Exhaled NO, nitrite/nitrate levels, allergy, rhinitis and asthma in the EGEA study

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SUPPLEMENTARY DATA

Phenotypes

Inclusion criteria used to define asthma in probands were based on self-reported answers to the four questions: "*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 their medical record. Asthma in relatives of probands was defined as a positive answer to at least one of the first two questions. Among subjects with asthma, "current asthma" was defined by a report of respiratory symptoms in the past 12 months (wheeze, nocturnal chest tightness, attacks of breathlessness following strenuous activity, at rest or at night time, and asthma attacks) or use of inhaled and/or oral medicines because of breathing problems.

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. The atopic burden assessed by the number of SPT+ (SPTQ, skin prick test quantitative score) is an indicator of allergy previously shown to have good biometric properties [1]. Subjects were also classified according to the number of SPT+ as none, 1, 2, and >2.

Current rhinitis was defined by a positive answer to one of the two questions: *"Have you ever had rhinitis?" or " Have you ever had hay fever?"* and a positive answer to *"have you had sneezing problems or a runny nose in the past 12 months?"* Allergic rhinitis was defined as having both current rhinitis and one or more SPT+. Subjects were also classified in three groups as non sensitized (no SPT+), sensitized only (having one or more SPT+ and no current rhinitis) and as having allergic rhinitis (one or more SPT+ and current rhinitis).

Asthma control was assessed by combining epidemiological data following as much as possible the 2006-2009 GINA (Global INitiative for Asthma) guidelines [2]. Asthma was defined as 1) controlled if all the following features, assessed on average over the past 3 months except for FEV1, were present: trouble with breathing less than once a week, no asthma attacks, no nocturnal symptoms, short acting β2-agonists use twice or less per week in the last 3 months, no use of oral steroids in the last 12 months, FEV1 ≥80% of predicted value, 2) partly-controlled if 1 or 2 of the above features were absent, 3) uncontrolled if ≥3 of these features were absent or if respiratory problems had caused hospital/emergency department admissions in the last 12 months, or if oral steroids had been used in the last 12 months or ≥12 asthma attacks in the past 3 months.

The asthma symptomatic score, proposed by Pekkanen *et al.* [3], varying from 1 to 5, is based on the number of asthma symptoms: wheeze and breathlessness, woken with chest tightness, woken by an attack of shortness of breath, attack of shortness of breath at rest, attack of shortness of breath after exercise.

A lung function test with methacholine challenge was performed using a standardized protocol with similar equipment across centres according to the ATS/ERS guidelines [4]. Methacholine challenge was performed unless baseline FEV1 <80% predicted.

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

Eosinophil count (EOS) was obtained from white blood cell count.

Exhaled Breath condensate collection

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 (http://www.afaq.org/certification=262711141114). Plasma aliquots were stored from 1.7 to 5.3 years and EBC samples from 1.8 to 5.4 years until analysis.

Measurement of total nitrite/nitrate level

Total NO_2^-/NO_3^- levels were measured in plasma and EBC by the Griess reaction [5]. Briefly, NO_3^- was reduced to NO_2^- by adding NO_3^- reductase (25 mU/ml) and NADPH 20 mM at room temperature. After 3 hours, samples were deproteinized by adding a solution of $ZNSO_4$ 30% and centrifuged. Griess reagent (0.1% naphthalethylene-dimine and 1 sulfanilamide in 5% H_3PO_4) was added to supernatants. The optical density at 560 nm was measured using a microplaque reader. NO_2^- levels were calculated by comparison with optical density 560 of

standard sodium NO_2^- solutions. All measurements were done in duplicate. Analytical intra-run imprecision was below 3%. Measurements with a coefficient of variation >15% and extreme outliers (n=7) were excluded from the analyses. Protein concentration in EBC was determined according to Smith *et al.* [6]. Total NO_2^-/NO_3^- level levels were expressed as μM in plasma and as μmol/mg of proteins in EBC [7].

Measurement of FeNO level

Online (Paris and Montpellier) or off-line (Grenoble) measurements were realized according to American Thoracic Society/European Respiratory Society recommendations for standardized procedures before other pulmonary function tests [8]. Participants avoided food and beverages (including alcohol), and smoking tobacco at least 1 hour prior to testing. Upper or lower respiratory tract infections within 4 weeks were recorded. Participants, seated comfortably in the upright position without a nose clip, were asked to exhale at a constant flow against an oral pressure of at least 5 cm of H_2O. Measurements were taken for at least 6 seconds. Three NO analyzers were used in this study; Endono 8000 (Seres, Aix-en-Provence, France) in Paris, Aerocrine (Aerocrine AB, Solna, Sweden) in Montpellier and Topaze 2020 (Cosma SA, Igny, France) in Grenoble. The three analyzers were calibrated with gas containing NO (Air Liquide, France). For Endono 8000 and Topaze 2020 analyzers, the flow rate was calibrated daily. For the Aerocrine analyzer, the flow rate was calibrated every 13 days according to manufacturer standards. Scrubbed air (0 ppb of NO) was inhaled before measurements. Repeated reproducible exhalations were performed to obtain at least 2 NO plateau values that agree within 10% of each other. Depending on the analyzer, 2 or 3 measurements at the 50mL/s exhalation flow rate were recorded. In Grenoble (offline method) and Montpellier (online method), 3 measurements performed only at the 50 mL/s flow rate were recorded. In Paris (online method), measurements were performed at 3 exhalation flow rates: 25, 50 and 100 mL/s, and only 2 measurements were recorded at each flow rate to avoid tiring out the participants. Measurements of F_{ENO} at the 50mL/s flow rate on each of the 3 analyzers were combined. Participants with only 1 F_{ENO} measurement or poor calibration were excluded from the analyses (5%).

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Figure. S1. Associations between total nitrite-nitrate (NO_2^-/NO_3^-) levels (FIG1A) and FE_{NO} levels (FIG1B) and allergic sensitization expressed as the number of positive Skin Prick Tests (SPTQ).

Results are expressed as log10 (NO_2^-/NO_3^-) and log10 (FE_{NO}). Trend p values.

The horizontal line indicates the median. The top and the bottom borders of each box mark the 75th and the 25th percentiles, respectively. The whiskers correspond to 1.5*interquartile range and the circles above and below each box mark the maximum and minimum values.

Table S1. Comparison of main characteristics between subjects included in the present study, and non selected subjects and all subjects

	Subjects included in the present analyses (n=523)		Non selected subjects (n=1047)		All subjects (n=1570)	
	n	percent	n	percent	n	percent
Age, year, mean (SD)	523	39.9 (16.6)	1047	44.9 (16.2)†	1570	43.2 (16.5)
Sex, women	267	51	528	50.4	795	50.6
Smoking habits						
Never smokers	270	51.6	509	48.6	779	49.6
Ex-smokers	132	25.3	290	27.7	422	26.9
Current smokers	121	23.1	240	22.9	361	23.0
Asthma						
Ever asthma	255	48.7	428	40.9†	683	43.5
Current asthma	212	40.5	389	37.1	558	35.5
FEV_1 % predicted, n, mean (SD)	523	103.4 (17.2)	864	101.8 (18.5)	1387	102.4 (18.0)
Methacholine test*, PD20≤4 mg, %	377	45.4	489	42.9	866	44.0
SPT+#	304	58.1	428	40.9	732	46.6
SPTQ, median [Q1-Q3]	523	1 [0-3]	780	1 [0-2]	1303	1 [0-2]
Current rhinitis	205	39.2	348	33.2‡	553	35.2
FeNO, ppb, Gmean [Q1-Q3]	523	15.1 [10.0-23.0]	186	15.5 [11.5-22.5]	709	15.2 [10.4-22.6]
EBC NO_2-NO_3, µmol/mg, GM [Q1-Q3]	523	2.25 [1.12-4.98]	449	1.45 [0.73-2.81]†	972	1.84 [0.90-3.89]
Plasma NO_2-NO_3, µM, GM [Q1-Q3]	510	39.1 [28.0-53.7]	683	33.3 [23.5-47.5]†	1193	35.7 [25.4-50.8]
IgE, IU/ml, GM [Q1-Q3]	523	81.7 [28.8-226]	873	69.0 [23.3-194]‡	1396	73.5 [25.2-209]

Eosinophils, cells/mm^3, GM [Q1-Q3]	505	170 [100-280]	864	161 [100-260]	1369	164 [100-260]

SPTQ: number of positive skin prick tests; GM= geometric mean, Q1-Q3= first and third quartile.
*Methacholine challenge test was not performed if baseline FEV_1 <80% predicted.
#Skin Prick Test positivity (SPT+) was defined by a mean wheal diameter ≥3mm than the negative control for at least one of 12 aeroallergens.
Current rhinitis was defined by a positive answer to one of the two questions: *"Have you ever had rhinitis?" or " Have you ever had hay fever?"* and a positive answer to "have you had sneezing problems or a runny nose in the past 12 months?" .
P values comparing the distributions of the characteristics between the subjects included in the present study with those who were not selected were obtained from independent T Tests for continuous and Chi2 test for qualitative variables. P values >0.05 are not shown.
†P<0.005; ‡0.02≤P≤0.005

Figure S1A and S1B