ORIGINAL ARTICLE

Urinary Leukotriene E_4 and Prostaglandin D_2 Metabolites Increase in Adult and Childhood Severe Asthma Characterized by Type 2 Inflammation

A Clinical Observational Study

Johan Kolmert[1,2,3], Cristina Gómez[1,2,3], David Balgoma[1,2,3], Marcus Sjödin[1,2,3], Johan Bood[1,3,4], Jon R. Konradsen[3,5,6], Magnus Ericsson[7], John-Olof Thörngren[7], Anna James[1,3], Maria Mikus[1,3], Ana R. Sousa[8], John H. Riley[8], Stewart Bates[8], Per S. Bakke[9], Ioannis Pandis[10], Massimo Caruso[11,12], Pascal Chanez[13], Stephen J. Fowler[14], Thomas Geiser[15], Peter Howarth[16], Ildikó Horváth[17], Norbert Krug[18], Paolo Montuschi[19], Marek Sanak[20], Annelie Behndig[21], Dominick E. Shaw[22], Richard G. Knowles[23], Cécile T. J. Holweg[24], Åsa M. Wheelock[25], Barbro Dahlén[3,4], Björn Nordlund[5,6], Kjell Alving[26], Gunilla Hedlin[3,5,6], Kian Fan Chung[10], Ian M. Adcock[10], Peter J. Sterk[27], Ratko Djukanovic[16], Sven-Erik Dahlén[1,3*], and Craig E. Wheelock[2,3*]; on behalf of the U-BIOPRED Study Group

[1]The Institute of Environmental Medicine, [2]Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, [3]The Center for Allergy Research, [5]Department of Women's and Children's Health, and [25]Respiratory Medicine Unit, Department of Medicine, Solna Campus, and Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; [4]Department of Medicine and [7]Department of Clinical Pharmacology, Huddinge Campus, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden; [6]Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; [8]GlaxoSmithKline, London, United Kingdom; [9]Institute of Medicine, University of Bergen, Bergen, Norway; [10]National Heart and Lung Institute and Department of Computing & Data Science Institute, Imperial College London, London, United Kingdom; [11]Department of Clinical and Experimental Medicine and [12]Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy; [13]Clinique des Bronches, Allergies et Sommeil, Aix Marseille Université, Assistance Publique des Hôpitaux de Marseille, Marseille, France; [14]Division of Infection, Immunity and Respiratory Medicine, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, and Manchester Academic Health Science Centre and National Institute for Health Research Biomedical Research Centre, Manchester University Hospitals National Health Service Foundation Trust, Manchester, United Kingdom; [15]Department of Pulmonary Medicine, University Hospital Bern, Bern, Switzerland; [16]Faculty of Medicine, Southampton University, and National Institute for Health Research Southampton Respiratory Biomedical Research Center, University Hospital Southampton, Southampton, United Kingdom; [17]Department of Pulmonology, Semmelweis University, Budapest, Hungary; [18]Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany; [19]Department of Pharmacology, Catholic University of the Sacred Heart, and Agostino Gemelli University Hospital Foundation, IRCCS, Rome, Italy; [20]Department of Internal Medicine, Medical College, Jagiellonian University, Cracow, Poland; [21]Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden; [22]Nottingham National Institute for Health Research Biomedical Research Centre, University of Nottingham, United Kingdom; [23]Knowles Consulting, Stevenage Bioscience Catalyst, Stevenage, United Kingdom; [24]Genentech Inc., South San Fransisco, California; [26]Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden; and [27]Department of Respiratory Medicine, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, the Netherlands

ORCID IDs: 0000-0001-7116-6583 (J.K.); 0000-0001-6560-6124 (M.M.); 0000-0002-8013-2745 (Å.M.W.); 0000-0003-2101-8843 (I.M.A.); 0000-0002-4993-4002 (S.-E.D.); 0000-0002-8113-0653 (C.E.W.).

Abstract

Rationale: New approaches are needed to guide personalized treatment of asthma.

Objectives: To test if urinary eicosanoid metabolites can direct asthma phenotyping.

Methods: Urinary metabolites of prostaglandins (PGs), cysteinyl leukotrienes (CysLTs), and isoprostanes were quantified in the U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) study including 86 adults with mild-to-moderate asthma (MMA), 411 with severe asthma (SA), and 100 healthy control participants. Validation was performed internally in 302 participants with SA followed up after 12–18 months and externally in 95 adolescents with asthma.

Measurement and Main Results: Metabolite concentrations in healthy control participants were unrelated to age, body mass index, and sex, except for the PGE_2 pathway. Eicosanoid concentrations were generally greater in participants with MMA relative to healthy control participants, with further elevations in participants with SA. However, PGE_2 metabolite concentrations were either the same or lower in male nonsmokers with asthma than in healthy control participants. Metabolite concentrations were unchanged in those with asthma who adhered to oral corticosteroid treatment as documented by urinary prednisolone detection, whereas those with SA treated with omalizumab had lower concentrations of LTE_4 and the PGD_2 metabolite 2,3-dinor-11β-$PGF_{2\alpha}$. High concentrations of LTE_4 and PGD_2 metabolites were associated with lower lung function and increased amounts of exhaled nitric oxide and eosinophil markers in blood, sputum, and urine in U-BIOPRED participants and in adolescents with asthma. These type 2 (T2) asthma associations were reproduced in the follow-up visit of the U-BIOPRED study and were found to be as sensitive to detect T2 inflammation as the established biomarkers.

Conclusions: Monitoring of urinary eicosanoids can identify T2 asthma and introduces a new noninvasive approach for molecular phenotyping of adult and adolescent asthma.

Clinical trial registered with www.clinicaltrials.gov (NCT 01976767).

Keywords: severe asthma; urinary eicosanoid metabolites; U-BIOPRED; type 2 inflammation; mass spectrometry

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Many of the new biologic treatments of asthma target type 2 (T2) asthma, in which mast cells, eosinophils, and the cytokines IL-4, IL-5, and IL-13 mediate central components of the inflammatory reactions (1). However, stratification of patients for treatment is at present limited to measures of blood or sputum eosinophils, fractional exhaled nitric oxide (F_{ENO}), or protein markers in blood such as total IgE or periostin that do not provide consistent information (2). There is accordingly an unmet need to identify new predictive biomarkers to improve stratification of patients by pathobiologic mechanisms and to aid selection of treatments.

Herein we report data from the pan-European U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes) study on potential urinary lipid biomarkers of asthma (3). Leukotrienes (LTs), prostaglandins (PGs), and related arachidonic acid derivatives, collectively termed eicosanoids, are fundamental signaling molecules in human biology (4) that have been implicated in the pathophysiology of asthma. Specifically, the biologically active eicosanoids may exert pro- and antiinflammatory actions, and many cause bronchoconstriction (Figure 1). Whereas prostaglandins and thromboxane A_2 (TXA_2) are biosynthesized in enzymatic reactions initially catalyzed by either of two cyclooxygenases (COX-1 or COX-2), the LTs are generated via a pathway initiated by the 5-LOX (5-lipoxygenase) enzyme. Cysteinyl LTs (CysLT; LTC_4, LTD_4, and LTE_4) are potent bronchoconstrictive (5) and proinflammatory mediators (6); $CysLT_1$ receptor antagonists and the 5-LOX inhibitor zileuton are used for the treatment of asthma (7, 8). PGD_2 is the major COX product in mast cells with bronchoconstrictive and proinflammatory actions (9, 10) and is investigated as a potential new target for asthma therapy. The isoprostanes are primarily generated nonenzymatically under conditions of oxidative stress (11), and reported biologic effects in the airways suggest that they may contribute to the pathophysiology of asthma (12).

The eicosanoids are short-lived in the tissues in which they are biosynthesized and are rapidly removed from circulation for excretion by the kidney. The amounts excreted into the voided urine therefore represent an integration of the systemic load since the previous emptying of the urinary bladder. The measurement of metabolites in the urine that reflect activation of the different biosynthetic pathways is a reliable method to assess *in vivo* production of primary eicosanoids (13–15). In contrast, their concentrations in blood are low and fluctuating, and interpretations can be complicated by artifactual formation during sampling.

In the current study, we present the largest evaluation to date of multiple urinary eicosanoid metabolites present in healthy adults and adults with asthma. We show that profiling of lipid mediators in the urine provides a valuable noninvasive approach for molecular phenotyping of asthma and, in particular, provide data from two patient cohorts demonstrating that urinary LTE_4 and metabolites of PGD_2 correlate with eosinophilic T2 inflammation. Some of the results from these studies have been previously reported in the form of abstracts (16, 17).

Methods

Cohort Descriptions

The U-BIOPRED study (clinicaltrials.gov identifier NCT 01976767) was approved by the ethics committees at each of the 16 clinical sites and included adult participants aged 18–79 years with either controlled mild-to-moderate asthma (MMA; $n = 86$), or severe asthma (SA; $n = 411$), classified according to the international guidelines for SA (18). The participants with SA were stratified by smoking status into smokers ($n = 109$), including current or past smokers (>5 pack-years), and nonsmokers. Clinical study data for the baseline cross-sectional examination have been published previously (3), but essential outcomes

*Co–senior authors.

A complete list of U-BIOPRED Study Group members may be found before the beginning of the References.

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Correspondence and requests for reprints should be addressed to Craig E. Wheelock, Ph.D., Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Biomedicum Quarter 9A, 171 77 Stockholm, Sweden. E-mail: craig.wheelock@ki.se.

This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

At a Glance Commentary

Scientific Knowledge on the Subject: Eicosanoids may exert pro- and antiinflammatory actions contributing to the pathobiology of asthma. However, their relative abundance in individuals with asthma and association with type 2 (T2) asthma are unclear. In addition, the influence of oral corticosteroid (OCS) treatment on eicosanoid concentrations is debated.

What This Study Adds to the Field: Urinary concentrations of 11 eicosanoid metabolites were quantified in 597 individuals participating in the U-BIOPRED study. From normal values established in 100 healthy participants, we observed a progressive increase in most metabolites in relation to asthma severity. We demonstrate that eicosanoid concentrations were independent of OCS treatment, whereas participants on anti-IgE therapy had lower concentrations of leukotriene E_4 (LTE_4) and prostaglandin D_2 (PGD_2) metabolites. Moreover, a strong relationship between LTE_4 and PGD_2 metabolites with markers of T2 inflammation was validated internally and externally using adolescents with severe or controlled persistent asthma. An exploratory benchmarking analysis suggested that the strength of the association of urinary LTE_4 and T2 asthma was in the same range as for blood eosinophils and fractional exhaled nitric oxide. We propose that urinary LTE_4 and PGD_2 metabolites should be explored as new noninvasive biomarkers to guide molecular phenotyping of asthma and the selection of biologics targeting T2 inflammation.

including medication use are described in Table 1. The level of treatment with inhaled corticosteroids (ICS) was one inclusion criterion (≤500 μg fluticasone equivalents/d in MMA and ≥1,000 μg fluticasone equivalents/d in SA). A total of 41% of those with SA were prescribed oral corticosteroids (OCS), and 13% were treated with omalizumab. No participants were treated with 5-LOX inhibitors or prescribed nonsteroidal antiinflammatory drugs for regular use. A control group of healthy participants ($n = 100$) was included. The findings in the baseline study were internally validated in 302 participants with SA followed up in a longitudinal visit after 12–18 months. External validation was conducted in 95 adolescent participants aged 10–16 years with SA or controlled persistent asthma from the Swedish Search cohort. Subject characteristics including use of ICS as budesonide equivalents are summarized in Table 2 (19). The Swedish Search study was approved by the regional board of ethics at Karolinska Institutet (no. 2006/1324-31/1). Written informed consent was obtained from all participants or their guardians. In all studies, spot samples of urine were collected and stored at −80°C without additives until analysis.

Quantification of Eicosanoid Metabolites

For the mass-spectrometry analysis, urine samples were randomly distributed into batches of 24 samples, each of which included one quality-control reference sample. Eicosanoid metabolites (Figure 1) and creatinine were quantified as previously published (20). Among the 13 metabolites representing key pathways (Figure 1), 2,3-dinor-6-keto-$PGF_{1\alpha}$ and TXB_2 displayed unacceptable technical variability (>40% coefficient of variation across batches) and were excluded from the final analysis. Missing values (i.e., lower than the limit of quantification) occurred in <0.6% and 3.2% of the baseline and longitudinal samples, respectively. In adolescent urine samples, the PGD_2 and CysLT metabolites were quantified using enzyme immunoassays from Cayman Chemical, as previously

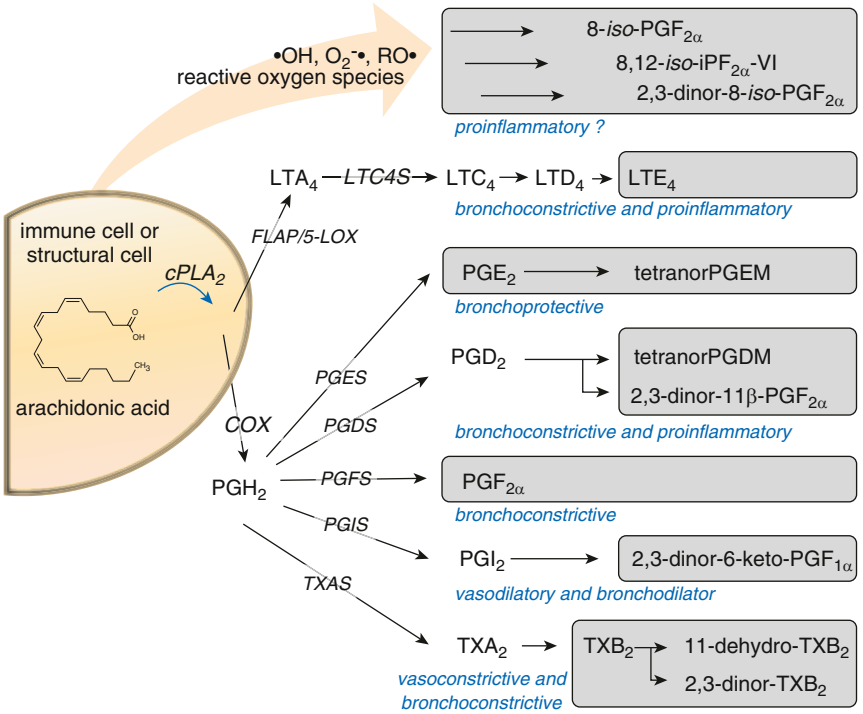

Figure 1. Schematic overview of arachidonic acid–derived lipid mediators (eicosanoids) following both enzymatic and nonenzymatic metabolism. Blue text indicates the known or proposed biologic effect of the indicated pathway. Gray boxes highlight eicosanoids quantified in urine from participants in the U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) study by UPLC-MS/MS. *5-LOX* = 5-lipoxygenase; *COX* = cyclooxygenase; *cPLA2* = cytosolic phospholipase A_2; *FLAP* = five lipoxygenase-activating protein; $iPF_{2\alpha}$ = isoprostane-$F_{2\alpha}$; LT = leukotriene; LTC4S = LTC4-synthase; PG = prostaglandin; PGDS = PGD-synthase; PGES = PGE-synthases; PGFS = PGF-synthase; PGIS = PGI-synthase; tetranorPGDM = tetranor PGD_2 metabolite; tetranorPGEM = tetranor PGE_2 metabolite; TX = thromboxane; TXAS = TXA-synthase; UPLC–MS/MS = ultraperformance liquid chromatography–tandem mass spectrometry.

Table 1. U-BIOPRED Study Characteristics of 597 Participants Used for Urinary Eicosanoid Metabolite Profiling

	Healthy Nonsmoking Participants		Mild-to-Moderate Nonsmoking Asthma		Severe Nonsmoking Asthma		Smokers and Ex-Smokers with Severe Asthma		
	Median (IQR) or %	*n*	Median (IQR) or %	*n*	Median (IQR) or %	*n*	Median (IQR) or %	*n*	*P* Value
Participants, *n*	100	—	86	—	302	—	109	—	—
Age, yr	35 (27–49)	100	43 (28–53)	86	53 (43–62)	302	55 (48–61)	109	<0.0001
Sex, % F	39%	—	50%	—	66%	—	51%	—	<0.0001
BMI, kg/m^2	24.6 (22.8–27.5)	100	24.8 (23.1–28.8)	86	27.8 (24.6–33.7)	302	28.9 (25.2–32.6)	109	<0.0001
FEV_1%*	102 (94–110)	100	92 (76–100)	85	67 (51–85)	299	66 (53–78)	109	<0.0001
Exacerbations, *n*	NA	—	0.0 (0–1.0)	86	2.0 (1.0–3.0)	301	2.0 (1.0–4.0)	109	<0.0001
Smoking history, pack-years)	1 (0−4)	20	4 (1–5)	13	2 (1–4)	43	17 (10−26)	109	<0.0001
ACQ-5	NA	—	0.8 (0.3–1.4)	83	2.2 (1.4–3.0)	291	2.2 (1.4–3.0)	106	<0.0001
AQLQ	NA	—	6.2 (5.4–6.5)	82	4.6 (3.6–5.4)	299	4.4 (3.5–5.3)	105	<0.0001
Daily to weekly OCS use	NA	—	NA	—	41%	124	40%	44	—
OCS, mg eq.	NA	—	NA	—	12 (8–20)	145	16 (10–25)	58	0.1374
Omalizumab users	NA	—	NA	—	13%	40	13%	14	—
Comb. atopy, % positive	40%	40	90%	77	74%	224	62%	68	<0.0001
F_{ENO}, ppb	19 (13–29)	95	25 (18–52)	85	27 (16–48)	281	23 (12–43)	103	0.0005
Periostin, ng/ml	50 (44–57)	88	49 (41–55)	71	50 (42–60)	250	44 (36–59)	85	0.0339
Sputum eosinophils, %	0.3 (0.2–0.9)	18	1.3 (0.7–3.9)	35	4.1 (1.3–26.5)	109	4.5 (1.1–13.8)	49	<0.0001
Blood eosinophil, counts/μl	100 (90–200)	100	200 (100–300)	86	200 (100–400)	294	220 (110–405)	105	<0.0001
Serum IL-13, pg/ml	0.4 (0.3–0.6)	87	0.6 (0.4–0.9)	70	0.6 (0.3–1.1)	245	0.5 (0.3–1.1)	82	0.0004
Serum total IgE, IU/ml	23 (9–63)	97	89 (50–244)	83	119 (45–347)	295	124 (59–343)	106	<0.0001
hsCRP, mg/L	0.8 (0.3–1.6)	97	0.8 (0.4–2.1)	85	2.1 (0.9–4.9)	295	2.3 (1.1–4.8)	109	<0.0001

Definition of abbreviations: ACQ-5 = Asthma Control Questionnaire mean of 1–5; AQLQ = Asthma Quality Of Life Questionnaire total mean; BMI = body mass index; Comb. = combined; F_{ENO} = fractional exhaled nitric oxide; hsCRP = high-sensitivity C-reactive protein; IQR = interquartile range; mg eq. = dose equivalents normalized to milligrams of prednisolone; NA = not applicable; OCS = oral corticosteroids; ppb = parts per billion; U-BIOPRED = Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes.
Significance was evaluated using a Kruskal-Wallis nonparametric test or a chi-square test for categorical variables.
*Prebronchodilator FEV_1%.

described (9). Eosinophil-derived neurotoxin (EDN) was measured according to the manufacturer's instruction using a kit from Medical and Biological Laboratories Co.

Data Analysis

Associations between U-BIOPRED baseline or longitudinal eicosanoid concentrations and clinical or hematological markers of asthma were evaluated using an extreme-value approach, in which participants with asthma were stratified by high (75th percentile) or low (25th percentile) urinary concentrations of LTE_4, combined PGD_2 metabolites (*c*-PGD_2), and a combined isoprostane variable. The composite variables were created by $\log_2$ transformation, followed by scaling each analyte to unit variance (i.e., *z* score) before summation at the subject level. The same associations were evaluated for CysLTs and PGD_2 metabolites in the Swedish Search study. Eicosanoid variables followed a nonnormal distribution. Outcome variables were collected from the U-BIOPRED tranSMART database. Because of the exploratory nature of this study, unadjusted *P* values were used and P values < 0.05 were considered significant using the Kruskal-Wallis, Mann-Whitney *U*, or chi-square test. Extreme-value analysis was performed using R (version 3.4.4; CRAN Network) and statistical evaluation was performed in GraphPad Prism (version 8; GraphPad). Multivariate correlation analysis between variables used to calculate the Refractory Asthma Stratification Program (RASP) T2 severity score (blood eosinophils, F_{ENO}, and serum periostin) (21), and the three urinary metabolites of interest (LTE_4, tetranor PGD_2 metabolite [tetranorPGDM], and 2,3-dinor-11β-$PGF_{2\alpha}$) was performed using partial least-squares regression in SIMCA-P (Sartorius, Umetrics). The correlation between the two data blocks (inner relation) was calculated as the Pearson *r* between the resulting latent variables, as previously described (22), with $P < 0.05$ considered significant.

Results

Urinary Excretion of Eicosanoid Metabolites in Healthy Participants

The highest urinary concentrations were those of the main metabolite of PGE_2, tetranor PGE_2 metabolite (tetranorPGEM) (Figure 2 and Table 3), which was the only metabolite to display meaningful sex differences, with median values among men being approximately twice those of women (1,510 ng/mmol creatinine for men vs. 701 ng/mmol creatinine for women; $P < 0.01$; Figure 2 and Table 3).

Isoprostanes constituted the second most abundant group of metabolites (Figure 2 and Table 3). The median concentrations of 8,12-*iso*-isoprostane-$F_{2\alpha}$-VI (8,12-*iso*-i$PF_{2\alpha}$-VI) were highest, followed by 2,3-dinor-8-*iso*-$PGF_{2\alpha}$, whereas the commonly measured 8-*iso*-$PGF_{2\alpha}$ was the least abundant, accounting for only ∼4% of total isoprostane concentrations. There was a small sex difference for the median concentrations of 2,3-dinor-8-*iso*-$PGF_{2\alpha}$ (151 ng/mmol creatinine for men vs. 214 ng/mmol creatinine for women; $P < 0.05$).

Table 2. Clinical Characteristics of 95 Adolescent Participants in the Swedish Search Study Stratified by Asthma Severity

	Controlled Persistent Asthma	Severe Asthma	*P* Value
Number	38	57	—
Age, yr	14.2 (11.5–16.2)	13.5 (10.6–15.6)	0.370
Sex, % F	39	42	0.798
FEV_1%*	90 (82–100)	82 (70–94)	0.009
Asthma Control Test	23 (22–24)	18 (15–19)	<0.0001
Methacholine, DRS	3 (0.4–30)	18 (2–61)	0.006
Atopic, %	84	83	0.910
Respiratory allergy, %	76	77	0.870
Food allergy, %	53	37	0.140
Exacerbations in previous 12 mo	0 (0–1)	5 (3–10)	<0.0001
ICS, μg budesonide equivalents	320 (190–400)	800 (800–800)	<0.0001
Antileukotriene users (montelukast), *n*	0	46	—
Serum total IgE, IU/ml	290 (81–765)	283 (118–853)	0.609
FE_{NO}, ppb	17 (10–26)	22 (10–40)	0.166
Blood eosinophils, counts/μl	200 (100–325)	300 (200–585)	0.008
Serum periostin, ng/ml	90 (76–119)	84 (56–106)	0.190
EDN, ng/mmol creatinine	116 (73–145)	114 (88–173)	0.357
CysLTs, ng/mmol creatinine	100 (77–133)	112 (81–162)	0.188
PGD_2 metabolites, ng/mmol creatinine	65 (44–82)	67 (49–93)	0.359

Definition of abbreviations: CysLT = cysteinyl leukotriene; DRS = methacholine slope of dose–response; EDN = eosinophil-derived neurotoxin; FE_{NO} = fractional exhaled nitric oxide; ICS = inhaled corticosteroids; PGD_2 = prostaglandin D_2; ppb = parts per billion.
All values are given as median (interquartile range) unless otherwise specified. Group comparisons were performed by using Mann-Whitney *U* test or chi-square test for categorical variables. "Atopic" refers to being sensitized to at least one food or respiratory allergen. "Respiratory allergy" refers to being sensitized (>0.35 kuA/L) to one or more of the following allergen sources: cat, dog, horse, timothy, birch, mugwort, *Dermatophagoides pteronyssinus*, and *Cladisporium*. "Food allergy" refers to being sensitized (>0.35 kuA/L) to one or more of the following allergen sources: milk, egg, wheat, peanut, soya, and cod. Concentrations of CysLTs and PGD_2 metabolites were measured by enzyme immunoassay.
*Prebronchodilator FEV_1%.

The two major metabolites of PGD_2, tetranorPGDM and 2,3-dinor-11β-$PGF_{2\alpha}$, were found in a similar range as the two 8-*iso*-isoprostanes (Figure 2 and Table 3). The panel did not include downstream metabolites of $PGF_{2\alpha}$, but the parent compound was consistently detected at concentrations ~10-fold higher than those of primary PGE_2 (Figure 2 and Table 3). Concentrations of the sequential metabolites of TXA_2, 11-dehydro-TXB_2 and 2,3-dinor-TXB_2, were similar in abundance to PGE_2 but were less abundant than $PGF_{2\alpha}$ (Figure 2 and Table 3). The least abundant analyte was the terminal metabolite of the CysLTs, LTE_4 (median concentration, 3.1 ng/mmol creatinine) (Figure 2 and Table 3).

None of the eicosanoid metabolites in healthy participants showed biologically meaningful correlations with age or body mass index (BMI; data not shown). In addition, except for the metabolites mentioned above, there were no concentration differences in relation to sex.

Comparison of Concentrations between the Study Groups

For five of the six pathways quantified (PGD_2, $PGF_{2\alpha}$, TXA_2, isoprostanes, and CysLTs), there was a general pattern of progressively higher concentrations from healthy to SA; group median concentrations in those with MMA were higher compared with those of healthy participants, and further elevations were evident in participants with SA in comparison with those with MMA

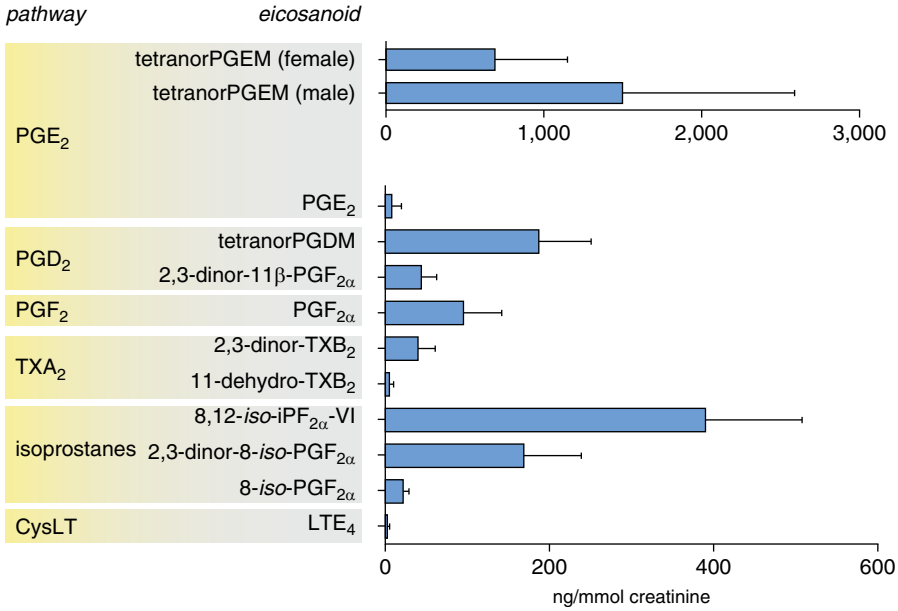

Figure 2. Median (interquartile range) urinary concentration of individual eicosanoids in the U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) adult baseline healthy participant (healthy control) group ($n = 100$). TetranorPGEM concentrations are stratified by sex. CysLT = cysteinyl LT; $iPF_{2\alpha}$ = isoprostane-$F_{2\alpha}$; LT = leukotriene; PG = prostaglandin; tetranorPGDM = tetranor PGD_2 metabolite; tetranorPGEM = tetranor PGE_2 metabolite; TX = thromboxane.

Table 3. Median (IQR) Urinary Eicosanoid Metabolite Concentrations (ng/mmol Creatinine) in U-BIOPRED Healthy Participants and Participants with Asthma Sorted in Order of Descending Concentration

	Healthy Nonsmoking Participants (*HC*) (*n* = *100*) [*Median (IQR)*]	MMA (*n* = *86*)		SAn (*n* = *302*)		SAs/ex (*n* = *109*)	
		Median (IQR)	HC vs. MMA *P* Value	Median (IQR)	MMA vs. SAn *P* Value	Median (IQR)	SAn vs. SAs/ex *P* Value
TetranorPGEM (male)	1,510 (989–2,588)	925 (677–1,576)	<0.001	1,196 (835–1,922)	0.043	1,665 (1,084–2,303)	0.025
TetranorPGEM (female)	701 (482–1,152)	656 (433–899)	0.498	698 (533–1,166)	0.230	1,021 (582–1,489)	0.013
8,12-*iso*-$iPF_{2\alpha}$-VI	392 (293–508)	384 (281–506)	0.570	387 (265–549)	0.669	367 (273–508)	0.484
TetranorPGDM	188 (139–250)	204 (138–285)	0.486	268 (190–368)	<0.001	297 (204–410)	0.083
2,3-dinor-8-*iso*-$PGF_{2\alpha}$	171 (124–239)	163 (113–221)	0.390	191 (135–291)	0.009	208 (150–314)	0.153
$PGF_{2\alpha}$	97 (75–142)	105 (79–142)	0.460	116 (86–158)	0.039	117 (86–153)	0.647
2,3-dinor-11β-$PGF_{2\alpha}$	46 (31–62)	47 (31–68)	0.833	58 (39–80)	0.001	62 (46–83)	0.077
2,3-dinor-TXB_2	42 (30–61)	42 (27–60)	0.908	45 (30–67)	0.567	58 (36–83)	0.006
8-*iso*-$PGF_{2\alpha}$	23 (18–29)	24 (18–33)	0.897	27 (20–39)	0.014	30 (25–43)	<0.001
PGE_2	10.0 (5.8–20.0)	10.0 (6.0–15.0)	0.205	15.0 (8.3–23.0)	<0.001	14.0 (8.9–24.0)	0.779
11-dehydro-TXB_2	6.8 (3.4–11.0)	6.8 (4.6–9.3)	0.741	8.3 (4.8–13.0)	0.020	9.1 (6.4–14.0)	0.092
LTE_4	3.1 (1.9–4.9)	4.5 (3.1-7-0)	<0.001	6.3 (3.9–11.0)	<0.001	7.3 (4.3–12.0)	0.304

Definition of abbreviations: HC = healthy control participants; $iPF_{2\alpha}$ = isoprostane-$F_{2\alpha}$; IQR = interquartile range; LTE_4 = leukotriene E_4; MMA = participants with mild-to-moderate asthma; PG = prostaglandin; SAn = nonsmokers with severe asthma; SAs/ex = smokers or ex-smokers with severe asthma; tetranorPGDM = tetranor PGD_2 metabolite; tetranorPGEM = tetranor PGE_2 metabolite; TXB_2 = thromboxane B_2; U-BIOPRED = Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes.
Significance was determined by using a nonparametric Mann-Whitney *U* test.

(Figure 3 and Table 3). The observed concentrations occasionally reached statistical significance among healthy participants, participants with MMA, and the two groups with SA, primarily among healthy participants and either or both of the groups with SA.

The concentrations of LTE_4 were significantly higher in all asthma groups relative to healthy participants, with the strongest difference for LTE_4 (median fold change [MFC] ≥ 2.0; $P < 0.0001$) in either SA group (Figure 3D and Table 3). The PGD_2 metabolites (tetranorPGDM and 2,3-dinor-11β-$PGF_{2\alpha}$) were also elevated in relation to asthma severity (healthy participants vs. either SA group, MFC ≥ 1.4; $P < 0.001$; healthy participants vs. either SA group, MFC ≥ 1.3; $P < 0.001$, respectively) (Figures 3A and 3B and Table 3).

Two isoprostanes t(8-*iso*-$PGF_{2\alpha}$ and 2,3-dinor-8-*iso*-$PGF_{2\alpha}$) were significantly elevated in the group with SA, with 8-*iso*-$PGF_{2\alpha}$ in addition showing a linear increase with asthma severity. The increase in 8-*iso*-$PGF_{2\alpha}$ was to a great extent driven by the women, a trend also shown for its metabolite 2,3-dinor-8-*iso*-$PGF_{2\alpha}$ (*see* Table E1 in the online supplement). The median level of the most abundant isoprostane, 8,12-*iso*-$iPF_{2\alpha}$-VI, was, however, the same in all four study groups (Figures 3G–3I and Table 3).

In distinct contrast to the other metabolites, primary PGE_2 was lower in those with MMA than in healthy participants, but reached higher concentrations in SA compared with healthy participants (Figure 3J). Moreover, in men, the main metabolite tetranorPGEM was also lower in those with MMA than in healthy participants, and its concentrations in either of the groups with SA were no different from those of healthy participants (Figure 3K and Table 3). The same numerical trends were observed for tetranorPGEM in women, although only smokers with asthma were statistically different from healthy participants. Among both women and men, smokers with SA had higher concentrations of tetranorPGEM than the corresponding nonsmokers.

Influence of OCS Treatment

The use of prescribed OCS was similar in smokers and nonsmokers with SA (40% and 41%, respectively; Table 1), with the vast majority receiving daily treatment (122 of 124 nonsmokers and 43 of 44 smokers). However, for 9 of the 11 measured metabolites, there were no differences in the concentrations in urine between those with asthma who reported receiving OCS and those stating no use (Table 4). The exceptions were 2,3-dinor-TXB_2 and 8,12-*iso*-$iPF_{2\alpha}$-VI, both of which exhibited slightly lower concentrations (12–13%) among the participants prescribed OCS.

The virtual absence of steroid influence on urinary eicosanoid metabolite concentrations was strengthened when the prescription information was combined with data on the actual detection of prednisolone metabolites in urine (Table 4). Stratifying participants according to this stricter classification again demonstrated no differences between groups for the majority of measured metabolites in the SA participants. The observed lower concentrations of the same two metabolites (2,3-dinor-TXB_2 [13% lower] and 8,12-*iso*-$iPF_{2\alpha}$-VI [20% lower]) were replicated in this smaller, but objectively verified, group of OCS users (Figure 4A). Consistent with the overall findings, there were no signs of dose-related effects of steroid treatment on urinary concentrations of CysLTs or PGD_2 metabolites when adult (U-BIOPRED) and

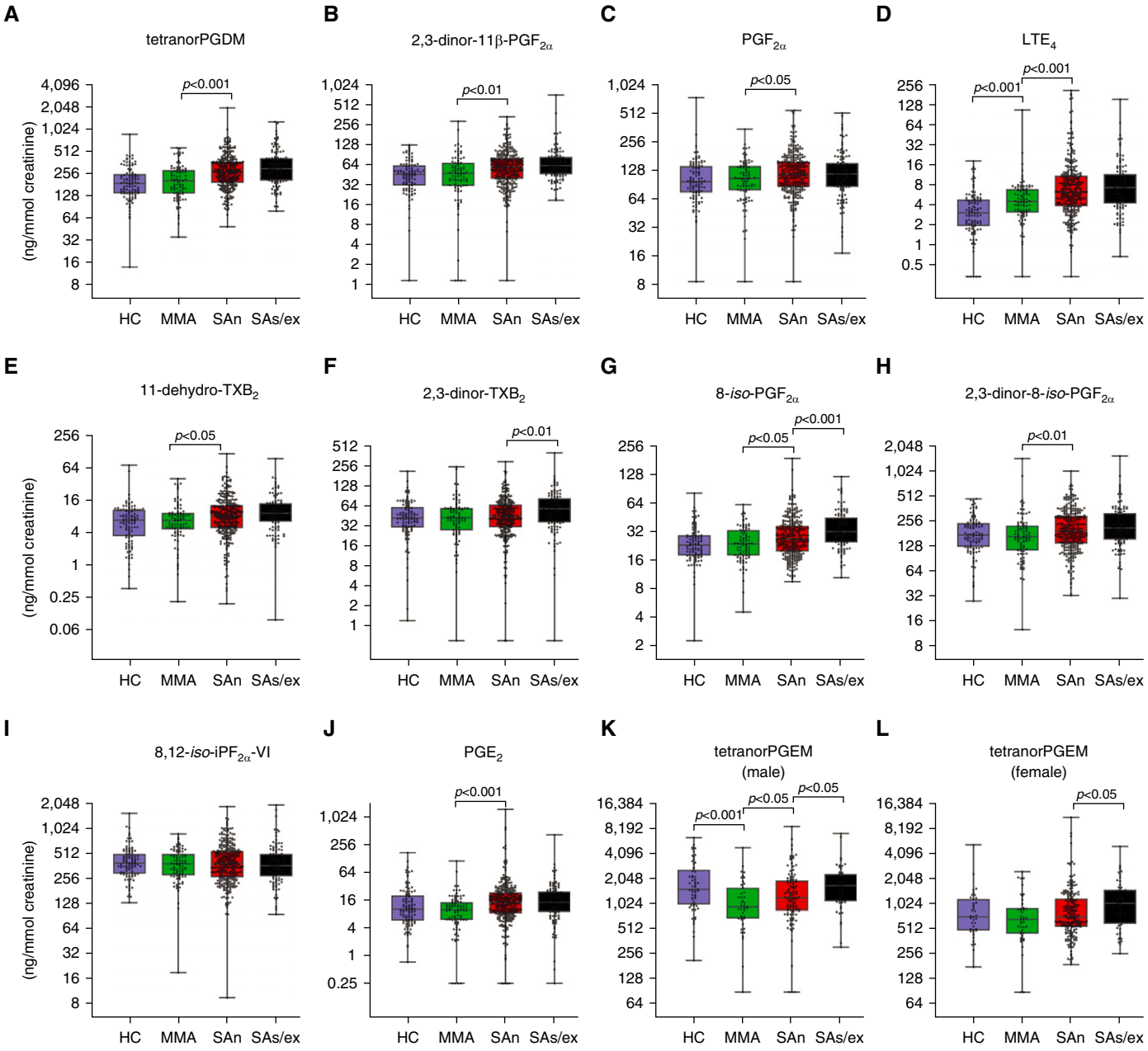

Figure 3. Distribution of urinary eicosanoid concentrations in HC ($n = 100$), MMA ($n = 86$), SAn ($n = 302$), and SAs/ex ($n = 109$) for (*A* and *B*) PGD_2 metabolites, (*C*) $PGF_{2\alpha}$, (*D*) LTE_4, (*E* and *F*) thromboxane metabolites, (*G–I*) isoprostanes, (*J*) PGE_2, and (*K* and *L*) and PGE_2 metabolites. Data are plotted on a $\log_2$ scale. Boxes highlight the interquartile range with the group median; bars display the total distribution range (minimum to maximum). Significant group differences are indicated by *P* values determined by the Mann-Whitney *U* test. HC = healthy control participants; $iPF_{2\alpha}$ = isoprostane-$F_{2\alpha}$; LT = leukotriene; MMA = participants with mild-to-moderate asthma; PG = prostaglandin; SAn = nonsmokers with severe asthma; SAs/ex = smokers or ex-smokers with severe asthma; tetranorPGDM = tetranor PGD_2 metabolite; tetranorPGEM = tetranor PGE_2 metabolite; TX = thromboxane.

adolescent (Swedish Search, *see* below) participants were subdivided into three urine-prednisolone-concentration groups or by three budesonide-dose groups, respectively (Figures 4B and 4C).

Relation between Eicosanoid Metabolites and Omalizumab Treatment

In view of the report that omalizumab treatment may decrease the concentrations of urinary LTE_4 and metabolites of PGD_2 (23), we performed a subgroup analysis of 52 individuals with SA on omalizumab treatment in the year before the study. The serum IgE concentrations were matched using a (1:2) case-control design with no difference in standard asthma

Table 4. Evaluation of the Effect of OCS on U-BIOPRED Urinary Eicosanoid Metabolite Concentrations in Participants with Severe Asthma

Pathway and Metabolite	OCS Usage according to Medical History*			Combined Criteria: Reported Daily OCS (Yes or No) and Detection of Urinary Prednisolone or Its Metabolites† (Yes or No)‡		
	No (*n* = 198)	Yes (*n* = 168)	*P* Value	No/No (*n* = 167)	Yes/Yes (*n* = 90)	*P* Value
PGD_2						
TetranorPGDM	283 (195–387)	268 (203–368)	0.927	285 (195–386)	272 (216–399)	0.528
2,3-dinor-11β-$PGF_{2\alpha}$	60.8 (39.3–78.4)	56.5 (39.5–80.4)	0.778	60.5 (42.8–78.5)	56.6 (37.3–80.9)	0.641
PGE_2						
TetranorPGEM, M§	1,177 (809–2,068)	1,291 (901–1,952)	0.878	1,416 (912–2,113)	1,355 (900–1,970)	0.813
TetranorPGEM, F§	778 (552–1,230)	761 (499–1,343)	0.761	790 (555–1,246)	743 (501–1,313)	0.689
PGE_2	13.3 (7.8–24.8)	15.8 (9.2–23.9)	0.343	14.2 (8.9–25.5)	15.0 (8.3–24.1)	0.654
$PGF_{2\alpha}$						
$PGF_{2\alpha}$	116 (80–155)	118 (88–164)	0.250	116 (82–156)	114 (83–168)	0.771
TXA_2						
11-dehydro-TXB_2	8.9 (4.9–13.2)	8.3 (4.7–12.4)	0.557	8.5 (4.9–12.5)	8.7 (5.5–12.6)	0.723
2,3-dinor-TXB_2	50.0 (33.6–76.3)	44.7 (30.5–61.0)	0.017	51.8 (34.0–77.1)	45.1 (30.5–58.1)	0.044
Isoprostanes						
8-*iso*-$PGF_{2\alpha}$	26.7 (19.5–36.2)	28.9 (21.7–40.8)	0.063	26.8 (20.0–37.0)	28.8 (21.5–39.0)	0.349
2,3-dinor-8-*iso*-$PGF_{2\alpha}$	202 (136–295)	183 (134–290)	0.314	204 (137–295)	170 (121–271)	0.058
8,12-*iso*-i$PF_{2\alpha}$-VI	407 (282–550)	350 (248–498)	0.038	419 (295–550)	335 (244–517)	0.036
Cysteinyl LTs						
LTE_4	6.9 (4.1–10.2)	6.3 (3.9–12.2)	0.719	6.7 (4.3–9.9)	6.5 (4.1–12.4)	0.572

Definition of abbreviations: i$PF_{2\alpha}$ = isoprostane-$F_{2\alpha}$; LTE_4 = leukotriene E_4; OCS = oral corticosteroids; PG = prostaglandin; tetranorPGDM = tetranor PGD_2 metabolite; tetranorPGEM = tetranor PGE_2 metabolite; TX = thromboxane; U-BIOPRED = Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes.

Statistical comparison was performed using two different criteria.*‡ Data are presented as median (interquartile range). The nonparametric Mann-Whitney *U* test was used.

*Participant stratification by reported use of OCS (yes vs. no). Participants reporting daily to weekly OCS are classified as "yes," whereas participants reporting no or previous OCS use are classified as "no."

†Positive detection was defined by the presence of prednisolone or prednisone, methylprednisolone, 16α-OH-prednisolone, 20β-dihydroprednisolone, or desacetyl deflazacort in urine.

‡Participant stratification by reported daily OCS usage plus detection of prednisolone† in urine (yes/yes vs. no/no).

§For tetranorPGEM, the number of participants was as follows for participant stratification by reported use of OCS: male, no = 73 and yes = 69; female, no = 125 and yes = 99; and as follows for participant stratification by reported daily OCS usage plus detection of prednisolone in urine: male, no/no = 64 and yes/yes = 41; female, no/no = 103 and yes/yes = 49.

medication usage, including OCS and ICS, antileukotriene, or long-acting β_2-agonist (Table E2). The omalizumab group had lower concentrations of the early PGD_2 metabolite 2,3-dinor-11β-$PGF_{2\alpha}$ ($P < 0.05$), LTE_4 ($P < 0.01$), and 11-dehydro-TXB_2 ($P < 0.01$), with a tendency of fewer high values in this group (Figure 5 and Table E3). There were no other group differences with respect to common T2 markers, including blood eosinophils, serum periostin, and F_{ENO} (Table E2).

Extreme-Value Analysis in U-BIOPRED

The majority of the monitored eicosanoid pathways evidenced progressive increases with asthma severity but also showed considerable overlap between the study groups (Figure 3). We therefore further stratified the individuals with asthma according to high (75th) and low (25th) percentile distribution for each eicosanoid pathway to identify associations between urinary metabolite concentrations and clinical outcomes as well as other biomarkers.

The most prominent associations in this extreme group comparison were found between high urinary LTE_4 and low lung function as well as typical T2 inflammation markers such as F_{ENO}, blood and sputum eosinophils, and serum periostin and IL-13 (Figure 6). In addition, high urinary LTE_4 showed a significant association with high serum IgE, as well as with T2-associated CCL-18 (Table E4). A number of other serum markers were higher in the high-LTE_4 group, including CCL-26 (eotaxin-3), CCL-17 (TARC), MMP-3, IL-1α, and TNFα (Table E4).

The pattern of high *c*-PGD_2 was similar to the T2 profile and the low lung function associated with high LTE_4 (Figure 6), although values for F_{ENO} and serum periostin did not reach significance. Higher urinary *c*-PGD_2 was also associated with poorer Asthma Control Questionnaire mean of 1–5 (ACQ-5) and Asthma Quality of Life Questionnaire total mean (AQLQ) scores and higher hsCRP (high-sensitivity C-reactive protein) but greater reversibility. The group with high urinary PGD_2 metabolite concentrations displayed greater concentrations of several cytokines, including CCL-18 (Table E4). The participants in the groups with high LTE_4 or high composite PGD_2 received OCS more frequently, together with more frequent detection of urinary OCS. However, the high-LTE_4 and *c*-PGD_2 groups were composed of more participants with SA (Table E5).

The high-isoprostane group (combined isoprostanes) included more women (81% vs. 40% in the low-isoprostane group) and had higher BMI; higher hsCRP; more frequent exacerbations; and poorer results from the ACQ-5, AQLQ, and hospital anxiety and depression scale but had lower F_{ENO} (Tables E4 and E5). High values for

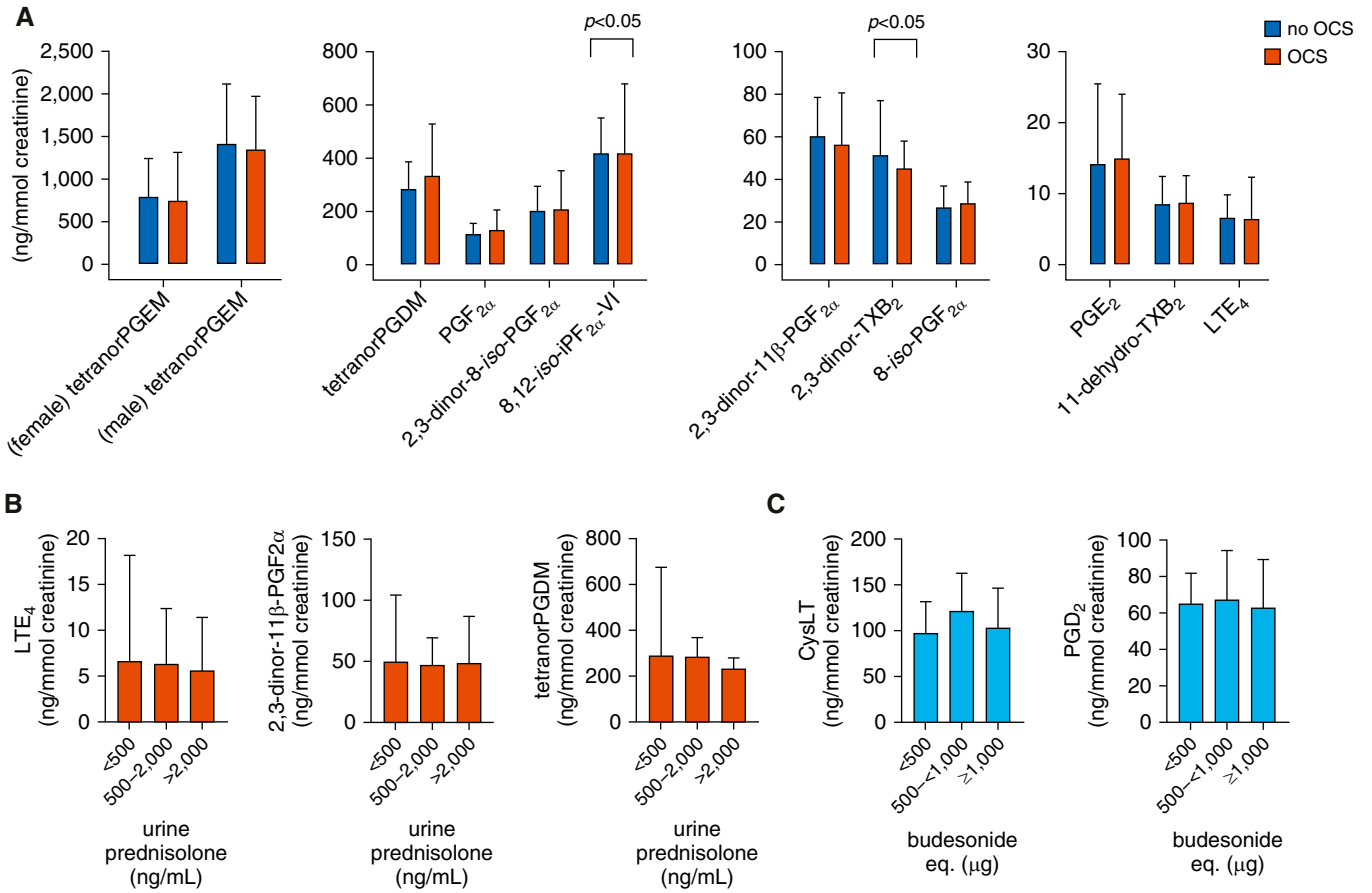

Figure 4. Effect of oral corticosteroids (OCS) on observed urinary eicosanoid concentrations. (*A*) Stratification of adult U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) participants with severe asthma according to reported daily use of OCS and detection of prednisolone or prednisolone metabolites in urine (OCS, $n = 90$; no OCS, $n = 167$) (data from Table 4). (*B*) Median (interquartile range [IQR]) of urinary LTE_4, tetranorPGDM, and 2,3-dinor-11β-$PGF_{2\alpha}$ concentrations across participants with detectable urinary prednisolone in the adult U-BIOPRED study. Of the 90 individuals in which prednisolone or its metabolites were detected, parent prednisolone was only detected in 68 individuals. Quantified urinary prednisolone is stratified as follows: <500 ($n = 17$), 500–2,000 ($n = 27$), and >2,000 ng/ml ($n = 24$). (*C*) Median (IQR) of urinary concentration of CysLT and PGD_2 metabolites across the three inhaled corticosteroid budesonide dose groups in adolescent children from the Swedish Search study. Budesonide eq. is stratified as follows: <500 ($n = 38$), 500–<1,000 ($n = 46$), and ⩾1,000 μg ($n = 11$). CysLT = cysteinyl LT; eq. = equivalents; $iPF_{2\alpha}$ = isoprostane-$F_{2\alpha}$; LT = leukotriene; PG = prostaglandin; tetranorPGDM = tetranor PGD_2 metabolite; tetranorPGEM = tetranor PGE_2 metabolite; TX = thromboxane.

tetranorPGEM in women were related to low lung function but were not related to the sets of typical T2 markers (Table E4). In men, the variability of this metabolite was greater, and there were only a few associations with serum proteins (Table E4).

Internal Validation of U-BIOPRED Findings at 12- to 18-Month Follow-up

Longitudinal samples from 302 (73.5%) of those with SA were employed as an internal validation to test the observed relationship at baseline between urinary LTE_4 and metabolites of PGD_2 and the T2 markers. The main clinical outcomes did not change at the longitudinal time point, including the incidence of OCS treatment (Table 5). The primary findings were confirmed, demonstrating the temporal stability of the eicosanoid T2 signature (Figure 6). For LTE_4, >95% of the participants had values within 1 SD between the two time-points, whereas for the PGD_2 metabolites, this measure was only slightly lower (87.4%) (Table E6). Moreover, repeating the extreme-value analysis confirmed that high urinary LTE_4 and *c*-PGD_2 were significantly associated with blood and sputum eosinophils and serum IL-13 at the longitudinal visit (Figure 6). A nominal decrease in FEV_1% was observed for both participants with high LTE_4 and participants with high *c*-PGD_2, whereas FE_{NO} and serum periostin were strongly elevated in the group with high LTE_4 but were not strongly elevated in the group with high *c*-PGD_2.

External Validation of U-BIOPRED Findings in the Adolescent Swedish Search Cohort

In this external validation cohort of 57 school-aged participants with SA and 38 participants with controlled persistent asthma, there were significant differences in clinical outcomes at the group level (Table 2). However, the median concentrations of urinary CysLTs and PGD_2 metabolites as well as the eosinophil marker EDN did not differ between the two study groups (Table 2). In contrast,

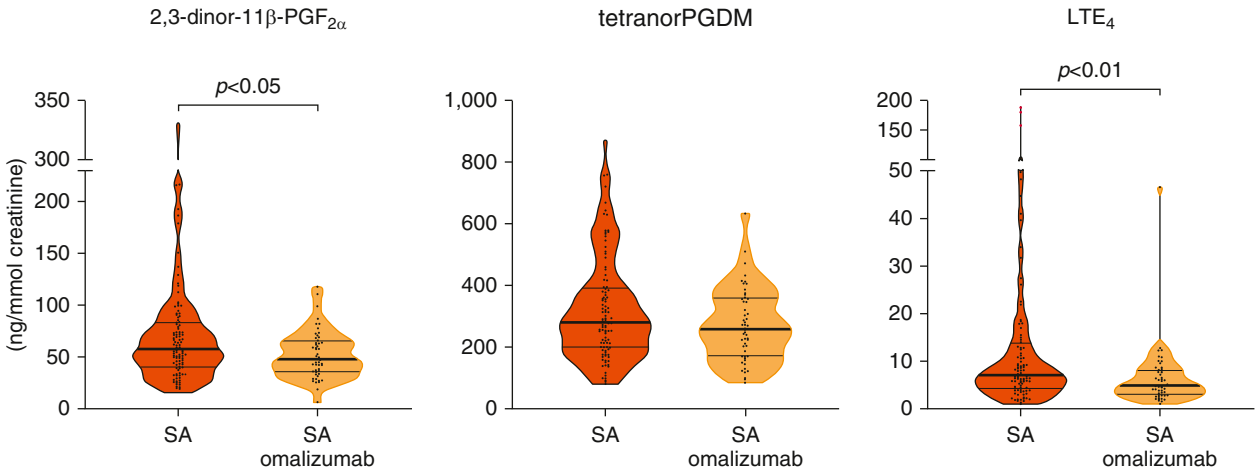

Figure 5. Participants with SA from the adult U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) study with a history of omalizumab treatment ($n = 52$) were compared in a case-control design (1:2) to a group ($n = 104$) matched for similarities in serum IgE. The violin-plot horizontal lines correspond to group median and interquartile-range values for the two PGD_2 metabolites and LTE_4. Group comparisons were evaluated by the Mann-Whitney *U* test (data from Table E3). LT = leukotriene; PG = prostaglandin; SA = severe asthma; tetranorPGDM = tetranor PGD_2 metabolite.

performing the same extreme group comparison as in U-BIOPRED uncovered clinically meaningful differences between participants allocated to the high and low quartiles for urinary concentrations of CysLTs and PGD_2 metabolites (Figure 7 and Table E7). The high-level groups displayed the same associations with lower FEV_1% and elevated T2 markers (e.g., blood eosinophils, serum IgE, and urinary EDN—a surrogate marker for eosinophil activation) (24) as those shown in the U-BIOPRED study. In addition, the high-LTE_4 and high-PGD_2-metabolite groups had increased methacholine responsiveness (Table E7). There was a numerical, albeit small, increase in F_{ENO}, whereas serum periostin concentrations were not different (Figure 6). There was no treatment bias between the groups (Table E7).

Comparison of Urinary LTE_4 and PGD_2 Metabolites with Established T2 Markers

To determine the performance of urinary eicosanoids as markers for T2 asthma, LTE_4 and PGD_2 metabolites were benchmarked against established markers. First, we constructed a heat map in which each cell represented the percentage overlap between two compared biomarkers (Figure 8). Cutoffs for blood eosinophils, F_{ENO}, periostin, and serum IgE were selected from previous work in U-BIOPRED and other consortia (21), and the data in the group of healthy participants were used to define high LTE_4 and *c*-PGD_2 (*see* Figure 8). Generally, T2 cutoffs identified similar percentages of individuals with high LTE_4, blood eosinophils and F_{ENO}, whereas the percentages were slightly lower for periostin and IgE (Figure 8A). Urinary *c*-PGD_2 showed slightly less overlap with the other selected markers but showed high interrelation with LTE_4. The same relationships were observed for the group with SA at the 12- to 18-month longitudinal visit (Figure 8B).

Next, multivariate correlation analysis was preformed between the latent variable representing the concentrations of the markers used to calculate the RASP T2 score (blood eosinophils, F_{ENO}, and periostin) (21) and the latent variable representing the concentrations of three urinary metabolites (LTE_4, tetranorPGDM, and 2,3-dinor-11β-$PGF_{2\alpha}$). The RASP score combines blood eosinophils, F_{ENO}, and serum periostin values into one digit (2 = high; 1 = intermediate; 0 = low). Complete data sets were obtained for 364 of those with asthma at the baseline visit (Table E5) and for 202 of the participants with SA who attended the follow-up visit. The correlation for participants with a high RASP score was significant across all U-BIOPRED asthma groups, both at baseline (Figure 9A; $n = 112$; $r = 0.45$; $P < 0.00001$) and at the longitudinal follow-up (Figure 9B; $n = 52$; $r = 0.48$; $P = 0.0003$). In contrast, the correlation for participants with an intermediate (Figure 9C; $n = 200$; $r = 0.20$) or low (Figure 9D; $n = 75$; $r = 0.22$) RASP score displayed essentially no relevant correlation with the urinary eicosanoids, as evidenced by the flat slopes ($y = 0.04x$). Taken together, the findings demonstrate a strong relationship between established T2 markers and urinary LTE_4 and demonstrate a fair relation to the PGD_2 metabolites.

Discussion

This report presents the largest data set to date on urinary lipid-mediator metabolites in participants with asthma. The results were obtained with an analytical platform specifically designed for the U-BIOPRED study (20). The selected 11 urinary eicosanoid metabolites monitored six major pathways (Figure 1) in samples from 597 individuals, of whom 100 were healthy participants providing an estimation of reference values. It was discovered that high urinary concentrations of LTE_4 and the main PGD_2 metabolites were associated with asthma severity and markers of T2 airway inflammation. The finding was internally validated in a follow-up study of 302 of the adults with SA 12–18 months after the original study and was externally validated in an adolescent population with asthma. Moreover, in both cohorts, we show that the urinary concentrations of eicosanoid metabolites were unrelated to the degree of treatment with inhaled or oral glucocorticosteroids, substantiating findings in previous smaller studies

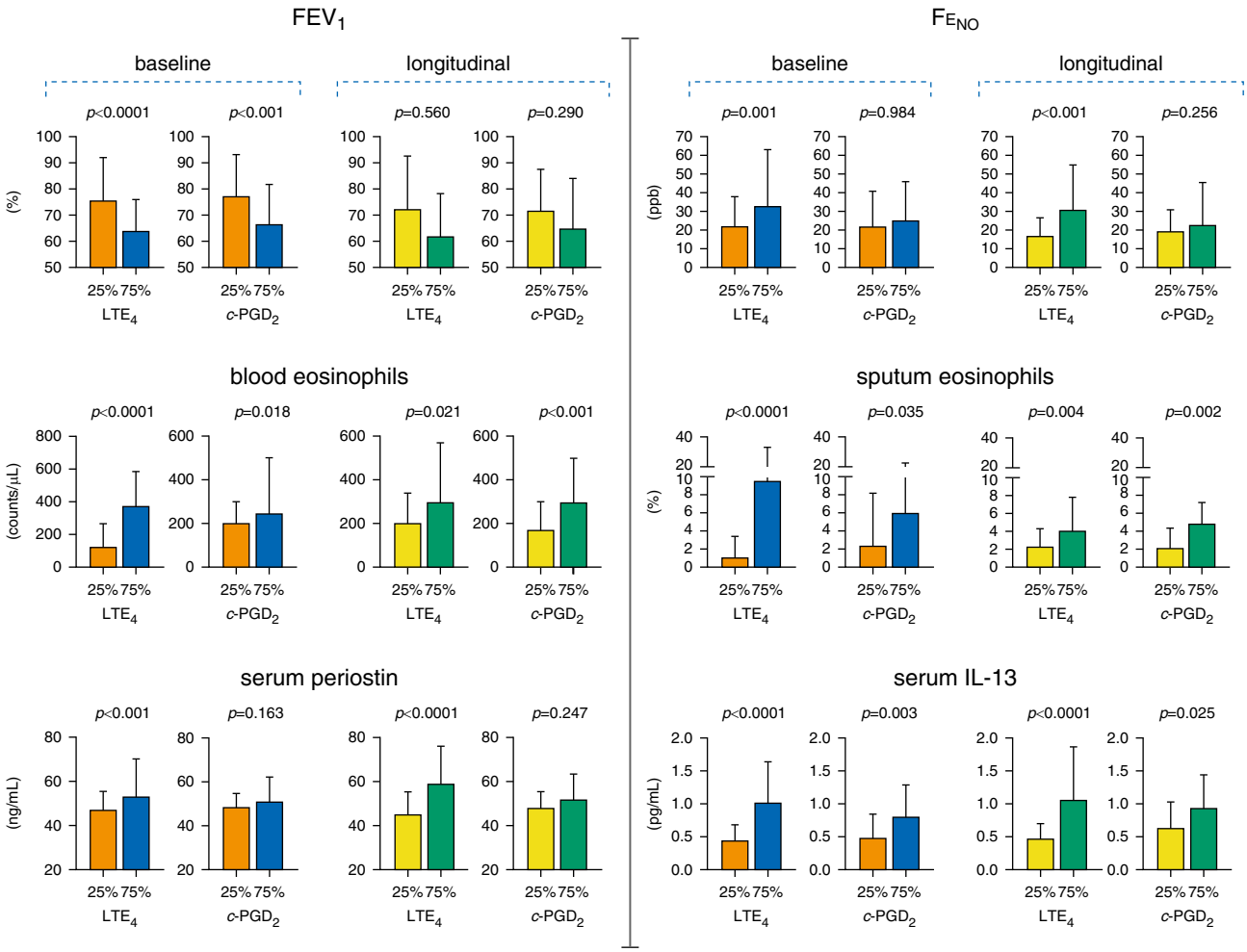

Figure 6. Selection of U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) adult participants with mild-to-moderate asthma, nonsmokers with severe asthma, and smokers/ex-smokers with severe asthma at baseline using the 25th and 75th concentration percentile of urinary LTE_4 and calculated composite (log_2-transformed and *z*-scored) variables for PGD_2 metabolites (*c*-PGD_2). A complete list of significant associations is provided in Table E6. At the 12- to 18-month follow-up visit (longitudinal), 302 participants with severe asthma were used for internal validation of the type 2 associations. Data are presented as the median and interquartile range and were evaluated by using the Mann-Whitney *U* test. *c*-PGD_2 = combined PGD_2 metabolites; F_{ENO} = fractional exhaled nitric oxide; LTE_4 = leukotriene E_4; PGD_2 = prostaglandin D_2; ppb = parts per billion.

suggesting that steroids do not affect eicosanoid biosynthesis *in vivo*.

Our previous studies have shown that measurements of urinary eicosanoids in association with bronchial challenges can elucidate mediator mechanisms (13, 14, 25). There are three main explanations for why the excretion of urinary eicosanoid metabolites reflects inflammatory processes in the airways. First, eicosanoids are a dynamic class of mediators with rapid turnover from the biosynthesis of the biologically active substances in immune-competent and structural cells of the airway tissue to the excretion of inactive metabolites in the urine. Second, the airway mucosa is highly perfused with a large total surface area. Inflammatory reactions in the lower respiratory tract that stimulate the biosynthesis of a particular eicosanoid will therefore be rapidly mirrored in the blood and almost immediately reflected by the appearance of these metabolites in the urine. In contrast, local inflammatory reactions in mucosal tissues with less surface area, such as the nasal airways, do not lead to distinguishable changes in urinary eicosanoid concentrations (26). Third, the source of eicosanoid metabolites in urine may be interpreted with considerable confidence by considering the physiological context. For example, in individuals with ischemic heart disease, increased urinary excretion of metabolites of TXA_2 reflects ongoing platelet activation (27). Likewise, in systemic mastocytosis (28), the concentrations of the urinary PGD_2 metabolites and the major histamine metabolite are increased because of an extrapulmonary supply. In the context of asthma, multiple studies with repeated urine sampling during bronchoprovocations with allergen, exercise, or aspirin (in individuals with aspirin-intolerant asthma) have consistently documented increased concentrations of LTE_4 and PGD_2 metabolites in the urine in correlation with the induced airflow obstruction (9, 14, 25).

Initially, normal values for the urinary eicosanoids were established in the

Table 5. Clinical and Biochemical Characteristics of 302 Adult Participants with Severe Asthma at Baseline and at the 12- to 18-Month Longitudinal Visit in the U-BIOPRED Study

	Baseline	Longitudinal	*P* Value
Sex, % F	60%	60%	—
FEV_1%*	65 (19–82)	64 (16–82)	0.100
F_{ENO}, ppb	26 (15–46)	23 (15–41)	0.251
Blood eosinophils, counts/μl	200 (100–400)	200 (100–400)	0.592
Sputum eosinophils, %	2.8 (0.4–13)	2.0 (0.4–11)	0.442
Serum IL-13, pg/ml	0.58 (0.30–1.14)	0.65 (0.32–1.23)	0.576
Serum periostin, ng/ml	49 (40–60)	51 (42–63)	0.196
ACQ-5	2.2 (1.4–3.0)	2.0 (1.0–3.2)	0.449
AQLQ	4.5 (3.5–5.5)	4.4 (3.5–5.4)	0.515
OCS detected, % yes†	28	32	0.368
Urine prednisolone, ng/ml	1,391 (498–2,842)	1,250 (664–2,724)	0.931
Urine LTE_4, ng/mmol creatinine	6.4 (3.9–11.3)	6.2 (3.6–10.4)	0.211
Urine tetranorPGDM, ng/mmol creatinine	279 (198–382)	261 (174–384)	0.131

Definition of abbreviations: ACQ-5 = Asthma Control Questionnaire mean of 1–5; AQLQ = Asthma Quality of Life Questionnaire total mean; F_{ENO} = fractional exhaled nitric oxide; LTE_4 = leukotriene E_4; OCS = oral corticosteroids; ppb =parts per billion; tetranorPGDM = tetranor prostaglandin D_2 metabolite; U-BIOPRED = Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes.
All values are given as the median (interquartile range). Group comparisons were performed by Mann-Whitney *U* test.
*Prebronchodilator FEV_1%.
†Positive detection was defined by the presence of prednisolone or prednisone, methylprednisolone, 16α-OH-prednisolone, 20β-dihydroprednisolone, or desacetyl deflazacort in urine.

100 healthy participants (Figure 2). The observed concentrations ranged from a few ng/mmol creatinine for LTE_4 to thousands of ng/mmol creatinine for the most abundant metabolite tetranorPGEM (Figure 2 and Table 3). Although concentrations of the isoprostane 2,3-dinor-8-*iso*-$PGF_{2\alpha}$ and 2,3-dinor-TXB_2 were somewhat higher in women, the only pronounced sex difference was for tetranorPGEM, for which men had about twice the concentrations of women. The higher concentration of tetranorPGEM in men confirms previous findings in smaller studies (25, 29) and likely reflects a high level of biosynthesis in male accessory genital glands.

Next, eicosanoid concentrations were evaluated in the groups with asthma. A progressive increase was observed in most eicosanoid metabolites from healthy participants to participants with asthma, and in relation to asthma severity. This trend was particularly significant for LTE_4 and the two PGD_2 metabolites but was also significant for metabolites of TXA_2 originating from platelets, in keeping with suggestions of enhanced platelet activation in asthma (30). Whereas the biosynthesis of PGD_2 in humans occurs predominantly in mast cells, CysLTs are generated by activated mast cells and eosinophils, as well as in transcellular interactions (31).

The one exception to the general trend for higher concentrations of urinary eicosanoid metabolites in asthma was tetranorPGEM, which evidenced significantly lower median concentrations in men with MMA and a similar, albeit nonsignificant, decrease in women. The concentrations of tetranorPGEM were often numerically lower in nonsmokers with asthma (both mild and severe) than in healthy participants. Because PGE_2 has antiasthmatic effects, including stabilization of mast cells and inhibition of type 2 innate lymphoid cells (32–34), it could be speculated that decreased production of PGE_2 in the airways might be one of several deficiencies associated with asthma.

One confounding factor in biomarker studies is that treatment with corticosteroids may modulate observed concentrations. This large study, however, documents that the majority of urinary eicosanoid metabolites were unaffected by steroid use, whether by following stratification of patients by historical prescription of OCS or by objectively quantifying metabolites of urinary prednisone. The latter approach has, to the best of our knowledge, not been used previously in studies of eicosanoid metabolites in urine. The slightly lower (10–20%) values of 2,3-dinor-TXB_2 and 8,12-*iso*-$iPF_{2\alpha}$-VI in OCS users warrant replication in an interventional trial but appear to be relatively unimportant. The same responses were observed in the adolescent participants, in whom no significant change in urinary PGD_2 metabolites or CysLT was observed in relation to the dose of ICS. Our findings therefore confirm and extend previous data from smaller studies indicating that the biosynthesis of PGs and CysLTs are not affected by corticosteroids (35–39). These observations in turn provide the rationale for add-on treatments with antileukotrienes and other drugs that target specific eicosanoids.

Anti-IgE treatment is another therapeutic modality targeting T2 inflammation. In U-BIOPRED, 52 of those with SA had been prescribed omalizumab. We therefore performed a case-control substudy, which found that the concentrations of LTE_4 and metabolites of PGD_2 and TXA_2 were lower in the omalizumab group compared with matched control patients. These associations agree with a previously published open-label study (23) with findings recently confirmed in a controlled trial (40). Interestingly, in the controlled study, the effect on basal excretion of LTE_4 and tetranorPGDM was of the same order of magnitude as the difference we here report between the omalizumab group and the matched control participants in U-BIOPRED. For example, we found 30% lower values for

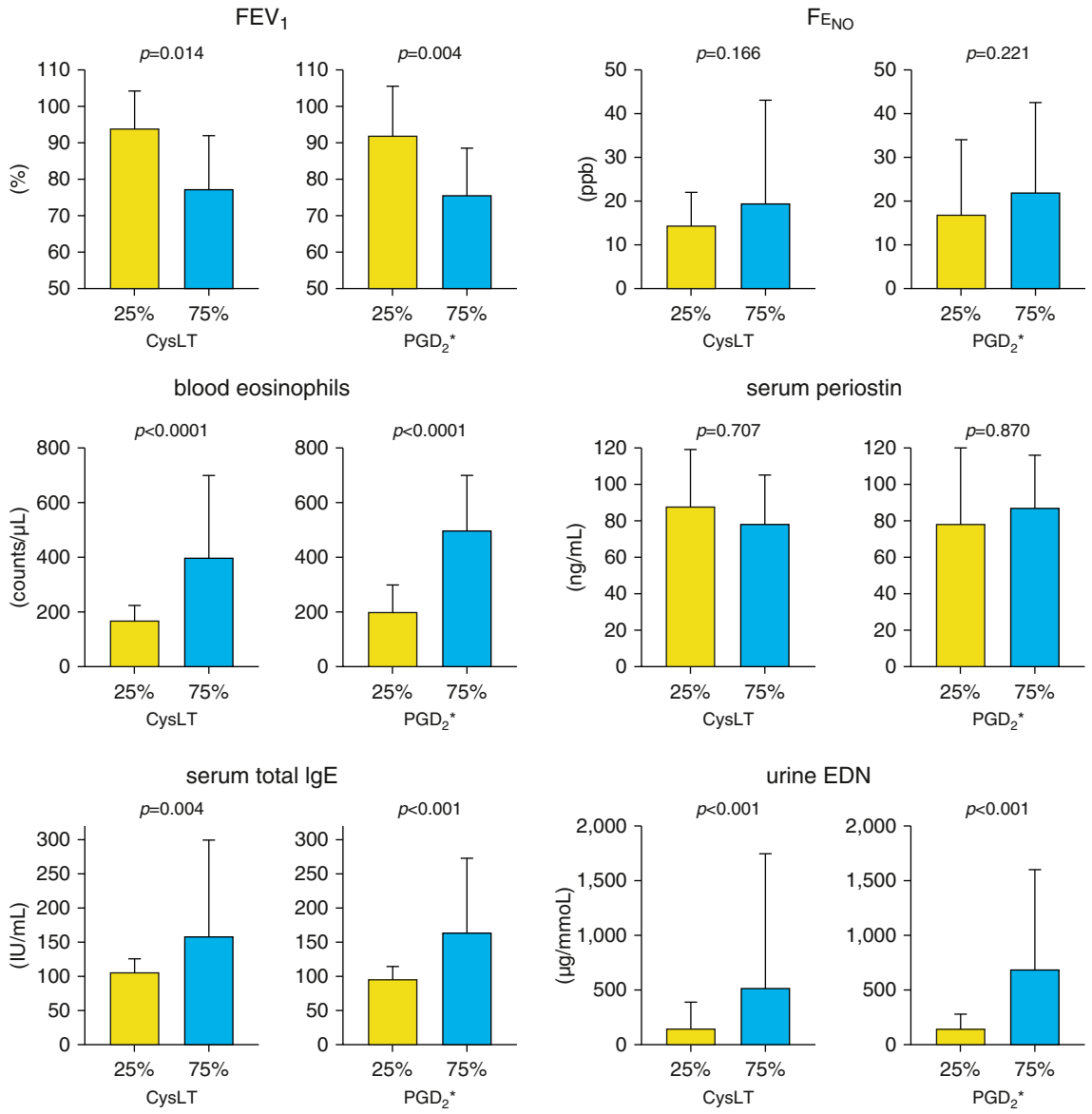

Figure 7. Validation of adult type 2 associations in adolescent participants from the Swedish Search study. Participants with severe or controlled persistent asthma were stratified using the 25th and 75th concentration percentile of urinary metabolites of PGD_2 and CysLTs. For each percentile, the corresponding variable group median (interquartile range) differences were evaluated by the Mann-Whitney *U* test. *Total urinary PGD_2 metabolites were determined by enzyme immunoassay, which has a 9:1 binding ratio toward 2,3-dinor-11β-$PGF_{2\alpha}$:11β-$PGF_{2\alpha}$ as determined by a cross-reactivity test and ultraperformance liquid chromatography–tandem mass spectrometry (9). CysLT = cysteinyl leukotrienes; EDN = eosinophil-derived neurotoxin; F_{ENO} = fractional exhaled nitric oxide; PGD_2 = prostaglandin D_2; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; ppb = parts per billion.

LTE_4, and they reported a 21% reduction after 3 months of treatment.

Although concentrations of most urinary eicosanoids were broadly related to the presence of disease and its severity, significant overlap was observed across the groups (Figure 3). To more clearly identify associations between high concentrations of urinary eicosanoids and other asthma biomarkers, as well as clinical outcomes, an extreme group analysis of the data was performed. The consistent finding was that high concentrations (i.e., upper quartile) of LTE_4 in particular, but also of composite PGD_2 metabolites, strongly associated with T2 biomarkers, including blood and sputum eosinophils, serum periostin and IL-13, and high F_{ENO}. The participants with the highest concentrations of urinary LTE_4 and *c*-PGD_2 metabolites also had worse lung function (FEV_1 = 64% and 67% predicted vs. 76% and 77% in the patients in the lowest quartiles for these two metabolites; Figure 6). The lower lung function associated with elevated concentrations of the two eicosanoids presumably relates to the fact that both compounds are potent mediators of airway obstruction.

A Baseline

T2 biomarkers	*blood eosinophils*	*F_{ENO}*	*serum periostin*	*serum IgE*	*urine LTE_4*	*urine c-PGD_2*	*no. of stratified participants out of 497 asthmatics*
blood eosinophils ≥ 300		58%	53%	53%	62%	40%	*n* =195
F_{ENO} ≥ 30	56%		46%	49%	54%	32%	*n* =202
serum periostin ≥ 55	64%	55%		47%	62%	35%	*n* =125
serum IgE ≥ 150	50%	48%	39%		48%	30%	*n* =204
urine LTE_4 ≥ 6.4	51%	47%	45%	42%		47%	*n* =234
urine *c* -PGD_2 ≥ 0.92	48%	40%	39%	38%	67%		*n* =163

B Longitudinal

T2 biomarkers	*blood eosinophils*	*F_{ENO}*	*serum periostin*	*urine LTE_4*	*urine c-PGD_2*	*no. of stratified participants out of 302 asthmatics*
blood eosinophils ≥ 300		44%	49%	58%	31%	*n* =119
F_{ENO} ≥ 30	51%		48%	58%	22%	*n* =103
serum periostin ≥ 55	53%	46%		59%	24%	*n* =83
urine LTE_4 ≥ 6.4	48%	41%	43%		30%	*n* =145
urine *c* -PGD_2 ≥ 0.92	60%	37%	40%	69%		*n* =62

Figure 8. Relationship between proposed type 2 (T2) markers (*A*) at baseline and (*B*) at the 12- to 18-month longitudinal follow-up visit in the U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) study. The heat map displays the percentage overlap between high amounts of four common T2 markers and the two urinary eicosanoid markers leukotriene E_4 (LTE_4) and combined prostaglandin D_2 metabolites (*c*-PGD_2). Established cutoffs (21) for the T2 markers were used (blood eosinophils ⩾ 300 counts/μl, F_{ENO} ⩾ 30 ppb, periostin ⩾ 55 ng/ml, and IgE ⩾ 150 IU/ml). Cutoff values for the urinary metabolites were calculated from the median + 1 SD in the healthy control group (Table 3). Each cell represents the percentage of participants satisfying both cutoffs for a given comparison. For example, in the first row in *A*, 62% of the *n* = 195 participants with blood eosinophils ⩾ 300 counts/μl also have urinary LTE_4 ⩾ 6.4 ng/mmol creatinine. The total number of participants positive for each row criterion is displayed in the far-right column. Serum IgE data were only available at baseline. *c*-PGD_2 is a *z*-scored composite variable consisting of tetranorPGDM and 2,3-dinor-11β-$PGF_{2\alpha}$ (*see* Methods). F_{ENO} = fractional exhaled nitric oxide; ppb = parts per billion; tetranorPGDM = tetranor PGD_2 metabolite.

The strength of the discovered T2 association was first validated internally at the longitudinal visit, lending evidence to the existence of a stable biochemical and physiological T2 association. The long-term stability of profiles and concentrations of urinary eicosanoid metabolites in individuals has not been studied extensively. The results of the longitudinal follow-up 12 to 18 months after the baseline visit, however, demonstrated preserved excretion of high concentrations of urinary LTE_4 and tetranorPGDM in those with SA. This observation is in line with some treatment trials with 5-LOX inhibitors, suggesting that the fluctuations of urinary LTE_4 in the placebo arms are modest (8, 41, 42). There are recent data for 8-*iso*-$PGF_{2\alpha}$ and tetranorPGEM that document excellent stability over 5 months (43), and the recent controlled trial of omalizumab in individuals with aspirin-exacerbated respiratory disease showed no variability during the placebo period (40).

As atopy and T2 inflammation generally are more common in pediatric asthma, the T2 associations from U-BIOPRED were then externally validated in urine samples from an independent cohort of school-aged children with severe or controlled persistent asthma. All included T2 markers were significantly associated with urinary LTE_4 and PGD_2 metabolites in the adolescent cohort, except for F_{ENO} and periostin (Figure 7). Although nominal F_{ENO} amounts were higher, they did not reach significance, and serum periostin concentrations are not a useful T2 marker in children because of bone growth (44). The eosinophil-activation marker urinary EDN was used in the extreme-value analysis because sputum eosinophils were not available from the adolescent cohort, and the data suggest that this protein would be a useful component of a urinary T2 phenotyping panel.

The utility of urinary LTE_4 and PGD_2 metabolites to identify the T2 endotype was compared with the more traditional markers in an exploratory *post hoc* analysis (Figures 8 and 9). For this benchmarking screen, we used cutoffs for the current markers that are commonly applied in other studies, including U-BIOPRED (21). The cutoff values for LTE_4 and *c*-PGD_2 were defined by the baseline excretion concentrations in the group of healthy participants in U-BIOPRED. Generally, a majority of participants above each T2 biomarker cutoff presented elevated blood eosinophils and urinary LTE_4 to a similar degree, and these amounts were closely followed by F_{ENO}, serum periostin, and serum IgE, with fewer participants having high *c*-PGD_2 (Figure 8). Moreover, a multivariate analysis approach to benchmarking the urinary eicosanoids provided similar findings (Figure 9). In this case, the three RASP markers (blood eosinophils, F_{ENO}, and periostin) were combined into a single latent variable and compared with a combined latent

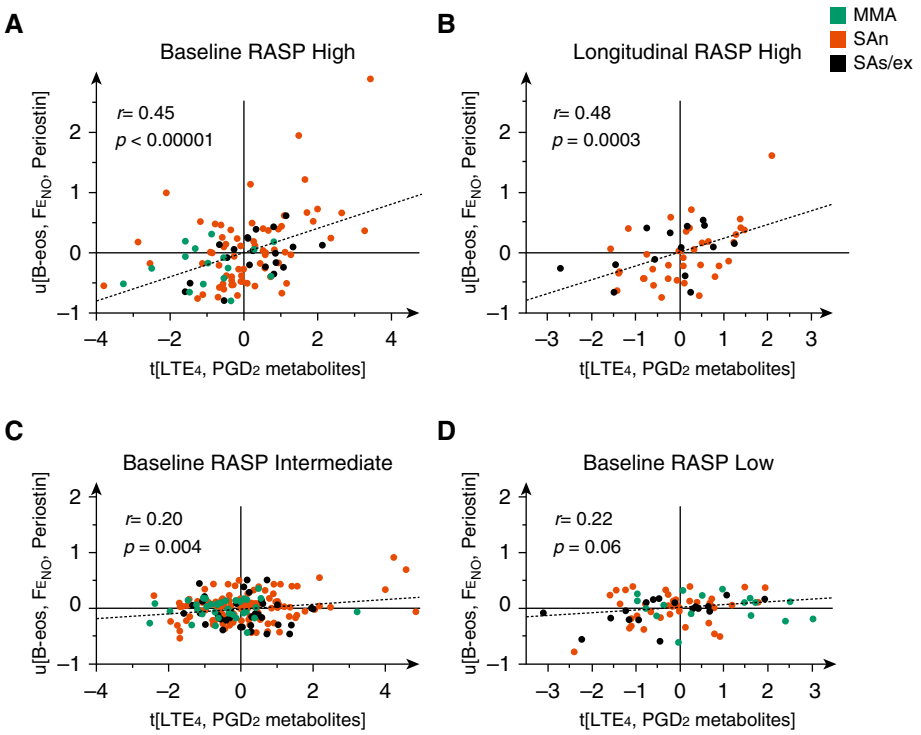

Figure 9. Multivariate correlation analysis between the latent variable consisting of the markers employed to calculate the Refractory Asthma Stratification Program (RASP) type 2 severity score (B-eos, F_{ENO}, and serum periostin) (21) and the latent variable representing concentrations of the three urinary eicosanoid metabolites: LTE_4, tetranorPGDM, and 2,3-dinor-11β-$PGF_{2\alpha}$. The correlation for participants with a high RASP score with urinary eicosanoid concentrations was significant across all U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) asthma groups at both (*A*) baseline ($n = 112$) and (*B*) the longitudinal follow-up ($n = 52$). In contrast, the correlation for baseline participants with a (*C*) intermediate ($n = 200$) or (*D*) low ($n = 75$) RASP score displayed no relevant correlation with the urinary eicosanoid concentrations, as evidenced by the flat slopes ($y = 0.04x$). PGD_2-metabolites include tetranorPGDM and 2,3-dinor-11β-$PGF_{2\alpha}$. B-eos = blood eosinophils; F_{ENO} = fractional exhaled nitric oxide; LTE_4 = leukotriene E_4; MMA = participants with mild-to-moderate asthma; PGD_2 = prostaglandin D_2; SAn = nonsmokers with severe asthma; SAs/ex = smokers or ex-smokers with severe asthma; t = scores vector for *x*-block, calculated from the variables defined in brackets; tetranorPGDM = tetranor PGD_2 metabolite; u = scores vector for *y*-block, calculated from the variables defined in brackets.

variable for the urinary eicosanoids (LTE_4, tetranorPGDM, and 2,3-dinor-11β-$PGF_{2\alpha}$). Participants with asthma with a high RASP score, indicating a high-T2 phenotype, correlated strongly with the urinary eicosanoid concentrations at both baseline and at the longitudinal follow-up. In contrast, participants with an intermediate or low RASP score displayed no relevant correlation.

The findings collectively further support the exploration of urinary LTE_4 and PGD_2 metabolites as noninvasive biomarkers of T2 inflammation. The use of eicosanoids as biomarkers should also be considered within the context that they are potent, biologically active mediators of inflammation and indicators of the activation of particular immune cells, especially eosinophils and mast cells. It is therefore warranted to test the use of urinary eicosanoids for stratification of patients for treatment with biologics that target T2 inflammation (e.g., anti–IL-5s, anti-IL4Rα, and antialarmins).

In contrast to the T2 asthma associations for PGD_2 and the CysLTs, high isoprostane concentrations were associated with a different phenotypic pattern. This group was enriched in women with high BMI, lower F_{ENO}, poor quality of life (AQLQ), more frequent exacerbations, elevated hsCRP, and less asthma control. This observation suggests a role for isoprostanes as markers of a non-T2 phenotype, presumably the phenotype of asthma dominated by women with a high BMI. The isoprostane 8-*iso*-$PGF_{2\alpha}$ is in particular considered to be a gold-standard marker of oxidative stress (45), which is at least partially due to the availability of an immunoassay. However, in the current study, the mass spectrometry–based quantification of 8-*iso*-$PGF_{2\alpha}$ accounted for <5% of the total observed isoprostane metabolites in the urine. Interestingly, 8-*iso*-$PGF_{2\alpha}$ exhibited a significant increase with asthma severity (Figure 3G), whereas the more abundant metabolites, 2,3-dinor-8-*iso*-$PGF_{2\alpha}$ and 8,12-*iso*-$iPF_{2\alpha}$-VI, had weaker responses. Future studies are needed to clarify the biologic indications of these species, including a screen of all 64 potential isoprostanes and their metabolites in urine (12), to determine the most appropriate analyte to serve as a marker of oxidative stress.

One limitation of the study was that concentrations of PGI_2 metabolites were lost because of technical problems, and the $PGF_{2\alpha}$ pathway was only assessed by the parent compound. The latter shortcoming has been mitigated in our updated version of the platform that includes downstream metabolites of $PGF_{2\alpha}$ (15). Although the stability of the key eicosanoid metabolites was demonstrated at 12–18 months of follow-up, there is a need for further studies of the long-term fluctuations of eicosanoid metabolites in urine in both health and disease. There is also emerging evidence suggesting that defective biosynthesis of specialized proresolving mediators (46) may be part of the SA pathobiology (47). However, to date, only cellular metabolism studies have been performed (48, 49), and the *in vivo* metabolism of these mediators remains to be determined before indicative markers may be followed in the urine.

We conclude that this study supports the premise that asthma phenotyping may be aided by measurements of indicative metabolites of lipid mediators in the urine. The discovery of strong associations among urinary LTE_4, PGD_2 metabolites, and markers of T2 inflammation warrant application in future treatment trials with biologics and strengthen evidence of the key role of mast cells and eosinophils in asthma. Urinary eicosanoid signatures could be particularly useful to guide selection for treatment in children with SA, in whom sputum induction and blood sampling can be challenging. The panel of urinary biomarkers could potentially be enhanced by standardized inclusion of

noneicosanoid markers such as, for example, histamine metabolites (50) and EDN (24). Because of its noninvasive nature, the measurement of urinary eicosanoids would be particularly useful at the point of primary care at which the vast majority of asthma patients are managed. ■

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U-BIOPRED Study Group members:
H. Ahmed, C. Auffray, A. T. Bansal, E. H. Bel, J. Bigler, B. Billing, F. Baribaud, H. Bisgaard, M. J. Boedigheimer, K. Bønnelykke, J. Brandsma, P. Brinkman, E. Bucchioni, D. Burg, A. Bush, A. Chaiboonchoe, C. H. Compton, J. Corfield, D. Cunoosamy, A. D'Amico, B. De Meulder, V. J. Erpenbeck, D. Erzen, K. Fichtner, N. Fitch, L. J. Fleming, E. Formaggio, U. Frey, M. Gahlemann, V. Goss, Y. Guo, S. Hashimoto, J. Haughney, P. W. Hekking, T. Higenbottam, J. M. Hohlfeld, A. J. Knox, N. Lazarinis, D. Lefaudeux, M. J. Loza, R. Lutter, A. Manta, S. Masefield, J. G. Matthews, A. Mazein, A. Meiser, R. J. M. Middelveld, M. Miralpeix, N. Mores, C. S. Murray, J. Musial, D. Myles, L. Pahus, S. Pavlidis, A. Postle, P. Powel, G. Praticò, M. PuigValls, N. Rao, A. Roberts, G. Roberts, A. Rowe, T. Sandström, J. P. R. Schofield, W. Seibold, A. Selby, R. Sigmund, F. Singer, P. J. Skipp, M. Smicker, K. Sun, B. Thornton, M. Uddin, W. M. van Aalderen, M. van Geest, J. Vestbo, N. H. Vissing, A. H. Wagener, S. S. Wagers, Z. Weiszhart, S. J. Wilson, and J. Östling.

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