Molecular Diagnosis of Eosinophilic Esophagitis by Gene Expression Profiling

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BACKGROUND & AIMS: Gene expression profiling provides an opportunity for definitive diagnosis, but has not yet been well applied to inflammatory diseases. Here we describe an approach for diagnosis of an emerging form of esophagitis, eosinophilic esophagitis (EoE), which is currently diagnosed by histology and clinical symptoms.

METHODS: We developed an EoE diagnostic panel (EDP) comprising a 96-gene quantitative polymerase chain reaction array and an associated dual-algorithm that uses cluster analysis and dimensionality reduction using a chain reaction array and an associated dual-algorithm that uses cluster analysis and dimensionality reduction using a cohort of randomly selected esophageal biopsy samples from pediatric patients with EoE (n = 15) or without EoE (non-EoE controls, n = 14) and subsequently vetted using a separate cohort of 194 pediatric and adult patient samples derived from both fresh or formalin-fixed, paraffin-embedded tissue: active EoE (n = 91), control (non-EoE and EoE remission, n = 57), histologically ambiguous (n = 34), and reflux (n = 12) samples.

RESULTS: The EDP identified adult and pediatric patients with EoE with approximately 96% sensitivity and approximately 98% specificity, and distinguished patients with EoE in remission from controls, as well as identified patients exposed to swallowed glucocorticoids. The EDP could be used with formalin-fixed, paraffin-embedded tissue RNA and distinguished patients with EoE from those with reflux esophagitis, identified by pH-impedance testing. Preliminary evidence showed that the EDP could identify patients likely to have disease relapse after treatment.

CONCLUSIONS: We developed a molecular diagnostic test (referred to as the EDP) that identifies patients with esophagitis in a fast, objective, and mechanistic manner, offering an opportunity to improve diagnosis and treatment, and a platform approach for other inflammatory diseases.

Keywords: EoE; GERD; Nonerosive Reflux Disease; Diagnostic Panel; Signature; Eosinophil; EoE Transcriptome; Fluidic Card.

Eosinophilic esophagitis (EoE) is a recently identified chronic, immune-mediated, clinicopathological, upper gastrointestinal (GI) disorder. It is characterized by esophageal dysfunction (eg, dysphagia) and eosinophilia of ≥15 eosinophils/high-power field (HPF) in patients for whom acid-induced esophageal injury has been excluded.1 The incidence of EoE has continued to increase since its initial characterization 2 decades ago,2 and EoE now accounts for approximately 10%–30% of chronic esophagitis refractory to proton pump inhibitor therapy and 7% of patients who undergo upper GI endoscopy.3 The treatment of EoE is distinct from other forms of esophagitis, such as gastroesophageal reflux disease (GERD), as effective management depends on elimination of the triggering food or the use of anti-inflammatory medications (eg, glucocorticoids). Until now, the only widely accepted means of diagnosing EoE was based on this histological analysis of esophageal biopsies together with clinical symptom evaluation,4 and it has been suggested that at least 5 biopsies, preferably from both the distal and proximal esophagus, are required to obtain sufficient sensitivity because of the patchiness of disease pathology.5–7 Unfortunately, esophageal eosinophilia is not specific to EoE, as it also occurs in other disease processes, including GERD, infections, and autoimmune diseases, rendering specificity of histology-based diagnosis problematic.8

One of the critical findings in understanding EoE pathogenesis was the discovery of the whole-genome messenger RNA esophageal expression profile (EoE transcriptome).9 The EoE transcriptome consists of approximately 500 EoE genes and has uncovered key pathogenic steps, such as the involvement of CCL3 in eosinophil accumulation and activation; the importance of peristin in facilitating eosinophil recruitment and tissue remodeling;10 the critical role of mast cells,11 T cells,12 and the local cytokine milieu13 in disease pathogenesis; and the importance of impaired local barrier function.14 Ideally, microarray expression can be used to provide diagnosis of EoE.9 However, the long turnaround time, the technical complexity of the messenger RNA microarray, and the associated high financial cost hinder practical clinical application for diagnostic purpose.

Abbreviations used in this paper: AUC, area under the curve; EDP, eosinophilic esophagitis diagnostic panel; EGID, eosinophilic-gastrointestinal disorder; EoE, eosinophilic esophagitis; FFPE, formalin-fixed, paraffin-embedded; GERD, gastroesophageal reflux disease; GI, gastrointestinal; HFP, high-power field; MII, multichannel intraluminal impedance; NL, normal; ROC, receiver operating characteristic.
To utilize the diagnostic strength from the microarray and reduce technical barriers, we developed a molecular EoE diagnostic panel (EDP) built on a Taqman-qPCR–based low-density array system. Here, we report 3 major steps in the development and application of the EDP. First, we successfully demonstrate that a selected representative EoE gene set (a 94-gene panel) is sufficient to provide EoE diagnosis with high sensitivity and specificity (92%–100%, 96%–100%, respectively), based on dual-computational algorithms in both pediatric and adult EoE. Second, we demonstrate that EoE remission patients can be readily distinguished from normal (NL) patients, which cannot be achieved by conventional methods. Third, we demonstrate the utility of the EDP with formalin-fixed, paraffin-embedded (FFPE)–derived tissue RNA and in distinguishing EoE from GERD, using pH-impedance testing to confirm the presence of acid-induced disease. Taken together, the EDP has promising future applications in the classification of eosinophagis and in advancing personalized medicine.

Materials and Methods

Patient Sample Selection

For algorithm-developing cohorts, NL patients were defined by the distal esophagus (where tissue RNA was obtained) having ≤1 eosinophil/HPF and by not being treated with swallowed or systemic steroids without EoE history. Patients with EoE were selected for having ≥15 eosinophil/HPF in the distal esophagus, typically when on proton pump inhibitor therapy and by not being treated with swallowed or systemic steroids. Patients with EoE remission were selected based on having partial (≤2 eosinophil/HPF) or complete (≤1 eosinophil/HPF) histological remission (mean 0.6 ± 0.7 eosinophil/HPF) after topical fluticasone propionate or budesonide treatment. The selection criterion was based on the peak eosinophil count in the distal esophageal biopsies, which is the only tissue type assayed in this report. All EoE patients tested were clinically symptomatic pediatric patients younger than 21 years old, with the exception of the adult transcriptome assay, which recruited 12 adults older than 22 years old. In a replication study and the overall study, control samples were defined as ≤2 eosinophil/HPF. For the collective FFPE diagnostic merit study and the impedance-guided study, NL was defined as 0 eosinophil/HPF with no history of EoE, and EoE was defined as ≥15 eosinophil/HPF on proton pump inhibitor therapy. For detailed clinical information, see Supplementary Table 3.

RNA Extraction and Reverse Transcription

Biopsy messenger RNA/microRNA extraction was carried out routinely by miRNeasy RNA extraction kit (217004; Qiagen, Valencia, CA); samples were archived in the −80°C eosinophil-gastrointestinal disorder (EoE) research sample library at Cincinnati Children’s Hospital Medical Center and registered in an electronic EoD database. An aliquot of RNA was reverse-transcribed to complementary DNA by the iScript cDNA Synthesis Kit (170-8891; Bio-Rad, Hercules, CA). Briefly, 500 ng RNA was mixed with the reaction mixture containing reverse transcriptase and deoxyribonucleotide triphosphates in a total volume of 20 μL; incubated at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min; and then kept at −20°C for storage.

Taqman qPCR Amplification With 384-Well Fluidic Cards

An aliquot of complementary DNA equivalent to 125–500 ng starting RNA was adjusted to 100 μL with H2O and mixed with 100 μL TaqMan Universal PCR Master Mix (4440040; Applied Biosystems, Carlsbad, CA) and loaded on fluidic cards. The standard amplification protocol consists of a ramp of 50°C for 2 minutes and a hot start of 94.5°C for 10 minutes, followed by 40 cycles of 30 seconds at 97°C and 1 minute at 59.7°C.

Esophageal pH Impedance-Guided EDP

We systematically searched the hospital’s (Cincinnati Children’s Hospital Medical Center) pH-multichannel intraluminal impedance (MII) results for patients who had the pH-MII performed for upper GI symptoms and also had an esophageal biopsy report available concurrently (gap between the 2 procedures was a mean ± 2 ± 2 days, with 2 exceptions of 65 and 143 days). These 38 patients were divided into 4 different study cohorts based on their histological report and concurrent esophageal impedance, namely NL (normal pathology, normal impedance), nonerosive reflux disease (normal pathology, abnormal impedance), GERD (abnormal pathology, normal impedance), and EoE (abnormal pathology ≥15 eosinophils/HPF, normal impedance). We defined an abnormal pH-MII result as having >80 reflux episodes in 24 hours, which is further defined as a retrograde decrease in impedance baseline that exceeded 50% of the distance between the baseline and the impedance nadir in at least the 2 distal channels. Similar criteria have recently been recognized.15

Results

Two Independent Algorithms, Cluster Analysis and EoE Score, Successfully Differentiate EoE From NL Patients

The Taqman amplification reagents for a panel of 94 representative EoE genes and 2 housekeeping genes were pre-embedded onto a 384-well fluidic card (Table 1, Supplementary Tables 1 and 2). Figure 1 offers a
schematic summary of the steps involved. For algorithm development, a random set of samples from pediatric patients including NL (n = 14) and EoE (n = 15) samples without exposure to topical or systemic glucocorticoid treatment were selected for initial EDP analysis (for clinical information see Supplementary Table 3). We identified 77 signifi-
cantly dysregulated genes out of the 94 genes after false discovery rate correction
(true discovery rate/C0 corrected P < .05 by 2-tailed t test, fold change
>2.0). As shown in Figure 2A, we initially examined NL
(blue branches) and EoE (red branches) samples with estab-
lished histological diagnosis and then setup a
2-dimensional dendrogram, clustering on both entities
(genes) and conditions (EoE status), based on a Pearson-
centered similarity algorithm. The clustering on the
Y-axis demonstrated that the up-regulated and down-
regulated EoE genes were well represented by the EDP
platform. Judging from the
first branch of the dendro-
gram on the X-axis (condition), the EoE pattern can be
readily recognized with a long-distance metrics separation
on top of the dendrogram. All of the NL samples clustered
together and all of the EoE samples grouped together
(Figure 2A). Multidimensional scaling analysis of the
77 differentially expressed EoE genes established a
3-dimensional plot based on Euclid distance between
samples (Figure 2B), which demonstrated a clear separa-
tion of EoE and NL samples.

We also developed a parallel dimensionality reduction
algorithm to calculate an EoE score (see Methods), with
the goal of developing a quantitative diagnostic cut-off. As
shown in Figure 2C, the EoE score of the 2 cohorts was
well separated with 100% disease prediction. Notably, the
EoE score cut-off (333) was determined through a larger-
scale study consisting of 132 patients (discussed later).

With the EoE score algorithm, receiver operating charac-
teristic (ROC) analysis demonstrated an excellent diag-
nostic merit with area under curve (AUC) being 1.00
(Figure 2D). There was a significant correlation between
the EoE score and esophageal eosinophil counts
(Figure 2E; P < .0001), a surrogate marker of disease
severity. Two mast cell markers, carboxypeptidase A3 and
tryptase, correlated well with each other (Figure 2F, left
indicating intra-panel reproducibility. Also, the gene for eosinophil lysophospholipase (CLC), the only highly expressed eosinophil granule protein gene, positively correlated with mast cell gene levels (Figure 2F, middle panel) and eosinophil counts (Figure 2F, right panel), corroborating earlier findings.11

In a replication study, we also performed EDP analysis on a biologically independent cohort of control (n = 14) and EoE (n = 18) patients (for clinical information, see Supplementary Table 3). The cluster analysis dendrogram predicted control (blue branch) vs EoE (red branch) with high accuracy (Supplementary Figure 1A), and the EoE

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Figure 2. Dual EDP algorithms for molecular diagnosis of EoE. (A) For the 94 EoE genes embedded, a statistical screening was performed between the 14 normal (NL) patients (blue branch) and 15 patients with EoE (red branch), resulting in 77 genes with false discovery rate—corrected P < .05 and fold change >2.0. Based on these 77 core genes, a heat map (red: up-regulated) was created, with the hierarchical tree (dendrogram) established on both gene entities and sample conditions. On the x-axis, the first branch of the top tree is utilized to predict EoE vs NL. (B) The 77-gene/dimension expression data on 14 NL controls (blue) and 15 patients with EoE (red) were reduced to 3-dimensional presentation by multidimensional scaling analysis for visual presentation of the expression distance between samples. (C) An EoE score was developed based on dimensionality reduction to distinguish EoE vs NL and quantify EoE disease severity. A diagnosis cut-off at EoE score = 333 (dashed line) was derived from later, larger-scale studies by ROC analysis. (D) ROC curve based on panel C and the EoE score = 333 cut-off, with an AUC of 1.0. (E) A linear correlation between eosinophils/HPF and EoE score, with Spearman r and P values shown. (F) To demonstrate gene amplification accuracy, representative linear regressions regarding mast cell gene intra-correlation (carboxypeptidase A3 [CPA3] vs tryptase [TPSB2]), mast cell gene/eosinophil gene inter-correlation (CPA3 vs CLC), and eosinophil gene/eosinophilia correlation (CLC vs eosinophils/HPF) are shown in the left, middle, and right panels, respectively. All scatter plots were graphed as mean ± SEM. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
The EoE score cut-off again resulted in an almost rectangular-shaped ROC curve with an AUC of 0.99 (Supplementary Figure 1C). Similarly, the EoE score strongly correlated with disease severity (Supplementary Figure 1D), as measured by eosinophils/HPF (Supplementary Figure 1E). To evaluate the inter-panel reproducibility, we selected 2 fresh RNA samples with intermediate transcriptome changes (up- and down-regulated genes), re-performed the reverse transcription and processed their complementary DNA 6 months later with different EDP fluidic cards. In order to confirm the highly reproducible gene expression signature for each gene at its raw CT level, we performed a Bland-Altman analysis on the raw CT value of each of the 96 genes (x-axis) and the CT value difference between the prior run and the average of both runs (y-axis) (Supplementary Figure 2A; test A, early run; test B, later run; blue, sample I; red, sample II). The flat line distribution along the x-axis (difference = 0) for both samples indicates that the raw CT value between different EDP amplifications are highly stable for the 96 embedded genes. To further test the correlation between the 2 independent EDP analyses, we performed the linear correlation of the 96 genes on the dual runs with Spearman r > 0.98 (95% confidence interval, Supplementary Figure 2B), indicating a highly correlated signature analysis for each gene.

**EDP Indicates a Comparable EoE Transcriptome Between Adult and Pediatric Samples**

We aimed to determine whether the EDP would be sufficient across age groups. Adult and pediatric patients with EoE (matched for eosinophil levels) were subjected to the EDP analysis (Supplementary Figures 3A and B). Notably, the 77 core gene expression signatures were largely comparable between pediatric and adult patients with EoE by both clustering and EoE score analyses (Supplementary Figures 3C and E). Multidimensional scaling analysis indicated 2 distinct groups when pediatric NL (blue dots) and EoE (red dots) samples were analyzed, indicating a large Euclid distance (Supplementary Figure 3D, left panel); in contrast, pediatric EoE (red) and adult EoE (blue) samples exhibited a mixed pattern due to a similar signature (Supplementary Figure 3D, right panel).

**EDP Identifies EoE Remission Status**

The dysregulated expression of a subset of the EoE transcriptome is resistant to steroid therapy, even when steroid therapy induces remission, i.e., the absence of tissue pathology including eosinophilia. In addition, there is a unique set of genes that are up-regulated in response to glucocorticoid exposure. Therefore, we included a selection of EoE remission genes, as well as several steroid-induced markers, in the EDP design, aiming to distinguish EoE remission and NL and to assess glucocorticoid exposure (for genes related to these purposes, see Supplementary Table 2). EDP analysis of esophageal biopsies from patients with EoE responding well to swallowed topical fluticasone propionate (Flovent, green) therapy or budesonide (Pulmicort, light blue) identified 44 and 28 dysregulated genes, respectively, with an overlap of 22 genes between the 2 steroids (Figure 3A). Within this 22-gene set, we derived a scoring algorithm, EoE remission score, to quantitatively differentiate between NL and EoE remission. Judged by an ROC-derived cut-off of EoE remission score of 74, NL and the 2 EoE remission cohorts were well discriminated (Figure 3B). The rectangular-shaped ROC curve demonstrated high diagnostic merit (Figure 3C). Notably, the 77-gene EoE definitive diagnosis cluster on the same panel could not distinguish the remission cohorts from the NL cohort, which is conceivable, as these are inactive samples (Figure 3D).

**EDP Analysis of Histologically Ambiguous Patients Identifies a Significant Subset of EDP-Positive Patients Associated With Worse Prognosis**

In clinical practice, when esophageal eosinophil numbers are less than the diagnostic threshold (eg, in the range of 6 to 14 eosinophils/HPF), a diagnostic dilemma is posed. In order to clarify potential EoE cases within this “subdiagnosis zone,” we utilized the EDP and the associated algorithms to assess the signature of this histologically ambiguous population in a cohort of 34 pediatric patients with eosinophil counts in the sub-diagnosis zone (6–14 eosinophils/HPF). Juxtaposition of the expression signatures of the histologically ambiguous patients with NL and EoE reference cohorts (Figure 4A) demonstrated that the ambiguous cohort had a unique molecular signature with EoE up-regulated genes modestly increased but EoE down-regulated genes largely unchanged. When analyzed by the EoE score algorithm, 47% of these patients (16 of 34) had a positive EDP score (Figure 4B). After dimensionality reduction, we further positioned this ambiguous cohort (green, n = 34) onto a 3-dimensional expression plot by multidimensional scaling (Figure 4C), demonstrating that the signature of these patients was distinguished from NL (blue) and EoE (red) reference cohorts with their Euclid distance closer to NL (Figure 4D). In order to study whether this initial signature change could be predictive for long-term prognosis of EoE, we examined the clinical outcomes of these 34 patients (with 6–14 eosinophils/HPF) by tracking their medical records and found that 69% of the EDP-positive patients and 33% of the EDP-negative patients developed active EoE (≥15 eosinophils/HPF) within 2 years (mean, 1.2 ± 0.5 years; range, 0.4–2 years; n = 17). In Figure 4E, by χ² test, there was a substantial risk for developing active EoE after a positive EDP (odds ratio = 4.4; P = .039).
**FFPE Compatibility and a Ph Impedance-Based Study for EoE vs GERD Differentiation by EDP Analysis**

We next explored whether the EDP was able to analyze FFPE sections from EoE and NL samples defined by histological criteria in clinically symptomatic EoE patients. Cluster analysis of NL (n = 21) and EoE (n = 24) FFPE samples demonstrated a pronounced separation between the 2 groups (Supplementary Figure 4A), resulting in a sensitivity of 96% and specificity of 100%. Likewise, the EoE score algorithm separated the same NL and EoE cohorts in the FFPE format with high merit (sensitivity 92%, specificity 100%). With an AUC of 0.96 from ROC analysis and an optimized cut-off of 355 (Supplementary Figure 4B), the performance of the EDP of FFPE samples proved to be comparable with fresh samples. In addition, we wanted to assess the reproducibility between 2 different section cuts from the same FFPE block, which is a function of both histological and technological variations. The 2 FFPE RNA extractions and EDP amplifications were performed at least 1 month apart. The raw CT values were 2-dimensionally plotted (cut 1 vs cut 2) to reveal the correlation of the adjacent section inputs (8 × 10 μm cut twice), which indicated a high reproducibility between different tissue inputs on the same archived tissue (Supplementary Figure 4C). Finally, we evaluated the correlation between a FFPE biopsy and a fresh biopsy taken simultaneously from the same patient; notably, a high correlation for individual gene expression was observed (Supplementary Figure 4D). Across larger cohorts, comparing the algorithm-developing cohort (fresh, n = 29) and the FFPE cohorts (n = 45), the dysregulation vectors (fold change EoE over NL; red indicating up-regulated genes; blue for down-regulated) robustly correlate between fresh and FFPE samples (r² = 0.87, Supplementary Figure 4E). Supplementary Figure 4F, left panel illustrates a representative 230–280 nm spectrometry result for RNA isolated from 80-μm FFPE sections with high RNA purity and microgram-level abundance. RNA integrity analysis by the Agilent 2100...
Bioanalyzer revealed substantial FFPE RNA degradation as a function of time (Supplementary Figure 4F, right panel); however, the EDP performance was unaffected, as the FFPE samples had a conserved signature pattern compared with fresh RNA, even though the RNA was 0–3 years old (Supplementary Figure 4A, B). Collectively, these

Figure 4. EoE transcriptome pattern in the patient population with ambiguous eosinophil (EOS) levels, 6–14 EOS/HPF. (A) To acquire the esophageal signatures of 34 patients with noticeable eosinophilia that did not exceed the diagnostic cut-off (6 < EOS/HPF < 15), EDP-based expression signatures were juxtaposed with the reference normal (NL, 0 EOS/HPF) and eosinophilic esophagitis (EoE, >15 EOS/HPF) cohorts. (B) The EoE score algorithm was utilized to assess EoE signature within the population with ambiguous EOS levels of 6–14 EOS/HPF (6–14/HPF). The EoE score scatter plot indicates that approximately 47% (16 of 34) patients in this subdiagnosis zone were EDP positive, as determined by the 333 EoE diagnostic cut-off (dashed line). (C) Multidimensional scaling analysis was carried out to visualize the expression difference between the 6–14 EOS cohort (green) and NL (blue), and EoE (red) reference cohorts. (D) The average (Avg) Euclid distances from the 6–14 EOS cohort to the NL and EoE cohorts, respectively, were graphed as mean ± 95% confidence interval, revealing their collective Euclid distance to NL and EoE reference cohorts, respectively, ***P < .001. (E) All 34 patients with ambiguous EOS levels (6–14 EOS/HPF) were clinically followed for 2 years based on their medical record. The association between EDP results and subsequent active EoE (>15 EOS/HPF) (in mean, 1.2 ± 0.5 years) was graphed in the \( \chi^2 \) table. The odds ratio of 4.4 was calculated based on the relative risk factor for EoE development, EDP-positive (EoE score <333) vs EDP-negative result, with a significant \( P \) value = .039 by \( \chi^2 \) test.
Overall EDP Analysis on a Larger Scale

We next aimed to evaluate the diagnostic merits of the EDP by examining the transcription signatures of 166 fresh RNA samples. Among the 166 patients, 132 had eosinophil counts in the ranges in which diagnosis was considered unambiguous (eg, ≤2 or ≥15 eosinophils/HPF), forming the control and EoE pool, respectively (for clinical information see Supplementary Table 3). The clustering analysis indicated the distinct cohorts of EoE transcriptome and NL-like transcriptome (Figure 6A). Under the first branch, the uniformity of tree color reflects diagnostic merit. Using the EoE score algorithm (Figure 6B) and ROC cut-off optimization (Figure 6C), we demonstrated that the EDP had an excellent coverage between sensitivity (92%) and specificity (96%) and an high AUC of 0.97 (Figure 6C and D). In order to assess whether EoE score could reflect disease severity as measured by histology (eg, eosinophil counts/HPF) for these 166 cases, we performed nonparametric regression analysis with the predicted model shown in red dots and the true values shown in blue dots (Figure 6E). The $r^2$ of 0.68 is indicative of a strong correlation between the 1-dimensional EoE score and tissue eosinophilia, which is the currently accepted gold standard for measurement of disease activity.

Discussion

EoE is an emerging and enigmatic disease, whose diagnostic standards still remain debatable. In this report, we demonstrated a readily performed molecular platform providing differential diagnosis of EoE vs NL and inactive EoE, EoE vs non erosive reflux disease/GERD, and EoE remission vs NL by dual-computational algorithms. The EDP panel was shown to be FFPE sample compatible with comparable merit to analysis of fresh tissue and applicable to pediatric and adult EoE. The EDP also demonstrated the prediscptive capacity for patients with subclinical histology, suggesting that these patients should be tightly monitored as an EoE high-risk population and that the EDP might potentially be used as a personal medicine prediction device. The EDP has the potential to overcome the limitations of histological analysis, as it provides potentially deeper insight into tissue processes that are not all visible microscopically or that can be microscopically patchy, highlighting the transformative value of using molecular parameters compared with histology for the diagnosis of inflammatory diseases. In addition, the EDP has the capacity to reveal EoE pathogenesis that could vary from patient to patient, forming the basis for practicing personal medicine. It is important to note that the EDP has a high performance using only 1 distal esophageal biopsy, even though consensus recommendations include procurement of 5 biopsies.

The EDP can readily differentiate topical steroid–induced EoE remission samples from NL samples by both algorithms—a capacity histology does not have.
and subjective steroid compliance can be objectively predicted with steroid-responding biomarkers. Besides the steroid intervention assessment, a future EDP study should also be performed in the context of diet retreatment. It would be interesting to prospectively evaluate the effects of multiple forms of diet therapy, such as elimination vs elementary, in reference to steroid effects. Importantly, it is proposed that, with a paired-sample

**Figure 6.** Overall assessment of EDP merit. To assess the dual algorithms of the EDP on a larger scale, we collected EDP signatures for a total of 166 patients, in which there are 82 patients with EoE (≥15 eosinophils/EOS)/HPF and 50 control patients (<2 EOS) by histology. (A) With the clustering algorithm by first branch, a double-clustered heat map (red branch, EoE; blue branch, control) indicates that EDP clustering algorithm is highly competent at positive prediction, with only one control misdiagnosed as EoE (single blue branch in the EoE cluster on the left). (B) EoE scores from histology-defined 50 control patients and 82 patients with EoE were plotted over the 333 diagnostic cut-off (determined here and used throughout this report), resulting in an optimized balance between sensitivity and specificity. The scatter plots were graphed as mean ± SEM. (C) The ROC curve of EDP diagnosis in (B) with 132 patients in reference to histological method. (D) On the basis of the EoE score analysis in (B) and (C) with the histology as gold standard, the EDP’s diagnostic merit was summarized in specificity vs sensitivity and positive predictive value vs negative predictive value, reflecting the diagnosing power in clinical practice. (E) The correlation between tissue eosinophilia (eosinophils/HPF) and EoE score was analyzed by LOWESS (Locally Weighted Scatterplot Smoothing) nonparametric regression and plotted with the predicted trend (red) and realistic values (blue), indicating a negative relationship with $r^2 = 0.68$ and reflecting disease severity.
study before and after EoE treatments, the EDP (in its current form or with modified composition) will predict which treatment is more effective (eg, reversing the EDP signature). This approach will be critically important for selection of the most effective intervention for EoE patients, which is currently not fully agreed on.

In clinical practice, physicians often face a diagnostic dilemma when patients with esophagitis have clinical symptoms comparable with EoE yet have endoscopic findings with <15 eosinophils/HPF. The cut-off value of 15 eosinophils/HPF has been questioned recently, even in a recent Consensus Report. Even within 1 biopsy, the eosinophil number varies from site to site. The EoE score algorithm indicated that 47% of the histologically ambiguous patients are molecularly equivalent to active EoE patients. Fifty percent of these histologically ambiguous patients later developed histologically active EoE within a 2-year period, indicating that these patients are at high risk for active EoE. A positive EDP coupled with subclinical nonremission histology (6–14 eosinophils/HPF) increased the likelihood of fully active EoE in the next 2 years, as indicated by the 4.4-fold odds ratio between EDP-positive and EDP-negative histologically ambiguous patients, demonstrating a potential predictive medicine capacity. Although these data are limited by the nonprospective design, they are consistent with the recent finding that long-term consequences of esophageal eosinophilia emerge at eosinophil levels >5 eosinophils/HPF and with another study showing that 36% of the “low-grade esophageal eosinophilia” cases (1–14 eosinophils/HPF) are truly EoE, as proved by subsequent repetitive endoscopy.

An EDP analysis of 177 patient samples (132 fresh and 45 FFPE) achieved the historically high approximately 98% specificity and approximately 96% sensitivity using the EoE score (ΔACT) algorithm. Notably, it is likely that the diagnostic merit of EDP is underestimated because the caveat for this study is using the histological method as the “gold standard.” As mentioned previously, the 15 eosinophil cut-off itself is debatable due to the heterogeneity of eosinophilia, the variability of counting, and shared eosinophilia with non-EoE diseases. On the other hand, although the EDP demonstrated unprecedented performance using samples from our institution and was able to perform well with fresh and FFPE samples from other sites in multi-centered studies (data not shown), an external validation will be helpful to strengthen the EDP application. With high diagnostic merit, a value for personalized medicine, and ability to distinguish EoE remission tissue from healthy tissue, the EDP represents the next generation for EoE diagnosis. Although histological diagnosis by eosinophil count and an empirical eosinophilia cut-off will still prove to be useful under certain circumstances, the EDP offers an accurate, rapid, informative, and low-cost diagnosis based on the EoE transcriptome as a biomarker for evaluating therapeutic interventions. Our results provide proof-of-principle for the application of tissue-based molecular diagnosis of inflammatory diseases, especially for esophagitis and a growing number of other eosinophil-associated inflammatory diseases, such as asthma.

Supplementary Material
Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.08.046.

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Conflicts of interest
These authors disclose the following: Marc E. Rothenberg serves as a consultant for Immune Pharmaceuticals and has an equity ownership; he also has a royalty from reslizumab, a drug being developed by Teva Pharmaceuticals. Neither of these interests is directly related to this article. Marc E. Rothenberg and Ting Wen are co-inventors for a pending patent based on the EDP test described here. The remaining authors disclose no conflicts.

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