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Abstract

Asthma is an oxidative stress-related disease but associations with asthma outcomes are poorly studied in adults.

We aimed to study the associations between several biomarkers related to oxidative stress and various asthma outcomes.

Cross-sectional analyses were conducted in 1388 adults (43 years old, 44% with asthma) from the Epidemiological Study on the Genetics and Environment of Asthma (EGEA2). Three blood antioxidant enzyme activities (biomarkers of response to oxidative stress) and exhaled breath condensate 8-isoprostanes and plasma Fluorescent Oxidation Products (FlOPs) levels (two biomarkers of damage) were measured. Associations between biomarkers and 1) ever asthma, and 2) among participants with asthma, asthma attacks, asthma control, and lung function, were evaluated by regression models adjusted for age, sex and smoking.

Biomarkers of response were unrelated to asthma outcomes. Higher 8-isoprostanes level was significantly associated with ever asthma (odds ratio for one interquartile range increase [95% CI] 1.28 [1.06-1.67]). Among participants with asthma, 8-isoprostanes level was negatively associated with adult-onset asthma (0.63 [0.41-0.97]) and FlOPs level was positively associated with asthma attacks (1.33 [1.07-1.65]), poor asthma control (1.30 [1.02-1.66]), and poor lung function (1.34 [1.04-1.74]).

Results suggest 8-isoprostanes to be involved in childhood-onset asthma and FlOPs to be linked to asthma expression.

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Key words: asthma characteristics, adults, biomarkers, oxidative stress, epidemiology

Take home message: Oxidative stress-related damages seem to be involved in asthma expression and control.

Plain language summary:

Asthma is an oxidative stress-related disease but few studies are available on the associations with asthma characteristics, particularly in adults. The aim of this study is to assess the associations between biomarkers of response or of damage related to oxidative stress and various asthma outcomes.

Results showed that biomarkers of response were not associated with any asthma outcomes. 8-isoprostanes level was higher in adults with ever asthma, while Fluorescent oxidation products (FlOPs) level, a global marker of oxidation process, was higher in adults with poor asthma outcomes and poor lung function.

Our study suggests 8-isoprostanes and FlOPs as biomarkers of interest for studying oxidative stress-related damage in chronic diseases such as asthma.

Introduction

Asthma is a chronic airway inflammatory disease affecting about 350 million people worldwide [1]. This heterogeneous disease [2, 3] is now studied at cellular and molecular levels, offering new opportunities for its prevention and control [4, 5]. Oxidative stress, which reflects an imbalance between increased exposure to reactive oxygen species (ROS) and antioxidant defense, is involved in the pathophysiological mechanism of asthma [6]. In asthma, ROS are produced endogenously by metabolic reactions [7, 8] and exogenously by environmental factors (e.g., air pollutants, smoking) [8, 9].

To neutralize overproduction of ROS, the organism develops antioxidant defense through an enzymatic system including superoxide dismutase (SOD) coupled to catalase and glutathione peroxidase (GPX) [10, 11]. These antioxidant enzymes, which are the first line of defense against the ROS, are biomarkers of interest in the response to oxidative stress [8]. In addition, ROS interact with lipids of cells membranes, especially arachidonic acid, releasing 8-isoprostanes, the end-products of lipid peroxidation [12]. However, few studies are available on the associations between biomarkers of response (SOD, GPX and catalase) [13–16] or biomarkers of damage (8-isoprostanes) [6, 12] and asthma characteristics particularly in adults, and their results are discordant.

Fluorescent oxidation products (FlOPs) level, a global biomarker of oxidation processes including protein and DNA oxidation and lipid peroxidation [17], is of growing interest in epidemiology. This biomarker of damage was found more recently to be associated with chronic diseases [18] such as coronary heart disease [19] and chronic kidney disease [17]. Nevertheless, to our knowledge, no studies have been conducted to evaluate the association between FlOPs and asthma.

Taking advantage of the extensive biological and phenotypic characterization of more than 1000 adults in the EGEA study, we aimed to investigate the associations between biomarkers of response and damage related to oxidative stress, measured from different biological compartments, and asthma outcomes.

Methods

Population and study design

The Epidemiological study on the Genetics and Environment of Asthma (EGEA) is a French cohort study with three surveys over 20 years. The first EGEA survey (EGEA1) included cases with asthma, recruited in five chest clinics, their first-degree relatives and population-based controls, recruited in the early 90's in 5 French cities (total: n=2047). A follow-up of the participants has been completed in 2003-2007 (EGEA2), including 1601 subjects with complete examination, almost exclusively adults. At each survey, all subjects responded to a questionnaire based on international standardized tools to diagnose asthma and to determine respiratory and allergic symptoms, treatments, and environmental exposures. The protocol and descriptive characteristics have been described previously [20, 21]. EGEA collection is certified ISO 9001 and referenced in the Biobank network [22].

The present analyses used data collected at EGEA2. Only adult participants ($\geq$ 16 years old) with available data on measurement of any biomarker and smoking status were included (n=1388, supplementary Figure E1). Among adults, participants not included in the analyses (n=183) were similar to those included regarding age, sex, smoking status, body mass index (BMI) and asthma characteristics (supplementary Table E1).

Asthma outcomes

Asthma cases were participants who had positive responses to four questions from the validated and standardized British Medical Research Council, European Coal and Steel Community, American Thoracic Society (ATS) and European Community Respiratory Health Survey questionnaires: *"Have you ever had attacks of breathlessness at rest with wheezing?"*, *"Have you ever had asthma attacks?"*, *"Was this diagnosis confirmed by a physician?"* and *"Have you had an asthma attack in the last 12 months?"*, or a positive response to at least two questions and a positive review of medical records. Asthma in first-degree relatives of cases was defined as a positive answer to at least one of the first two questions [21, 23]. Among the 614 participants with ever asthma included in the present analysis, 536 (87.3%) had a diagnosis of asthma confirmed by a physician. Among participants with ever asthma, **current asthma** was defined by the report of respiratory symptoms (wheeze, nocturnal chest tightness, attacks of breathlessness following strenuous activity, at rest or at nighttime) or asthma attacks or use of inhaled and/or oral medicines because of breathing problems in the past twelve months [24]. **Asthma control** has been assessed over a 3 month period as previously [25], as closely as possible from the Global Initiative for Asthma 2015 definition, based on frequency of daytime/nighttime symptoms, use of reliever medication and activity limitations. **Asthma exacerbations** were defined by hospital or emergency admissions because of respiratory problems or use of oral steroids for breathing difficulties in the past twelve months [26].

More details on the definition of asthma outcomes, lung function, medication use, allergic and inflammatory characteristics are provided in online supplementary materials.

Biomarkers related to oxidative stress

Biomarkers of response

Erythrocyte antioxidant enzyme activities were measured as previously [27, 28] according to standardized procedures. The enzymatic activity was expressed in U/g of hemoglobin (Hb) for SOD and GPX and in k/g Hb for catalase (1k corresponds to the rate constant of the first order reaction). All samples were analysed in duplicate or triplicate at the Laboratory of Biochemistry Molecular Biology of CHRU de Lille according to validated and standardized procedures. The coefficient of variation was <10% for each enzymatic assay.

Biomarkers of damage

Exhaled breath condensate (EBC) was collected with an RTubeTM according a standardized method as previously described [22]. Briefly, the RTube (TM) was rinsed with deionized water and dried thoroughly. Participants breathed orally at tidal volumes into a mouthpiece attached to a cold condenser (-20°C). They were seated comfortably with a headrest. All headrests and back seats were tilted slightly to avoid any saliva contamination during breathing maneuvers. Breathing was quiet and regular. After 15 minutes, EBC collection was immediately separated in aliquots and stored at -80°C according to standardized procedures.

8-isoprostanes level was measured in EBC with a specific enzyme immunoassay (EIA) kit (8-isoprostanes EIA kit, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. 50μL of unextracted EBC was assayed in duplicate and the 8-isoprostanes level was calculated from a calibration curve obtained from the eight calibration points (0.8-2.0-5.1-12.8-32-80-200-500 pg/mL where 0.8 pg/ml is the lower point). The lower limit of detection for 8-isoprostanes was 4.0 pg/mL and the intra-assay coefficient of variation was less than 20%.

Plasma FlOPs level was measured as previously described [18, 29]. Briefly, plasma was extracted into a mixture of ethanol/ether (3/1, v/v) and measured using a spectrofluorimeter (360nm excitation wavelength, 430nm emission wavelength). Fluorescence was expressed as a unit of relative fluorescence intensity per milliliter of plasma (RFU/mL).

Inter-plate variability, storage time and diurnal variation did not affect any of the biomarkers levels (data not shown). More details on blood and EBC collection as well as measurement of biomarkers are provided in online supplementary data.

Statistical analyses

SOD, GPX, FlOPs and 8-isoprostanes were log-transformed due to their skewed distribution and expressed as geometric mean (GM) and Q1-Q3.

Associations between each biomarker and age, sex, BMI and smoking status were studied in participants without asthma, in order to evaluate these associations independently of the disease.

We used regression models to investigate the associations between each biomarker and asthma ever in all participants; and between each biomarker and asthma characteristics, pulmonary function, allergic and inflammatory characteristics in participants with ever asthma. "Partly controlled" and "uncontrolled" asthma were regrouped in one class, due to the small number of asthmatics in the uncontrolled group.

Regression analyses were conducted using generalized estimated equations (GEEs) to take into account familial dependence between individuals. Multiple regression models considered age (continuous), sex and smoking status (never-, ex- or current smokers) as potential confounders. A

sensitivity analysis on the association between FlOPs level and asthma outcomes was performed with adjustment for heavy smoking expressed as number of cigarette packs per year or daily tobacco consumption (g/day) instead of smoking status.

To facilitate result interpretations, we rescaled the biomarker levels using interquartile range (IQR), defined as the distance between the 25th and 75th percentiles and compared participants with a typical "high" level of biomarker to participants with a typical "low" level.

Statistical analyses were performed using SAS statistical software (version 9.3; SAS Institute, Inc., Cary, NC, USA). A p-value of less than 0.05 was considered statistically significant.

Results

Characteristics of the participants

Table 1 shows the characteristics of the 1388 participants included in the analyses. Among them, 614 had ever asthma. Their mean age was 43 years, 51% were women and 23% were smokers. Participants with ever asthma were more often men (p=0.01) and younger than participants without asthma (p <0.0001). They also had higher rate of allergic sensitization, lower FEV_1 and higher bronchial hyperresponsiveness than participants without asthma (all p <0.0001).

Among participants with ever asthma, 89% had current asthma, 32% had partly controlled asthma and 11% had uncontrolled asthma, 34% had an age of asthma onset ≥16 years; in the past 12 months, 66% reported use of any asthma treatments (inhaled or oral medicines), 40% reported use of inhaled corticosteroids (ICS), 38% had asthma attacks, 14% reported having had asthma exacerbations and 78% reported having had respiratory symptoms.

In all participants, the geometric means [Q1; Q3] of biomarkers of oxidative stress were: 1218 [1036; 1449] U/g Hb for SOD, 39.1 [34.2; 45.3] U/g Hb for GPX; mean (SD) of catalase was 163 (40.4) k/g Hb. The geometric means of 8-isoprostanes were 2.88 [1.31; 6.66] pg/mL and 93.3 [79.9; 106] RFU/mL for FlOPs.

Description of biomarkers of oxidative stress in participants without asthma

Among biomarkers of response, catalase activity was higher among those with higher BMI (p_{trend}=0.02) and was higher in current and ex-smokers than in non-smokers (p_{trend}=0.004). However, no significant association was observed between catalase and heavy smoking. GPX activity was positively associated with age (p_{trend}=0.01) and was higher in women than in men ($p<0.0001$). SOD activity was negatively associated with age ($p_{trend}<0.0001$) and positively associated with BMI (p_{trend}=0.003) (supplementary table E2).

Among biomarkers of damage, 8-isoprostanes level was negatively associated with age (p_{trend}=0.01), and was higher in women than in men (p=0.04). FlOPs level was positively associated with age ($p_{trend}<0.0001$) and was higher in current and ex-smokers than in non-smokers ($p_{trend}<0.0001$) (supplementary table E3). Furthermore, in current and ex-smokers, FlOPs level was positively associated with the number of packs of cigarettes per year (p_{trend}=0.03); and in current smokers, FlOPs level was positively associated with daily tobacco consumption (p_{trend}=0.001) (supplementary figure E2).

Associations between biomarkers and asthma status

The distribution of each biomarker according to ever asthma is shown in figure 1 and figure 2. There was no significant difference in SOD, GPX or catalase activities according to ever asthma. In contrast, 8-isoprostanes level was higher and FlOPs level was lower in participants with ever asthma compared to those without asthma. After adjustment for age, sex and smoking status, only the association between 8-isoprostanes level and ever asthma remained significant (table 2).

Associations between biomarkers and asthma outcomes among participants with ever asthma

Among participants with ever asthma, there was no significant association between SOD, GPX and catalase activities and asthma outcomes or lung function (supplementary table E4).

In contrast, biomarkers of damage were associated with several asthma outcomes (table 2). Lower level of 8-isoprostanes was significantly associated with adult-onset asthma compared to childhood-onset. No other significant associations were found.

Higher FlOPs level was significantly associated with adult-onset asthma, poor asthma control, asthma attacks, any asthma treatment and use of inhaled corticosteroids in the past 12 months. Furthermore, a significant positive association was observed between FlOPs level and poor lung function. Consistently, FlOPs level was negatively correlated with FEV_1 (r=-0.16, p=0.0001). All these associations remained significant after adjustment for age, sex and smoking status, except for the association between FlOPs and adult-onset asthma. No significant association was found between FlOPs level and respiratory symptoms (table 2). Results were unchanged when we adjusted for age, sex and heavy smoking expressed as number of pack-years or daily tobacco consumption (data not shown).

Among participants with ever asthma, no significant association was observed between any biomarkers and bronchial hyperresponsiveness.

Associations between biomarkers of damage, allergic sensitization and markers of inflammation

In participants with ever asthma, we also studied the association between biomarkers of damage and allergic sensitization markers (total IgE or SPT+), and did not observed any significant association (table 3).

In addition, we studied the association between biomarkers of damage and blood neutrophil and eosinophil counts (table 3). No significant associations were observed between any biomarkers and eosinophil counts. However, a positive significant association was observed between FlOPs level and high neutrophil counts ($\geq$5000 *vs.* <5000 cells/mm^3). Consistently, we observed a positive correlation between FlOPs and neutrophils count (r=0.12, p=0.005).

Discussion

The present study investigated the associations between biomarkers related to oxidative stress and various asthma outcomes. We found no significant association between any biomarkers of response and asthma outcomes. Regarding biomarkers of damage, a positive and significant association was observed between 8-isoprostanes level and ever asthma. Among participants with ever asthma, we showed for the first time a significant and positive association between 8-isoprostanes and childhood-onset asthma, while significant positive associations were shown between FlOPs level and poor asthma control, asthma attacks, any asthma treatments, poor lung function and neutrophilic asthma.

The main strength of our study was the investigation of the association between several biomarkers either involved in the response to oxidative stress or related to its damages, and various asthma characteristics. Indeed, while biomarkers of response are intracellular and reflect activities of antioxidant enzymes [8], they take part at the beginning of the "oxidative stress chain" [30, 31] in the continuum between environment and the disease. On the other hand, FlOPs are a global marker of oxidative stress, reflecting a mixture of oxidation products from DNA, proteins, and lipids [18], and 8-isoprostanes are a specific products of lipid peroxidation [32]. These two biomarkers of damage take part at the end of the "oxidative stress chain" and are more likely to be associated with asthma. Furthermore, we studied oxidative stress at the systemic level and close to the lung in exhaled breath condensate. Most of the participants with asthma were recruited in chest clinics as asthma cases, with a careful procedure set up to include true asthmatics using standardized and validated questionnaires. Others were recruited as first-degree relatives of asthmatic cases, based on answers to questions on asthma diagnosis. This leads to a

group of asthmatics with wide range of severity and response to methacholine. The detailed phenotypic characterization included various asthma outcomes, which have been rarely studied in relation to oxidative stress biomarkers. No follow-up bias related to the asthma status and asthma-related phenotypes was shown in the EGEA study, and the adult asthmatics included in the present study are representative of the original study population of asthmatic cases and their first degree relatives with asthma. Oxidant /antioxidant status, and thus biomarker levels, depend on the ability of each individual to counter oxidative reactions involving genetic factors, lifestyle and environmental factors. Although not all these parameters were taken into account in our analysis and unmeasured confounding can never be ruled out, we have adjusted for relevant potential confounders such as age, sex and smoking status, thought to be associated with the biomarkers we studied.

We did not find any significant association between erythrocyte SOD, GPX and catalase activities and asthma characteristics, in contrast with previous smaller studies (n ~ 30 to 150). In these studies, lower GPX level was consistently associated with asthma [13–15, 33]; however, for SOD and catalase, contrasted results with increased [13, 14, 33] or decreased [15, 34] level according to asthma status were reported. Beside differences in technical measurements, discrepancies between results may be partly explained by differences in asthma outcomes (asthma, severe asthma or incident asthma) or in body compartments (erythrocytes, plasma or serum). The relationship between antioxidant enzymes and asthma is likely complex, involving several endogenous and exogenous factors that can influence biomarkers level. In addition, it is difficult to determine cross-sectionally whether variation in biomarker levels would be the cause or the consequence of asthma. Our null results, along with discrepant findings in the literature,

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suggest that biomarkers of response may not be the most relevant to study oxidative stress in asthma. However, longitudinal studies may provide more insight regarding this relationship.

We found a significant positive association between EBC 8-isoprostanes and ever asthma consistently with previous studies including studies of children and adults reviewed in the paper by Aldakheel et al. [12]. Overall, results suggest that 8-isoprostanes may be a relevant biomarker for studying asthma. Our findings also confirm that EBC, which shows local production of free radicals [6], is an effective noninvasive method for measuring biomarkers related to oxidative stress.

We also investigate the association between 8-isoprostanes and asthma outcomes among participants with ever asthma. Interestingly, among participants with asthma, we observed that those with childhood-onset asthma had higher 8-isoprostanes level than those with adult-onset asthma. However, we did not observe any significant association between 8-isoprostanes and other asthma outcomes. Further, we confirm the lack of association between 8-isoprostanes and lung function or bronchial hyperresponsiveness, consistently with previous studies [35, 36]. We hypothesized that the longer a person has had asthma (for example since childhood), the greater the tissue damage in the lungs, and that high 8-isoprostanes level reflects long-term damages or the "background" of the disease rather than its expression. However, we did not observe a significant association between asthma duration and 8-isoprostanes level (data not shown). Another hypothesis is that adult-onset asthma and childhood asthma, which are two different asthma phenotypes [4, 37], may have partly distinct biological mechanisms.

We found a negative association between FlOPs level and ever asthma, although this association was no longer significant after adjustment. Among participants with ever asthma, higher FlOPs level was significantly associated with poor asthma control, poor lung function and neutrophilic asthma. Consistently, we observed a significant positive association between FlOPs level, asthma attacks, use of any asthma treatments and use of ICS in the last 12 months. However, no significant association was found with current asthma, adult-onset asthma or respiratory symptoms. To the best of our knowledge, no study on the relationship between FlOPs and asthma has been published before. Nevertheless, a significant and positive association between FlOPs and others chronic inflammatory diseases such as chronic kidney disease (CKD) [17] or coronary heart disease [19] has been reported. Our results did not change after removing participants with history of CKD or cardiovascular diseases (data not shown).

Interestingly, a significant and positive association was observed between FlOPs and neutrophilic asthma suggesting that FlOPs may be related to a non-allergic phenotype. Furthermore, in the literature as in our analyses, FlOPs were shown to be associated with irritant exposures, such as tobacco smoke ([18] and online supplementary data) or occupational exposure to irritant chemicals (e.g., cleaning products) [29], which have also been associated with non-allergic asthma phenotypes [37–40].

Based on these findings, FlOPs appear to be a good biomarker for measuring oxidative stress, a pathophysiological mechanism related to several chronic diseases. In our study, some participants were not fasting at the time of the blood collection and some potential confounders such as cholesterol were not taken into account. Although more studies are needed for the standardization

of FlOPs measurement, our findings suggest that this oxidation marker is linked to asthma expression.

Associations of 8-isoprostanes and FlOPs with asthma outcomes appeared discrepant in our study. Indeed, after adjustment, only 8-isoprostanes were associated with ever asthma and associations with asthma outcomes also differed for these two biomarkers among participants with ever asthma. The exact mechanisms explaining these different results are beyond the scope of our epidemiological study. As noted earlier, these biomarkers belong to different biological processes, and were measured in different compartments. 8-isoprostanes are the main biomarkers of the lipid peroxidation, measured in the exhaled breath condensate – likely reflecting the composition of the airway lining fluid [6] – and may therefore better represent airway process and reflect lung inflammation. FlOPs level is a biomarker of several oxidation processes, reflecting a mixture of oxidation products from DNA, proteins and lipids. This biomarker has been measured in blood – an easily available source of a large amount of antioxidant defenses in the body [13] – and can therefore better reflect oxidation at the systemic level.

In addition, none of the biomarkers of response to oxidative stress were correlated with biomarkers of damages, and no correlation was observed between 8-isoprostanes measured in EBC and FlOPs measured in blood (data not shown). Overall, all these results highlight the interest to study biomarkers in several compartments belonging to different biological processes and suggest 8-isoprostanes and FlOPs to be complementary to assess asthma.

Conclusion

In summary, our results suggest that EBC 8-isoprostanes seem to be involved in childhood-onset asthma and FlOPs seem to be linked to asthma expression and control in adults. Immediate clinical implications could not be inferred from this epidemiologic study. However, if replicated, our findings will suggest that FlOPs levels may help identifying asthmatics with higher asthma burden. Further research is needed, especially through longitudinal studies, to determine the potential interest of measuring FlOPs level in clinical practice.

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Figure legends

Figure 1: Levels of biomarkers of response related to oxidative stress according to ever asthma.

Boxplots show the median (bar), the first and third quartiles (box), the 1st and 99th percentiles (fences) and the minimum and maximum (stars) of the biomarkers level for each asthma status. The y-axis to the left represents the raw biomarker concentration and the y-axis to the right represents the log10 concentration. Catalase was not log-transformed.

SOD: Superoxide dismutase; GPX: Glutathione peroxidase; GM: Geometric mean; Q1-Q3: first and third quartiles; SD: standard deviation.

Figure 2: Levels of biomarkers of damage related to oxidative stress according to ever asthma.

Boxplots show the median (bar), the first and third quartiles (box), the 1st and 99th percentiles (fences) and the minimum and maximum (stars) of the biomarkers level for each asthma status. Except for the 8-isoprostanes where the fences represent the 10th and 90th percentiles. The y-axis to the left represents the raw biomarker concentration and the y-axis to the right represents the log10 concentration.

FlOPs: Fluorescent Oxidation Products; GM: Geometric mean; Q1-Q3: first and third quartiles; SD: standard deviation.

Table 1: Description of participant characteristics, according to ever asthma (n=1388).

	All participants	Without ever asthma	With ever asthma	
	n=1388	n=774	n=614	p[†]
Sex, women, %	51.1	54.4	47.1	0.01
Age, mean (SD)	43.1 (16.5)	46.2 (15.9)	39.1 (16.3)	<0.0001
Body Mass Index, kg/m^2, %	n=1365	n=761	n=604	
< 20	10.6	9.33	12.2	
[20-25[	51.6	50.5	53.0	0.09
[25-30[	27.8	30.1	24.8	
≥ 30	10.0	10.1	9.93	
Smoking status, %				
Non smoker	50.1	50.1	50.2	
Ex-smoker	27.4	29.2	25.1	0.10
Current smoker	22.5	20.7	24.8	
Age of asthma onset, %	n=573		n=573	
< 4 years	30.5		30.5	
[4-16[years	35.1	-	35.1	
≥ 16 years	34.4		34.4	
Current asthma, %	n=572		n=572	
	88.8	-	88.8	
Asthma control, %	n=537		n=537	
Controlled	57.9		57.9	
Partly controlled	31.5	-	31.5	
Uncontrolled	10.6		10.6	
Asthma attacks (last 12 months), %	n=611		n=611	
	38.1		38.1	
Exacerbation (last 12 months), %	n=573		n=573	
	13.6	-	13.6	
Respiratory symptoms (last 12 months), %	n=1377	n=768	n=609	
	54.6	36.2	77.8	<0.0001
Use of any asthma treatments[§] (last 12 months), %	n=1388	n=774	n=614	
	34.8	10.3	65.6	<0.0001
Use of ICS (last 12 months), %	n=1381	n=772	n=609	
	19.6	3.24	40.4	<0.0001
FEV1 % predicted, mean (SD)	n=1360	n=757	n=603	
	102 (18.0)	107 (16.5)	96.9 (18.4)	<0.0001
FEV1 < 80% predicted, %	n=1360	n=757	n=603	
	9.7	5.42	15.1	<0.0001
Methacholine challenge[#], PD20 ≤ 4mg, %	n=851	n=487	n=364	
	44.3	26.9	67.6	<0.0001
Skin prick test positivity[**], %	n=1281	n=716	n=565	
	56.3	38.5	78.8	<0.0001
Total IgE	n=1383	n=772	n=611	
≥ 100 IU/mL, %	43.0	29.5	60.1	<0.0001
Eosinophils count				
≥ 250 cell/mm^3, %	26.9	17.2	39.2	<0.0001
Neutrophils count				
≥ 5000 cells/mm^3, %	20.0	19.2	21.0	0.42

ICS: Inhaled corticosteroids only or in combination with long actin B2-agonist; FEV1: Forced expiratory volume in one second; IgE: Immunoglobulin E ; § Asthma treatments were defined by the use of inhaled and/or oral medicines in the past twelve months because of breathing problems.; ** Skin Prick Test positivity (SPT+) was defined by a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens; PD20: provocative dose causing a 20% fall in FEV_1; #Methacholine challenge test was not performed if baseline FEV1 <80% predicted; † p-value of χ2 test for categorical variables and Student test for quantitative variables.

Table 2: Associations between biomarkers of damage related to oxidative stress and various asthma outcomes.

	8-isoprostanes (pg/mL)			Fluorescent oxidation products (RFU/mL)		
	n	OR (95% CI)*	OR$_{adjusted}$ (95% CI)*	n	OR (95% CI)*	OR$_{adjusted}$ (95% CI)*
Asthma ever (vs never asthma)	688	1.40 (1.17; 1.67)	1.28 (1.06; 1.55)	1325	0.84 (0.73; 0.96)	0.98 (0.86; 1.13)
Among participants with ever asthma						
Adult-onset asthma† (vs childhood-onset)	294	0.51 (0.35; 0.76)	0.63 (0.41; 0.97)	548	1.55 (1.25; 1.93)	1.08 (0.85; 1.38)
Current asthma (vs without current asthma)	295	0.65 (0.38; 1.13)	0.69 (0.40; 1.18)	547	1.44 (0.97; 2.16)	1.38 (0.92; 2.06)
Poor asthma control (vs controlled asthma)**	282	0.82 (0.59; 1.15)	0.86 (0.61; 1.22)	514	1.40 (1.11; 1.77)	1.30 (1.02; 1.66)
Asthma attacks in the last 12 months	315	0.80 (0.58; 1.10)	0.80 (0.57; 1.12)	584	1.27 (1.04; 1.55)	1.33 (1.07; 1.65)
Exacerbation in the last 12 months	297	0.75 (0.47; 1.20)	0.79 (0.50; 1.25)	549	1.18 (0.90; 1.56)	1.11 (0.81; 1.52)
Respiratory symptoms in the last 12 months	311	0.73 (0.35; 1.10)	0.76 (0.39; 1.13)	582	1.17 (0.87; 1.46)	1.11 (0.81; 1.41)
Any asthma treatments in the last 12 months§	315	0.77 (0.45; 1.09)	0.80 (0.47; 1.13)	587	1.34 (1.12; 1.56)	1.29 (1.05; 1.52)
Use of ICS in last 12 months	313	0.72 (0.51; 1.00)	0.80 (0.57; 1.11)	582	1.45 (1.19; 1.77)	1.30 (1.05; 1.60)
FEV$_1$ < 80% predicted	314	0.85 (0.52; 1.39)	1.01 (0.64; 1.60)	579	1.63 (1.28; 2.07)	1.34 (1.04; 1.74)
Methacholine challenge PD20 ≤ 4mg#**	193	0.92 (0.54; 1.30)	0.84 (0.42; 1.26)	354	0.97 (0.68; 1.26)	0.97 (0.65; 1.28)

*OR expressed for an increase corresponding to the value of the interquartile range (distance between the 25th and 75th percentile) of each biological markers; adjusted for age, sex and smoking status; **Results were unchanged when restricting analyses to participants with current asthma; †adult-onset asthma is defined to an age of asthma onset ≥ 16 years old and childhood-onset was the reference group; §Asthma treatments were defined by the use of inhaled and/or oral medicines in the past twelve months because of breathing problems.; ICS: inhaled corticosteroids only or in combination with long actin B2-agonist; FEV$_1$: Forced expiratory volume in one second; PD20: provocative dose causing a 20% fall in FEV$_1$; #Methacholine challenge test was not performed if baseline FEV1 <80% predicted.

Table 3: Associations between biomarkers of damage related to oxidative stress, markers of allergy and markers of inflammation in participants with ever asthma.

	8-isoprostanes (pg/mL)			Fluorescent oxidation products (RFU/mL)		
	n	OR (95% CI)*	OR$_{adjusted}$ (95% CI)*	n	OR (95% CI)*	OR$_{adjusted}$ (95% CI)*
Markers of allergy						
Total IgE ≥100 IU/mL	314	0.98 (0.72; 1.34)	0.85 (0.61; 1.18)	584	0.99 (0.82; 1.21)	1.17 (0.93; 1.47)
Positive SPT	297	0.92 (0.62; 1.37)	0.70 (0.47; 1.05)	546	0.86 (0.68; 1.08)	1.19 (0.90; 1.57)
Markers of inflammation						
Neutrophils ** **≥5000 cells/mm^3**	315	0.88 (0.62; 1.26)	0.94 (0.65; 1.36)	587	1.38 (1.11; 1.72)	1.34 (1.06; 1.70)
Eosinophils ** **≥250 cells/mm^3**	315	0.87 (0.63; 1.19)	0.84 (0.60; 1.16)	587	0.94 (0.78; 1.13)	1.02 (0.84; 1.25)

* OR expressed for an increase corresponding to the value of the interquartile range (distance between the 25th and 75th percentile) of each biological markers; adjusted for age, sex and smoking status; **Results were unchanged when restricting analyses to participants with current asthma; SPT: skin prick test;   IgE: Immunoglobulin E.

ONLINE SUPPLEMENTARY MATERIALS

Oxidative stress biomarkers and asthma characteristics in adults of the EGEA study

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Methods

Asthma outcomes

Asthma control has been assessed over 3 month period, using responses to EGEA2 survey questions to approximate the Global Initiative for Asthma 2015 definition as closely as possible and as previously used [1]. Participants were defined as having controlled, partly controlled, and uncontrolled asthma if they had none, 1 to 2, or 3 to 4 of the following criteria, respectively: frequent daytime symptoms (defined by ≥1 asthma attacks or ≥1 episodes of trouble breathing per week in the past 3 months), any nighttime symptoms (defined as waking because of asthma or an attack of shortness of breath in the last 3 months), frequent use of reliever medication (defined, on average, as more than twice a week in the past 3 months), and any activity limitation (defined by the following answers: ''totally limited,'' ''extremely limited,'' ''very limited,'' ''moderate limitation,'' and ''some limitation'' to the question *''Overall, among all the activities that you have done during the last two weeks, how limited have you been by your asthma?''*).

Lung function, allergic and inflammatory characteristics

A lung function test with spirometry and methacholine challenge was performed using standardized protocol with similar equipment across centers according to the American Thoracic Society / European Respiratory Society guidelines [2]. Forced expiratory volume in one second (FEV_1) percent predicted value was based on Quanjer et *al*. reference equations [3]. For participants with a FEV1 ≥80% of the predicted value, a methacholine bronchial challenge test was performed (maximum dose 4mg) using a Biomedin spirometer (Biomedin

Srl, Padua, Italy) in all centers, except in Lyon, where a Pneumotach Jaeger spirometer (Jaeger) was used [4].

Allergic sensitization was defined by a positive skin prick test (SPT+) with a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens (indoor: cat, *Dermatophagoides pteronyssinus*, *Blattela germanica*, outdoor: olive, birch, *Parieteria judaica*, timothy grass, *Cupressu*s and ragweed pollen, and molds: Aspergillus, *Cladosporium herbarum*, *Alternaria tenuis*). Subjects were classified as sensitized if they have one or more SPT+ [5].

Total Immunoglobulin E (IgE) determination was assessed by UniCAP system (Pharmacia®) from blood samples in a centralized laboratory, and expressed in international units (IU) per milliliter.

Blood inflammatory patterns were defined from white blood cell (WBC) counts according to eosinophil (EOS) and neutrophil (NEU) counts cut-off points previously described[6, 7]. Briefly, the cut-off point for eosinophils (250 EOS/mm^3) is the one commonly used in epidemiology, and corresponded to the 75th percentile in the 1356 adults at the EGEA1 study. A cut-off point of 5000 NEU/mm^3 was chosen that corresponded to the 75th percentile of the distribution.

Determination of biomarkers of oxidative stress

Blood collection

Blood samples were collected into 5 mL Vacutainer tubes containing heparinate as an anticoagulant (Becton Dickinson, USA). On the same day, corresponding plasma and glycerolized red blood cells (RBCs) were prepared and stored at -80°C. Briefly, 38%

wt/vol glycerol was added progressively to an equal volume of RBCs to obtain a final concentration of 19% glycerol. Glycerolized RBCs aliquots were stored from 2 to 10 years until analysis. Immediately after thawing at room temperature, RBCs were diluted by addition of ice-cold distilled water (1:1); the resulting hemolysate was centrifuged at 3000×g for 10 min at +4°C to remove unbroken cells and large cell debris. Dialysis of the supernatant was performed on D-Tube96™DialyzerMini device (Novagen,Madison,WI,USA). Each kit contains a floating rack allowing dialysis up to 96 samples (10–250 μl) through a dual membrane with molecular weight cutoffs of 6–8 kDa or 12–14 kDa. The 6–8 kDa cutoff was chosen to remove glycerol. 170 μL of supernatant was carefully added to D-Tube96™ previously prehydrated for 10min with distilled water. The floating rack was placed in beaker containing 2 L of 0.1 Msodiumphosphate buffer (pH 7.4) and a stir bar [8].

Measurement of antioxidant enzyme activities

All assays were carried out at the Laboratory of Biochemistry Molecular Biology of CHRU de Lille according to validated and standardized procedures. Catalase activity was measured according to the method by Aebi [8, 9]. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were estimated using commercial available kits (Randox Laboratories, Mauguio, France) according to the method by Paglia and Valentine [8, 10] and McCord and Fridovich [8, 11] respectively.

Total hemoglobin (Hb) concentration was measured by the cyanmethemoglobin method [8, 12]. Activities were expressed as U/g Hb (SOD, GPx) or k/g Hb (catalase). All samples were

analyzed in duplicate or triplicate and analytical intra-run imprecisions (CV) were below 10%.

Results

Description of medication data

Regarding medication for asthma, 59.6% of the participants did not take any medication in the last twelve months. Among those who reported medication use in the last twelve months, 39.2% reported ICS use only and 60.8% reported ICS use in combination with long acting B2-agonists (LABA). In the last three months, 23.3% reported regular use of ICS (ICS or ICS+LABA), and 11.6% reported irregular use of ICS (ICS or ICS+LABA). Median [min-max] main daily ICS dose over the past twelve month was of 250 [50-500] μg of beclomethasone equivalent.

Associations between FlOPs level and ICS use

We performed supplementary analyses between medication expressed in three classes (no use, ICS only and ICS in combination with LABA) and FlOPs level, and found a positive and significant association: geometric mean [Q1; Q3] of FlOPs level was 88.9 [76.7; 100], 93.2 [81.5; 103] and 97.1 [79.7; 109] RFU/mL in no use, ICS only and ICS+LABA respectively, P for trend=0.01. In addition, we studied the association between FlOPs and ICS dose (μg) over the past twelve months and the ICS use in the last three months expressed as no use/ irregular use / regular use) and found that FlOPs level was positively associated with the ICS use

(geometric mean [Q1; Q3] of FlOPs level was 88.8 [76.5; 100], 90.9 [76.6; 103] and 100 [83.3; 110] RFU/mL in the class of no use, irregular use and regular use respectively (p<0.001)). We did not find a correlation between FlOPs level and ICS dose.

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Figure legends:

Figure E1: Flow chart of the population studied

Figure E2: Boxplots of plasma Fluorescent oxidation products level, in non-asthmatic participants according to smoking status, number of cigarette packs per year in current and ex-smokers and tobacco daily consumption in current smokers.

FlOPS: Fluorescent Oxidation Products; Boxplots show the median (bar), the first and third quartiles (box), the first and 99th percentiles and the minimum and maximum (fences) for each category. Except in non-smokers, ex-smokers, smokers and in the first category (<10 packs/year) of current and ex-smokers where the fences represent the 1st and 99th percentiles and the stars represent the minimum and maximum.

Table E1: Characteristics of the participants included or non-selected in the analyses.

	All participants n=1571		Participants included n=1388		Participants non selected n=183		
	n	Percent, mean (SD)	n	Percent, mean (SD)	n	Percent, mean (SD)	p†
Sex, women	1571	50.6	1388	51.2	183	46.5	0.23
Age, mean (SD)	1571	42.8 (16.5)	1388	43.0 (16.5)	183	40.7 (16.9)	0.08
Smoking status							
Non smoker	780	49.9	696	50.1	84	48.0	
Ex-smoker	422	27.0	380	27.4	42	24.0	0.24
Current smoker	361	23.1	312	22.5	49	28.0	
Body Mass Index, kg/m^2							
< 20	149	10.7	145	10.6	4	14.8	
[20-25[	717	51.5	704	51.6	13	48.2	0.11
[25-30[	383	27.5	379	27.8	3	14.8	
≥ 30	143	10.3	137	10.0	6	22.2	
Ever asthma	1571	43.5	1388	44.2	183	37.7	0.09
Current asthma	630	88.6	572	88.8	58	86.2	0.55
Asthma control							
Controlled	334	57.8	311	57.9	23	56.1	
Partly controlled	179	31.0	169	31.5	10	24.4	0.19
Uncontrolled	65	11.2	57	10.6	8	19.5	
Skin prick test positivity*	1303	56.2	1281	56.3	22	50.0	0.56
Total IgE, ≥ 100 IU/mL	1396	43.0	1383	43.0	13	46.1	0.82
FEV1 % predicted, mean (SD)	1387	102 (18.0)	1360	102 (18.1)	27	97.6 (15.1)	0.16
FEV1 < 80% predicted	1387	9.60	1360	9.70	27	3.70	0.29
Methacholine challenge**, PD20 ≤4mg, %	866	44.0	851	44.3	15	26.7	0.17

FEV1: Forced expiratory volume in one second; IgE: Immunoglobulin E; * Skin Prick Test positivity (SPT+) was defined by a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens;**Methacholine challenge test was not performed if baseline FEV1 <80% predicted; PD20: provocative dose causing a 20% fall in FEV$_1$;† p-value of χ2 test for categorical variables and Student test for quantitative variables.

Table E2: Levels of biomarkers of response according to sociodemographic characteristics among participants without ever asthma (N=774).

		Catalase (k/g)			GPX (U/g)			SOD (U/g)	
	n	Mean (SD)	p $p_{adjusted}$*	n	GM (Q1. Q3)	p $p_{adjusted}$*	n	GM (Q1. Q3)	p $p_{adjusted}$*
Age (years)									
[16-25[	93	169 (34.6)		93	38.9 (33.7 ; 44.0)		93	1265 (1083; 1517)	
[25-35[	133	154 (44.2)	**0.03**	134	37.0 (32.5 ; 42.5)	**0.001**	134	1260 (1083; 1511)	**0.01**
[35-45[	93	163 (38.6)	**0.04**	96	38.9 (33.3 ; 44.0)	**0.01**	96	1244 (1044; 1509)	**0.003**
[45-55[	181	168 (34.9)		182	40.6 (35.7 ; 46.7)		182	1204 (1206; 1413)	
≥ 55	262	162 (40.8)		266	40.1 (35.0 ; 46.4)		265	1170 (960 ; 1410)	
p for trend adjusted*			0.94			**0.01**			**<0.0001**
Sex									
Men	349	163 (39.4)	0.77	349	37.8 (33.1 ; 43.8)	**< 0.0001**	350	1226 (1027; 1474)	0.31
Women	413	164 (39.3)	0.30	420	40.7 (35.7 ; 46.7)	**< 0.0001**	420	1204 (1021; 1453)	0.71
BMI (kg/m^2)									
< 20	71	165 (33.9)		71	39.8 (35.4; 44.8)		71	1194 (1009; 1421)	
[20-25[	376	160 (39.7)	0.07	382	39.2 (33.8; 45.7)	0.87	382	1193 (1011; 1411)	0.28
[25-30[	226	165 (41.1)	0.06	227	39.7 (35.1; 45.8)	0.62	228	1232 (1034; 1470)	0.02
≥ 30	76	171 (37.1)		76	39.6 (35.8; 45.2)		76	1247 (1034; 1606)	
p for trend adjusted*			**0.02**			0.73			**0.003**
Smoking status									
Non smoker	380	159 (39.3)		385	39.6 (34.9 ; 44.8)		386	1216 (1022; 1474)	
Ex-smoker	225	167 (40.0)	**0.02**	225	39.7 (34.0 ; 46.5)	0.13	225	1190 (1028; 1441)	0.24
Current smoker	157	167 (37.7)	**0.02**	159	38.2 (33.6 ; 44.0)	0.59	159	1242 (1033; 1452)	0.42
p for trend adjusted*			**0.004**			0.47			0.70

GPX: glutathione peroxidase; SOD: superoxide dismutase; GM: geometric mean; BMI: Body Mass Index.

*Results were adjusted for age, sex and smoking status.

Table E3: Levels of biomarkers of damage according to sociodemographic characteristics among participants without ever asthma (N=774).

	n	8-isoprostane (pg/mL) GM (Q1 ; Q3)	p $p_{adjusted}$*	n	FlOPs (RFU/mL) GM (Q1. Q3)	p $p_{adjusted}$*
Age (years)						
[16-25[	52	3.97 (1.75 ; 7.67)		90	86.5 (74.9 ; 96.3)	
[25-35[	65	2.73 (1.14 ; 8.08)	**0.04**	129	89.3 (77.2 ; 101)	**< 0.0001**
[35-45[	47	1.98 (0.84 ; 5.28)	**0.03**	91	92.0 (81.4 ; 101)	**< 0.0001**
[45-55[	96	2.52 (1.21 ; 5.70)		177	96.9 (83.4 ; 112)	
≥ 55	113	2.01 (0.74 ; 4.66)		251	100 (87.0 ; 113)	
p for trend adjusted*			**0.01**			**<0.0001**
Sex						
Men	164	2.08 (1.05 ; 5.18)	**0.04**	335	93.1 (80.7 ; 105)	**0.07**
Women	209	2.82 (1.14 ; 6.12)	**0.02**	403	95.9 (82.3 ; 109)	**0.03**
BMI (kg/m^2)						
< 20	38	2.39 (0.74 ; 9.03)		68	90.9 (76.5 ; 102)	
[20-25[	193	2.67 (1.10 ; 6.62)	0.70	363	95.1 (82.7 ; 108)	0.17
[25-30[	100	2.17 (1.19 ; 4.35)	0.56	226	94.0 (80.3 ; 109)	0.34
≥ 30	42	2.38 (1.09 ; 5.67)		73	98.4 (85.2 ; 107)	
p for trend adjusted*			0.45			0.91
Smoking status						
Non smoker	176	2.62 (1.15 ; 6.15)		367	91.8 (79.4 ; 103)	
Ex-smoker	124	2.16 (1.00 ; 5.24)	0.45	220	98.0 (85.2 ; 113)	**0.001**
Current smoker	73	2.65 (1.22 ; 5.44)	0.92	151	96.9 (85.1 ; 111)	**0.0001**
p for trend adjusted*			0.80			**<0.0001**

FlOPS : Fluorescent oxidation products ; GM: geometric mean ; BMI: Body Mass Index.

*Results were adjusted for age, sex and smoking status.

Table E4: Associations between biomarkers of response related to oxidative stress, asthma and various asthma outcomes.

	Superoxide dismutase (U/g)			Glutathione peroxidase (U/g)			Catalase (k/g)		
	n	OR (95% CI)*	OR$_{adjusted}$ (95% CI)*	n	OR (95% CI)*	OR$_{adjusted}$ (95% CI)*	n	OR (95% CI)*	OR$_{adjusted}$ (95% CI)*
Asthma ever *(vs never asthma)*	1377	1.04 (0.91; 1.20)	0.96 (0.83; 1.12)	1378	0.91 (0.81; 1.03)	1.01 (0.89; 1.15)	1368	0.97 (0.84; 1.11)	0.97 (0.85; 1.12)
Among participants with ever asthma									
Current asthma *(vs without current)* asthma	567	1.04 (0.74; 1.46)	1.08 (0.76; 1.54)	568	0.85 (0.62; 1.17)	0.83 (0.59; 1.15)	565	0.96 (0.66; 1.38)	0.93 (0.64; 1.35)
Asthma attacks in the last 12 months	604	0.95 (0.68; 1.21)	0.91 (0.64; 1.19)	606	0.95 (0.74; 1.15)	0.90 (0.68; 1.11)	603	1.10 (0.88; 1.32)	1.13 (0.91; 1.35)
Poor asthma control** *(vs controlled asthma)*	532	0.87 (0.67; 1.13)	0.91 (0.70; 1.19)	533	0.87 (0.70; 1.09)	0.82 (0.65; 1.04)	530	1.00 (0.79; 1.28)	1.01 (0.79; 1.29)
FEV$_1$ <80% predicted**	596	0.98 (0.69; 1.40)	1.15 (0.77; 1.73)	598	1.21 (0.93; 1.57)	1.12 (0.84; 1.50)	595	1.04 (0.77; 1.39)	1.02 (0.76; 1.36)

*OR expressed for an increase corresponding to the value of the interquartile range (distance between the 25th and 75th percentile) of each biological marker;

Results were adjusted for age, sex and smoking status; **Results were unchanged when restricting analyses to participants with current asthma;

FEV$_1$: Forced expiratory volume in one second.