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Title: Nasal and Pharyngeal Eosinophil Peroxidase Levels in Adults with Poorly Controlled Asthma Correlate with Sputum Eosinophilia

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Short title: Nasal and Pharyngeal EPX in Adults with Asthma

Key Words: asthma; diagnostics; eosinophil granule proteins; eosinophils; sputum

Abstract

The objective of the study was to compare nasal, pharyngeal, and sputum eosinophil peroxidase (EPX) levels with induced sputum eosinophil percentage in 10 adults with poorly controlled asthma and 10 normal controls. EPX was measured using an ELISA and normalized for grams of protein for nasal and pharynx specimens and for mL-gram of protein for sputum. Sputum EPX levels were statistically different between asthma and control. This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12817

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subjects ($p=0.024$). EPX levels measured in the nasal and pharyngeal swab samples derived from the same patients were also different between asthma and control subjects, each displaying a high degree of significance ($p=0.002$). Spearman's correlation coefficients for nasal EPX and pharyngeal EPX levels compared to induced sputum eosinophil percentage were 0.81 ($p=0.0007$) and 0.78 ($p=0.0017$), respectively. There is a strong association in a given patient between both nasal and pharyngeal EPX levels and the eosinophil percentage of induced sputum.

Systematic review and meta-analyses of available studies suggest that titrating inhaled corticosteroids using sputum eosinophil percentage is an effective strategy to reduce the frequency of asthma exacerbations.(1) However, the required technical expertise and other confounding factors (e.g., labor-intensive character) associated with sputum eosinophil measurements have limited their use in clinical practice. Several proposed surrogate biomarkers of airway eosinophilia, including the fractional exhaled nitric oxide (FeNO), circulating blood eosinophil levels, and serum Immunoglobulin E, were compared in a systematic diagnostic testing accuracy review/meta-analysis and were found to have moderate sensitivity and specificity for predicting the presence of eosinophils in induced sputum (i.e., lower airway eosinophilia).(2) Therefore, a less invasive, technically simpler, and more precise surrogate biomarker would represent an un-met need to facilitate widespread implementation of management algorithms that incorporate eosinophil biomarkers.

A surprisingly high correlation between lower airway eosinophilia and the presence of eosinophils in nasal lavage was previously demonstrated with a receiver operating characteristic (ROC) curve whose area was 0.84 ($p<0.001$) using induced sputum as the comparative measure.(3) In contrast, the relationship between sputum eosinophilia and the presence of pharyngeal eosinophils has not been extensively studied. Eosinophil peroxidase (EPX) is a secondary granule protein uniquely secreted by eosinophils that we have shown is easily detected and quantified using a novel enzyme-linked immunosorbent assay (ELISA).(4) In particular, our previous studies of respiratory patients have shown that sputum EPX levels strongly correlate with eosinophil cell counts in sputum ($r=0.84$).(5) The objective of this current study was to expand our assessments of asthma patients and determine if EPX was detectable in topical swabs of the mucosal surfaces from the nasal and pharyngeal cavities of asthma patients as a means of providing a minimally invasive and technically simpler point-of-care diagnostic assay.

Adult participants were identified from pulmonary and allergy-immunology subspecialty clinics from April 1, 2014 through March 31, 2015. Inclusion criteria for the case group of poorly-controlled asthma patients were provider-diagnosed asthma, FEV₁ >1.0 Liter, Asthma Control Test (6) (ACT)TM <20, and FeNO (7) >50 ppb; exclusion criteria were a diagnosis of COPD or evidence of bronchiectasis. Control participants were age-matched to asthma participants (within 10 years) and were excluded if they had a history of rhinitis, sinusitis, asthma, or any eosinophil disorder. Three specimen types were collected from each participant: *(i)* Initially, a nasal specimen was collected using a sterile polyester fiber-tipped swab passing 5 times along the nasal floor and below the inferior turbinate bilaterally. *(ii)* A sterile polyester fiber-tipped swab was rubbed across the oropharynx 5 times. *(iii)* Participants underwent a sputum induction as previously described.⁽⁵⁾ The measurement of EPX levels has been previously described (4, 5) and details regarding our current protocols and methods are included as an online supplement. EPX levels between asthma and control subjects were compared using the Wilcoxon signed-rank test; Spearman's test was used to assess correlations between EPX levels and sputum eosinophil percentages. This study was approved by the Mayo Clinic IRB (14-001168).

A summary of the participant demographics, clinical information, and laboratory data are displayed in **Table 1**, with equal numbers of females and males in the asthma and control subject groups. The mean age ($\pm$ SD) of the asthma subjects was 48 years old ($\pm$ 8.7) and for controls was 43 ($\pm$ 13.6). Three of the subjects with asthma were using intranasal corticosteroids and 6 were using inhaled corticosteroids at the time samples were collected. All 10 of the asthma subjects had a diagnosis of rhinitis or sinusitis, while these diagnoses were excluded in the control subjects. None of the subjects smoked cigarettes. The mean ACT score ($\pm$ SD) in the asthma group was 10.2 ($\pm$ 3.2) with <20 considered to be poorly controlled asthma. The mean FeNO measurements ($\pm$ SD) in the asthma group was 93ppb ($\pm$ 40.3) with >39ppb considered to be abnormal. Mean pre-bronchodilator FEV₁ % predicted ($\pm$ SD) was 64% ($\pm$ 17.3%) for the asthma subjects and 101% ($\pm$ 9.8%) for the control group. Induced sputum eosinophilia was a highly significant metric ($p=0.0017$) stratifying asthma vs. control subjects with all but one of the enrolled subjects in the asthma group having an induced sputum eosinophil percentage >3% (**Figure 1(A)**). It is noteworthy that even in this structured setting, recovery and assessment of induced sputum from patients (particularly control subjects) was difficult with only 4 of 10 subjects capable of producing the necessary sample. As we have previously demonstrated, sputum EPX levels measured by our novel ELISA were statistically different between asthma and control subjects ($p=0.024$) (**Figure 1(B)**). More importantly, EPX levels measured in the nasal and pharyngeal swab samples derived from the same patients were also different between asthma and control subjects,

each displaying a high degree of significance ($p=0.002$) (**Figure 1(B)**). Further statistical assessments of the data showed that Spearman's correlation coefficients for nasal EPX and pharyngeal EPX levels compared to induced sputum eosinophil percentage were 0.81 ($p=0.0007$) and 0.78 ($p=0.0017$), respectively (**Figure 1(C)**). Indeed, the areas under ROC curves of these sample sources relative to induced sputum eosinophil percentage were each 0.89 ($p=0.003$).

We recognize that there are several limitations to keep in mind when considering the results of this study. For example, poorly controlled asthma patients were selected for study inclusion using elevated FeNO measurements so our findings may not apply to the broader asthma population, which includes patients with non-eosinophilic asthma. In addition, some of the patients had very high sputum eosinophil percentages and could possibly have diagnoses in addition to asthma such as allergic bronchopulmonary aspergillosis or eosinophilic granulomatosis with polyangiitis. Finally, the ability to report the best cut-off levels for EPX measurements and analyze potentially confounding factors such as nasal steroid medications was limited by sample size.

The primary findings of this study are two-fold: **(1)** Nasal and pharyngeal EPX levels are elevated in poorly controlled asthma patients with elevated FeNO levels compared to normal control subjects and **(2)** There is a strong association in a given patient between both nasal and pharyngeal EPX levels and the eosinophil percentage of induced sputum. The demonstration that EPX levels in these minimally invasive and easily accessible samples are surrogate biomarkers for lower airway eosinophilia is significant. That is, eosinophil percentage of induced sputum, albeit a technically difficult measurement not widely adopted into clinical practice, is nonetheless considered a “gold standard” metric for adjusting asthma therapy. Thus, the use of nasal or pharyngeal swabs may represent a clinically relevant diagnostic metric whose simplicity of use would provide a novel point-of-care assay in the management of asthma patients. This is particularly true for the management of pediatric patients where the diagnostic tools available to clinicians (e.g., questionnaires and the use of pulmonary inflammatory assessments) are limited relative to adults.

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Figure Legend

Figure 1. Nasal and pharyngeal EPX levels represent diagnostic metrics that significantly correlate with the lower airway eosinophilia observed in poorly controlled asthmatics. (A) The presence of sputum eosinophils stratifies asthma from control subjects. (B) EPX levels in sputum as well as the mucosal surfaces of the nasal and pharyngeal cavities of asthma patients are each statistically different between asthma and control subjects. (C) Spearman correlation demonstrates that both nasal and pharyngeal EPX assessments increase as a function of induced sputum eosinophilia.

References

1. Petsky HL, Kynaston JA, Turner C, Li AM, Cates CJ, Lasserson TJ, et al. Tailored interventions based on sputum eosinophils versus clinical symptoms for asthma in children and adults. The Cochrane database of systematic reviews 2007(2):CD005603.
2. Korevaar DA, Westerhof GA, Wang J, Cohen JF, Spijker R, Sterk PJ, et al. Diagnostic accuracy of minimally invasive markers for detection of airway eosinophilia in asthma: a systematic review and meta-analysis. The Lancet. Respiratory medicine 2015;3(4):290-300.
3. Amorim MM, Araruna A, Caetano LB, Cruz AC, Santoro LL, Fernandes AL. Nasal eosinophilia: an indicator of eosinophilic inflammation in asthma. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology 2010;40(6):867-74.
4. Ochkur SI, Kim JD, Protheroe CA, Colbert D, Moqbel R, Lacy P, et al. The Development of a Sensitive and Specific ELISA for Mouse Eosinophil Peroxidase: Assessment of Eosinophil Degranulation Ex Vivo and in Models of Human Disease. Journal of Immunological Methods 2012;375(1-2):138-47.
5. Nair P, Ochkur SI, Protheroe CA, Radford K, Efthimiadis A, Lee NA, et al. Eosinophil Peroxidase in Sputum Represents a Unique Biomarker of Airway Eosinophilia. Allergy 2013;68:1177-1184.
6. Nathan RA, Sorkness CA, Kosinski M, Schatz M, Li JT, Marcus P, et al. Development of the asthma control test: a survey for assessing asthma control. The Journal of allergy and clinical immunology 2004;113(1):59-65.
7. Dweik RA, Boggs PB, Erzurum SC, Irvin CG, Leigh MW, Lundberg JO, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. Am J Respir Crit Care Med 2011;184(5):602-15.

Table 1. Clinical Characterization of Participating Control and Asthma Patients

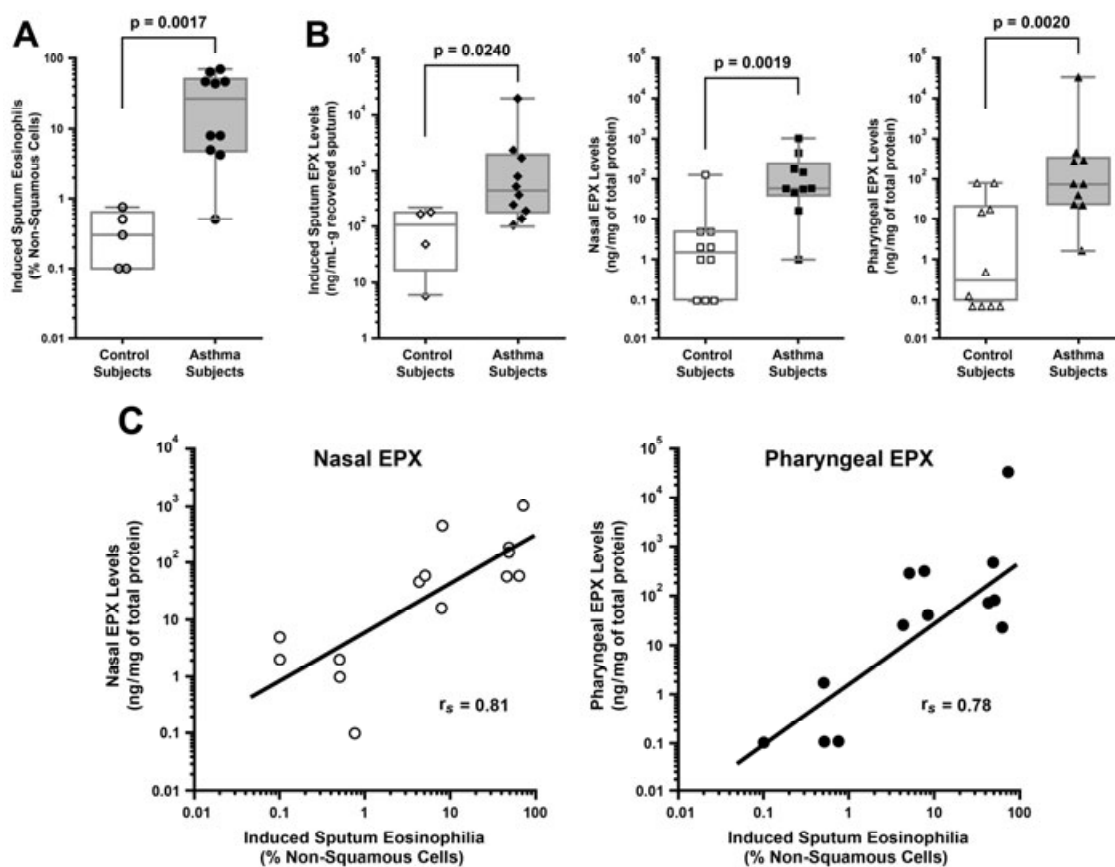
Age	Subject Type	Gender	Nasal Steroid	Inhaled Steroid	ACT _M score (% predicted)	FEV ₁	FENO (ppb)	Sputum Eosinophil (% non-squamous cells)	Sputum EPX (ng/mL-g protein)	Nasal EPX (ng/mg protein)	Pharyngeal EPX (ng/mg protein)
61	Asthma	Male	Y	Y	17	72	53	4.3	532	46	24
53	Asthma	Male	Y	Y	5	57	141	45.5	806	57	74
52	Asthma	Female	-	-	11	57	77	62.7	369	58	22
50	Asthma	Female	Y	Y	10	69	73	48	20196	151	76
46	Asthma	Male	-	-	8	75	175	8	191	446	39
56	Asthma	Male	-	Y	12	37	104	5	247	59	274
38	Asthma	Male	-	-	9	43	70	0.5	1735	1	1.63
42	Asthma	Female	-	Y	10	66	70	7.8	106	16	303
47	Asthma	Female	-	-	12	99	115	48	139	182	469
32	Asthma	Female	-	Y	8	65	52	70.8	2374	1026	33487
25	Control	Female	-	-	NP	124	NP	0.75	6	0	0
36	Control	Female	-	-	NP	105	NP	0.3	NA	0	15
37	Control	Female	-	-	NP	99	NP	NA	NA	5	80
36	Control	Female	-	-	NP	97	NP	NA	NA	1	0.49
63	Control	Male	-	-	NP	91	NP	NA	NA	5	80
44	Control	Male	-	-	NP	107	NP	NA	NA	127	17
58	Control	Male	-	-	NP	100	NP	0	167	5	0
56	Control	Male	-	-	NP	104	NP	NA	NA	1	0.13
25	Control	Male	-	-	NP	89	NP	0.5	183	2	0
52	Control	Female	-	-	NP	96	NP	0	48	2	0

NA = Not Available

NP = Not Performed

Y = Yes

- = No



Nasal and Pharyngeal Eosinophil Peroxidase Levels in Adults with Poorly Controlled Asthma Correlate with Sputum Eosinophilia

ONLINE SUPPLEMENTARY MATERIALS AND METHODS

Sputum processing and cell/supernatant preparation. The entire sputum expectorates (spontaneous or induced) were transferred to a Petri dish and the macroscopic appearance recorded. All portions free of salivary contamination (selected portion), confirmed by inverted microscopy, were placed in a pre-weighed 15mL polystyrene tube and the weight in mg recorded. The sputum was treated with freshly prepared 0.5% dithiothreitol (DTT) which was added to the sputum at a final concentration of 0.1%. The sputum was gently aspirated in and out of a pipette, and vortexed for 15 seconds to ensure mixing. The tube was then be placed on a bench rocker (Dade Tube Rocker, Baxter Diagnostics Corporation, Miami, Florida) and rocked for 15 minutes. A further 4 volumes of phosphate buffered saline were added to the suspension and it was filtered through 60µm nylon net filters (Millipore, Billerica, MA), to remove debris. Total cell count (TCC) and viability (V) were performed simultaneously in a Neubauer hemocytometer. Viability of cells was assessed by the Trypan blue exclusion. The recovered sputum cell suspensions were adjusted to 1.0×10^6 cells/mL and 60µl of these cell suspensions were used to generate cytopsin preparations with a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickley, PA); spinning at 450rpm for 6 minutes. The slides were air dried and stained with Wright's-Giemsa. Differential cell counts were performed on 400 non-squamous cells. The sputum cellularity was expressed per mg of sputum. The remaining cell suspensions were spun at 600g for 4 minutes to generate cell-free supernatants that were aspirated and stored at -80°C for later analysis.

EPX monoclonal antibody-based ELISA. The basic Sandwich/Capture ELISA protocol was created as a modification of our previously published protocol for the laboratory-based detection and quantification of EPX in fluid samples from patient sources.(4, 5) Briefly, micro-titer plates were pre-treated with 4µg/mL anti-EPX monoclonal antibody MM25-429.1.8 (capture antibody) in 100µL of Coating Solution at 4°C overnight. Coated wells of the micro-titer plates were cleared of unbound antibody with 4-5 cycles of rinsing using Wash Solution. Potential areas of non-specific binding in each well of the plate were blocked by a 30 minute room temperature pre-incubation with 300µL of Diluent/Blocking Solution that was itself previously diluted 1/10. Following the incubation to block the wells, the Diluent/Blocking Solution was removed and

100µL of sample (and EPX standards) were added and the plate was incubated at room temperature without shaking for 1.5-2 hours. The wells of the micro-titer plate were cleared of unbound target antigen with 4-5 cycles of rinsing using Wash Solution. 100µL of biotinylated anti-EPX monoclonal antibody MM25-82.2.1 (detection antibody) was added to each well at a final concentration of 0.8µg/mL and the plate was incubated at room temperature for 1.5-2 hours. The wells of the micro-titer plate were cleared of unbound detection antibody with 4–5 cycles of rinsing using Wash Solution; 100µL of Streptavidin-AP (diluted 1/500 in 1% BSA, 0.05% Tween-20, 0.025M Tris-HCL pH 7.4, 0.5M NaCl) was added to each well and the plate was incubated at room temperature for 30 minutes. The wells of the micro-titer plate were cleared of unbound Streptavidin-AP with 4-5 cycles of rinsing using Wash Solution. 100µL of BluePhos™ substrate was added to each well and the plate was incubated at 37°C for 1 hour with gentle rotation. The colorimetric reaction was terminated with the addition of 100µL of Stop Solution. Absorbance of individual wells of the plate was determined at a wavelength of 630nm.

References

1. Petsky HL, Kynaston JA, Turner C, Li AM, Cates CJ, Lasserson TJ, et al. Tailored interventions based on sputum eosinophils versus clinical symptoms for asthma in children and adults. The Cochrane database of systematic reviews 2007(2):CD005603.
2. Korevaar DA, Westerhof GA, Wang J, Cohen JF, Spijker R, Sterk PJ, et al. Diagnostic accuracy of minimally invasive markers for detection of airway eosinophilia in asthma: a systematic review and meta-analysis. The Lancet. Respiratory medicine 2015;3(4):290-300.
3. Amorim MM, Araruna A, Caetano LB, Cruz AC, Santoro LL, Fernandes AL. Nasal eosinophilia: an indicator of eosinophilic inflammation in asthma. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology 2010;40(6):867-74.
4. Ochkur SI, Kim JD, Protheroe CA, Colbert D, Moqbel R, Lacy P, et al. The Development of a Sensitive and Specific ELISA for Mouse Eosinophil Peroxidase: Assessment of Eosinophil Degranulation Ex Vivo and in Models of Human Disease. Journal of Immunological Methods 2012;375(1-2):138-47.
5. Nair P, Ochkur SI, Protheroe CA, Radford K, Efthimiadis A, Lee NA, et al. Eosinophil Peroxidase in Sputum Represents a Unique Biomarker of Airway Eosinophilia. Allergy 2013;68:1177–1184.
6. Nathan RA, Sorkness CA, Kosinski M, Schatz M, Li JT, Marcus P, et al. Development of the asthma control test: a survey for assessing asthma control. The Journal of allergy and clinical immunology 2004;113(1):59-65.
7. Dweik RA, Boggs PB, Erzurum SC, Irvin CG, Leigh MW, Lundberg JO, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. Am J Respir Crit Care Med 2011;184(5):602-15.