

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 (FEV₁) percent predicted value was based on Quanjer et al. reference equations [3]. For participants with a FEV₁ $\geq 80\%$ 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 ≥ 3 mm than the negative control for at least one of 12 aeroallergens (indoor: cat, *Dermatophagoides pteronyssinus*, *Blattella germanica*, outdoor: olive, birch, *Parietaria judaica*, timothy grass, *Cupressus* 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/mm³) 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/mm³ 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 M sodium phosphate 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 FIOPs 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 FIOPs level, and found a positive and significant association: geometric mean [Q1; Q3] of FIOPs 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 FIOPs 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 FIOPs 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		p [†]
	n	Percent, mean (SD)	n	Percent, mean (SD)	n	Percent, mean (SD)	
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/m²							
< 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 FEV1; † p-value of χ^2 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/m²)									
< 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).

	8-isoprostane (pg/mL)			FIOPs (RFU/mL)		
	n	GM (Q1 ; Q3)	p P _{adjusted} *	n	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/m²)						
< 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

FIOPS : 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) <i>Among participants with ever asthma</i>	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)
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)
FEV₁ <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;

FEV₁: Forced expiratory volume in one second.