Eoxins: A new inflammatory pathway in childhood asthma

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Background: Increased levels of leukotrienes (LTs) in exhaled breath condensate (EBC) are associated with asthma and bronchial hyperresponsiveness (BHR), whereas eicosanoids generated through the 15-lipoxygenase (LO) pathway (15-hydroxyeicosatetraenoic acid [HETE] and eoxins) have been less studied.

Objective: We investigated whether metabolites of the 5- and 15-LO pathways in EBC are associated with childhood asthma, asthma severity, and clinical parameters.

Methods: The present study included 131 school-aged children (27 children with problematic severe asthma, 80 children with mild-to-moderate asthma, and 24 healthy children) from the Severe Asthma Recognized in Childhood study and 19 children with other nonasthmatic chronic lung diseases. Clinical work-up included spirometry, fractional exhaled nitric oxide measurements, skin prick testing, and methacholine challenge. Eicosanoids were analyzed in EBC by using mass spectrometry and are reported as concentrations (in picograms per milliliter) and eicosanoid/palmitic acid (PA) ratios.

Results: Eoxin C₄/PA, eoxin D₄/PA, eoxin E₄/PA, 15-HETE/PA, and LTC₄/PA ratios were significantly increased in asthmatic versus healthy children. Eoxin D₄/PA and LTE₄/PA ratios were also significantly higher in children with BHR. A nonsignificant trend was observed toward higher eoxin/PA ratios with increasing asthma severity. In contrast to asthma, children with chronic lung disease had the highest 15-HETE/PA, LTC₄/PA, LTE₄/PA, and LTB₄/PA ratios.

Conclusion: The results point to increased activity of the 15-LO inflammatory pathway in childhood asthma. Mass

spectrometric analyses of EBC demonstrate that increased eoxin levels not only accompany the increased 5-LO product LTC₄ but are also associated with BHR. These markers might represent a new therapeutic target for asthma treatment. (J Allergy Clin Immunol 2010;126:859-67.)

Key words: Exhaled breath condensate, leukotrienes, 15-hydroxyeicosatetraenoic acid, eoxins, asthma, child

Inflammation is a key element in asthma pathophysiology.¹ Understanding the underlying inflammatory processes might help tailor individualized anti-inflammatory treatment. Eicosanoids, including prostanoids, leukotrienes (LTs), and lipoxins, are nonspecific products of arachidonic acid catabolism present in a number of inflammatory responses.

Cysteinyl leukotrienes (CysLTs: LTC₄, LTD₄, and LTE₄) and LTB₄ are generated through the 5-lipoxygenase (LO) pathway.² Increased levels in exhaled breath condensate (EBC) of asthmatic children are found in some studies³⁻⁶ but are inconsistently reproduced. The biological role of the 15-LO pathway mediators is less clear, although 15-LO and its major arachidonic metabolite, 15-hydroxyeicosatetraenoic acid (15-HETE), might be involved in asthma pathophysiology. IL-4 and IL-13 upregulate 15-LO in human airway epithelial cells and macrophages *in vitro*.^{7,8} Overexpression of 15-LO, as seen in endobronchial biopsy specimens of asthmatic subjects,⁹ induces chemokine release in cultured airway epithelial cells⁸ and upregulates MUC5AC expression, indicating a putative role in mucin production.¹⁰ Increased levels of 15-HETE are found in bronchoalveolar lavage (BAL) fluid from persistently wheezing children¹¹ and asthmatic adults⁹ and in induced sputum.⁷

The end metabolites of the 15-LO pathway, previously known as 14,15-LTs and recently renamed eoxins C₄, D₄, and E₄, have proinflammatory properties.¹² Eoxins resemble CysLTs but have a different 3-dimensional structure (Fig 1)¹³ and are formed in eosinophils by means of activation of a different signal transduction pathway.¹² Eoxin C₄ was recently detected in BAL fluid from 3 asthmatic adults,¹⁴ whereas the eoxins' relationship to childhood asthma is unknown.

The clinical usefulness of EBC in asthma management is unclear.¹⁵ EBC has an attractive potential, being noninvasive, and might provide more differentiated inflammatory profiles than fractional exhaled nitric oxide (FE_{NO}).¹⁶ Although low biomarker concentrations limit the sensitivity of conventional assays, mass spectrometry (MS) offers the advantage of highly sensitive and specific analyses.^{5,17}

We aimed primarily to investigate whether metabolites of the 5- and 15-LO pathways in EBC are increased in asthmatic subjects. Secondly, we aimed to assess whether EBC eicosanoids are related to asthma severity or linked to clinical characteristics of childhood asthma, including lung function, bronchial hyperresponsiveness (BHR), FE_{NO}, and allergic sensitization.

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Abbreviations used

BAL:	Bronchoalveolar lavage
BHR:	Bronchial hyperresponsiveness
BMI:	Body mass index
CysLT:	Cysteinyl leukotriene
EBC:	Exhaled breath condensate
ECP:	Eosinophilic cationic protein
FEF ₂₅₋₇₅ :	Forced expiratory flow during 25% to 75% of FVC
FE _{NO} :	Fractional exhaled nitric oxide
FVC:	Forced vital capacity
HETE:	Hydroxyeicosatetraenoic acid
ICS:	Inhaled corticosteroid
LO:	Lipoxygenase
LT:	Leukotriene
LTRA:	Leukotriene receptor antagonist
MS:	Mass spectrometry
PA:	Palmitic acid
SEARCH:	Severe Asthma Recognized in Childhood

METHODS**Study design**

The cross-sectional Severe Asthma Recognized in Childhood (SEARCH) study was performed at Oslo University Hospital, Norway (2006-2007), including children (6-18 years) with problematic severe asthma and age-matched children with mild-to-moderate asthma and healthy children. An additional population of 19 children with chronic lung diseases was subsequently included for comparison with asthmatic and healthy children, all recruited during the same time period from the tertiary pediatric unit at Oslo University Hospital.

EBC analyses were performed in the University Department of Medicine, Krakow, Poland (2009). The study was approved by the Regional Committee for Medical Research Ethics and registered in the Norwegian Biobank Registry. Written informed consent was obtained for all participants.

Study population and recruitment

The present study consisted of the 131 SEARCH children (39% girls; Table 1 and see Table E1 and Fig E1, which are available in this article's Online Repository at www.jacionline.org) recruited through 4 hospital databases (107 with asthma, 27 of which had problematic severe asthma) or randomly selected healthy children (n = 24) through the Norwegian National Registry. Details of patient recruitment are provided in the Methods section of this article's Online Repository at www.jacionline.org. The primary diagnoses of the 19 children with chronic lung disease (9-18 years, 5 girls) were bronchiectasis (n = 6), primary ciliary dyskinesia (n = 6), bronchopulmonary dysplasia (n = 3), chronic obstructive pulmonary disease (n = 1), chronic atelectasis (n = 1), cystic fibrosis (n = 1), and bronchiolitis obliterans (n = 1). All but one child with chronic lung disease used inhaled corticosteroids (ICSs), and 5 used leukotriene receptor antagonists (LTRAs; Table 1).

Inclusion criteria

Asthma. Asthmatic children required a specialist's diagnosis of asthma plus use of antiasthma treatment, the presence of asthma symptoms within the preceding year, or both. Children with vocal cord dysfunction, cystic fibrosis, bronchiectasis and other chronic lung diseases, immunodeficiencies, or significant neurologic dysfunction were excluded from the SEARCH study.

Problematic severe asthma.¹⁸ Problematic severe asthma was defined as asthmatic patients who satisfied the following predefined criteria, ascertained by means of a structured interview:

- Medication at enrollment and during at least 6 of the preceding 12 months (both required):
 - Daily high-dose (≥ 800 $\mu\text{g/d}$ budesonide equivalent) ICS treatment
 - Regular long-acting inhaled β_2 -agonist, LTRA, or both treatment

B. Asthma symptoms during the preceding year (≥ 1 required):

- Exacerbations:
 - ≥ 1 Emergency hospitalization
 - ≥ 1 Course of doctor-administered oral corticosteroid treatment
 - ≥ 2 Emergency outpatient visits
 - Exacerbations of asthma more than once a month on average (≥ 12 per year)
- Chronic asthma symptoms (present more than twice a week for at least 3 consecutive months):
 - Asthma symptoms limiting participation in daily activities (including sport and leisure)
 - Nocturnal awakenings because of asthma symptoms

Mild-to-moderate asthma. Mild-to-moderate asthma was defined as all asthmatic patients not meeting the inclusion criteria for problematic severe asthma, irrespective of current asthma treatment and symptom control. Further subclassification according to current regular ICS use was done in some analyses.

Healthy. Healthy subject were those with no doctor's diagnosis of asthma or symptoms indicating obstructive airway disease or other chronic lung disease and no current rhinoconjunctivitis (definition provided in the Methods section of this article's Online Repository), although atopic dermatitis was permitted.

Methods

Study examinations were performed during 2 to 3 visits, usually within a 2-week period, at least 2 weeks after airway infections or asthma exacerbations. Repeated visits were delayed accordingly, if necessary. A combined patient/parental structured interview included questions on demographic data, atopic disease, current and previous asthma medication, asthma exacerbations, nocturnal and daytime asthma symptoms, and health resource use. Age- and sex-adjusted body mass index (BMI) percentiles were calculated according to published algorithms.¹⁹ The study protocol also included spirometry,²⁰ tests of bronchial responsiveness to methacholine, skin prick tests, FE_{NO} measurements, and blood sampling (see the Methods section of this article's Online Repository for more information).

EBC sampling

EBC samples were collected in accordance with American Thoracic Society/European Respiratory Society guidelines²¹ by using an ECoScreen Jaeger condensing chamber (GmbH Hoechberg, Germany) with use of a nose clip. Participants performed tidal ventilation for 15 minutes through a mouthpiece connected to the condenser through a 2-way nonbreathing valve and a salivary trap. Mouth rinse was performed before sampling, and participants were instructed to swallow saliva periodically. EBC samples (1-2 mL) were immediately stored at -80°C in polypropylene microcentrifuge tubes (Eppendorf tubes).

HPLC-tandem MS

EBC sample preparation is presented in the Methods section of this article's Online Repository. Ten microliters of methanol EBC extract was injected on a reverse phase column (Zorbax Eclipse XDB C-18; Agilent Technologies, Santa Clara, Calif), stabilized thermally (37°C), and eluted with a gradient consisting of 2 mobile phases, acetonitrile/water/acetic acid (20/80/0.01) and acetonitrile/isopropanol/acetic acid (55/45/0.01), by using the flow rate of 0.11 mL/min on HPLC equipped with an autosampler (Shimadzu Sil-2-AC). Analytes were measured by using multiple reactions monitoring mode tandem MS (Qtrap 4000; Applied Biosystems, Foster City, Calif) equipped with an electrospray ion source in 3 separate runs. Positive ionization was used to quantify eoxins, LTC₄, and LTD₄. Negative ionization was used for LTE₄, LTB₄, and HETEs. An example of MS analysis of eicosanoids is presented in Fig 2. Palmitic acid (PA) was measured after its derivatization into pentafluorobenzyl ester and purification with thin-layer chromatography by using a gas chromatography negative-ion chemical ionization MS (GC-NICI-MS).

FIG 1. Overview of the biosynthesis of LTs and eoxins.

A single quadrupole instrument (Engine 5989B series II; Hewlett Packard, Palo Alto, Calif) was used in selected ion-monitoring mode. The method currently used has recently been described.²²

PA was used to adjust for EBC dilution. The concentration of PA was similar in children with other chronic lung diseases and healthy children but decreased with asthma severity in the asthmatic group, possibly enhancing the ratio differences in outcomes between asthmatic and healthy children. Therefore the reported values of EBC eicosanoids include both eicosanoid concentrations (in picograms per milliliter) and eicosanoid/PA ratios.

The MS analyses of the EBC were performed by technicians blinded to all clinical data. No significant time dependency was found for the analyzed eicosanoid levels relative to the patient groups (data not shown).

Statistical analyses

Results are presented as means (95% CIs) or counts (percentages). Eicosanoids, eosinophilic cationic protein (ECP), IgE, and FE_{NO} measurements are given as geometric means with 95% CIs. One-way ANOVA was used for comparison of normally distributed or ln-transformed data with the Tukey post-hoc

test. The Mann-Whitney test was used for comparisons of nontransformed data; categorical variables were compared by using the Pearson χ^2 test or Fisher exact test. Correlation analyses were performed with the Pearson or Spearman tests as appropriate with respect to data distribution.

Analysis of covariance was used to assess the influence of multiple explanatory factors (age, sex, BMI percentile, disease status [asthma or healthy chronic lung disease], FEV₁ [percent predicted], forced vital capacity [FVC; percent predicted], forced expiratory flow during 25% to 75% of FVC [FEF₂₅₋₇₅; percent predicted], FEV₁/FVC ratio, allergic sensitization, regular ICS use, and LTRAs) in the last 12 months on eicosanoid concentrations and eicosanoid/PA ratios. A step-down Hosmer procedure was used, excluding variables not significant at the .05 levels. All nonsignificant variables were tested for confounding (ie, a change in the estimate of greater than 25%). The model was tested for interactions. Least-square means with 95% CIs were calculated from the final model. Cook's D and Jackknife residuals were used to assess the appropriateness of the models. Statistical analyses were performed with SPSS Version 16.0 software (SPSS, Chicago, Ill) and SAS software (SAS Institute, Inc, Cary, NC). A 2-tailed probability of .05 or less was the threshold for significance in all comparisons.

TABLE I. Patients' characteristics and clinical data

	Healthy subjects (n = 24)	Asthmatic subjects (n = 107)	Subjects with chronic lung disease (n = 19)	Overall P value
Female subjects, n (%)	16 (67)	35 (33)	5 (26)	.005
Age (minimum maximum; y)	11.4 (6-17)	10.9 (6-18)	12.8 (9-18)	.04
BMI percentile (median [IQR])	61 (51)	49 (48)	31 (89)	.65
ICS use, intermittent, n (%)*	0	25 (23)	0	<.001
ICS use, regular, μ g (%)				
<400	0	16 (15)	5 (26)	
400-800	0	21 (20)	5 (26)	
$\geq$ 800	0	39 (36)	8 (42)	
LTRA, n (%)*	0	38 (36)	5 (26)	<.001
LABA, n (%)*	0	69 (64)	10 (53)	<.001
FEV ₁ (% predicted [SD])†	99 (9)	95 (15)	67 (28)	<.001
FEV ₁ /FVC ratio (SD)	0.89 (0.07)	0.85 (0.07)	0.70 (0.16)	<.001
FE _{NO} ppb (geometric mean)	6.5 (4.5-9.5)	13.9 (11.2-17.2)	6.1 (4.6-8.0)	.001
PD ₂₀ $\leq$ 8 μ mol (%)‡	7 (37)	68 (64)	4 (31)	.003
SPT, any positive, n (%)	5 (21)	73 (68)	2 (11)	<.001
Total IgE kU/L (geometric mean)	68 (36-129)	247 (174-351)	28 (10-78)	<.001
ECP μ mol (geometric mean)	8.9 (6.4-12.3)	15.6 (13.1-18.7)§	15.8 (11.2-2.2)	.02

Results are represented as means (95% CIs) or counts unless otherwise stated. Significance figures are derived by using ANOVA among the 4 populations for continuous variables or χ^2 tests for proportions.

BMI, body mass index¹⁹; IQR, interquartile range; SPT, skin prick test.

*Antiasthma medication within 12 months preceding inclusion to the study and ICS dosage referring to budesonide equivalent daily dose. Intermittent ICS use implies ICS use of short duration during asthma exacerbations and not within 2 weeks before inclusion.

†Reference values by Stanojevic et al.²⁰

‡Two children with asthma and 4 children with other chronic lung diseases were too sick to perform PD₂₀ measurement (FEV₁ <70% of predicted value).

§Significant differences at $P < .05$ versus healthy children.

||Significant difference at $P < .01$ versus healthy children.

RESULTS

Patients' characteristics and clinical data are presented in Table I and by asthma subgroups in Table E1. Compared with healthy children, children with asthma had significantly lower lung function, except FEV₁; higher FE_{NO}; more often BHR and allergic sensitization; and increased total IgE and ECP levels. A reduced FEV₁/FVC ratio was the only measurement differentiating problematic severe versus ICS-treated mild-to-moderate asthma (see Table E1). The additional control group of children with other chronic lung diseases had significantly lower lung function than the 2 other groups (Table I).

Eoxin C₄/PA, eoxin D₄/PA, eoxin E₄/PA, and 15-HETE/PA ratios correlated significantly with LTC₄/PA, LTD₄/PA, LTE₄/PA, and 5-HETE/PA ratios, respectively (see Fig E2 and Table E2 in this article's Online Repository at www.jacionline.org).

Eicosanoids and asthma

Group comparisons demonstrated that asthmatic compared with healthy children had significantly increased concentrations of LTC₄ (as the only eicosanoid) and significantly higher eoxin C₄/PA, eoxin D₄/PA, eoxin E₄/PA, 15-HETE/PA, LTC₄/PA, and LTB₄/PA ratios (Table II).

Eicosanoid concentrations and eicosanoid/PA-ratios in EBC are given for healthy children and children with asthma, as well as other chronic lung diseases, in Table II.

Eicosanoids, sex, age, and BMI (n = 150)

No significant sex-based differences were found for eicosanoid concentrations, but boys had higher LTC₄/PA ratios (1.9 vs 1.4×10^{-6} , $P = .048$) than girls. A similar nonsignificant trend was found for eoxin D₄/PA ratios (1.7 vs 1.3×10^{-6} , $P = .051$). A weak correlation was found between eoxin E₄ concentration

and age, ($\rho = -0.20$, $P = .02$), whereas LTD₄ ($r = -0.17$, $P = .04$) and LTE₄ ($r = -0.17$, $P = .03$) concentrations correlated weakly with BMI. No significant correlations were found between eicosanoid/PA ratios, age, or BMI.

Eicosanoids, BHR, lung function, allergic sensitization, and inflammatory markers in asthmatic and healthy children

In children with BHR, eoxin D₄ ($P = .007$), eoxin E₄ ($P = .02$), and LTE₄ ($P = .01$) concentrations were significantly increased, as were eoxin D₄/PA ($P = .03$) and LTE₄/PA ($P = .046$) ratios compared with values seen in children without BHR. A similar nonsignificant trend was found for eoxin E₄/PA ratios ($P = .06$, Fig 3 and see Fig E3 in this article's Online Repository at www.jacionline.org). The dose-response slope_{methacholine} correlated significantly but weakly with the LTE₄/PA ratio ($r = 0.20$, $P = .03$). Only the LTD₄/PA ratio correlated significantly with FEV₁ ($r = 0.20$, $P = .03$), whereas no correlations were found between eicosanoid levels and FEV₁/FVC ratios. Children with allergic sensitization had higher LTE₄/PA ratios than those without (2.7 vs 2.0×10^{-6} , $P = .03$). No significant correlation was found between eicosanoid levels in EBC and FE_{NO}, total IgE, or serum ECP levels.

Multifactor analyses (n = 150)

Multifactor analyses on eicosanoid/PA ratios demonstrated asthma to be significantly associated with increased eoxin E₄/PA and eoxin D₄/PA ratios in EBC compared with those seen in healthy children and children with chronic lung diseases. Least-squares means (with 95% CIs) of eicosanoid concentrations and eicosanoid/PA ratios are presented in Figs 4 and 5 for the final model of the multifactor analyses (initially including sex, age,

FIG 2. Overlaid HPLC-tandem MS spectrograms of eicosanoids in 1 EBC sample. Eoxins (*EXs*) C_4 , D_4 , and E_4 and $CysLTs$ were measured by using the positive ionization mode and multiple reaction monitoring. Retention times are given aside peaks and verified by using commercially available nondeuterated eoxins (Cayman Chemical Co, Ann Arbor, Mich). Additional details are shown in the Results section of this article's Online Repository.

BMI, lung function, allergic sensitization, and disease status adjusted for LTRA use and regular ICS treatment). Including BHR to methacholine in the initial regression model did not alter the least-squares means and was subsequently excluded from further analyses because of a lack of data in 23 children unable to undergo testing.

Compared with healthy children, EBC from children with asthma had significantly higher LTC_4 concentrations ($P < .0001$), as well as significantly higher eoxin C_4/PA ($P = .008$), eoxin D_4/PA ($P = .005$), eoxin E_4/PA ($P = .007$), 15-HETE/ PA ($P = .006$), and LTC_4/PA ($P = .0004$) ratios.

Subsequent analyses comparing EBC from children with asthma versus other chronic lung diseases demonstrated significantly higher eoxin D_4/PA and eoxin E_4/PA ratios but lower concentrations of eoxin C_4 , 15-HETE, LTC_4 , LTD_4 , LTE_4 , and LTB_4 , as well as lower 15-HETE/ PA , LTC_4/PA , LTE_4/PA , and LTB_4/PA ratios in asthmatic subjects. Overall, children with other chronic lung diseases had different patterns of eicosanoids in EBC compared with those seen in both asthmatic and healthy children, with significantly higher concentrations of eoxin C_4 , 15-HETE, LTC_4 , LTD_4 , LTE_4 , and LTB_4 , as well as 15-HETE/ PA , LTC_4/PA , LTE_4/PA , and LTB_4/PA ratios. Group comparisons (ANOVA analyses) showed similar results (see the Results section of this article's Online Repository).

Lung function measures were inconsistently associated with eicosanoids. Although FEV_1 and FEF_{25-75} were positively associated with LTB_4/PA ($P = .04$) and 15-HETE/ PA ($P = .006$) ratios, respectively, FVC was negatively associated with both LTB_4 concentrations and PA ratios, (both $P = .04$) but positively associated

with LTD_4 ($P = .01$) concentrations. An inverse association was found for FEV_1/FVC and LTB_4/PA ratios ($P = .03$).

Allergic sensitization was associated with increased LTE_4 concentrations ($P = .01$) and LTE_4/PA ratios ($P = .04$) and LTB_4 concentrations ($P = .04$). Age was associated with decreased concentrations of eoxin C_4 ($P = .05$) and LTD_4 ($P = .01$), and BMI was associated with decreased LTD_4 concentrations ($P = .002$). LTRA use was not significantly associated with eicosanoid concentrations in EBC but with increased eoxin E_4/PA ($P = .008$) and LTE_4/PA ($P = .01$) ratios, whereas regular ICS treatment was associated with decreased LTE_4 concentrations and LTE_4/PA ratios ($P = .02$).

Eicosanoid/ PA ratios and asthma severity subgroups

Eoxin/ PA ratios within asthma subgroups and healthy children are presented in Fig E4 (available in this article's Online Repository at www.jacionline.org), and background data on eoxin concentrations and eoxin/ PA ratios in the asthma subgroups are provided in Table E3 (available in this article's Online Repository at www.jacionline.org). Although eoxin/ PA ratios were highest in patients with problematic severe asthma, differences compared with all other asthmatic children did not reach statistical significance (eoxin C_4 : 1.0 vs 0.8×10^{-6} , $P = .54$; eoxin D_4 : 2.3 vs 1.7×10^{-6} , $P = .10$; and eoxin E_4 : 5.1 vs 3.9×10^{-6} , $P = .31$). Of the SEARCH children, all 10 subjects with the highest levels of 3 or 4 of the eoxin/ PA , 15-HETE/ PA , or both ratios had asthma (4 problematic severe), 7 used regular ICSS, 5 used LTRAs, and only 1 of the 10 had a negative PD_{20} methacholine challenge test.

The LTC_4/PA ratio was significantly increased in children with problematic severe asthma (2.0×10^{-6} [95% CI, 1.3 - 2.9×10^{-6}], $P = .008$) and in children with mild-to-moderate asthma with (1.8×10^{-6} [95% CI, 1.4 - 2.3×10^{-6}], $P = .007$) and without (1.6×10^{-6} [95% CI, 1.1 - 2.3×10^{-6}], $P = .048$) regular ICS use compared with healthy children (0.8×10^{-6} [95% CI, 0.5 - 1.2×10^{-6}]).

DISCUSSION

The present cross-sectional study, using MS methodology, is the first to demonstrate the presence of eoxins and 15-HETE, inflammatory metabolites of the 15-LO pathway, in EBC and their association with childhood asthma and BHR.

The significantly increased PA ratios of all 15-LO pathway metabolites (15-HETE/ PA , eoxin C_4/PA , eoxin D_4/PA , and eoxin E_4/PA ratios) in asthmatic compared with healthy children and increased eoxin D_4 and E_4 concentrations in children with BHR (Fig 3) suggest that these metabolites are involved in asthma inflammation in children. Moreover, to substantiate the likelihood that these were in fact related to asthma, we added a second control group of children with other chronic lung diseases. The multivariate analyses demonstrated different eicosanoid patterns in EBC of children with asthma versus those with other chronic lung disease or healthy children (Figs 4 and 5), showing eoxin D_4/PA and eoxin E_4/PA ratios to be significantly increased in asthmatic children, whereas eoxin C_4/PA and 15-HETE/ PA ratios were highest in children with chronic lung diseases (Fig 4).

To our knowledge, eoxins have not previously been described in children. Our results are, however, supported by adult studies detecting eoxin C_4 (but not eoxins D_4 or E_4) in BAL fluid of

FIG 3. Eoxins D₄ and E₄ concentrations and PA ratios (eicosanoid/PA $\times 10^{-6}$) in children with BHR to methacholine ($PD_{20} \leq 8 \mu\text{mol}$ methacholine). Values are given as natural logarithms and back-transformed values (geometric means with 95% CIs).

subjects with various respiratory diseases, including asthma.¹⁴ 15-HETE has also been found to correlate with increasing asthma severity and subbasement membrane thickness.⁹ Biological plausibility of the 15-LO pathway being involved in respiratory inflammation is supported by a study demonstrating that mice deficient in 12/15-LO (the analog to human 15-LO-1) had attenuated allergic inflammation compared with that seen in wild-type mice.²³

The present study suggests involvement of a new pathway in asthmatic airway inflammation in children. This might pose an alternative explanation for the lack of an LTRA treatment response in many asthmatic children,^{24,25} as well as suggesting a new pathway for the development of new anti-inflammatory drugs for childhood asthma.

Significantly increased LTC₄/PA ratios in asthmatic children is in line with previous studies on CysLTs,^{3,4,6,26} yet separate detection of LTC₄ (or LTD₄) was not performed in these studies. In the present compared with previous pediatric studies,^{5,27,28} asthma status was not significantly associated with LTE₄ (or LTB₄). Because most previous reports on CysLTs in childhood asthma have used enzyme immunoassay,^{3,4,6,26-28} direct comparisons with our study are difficult. Somewhat surprisingly, EBC of children with other chronic lung diseases had significantly increased LTC₄/PA, LTE₄/PA, and LTB₄/PA ratios, as well as increased concentrations of all LTs, compared with those seen in asthmatic and healthy children (Fig 5). However, the present study was not designed to investigate the role of eicosanoids in a wide spectrum of lung diseases, although this clearly warrants further study.

FIG 4. Least-squares means and 95% CIs of eicosanoids of the 15-LO pathway (15-HETE and eoxins C₄, D₄, and E₄), both concentrations and PA ratios (eicosanoid/PA × 10⁻⁶).

FIG 5. Least-squares means and 95% CIs of eicosanoids of the 5-LO pathway (5-HETE, LTC₄, LTD₄, and LTE₄; **A**) and LTB₄ (**B**), both concentrations and PA ratios (eicosanoid/PA × 10⁻⁶).

TABLE II. Values (geometric means and 95% CIs) of eicosanoids in EBC of healthy children and children with asthma and other chronic lung diseases: Concentrations (in picograms per milliliter) and adjusted PA ratios*

Eicosanoid	Healthy subjects	Asthmatic subjects	P value vs healthy children	Chronic lung disease	Overall P value
15-HETE					
pg/mL	1.81 (1.26-2.61)	2.02 (1.78-2.30)	.50	7.33 (5.37-10.01)	<.001
Ratio	3.39 (2.37-4.84)	5.11 (4.35-5.99)	.03	12.85 (10.18-16.21)	<.001
Eoxin C ₄					
pg/mL	0.28 (0.22-0.35)	0.34 (0.29-0.40)	.22	0.49 (0.36-0.67)	.03
Ratio	0.52 (0.40-0.68)	0.86 (0.73-1.03)	.009	0.86 (0.63-1.19)	.03
Eoxin D ₄					
pg/mL	0.56 (0.39-0.80)	0.72 (0.62-0.83)	.16	0.50 (0.35-0.70)	.09
Ratio	1.04 (0.70-1.56)	1.81 (1.53-2.13)	.006	0.87 (0.62-1.22)	<.001
Eoxin E ₄					
pg/mL	1.21 (0.90-1.62)	1.66 (1.36-2.03)	.16	1.11 (0.87-1.40)	.11
Ratio	2.25 (1.70-3.00)	4.19 (3.36-5.22)	.01	1.94 (1.55-2.43)	.001
5-HETE					
pg/mL	1.69 (1.24-2.31)	1.75 (1.51-2.02)	.85	2.35 (1.62-3.42)	.27
Ratio	3.17 (2.39-4.20)	4.42 (3.75-5.21)	.08	4.12 (3.05-5.57)	.19
LTC ₄					
pg/mL	0.44 (0.31-0.63)	0.70 (0.60-0.81)	.01	1.92 (1.26-2.91)	<.001
Ratio	0.82 (0.54-1.24)	1.76 (1.48-2.11)	<.001	3.36 (2.28-4.95)	<.001
LTD ₄					
pg/mL	0.49 (0.37-0.66)	0.49 (0.43-0.56)	.97	0.73 (0.58-0.92)	.054
Ratio	0.92 (0.66-1.28)	1.24 (1.06-1.44)	.11	1.27 (1.03-1.58)	.22
LTE ₄					
pg/mL	1.11 (0.83-1.50)	0.98 (0.87-1.11)	.42	1.84 (1.41-2.40)	.001
Ratio	2.08 (1.65-2.62)	2.47 (2.14-2.86)	.30	3.23 (2.45-4.24)	.13
LTB ₄					
pg/mL	2.80 (2.22-3.54)	2.91 (2.61-3.25)	.77	9.26 (5.89-14.54)	<.001
Ratio	5.24 (3.91-7.04)	7.33 (6.33-8.50)	.05	16.22 (9.90-26.60)	<.001
PA					
ng/mL	535 (433-660)	395 (361-433)	.006	570 (461-705)	.001

*Eicosanoid/PA ratio ($\times 10^{-6}$).

The lack of significant associations between eoxin/PA and CysLT/PA ratios and asthma severity contrasts with previous pediatric studies^{3,6} and might be due to the phenotypic heterogeneity of asthma or possibly to the relatively small number with severe problematic asthma. Nevertheless, children with the highest eoxin levels all had asthma, the majority with severe disease, ICS/LTRA use, or both, suggesting a potential role for eoxins in asthma severity.

Except for the inconsistent association between LTB₄ and lung function (FEV₁, FVC, and FEV₁/FVC ratio) between LTD₄ concentration and FVC and between 15-HETE/PA ratio and FEF₂₅₋₇₅, prebronchodilator and postbronchodilator lung function were not associated with EBC eicosanoids, which is in line with other pediatric studies (data not shown). The positive association between BHR and LTE₄ in EBC is supported by 1 pediatric study.²⁹ Lack of association between eicosanoid ratios and FE_{NO} is also in accordance with most,^{3,5,26} but not all,⁶ previous pediatric reports. Allergic sensitization was associated with increased LTE₄ and LTB₄ values only, supporting the suggestion that CysLTs (and eoxins) are less related to allergic sensitization than is FE_{NO}.⁴ Small population size prevented subgroup analyses on other allergic traits.

Although anti-inflammatory treatment might confound associations between EBC eicosanoids, asthma status, and asthma severity, regular ICS use did not significantly affect eoxin/PA ratios in EBC. The association between ICS use and reduced LTE₄ values is supported by some,^{28,30} but not all,⁴ previous pediatric studies. On the other hand, the association between LTRA

use and increased levels of both 5-LO and 15-LO pathways (LTE₄ and eoxin E₄) in this population, selected based on criteria other than use of LTRAs, contrasts with previous reports on LTs.^{27,31,32} Because LTRAs were predominantly used in children also using regular ICSs, it might be a surrogate marker for increased asthma severity. In addition, one cannot exclude the possibility that LTRA use, previously shown to have a selective inhibitory effect on 5-LO *in vitro*,³³ shifts eicosanoid production in the direction of the 15-LO pathway. More studies are required to elucidate this issue.

MS analysis in EBC is highly sensitive and superior to other assays, such as enzyme immunoassay.^{21,34} Because some of the observed variability in EBC solute concentrations might reflect differences in airway lining fluid dilution, adjusting for dilutions by using markers such as urea, cations, total protein, or in this case PA has been recommended.^{21,22,35} Interestingly, although PA was reasonably normally distributed among all samples, reanalysis stratified by healthy and asthmatic children demonstrated clear deviations from normality in the latter study group (decreasing PA concentrations with increasing asthma severity, see Table E3). Therefore a second control group (other chronic lung diseases) was included in the study, in which the distribution of PA concentrations was similar to that of healthy children. Although there is no consensus on what diluent indicator to use in EBC or whether this is necessary, the use of PA is supported by studies demonstrating a correlation between LT levels in EBC and BAL fluid only when adjusted values were calculated.³⁶ PA is a main constituent of lung surfactant, but the method of sample

extraction and PA analysis implied measurement of the free fatty acid component only. The relatively constant plasma level of free fatty acid diffuses freely across biological membranes and proved, in our hands, more convenient than urea concentration.³⁷ All EBC analyses were performed by a scientific team blinded to subject characteristics, thus reducing the possibility of interpretation bias. Without measurement of amylase concentration, salivary contamination of EBC cannot be ruled out,¹⁶ but the risk was minimized with swallowing and the use of a salivary trap.²¹

In conclusion, the results point to increased activity of the 15-LO inflammatory pathway in childhood asthma. MS analyses of EBC demonstrate that increased eoxin levels accompany the increased 5-LO product LTC₄ and are associated with BHR. These markers of the 15-LO pathway might represent a new therapeutic target for asthma treatment.

The research team, including Torill Tørres, Solveig Knutsen, Eli Schrøder, and Amund Riiser, assisted in data collection.

Clinical implications: The present study is the first to demonstrate an association between eoxins and asthma and BHR in children, potentially signifying a new therapeutic target for asthma treatment.

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METHODS

Recruitment and study population

Children with a registered specialist's diagnosis of asthma were identified and were eligible for inclusion to the study. Patients' charts from 2 university clinics (Ullevål and Rikshospitalet) and 2 county hospitals (Lillehammer and Fredrikstad) were systematically reviewed, identifying patients receiving high-dose ICSs (≥ 800 μg of budesonide equivalent), oral steroid therapy, or both within the preceding year. A corresponding number of age-matched but otherwise unselected asthmatic control subjects were identified from the remaining outpatient population. Age-matched non-asthmatic control subjects with a registered address in Oslo were randomly identified from the Norwegian National Registry. In children with mild-to-moderate asthma overall, use of ICS treatment (intermittent or regular) was reported in 93%, use of LTRAs in 23%, and use of long-acting inhaled β_2 -agonists in 55%.

Participants were classified as having current rhinoconjunctivitis if they had at least 1 major and 1 minor criterion (other than a major criterion) within the last 12 months. Major criteria were a doctor's diagnosis of hay fever or at least 2 of the following: rhinorrhea, itchy/running eyes, nasal congestion, or sneezing. Minor criteria (at least 1 of the following) were (1) rhinorrhea, itchy/running eyes, nasal congestion, or sneezing or (2) treatments for eye/nose symptoms: local or systemic antihistamines, local or systemic corticosteroids, sodium cromoglycate, LT antagonists, and immunotherapy.

Methods

Sample preparation. After thawing, the pH of the EBC was adjusted to 3.5 by using acetic acid (2 μL), and internal deuterated standards, comprising 5-HETE- d_8 , 15-HETE- d_8 , LTD $_4$ - d_5 , LTE $_4$ - d_3 , LTB $_4$ - d_4 (0.5 ng), and PA- d_4 (40 ng) in methanol, were added. The sample was extracted twice with 1 mL of tertiary butyl methyl ether. Organic phases were combined and evaporated under nitrogen at room temperature. Residues were dissolved in methanol (60 μL) and immediately used for further analyses.

Example of eicosanoid measurements

For more information, see Fig 2. Two deuterated internal standards (LTD $_4$ - d_5 and LTE $_4$ - d_3 , 0.5 ng each) were added to 1 mL of the condensate. Eoxins C $_4$, D $_4$, and E $_4$ and CysLTs LTC $_4$ and LTD $_4$ - d_5 were measured by using the positive ionization mode and multiple reaction monitoring (eoxins: 626.4 $\rightarrow$ 205.3, 497.4 $\rightarrow$ 205.2, and 440.2 $\rightarrow$ 205.2 M/z; CysLTs: 626.4 $\rightarrow$ 308.3 and 502.4 $\rightarrow$ 199.4 M/z). Native LTE $_4$ was measured by using negative ionization and fragmentation of ions (438.4 $\rightarrow$ 333.3 M/z), whereas LTE $_4$ - d_3 standard was measured by using both positive and negative ionization modes (443.4 $\rightarrow$ 192.4 and 441.4 $\rightarrow$ 336.2 M/z). Vertical scale of individual spectrograms was changed to relative intensity of counts per second.

Lung function was measured based on forced expiratory flow-volume curves using a Sensor Medics Vmax 229 series with Autobox (VIASYS Healthcare, Inc, Yorba Linda, Calif), according to American Thoracic Society/European Respiratory Society guidelines.^{E1} Reported values included FEV $_1$ and the FEV $_1$ /FVC ratio, both expressed as percent predicted according to recently published reference algorithms by Stanojevic et al.^{E2}

BHR was determined based on PD $_{20}$ methacholine by using a Spira nebulizer (Spira Respiratory Care Centre, Hämeenlinna, Finland).^{E3} Two patients were excluded from methacholine provocation because of a baseline FEV $_1$ of less than 70%, and 15 BHR tests, mostly in children aged 6 to 7 years, were incomplete or discarded because of unstable lung function technique. BHR was defined as PD $_{20}$ of 8 μmol or less methacholine. The dose-response slope_{methacholine} was determined by using the following formula: Percentage decrease in FEV $_1$ /Cumulated dose methacholine (in micromoles).^{E4}

Skin prick tests to 13 common allergens were performed with Soluprick allergens (ALK-Abelló, Hørsholm, Denmark). Wheal diameters of 3 mm or more larger than those elicited by the negative control (isotonic saline) were considered positive. The following allergens were used: domestic mite (*Dermatophagoides pteronyssinus*); dog, cat, and rabbit dander; birch, timothy, and mugwort pollen; *Cladosporium herbarum*; *Alternaria alternata*; egg white; milk; peanut; and codfish.

Short-acting inhaled β_2 -agonists were withheld for 8 hours, and long-acting inhaled β_2 -agonists/LTRAs were withheld for 48 hours before lung function measurements and methacholine bronchial challenge tests, whereas systemic antihistamines were withheld for 5 days before skin prick testing.

FE $_{NO}$ was recorded before lung function measurement by using the Eco Medics CLD 88 Exhalyzer (Eco Medics AG, Duernten, Switzerland), according to American Thoracic Society/European Respiratory Society recommendations.^{E5}

Serum ECP and total IgE levels were analyzed by using a fluoroenzyme immunoassay, according to the manufacturer's recommendations (ImmunoCAP; Phadia AB, Uppsala, Sweden).

RESULTS

Eicosanoids and medication in children with asthma

Regular use of ICSs was not significantly associated with eicosanoid levels, except a lower LTE $_4$ concentration seen in children receiving ICS treatment (0.9 vs 1.2 pg/mL, $P = .02$).

Asthmatic children receiving LTRAs had higher eicosanoid levels than those without, with differences reaching statistical significance for eoxin E $_4$ (2.1 vs 1.4 pg/mL, $P = .03$) and 5-HETE (2.2 vs 1.6 pg/mL, $P = .04$) concentrations, as well as eoxin E $_4$ /PA (5.9 vs 3.5×10^{-6} , $P = .02$), 5-HETE/PA (6.0 vs 3.7×10^{-6} , $P = .002$), and LTE $_4$ /PA ratios (3.0 vs 2.2×10^{-6} , $P = .05$). When this analysis was limited to patients with regular ICS use, differences in eicosanoid levels in patients using versus those not using LTRAs were less pronounced, reaching significance for 5-HETE/PA ratios only (5.9 vs 3.8×10^{-6} , $P = .03$).

ANOVA analyses of eicosanoid concentrations and eicosanoid/PA ratios in the 3 groups of children (healthy children, asthmatic children, and children with other chronic lung diseases)

Eoxin concentrations. Compared with healthy children, children with other chronic lung diseases had significantly higher concentrations of eoxin C $_4$ ($P = .03$) and 15-HETE ($P < .001$) only. 15-HETE concentrations were significantly higher in children with other chronic lung diseases compared with asthmatic children, whereas there was no significant difference for eoxin C $_4$.

LT concentrations. The LT concentrations demonstrated that compared with healthy children, only LTC $_4$ concentrations were increased in asthmatic children, whereas in children with other chronic lung diseases, LTC $_4$ ($P < .001$), LTE $_4$ ($P = .03$), and LTB $_4$ ($P < .001$) concentrations were significantly increased. Compared with asthmatic children, children with chronic lung disease had significantly higher LTC $_4$ ($P < .001$), LTD $_4$ ($P = .045$), LTE $_4$ ($P < .001$), and LTB $_4$ ($P < .001$) concentrations.

Eicosanoid/PA ratios. Asthma was associated with significantly increased eoxin C $_4$ /PA, eoxin D $_4$ /PA, and eoxin E $_4$ /PA ratios, whereas the 15-HETE/PA ratio only was significantly higher in children with other chronic lung diseases compared with healthy children. Children with asthma had significantly higher eoxin D $_4$ /PA and eoxin E $_4$ /PA ratios but significantly lower 15-HETE/PA ratios compared with patients with chronic lung disease.

Asthmatic children had significantly higher LTC $_4$ /PA ratios, whereas children with other chronic lung diseases had significantly higher LTC $_4$ /PA and LTB $_4$ /PA ratios compared with healthy children. Compared with children with other chronic lung diseases, asthmatic children had significantly lower LTC $_4$ /PA and LTB $_4$ /PA ratios.

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FIG E1. Flow chart of the inclusion algorithm of the 131 SEARCH children.

FIG E2. Significant correlations between eoxin C_4/PA , eoxin D_4/PA , eoxin E_4/PA , and 15-HETE/PA ratios (eoxin/PA $\times 10^{-6}$) with the respective LTC_4/PA , LTD_4/PA , LTE_4/PA , and 5-HETE/PA ratios (LT/PA $\times 10^{-6}$) in the SEARCH children ($n = 131$). Ratios are given as natural logarithms, and the Pearson test was used for correlation analyses (correlation lines with 95% CIs).

FIG E3. LTE_4 concentrations and PA ratios (eicosanoid/PA $\times 10^{-6}$) in children with BHR to methacholine ($PD_{20} \leq 8 \mu\text{mol}$ methacholine). Values are given as natural logarithms and back-transformed values (geometric means with 95% CIs).

FIG E4. Eoxin/PA and 15-HETE/PA ratios (eoxin/PA $\times 10^{-6}$) are demonstrated in children with problematic severe asthma, children mild-to-moderate asthma with or without regular ICS use, and healthy nonasthmatic children. Values are given as natural logarithms and back-transformed values (geometric means with 95% CIs). Group comparisons by means of 1-way ANOVA and *P* values are given for pairwise multiple comparisons by using the Tukey test.

TABLE E1. Patients' characteristics and clinical data of the asthmatic subgroups of children

	Mild-to moderate asthma		Problematic severe asthma (n = 27)
	No regular ICSs (n = 31)	Regular ICSs (n = 49)	
Female subjects, n (%)	11 (35)‡	16 (33)‡	8(30)‡
Age (minimum-maximum; y)	10.8 (6-17)	10.6 (6-18)	11.5 (6-17)
BMI percentile (SD)	51 (28)	49 (27)	54 (33)
ICS use, = intermittent, n*	0	0	0
ICS use (µg) regular, n (%)			
<400	0	16 (33)	0
400-800	0	21 (43)	0
≥800	0	12 (24)	27 (100)
LTRA, n (%)*	5 (16)§	13 (27)§	20 (74)
LABA, n (%)*	11 (36)§	33 (67)§	25 (93)
FEV ₁ (% predicted [SD])†	93 (15)	98 (13)	92 (17)
FEV ₁ /FVC ratio (SD)	0.85 (0.08)	0.87 (0.06)§	0.82 (0.08)‡
FE _{NO} ppb (geometric mean)	18.2 (12.9-25.8)‡	11.1 (7.9-15.8)	15.2 (9.9-23.1)‡
PD ₂₀ ≤8 µmol (%)	21 (78)‡	31 (69)‡	16 (70)‡
SPT, any positive, n (%)	21 (68)‡	35 (71)‡	17 (63)‡
Total IgE kU/L (geometric mean)	233 (114-475)	194 (120-315)	416 (191-906)‡
ECP µmol (geometric mean)	13.0 (9.4-17.8)	16.4 (12.5-21.6)‡	17.5 (11.9-25.7)‡

Results are represented as means (95% CIs) or counts unless otherwise stated. Significance figures are derived by using ANOVA among the 4 populations for continuous variables or χ^2 tests for proportions.

BMI, Body mass index^{E6}; SPT, skin prick test.

*Antiasthma medication within 12 months preceding inclusion to the study and ICS dosage referring to budesonide equivalent daily dose. Intermittent ICS use implies ICS use of short duration during asthma exacerbations and not within 2 weeks before inclusion.

†Reference values by Stanojevic et al.^{E2}

‡Significant differences ($P < .05$) versus healthy subjects.

§Significant difference ($P < .05$) versus children with problematic severe asthma.

TABLE E2. Correlations between eoxins C₄, D₄, and E₄ and 15-HETE concentrations and PA ratios (eoxin/PA × 10⁻⁶) with the respective LTC₄, LTD₄, LTE₄, and 5-HETE concentrations and PA ratios in the SEARCH children (n = 131)

Disease groups	Eoxin vs LT	Coefficient* (pg/mL)†	P value	Coefficient* ratio‡	P value
Healthy children	Eoxin C ₄ /LTC ₄		NS		NS
	Eoxin D ₄ /LTD ₄		NS	r = 0.43	.04
	Eoxin E ₄ /LTE ₄		NS		NS
	15-HETE/5-HETE		NS	ρ = 0.46	.02
Children with mild-to-moderate asthma, ICS –	Eoxin C ₄ /LTC ₄	r = 0.50	.006	r = 0.71	<.001
	Eoxin D ₄ /LTD ₄	r = 0.64	<.001	r = 0.80	<.001
	Eoxin E ₄ /LTE ₄	r = 0.34	.02	r = 0.36	.05
	15-HETE/5-HETE	ρ = 0.59	<.001	ρ = 0.69	<.001
Children with mild-to-moderate asthma, ICS+	Eoxin C ₄ /LTC ₄	r = 0.53	<.001	r = 0.64	<.001
	Eoxin D ₄ /LTD ₄		NS	r = 0.76	<.001
	Eoxin E ₄ /LTE ₄	r = 0.34	.02	r = 0.55	<.001
	15-HETE/5-HETE	ρ = 0.37	.009	ρ = 0.62	<.001
Children with problematic severe asthma	Eoxin C ₄ /LTC ₄		NS	r = 0.44	.03
	Eoxin D ₄ /LTD ₄		NS	r = 0.40	.04
	Eoxin E ₄ /LTE ₄		NS	r = 0.57	.002
	15-HETE/5-HETE		NS	ρ = 0.51	.007
Children with chronic lung disease	Eoxin C ₄ /LTC ₄		NS		NS
	Eoxin D ₄ /LTD ₄		NS		NS
	Eoxin E ₄ /LTE ₄	r = 0.49	.03	r = 0.49	.03
	15-HETE/5-HETE	ρ = 0.48	.04	ρ = 0.48	.04

NS, not significant.

*Correlation coefficients: Pearson *r* and Spearman *ρ*.

†Correlation between eicosanoid concentrations (picograms per milliliter).

‡Correlation between eicosanoid/PA ratios × 10⁻⁶.

TABLE E3. Values of eicosanoids in EBC of children within subgroups of asthma: Concentrations (in picograms per milliliter) and PA ratios*

Eicosanoid	Healthy	Mild-to-moderate asthma, ICS–	Mild-to-moderate asthma, ICS+	Severe asthma	P value*
15-HETE					
pg/mL	1.81 (1.26-2.61)	2.19 (1.76-2.73)	2.19 (1.81-2.66)	1.59 (1.20-2.10)	.20
Ratio	3.39 (2.37-4.84)	5.27 (3.95-7.02)	5.43 (4.20-7.01)	4.41 (3.23-6.04)	.13
Eoxin C ₄					
pg/mL	0.28 (0.22-0.35)	0.30 (0.22-0.39)	0.37 (0.30-0.45)	0.35 (0.24-0.49)	.36
Ratio	0.52 (0.40-0.68)	0.72 (0.50-1.02)	0.93 (0.73-1.17)	0.95 (0.65-1.39)	.032
Eoxin D ₄					
pg/mL	0.56 (0.39-0.80)	0.67 (0.52-0.86)	0.69 (0.56-0.84)	0.84 (0.56-1.24)	.35
Ratio	1.04 (0.70-1.56)	1.62 (1.21-2.17)	1.71 (1.36-2.14)	2.29 (1.53-3.41)	.017
Eoxin E ₄					
pg/mL	1.21 (0.90-1.62)	1.60 (1.02-2.49)	1.61 (1.22-2.12)	1.86 (1.26-2.76)	.49
Ratio	2.25 (1.70-3.00)	3.84 (2.47-5.99)	3.98 (2.90-5.48)	5.10 (3.22-8.08)	.06
5-HETE					
pg/mL	1.69 (1.24-2.31)	1.65 (1.24-2.21)	1.89 (1.58-2.26)	1.61 (1.09-2.38)	.80
Ratio	3.17 (2.39-4.20)	3.98 (2.89-5.49)	4.68 (3.76-5.83)	4.48 (3.00-6.71)	.28
LTC ₄					
pg/mL	0.44 (0.31-0.63)	0.67 (0.52-0.85)	0.71 (0.57-0.90)	0.71 (0.50-1.02)	.09
Ratio	0.82 (0.54-1.24)	1.61 (1.14-2.26)	1.78 (1.37-2.31)	1.95 (1.32-2.87)	.005
LTD ₄					
pg/mL	0.49 (0.37-0.66)	0.57 (0.44-0.75)	0.51 (0.43-0.61)	0.38 (0.29-0.50)	.14
Ratio	0.92 (0.66-1.28)	1.37 (0.99-1.91)	1.26 (1.02-1.56)	1.04 (0.77-1.42)	.23
LTE ₄					
pg/mL	1.11 (0.83-1.50)	1.23 (0.98-1.56)	0.89 (0.73-1.06)	0.93 (0.72-1.19)	.11
Ratio	2.08 (1.65-2.62)	2.97 (2.32-3.80)	2.17 (1.71-2.76)	2.54 (1.94-3.31)	.20
LTB ₄					
pg/mL	2.80 (2.22-3.54)	2.81 (2.22-3.57)	3.10 (2.62-3.67)	2.70 (2.25-3.23)	.74
Ratio	5.24 (3.91-7.04)	6.77 (4.88-9.39)	7.67 (6.13-9.61)	7.39 (5.86-9.32)	.23
PA					
ng/mL	535 (433-660)	415 (348-496)	404 (350-466)	360 (307-422)	.03

*Eicosanoid/PA ratio ($\times 10^{-6}$).