



Eosinophil Quantitated Urine Kinetic A novel assay for assessment of eosinophilic esophagitis

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ABSTRACT

Background: Eosinophilic esophagitis (EoE) is a chronic disease that requires long-term medical management and monitoring. The eosinophil count determined during esophageal biopsy remains the gold standard for diagnosis and monitoring of EoE. Although markers of eosinophil degranulation correlate with symptoms, eosinophil counts do not correlate. Development of a noninvasive, cost-effective biomarker of eosinophil activation for the evaluation of EoE is an unmet medical need.

Objective: To conduct a proof-of-concept study to evaluate the potential for measuring urinary 3-bromotyrosine (3-BT) levels in creatinine normalized urine for quantifying eosinophil degranulation in EoE disease.

Methods: A mass spectrometry–based method of measuring normalized 3-BT levels, the Eosinophil Quantitated Urine Kinetic (EoQUIK), was developed, and proof-of-concept evaluation was performed for patients with EoE (n = 27), atopic controls (n = 24), and nonatopic controls (n = 24).

Results: EoQUIK revealed that median normalized 3-BT levels were increased 93-fold in patients with EoE compared with nonatopic controls ($P = .01$) and increased 13-fold in patients with EoE compared with atopic controls ($P = .01$). Cutoff thresholds were selected for EoQUIK that yielded a specificity of 100% and a negative predictive value of 100% for nonatopic controls and a specificity of 79% and a negative predictive value of 90% for atopic controls. In a logistic regression model, a urine 3-BT level greater than 20 pg per 400 mg of creatinine increased the odds of a patient having EoE by 4.8 (95% confidence interval, 1.14–20.5; $P = .03$) when compared with atopic controls after controlling for race and sex.

Conclusion: These data provide proof of concept that EoQUIK can potentially be a useful noninvasive clinical tool in the evaluation of possible EoE.

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Introduction

The current consensus guidelines define eosinophilic esophagitis (EoE) as “a chronic, immune/antigen-mediated esophageal disease characterized clinically by symptoms related to esophageal dysfunction and histologically by eosinophil-predominant inflammation.”¹ EoE is a clinicopathologic diagnosis based on symptoms, endoscopic findings, and histologic findings. In the clinical

evaluation of possible EoE, it is important to rule out other causes of esophageal eosinophilia, such as proton pump inhibitor responsive esophageal eosinophilia, primary hypereosinophilic syndrome, drug hypersensitivity, infection, Crohn disease, celiac disease, and connective tissue disease.¹ Currently, diagnostic confirmation of EoE is achieved in a symptomatic patient with esophageal eosinophilia by demonstrating persistent esophageal eosinophilia after empiric treatment with high-dose proton pump inhibitors for 6 weeks to rule out gastrointestinal reflux disease and proton pump inhibitor–responsive esophageal eosinophilia.²

Once the diagnosis of EoE is confirmed and treatment initiated, many centers repeat endoscopy with biopsy in 6 to 8 weeks to assess improvement. Currently, there are no guidelines for the

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frequency of biopsies for longitudinal follow-up of these patients. Symptoms often do not correlate with esophageal histologic findings.³ This fact paired with the fibrostenotic potential of uncontrolled EoE requires at least periodic histologic evaluation in patients with EoE. Currently, there is no reliable noninvasive assay to monitor esophageal eosinophilia in the long term, although some assays are under investigation.⁴

Endoscopy and biopsy are imperfect tests for the long-term assessment of esophageal eosinophilia. In addition to the cost and risk associated with the procedure, endoscopic biopsy samples a very small surface area of the esophageal mucosa, and eosinophilia is not uniformly distributed throughout the esophagus in EoE. Five 2-mm biopsy specimens represent less than 0.7% of the esophageal mucosa in a 20- to 25-cm esophagus.⁵ There is not a standardized high-power field area, and current diagnostic thresholds for eosinophils per high-powered field are not validated. The development of a noninvasive assay to determine disease activity in EoE has 2 potential applications: (1) aiding in the initial evaluation of patients with symptoms consistent with EoE and (2) evaluating disease progression and response to therapy in patients with EoE in the long term. Thus, development of an accurate method to non-invasively measure EoE disease activity is a major unmet medical need.

3-Bromotyrosine (3-BT) is a chemical marker of eosinophil activation. Eosinophil peroxidase resides in a matrix of cytoplasmic granules within eosinophils and is released when eosinophils are activated. Eosinophil peroxidase halogenates tyrosine residues in plasma proteins and generates 3-BT. Wedes et al⁶ found that urinary levels of 3-BT, a noninvasive marker of eosinophil-catalyzed oxidation, strongly predict the presence of asthma. In patients with EoE, the presence of extracellular eosinophil granules and deposition of eosinophil peroxidase suggest a similar activation of eosinophils. Therefore, urinary 3-BT may be a useful biomarker for disease activity in EoE. However, urine analysis cannot provide any information on the cellular origin of 3-BT, so studies examining the role of urinary 3-BT measurement in the management of EoE need to consider other possible sources, especially considering that approximately two-thirds of patients with EoE have another atopic disease.

We conducted a proof-of-concept study to evaluate the potential for measuring 3-BT in urine as a noninvasive method for detecting eosinophil degranulation in EoE. A mass spectrometry-based quantitative method for measuring 3-BT was developed and tested with urine samples from patients with EoE, atopic controls, and nonatopic controls in a pilot study.

Methods

Ethics Statement

Urine samples were obtained from consented patients as part of their standard-of-care visit at the Children's Hospital of The King's Daughters clinics under an Eastern Virginia Medical School Institutional Review Board–approved protocol. Written informed consent was obtained. Atopic control urine samples were obtained from consented patients during routine visits to the allergy clinic. Nonatopic control urine samples were obtained from consented healthy volunteers without a diagnosed allergic condition. Clinical data that were associated with each urine sample were obtained under the same institutional review board protocol.

Urine Samples

Urine samples were placed on ice and then frozen at -80°C . Urine samples were thawed, aliquoted, subject to centrifugation for 5 minutes at $450\times g$, and then measured for creatinine concentration using the enzymatic slide method (Ortho Diagnostic Vitros,

Raritan, New Jersey) in the clinical laboratory at Children's Hospital of The King's Daughters. The urine samples were normalized based on creatinine concentration, and 400-mg equivalent total urine protein samples from each patient were processed for downstream analysis. Urinary levels of 3-BT obtained after hydrolysis of precipitated urine proteins were analyzed by selected ion monitoring (SIM) using a LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts). The intensities of extracted ion chromatograms from the SIM scans from serial dilutions of synthetic stable isotope labeled 3-BT were used to generate standard curves for relative quantitation of endogenous 3-BT in urine samples using extracted ion chromatograms from the SIM scans of individual urinary hydrolysates.

Clinical Data

Patients denoted as having EoE met the clinical and biopsy criteria. Although this proof-of-concept study was not powered to evaluate 3-BT in the context of detailed clinical data, we wished to evaluate whether there were any striking associations with clinical details that could inform future studies. Urine samples from patients with EoE were collected on the day of endoscopy. Clinical data were obtained from data entered into the patient medical record for the visit when the urine sample was obtained. Clinical data obtained included demographics, other allergic diagnoses, corticosteroids and route of administration, elimination diet, and the physician's assessment of the patient's disease control. Endoscopic and histologic data were collected, including eosinophils per high-power field (HPF), basal cell hyperplasia, and mucosal appearance. The biopsy result was considered positive if more than 15 eosinophils per HPF were noted on proximal or distal biopsy.

Statistical Analysis

The data were analyzed using SAS statistical software, version 9.4 (SAS Institute Inc, Cary, North Carolina), and SPSS statistical software, version 19 (SPSS Inc, Chicago, Illinois). The level of significance was set at .05, and all hypothesis tests were 2-sided. Descriptive statistics (mean, median, and interquartile range [IQR]) were reported for 3-BT stratified by clinical measures and compared using the Wilcoxon-Mann-Whitney test. Pearson and Spearman correlation coefficients were reported, where appropriate, for 3-BT and the clinical measures. To distinguish among the subgroups, 3-BT thresholds were reported along with the calculated sensitivity and specificity. Multiple logistic regression models were used to calculate the odds ratios for associations between urine 3-BT levels and EoE, after controlling for confounders.

Results

Study Population

This proof-of-concept study included urine samples from 75 participants providing one samples each. The participants included 27 patients with EoE, 24 atopic controls, and 24 nonatopic controls. The demographics for each group are given in Table 1. There were fewer white race participants in the EoE group than in the control groups. The EoE population in this study frequently had diagnoses

Table 1
Demographic Characteristics of the Controls and Patients With EoE

Demographic Characteristic	Nonatopic Controls (n = 24)	Atopic Controls (n = 24)	Patients With EoE (n = 27)
Age range, y	3–31	4–13	2–18
Sex, % female	63	50	68
Race, % white	90	38	82

Abbreviation: EoE, eosinophilic esophagitis.

of other atopic conditions similar to what has been previously reported.⁷ The frequency of different types of atopic diseases and use of corticosteroids in the EoE group were compared with the nonatopic control group in Table 2. Asthma was well represented in the atopic control group (75%), which is of particular interest because this population has been associated with an increased incidence of EoE.⁸ Clinical characteristics of the EoE group are given in Table 3.

EoQUIK Normalized 3-BT Measurements

The normalized 3-BT measurement for each urine sample is shown in Figure 1A. Each bar shows the 3-BT measurement for each group in ascending value. The nonatopic control group had very low levels of 3-BT. For statistical comparison, these values are shown in box and whisker plots in Figure 1B, showing the IQR, median, and mean. These data are skewed particularly for the atopic controls, making comparisons of medians most appropriate. The median urinary 3-BT level was 93-fold higher for all patients with EoE compared with nonatopic controls ($P = .01$). Nonatopic controls had a median 3-BT level of 1.81 pg per 400 mg of creatinine (IQR, 0.99–3.8 pg per 400 mg of creatinine) compared with patients with EoE, who had a median 3-BT level of 169.5 pg per 400 mg of creatinine (IQR, 40.74–412.86 pg per 400 mg of creatinine). The median urinary 3-BT level was 13-fold higher for all patients with EoE compared with atopic controls ($P = .01$). Atopic controls had a median 3-BT of 12.87 pg per 400 mg of creatinine (IQR, 3.51–114.69 pg per 400 mg of creatinine). These data suggest that EoQUIK measurement of urinary 3-BT is very low in nonatopic controls compared with nearly all patients with EoE with elevated 3-BT levels.

To further evaluate the potential utility of EoQUIK for discriminating patients with EoE, we tested sensitivity, specificity, and negative predictive value (NPV) based on threshold cutoff values (Table 4). A threshold cutoff of 17 was used, which yielded a specificity of 100% and an NPV of 100% when comparing patients with EoE with nonatopic controls. Comparing patients with EoE and atopic controls, a threshold cutoff of 145 yielded a specificity of 79% and an NPV of 90%. In a logistic regression model, a urine 3-BT level of greater than 20 pg per 400 mg of creatinine increased the odds of a patient having EoE by 4.8 (95% CI, 1.14–20.5; $P = .03$) when compared with atopic controls after controlling for race and sex. Although this is a small sample size, these results suggest that EoQUIK may have utility in terms of excluding or at least providing fairly robust evidence against the diagnosis of EoE when it is a diagnosis under consideration.

Urine 3-BT Levels and Clinical Characteristics

To further evaluate elevated 3-BT levels in the atopic control group, we stratified this group by those with 3-BT levels greater than or equal to the threshold cutoff of 145 pg per 400 mg of creatinine and those below (Table 5). The various atopic diagnoses were similarly represented in the high and low 3-BT groups,

Table 3

Characteristics of the 27 Patients With EoE

Characteristic	Patients With EoE, %
New EoE diagnosis	7
PPI use	61
Elimination diet	89
Uncontrolled EoE	39
>15 eosinophils HPF	56

Abbreviations: EoE, eosinophilic esophagitis; HPF, high-power field; PPI, proton pump inhibitor.

including asthma. Asthma was a diagnosis for 79% of atopic patients with low 3-BT levels and a diagnosis for 60% of those with high 3-BT levels. Thus, asthma was not overrepresented in the high 3-BT group, suggesting that in this small sample size asthma was not associated with high 3-BT.

Atopic controls with flares of their atopic disease at the time of their urine samples were also evaluated. Of the atopic controls, 14 were experiencing a flare compared with 10 who were not. The median 3-BT level of those experiencing a flare was 39.2 pg per 400 mg of creatinine compared with stable atopic controls, who had a median 3-BT level of 12.9 pg per 400 mg of creatinine ($P = .24$). Thus, atopic controls experiencing a flare of their atopic disease at

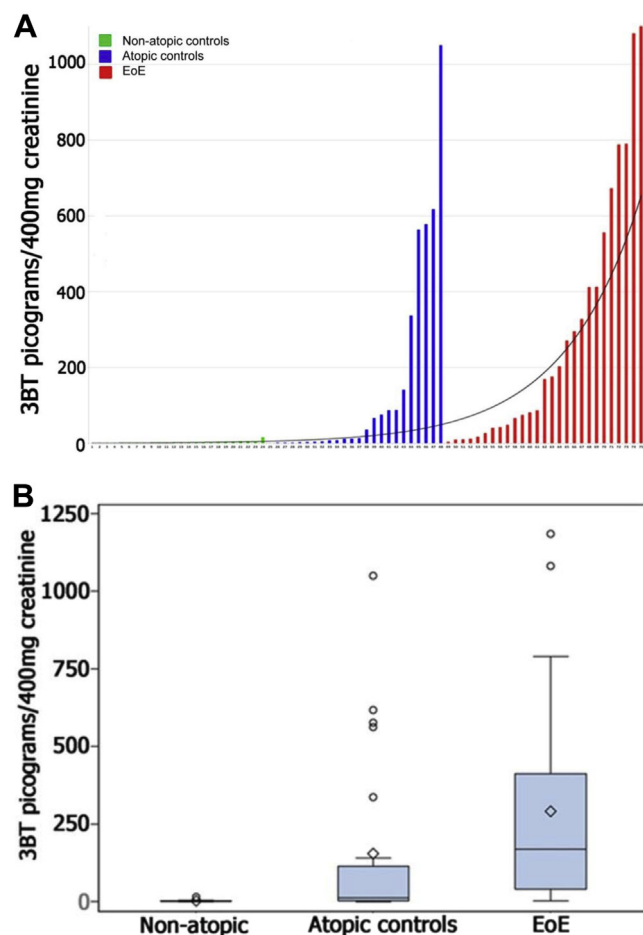


Figure 1. Normalized 3-BT levels in urine samples of non-atopic controls ($n = 24$), atopic controls ($n = 24$), and subjects with EoE ($n = 27$). (A) Individual 3-BT levels for each subject. (B) Box and whisker plot of 3-BT levels in urine samples of non-atopic controls ($n = 24$), atopic controls ($n = 24$), and subjects with EoE ($n = 27$). Box shows interquartile range, whiskers indicate $1.5 \times \text{IQR}$, and diamond indicates mean.

Table 2

Characteristics of Atopic Controls and Patients With EoE

Condition or Treatment	Atopic Controls, % ($n = 24$)	Patients with EoE, % ($n = 27$)
Allergic rhinitis	92	74
Atopic dermatitis	50	29
Food allergies	38	48
Asthma	75	37
Oral corticosteroids	4	0
Nasal corticosteroids	33	11
Inhaled corticosteroids	58	29

Abbreviation: EoE, eosinophilic esophagitis.

Table 4
Sensitivity, Specificity, and NPV

Group	Sensitivity, %	Specificity, %	NPV, %
Patients with EoE vs nonatopic controls (threshold cutoff = 17 pg of 3-BT per 400 mg of creatinine)	81	100	100
Patients with EoE vs atopic controls (threshold cutoff = 145 pg of 3-BT per 400 mg of creatinine)	67	79	90

Abbreviations: 3-BT, 3-bromotyrosine; EoE, eosinophilic esophagitis; NPV, negative predictive value.

the time of urine sample were not associated with a statistically significant difference in 3-BT for this small sample size.

This study was not powered for correlative analyses between 3-BT levels and clinical characteristics. We conducted subanalyses to assess whether marked associations were present that could inform future studies. Eosinophils per HPF on biopsy specimens did not correlate with urine 3-BT level in this limited data set, which is not surprising given that eosinophils per HPF did not correlate between proximal and distal biopsy specimens. There appeared to be a nonsignificant trend toward increased median 3-BT level and furrowing ($n = 176$) compared with no furrowing ($n = 57$) ($P = .40$). In addition, there was a nonsignificant trend toward increased median 3-BT level and being considered to have uncontrolled EoE ($n = 224$) compared with well-controlled EoE ($n = 71$) ($P = .30$). Thus, no strong clinical correlations were associated with urine 3-BT in this small data set, as anticipated.

Discussion

Currently, the diagnosis and monitoring of EoE predominantly rely on symptoms and endoscopy with biopsies. The limitations of endoscopy, biopsy, and histologic analysis are well documented, including the risk of sampling error and the lack of correlation of eosinophils per HPF with clinical disease severity.⁹ Martin et al⁹ found that although staining esophageal biopsy specimens for eosinophil peroxidase, a marker of eosinophil degranulation, correlates with symptoms reported on the Pediatric Eosinophilic Esophagitis Symptom Severity Module, version 2.0, eosinophils per HPF on histologic analysis did not correlate with symptoms. These studies suggest that eosinophil degranulation rather than absolute number is important in the pathogenesis of EoE. This makes sense because it is release of the harsh toxins from the eosinophil granules that produce cellular damage and not the mere presence of eosinophils. Presumably repetitive exposure of esophageal tissues to the eosinophil granule toxins produce the symptoms and eventually, if uncontrolled, lead to dysphagia and esophageal fibrosis.¹⁰ Thus, the development of a noninvasive method to

measure eosinophil degranulation in EoE remains a major unmet medical need.

Currently, multiple methods are being developed for evaluating EoE disease activity, including noninvasive and somewhat invasive approaches. Three less invasive approaches that obtain samples from the esophagus but without the cost and sedation required with endoscopy and biopsy are being evaluated. Twenty individuals were studied to evaluate esophageal cytology via Cytosponge (Medtronic GI Solutions, Minneapolis, Minnesota). This study found a sensitivity of 83% and a specificity of 57% for EoE.¹¹ Esophageal brushing, another less invasive method to study esophageal cytology, was studied in 28 patients and yielded a sensitivity of 67% to 72% with a specificity of 61% to 75%.¹² The esophageal string test that measures major basic protein 1, eosinophil-derived neurotoxin, eosinophil cationic protein, eosinophil peroxidase, and Charcot-Leyden crystal protein ($N = 41$) was found to correlate with peak and mean eosinophil counts on biopsy.⁴ These proof-of-concept studies reveal the potential of these modalities in the long-term management of EoE, but these methods are inconvenient and at least mildly unpleasant. In addition, sample sizes studied were small, necessitating larger validation studies. A less invasive study of a panel of serum biomarkers found that none of the markers studied correlate with disease, including interleukin (IL) 4, IL-5, IL-6, IL-9, IL-13, transforming growth factor (TGF) α , TGF- β , tumor necrosis factor α , eotaxin-1, eotaxin-2, eotaxin-3, thymic stromal lymphopoietin, major basic protein, and eosinophil-derived neurotoxin.¹³ The eosinophilic diagnostic panel, a 96-gene quantitative polymerase chain reaction array performed on esophageal biopsy specimens in 177 individuals, was able to identify EoE in remission vs control with a sensitivity of 96% and a specificity of 98%.¹⁴

In developing EoQUIK, we had concerns about whether other atopic diseases, as has been found for asthma,⁶ would reveal elevated urinary 3-BT as a result of eosinophil degranulation in sites other than the esophagus. In comparing nonatopic controls with patients with EoE, the discriminatory power of EoQUIK was excellent. As expected, many of the atopic controls had elevated 3-BT levels when compared with nonatopic controls. This necessitated picking a higher threshold cutoff for atopic patients. With a higher threshold cutoff for atopic patients, EoQUIK was still able to provide very good discriminatory power in terms of specificity and NPV. Of interest, asthma was not overrepresented among atopic controls with 3-BT levels above the threshold cutoff compared with atopic controls with low 3-BT levels. This finding suggests that asthmatic patients do not need to be excluded from testing with EoQUIK if there is a suspicion for EoE.

These data provide proof of concept that testing normalized 3-BT in urine by the EoQUIK method can provide a noninvasive method to evaluate eosinophil degranulation in EoE. Although the sensitivity of EoQUIK is modest in discriminating EoE, a high 3-BT level clearly increases the odds that EoE may be present and, thus, the index of suspicion. Of note, the atopic control in this study with the highest 3-BT level has subsequently been diagnosed as likely having EoE. Analysis of atopic controls experiencing flares of their atopic disease had only a small and nonstatistically significant difference in median 3-BT compared with stable atopic patients. Thus, it is unclear whether EoQUIK might be less useful for atopic patients during flares of their atopy. Overall, the specificity and NPV of EoQUIK appear to be outstanding in nonatopic patients and very good even in patients with other atopic conditions. Thus, EoQUIK could potentially be of clinical benefit in providing evidence that EoE is unlikely (ie, a low urine 3-BT level) when EoE is a diagnostic consideration.

Another potential use for EoQUIK may be in monitoring disease activity and response to therapeutic interventions in the individual with EoE. We could not evaluate for this in this study population

Table 5
Characteristics of Atopic Controls With High 3-BT Levels (≥ 145 pg per 400 mg of Creatinine) vs Atopic Controls With Low 3-BT Levels (< 145 pg per 400 mg of Creatinine)

Condition or Treatment	All Atopic Controls, % ($n = 24$)	Atopic Controls With Low 3-BT Levels, % ($n = 19$)	Atopic Controls With High 3-BT Levels, % ($n = 5$)
Allergic rhinitis	92	95	80
Atopic dermatitis	50	47	60
Food allergies	38	32	60
Asthma	75	79	60
Oral corticosteroids	4	4	0
Nasal corticosteroids	33	26	60
Inhaled corticosteroids	58	58	60

Abbreviation: 3-BT, 3-bromotyrosine.

because each sample was from a separate person. Thus, the study design did not provide the power to evaluate for correlations with measures of disease severity. However, these proof-of-concept data suggest that a future study to evaluate patients longitudinally and make correlations with EoE disease activity is warranted.

In summary, these data suggest that measuring normalized urine 3-BT via the EoQUIK method may become a useful noninvasive tool in discriminating patients with EoE. Larger clinical studies are needed to validate these proof-of-concept data.

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