Noninvasive Biomarkers Identify Eosinophilic Esophagitis: A Prospective Longitudinal Study in Children

Short Running Title: Noninvasive peripheral biomarkers in EoE

Joshua B. Wechsler, MD, MSCI[1,2] Steven J. Ackerman, PhD,[3] Mirna Chehade, MD, MPH,[4] Katie Amsden, MPH[2] Mary E. Riffle,[4] Ming-Yu Wang, MD,[2] Jian Du,[3] Matt L. Kleinjan,[3] Preeth Alumkal,[3] Elizabeth Gray, MS,[1,5] Kwang-Youn A. Kim, PhD,[1,5] Barry K. Wershil, MD,[1,2,7] Amir F. Kagalwalla, MBBS[1,2,6,7]

[1] Northwestern University Feinberg School of Medicine, Chicago, Illinois

[2] Eosinophilic Gastrointestinal Diseases Program, Division of Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, Illinois, USA

[3] Department of Biochemistry and Molecular Genetics, University of Illinois-Chicago, Chicago, Illinois, USA

[4] Mount Sinai Center for Eosinophilic Disorders, Icahn School of Medicine at Mount Sinai, New York, New York, USA

[5] Department of Preventive Medicine, Feinberg School of Medicine, Chicago, Illinois

[6] John H. Stroger Hospital of Cook County, Chicago, Illinois, USA

[7] Co-senior authors

Corresponding Authors:
Steven J. Ackerman, PhD (guarantor of the article)
Department of Biochemistry and Molecular Genetics, College of Medicine, University of Illinois at Chicago
MBRB 2074, MC669
900S. Ashland Ave
Chicago, IL 60607
E-mail: sackerma@uic.edu

Specific author contributions: Steven Ackerman is the guarantor of the manuscript. Dr. Wechsler recruited patients, collected data, supervised biomarker quantification, supervised biostatistical analysis, drafted and critically revised the manuscript. Steven

Ackerman conceived the study, supervised biomarker quantification, reviewed data analysis, drafted and critically revised the manuscript, Mirna Chehade recruited patients, collected data, reviewed data analysis, and critically revised the manuscript, Katie Amsden recruited patients, and collected data. Mary Ellen Riffle recruited patients and collected data. Ming-Yu Wang performed quantification of biomarkers. Jian Du performed quantification of biomarkers. Matt Kleinjan performed quantification of biomarkers. Preeth Alumkal performed quantification of biomarkers. Elizabeth Gray performed biostatistical analysis. Kwang-Youn Kim supervised and conducted biostatistical analysis. Barry Wershil conceived the study, recruited patients, reviewed data analysis, and critically revised the manuscript. Amir Kagalwalla obtained funding, conceived the study, recruited patients, collected data, reviewed data analysis, drafted and critically revised the manuscript, and oversaw study conduct. All authors have reviewed and approved the final version of the manuscript.

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Abbreviations: AEC, absolute eosinophil count; PEC, peak esophageal eosinophil count; EoE, eosinophilic esophagitis; eos, eosinophil; EST, esophageal string test; EGD, esophagogastroduodenoscopy; CLC/GAL-10, Charcot-Leyden Crystal protein/Galectin-10; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; MBP-1, major basic protein-1; MMP-9, matrix-metalloproteinase-9; OPN, osteopontin; ROC, receiver operating characteristics, AUC, area under curve, hpf, high-powered field.

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Abstract (word counts: 250)

Background: Esophageal histology is critical for diagnosis and surveillance of disease activity in eosinophilic esophagitis (EoE). A validated noninvasive biomarker has not been identified. We aimed to determine the utility of blood and urine eosinophil-associated proteins to identify EoE diagnosis and predict esophageal eosinophilia.

Methods: Blood and urine were collected from children undergoing endoscopy with biopsy. Absolute eosinophil count (AEC), plasma eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), major basic protein-1 (MBP-1), galectin-10 (CLC/GAL-10), Eotaxin-2 and Eotaxin-3, and urine osteopontin (OPN) and matrix metalloproteinase-9 (MMP-9) were determined. Differences were assessed between EoE and control, and with treatment response. The capacity to predict EoE diagnosis and esophageal eosinophil counts was assessed.

Results: 183 specimens were collected from 56 EoE patients and 15 non-EoE patient controls; 33 EoE patients had paired pre- and post-treatment specimens. Plasma (CLC/GAL-10, ECP, EDN, Eotaxin-3, MBP-1) and urine (OPN) biomarkers were increased in EoE compared to control. A panel comprising CLC/GAL-10, Eotaxin-3, ECP, EDN, MBP-1, and AEC was superior to AEC alone in distinguishing EoE from control. AEC, CLC/GAL-10, ECP, and MBP-1 were significantly decreased in patients with a good response to treatment compared to patients with a poor response. AEC, CLC/GAL-10, ECP, EDN, OPN, and MBP-1 each predicted esophageal eosinophil counts utilizing mixed models controlled for age, gender, treatment and atopy; AEC combined with MBP-1 best predicted the counts.

Conclusions: We identified novel panels of eosinophil-associated proteins that along with AEC are superior to AEC alone in distinguishing EoE from control and predicting esophageal eosinophil counts.

Key Words: eosinophilic esophagitis; noninvasive; biomarker; panel; inflammation; eosinophil-derived; blood; plasma; urine

INTRODUCTION

Upper endoscopy with multiple biopsies is required for diagnosis and surveillance of eosinophilic esophagitis (EoE) to identify the maximal density of eosinophils.[1] This procedure is invasive, time consuming and expensive with lost time from school and work. Currently, there are no validated noninvasive tests to assess disease activity that are well-correlated with esophageal eosinophilia.

In EoE, chronic dietary antigen exposure leads to production of chemokines Eotaxin-3 (CCL26) and -2 (CCL24) which drive sustained eosinophilic inflammation.[2] Eosinophil-derived granule proteins such as major basic protein-1 (MBP-1), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPX), eosinophil cationic protein (ECP) and Charcot-Leyden Crystal protein/Galectin-10 (CLC/GAL-10) are present in esophageal tissue and luminal secretions of EoE subjects.[3, 4] Prospective cross-sectional studies have identified promising noninvasive biomarkers in the plasma and stool.[5-7] Absolute eosinophil count (AEC) correlates with esophageal eosinophilia,[6, 8, 9] and several eosinophil granule cationic proteins including ECP and EDN, and chemokine Eotaxin-3 distinguish active EoE from control.[6-8] However, an optimal individual noninvasive biomarker has not been established, such as calprotectin in inflammatory bowel disease.[10, 11] Additionally, it is unclear whether a panel of noninvasive biomarkers is more effective than a single biomarker to identify esophageal eosinophilia. We hypothesized that a panel of biomarkers would be superior to AEC to differentiate EoE from non-EoE patient controls and to predict peak esophageal eosinophilia longitudinally.

METHODS

Study Population

We conducted a prospective, longitudinal cohort study of children ages 1-18 years undergoing routine outpatient esophagogastroduodenoscopy (EGD) with biopsies for suspected or previously diagnosed EoE. Subjects were recruited from January 2011 to December 2015 at Ann & Robert H. Lurie Children's Hospital of Chicago, Northwestern University Feinberg School of Medicine and Mount Sinai Medical Center, Icahn School of Medicine at Mount Sinai, New York. Parents were consented prior to the EGD for sample collection. The study was approved by the Institutional Review Boards of Lurie Children's Hospital and Mount Sinai Medical Center.

Case Definition

Diagnosis of EoE in children was based on presence of symptoms of esophageal dysfunction and esophageal biopsies with at least 15 eosinophils per high powered field (eos/hpf).[1] Patients were treated with twice daily proton pump inhibitor (PPI) for 8 weeks prior to their diagnostic endoscopy per the 2011 consensus recommendations for EoE diagnosis during that time.[1] Other causes of esophageal eosinophilia were excluded such as medication, infection, or graft vs. host disease. Several patients had a history of co-morbid celiac or inflammatory bowel disease that were not active at the time of the diagnostic endoscopy for EoE. Samples were also collected from children previously diagnosed with EoE undergoing endoscopy to assess response to either dietary elimination, food re-introduction or ‘topical’ corticosteroid treatment. Treatment responders and non-responders were patients on diet elimination or swallowed steroid treatment with <15 eos/hpf (inactive EoE, responder) or ≥15 eos/hpf (active EoE, non-

responder) on post-treatment esophageal biopsy. Non-EoE patient controls (referred to as 'controls') comprised participants undergoing diagnostic endoscopy for symptoms of esophageal dysfunction with histologically normal esophageal biopsies. Control subjects included those with co-morbid atopic disease, but without another intestinal inflammatory disease, autoimmune illness, or history of neoplasm or transplant. Controls were found to have dyspepsia or non-erosive reflux disease (NERD). Subjects with PPI responsive EoE were excluded.

Histologic Analysis

Four esophageal biopsies were obtained from 2 levels of the esophagus for standard of care assessment with hematoxylin & eosin by a pathologist. Eosinophilic inflammation was reported as peak esophageal eosinophil count (PEC) assessed at high power magnification (0.23 mm^2).

Sample Collection

Sample collection occurred longitudinally for EoE patients. A urine sample was collected the morning of endoscopy and blood was drawn when an intravenous line was placed for anesthesia. We collected 5 mL each for plasma and serum, along with 3 mL collected in potassium ethylenediaminetetraacetic acid (EDTA) and immediately processed for the peripheral absolute eosinophil count (AEC). Samples were given a unique coded study ID blinded to case/control as well as pre/post-treatment status.

Specimen Processing

Serum was obtained by allowing the blood to clot for 30 minutes before centrifuging at 1100 RCF for 15 minutes at room temperature. Aliquots were stored frozen at -70°C until biomarker analysis. Plasma was obtained by centrifuging blood samples collected in EDTA tubes within 15 min at 1100 RCF for 15 minutes at room temperature and extracted plasmas were aliquoted and stored frozen at -70°C until they were processed. Urine samples were centrifuged twice at 1400 RCF for 10 minutes at 4°C aliquoted, frozen and stored at -70°C until analysis.

Measurement of biomarkers

Analysis of samples was conducted at Lurie Children's Hospital and at the University of Illinois at Chicago. Samples were batch analyzed with a single thaw by ELISA using commercial kits for: eosinophil-derived neurotoxin (EDN) (7830, MBL International, Woburn, MA, USA), eosinophil cationic protein (ECP) (7618E, MBL International, Woburn, MA), Eotaxin-2 (DCC240B, R&D Systems, Minneapolis, MN), Eotaxin-3 (DCC260, R&D Systems, Minneapolis, MN), osteopontin (OPN) (DOST00, R&D Systems, Minneapolis, MN), and matrix metalloproteinase 9 (MMP-9) (DMP900, R&D Systems, Minneapolis, MN). Major basic protein 1 (MBP-1) and galectin-10 (CLC/GAL-10) utilized an in-house ELISA performed in the laboratory of Dr. Ackerman as previously described.[3] Urine creatinine was measured using a commercial ELISA kit (KGE005, R&D Systems, Minneapolis, MN) to normalize OPN & MMP-9 levels.

Pilot Biomarker Assessment

During the discovery phase, 10 serum, plasma and urine aliquots from subjects with active EoE and 10 non-EoE patient controls were analyzed for eosinophil proteins (CLC/GAL-10, ECP, EDN, MBP-1), eosinophil-associated chemokines (Eotaxin-2, Eotaxin-3) and cytokines (IL-17 and TSLP). Ten serum aliquots from children with active EoE and 10 controls were also analyzed for mast cell-associated enzymes (tryptase-alpha/beta 1, carboxypeptidase A3, matrix metalloproteinase-9). Urine from 10 subjects with EoE and 10 controls were analyzed for osteopontin (OPN) and matrix metalloproteinase-9 (MMP-9). Selection of the above biomarkers for analysis was based on previously published literature that demonstrated elevated levels of these proteins in esophageal tissue, blood and/or esophageal string test (EST) of subjects with EoE.[3-9] Laboratory personnel were blinded to the status of the samples. Only biomarkers that demonstrated at least a two-fold increase in plasma, serum or urine compared to controls were further analyzed in the full study cohort. Based on these findings, CLC/GAL-10, ECP, EDN, MBP-1, Eotaxin-2, Eotaxin-3, OPN and MMP-9 were identified as the most promising biomarkers for subsequent full analysis. Plasma, rather than serum, was chosen for all subsequent biomarker assays to avoid potential coagulation-induced non-specific increases in biomarker levels in the blood samples, as demonstrated for EDN and ECP (see Figure S1) and CLC/GAL-10 (data not shown).

Statistical Analysis

Statistical analyses were performed using R version 3.4.3 with alpha of 0.05 used to determine statistical significance. Differences in binary patient characteristics were

224 determined by Wilcoxon rank-sum and Fisher's exact test as appropriate. The sample
225 size was determined with the assumption that the standardized mean of any single
226 biomarker is 50% reduced after treatment, and the standardized mean of the biomarker
227 is 1.2 with the same standard deviation of 1 before treatment and an effect of size of 0.7.
228 Based on this calculation, 29 subjects were needed to have 80% power to detect this
229 difference using two-sided Wilcoxon signed-rank test with the overall type I error rate of
230 5%. Biomarker levels between EoE and patient controls were compared by non-
231 parametric Wilcoxon rank-sum test. Paired, non-parametric Wilcoxon signed-rank test
232 was used to assess differences between biomarker levels pre- and post-treatment.
233 Spearman correlations were determined between biomarkers and AEC. Zero-inflated
234 Poisson models controlling for age, gender, treatments, and atopy were fit to estimate the
235 association between a panel of biomarkers and peak esophageal eosinophil counts, using
236 the mixed_model function in the R package GLMMadaptive. Each biomarker was first
237 assessed in the model univariately followed by a combination of biomarkers (e.g. AEC
238 and MBP-1). Random forest, a classification method using decision trees, was used to
239 assess a panel of biomarkers to predict EoE diagnosis, using the randomForest function
240 in the R package randomForest. The area under the curve for receiver operating
241 characteristics was determined for differentiation of EoE from control.

242

243 **Additional methods regarding collection of demographics, medical history,**
244 **endoscopic findings, and symptoms in Supplemental Materials.**

245

246 **RESULTS**

Patient Characteristics

71 patients were enrolled and underwent at least one upper endoscopy with biopsies, with collection of plasma, serum, and urine specimens. Among the 71 subjects, 15 were non-EoE patients controls (referred to as 'controls'), 15 patients had known EoE, while 41 had a diagnostic endoscopy while on high dose PPI (1-2 mg/kg/day), which identified active EoE (≥15 eos/hpf) (referred to as 'diagnostic EoE'). Among the EoE patients, specimens were collected at a total of 183 endoscopies. A consort diagram is shown in Figure 1. Compared to control, there were no significant differences in age, race, ethnicity, or atopic conditions except for increased allergic conjunctivitis (diagnostic EoE: 27% vs. control: 6%, $p<0.05$) (Table 1). The diagnostic EoE patients had a significant increase in endoscopic findings: edema, exudate and furrows ($p<0.001$), and a trend toward increased dysphagia (41% vs. 13%, $p=0.06$) and feeding aversion (31% vs. 7%, $p=0.05$) compared to control who had increased abdominal pain (73% vs. 31% $p<0.01$). The median [IQR] of PEC for diagnostic EoE was 60 [35, 90] compared to control which was 0 [0, 6] ($p<0.001$) (Figure S2A).

Comparison of biomarkers in diagnostic EoE and control

To address whether a non-invasive biomarker could serve as a screen for EoE, we assessed differences between the plasma and urine eosinophil-associated proteins between the diagnostic EoE and control patients. As expected, AEC was increased in diagnostic EoE compared to control (median [IQR], 445 [288, 653] vs. 160 [85, 199] thousands/μL, $p<0.001$) (Figure 2A). Notably, we found several plasma eosinophil-

associated proteins increased in diagnostic EoE compared to control: CLC/GAL-10 (19.7 [10.3, 32.1] vs. 7.5 [5.8, 9.7] ng/mL, p<0.001), ECP (3.9 [2.5, 5.4] vs. 1.2 [0.8, 2.5] ng/mL, p<0.001), EDN (20.0 [15.7, 25.5] vs. 12.8 [11.2, 14.6] ng/mL, p<0.001), Eotaxin-3 (5.5 [0.0, 14.2] vs. 0 [0, 2.7] pg/mL, p<0.01), MBP-1 (751 [555, 1104] vs. 497 [427, 588] ng/ml, p<0.01) (Figure 2B-F). Urine OPN was elevated in diagnostic EoE compared to control (19.4 [12.0, 29.0] vs. 8.9 [6.0, 17.8] ng/mL, p<0.05, Figure S2D). We next assessed the receiver operating characteristics (ROC) of individual biomarkers to distinguish diagnostic EoE from control. Several plasma eosinophil-associated proteins had AUC over 0.75; AEC had an AUC of 0.9 (Figure S3). We determined the optimal cut-point for each biomarker, along with sensitivity, specificity, positive/negative predictive value (Table S1). Together, these findings validate prior publications[6, 8, 9] and identify novel biomarkers, particularly CLC/GAL-10, with potential as a screen for EoE.

Utility of single or multiple biomarkers for EoE diagnosis

We next sought to determine the utility of combinations of biomarkers to differentiate EoE diagnosis from control. We performed random forest, which utilizes machine learning and decision trees to determine an optimal combination of factors to predict an outcome by bootstrapping the decision trees with random portions of the dataset. The urine biomarkers were not included as samples were not available for all patients. We assessed four scenarios: 1) all plasma biomarkers including AEC, 2) significant biomarkers (determined by random forest) including AEC, 3) AEC alone, and 4) significant biomarkers (determined by random forest) without AEC. The Receiver Operating

Characteristic (ROC) graphs for each model along with performance characteristics are shown in Figure 3. Notably, the panel of biomarkers determined to be significant by random forest (CLC/GAL-10, ECP, EDN, Eotaxin-3, and MBP-1) along with AEC were found to best predict EoE with an error rate of 16% (sensitivity: 89%, specificity 79%, PPV: 73%, NPV: 91%), and an AUC of 0.9 (Figure 3B). This was more optimal compared to AEC (Figure 3C) or the significant biomarkers alone (Figure 3D). Thus, we identified a panel of plasma eosinophil-associated proteins that along with AEC may be suitable to screen for EoE.

Effect of treatment on biomarkers

We next sought to determine how the eosinophil-associated proteins changed with EoE-directed treatment. Among the 33 patients with paired specimens before and after treatment, 9 were treated with swallowed corticosteroids and 24 with elimination diet. The median [IQR] PEC for these 33 patients before and after treatment was 60 [30, 95] and 6 [0, 20], respectively (Table S2, $p<0.001$). Most biomarkers were significantly reduced after treatment (Table S2). Among this paired group (Figure S4A), 23 were treatment responders (PEC <15 eos/hpf on biopsy after treatment) and 10 were non-responders (PEC at least 15 eos/hpf after treatment). We found a very significant reduction in AEC, along with plasma CLC/GAL-10, ECP, and MBP-1 in histologic responders compared to non-responders (Figure 4), while a small but significant decrease was seen in EDN (Figure S4B), and no significant change in plasma eotaxin-2, eotaxin-3 and urine MMP-9

and OPN (Figure S4C-F). Thus, we identified a novel treatment responsiveness of plasma CLC/GAL-10, and validated this responsiveness in AEC, and plasma ECP and MBP-1.

Utility of single or multiple biomarkers to predict esophageal eosinophil count longitudinally

We next interrogated the full longitudinal EoE cohort to determine the ability of one or more eosinophil-associated proteins to predict PEC. To determine whether atopic diseases (asthma, conjunctivitis, eczema, and rhinitis) or medications (antihistamine, inhaled steroid, intranasal steroid, montelukast, topic steroids, and proton pump inhibitor) influenced disease activity or biomarker level, we performed mixed effects models with the log of each biomarker as the dependent variable. This identified antihistamine treatment along with a diagnosis of asthma, eczema and rhinitis as potential confounders (Table S3, S4A/B). We next utilized mixed models to assess the ability of the eosinophil-associated proteins to predict PEC in the longitudinal cohort. A Zero-Inflated Poisson (ZIP) mixed model was utilized to account for the significant number of time points with a PEC of 0. Unadjusted models for each individual biomarker (Table 2) identified AEC, CLC/GAL-10, ECP, EDN, and MBP-1 as significant predictors of PEC. When we adjusted for antihistamine treatment, diagnosis of asthma, eczema or rhinitis, as well as age and gender, the model estimates of AEC, MBP-1 and CLC/GAL-10 increased with lower standard error and p-values (Table 2). AEC was the strongest predictor followed by MBP-1. Finally, we assessed whether AEC, AEC+MBP-1 (combination 1), or AEC+CLC/GAL-10+ECP+EDN+MBP-1 (combination 2) was superior to predict PEC in ZIP mixed models

(Table S5). AEC+MBP-1 better predicted PEC than AEC alone ($p<0.005$) but was no different than the larger combination (Table S5). Thus, we identified CLC/GAL-10 as a novel predictor of esophageal eosinophilia. In addition, we validated AEC and MBP-1 as predictors of esophageal eosinophilia, with improved utility in combination.

DISCUSSION

In this prospective, longitudinal pediatric study, we identified several plasma and urine biomarkers with potential as noninvasive measures in the diagnosis and surveillance of EoE. While prior studies have typically assessed serum, our study assessed a panel of plasma and urine biomarkers in addition to Absolute Eosinophil Count (AEC). We found a combination of plasma CLC/GAL-10, ECP, EDN, Eotaxin-3, and MBP-1, along with AEC was superior to AEC alone in differentiating EoE diagnosis from control. The combination of AEC and plasma MBP-1 better predicted Peak Eosinophil Count (PEC) than AEC alone longitudinally in children with EoE. While additional validation is necessary, these novel findings of unique plasma biomarkers, and 2 panels of noninvasive measures are promising potential tools for EoE screening and surveillance.

Many studies have assessed absolute eosinophil count (AEC) as a biomarker in EoE. We found AEC was effective at identifying EoE diagnosis (AUC 0.9), and highly predictive of peak esophageal eosinophil count. Min SB et al. found AEC to be higher in 46 EoE adults and children compared to 53 controls, including predictability toward treatment response.[8] Schlag et al., found AEC correlated with esophageal eosinophil

density after treatment in adults.[9] Both studies assessed AEC in patients treated with swallowed corticosteroids. Konikoff et al. reported greater utility of AEC compared to EDN and Eotaxin-3, for identifying EoE disease activity and differentiating from control.[6] Our prospective study extends this previous work with more children with EoE, and inclusion of patients on elimination diets and swallowed corticosteroids. Together, this supports the utility of measuring AEC as a screen for EoE and for surveillance.

Unlike previous work,[6, 8, 12-14] we found CLC/GAL-10, an eosinophil cytosolic protein,[15] and MBP-1, a cytotoxic eosinophil granule cationic protein, were useful for EoE diagnosis, while Eotaxin-3, an eosinophil chemokine,[16] alone was less useful.[6] This is the first study to demonstrate the utility of plasma CLC/GAL-10 as an EoE biomarker. Furthermore, our findings validate the utility of eosinophil granule-associated cationic ribonucleases, EDN (*RNASE2*)[6, 8] and ECP (*RNASE3*),[8, 17] as biomarkers with potential utility to screen for EoE diagnosis. These findings reflect the ongoing recruitment and activation state of eosinophils during active EoE. We further identified a panel (AEC, CLC/GAL-10, ECP, EDN, Eotaxin-3, and MBP-1) that best predicted EoE diagnosis compared to AEC alone utilizing random forest, a data mining and machine learning analysis,[18] which bootstraps random chunks of data for decision trees to increase generalizability. This novel panel warrants further prospective studies. In addition to differences between active EoE and non-EoE controls, we found AEC, CLC/GAL-10, ECP, and MBP-1 distinguished treatment responders from non-responders, a novel finding with potential utility in treatment trials. Finally, we utilized Zero Inflated Poisson mixed models to assess the ability of the biomarkers to predict esophageal eosinophilia in the longitudinal EoE cohort. These models enhance generalizability by accounting for

clustering within subjects, and the relatively small range of eosinophil counts that identify inactive disease. This considers the dramatic difference that true remission may have relative to partial remission or active EoE. Individual biomarkers (AEC, CLC/GAL-10, ECP, EDN, and MBP-1) correlated with PEC longitudinally, and we identified the combination of AEC and MBP-1 was significant compared to AEC alone to predict esophageal eosinophilia, a novel finding. Together, our findings identify biomarker panels to screen for esophageal eosinophilia and should be validated in a large longitudinal cohort.

Another key strength of our study was the methods used for both sample collection and biomarker detection. Specifically, the rapid processing and use of plasma, rather than serum, to minimize clotting-induced non-specific biomarker "secretion" from eosinophils in the blood sample itself, was used to avoid biomarker levels (ECP and EDN; Figure S1) and CLC/GAL-10 (not shown) being simple "surrogates" of the AEC at the time of blood draw. For MBP-1 analysis, reduction and alkylation of the sample was performed prior to ELISA, and is established to maximize recovery and detection of MBP-1,[3, 17, 19] a potential limitation of prior work.[14] Thus, our approach is novel in peripheral protein biomarker detection in EoE, and an important contribution to the field.

Several studies have assessed potential biomarkers of EoE in urine. Cunnion KM et al. recently described a mass spectrometry-based method of measuring urinary 3-bromotyrosine, which showed excellent sensitivity and specificity in untreated EoE patients with active disease vs. atopic and non-atopic controls.[20] Lexmond et al., however found no utility in measuring urinary leukotriene E4.[20, 21] We found urine matrix metalloproteinase (MMP-9), a zinc-dependent endopeptidase thought to be involved in

remodeling,[22] was not useful to assess EoE disease activity. Osteopontin (OPN), an integrin-binding cell adhesion molecule expressed by a wide variety of immune cells,[23] weakly predicted esophageal eosinophil counts. Fewer urinary than plasma specimens were collected in this study, thus more validation studies regarding OPN are warranted to confirm its utility as a biomarker.

There were a number of weaknesses in our study. While we achieved power for the primary aim, a larger sample size in the paired longitudinal cohort may have found greater differences between treatment responders and non-responders. Nonetheless, we did not identify significant confounders, including atopy, gender or type of treatment (data not shown). While atopic diseases aside from EoE could be a source of elevated peripheral biomarkers, this study could not assess this directly as we measured atopic disease prevalence and medication use, but not disease severity. As all EoE patients were treated with a PPI prior to diagnostic endoscopy, we were not able to assess the effect of the PPI on these biomarkers. Direct comparison of patients treated with either elimination diet or swallowed corticosteroids could not be made since the study was not powered to detect this. In addition, we were not able to assess the relationship of the biomarkers to symptoms given the lack of an available validated tool at the time of the study, and broad range of symptoms in children. Finally, our reliance on peak esophageal eosinophil counts (PEC) as opposed to a composite histological score[24] may be a limitation, as it could underestimate the burden of eosinophilia in an entire biopsy. Utilization of the EoE histological scoring system[24] would be beneficial for validation studies.

In conclusion, we have identified a novel panel of plasma (CLC/GAL-10, ECP, EDN, Eotaxin-3 and MBP-1) biomarkers, which along with absolute eosinophil count (AEC), are useful in identifying untreated EoE from non-EoE controls. In addition, AEC, and plasma CLC/GAL-10, ECP, EDN, and MBP-1 each predicted esophageal eosinophilia, while the combination of AEC and MBP-1 was most optimal. Future, large prospective studies are needed to address the feasibility and applicability of these biomarker panels as a screening tool for clinicians to identify subjects for EGD referral to confirm EoE and to monitor treatment response.

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Figure Legends

Figure 1. Consort diagram for EoE and non-EoE patient controls. Patient recruitment, reasons for inclusion/exclusion, and treatment status are noted.

Figure 2. Increased AEC and plasma CLC/GAL-10, ECP, EDN, Eotaxin-3, and MBP-1 in children with EoE at diagnostic endoscopy compared to controls. Comparisons made by non-parametric t-test of absolute eosinophil count (A), and plasma levels of CLC/GAL-10 (B), ECP (C), EDN (D), Eotaxin-3 (E), and MBP-1 (F). **=$p<0.01$, ***=$p<0.001$

Figure 3. A panel of biomarkers is superior to AEC alone to identify EoE diagnosis. Receiver Operating Characteristics (ROC) from random forest analysis for distinguishing EoE from non-EoE controls. Panels are All Biomarkers (A), Significant Biomarkers (B), AEC alone (C), and Significant Biomarkers without AEC (D). Significant biomarkers were determined by random forest and are CLC/GAL-10, ECP, EDN, Eotaxin-3, and MBP-1. Area under the curve (AUC) is shown for each of the indicated biomarker combinations, along with probabilities along the ROC curves; all curves significant at $p<0.001$.

Figure 4. **Significant Reduction in AEC and Plasma CLC/GAL-10, ECP, and MBP-1 in Histologic Responders to Treatment for Eosinophilic Esophagitis.** Plasma eosinophil-associated proteins were measured by ELISA., and compared by Wilcoxon signed-rank test in paired EoE patients before (red) and after (blue) treatment with diet elimination or swallowed steroids. Patients grouped by post-treatment histologic response (responder PEC <15, non-responder PEC: 15 or more). There was a significant

reduction in AEC (A), CLC/GAL-10 (B), ECP (C), and MBP-1 (D) in histologic responders.

*=p<0.05, **=p<0.01, ***=p<0.001

Table 1. Patient characteristics for diagnostic EoE and non-EoE patient controls

	EoE (n=41)	Control (n=15)	p-value[1]
Age (years, Median [IQR])	8.8 [5.6,14.0]	6.9 [5.9, 12.9]	0.52
Demographics, n (%)			
Male	32 (78)	7 (47)	**0.05**
Hispanic	4 (10)	2 (13)	0.64
Asian	3 (7)	3 (20)	0.14
Black	3 (7)	0 (0)	0.56
White	36 (88)	12 (80)	0.67
Medical History, n (%)			
Atopic	32 (78)	10 (67)	0.49
Asthma	16 (39)	5 (33)	0.76
Conjunctivitis	2 (5)	4 (27)	**0.04**
Eczema	17 (41)	6 (40)	1.00
Food Allergy	13 (32)	1 (7)	0.08
Allergic Rhinitis	25 (61)	8 (53)	0.76
Drug Allergy	5 (13)	5 (33)	0.12
GERD	6 (15)	4 (27)	0.43
Celiac	3 (7)	0 (0)	0.56
IBD	1 (2)	0 (0)	1.00
Visual Findings, n (%)			
Edema	33 (80)	0 (0)	**<0.001**
Exudate	26 (63)	0 (0)	**<0.001**
Furrow	38 (93)	2 (13)	**<0.001**
Rings	10 (24)	1 (7)	0.26
Stricture	0 (0)	0 (0)	ND
Symptoms, n (%)			
Abdominal Pain	11 (27)	11 (73)	**0.004**
Chest Pain	4 (10)	2 (13)	0.65
Dysphagia	18 (44)	2 (13)	0.06
Early Satiety	12 (29)	3 (20)	0.74
Feeding Aversion	14 (34)	1 (7)	**0.05**
Food Impaction	8 (20)	3 (20)	1.00
FTT	9 (22)	1 (7)	0.26
Gagging	10 (24)	1 (7)	0.26
Heartburn	5 (12)	3 (20)	0.67
Nausea	13 (32)	4 (27)	1.00
Odynophagia	5 (12)	0 (0)	0.31
Pockets or spits out food	7 (17)	4 (27)	0.46
Slow eating	15 (37)	4 (27)	0.54
Vomiting	9 (22)	4 (27)	0.73

[1]Comparisons made by student's t-test for age and Fisher's Exact test for remaining dichotomous variables.

Table 2. Individual and Combinations of Eosinophil-Associated Plasma Proteins Predict Eosinophil Count

Biomarker	Unadjusted			Adjusted		
	Estimate[1]	Std. Err	p-value	Estimate[2]	Std. Err	p-value
AEC	2.39	0.55	**<0.0001**	2.52	0.55	**<0.0001**
CLC/GAL-10	0.44	0.13	**<0.001**	0.50	0.13	**<0.0001**
ECP	0.50	0.14	**<0.001**	0.59	0.16	**<0.001**
EDN	0.49	0.21	**<0.05**	0.67	0.26	**<0.05**
Eotaxin-2	0.01	0.03	0.65	0.01	0.04	0.72
Eotaxin-3	0.06	0.06	0.31	0.07	0.07	0.32
MBP-1	0.66	0.61	**<0.001**	0.89	0.23	**<0.0001**
MMP-9	0.51	0.51	0.60	0.99	1.17	0.40
OPN	0.25	0.25	0.09	0.26	0.15	0.08
Combination 1						
AEC	2.07	0.60	**<0.001**	2.00	0.57	**<0.001**
MBP-1	0.35	0.20	0.07	0.51	0.21	**<0.05**
Combination 2						
AEC	1.73	0.66	**<0.01**	1.73	0.67	**<0.01**
CLC/GAL-10	0.12	0.15	0.42	0.10	0.16	0.52
ECP	0.15	0.18	0.39	0.18	0.19	0.33
EDN	-0.08	0.24	0.73	-0.08	0.28	0.78
MBP-1	0.25	0.21	0.23	0.48	0.25	0.05

[1]Estimated mean effect of biomarker(s) to predict log PEC assessed in Zero-Inflated Poisson Mixed Models. Significant biomarkers are bolded ($p<0.05$).

[2]Estimated mean effect of biomarker(s) to predict log PEC assessed in Zero-Inflated Poisson Mixed Models which control for age, gender, atopic diseases/medications. Significant biomarkers are bolded ($p<0.05$).

547 Figure 1
548

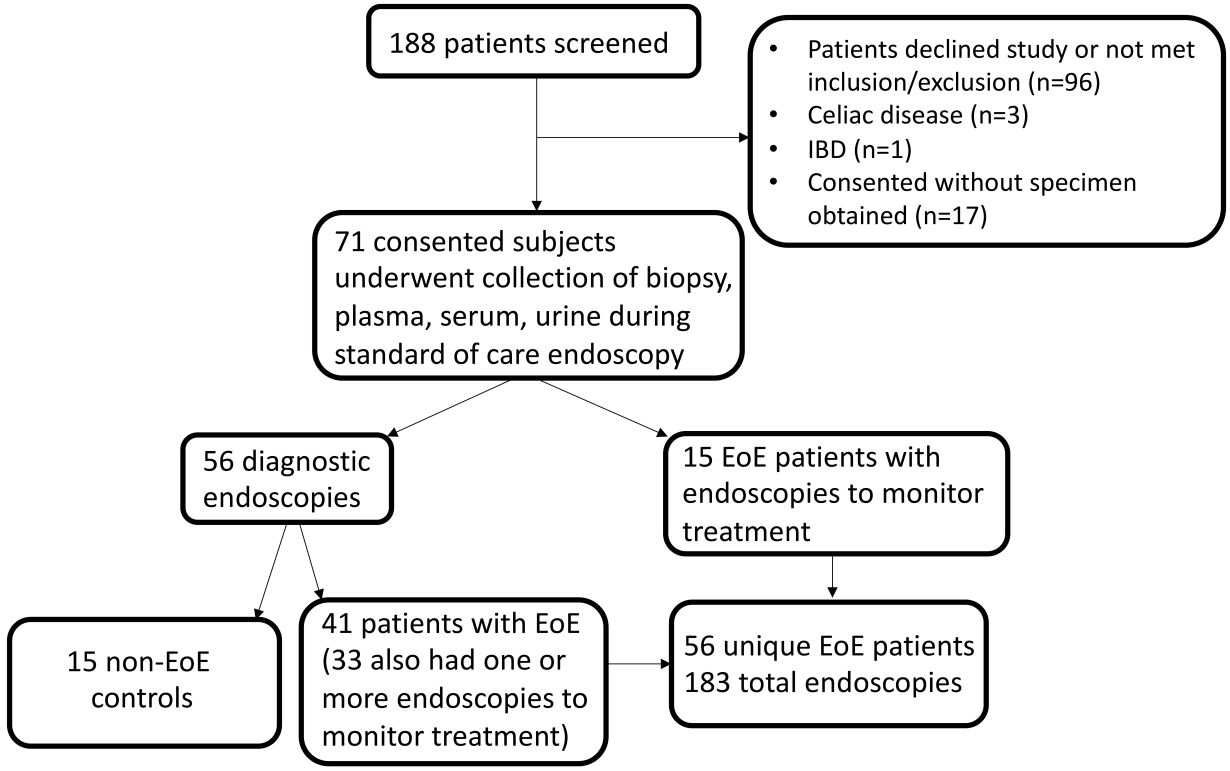

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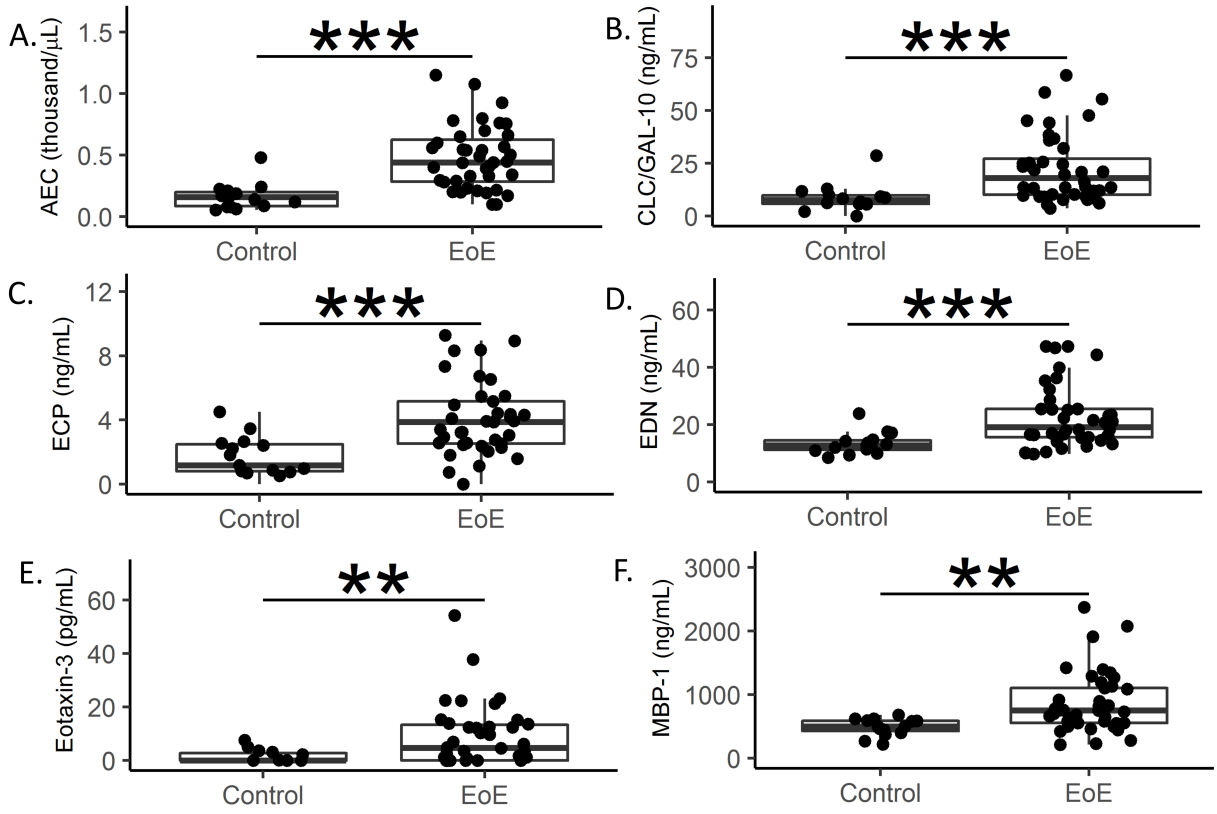
Figure 2


A.
AEC (thousand/μL)
1.5
1.0
0.5
0.0
***
Control
EoE
B.
CLC/GAL-10 (ng/mL)
75
50
25
0
***
Control
EoE
C.
ECP (ng/mL)
12
8
4
0
***
Control
EoE
D.
EDN (ng/mL)
60
40
20
0
***
Control
EoE
E.
Eotaxin-3 (pg/mL)
60
40
20
0
**
Control
EoE
F.
MBP-1 (ng/mL)
3000
2000
1000
0
**
Control
EoE

553 Figure 3
554

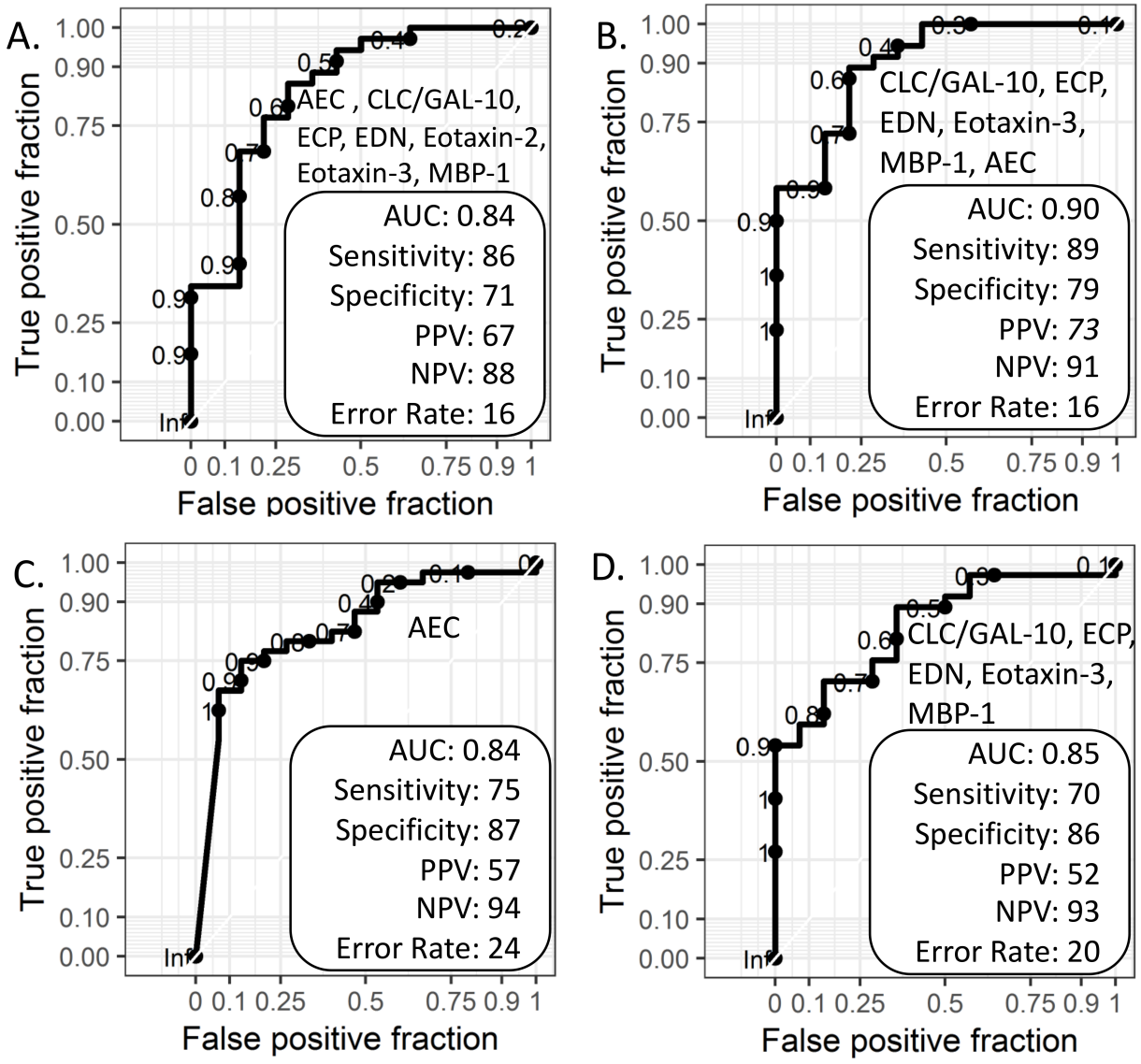


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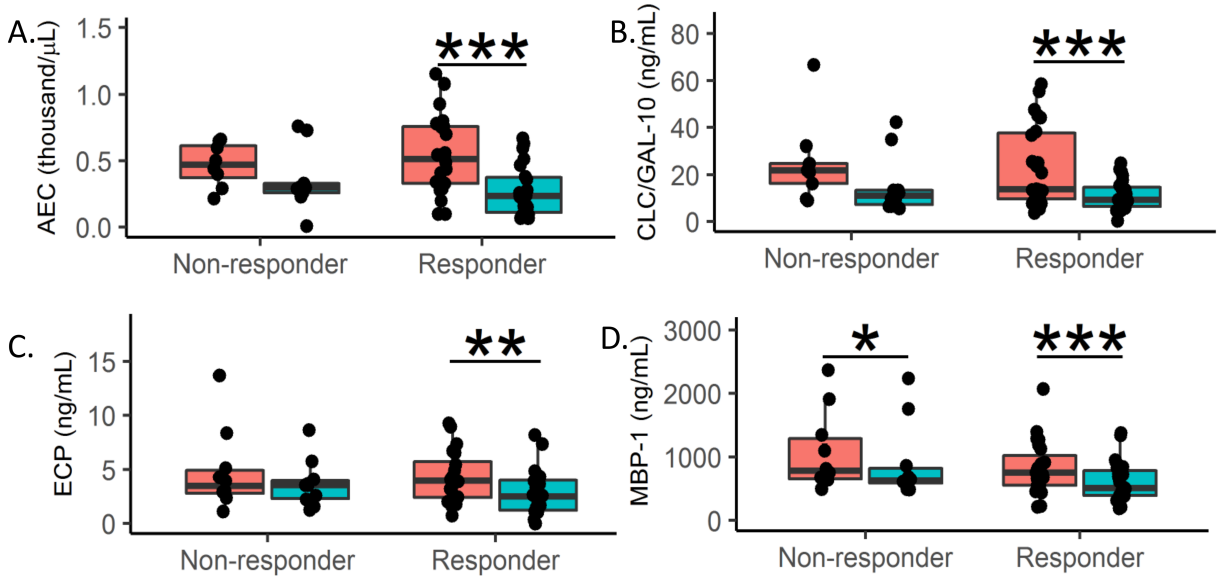
Figure 4


A.
AEC (thousand/μL)
1.5
1.0
0.5
0.0
***
Non-responder
Responder
B.
CLC/GAL-10 (ng/mL)
80
60
40
20
0
***
Non-responder
Responder
C.
ECP (ng/mL)
15
10
5
0
**
Non-responder
Responder
D.
MBP-1 (ng/mL)
3000
2000
1000
0
*
***
Non-responder
Responder