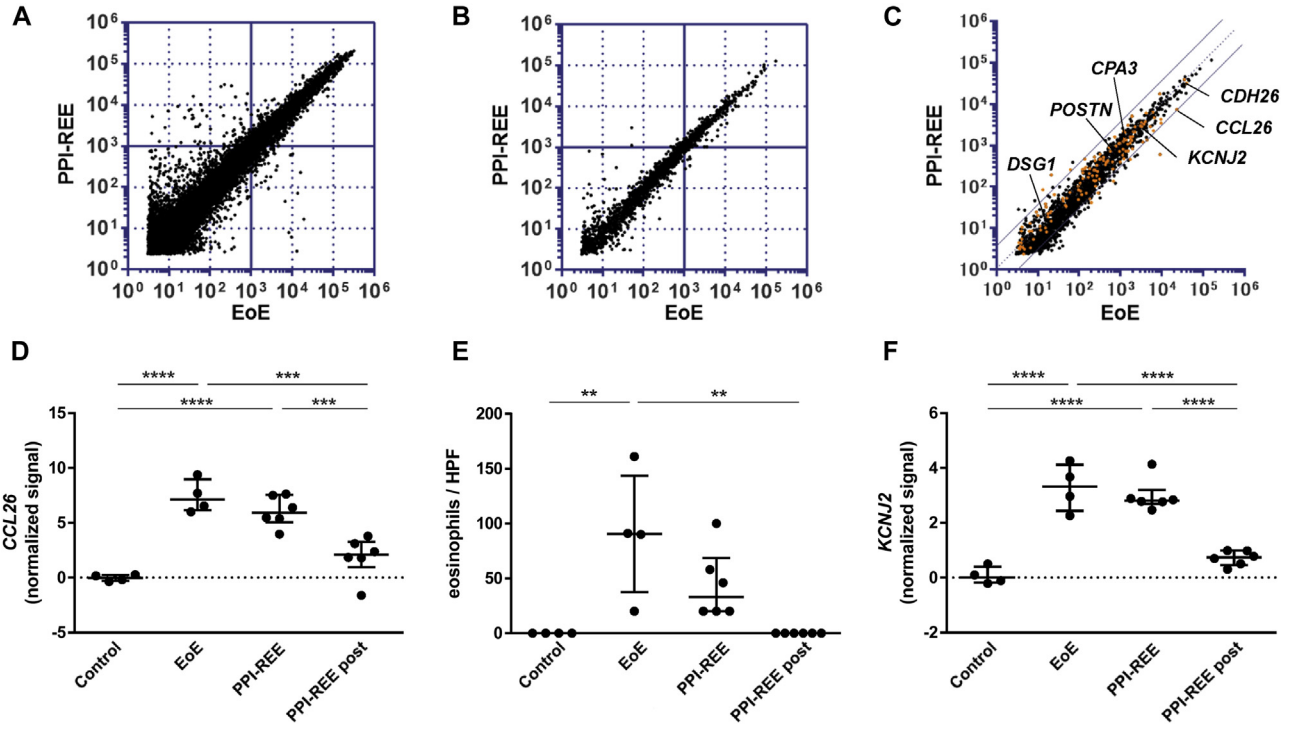

FIG 2. Comparison between EoE and PPI-REE transcriptome genes identified by microarray analysis. Spearman correlation comparing absolute raw values for all genes subjected to microarray **(A)**, all transcription factor gene expression levels **(B)**, and the 2097 differentially dysregulated genes **(C)**. Black, $P < .05$, >2-fold; orange, EoE diagnostic panel gene. **D, E,** and **F,** Eotaxin-3 (*CCL26*) mRNA expression, esophageal eosinophil counts, and *KCNJ2* mRNA expression in indicated groups are shown (****$P < .0001$, ***$P = .001$, and **$P < .01$). *PPI-REE post,* Post-PPI treated patients.

into question the current guideline position that the PPI response points to the existence of 2 distinct diseases. Finally, we note that this study has a small sample size, which means that it may lack the power to detect true effects. A follow-up study with a larger sample size is thus warranted.

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Does size really matter?: Relationship of particle size to lung deposition and exhaled fraction

To the Editor:

Particle size is the major determinant in the deposition and distribution of inhaled drug within the lungs and hence is related to local efficacy. The particle size distribution of an aerosol is usually expressed in terms of its mass median aerodynamic diameter (MMAD). Particles deposit in the respiratory tract by inertial impaction (3-6 μm), sedimentation (1-3 μm), and diffusion (<1 μm). To reach the lower respiratory tract past the carina, the MMAD of inhaled particles should be less than 5 μm in diameter; specifically, the particle size with the most efficient deposition in the small airways, so-called extrafine particle fraction, is said to be less than 2 μm.[1] It has been demonstrated that smaller particles of inhaled salbutamol achieve greater overall lung deposition, along with greater peripheral lung distribution.[2] Moreover, smaller particles of long-acting beta-agonist are also associated with improved small airways responses measured by impulse oscillometry.[3]

Although it is widely accepted that for efficient lung deposition, the MMAD should be lower than 5 μm, it is more controversial what happens to small particles with MMAD lower than 1 μm. It is conventionally believed that particles lower than 1 μm are mainly exhaled and therefore may not able to elicit any therapeutic activity within the lungs, because of their extremely low settling velocity. Conversely, it could be argued that although small particles have a greater potential to be exhaled, this is counterpoised by their capability to be distributed throughout the whole lungs and reach the distal airways with a high pulmonary deposition. We have therefore evaluated the relationship between *in vitro* MMAD and *in vivo* lung deposition (LD), and the exhaled fraction (EF) expressed as fraction of the delivered dose, using pooled analysis from relevant literature using scintigraphic studies conducted in healthy volunteers and patients with asthma using pressurized metered dose inhalers (pMDI) or dry powder inhaler (DPI). Moreover, the relationship between the ratio of EF to LD and MMAD was also evaluated.

We used pooled data (see Table E1 in this article's Online Repository at www.jacionline.org) from 18 studies, comprising 32 separate inhaled formulations, in healthy subjects (n = 173) and patients with asthma (n = 124); 21 formulations in healthy volunteers, 15 with pMDIs and 6 with DPIs, and 11 formulations in patients with asthma, 8 with pMDIs and 3 with DPIs, were evaluated. All participants were aged 18 to 65 years, and patients with asthma had a mean FEV_1 of 85% predicted. We combined the data in patients with asthma with that of healthy volunteers. All subjects were nonsmokers.

LD increased in relation to decreased MMAD, such that when the MMAD was around 1 μm the LD exceeded 50% of the delivered dose and became markedly lower when the MMAD approached 4 μm (Fig 1, *A*). Pointedly, this pattern was similar in healthy subjects and patients with asthma. The EF remained low irrespective of particle size. The mean amount of particles exhaled amounted to approximately 5% of the emitted dose, thus demonstrating that such extrafine particles are suitable for inhalation. Furthermore, the ratio of the amount exhaled to the amount deposited in the lung was found to be independent from the MMAD (Fig 1, *B*).

Our findings are consistent with previous data with inhaled salbutamol, which similarly demonstrated that total LD was greatest in particles with an MMAD of 1.5 μm compared with particles of 3 and 6 μm.[4] It could be argued that the ability to correctly perform the inhalation including an adequate breath-holding is likely to decrease the amount of exhaled drug. In this regard, in all the included studies in our cohort, inhaler technique with a given device was standardized. Previous data[5] have demonstrated that extrafine particles are able to reach the small airways more effectively and hence achieve better drug distribution throughout the whole bronchial tree. Interestingly, they also indicate that the EF is approximately 12% of the dose deposited in the lung and is totally independent from the MMAD, further confirming that extrafine particles are suitable for inhalation. The clinical relevance of extrafine particles of lower than 2 μm has been demonstrated by Nicolini et al[6] who compared extrafine (MMAD 1.1 μm) versus coarse particle hydrofluoroalkane (HFA)-beclometasone (MMAD 3.5 μm) in individuals with asthma, and demonstrated the extrafine particles significantly reduced both bronchial and alveolar exhaled nitric oxide unlike coarse particles, which reduced only bronchial exhaled nitric oxide. Moreover, extrafine HFA-flunisolide (MMAD 1.2 μm) has been shown to reduce histological evidence of eosinophilic and IL-5–mediated inflammation in peripheral and central airways after 6 weeks of treatment.[7] If these extrafine particles were mostly exhaled, as conventionally believed, one would not expect there to be an improvement in small airway inflammation as evidenced invasively and noninvasively. It should be appreciated that there will be a normal distribution of particle size around the MD such that with an MMAD of 1.1 μm there will be a proportion of particles lower than 1 μm. In this regard, such smaller particles may be absorbed from the alveoli and potentially increase systemic adverse effects, although such alveolar deposition might also contribute to antiasthmatic efficacy as shown in association with the nocturnal phenotype.[8] Our cohort of patients with asthma had an FEV_1 of 85% predicted; in this regard, it has been shown that there is evidence of small airways dysfunction in terms of a raised peripheral airways resistance occurring in approximately half of the patients who have a preserved FEV_1 of more than 80% predicted.[9]

In summary, the present data demonstrate that the EF/LD ratio is independent from the MMAD, suggesting that extrafine particles will not be associated with an appreciably higher exhaled fraction. Further prospective studies are required to

METHODS

Patient selection

We prospectively recruited patients with esophageal eosinophilia from April 2014 to July 2016. Among 16 patients who underwent gastrointestinal endoscopy before PPI treatment, 10 patients showed dense eosinophilia in the esophageal epithelium and were included in this study. RNA specimens extracted from esophageal biopsy specimens from patient 1 to 10 were of suitable RNA quality and quantity for microarray analysis. Patients 5 to 10 were paired PPI-REE post-PPI treated patients (PPI-REE post). As healthy volunteer controls, previously reported subjects (n = 4, patients 11-14) were also enrolled for microarray analysis.[E1] The clinical characteristics for the patients are presented in Table E1.

Microarray analysis inclusion criteria

EoE and PPI-REE were diagnosed in accordance with the 2011 consensus guidelines,[E2] and subjects with other possible causes of esophageal eosinophilia were excluded. Control specimens were defined as less than 1 eosinophil/hpf histologically, with no history of EoE or treatment with oral or systemic steroids. All esophageal biopsy specimens were obtained at the Department of Gastroenterology and Hepatology, Shimane University Hospital (Shimane, Japan). Informed consent was obtained from each study subject.

Specimens were placed in RNAlater solution (Qiagen, Valencia, Calif) at room temperature after biopsy and then stored at −80°C until gene expression profiling. As described previously,[E1,E3] microarray analysis (Agilent Technologies, Santa Clara, Calif) was performed according to the manufacturer's instructions. Briefly, total RNA was extracted using an RNeasy Micro kit (Qiagen) and then evaluated with an Agilent Bioanalyzer and an RNA 6000 Nano kit (Agilent Technologies). The gene expression profiles were assessed using microarray technology with Agilent SurePrint G3 Human GE 8 x 60k. Data analysis was performed using GeneSpring software version 12.5 (Agilent Technologies). To normalize variation in the staining intensity between microarrays, the average difference for all genes on a given microarray was divided by the median of all measurements on the microarray. Genes in the patients with EoE or PPI-REE that showed a significant difference in signal intensity compared with the same genes in the controls ($P < .05$, t test with Benjamini-Hochberg false-discovery rate correction) were considered to be upregulated or downregulated. Hierarchical clustering was performed using the gene expression data, contrasting patients with EoE, patients with PPI-REE, and controls.

Statistical analyses

Differences between the study groups were determined using the 1-way ANOVA followed by the Bonferroni multiple comparison posttest. Correlations were calculated using Spearman r value. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc, San Diego, Calif). A P value of less than .05 was considered significant.

Ethical approval

This study was performed according to a protocol approved by the institutional review board of National Center for Child Health and Development, Tokyo, Japan (acceptance no. 725).

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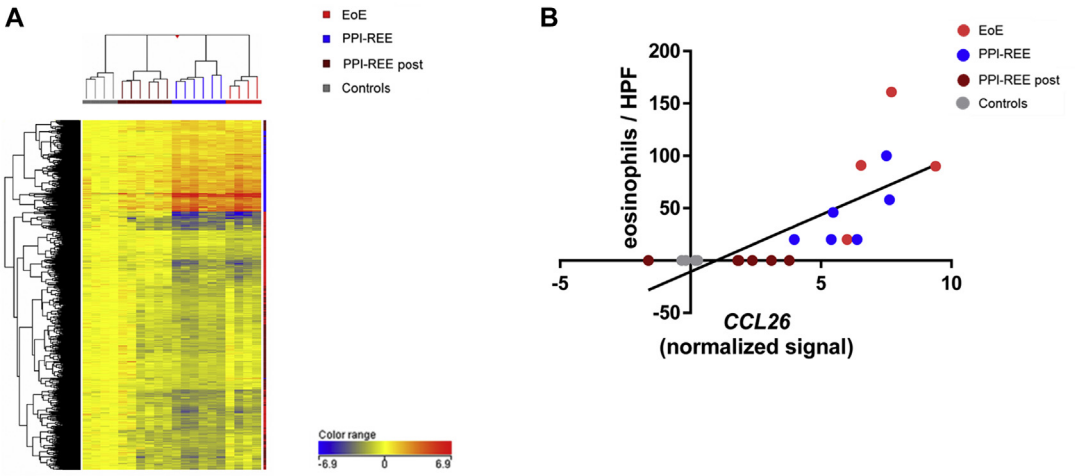

FIG E1. Comparison of esophageal transcriptomes using 2097 genes. **A,** Heatmap showing 2097 differentially dysregulated gene expression profiles ($P < .05$, >2-fold). Clustering analysis within each group was performed; each column represents an individual patient or control. **B,** Correlations between the *CCL26* level and eosinophil count for all the samples included in this study ($r = 0.91$; $P < .0001$).

TABLE E1. Clinical characteristics

							Clinical symptoms			Endoscopic findings				Eosinophils		Atopic history			
No.	Age (y)	M/F	Race	Medication at biopsy	Diagnosis	Response to PPI therapy	Dysphagia	Heartburn	Other esophageal symptom	Furrow	Concentric ring	White plaque	Stricture	Histology eosinophils/hpf	Peripheral blood eosinophils (/μL)	BA	AR	AD	FA
1	71	F	Asian	−	EoE	−	−	−	+	−	−	−	−	161	868	+	−	−	−
2	66	F	Asian	−	EoE	−	+	−	+	+	−	−	−	91	546	−	−	−	+
3	38	M	Asian	−	EoE	−	+	−	+	+	−	+	−	90	720	−	+	+	−
4	43	F	Asian	−	EoE	−	+	−	−	−	+	−	−	20	218	−	+	−	+
5	53	M	Asian	−	PPI-REE	+	+	+	−	+	+	+	−	20	298	−	+	−	−
6	43	M	Asian	−	PPI-REE	+	+	+	−	+	−	+	−	58	179	−	+	−	+
7	41	M	Asian	−	PPI-REE	+	−	−	−	+	+	+	−	46	319	−	+	+	−
8	76	F	Asian	−	PPI-REE	+	−	−	+	−	+	+	−	20	80	−	−	−	−
9	71	M	Asian	−	PPI-REE	+	+	−	−	+	+	+	−	100	81	−	+	−	+
10	48	M	Asian	−	PPI-REE	+	+	−	−	+	+	−	−	20	280	−	+	−	−
11	59	M	Asian	−	Control	NA	−	−	−	−	−	−	−	<1	NA	−	−	−	−
12	42	M	Asian	−	Control	NA	−	−	−	−	−	−	−	<1	NA	−	−	−	−
13	44	M	Asian	−	Control	NA	−	−	−	−	−	−	−	<1	NA	−	−	−	−
14	53	F	Asian	−	Control	NA	−	−	−	−	−	−	−	<1	NA	−	−	−	−

AD, Atopic dermatitis; *AR*, allergic rhinitis; *BA*, bronchial asthma; *F*, female; *FA*, food allergy; *M*, male; *NA*, not applicable.

TABLE E2. List of the 35 significant expression profiles ($P < .05$, >2-fold change)* of EoE/PPI-REE

	Fold change	Raw signal			
Probe name	EoE/PPI-REE	EoE	PPI-REE	Gene symbol	Gene name
A_33_P3350306	10.4	39.8	3.6	ZNF589	Zinc finger protein 589
A_19_P00317960	6.3	21.7	3.2	KC6	Keratoconus gene 6
A_19_P00806092	5.3	19.5	3.4		
A_19_P00327175	4.9	20.2	3.8		
A_33_P3272493	4.9	32.4	6.2	CD209	CD209 molecule
A_19_P00315832	4.7	151.0	30.2		
A_19_P00806685	4.6	52.5	10.6		
A_24_P13831	4.4	15.4	3.3	SNX20	Sorting nexin 20
A_19_P00322426	4.0	14.4	3.4	LOC441204	Hypothetical LOC441204
A_19_P00807468	3.8	39.1	9.6	lnc-HIST1H1A-1	lnc-HIST1H1A-1:2
A_33_P3397127	3.7	27.2	6.9		
A_23_P22761	3.7	20.9	5.3	SHOX	Short stature homeobox
A_33_P3234031	3.6	21.0	5.5	lnc-TBC1D29-1	lnc-TBC1D29-1:1
A_33_P3389336	3.6	20.6	5.4	LOC101929918	Hypothetical LOC101929918
A_19_P00808669	3.2	49.6	14.4		
A_33_P3365142	3.2	27.5	8.2	GAD1	Glutamate decarboxylase 1
A_19_P00331739	3.1	16.9	5.1		
A_33_P3423171	3.1	9.1	2.7	WDR6	WD repeat domain 6
A_33_P3318642	3.1	11.9	3.6	MC1R	Melanocortin 1 receptor
A_33_P3228600	3.1	11.2	3.4	CPA5	Carboxypeptidase A5
DCP_20_7	2.9	99.7	32.1		
A_33_P3404566	2.9	21.0	6.9		
A_23_P397391	2.8	49.9	16.6	FFAR2	Free fatty acid receptor 2
A_33_P3225507	2.8	109.3	36.5	OR10G2	Olfactory receptor, family 10, subfamily G, member 2
A_19_P00811814	2.7	8.9	3.1		
A_19_P00326972	2.6	169.1	60.6		
A_33_P3272628	2.6	21.5	7.7	FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide
A_33_P3327818	2.6	1452.7	524.4		
A_33_P3295690	2.5	31.4	12.0	C16orf90	Chromosome 16 open reading frame 90
A_23_P208373	2.5	81.7	31.3	CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6
A_33_P3287105	2.4	565.0	221.6		
A_19_P00322337	2.4	42.8	17.1	lnc-CBLB-4	lnc-CBLB-4:1
A_24_P132633	2.2	19.5	8.5	TMEM252	Transmembrane protein 252
A_33_P3341154	2.1	40.3	17.7	INTS1	Integrator complex subunit 1
A_19_P00316288	−3.0	7.4	21.2	IPW	Imprinted in Prader-Willi syndrome

*With Westfall-Young false-discovery rate correction.