

Arachidonic acid metabolites and enzyme transcripts in asthma are altered by cigarette smoking

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Abstract

Background: Arachidonic acid metabolites are implicated in the pathogenesis of asthma although only limited information is available on the impact of current smoking history on these metabolites. The aim of the study was to examine the effect of smoking status on urinary, sputum, and plasma eicosanoid concentrations and relevant enzyme transcripts in asthma.

Methods: In 108 smokers and never smokers with asthma and 45 healthy controls [smokers and never smokers], we measured urinary tetranor prostaglandin (PG)_{D2} (PGDM) and leukotriene (LT)_{E4}, induced sputum fluid LTB₄, LTE₄, PGD₂, and PGE₂, plasma secretory phospholipase A₂ (sPLA₂), and 11β prostaglandin F_{2α} (11βPGF_{2α}), and, in a subgroup with severe asthma, airway leukocyte and epithelial cell mRNA expression levels of arachidonic acid metabolic enzymes.

Results: Smokers with asthma had higher urinary LTE₄; 83 (59, 130) vs 59 (40, 90) pg/mg creatinine, $P = 0.008$, and PGDM; 60 (35, 100) vs 41 (28, 59) ng/mg creatinine, $P = 0.012$ concentrations, respectively, and lower sputum PGE₂ concentrations 80 (46, 157) vs 192 (91, 301) pg/ml, $P = 0.001$ than never smokers with asthma. Sputum LTB₄ ($P = 0.013$), and plasma 11βPGF_{2α} ($P = 0.032$), concentrations, respectively, were increased in smokers with asthma compared with healthy smokers. Asthma-specific and smoking-related increases (>1.5-fold expression) in arachidonate 15-lipoxygenase and gamma-glutamyltransferase transcripts were demonstrated.

Conclusions: Several arachidonic acid metabolites and enzyme transcripts involving both lipoxygenase and cyclooxygenase pathways are increased in smokers with asthma and differ from never smokers with asthma. Possibly targeting specific lipoxygenase and cyclooxygenase pathways that are activated by asthma and cigarette smoking may optimize therapeutic responses.

The prevalence of active smoking in adults with asthma is similar to the general population, with rates ranging from under 20% to over 35% (1, 2). Cigarette smoking has important effects on symptoms and airway inflammation in asthma. Smokers have poor levels of asthma control, increased rates of healthcare utilization, and attenuated responses to corticosteroids compared with never smokers with asthma (1, 3). Altered airway inflammation is evident in

this group with decreased sputum eosinophils and increased airway mast cells compared with never smokers with asthma (1, 3, 4). There is a need for a greater understanding of the mechanisms of disease and treatment options for patients with asthma who smoke.

Metabolites of 5-lipoxygenase and cyclooxygenase are implicated in the pathogenesis of asthma (5), although there is limited published information on the influence of cigarette

smoking status on these pathways (6–9). Cigarette smoking is associated with increase urinary leukotriene (LT) E_4 production in healthy subjects in some (6), but not in all studies (7), in smokers with asthma compared with non-smokers with asthma (7), and in children with asthma exposed to second-hand smoke who are at increased risk of asthma exacerbations (8). Exhaled breath condensate LTD $_4$ concentrations are higher in smokers with asthma compared with nonsmokers with asthma (9). Tobacco smoke exposure *in vitro* induces cyclooxygenase (COX)-2 and prostaglandin (PG) E_2 receptor expression and promotes the release of PGE $_2$ in neutrophils and alveolar macrophages (10). Understanding the influence of cigarette smoking on 5-lipoxygenase and cyclooxygenase pathways in asthma is important as it may help stratify patients for therapies targeting these pathways (11–13).

Previous studies have examined a relatively limited range of arachidonic acid metabolites in biological samples. In this study, we sought to determine the influence of smoking on the levels of several arachidonic acid metabolites in urine, sputum, and blood, and mRNA expression levels of key metabolic enzymes in airway cells in smokers and never smokers with asthma and in healthy controls. The hypothesis was that cigarette smoking increased 5-lipoxygenase and cyclooxygenase pathways, as assessed by urinary LTE $_4$ and PGDM concentrations, in smokers with asthma compared with never smokers with asthma. Measurements of sputum and plasma eicosanoid concentrations and relevant enzyme transcripts were considered exploratory.

Methods

Subjects and study design

A cross-sectional study was performed in subjects with asthma and healthy controls as part of the Glasgow COPD and Asthma Biomarker study (14). Clinical, physiological, and sputum cell count measurements were taken. Participants were recruited with mild, moderate and severe persistent asthma (GINA classification) (15), (both current smokers and never smokers) and healthy smokers and never smokers. **Asthma:** Age range 18–75 years; Duration of asthma ≥ 6 months; Diagnosis confirmed by airway hyper-responsiveness ($\geq 20\%$ drop in FEV $_1$ at a methacholine dose of ≤ 8 mg/ml) or when FEV $_1$ $< 60\%$ predicted, by evidence of a $\geq 12\%$ and 200 ml increase in FEV $_1$ following 2.5 mg nebulized salbutamol. Patients with aspirin-intolerant asthma were not recruited. **Healthy Controls** (smokers and never smokers): age range 18–80 years; no known respiratory disease or chronic respiratory symptoms; normal spirometry. All subjects with asthma were on stable medication for 4 weeks and not on daily oral steroids, and without exacerbation of disease for 4 weeks. Definition of smokers is at least 10 pack-years and currently smoking 5 or more cigarettes per day; smoking history was confirmed by serum cotinine concentration (Table 1). The West Glasgow Research Ethics Committee approved the study and all patients gave written informed consent.

Measurements

Clinical and lung function measurements

Asthma Control Questionnaire (ACQ) results were recorded (16). Exacerbation in the previous year was defined by use of emergency oral steroids. Spirometry and airway hyper-responsiveness were measured in concordance with relevant guidelines. Lung volumes and diffusing capacity for carbon monoxide (DLco) were measured using the body box technique (Zan500 Body Plethysmography, nSpire Health Limited, Hertford, UK).

Sputum induction

Sputum induction was performed using hypertonic saline and processed to collect sputum cells and fluid as previously described (14). Samples with cell viability $< 40\%$ or squamous epithelial cells $> 80\%$ were discarded.

Sample collection

Blood for plasma, nasal respiratory epithelial cells by curettage and spot urine samples were collected. All fluids were stored at -80°C until use. For transcriptome analysis, induced sputum cells and nasal samples from healthy never smokers ($n = 15$), healthy smokers ($n = 13$), smokers with severe asthma ($n = 15$), and never smokers with severe asthma ($n = 15$) were collected into Qiazol (Qiagen Ltd., Crawley, UK) and RNA-later (Invitrogen Ltd., Paisley, UK), respectively, to stabilize RNA which was then isolated (Ambion mirVana RNA kit; Applied Biosystems, Warrington, UK) and stored at -80°C until use. RNA quality was assessed by Bioanalyser microfluidic chips (Agilent, Santa Clara, CA, USA), and RNA with Integrity Number < 6.4 was discarded.

RNA expression levels for enzymes key to arachidonic acid metabolism

Expression levels were measured using Affymetrix U133 + 2 chips and compared after global normalization.

Urinary, sputum fluid, and plasma arachidonic acid metabolites

Urine concentrations of tetranor prostaglandin D $_2$ (PGDM; 11, 15-Dioxo-9 α -hydroxy-2, 3, 4, 5-tetranorprostan-1, 20-dioic acid) and leukotriene (LT) E_4 were measured by LC-MS/MS and normalized with urinary creatinine (also measured by LC-MS/MS). Plasma concentrations of 11 β -PGF $_{2\alpha}$ and secreted phospholipases (sPLA) $_2$ and sputum fluid concentrations of LTB $_4$, LTE $_4$, PGD $_2$, and PGE $_2$ were measured by enzyme immunoassay (Cayman Chemicals, Cambridge Biosciences, Cambridge, UK).

Serum cotinine

It was measured by enzyme immunoassay (Calbiotec, Spring Valley, CA, USA).

Analysis

Median and interquartile range were estimated for each biomarker and for the smoking and severity disease subgroups. T-test, Wilcoxon test, ANOVA, Kruskal–Wallis test, and exact

Table 1 Baseline demographics

	Asthma			Healthy control		
	Never smoker	Smoker	<i>P</i> -value	Never smoker	Smoker	<i>P</i> -value
Number	53	56		26	20	
Age (years)	49 (38, 54)	47 (40, 53)	0.806	49 (42, 56)	53 (45, 58)	0.625
Gender						
Male, <i>n</i>	22	27	0.564	8	9	0.368
Female, <i>n</i>	31	29		18	11	
Disease Duration (years)	21 (9.0, 36.0)	18.5 (10.0, 31.3)	0.443	—	—	
Pack-years smoked	—	32.5 (21.8, 60)		—	31.0 (24.5, 38)	
Serum cotinine ng/ml	0.4 (0.2, 0.7)	32.4 (23.5, 45.0)	<0.001	0.9 (0.2, 1.1)	29.1 (15.8, 44.7)	<0.001
FEV ₁ % pred postbronchodilator	90.0 (78.1, 98.6)	81.5 (69.4, 99.2)	0.339	—	—	
FEV ₁ /FVC postbronchodilator	78.5 (67.5, 82.5)	71.5 (65.4, 79.3)	0.128	—	—	
DLco % pred	87.5 (79, 95.4)	75.4 (65.1, 85.3)	<0.001	—	—	
Beclometasone equivalent dose of inhaled steroid	800 (400, 1000)	800 (700, 1000)	0.975	—	—	
Emergency oral steroids in last year: yes/no	14 : 38	14 : 42	0.820			
Sputum Neutrophil %	40.8 (29.1, 66.1)	50.5 (35.1, 65)	0.399	46.5 (27.8, 56.4)	49.3 (33.1, 66.6)	0.271
Sputum Eosinophil %	1.34 (0.6, 2.8)	0.66 (0.4, 2.2)	0.520	0.53 (0.3, 0.8)	0.42 (0.2, 1)	0.417

Data depicted as Median (IQR) unless otherwise specified.

Bold values indicate $P < 0.05$.

FEV₁, forced expired volume in one-second; FVC, forced vital capacity; DLco % pred, diffusing capacity for carbon monoxide, corrected for hemoglobin, and carboxyhemoglobin as a percentage of the predicted value.

Fisher test were used for comparisons of patient groups as appropriate. Multivariate regression analysis was used to accommodate the effects across smoking status on asthma severity, lung function, and exacerbation. Analyses were carried out in R version 2.11.0 (17).

Results

Baseline characteristics

The never smokers with asthma and smokers with asthma were well matched for age, gender, duration of asthma, post-bronchodilator spirometry, inhaled dose of corticosteroid, and percent sputum neutrophil and eosinophil count (Table 1). Smokers with asthma had a lower DLco% predicted (87.5% vs 75.4%, $P < 0.001$).

Arachidonic acid metabolite measurements in urine, sputum, and plasma in asthma and healthy control groups according to smoking status

Urinary LTE₄ and PGDM concentrations were both significantly increased in smokers with asthma compared with never smokers with asthma ($P = 0.008$ and $P = 0.012$, respectively) and similar to healthy smokers (Table 2, Fig. 1). Sputum PGE₂ concentration was significantly lower in smokers with asthma compared with never smokers with asthma ($P = 0.001$) and similar to healthy smokers (Table 2, Fig. 2). Sputum LTB₄ concentration was significantly increased in smokers with asthma compared with healthy smokers ($P = 0.013$). Sputum LTE₄ and PGD₂ concentrations in

smokers with asthma were similar to never smokers with asthma. Plasma 11βPGF_{2α} concentration was increased in smokers with asthma compared with healthy smokers ($P = 0.032$) (Table 2, Fig. 3). Plasma sPLA₂ and 11βPGF_{2α} concentrations in smokers with asthma were similar to never smokers with asthma. When patients were stratified across smoking status, the concentrations of arachidonic acid metabolite measurements in urine, sputum, and plasma were equivalent between GINA categories. There was no association between arachidonic acid metabolites and % predicted FEV₁, but urine PGDM was associated with DLCO ($P = 0.029$). Sputum LTB₄ ($P < 0.001$) and LTE₄ ($P = 0.002$) in never smokers with asthma were associated with asthma exacerbation in the previous year.

Urinary LTE₄ concentration and plasma sPLA₂ concentration were increased in healthy smokers compared with healthy never smokers ($P < 0.001$ and $P = 0.036$, respectively) (Table 2, Figs 1 and 3, respectively). In smokers with asthma, sputum percent eosinophils were directly associated with urinary LTE₄ and PGDM [r-value and 95% bootstrap confidence interval; 0.328 (0.044, 0.586) and 0.289 (0.022, 0.530), respectively] and plasma 11βPGF_{2α} concentration [0.37 (0.04, 0.66)]. There was an association between sputum LTE₄ concentration and DLCO ($P = 0.035$). Sputum percent neutrophils were not associated with any arachidonic acid metabolite including sputum LTB₄ concentrations [0.28 (−0.07, 0.56)]. Urinary LTE₄ and PGDM concentration did not correlate with serum cotinine or pack-years. In never smokers with asthma, there was no association between any of the eicosanoid metabolites with FEV₁, DLCO or with sputum percent eosinophils. An increase in plasma sPLA₂ concentrations in

Table 2 Concentrations of urine LTE₄ and PGDM and, sputum, PGE₂, PGD₂, LTE₄, LTB₄, and plasma sPLA₂ and 11βPGF_{2α} in asthma and healthy control groups according to smoking status

	Asthma			Smoker vs healthy smoker <i>P</i> -value	Healthy control		
	Never smoker	Smoker	Never smoker vs smoker <i>P</i> -value		Never smoker	Smoker	Never smoker vs smoker <i>P</i> -value
Urine LTE ₄ (pg/mg creatinine)	59 (40, 96)	83 (59, 130)	0.008	0.571	52 (38, 63)	75 (56, 110)	<0.001
Urine PGDM (ng/mg creatinine)	41 (28, 59)	60 (35, 100)	0.012	0.055	30 (21, 55)	39 (32, 53)	0.334
Sputum PGE ₂ (pg/ml)	192 (91, 301)	80 (46, 157)	0.001	0.319	120 (94, 332)	111 (51, 191)	0.417
Sputum PGD ₂ (pg/ml)	215 (133, 295)	188.5 (123.5, 267)	0.476	0.785	220 (125, 312)	173 (136, 310)	0.773
Sputum LTE ₄ (pg/ml)	57 (29, 80)	66 (45, 107)	0.168	0.140	47 (22, 85)	53 (34, 71)	0.929
Sputum LTB ₄ (pg/ml)	409 (336, 577)	440 (336, 577)	0.764	0.013	352 (262, 412)	323 (264, 371)	0.422
Plasma sPLA ₂ (pg/ml)	224 (138, 275)	234 (147, 304)	0.637	0.516	101.75 (58, 235)	262 (205, 285)	0.036
Plasma 11βPGF _{2α} (pg/ml)	106 (56, 211)	120 (63, 245)	0.424	0.032	89 (49, 131)	61 (29, 97)	0.456

Data depicted as Median (IQR).

Bold values indicate $P < 0.05$.

Urine concentrations of leukotriene (LT) E₄ metabolites; tetranor prostaglandin D₂ (PGDM, 11,15-dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid); PG, prostaglandin; LT, leukotriene; sPLA₂, secreted phospholipases; 11βPGF_{2α}, 11β prostaglandin F_{2α}.

healthy smokers ($P = 0.036$) and in the combined asthma group ($P = 0.047$) compared with healthy never smokers was also evident.

Correlation between arachidonic acid metabolite measurements

In smokers with asthma, urinary LTE₄ concentration was directly associated with sputum PGE₂ [0.42 (0.11, 0.63)], but not sputum LTE₄. Sputum LTE₄ concentration was directly associated with sputum LTB₄ [0.56 (0.25, 0.78)]. In never smokers with asthma, urinary PGDM concentration was directly associated with plasma 11βPGF_{2α} [0.32 (0.03, 0.61)], sputum LTE₄ concentration was directly associated with sputum LTB₄ [0.41 (0.07, 0.65)], and plasma sPLA₂ concentration was directly associated with sputum PGD₂ [0.65 (0.40, 0.82)] and sputum PGE₂ [0.42 (0.03, 0.71)].

Expression levels of mRNA for eicosanoid metabolic enzymes in airway leukocytes and respiratory epithelium (Table 3)

In both severe asthma groups, there was increased relative expression of arachidonate 15-lipoxygenase (ALOX15), ALOX15B, and gamma-glutamyltransferase-like activity 1 (GGT5). In smokers with severe asthma, there was increased relative expression of prostaglandin-endoperoxide synthase 1 [(PTGS1) (COX-1)] and decreased expression of prostaglandin D₂ synthase, hematopoietic (HPGDS). In healthy subjects, there was smoking-related increased relative expression of ALOX15, ALOX15B, and gamma-glutamyltransferase 1 (GGT1).

Potential effect of drug therapy on arachidonic acid metabolite concentrations

Inhaled corticosteroids

No significant associations were found between the doses of inhaled corticosteroids with the arachidonic acid metabolite concentrations in urine, plasma, or sputum fluid or on expression levels (data not shown).

Aspirin

Within the whole study group, 18 subjects were taking regular aspirin. Using chi-squared analysis, there were more subjects taking aspirin in the asthma ($n = 12$) categories than the healthy subjects ($n = 6$), ($P < 0.001$), but there was no significant difference according to smoking category ($P = 0.318$). The only effect was a significantly reduced plasma 11βPGF_{2α} among those taking aspirin compared with those who were not (medians; 54 vs 123 pg/ml, $P < 0.001$).

Other nonsteroidal anti-inflammatory drugs (NSAIDs)

There were 20 subjects taking NSAIDs drugs (healthy $n = 2$, asthma $n = 18$). Using chi-squared analysis, there was no significant difference between the use of NSAIDs and disease category ($P = 0.06$) or smoking category ($P = 0.628$).

Summary of all results

To summarize the data, the relative differences of lipoxygenase and cyclooxygenase pathway metabolite concentrations

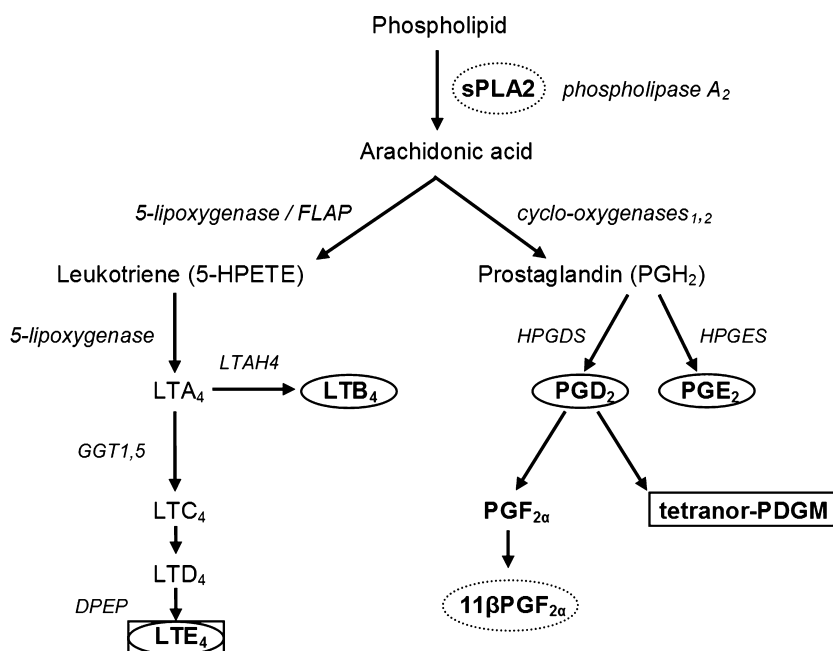


Figure 1 A summary pathway map illustrating the principal prostaglandin and leukotriene products of arachidonic acid metabolism. The compounds in bold font were measured in this study; those highlighted in solid-line ovals were quantified in sputum fluid, those highlighted in broken-line ovals were quantified in serum, and those highlighted in rectangles were quantified in urine. The relevant metabolic enzymes are indicated in italic font. Arachidonic acid is a polyunsaturated fatty acid present in the phospholipids of cell membranes. It is freed by phospholipase A_2 and becomes available for metabolism by cyclooxygenases (COX-1 and COX-2; prostaglandin G/H synthases; PTGS1 and PTGS2) to generate the prostaglandins, or by lipoxygenase (5-lipoxygenase and 5-lipoxygenase accessory protein; FLAP) to generate arachidonic acid

5-hydroperoxide (5-hydroperoxyeicosatetraenoic acid; 5-HPETE) which is an intermediate in the production of leukotriene A_4 . LTA_4 is converted to LTB_4 by leukotriene A_4 hydrolase (LTAH4) or converted by gamma-glutamyl transferase (GGT1 and GGT5) to LTC_4 . This is converted to LTD_4 by removal of glutamic acid, and LTD_4 is converted to LTE_4 by dipeptidase (DPEP). Prostaglandin metabolism was monitored by production of PGD_2 and PGE_2 which are produced from PGH_2 by the action of prostaglandin D synthase (HPGDS) and prostaglandin E synthase (HPGES), respectively. The primary metabolite of PGD_2 in plasma is 11β PGF $_{2\alpha}$, and in urine is tetranor-PDGM (metabolite (PGDM; 9 α -hydroxy-11,15-dioxo-13,14-dihydro-2,3,4,5-tetranor-prostan-1,20-dioic acid)).

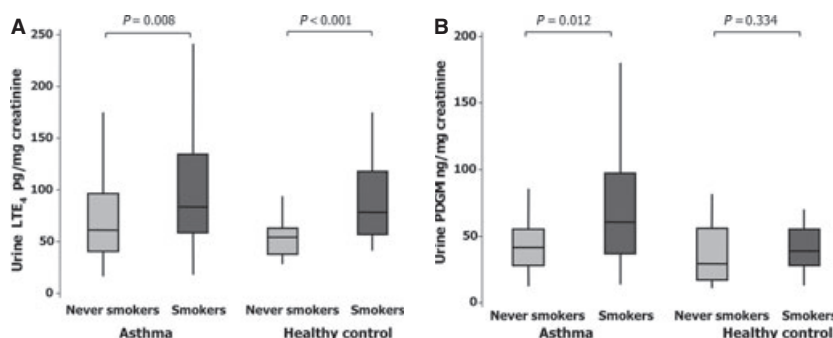


Figure 2 Urinary LTE_4 concentration (pg/mg creatinine) (A) and urinary PGDM concentration (ng/mg creatinine) (B) in asthma and healthy controls; never smokers and smokers (box, median, IQR; whiskers, range). Urinary LTE_4 and PGDM concentrations were both significantly increased in smokers with asthma compared with

never smokers with asthma ($P = 0.008$ and $P = 0.012$, respectively) and similar to healthy smokers. Urinary LTE_4 concentrations were increased in healthy smokers compared with healthy non-smokers ($P < 0.001$).

between the clinical groups were expressed as a simple fold-change compared with healthy never-smoker control levels (Table 4). The urinary PGDM was increased in smokers with

asthma, but not healthy smokers, whereas the urinary LTE_4 was also increased in patients with asthma who smoked as well as healthy smokers. The plasma 11β PGF $_{2\alpha}$ was

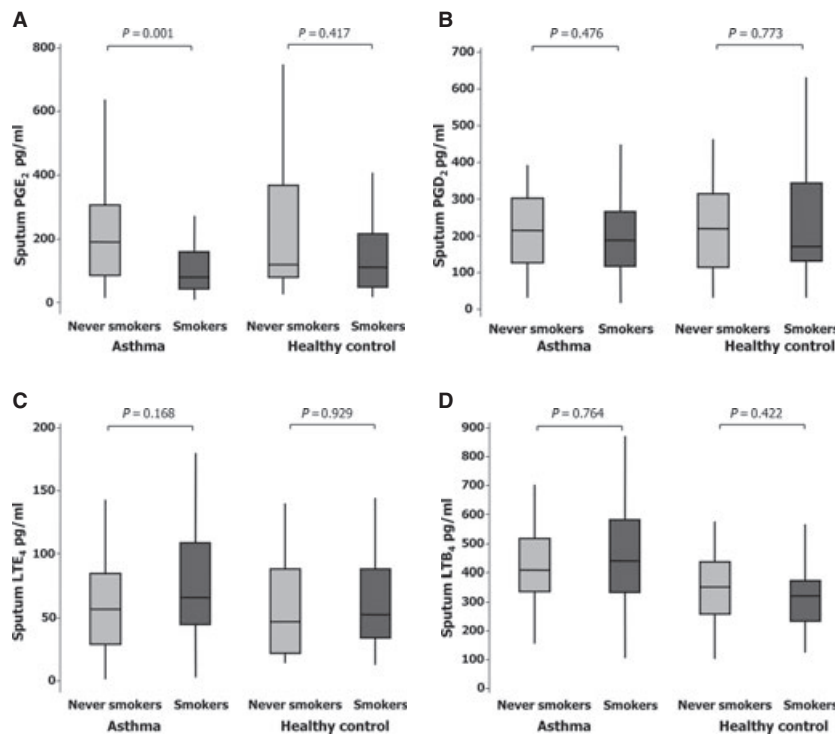


Figure 3 Sputum supernatant concentration (pg/ml) of PGE₂ (A), PGD₂ (B), LTE₄ (C), LTB₄ (D) in asthma and healthy controls; never smokers and smokers (box, median, IQR; whiskers, range). Sputum PGE₂ concentration was significantly lower in smokers with asthma compared with never smokers with asthma ($P = 0.001$)

and similar to healthy smokers. Sputum LTB₄ concentration was significantly increased in smokers with asthma compared with healthy smokers ($P = 0.013$). Sputum LTE₄ and PGD₂ concentrations in smokers with asthma were similar to never smokers with asthma.

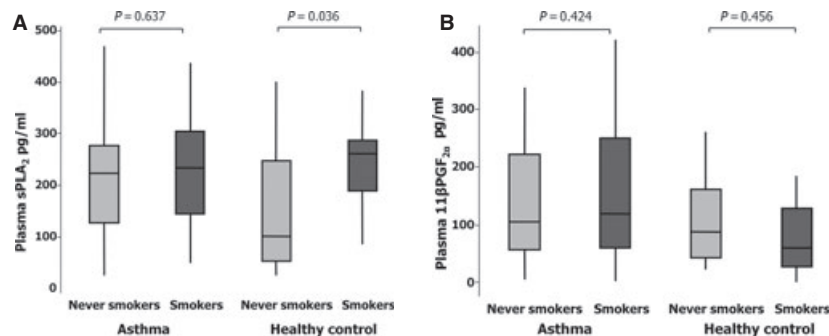


Figure 4 Plasma concentration (pg/ml) of sPLA₂ (A) and 11βPGF_{2α} (B) in patients with asthma and healthy controls; never smokers and smokers (box, median, IQR; whiskers, range). Plasma

11βPGF_{2α} concentration was increased in smokers with asthma compared with healthy smokers ($P = 0.032$).

increased in patients with asthma who smoked. Sputum LTE₄ was higher in patients with asthma irrespective of smoking. Sputum LTB₄ was higher in asthma never-smokers. Sputum PGE₂ was reduced in smokers with asthma.

To summarize the transcript data, most information content was associated with ALOX15, ALOX15B, GGT5, and PTGS1 (COX-1) in leukocytes from induced sputum. Expression levels of ALOX15 and GGT5, and of ALOX15B and COX-1 in the patients with asthma correlated ($P = 0.007$ and

$P = 0.001$, respectively), but not in healthy subjects ($P = 0.232$ and $P = 0.011$, respectively), suggesting coordinated increased expression of lipoxygenase and cyclooxygenase pathways in asthma.

An integration of biomarker and transcript data in the whole study group showed a positive correlation between ALOX15 and urinary LTE₄ ($r = 0.348$, $P = 0.008$), predominantly among asthmatic never-smokers ($r = 0.696$, $P = 0.004$), suggesting coordinated activation of

Table 3 Messenger RNA changes in representative genes involved in the leukotriene and prostaglandin pathways expressed as fold-change compared with healthy never-smoker control subjects. Levels greater than 1.5 were considered to be significantly increased and levels less than 0.5 significantly decreased

Representative probe	Induced sputum leukocytes			Nasal respiratory epithelium		
	Healthy smokers	Asthma never-smokers	Asthma smokers	Healthy smokers	Asthma never-smokers	Asthma smokers
Leukotriene: metabolic enzymes						
ALOX5	1.2	0.9	1.2	0.7	1.5	0.8
ALOX5AP	1.2	0.9	1.3	0.7	1.7	0.8
ALOX15	1.6	7.1	2.7	1.0	1.0	0.9
ALOX15B	1.9	1.8	2.9	1.9	2.0	2.3
LTA4H	1.1	0.7	1.1	1.0	1.1	1.0
GGT1	1.6	1.0	1.5	1.1	1.7	1.5
GGT5	1.2	4.1	3.9	0.6	2.7	1.0
DPEP1	1.4	1.1	1.1	1.5	1.5	1.4
DPEP2	0.8	0.8	0.8	0.6	1.4	0.7
DPEP3	0.9	1.4	1.1	0.9	1.4	1.0
Prostaglandin: metabolic enzymes						
PTGS1 (COX-1)	1.4	1.1	1.6	0.8	1.2	0.9
PTGS2 (COX-2)	1.0	0.9	1.1	1.0	0.8	0.9
HPGDS	0.7	0.7	0.4	0.7	1.8	0.9
PTGDS	0.9	1.2	0.8	0.6	1.5	0.7
PTGIS	0.9	0.6	0.7	1.1	1.0	1.1

Levels greater than 1.5 were considered to be significantly increased and levels less than 0.5 significantly decreased (bold values).

ALOX5, arachidonate 5-lipoxygenase (representative Affymetrix gene probe number 214366_s_at); ALOX5AP, arachidonate 5-lipoxygenase-activating protein (204174_at); ALOX15, arachidonate 15-lipoxygenase (207328_at); ALOX15B, arachidonate 15-lipoxygenase, second type (206714_at); LTA4H, leukotriene A4 hydrolase (208771_s_at); GGT1, gamma-glutamyltransferase 1 (211417_x_at); GGT5, gamma-glutamyltransferase-like activity 1 (205582_s_at); DPEP1, dipeptidase 1 (renal) (205983_at); DPEP2, dipeptidase 2 (219452_at); DPEP3, dipeptidase 3 (220179_at); PTGS1, prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase; COX-1) (205128_x_at); PTGS2, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase; COX-2) (204748_at); HPGDS, prostaglandin D2 synthase, hematopoietic (206726_at); PTGDS, prostaglandin D2 synthase 21 kDa (brain) (211748_x_at); PTGIS, prostaglandin I2 (prostacyclin) synthase (211892_s_at).

Table 4 Summary of relative concentrations of arachidonic acid metabolites in urine, plasma, and sputum fluid expressed as fold-change compared with healthy never-smoker control levels

	Healthy smokers	Asthma never-smokers	Asthma smokers
uPDGM	1.3	0.8	1.9
uLTE ₄	2.0	1.5	1.7
pSPLA2	1.7	1.5	1.5
p11βPGF _{2α}	1.5	0.9	4.9
sLTB ₄	0.9	1.6	1.4
sLTE ₄	1.0	2.0	1.6
sPGD ₂	0.8	0.9	0.7
sPGE ₂	0.6	1.2	0.3

Levels greater than 1.5 were considered to be significantly increased and levels less than 0.5 significantly decreased (bold values).

the lipoxygenase 5 and 15 pathways. The expression level of GGT5 was increased in asthmatic never-smokers, but this did not correlate with LTE₄ in sputum or urine in any of the clinical or smoking categories.

Discussion

Cigarette smoking is known to have adverse effects on asthma control and airway inflammation in asthma (1, 3, 4). In the present study, we sought to determine the influence of cigarette smoking on the concentrations of arachidonic acid metabolites in urine, sputum, and blood, and mRNA expression levels of metabolic enzymes in airway cells in asthma and healthy subjects. The study demonstrates for the first time that a range of arachidonic acid metabolites and enzyme transcripts involving both lipoxygenase and cyclooxygenase pathways differ in smokers with asthma compared with never smokers with asthma. These findings are important because cigarette smoking is common in asthma and there is a lack of understanding of mechanisms of diseases and best treatment options for this group of patients.

The measurement of urinary LTE₄ concentration has been widely used as an indirect index of leukotriene production in adults with chronic asthma (18–20). We found that urinary LTE₄ concentrations are increased in smokers with asthma and healthy smokers compared with never-smoking controls, confirming previous findings in smokers with asthma (7) and in healthy smokers (6). In contrast, sputum LTE₄ levels in

smokers with asthma were similar to never smokers with asthma. The reason for the apparent discrepancy between increased urinary and normal sputum LTE_4 results is unclear, but could be explained by instability of LTE_4 in sputum or because we measured sputum LTE_4 alone rather than cysteinyl leukotrienes (LTC_4 , LTD_4 and LTE_4), because exhaled breath condensate LTD_4 , but not LTE_4 , concentrations are increased in smokers with asthma compared with never smokers with asthma (9). Alternatively increased urinary LTE_4 concentrations in smokers could reflect leukotriene production from extra-pulmonary sites, although the finding of increased arachidonate 15-lipoxygenase (ALOX15) expression in sputum leukocytes and nasal respiratory epithelium in smokers, particularly in those with asthma, suggests the respiratory tract does contribute to increased LTE_4 synthesis in smokers with asthma. In support of our findings and this hypothesis, 15-lipoxygenase isoform activity is increased in bronchial biopsies from smokers with chronic bronchitis (21). ALOX15-derived lipid mediators are the most abundant eicosanoids in bronchoalveolar fluid before and after allergen challenge (22), and increased expression and activity of ALOX15 is associated with increased pro-inflammatory chemokine release and mucin gene upregulation contributing to the pathogenesis of asthma (23, 24). Although we found increased expression of this enzyme in smokers with asthma, the highest expression levels were found in never smokers with asthma.

Sputum LTB_4 concentrations were increased in smokers with asthma compared with healthy smokers, and concentrations were directly associated with sputum LTE_4 concentrations. Leukotriene B_4 is a potent chemotactic factor for neutrophils as well as also being implicated in causing eosinophil chemotaxis, mucus secretion, and airway hyper-reactivity (5). The lack of a significant association between sputum LTB_4 concentrations and the proportion of sputum neutrophils suggests that other chemotactic factors, for example IL-8 and IL-17, or pro-survival factors (25) are likely to contribute more to the complex mechanisms of sputum neutrophilia. Interestingly, increased sputum LTB_4 and LTE_4 concentrations were strongly associated with severe exacerbations in the last year, although only in never smokers with asthma.

In support of increased cyclooxygenase (COX) pathway activity in smokers with asthma, COX-1 mRNA expression was greater in this group, extending previous findings of increased immunoreactive COX-1 reported in nonsmokers with asthma (26). COX-2 (PTGS2) mRNA expression, however, was normal in our subjects' sputum leukocytes and nasal respiratory epithelium, whereas others have reported increased immuno-reactive COX-2 in sputum and bronchial biopsy cells from patients with asthma, possibly because these patients were inhaled steroid naïve at the time of testing (26, 27). PGE_2 is synthesized via the COX pathway and is released from epithelial cells and alveolar macrophages. In asthma, PGE_2 is thought to act as an anti-inflammatory mediator (20), and its biological effects include airway smooth muscle relaxation as well as the inhibition of cholinergic nerve contraction of human airways and the release of mediators from eosinophils, mast cells and neutrophils.

Interestingly, the concentrations of sputum PGE_2 were lower in smokers with asthma, which could reduce the anti-inflammatory effects of PGE_2 in this group. PGD_2 is a lipid mediator synthesized via COX pathways and released mainly by mast cells, but also by other inflammatory cells including eosinophils (28). PGD_2 is rapidly metabolized to $9\alpha,11\beta\text{-PGF}_{2\alpha}$, $2,3\text{-dinor-}9\alpha,11\beta\text{-PGF}_{2\alpha}$, and tetranor-PGDM, which are all excreted in urine. Tetranor-PGDM is the most abundant PGD_2 metabolite in urine (29) and as such has an advantage over other urinary biomarkers of PGD_2 synthesis. In this study, using a sensitive, reproducible, and accurate assay to measure urinary tetranor-PGDM (30), we found increased concentrations in smokers with asthma compared with never smokers with asthma. The concentrations of plasma $9\alpha,11\beta\text{-PGF}_{2\alpha}$, a metabolite of PGD_2 (19), were also significantly higher in smokers with asthma compared with healthy smokers.

It is likely that a range of different inflammatory airway cells contribute to altered arachidonic acid metabolite synthesis in smokers with asthma. Sputum eosinophils were associated with increased urinary PGDM, urinary LTE_4 , and plasma $11\beta\text{-PGF}_{2\alpha}$ concentrations in smokers with asthma, suggesting that eosinophils may contribute to the increased production of arachidonic acid metabolites, although the total number of airway eosinophils is usually suppressed in these patients (1, 3, 4). An association between sputum eosinophil count and urinary LTE_4 has been reported previously (7). Airway mast cell numbers are increased in smokers with asthma and are another potential source for arachidonic acid metabolite production (4).

We recognize that there were limitations in the study, such as the lack of bronchial biopsy data to confirm the findings from the other biological samples. The study design was cross-sectional, and further prospective studies are indicated to help define the stability of biomarkers of arachidonic acid metabolism in smokers with asthma and their relationship to clinical outcomes. It is possible that inhaled corticosteroids and/or nonsteroidal anti-inflammatory drug treatment might alter arachidonic acid metabolite concentrations, although we found no global effects on these measurements. The analysis of the data was exploratory involving multiple statistical testing, and as such the results will require to be confirmed by further studies and may provide pilot data for appropriate future power analysis. In addition, the study may have been underpowered for some measurements, for example the transcript data were based on group sizes of 15 patients.

Therapies that target specific lipoxygenase and cyclooxygenase pathways such as leukotriene receptor antagonists, 5-lipoxygenase-activating protein (FLAP) inhibitors that inhibit the production of the cysteinyl leukotrienes and prevent the formation of LTB_4 , or antagonists of the chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH₂) receptor that inhibit the pro-inflammatory effects of PGD_2 maybe too specific in their site of action to produce maximal clinical benefit in certain phenotypes of asthma such as smokers. We speculate that targeting both pathways together may optimize therapeutic responses. Future prospective studies are required to investigate this hypothesis.

In conclusion, the results of this study demonstrate that several arachidonic acid metabolites and enzyme transcripts involving both lipoxygenase and cyclooxygenase pathways differ in smokers with asthma compared with never smokers with asthma. Targeting specific lipoxygenase and cyclooxygenase pathways that are activated by asthma and by cigarette smoking may optimize therapeutic responses.

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Author contributions

Neil C. Thomson and Rekha Chaudhuri conceived the study, participated in its design, contributed to interpretation of the data, drafted the article for important intellectual

property, and gave final approval of the version to be published. Mark Spears participated in its design, contributed to interpretation of the data, drafted the article for important intellectual property, and gave final approval of the version to be published. C. Martina Messow and Scott Jelinsky performed the statistical analysis, contributed to interpretation of the data, drafted the article for important intellectual property, and gave final approval of the version to be published. Gino Miele, Karl Nocka, Eddie Takahashi, Omar J. Hilmi, and Malcolm C. Shepherd contributed to interpretation of the data, drafted the article for important intellectual property, and gave final approval of the version to be published. Douglas K. Miller conceived the study, participated in its design, contributed to interpretation of the data, and gave final approval of the version to be published. Charles McSharry participated in its design, contributed to interpretation of the data, drafted the article for important intellectual property, and gave final approval of the version to be published.

Conflicts of interest

Gino Miele and E. Takahashi were employees of Wyeth Pharmaceuticals at the time of the study and are currently employed by Epistem Ltd and PDP, respectively. Karl Nocka and Scott Jelinsky are employees of Pfizer and own stock in the company. There is no financial gain to the company expected through publication of this article. Douglas Millar, at the time when this work was performed, was an employee and stockholder of Pfizer, who gave financial support for the study. Neil Thomson, Rekha Chaudhuri, Mark Spears, Martina Messow, Malcolm Shepherd, Omar Hilmi, and Charles McSharry have no conflict of interest to declare regarding the article.

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