DISEASES OF THE ESOPHAGUS

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Rapid Review

Normalized serum eosinophil peroxidase levels are inversely correlated with esophageal eosinophilia in eosinophilic esophagitis

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SUMMARY. Eosinophil peroxidase is an eosinophil-specific, cytoplasmic protein stored in the secondary granules of eosinophils. While eosinophil peroxidase deposition is increased in the esophagus in eosinophilic esophagitis (EOE), its potential role as a peripheral marker is unknown. This study aims to examine the relationship between serum eosinophil peroxidase and esophageal eosinophilia in eosinophilic esophagitis. Prospectively collected serum from 19 subjects with incident EoE prior to treatment and 20 non-EoE controls were tested for serum eosinophil peroxidase, eosinophilic cationic protein, and eosinophil derived neurotoxin using ELISA. Matching esophageal tissue sections were stained and assessed for eosinophil peroxidase deposition using a histopathologic scoring algorithm. Mean peripheral blood absolute eosinophil counts in eosinophilic esophagitis subjects were significantly elevated compared to controls (363 vs. 195 cells/μL, $P = 0.008$). Absolute median serum eosinophil peroxidase, eosinophil cationic protein, and eosinophil derived neurotoxin did not differ between groups; however, when normalized for absolute eosinophil counts, eosinophilic esophagitis subjects had significantly lower median eosinophil peroxidase levels (2.56 vs. 6.96 ng/mL per eos/μL, $P = 0.002$, AUC 0.79 (0.64, 0.94 95% CI)). Multivariate analysis demonstrated this relationship persisted after controlling for atopy. Esophageal biopsies from eosinophilic esophagitis subjects demonstrated marked eosinophil peroxidase deposition (median score 46 vs. 0, $P < 0.0001$). Normalized eosinophil peroxidase levels inversely correlated with esophageal eosinophil density ($r = -0.41$, $P = 0.009$). In contrast to marked tissue eosinophil degranulation, circulating eosinophils appear to retain their granule proteins in EoE. Investigations of normalized serum eosinophil peroxidase levels as a biomarker of EoE are ongoing.

KEY WORDS: biomarker, eosinophilic esophagitis, eosinophils.

ABBREVIATIONS: AEC: absolute eosinophil count (peripheral blood); ECP: eosinophil cationic protein; EDN: eosinophil derived neurotoxin; EoE: eosinophilic esophagitis; EPX: eosinophil peroxidase; EPX-mAb: monoclonal antieosinophil peroxidase antibody; ESGPs: eosinophil secondary granule proteins; GERD: gastroesophageal reflux disease; Hpf: high-powered field; PPI: proton pump inhibitor; PPI-REE: PPI-responsive esophageal eosinophilia

INTRODUCTION

Eosinophilic esophagitis (EoE) is an immune-mediated, allergen-driven condition characterized by eosinophilic inflammation with resultant tissue remodeling, fibrosis, and esophageal dysfunction. Epidemiologic studies suggest that the prevalence of EoE has increased almost 20-fold over the past 15 years.[1] The current standard of care requires the diagnosis of EoE be confirmed by histologic evidence of an eosinophil infiltrate acquired by invasive means—upper endoscopy and esophageal biopsy.[2] Following diagnosis, a subsequent esophageal biopsy is required to assess response to therapy. Moreover, skin prick testing,[3,4] serum IgE testing,[5,6] and atopy patch testing[7] do not reliably identify common triggers. Consequently, culprit foods can only be objectively identified by dietary elimination followed by serial reintroduction and repeated endoscopic biopsies[8] resulting in substantial cost and morbidity.

To date, efforts to validate rapid, minimally invasive, diagnostic markers of EoE have been met with limited success.[9,10] Multiple studies have shown that EoE is characterized by a significant eosinophilic infiltration that is often accompanied by elevated levels of tissue degranulation leading to the release and tissue deposition of eosinophil secondary granule proteins (ESGPs).[11-13] Given the central role of eosinophils in EoE disease pathogenesis, ESGPs are likely candidate biomarkers reflective of eosinophil-mediated events occurring in the esophageal mucosa of these patients. Eosinophil cationic protein (ECP),[14,15] eosinophil-derived neurotoxin (EDN),[16,17] and eosinophil major basic protein (MBP)[9] have all been evaluated as serologic markers of disease with mixed results. Though some studies have shown differences in serum eosinophil granule protein levels between EoE subjects and controls, peripheral absolute eosinophil counts (AEC) more closely mirror esophageal eosinophil density.[18] Because AEC are often elevated in patients with other atopic diseases, there may be considerable overlap among individuals with EoE.

Unlike ECP and EDN, which are ribonucleases expressed other leukocytes, EPX is exclusively synthesized and released by eosinophils.[19] We have demonstrated the utility of immunohistochemical staining for eosinophil peroxidase (EPX) in EoE and developed a novel scoring system to quantify esophageal eosinophilia.[20] Importantly, we have shown that tissue EPX deposition is a marker of eosinophil activity and correlates better with symptoms of dysphagia than esophageal eosinophil counts.[21] We have developed a sensitive, eosinophil-specific sandwich ELISA for measurement of EPX in the peripheral blood,[19] but this has yet to be examined in EoE. In a previous blinded analysis of serum samples at Mayo Clinic, we found a linear relationship with between serum EPX levels and AEC; however, in some individuals serum EPX levels were disproportionate to peripheral blood AEC (unpublished data). Thus, we hypothesized that by normalizing for absolute eosinophil counts we might distinguish patients with active EoE. The objective of this study is to evaluate the relationship between serum EPX levels and EoE disease activity for the first time.

METHODS

Study population

This is a study of prospectively banked samples collected from adult subjects at the University of North Carolina over a two year period, from July 2011 to December 2013, as previously described.[9,22,23] Adult patients (ages 18–80) referred for outpatient esophagogastroduodenoscopy were eligible if they had symptoms of esophageal dysfunction including dysphagia, food impaction, heartburn, reflux, or chest pain. Exclusion criteria included the following: known diagnosis of EoE or another eosinophilic gastrointestinal disorder; gastrointestinal 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 provide consent. Normal controls were not recruited because asymptomatic subjects are unlikely to undergo upper endoscopy and comparisons with EoE subjects would likely have little clinical relevance for biomarker assessment. Written informed consent was obtained from subjects for storage of banked specimens prior to endoscopy. A waiver of consent was obtained for utilization of banked specimens. This study was approved by the UNC Institutional Review Board.

Case definitions, clinical data, and biospecimen collection

EoE was diagnosed by consensus guidelines (symptoms of esophageal dysfunction and >15 eos/hpf in esophageal biopsies after an 8-week trial of high dose proton pump inhibitor (PPI) therapy).[2] All cases of EoE were incident cases and did not receive treatment with steroids or dietary elimination prior to enrollment. Baseline data were obtained following the PPI trial at the time of confirmatory endoscopy. Non-EoE controls selected for this study were subjects with dysphagia or gastroesophageal reflux disease (GERD) and normal biopsies with 0 eos/hpf. Subjects with esophageal eosinophil counts greater than 0, but less than 15 were excluded in order to create clearly distinct case/control groups. Controls with symptoms concerning for esophageal

dysfunction, but no histologic evidence of EoE was selected for comparison with EoE subjects because the goal was to evaluate ability of serum EPX to distinguish between these two groups. Subjects with PPI-responsive esophageal eosinophilia (PPI-REE) were excluded from this study. Controls were classified by atopic status based on the presence of asthma, allergic rhinitis, eczema, or food allergy.

Clinical data were collected through standardized case report forms and a prospectively administered questionnaire to assess demographics, medical history, symptoms, allergic conditions, indications for endoscopy, endoscopic findings, and final diagnoses. Food allergies were assessed by subject self-report.

At the time of endoscopy, five esophageal biopsies were obtained from the proximal (2 biopsies), mid (1 biopsy), and distal (2 biopsies) esophagus to maximize diagnostic sensitivity. Gastric and duodenal biopsies were also collected to exclude concomitant eosinophilic gastroenteritis. Esophageal eosinophil counts were quantified using a validated methodology previously described.[24] Briefly, slides were blinded, digitized, and reviewed with Aperio ImageScope (Aperio Techologies, Vista, CA). Maximum eosinophil density (eos/mm^2) was determined by examination of five microscopy fields from each of the five biopsies obtained. Eosinophil density was converted to an eosinophil count (eos/hpf) using a hpf of 0.24 mm.[2,24]

Also at the time of endoscopy, a blood sample was obtained immediately prior to the procedure. Blood and additional esophageal biopsies were obtained and stored in the University of North Carolina EoE Patient Registry and Biobank. Serum was isolated by centrifugation of blood samples, aliquoted, and stored at -80°C. A clinical sample was also obtained for complete blood cell count and differential, to quantify the peripheral eosinophil count. Study personnel were blinded as to case/control status during sample analysis.

EPX Immunohistochemistry

Infiltrating intact eosinophils and evidence of eosinophil degranulation (i.e. the presence of free cytoplasmic granules and/or tissue deposition of eosinophil granule proteins) were assessed by immunohistochemistry using a mouse monoclonal anti-eosinophil peroxidase antibody (EPX-mAb) as previously described.[20] The same slides used for traditional hematoxylin and eosin staining were stained for EPX to allow for direct comparison.

Measurement of serum EPX

We utilized the previously published sandwich EPX ELISA protocol[19,25,26] with modifications for serum samples. Reagents include KPL preoptimized ELISA reagent system that includes Coating Solution Concentrate 10× (KPL, Cat # 50–84-00), 10% BSA Diluent/Blocking Solution Kit (KPL, Cat # 50–61-00), Wash Solution Concentrate 20X (KPL, Cat # 50–63-00), and BluePhos Microwell Phosphatase Substrate System (KPL, Cat # 50–88-00), Streptavidin-Alkaline Phosphatase (Strep-AP) from RD (R&D Systems, Minneapolis, MN, Cat # AR001), and Trizma hydrochloride buffer solution (Sigma-Aldrich, Cat # T2319–1L). EPX standards and serum samples were diluted 1/40–1/80 with Antibody Diluent (HAMA Blocker, Abcam ab193969). Briefly, anti-EPX antibody MM25–82.2.1 (detection antibody) was coated overnight at 4°C, washed four times, blocked for 30 minutes, and then samples were incubated at room temperature samples for ~1.5 hours. After washing four times, anti-EPX monoclonal antibody MM25–82.2.1 (detection antibody) was incubated for ~1.5 hours, washed four times and detected using Strep-AP with BluePhos substrate. The colorimetric reaction was terminated with Stop Solution. Absorbance of individual wells of the plate was determined at a wavelength of 630 nm. The EPX assay has a limit of detection (i.e. measureable signal three standard deviations above background) of 1.4 ± 0.2 ng/mL.

Measurement of serum ECP and EDN

Human serum ECP and EDN levels were determined by using the MBL International Corporation EDN ELISA kit (Code#: 7630) and Mesacup ECP ELISA kit (Code#: 7618E) according to the manufacturer's instructions. Serum was diluted to a final ratio of 1:5.44 in the diluent provided in kit. The minimum limits of detection were 0.125 ng/mL and 0.62 ng/mL for the ECP and EDN ELISA kits, respectively.

Esophageal biopsy EPX-mAb scoring

Slides were scored based on a scoring system previously described.[20] Briefly, EPX stains were assessed for (1) reproducibility (percent of all biopsies with significant eosinophil infiltration and/or degranulation), (2) patchiness (percent area of the maximally affected biopsy showing significant eosinophil infiltration and/or degranulation), (3) degranulation (level of degranulation observed in maximally affected biopsy), (4) peak eosinophil infiltrate: maximum single focus (number of intact eosinophils—peak value in a single 40× hpf), and (5) average eosinophil infiltrate: average of five designated foci (number of intact eosinophils—peak value in an average of five (5) 40× hpf) were observed.

Table 1 Characteristics of the study population

	Controls (n = 20)	EoE cases (n = 19)	P*
Mean age (±SD)	58.7 ± 12.5	34.9 ± 8.1	<0.001
Males (n, %)	5 (25)	9 (47)	0.15
White (n, %)	16 (80)	19 (100)	0.04
Symptoms			
Dysphagia	19 (95)	19 (100)	0.32
Heartburn	1 (5)	0 (0)	0.32
Atopic conditions (n, %)			
Asthma	8 (40)	8 (42)	0.89
Dermatitis	1 (5)	2 (11)	0.58
Seasonal allergies	8 (40)	12 (63)	0.25
Food allergies	3 (15)	4 (21)	0.73
Any atopic condition	10 (50)	14 (74)	0.25
Diagnoses			
EoE	0 (0)	19 (100)	–
GERD	6 (30)	0 (0)	–
Esophageal dysmotility	5 (25)	0 (0)	–
Functional	4 (20)	0 (0)	–
Schatzki's ring	3 (15)	0 (0)	
Normal	2 (10)	0 (0)	
Tissue eosinophil counts (max eos/hpf ± SD)	0 ± 0	157 ± 29.3	<0.0001
Peripheral blood absolute eosinophil counts (AEC) (mean cells/μL ± SEM)	195 ± 38.03	363.2 ± 46.65	0.008

EoE, eosinophilic esophagitis; eos/hpf, eosinophils per high powered field; SD, standard deviation.

Statistical analysis

Clinical characteristics of the cases and controls were summarized with descriptive statistics. Comparisons of mean total AEC were performed using a t-test and median normalized EPX, ECP, and EDN values were made using a Mann-Whitney test. Tissue EPX scores were compared using Wilcoxon signed rank test. Normalized EPX levels were compared using a multivariate analysis in order to control for atopic status. A subanalysis using pairwise comparisons was also performed by segregating the control subjects according to atopic status. ROC curves for each of the normalized serum granule protein levels were assessed. Correlations between ECP, EDN, EPX, and peak tissue eosinophil counts were obtained using Spearman's correlation analysis. Comparisons and plots were made with GraphPad Prism version 7.0 f for Windows, GraphPad software, San Diego, California, USA. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Clinical characteristics

Table 1 provides the clinical characteristics of the study population. By definition, all of the EoE cases were on PPI at the time of diagnosis when baseline samples were obtained. In the controls, 13 (65%) were on PPI at the time of the endoscopy. Dysphagia was the most common indication for endoscopy in both groups. Similar rates of atopic conditions were noted between groups and GERD was the most common diagnosis among the control subjects. Notably, EoE subjects had increased peak esophageal eosinophil counts with significantly higher AEC. None of the patients were being treated with systemic corticosteroids. Additionally, patients with asthma did not have active symptoms at the time of endoscopy. EoE subjects were younger and more likely to be Caucasian.

Immunohistochemistry assessments

Figure 1 shows hematoxylin and eosin stains and corresponding immunohistochemistry stains for eosinophil peroxidase in representative cases and control specimens. As expected, control subjects did not have evidence of esophageal eosinophilia (Fig. 1A) or EPX deposition (Fig. 1B). Subjects with EoE demonstrated a robust esophageal eosinophilia (Fig. 1C) often accompanied by evidence of extensive degranulation (i.e. extracellular matrix deposition of EPX) (Fig. 1D). Our EPX-based assessments of these patients were consistent with the previously established clinicopathologic diagnosis in all subjects with EoE and a summation of these scores is presented in Figure 2. Subjects with EoE had markedly elevated EPX scores compared to controls (i.e. eosinophil activity index scores of 46 vs. 0, respectively ($P < 0.0001$))

Serum EPX vs. AEC

Comparisons of absolute and normalized ESGP levels are presented in Figure 3, respectively. No significant differences in absolute ECP, EDN, or EPX were noted; however, when normalized for AEC, median EPX/AEC (2.56 (IQR 1.85–3.38) vs. 6.96 ng/mL per eos/μL (IQR 3.14–7.75), $P = 0.002$) and EDN/AEC ratios (0.07 (IQR 0.02–0.11) vs. 0.16 ng/mL per eos/μL (IQR 0.08–0.58), $P = 0.008$) were significantly lower in EoE subjects (Fig. 3)). Normalized ESGPs ratios were measured in ng/mL per eosinophils/μL. Significant differences between groups were not observed for ECP/AEC ratios (0.12 (IQR: 0.03–0.5) vs. 0.22 ng/mL per eos/μL (IQR 0.13–0.38), $P = 0.26$) (Fig. 3). A multivariate analysis was performed revealing the EPX/AEC ratio was inversely associated with EoE status regardless of the presence of atopy (unadjusted OR = 0.61, (0.44–0.85); OR adjusted for any atopy = 0.64 (0.46–0.89)) (Table 2). This means that every time the EPX/AEC ratio increases by one the odds of having EoE decrease by 0.36. A subanalysis dividing the control group by atopic status showed that both the AEC and EPX/AEC ratio distinguish subjects with EoE from non-atopic and atopic controls (eFigure 1, published online).
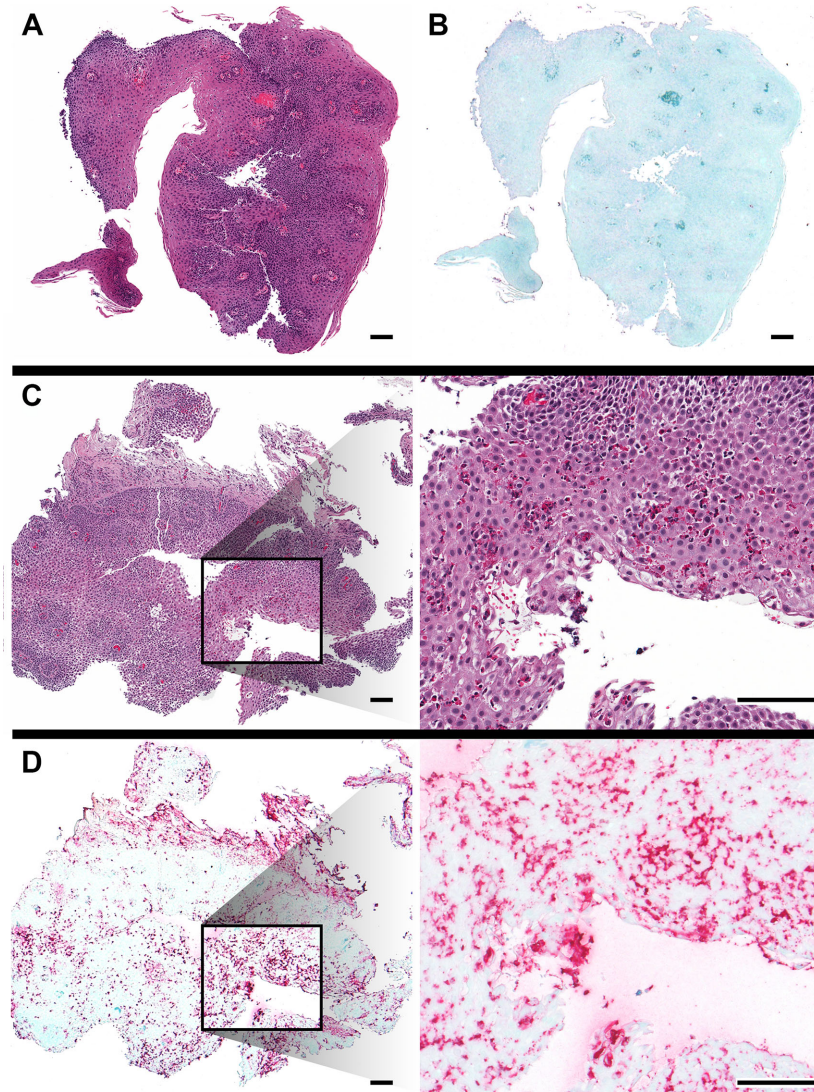
Fig. 1 Eosinophilic esophagitis (EoE) is associated with marked tissue deposition of eosinophil peroxidase (EPX). Hematoxylin and eosin stains of esophageal tissue from a control (A) and an EoE subject at low and high magnification (C). Corresponding immunohistochemistry stains for EPX (stained red) performed on the same slides (B and D).

Serum biomarkers of esophageal eosinophilia

Receiver operative characteristic (ROC) curves for AEC (AUC 0.79 (0.65, 0.94, 95% CI) $P = 0.002$), EPX/AEC (AUC 0.79 (0.64, 0.94 95% CI), $P = 0.002$) and EDN/AEC (AUC 0.74 (0.59, 0.90 95% CI), $P = 0.009$) were similar, suggesting each measure discriminated patients with and without EoE comparably (Fig. 4). The AUC for the ECP/AEC ratio was not significant (AUC 0.61, $P = 0.25$). Normalized serum EPX measurements inversely correlated with esophageal eosinophil density ($r = -0.41$, $P = 0.009$) whereas ECP/AEC and EDN/AEC ratios showed similar negative correlations that did not reach statistical significance. AEC also was also significantly elevated in EoE subjects vs. controls (363.2 vs. 195, $P = 0.008$, AUC 0.79 (0.65, 0.94 95% CI)) and

correlated positively with peak tissue eosinophil counts ($r = 0.39$, $P = 0.02$ (Fig. 5)).

DISCUSSION

The current standard of care for diagnosing EoE and monitoring response to therapy requires multiple endoscopies, but there is an urgent need for a non-invasive biomarker of disease activity. We sought to examine the relationship between and serum EPX levels and the esophageal eosinophilia occurring in EoE patients. We hypothesized, based on the literature of increased degranulation linked with eosinophil activation in diseased tissue, that serum ESGPs would be higher in EoE subjects. We did not observe significant
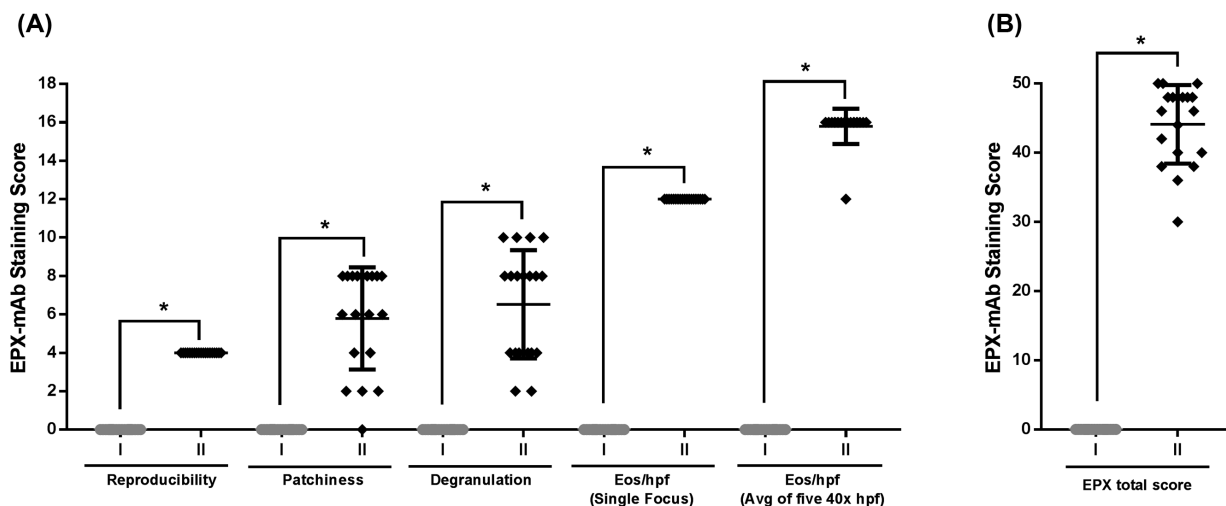
Fig. 2 Monoclonal antieosinophil peroxidase antibody (EPX-mAb)-based immunohistochemistry quantifies significant tissue degranulation in EoE. (A) Comparisons (Wilcoxon signed-rank test) of the median scores for individual EPX-mAb–based parameters associated with the controls (group 1) and EoE (group II) subjects. (B) Comparison of cumulative total median EPX-mAb-based staining scores.

differences in absolute serum EPX, EDN, or ECP, but noted an unexpected trend for lower EPX and EDN levels in subjects with EoE. Because AEC may not reliably discriminate subjects with EoE and subjects with other atopic diseases (i.e. asthma), we assessed whether ESGPs normalized by AEC would identify subjects with EoE. We found that EoE subjects had lower ESGP levels (EPX and EDN) per peripheral blood eosinophil in circulation and, thus, normalized circulating serum granule protein levels inversely correlated with esophageal eosinophil counts. This is the first study to examine serum EPX as a surrogate of esophageal eosinophilia.

Several groups have shown that AEC holds promise as a biomarker of EoE; moreover, peripheral blood eosinophils may prove useful in measuring response to therapy.[14,18,27] However, this metric may be confounded by comorbid allergic conditions resulting in significant overlap of AEC between atopic individuals and patients with EoE. Multiple investigators have also targeted ESGPs as biomarkers of EoE; however, the findings of these studies are also problematic. That is, several pediatric[16,17] and adult[14] studies have noted that EoE subjects have elevated absolute serum or plasma ECP and EDN levels. However, recent studies have suggested that not all of the ESGPs assessed (e.g. ECP[28] or EDN[29]) are eosinophil-specific. Moreover, baseline comparison of EDN and MBP in a large, prospective cohort showed no differences in serum MBP or EDN levels.[9] Decreases in serum ECP have also been described in EoE subjects responding to treatment in some studies[18] but not others.[27] Serum ESGP levels normalized by AEC were not assessed in any of these studies; therefore, the discrepancies among prior studies and the perceived lack of utility of these potential biomarkers may be explained by relative differences in AEC between case and control groups and/or simply the secretion of a given granule protein (e.g. ECP[19]) by other cell types.

The relationship between peripheral blood eosinophils and serum ESGP levels is still not entirely clear; however, the presence of detectable ESGP in the serum of healthy controls suggests at least some constitutive release by peripheral blood eosinophils. In patients with eosinophilic diseases, ESGP levels may reflect AEC.[30] Regardless of disease status, peripheral blood eosinophils do not display overt evidence of degranulation, piecemeal, or otherwise.[31] This is likely because the total amount of ESGPs in circulation is an exceedingly small fraction of the total ESGPs contained within circulating eosinophils (<0.2% of total available ESGPs).[19] Our observation that absolute serum EPX levels were no different, and possibly lower, in EoE subjects despite elevated AEC implies homeostatic mechanisms to maintain absolute serum ESGP levels in EoE.

We speculate the most likely rationale for this observation is that the one or more inflammatory signals in EoE patients is released into circulation and inhibits the constitutive ESGP release in peripheral blood eosinophils. This is consistent with findings in other allergic diseases (asthma, atopic dermatitis, allergic rhinitis, Churg Strauss) where circulating eosinophils show no evidence of cytolytic degranulation by electronic microscopy and display no overt morphologic differences with eosinophils from control subjects.[31] In contrast, many groups have reported that a majority of eosinophils infiltrating the esophageal mucosa of EoE patients display evidence of cytolysis (membrane disruption, free extracellular granule proteins)[13] and not piecemeal degranulation. That is, transmission electron microscopy of studies in EoE suggest that almost all invading eosinophils were characterized by marked
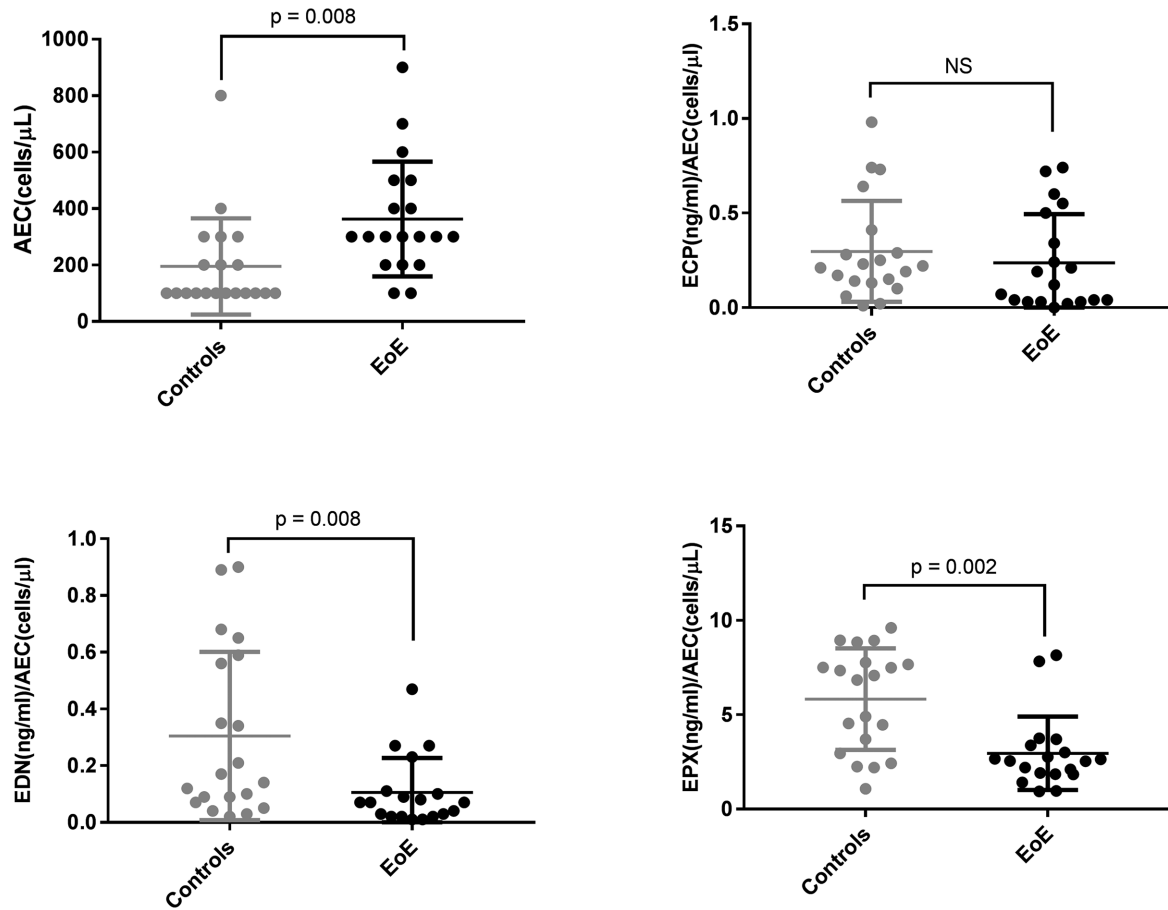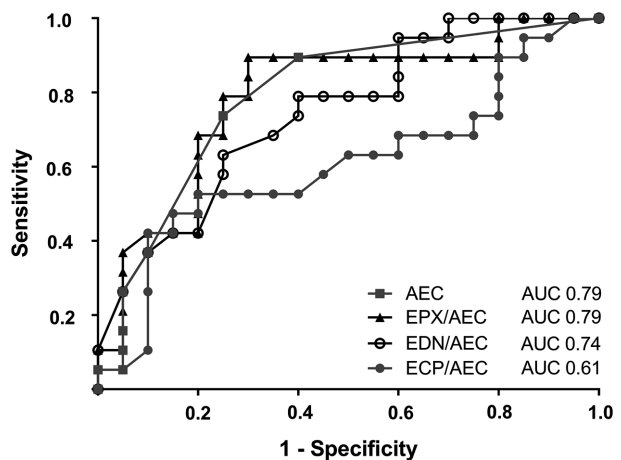
Fig. 3   EoE is associated with increased absolute eosinophil counts (AEC) but decreased serum eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPX) per AEC. Comparisons of mean AEC (t-test) and median serum ECP/AEC, EDN/AEC, EPX/AEC ratios (Mann-Whitney) shown.

Table 2   Multivariate analysis of EPX/AEC ratio as a marker of eosinophilic esophagitis

	OR	95% CI
Unadjusted	0.61	(0.44–0.85)
Adjusted for any atopic condition	0.64	(0.46–0.89)
Adjusted for asthma	0.63	(0.46–0.89)
Adjusted for eczema	0.64	(0.46–0.88)
Adjusted for allergic rhinitis	0.64	(0.46–0.89)
Adjusted for food allergy	0.64	(0.46–0.88)
Adjusted for PPI use	0.64	(0.46–0.88)

Fig. 4   Legend: Receiver operator characteristic curves for AEC, EPX/AEC, EDN/AEC, and ECP/AEC. The area under the curve was significant for the AEC, EPX/AEC ratio, and EDN/AEC ratio but not the ECP/AEC ratio.

cytoplasmic vesiculation and varying degrees of cytolysis,[13] suggesting that the esophagus is a terminal site of eosinophil effector activity in EoE. Though we observed decreased EPX/AEC and EDN/AEC ratios in subjects with EoE, we did not observe statistically significant differences in ECP/AEC ratios. Rather than differential granule protein release by eosinophils, this is likely due to ECP release by other cell types.[19] Regulation of peripheral ESGP levels may be a mechanism whereby eosinophils conserve granule proteins in order to maximize local effector function.

While interpreting our results, we acknowledge certain limitations. This was a small pilot study from a single center study of adult subjects; therefore, the results cannot be generalized to children and are not definitive given the preliminary nature of the data. Although some EoE subjects were followed after treatment, we did not obtain a subsequent AEC; consequently, we did not longitudinally
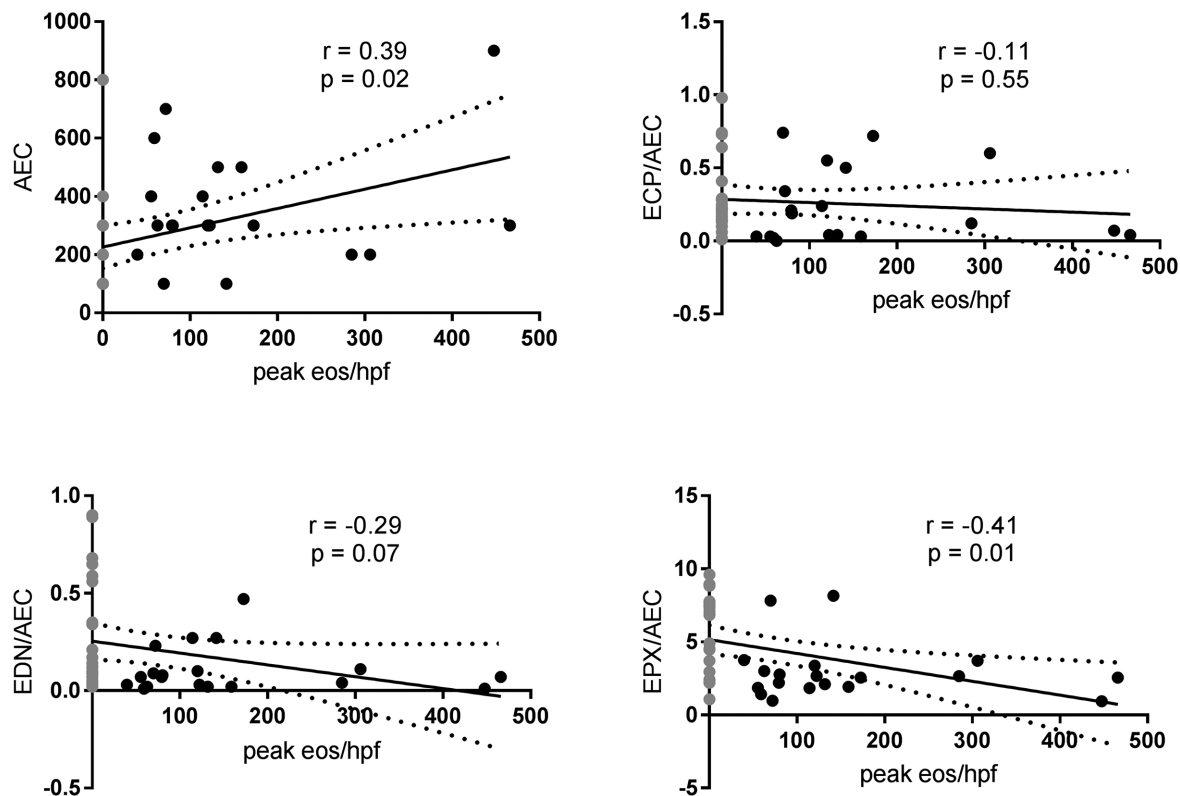
Fig. 5 EPX/AEC is inversely correlated with esophageal eosinophilia. Linear regression plots of AEC, ECP/AEC, EDN/AEC, and EPX/AEC vs. tissue eosinophils (peak eos/hpf). Spearman's rho values reported. Dotted lines represent 95% CI. Controls are denoted by gray circles and EoE subjects with black circles.

measure normalized serum granule protein levels from posttreatment specimens. In addition, while granule protein levels were measured for ECP, EDN, and EPX, we only assessed tissue degranulation by EPX staining. Finally, AEC levels were significantly lower in non-atopic controls and atopic subjects; therefore, differences in the ESGP/AEC ratios may be due to differences in AEC alone. While the AEC may be statistically higher in the EoE group, it is important to note that these values are still in the normal range. Therefore, the AEC alone cannot be used to diagnose or monitor treatment response in EoE (for example, a mean AEC of approximately 300 noted in the EoE group would not raise or lower the clinical suspicion for the condition); however, the ratio is discriminatory and may well have clinical utility, as this would not be decreased in non-EoE patients. In order to determine the utility of the EPX/AEC ratio as a diagnostic marker, we would need to perform a case control study of EoE subjects and atopic controls matched for AEC. The limitations of the study are balanced by a number of strengths including: the study's prospective design; utilization of banked samples collected and stored in a uniform fashion for all subjects; detailed clinical information; and blinded analysis of matched tissue and plasma samples. In addition, immunohistochemistry assessments for EPX were performed using the same slides stained for hematoxylin and eosin staining

allowing for direct comparison between EPX staining and histologic features.

In summary, we have shown in this small pilot cohort that absolute serum ESGP levels alone do not distinguish EoE subjects from controls; however, serum EPX/AEC and EDN/AEC ratios are significantly lower in EoE subjects. For the EPX/AEC ratio, this observation is independent of atopic status. Consistent with previous studies, we found that the AEC mirrors esophageal eosinophil density and that EPX/AEC is inversely correlated with peak tissue eosinophil counts. The inability of serum granule proteins alone to distinguish between EoE and non-EoE controls and monitor responsiveness to treatment has been shown before[9,27] but our finding that the normalization by peripheral eosinophil count, as well as the fact that the peripheral blood eosinophils in EoE do not appear to increase granule protein release, is an important observation that holds even with our small sample size. This has key implications not only for EoE but other allergic diseases characterized by eosinophilic inflammation. We speculate that constitutive release of ESGP by circulating eosinophils is actively inhibited in EoE. Future studies will include both mechanistic studies of EoE focusing on extracellular signaling pathways that regulate eosinophil degranulation. We also plan to conduct prospective, longitudinal clinical studies with atopic controls to

evaluate serum EPX/AEC in EoE subjects following treatment to determine its utility as a biomarker of eosinophilic inflammation.

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