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Utility of a non-invasive serum biomarker panel for diagnosis and monitoring of eosinophilic esophagitis: A prospective study

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Abstract

Objectives—Non-invasive biomarkers would be valuable for diagnosis and monitoring of eosinophilic esophagitis (EoE). The aim of this study was to determine the utility of a panel of serum biomarkers for the diagnosis and management of EoE.

Methods—We conducted a prospective cohort study of consecutive adults undergoing outpatient EGD. Incident cases of EoE were diagnosed per consensus guidelines; controls had GERD or dysphagia and did not meet EoE criteria. EoE cases were treated with topical steroids and had repeat endoscopy. Pre- and post-treatment serum samples were analyzed in a blinded fashion for: IL-4, IL-5, IL-6, IL-9, IL-13, TGF-α, TGF-β, TNF-α, eotaxin-1, -2, and -3, TSLP, major basic protein (MBP), and eosinophil-derived neurotoxin (EDN). Cases and controls were compared at baseline, and pre- and post-treatment assays were compared in cases.

Results—A total of 61 incident EoE cases and 87 controls were enrolled; 51 EoE cases had post-treatment serum analyzed. There were no significant differences in any of the biomarkers between

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None of the other authors have any relevant conflicts of interest to disclose.

EoE cases and controls at baseline. IL-13 and eotaxin-3 for cases and controls were 85 ±160 vs 43 ±161 pg/mL (p=0.12), and 41 ±159 vs 21 ±73 (p=0.30). There were no significant differences in assay values among cases before and after treatment. There were also no differences after stratification by atopic status or treatment response.

Conclusions—A panel of inflammatory factors known to be associated with EoE pathogenesis were not increased in the serum, nor were they responsive to therapy. None of these biomarkers are likely candidates for a serum test for EoE. Histologic analysis for diagnosis and management of EoE continues to be necessary, and novel, less invasive, biomarkers are needed.

Keywords

Eosinophilic esophagitis; biomarkers; serum; inflammation; cytokines

Introduction

The current diagnostic algorithm for eosinophilic esophagitis (EoE) requires upper endoscopy and biopsy, an invasive procedure, to assess for esophageal eosinophilia in patients with symptoms of esophageal dysfunction (1-3). In practice, several procedures are needed: the index endoscopy where the diagnosis is suspected, the follow-up endoscopy to confirm the diagnosis after a proton pump inhibitor (PPI) trial, and a third endoscopy to assess tissue response to therapy (1, 4). This approach is suboptimal due to high costs associated with the multiple procedures (5) as well as the possibility of procedural complications. Non-invasive biomarkers hold the potential to decrease costs and increase safety, but none have been clinically validated for routine use in EoE (6-8).

Candidate biomarkers could be selected from the pathogenesis of EoE, which is currently thought to involve a Th2-mediated response to allergens (9-12). A number of cytokines, including IL-4, IL-5, and IL-13 (13-18), chemokines such as eotaxin-3, which is the most highly upregulated gene in EoE (15, 19-21), and markers of eosinophil activation such as granule proteins (18, 22-24), have all been shown to be elevated in EoE as compared to controls. However, these findings have been generally reported in esophageal tissue, and data are primarily related to pathogenic studies in EoE (15, 19-21, 25-29). The true clinical utility of Th2-related cytokines, chemokines, and eosinophil granules as non-invasive serum biomarkers has yet to be demonstrated for either diagnosis or monitoring of treatment for EoE. Given the high cost of diagnosis and management of EoE using endoscopy findings, the translation of the research findings above into a viable serum test for the presence and/or severity of EoE would be of enormous value.

The aim of this study was to determine whether a panel of serum biomarkers based on the known pathogenesis of EoE could distinguish EoE from controls at baseline for diagnosis of EoE. We additionally sought to determine whether these biomarkers might have utility for monitoring EoE after treatment. We hypothesized that subjects with EoE would have significantly higher serum levels of one or more of these biomarkers, compared to clinically relevant non-EoE controls, and that these levels might decrease among the EoE cases after effective steroid therapy.

Methods

Study design, patients, clinical data, and follow-up

We conducted a prospective cohort study at University of North Carolina from July, 2011 through December, 2013. Consecutive adult patients (age 18-80 years) undergoing routine outpatient esophagogastroduodenoscopy (EGD) were approached if they had upper GI symptoms suggestive of esophageal dysfunction (e.g. dysphagia, food impaction, heartburn, reflux, chest pain). Subjects provided informed consent, including consent for future use of stored specimens, and were enrolled prior to the endoscopy. Subjects were excluded if they had a known (prevalent) diagnosis of EoE or a different eosinophilic gastrointestinal disorder (EGID), GI bleeding, active anticoagulation, known esophageal cancer, prior esophageal surgery, known esophageal varices, medical instability or multiple comorbidities precluding enrollment in the clinical opinion of the endoscopist, or inability to read or understand the consent form. This study was approved by the UNC Institutional Review Board and registered on clinicaltrials.gov (NCT 01988285).

Cases were diagnosed with EoE if they met consensus guidelines (1-3). Specifically, they were required to have at least one typical symptom of esophageal dysfunction; at least 15 eosinophils per high-power field (eos/hpf) on esophageal biopsy persisting after an 8 week PPI trial (20-40 mg twice daily of any of the available agents, prescribed at the discretion of the clinician); and other causes of esophageal eosinophilia excluded. Of note, baseline data for the EoE cases were obtained after the PPI trial, at the time of the confirmatory EGD, but prior to receiving the histologic results confirming the diagnosis or provision of EoE-specific treatment, so as to minimize potential recall bias. Controls were subjects who, after endoscopy and biopsy, did not meet clinical or histologic criteria for EoE. Subjects with PPI-responsive esophageal eosinophilia (PPI-REE) were not included in this study.

Clinical data were collected using a standardized case report form. Items recorded included demographics, symptoms, concomitant atopic diseases, indications for endoscopy, and endoscopic findings. Food allergy data was collected by patient self-report on a prospectively administered questionnaire, and could therefore include both food allergies and sensitizations. Systematic allergy testing was not a component of this study. During endoscopy, research-protocol esophageal biopsies were obtained (two from the proximal, one from the mid, and two from the distal esophagus) to maximize EoE diagnostic sensitivity (30, 31). Gastric and duodenal biopsies were also collected for research purposes to exclude concomitant eosinophilic gastroenteritis. Additional clinical biopsies were taken as indicated at the discretion of the endoscopist. Esophageal eosinophil counts were quantified by the study pathologists using our previously validated methods (32). In brief, slides were masked to case/control status, digitized, and reviewed with Aperio ImageScope (Aperio Technologies, Vista, CA). Five microscopy fields from each of the five biopsies were examined to determine the maximum eosinophil density (eosinophils/mm^2 [eos/mm^2]). So results could be compared to prior studies, eosinophil density was converted to an eosinophil count (eos/hpf) using a hpf size of 0.24 mm^2, the most commonly reported field size in the literature (33).

EoE cases were treated for 8 weeks as clinically indicated with topical corticosteroids (either oval viscous budesonide 1 mg twice daily or fluticasone from a multi-dose inhaler, 880 mcg twice daily) (34-36). At the end of the treatment period, repeat upper endoscopy with biopsy was performed, with collection of a second set of blood and tissue samples as noted above. A second blood sample was also collected for a subset of control subjects at least 2 months after baseline samples were collected to assess for stability in biomarkers over time.

Serum data and biomarkers

Prior to each procedure, a blood sample was obtained from all subjects and centrifuged. Serum was separated and aliquots were frozen and stored at −80°C. All samples were labelled with a unique study ID that was blinded as to case/control status, as well as to pre- or post-treatment status. After patient enrollment and follow-up were complete, samples were removed from the freezer, arranged in random order, thawed only once, and analyzed in a batch.

The serum analytes measured were: IL-4, IL-5, IL-6, IL-9, IL-13, TGF-α, TGF-β, TNF-α, eotaxin-1, -2, and -3, TSLP, major basic protein (MBP), and eosinophil derived neurotoxin (EDN). These were chosen based on prior demonstration of elevated levels in the esophageal tissue and/or in a peripheral source (6, 7, 13-19, 21-29, 37-40). IL-4, IL-5, IL-6, IL-9, IL-13, TGF-α, TNF-α, and eotaxin-1 were measured using an 8-plex panel (cat # HCYTOMAG-60K, Millipore, St. Charles, MO). Eotaxin-2, eotaxin-3, and TSLP were measured using a 3-plex panel (cat# HCYP2MAG-62K, Millipore). TGF-β (cat# 559119, BD Biosciences, San Diego, CA) was measured individually via ELISA, as were MBP (cat# ABIN1115874) and EDN (cat# ABIN858221, ABO). Samples were run in duplicate on 96-well plates with standards and positive/negative controls per manufacturer instructions. A Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA) was used to determine the mean fluorescence intensity of the multiplex assays. For a subset of EoE cases and non-EoE controls, immunohistochemical staining was used to measure tissue levels of MBP, eotaxin-3, and mast cell tryptase with methodology we have previously described (23, 24, 41).

Statistical analysis

Baseline clinical, endoscopic, and histologic characteristics of the cases and controls were described with bivariate analysis using Chi-square for categorical variables, and t-tests or Wilcoxon Rank-sum for continuous variables as appropriate. The mean baseline values of the individual serum biomarkers were compared between cases and controls using a 2 sample t-test, while baseline and follow-up values for the EoE cases were compared using a paired t-test or Wilcoxon Signed-rank as appropriate. Additional analyses were performed for the serum values after stratification for: 1) the presence of atopic diseases (ie asthma, atopic dermatitis, allergic rhinitis/sinusitis, and food allergy); and 2) histologic response to treatment for the EoE cases, defined as either <15 eos/hpf or <1 eos/hpf (4, 42). Because results were the same with both parametric and non-parametric testing, means and standard deviations are presented in the figures. Receiver operating characteristic (ROC) curves were constructed and areas under the curve (AUC) were calculated to determine the utility of the serum biomarkers, both individually and collectively, for distinguishing EoE cases from

controls at baseline and for monitoring response following treatment. The sample size was determined based on the ROC analysis. Enrolling at least 60 cases EoE and 60 controls was expected to provide >80% power to conclude that the AUC for an individual marker was significantly greater than 0.50 (43).

Results

Patient characteristics

A total of 61 EoE cases and 87 non-EoE controls were included in this study. Compared to controls, EoE cases at diagnosis were more likely to be younger (39 vs 52 years; $p < 0.001$), male (58% vs 42%; $p = 0.05$), white (94% vs 82%), and have an atopic disorder (74% vs 54%; $p = 0.01$) (Table 1). Dysphagia was common in both groups, but more common in the cases (97% vs 80%; $p = 0.002$). Heartburn was less common among cases (16% vs 67%; $p < 0.001$), as was the presence of a hiatal hernia (13% vs 57%; $p < 0.001$). As expected, the typical endoscopic findings of EoE were also more common in cases (Table 1). The mean of the maximum eosinophil counts in the cases was 146 eos/hpf, compared to 3 for the controls ($p < 0.001$). After treatment of the EoE cases, the mean eosinophil count decreased to 55 eos/hpf ($p < 0.001$ compared to baseline), with 55% achieving a histologic response of <15 eos/hpf (mean post-treatment eosinophil count of 3 eos/hpf), and 28% achieving normalization of the biopsies (<1 eos/hpf).

Baseline serum biomarkers

At baseline, there were no significant differences in any of the biomarkers between cases and controls (Figure 1). For example, mean values of IL-5, IL-13, eotaxin-3, and TSLP for cases and controls were 22 ±64 vs 10 ±47 pg/mL ($p=0.21$), 85 ±160 vs 43 ±161 ($p=0.12$), 41 ±159 vs 21 ±73 ($p=0.30$), and 15 ±33 vs 19 ±109 ($p=0.77$). Median values for the same comparisons did not change the results. ROC analysis confirmed that there was little diagnostic utility for the biomarkers either individually (AUCs ranging from 0.40 to 0.68) or in sum (AUC 0.69). There were also no differences between cases and controls after stratification by atopic status (data not shown). Of note, tissue levels of MBP, eotaxin-3, and mast cell tryptase were markedly elevated in esophageal biopsies from a subset of cases as compared to controls (Supplemental Table).

Post-treatment serum biomarkers

A total of 51 EoE cases had paired pre- and post-treatment serum available for analysis. There were no significant differences detected overall before and after treatment (Figure 2a). For example, values of IL-5, IL-13, eotaxin-3, and TSLP pre- and post-treatment were 19 ±57 vs 17 ±56 pg/mL ($p=0.81$), 71 ±152 vs 43 ±95 ($p=0.18$), 42 ±174 vs 28 ±76 ($p=0.41$), and 14 ±33 vs 18 ±40 ($p=0.29$). Median values for the same comparisons did not change the results. There were also no differences detected after stratification by treatment responder status, either at the <15 eos/hpf level (Figure 2b) or at the <1 eos/hpf level (data not shown). There were 17 controls with follow-up specimens, and there were no differences in any of the serum biomarker levels over time in this group (Supplemental Figure).

Discussion

Given the serial upper endoscopies required for diagnosis and monitoring of EoE, current clinical practice is cumbersome for patients, invasive, and expensive. Non-invasive methods for diagnosis and monitoring would be extremely valuable in this condition, and a serum test for a biomarker panel would be ideal. In this prospective study that collected clinical, endoscopic, histologic, and biologic data on incident cases of EoE and non-EoE controls, we investigated the utility of a large number of the most promising serum biomarkers for both diagnosis and monitoring of EoE. Despite choosing markers shown in multiple studies to be increased in esophageal tissue in EoE, we were unable to demonstrate any difference in the serum measures between cases and controls, nor did we find any that were responsive to treatment. These results were unaffected by atopy status of the cases and controls, or by the level of treatment response measured histologically.

Previous studies have identified multiple biomarkers that are characteristic of EoE at the esophageal tissue level. These include Th2-related cytokines, most notably IL-5 and IL-13, chemokines such as eotaxin-3, eosinophil granule proteins, microRNAs, and mast cells (13-23, 25-29, 37, 38, 41, 44-49). However, data on serum biomarkers are sparse and conflicting, and primarily come from either small studies examining mechanisms of EoE or sub-analyses of clinical trials focusing on a few cytokines, chemokines, or granule proteins. The preponderance of the data are also in children.

Konikoff and colleagues studied 47 children, 16 of whom had active EoE, and assessed a number of plasma biomarkers, including IL-5, eotaxin-1, -2, and -3, and EDN (19). They found that plasma EDN and eotaxin-3 correlated with tissue eosinophil levels and were also significantly increased in active EoE compared with controls, though the differences were mild (50.3 vs 31.1 ng/mL for EDN and 37.7 vs 11.5 pg/mL for eotaxin-3). Notably, they did not report differences in IL-5, eotaxin-1, or -2. Changes in eotaxin-1 and -3 were also not observed following treatment with an anti-IL-13 antibody in a recent trial (50). In contrast, a mechanistic study of the role of fibroblast growth factor by Huang and colleagues showed elevated levels of plasma IL-5 and IL-13 in 35 pediatric EoE cases compared with 8 healthy controls (51). IL-5 was also noted to be elevated in EoE compared to controls in a study of eosinophil function in 12 adults with EoE (40). Subbarao and colleagues studied serum IL-5 and EDN in 60 children with EoE and 20 controls and found that while EDN levels were significantly higher in cases compared to controls (23.5 vs 2.7 ng/mL), IL-5 levels were not (18). They also found that serum EDN levels significantly decreased after treatment. Of studies that have examined serum eosinophil cationic protein (ECP) after treatment for EoE, two studies showed that ECP decreased after treatment (52, 53) while one found no change (54). We are unaware of any prior published studies examining serum MBP in EoE. Our study does not confirm the previous findings related to EDN or IL-5, and suggests that none of the panel of cytokines, chemokines, and eosinophil granules that we examined has utility as a biomarker panel.

When interpreting the data from this study, there are potential limitations to consider. Because the results were not positive, there is the possibility of a type II error. However, this is the largest study to date assessing biomarkers for diagnosis and monitoring of EoE, and it

was powered to detect clinical meaningful differences based on ROC curve analysis. It is possible that different power calculation methods could have increased the sample size required, but given the lack of suggestive trends in our data, if any statistically significant differences between the assays could be detected with larger numbers of subjects, the clinical utility of such differences would be doubtful. Differences in specimen handling or degradation over time are unlikely to have contributed to our results, and analysis of a subset of controls with samples over time showed stability in the biomarker measures. All samples were handled identically, remained frozen until analysis, and were there to be degradation it would likely be non-differential for both the case and control groups. Finally, we did not include a PPI-REE group in the study, as the main goals were to develop biomarkers to detect EoE and monitor the response of EoE to treatment over time, not to distinguish EoE from PPI-REE. Therefore, we cannot comment on the utility of these serum biomarkers in patients with PPI-REE. While it has been recently reported that PPIs have anti-inflammatory and anti-eosinophilic effects independent of their anti-acid effect (55, 56), because the EoE cases in the present study all had a high tissue level of eosinophilic inflammation despite PPI use, it is not likely that PPIs impacted serum biomarker levels, but we are not able to test this directly with our study design.

Given the lack of signal in the serum for our biomarker panel, it may be that the brisk esophageal inflammation is not reflected systemically. While we did not measure the levels of the serum biomarkers in the esophageal biopsy specimens of the subjects included in this study either at baseline or after steroid treatment, we feel that it would be unlikely for baseline tissue levels to be low in these patients. For example, the EoE cases were highly inflamed, with an average peak esophageal eosinophil count of 146/hpf, and a subset of the EoE subjects who did have staining for MBP, eotaxin-3, and mast cell tryptase had markedly elevated tissue levels compared to controls. Minimally invasive diagnostic techniques are under development to sample the esophagus (57, 58), and these might be more amenable to a biomarker panel.

Our study also has a number of strengths. It was a prospective study specifically designed to evaluate biomarkers in a population of well-characterized incident EoE cases and non-EoE controls. The omission of prevalent cases makes it impossible that previous medical or dietary therapy for EoE would account for any observed differences between cases and controls. It is the largest study to date focusing on biomarkers for diagnosis and monitoring of EoE, and the only one of its kind to be done in an adult population. Uniform methods were used for case/control identification, sample handling, and analysis, and all baseline samples were obtained prior to the EoE diagnosis being known. Follow-up samples were obtained with identical methods as at baseline, and outcomes (eosinophil counts; biomarker levels) were quantified in blinded fashion, using rigorous, previously validated methods. These methodologic strengths make the data reliable and valid.

In conclusion, in this large prospective study, a panel of inflammatory markers associated with EoE pathogenesis and known to be elevated in esophageal tissue of patients with EoE were not increased in the serum of EoE patients at baseline compared to non-EoE controls. These markers also were unresponsive to treatment, even in the face of marked decrements in the esophageal eosinophil count. Therefore, none of these biomarkers are likely

candidates for a serum test. Instead, focus should move to novel blood-based biomarkers for diagnosing and monitoring EoE, as well as the development of more economical, non-endoscopic methods of sampling the esophageal mucosa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Study highlights

What is current knowledge?

- Diagnosis and management of eosinophilic esophagitis (EoE) currently requires repeated upper endoscopy with biopsies, an invasive and expensive procedure.
- Previous studies have suggested that inflammatory factors can be detected at elevated levels in the blood of patients with EoE, but their clinical utility for diagnosis and monitoring EoE is not established.

What is new here?

- This large prospective cohort study of adults with EoE analyzed a panel of serum biomarkers based on the known pathogenesis of EoE.
- There was no difference in serum biomarker levels of IL-4, IL-5, IL-6, IL-9, IL-13, TGF-α, TGF-β, TNF-α, eotaxin-1, -2, and -3, TSLP, major basic protein (MBP), or eosinophil-derived neurotoxin (EDN) in EoE cases compared to non-EoE controls at baseline.
- There was also no difference in the biomarker levels before or after treatment of EoE cases with topical corticosteroids.
- Novel serum biomarkers are needed for less invasive detection and monitoring of EoE.

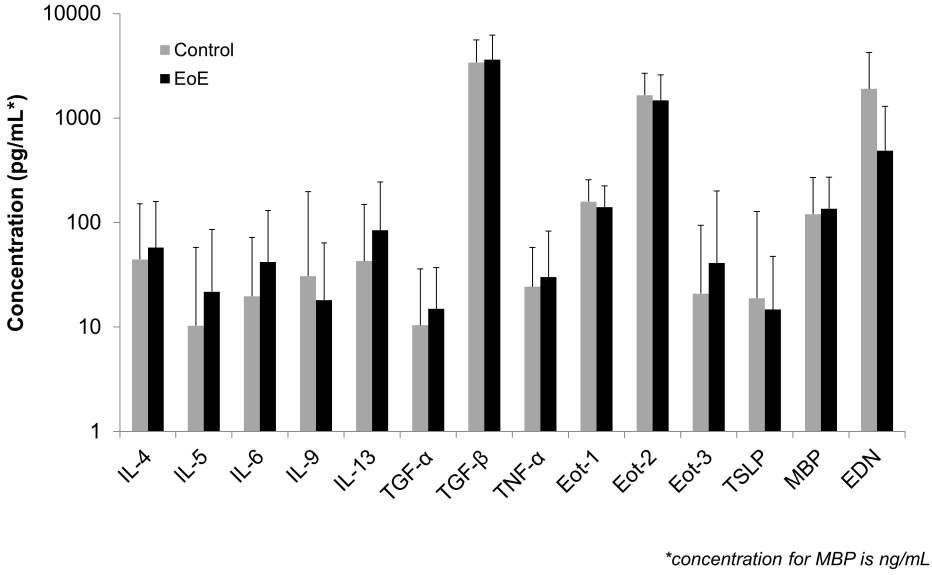

Figure 1.

Comparison of serum biomarkers for cases of EoE (black bars) and non-EoE controls (gray bars). The top of the bars represent the mean biomarker values, and the error bars represent the standard deviation. The y axis is on a log scale, and all concentrations are in pg/mL with the exception of major basic protein (MBP) which is in ng/mL.

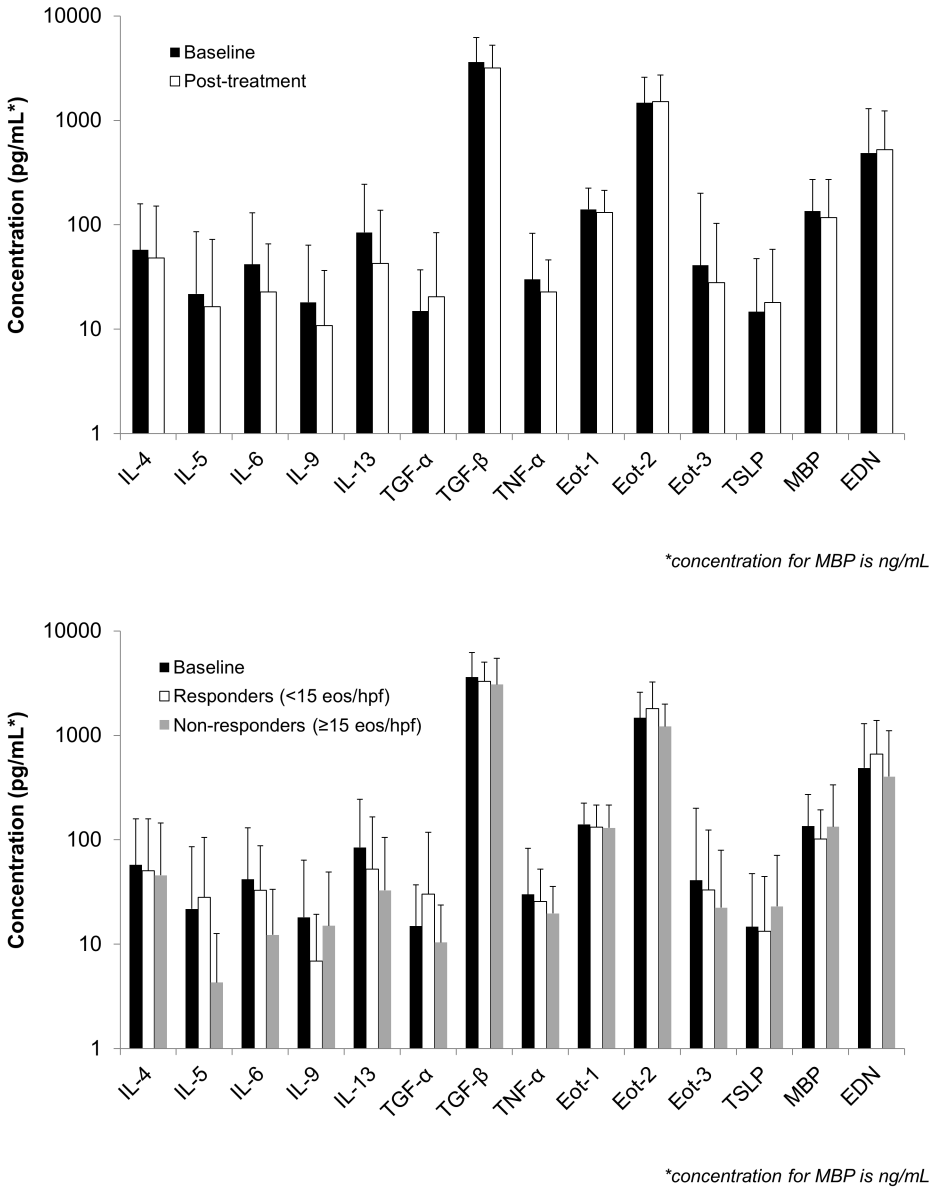

Figure 2.

(A) Comparison of serum biomarkers for cases of EoE before (black bars) and after (white bars) treatment with a topical corticosteroid. **(B)** Comparison for cases of EoE stratified by treatment response with pre-treatment (black bars), post-treatment responders at the <15 eos/hpf level (white bars), and non-responders at the ≥15 eos/hpf level (gray bars). For both panels, the top of the bars represent the mean biomarker values, the error bars represent the standard deviation, the y axis is on a log scale, and all concentrations are in pg/mL with the exception of major basic protein (MBP) which is in ng/mL.

Table 1

Clinical characteristics of EoE cases and non-EoE controls

	Non-EoE controls (n = 87)	EoE cases (n = 61)	p
Age at diagnosis (mean ± SD)	51.9 ± 13.2	38.8 ± 13.2	< 0.001
Male (n, %)	37 (42)	36 (58)	0.05
White (n, %)	73 (82)	58 (94)	0.04
Symptoms/EGD indication (n, %)			
Dysphagia	71 (80)	60 (97)	0.002
Heartburn	60 (67)	10 (16)	< 0.001
Abdominal pain	5 (6)	7 (11)	0.21
Nausea/vomiting	8 (9)	1 (2)	0.06
Atopic disorders (n, %)			
Asthma	19 (21)	19 (31)	0.20
Atopic dermatitis	7 (8)	4 (6)	0.74
Allergic rhinitis/sinusitis	40 (45)	40 (65)	0.02
Food allergies	10 (11)	28 (45)	< 0.001
Any atopic disease	48 (54)	46 (74)	0.01
EGD findings at baseline (n, %)			
Normal	13 (15)	3 (5)	0.06
Rings	8 (9)	47 (76)	< 0.001
Stricture	19 (21)	15 (24)	< 0.001
Narrowing	2 (2)	16 (26)	< 0.001
Furrows	3 (3)	55 (89)	< 0.001
Crêpe-paper	3 (3)	4 (6)	0.38
White plaques/exudates	3 (3)	27 (44)	< 0.001
Decreased vascularity	3 (3)	37 (60)	< 0.001
Erosive esophagitis	17 (19)	2 (3)	0.004
Schatzki's ring	13 (15)	7 (11)	0.55
Hiatal hernia	51 (57)	8 (13)	< 0.001
Baseline max eosinophil count (mean ± SD)	2.8 ± 6.8	146.3 ± 128.4	< 0.001
Median eosinophil count (IQR)	0 (0-2)	111 (60-165)	< 0.001
Follow-up max eosinophil count (mean ± SD)*	-	54.5 ± 87.9	-
Median eosinophil count (IQR)	-	9 (0-84)	-
Histologic response rates			
< 1 eos/hpf	-	17 (28)	-
< 15 eos/hpf	-	33 (55)	-

* n = 60 EoE cases with follow-up biopsy data