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

Rachel Nadif¹,², Marta Rava¹,², Brigitte Decoster³, Hélène Huyvaert³,⁴, Nicole Le Moual¹,², Jean Bousquet¹,²,⁵, Valérie Siroux⁶,⁷, Raphaëlle Varraso¹,², Isabelle Pin⁶,⁷,⁸, Farid Zerimech⁴, Régis Matran³

Affiliations:
¹Inserm, Centre for research in Epidemiology and Population Health (CESP), U1018, Respiratory and Environmental Epidemiology Team, F-94807, Villejuif, France
²Univ Paris-Sud, UMRS 1018, F-94807, Villejuif, France
³Univ Lille Nord de France, F-59000, Lille, France
⁴CHU, F-59000, Lille, France
⁵CHU Arnaud de Villeneuve, Montpellier, F-34295, France
⁶Inserm, U823, Epidémiologie environnementale appliquée à la reproduction et la santé respiratoire, Institut Albert Bonniot, F-38042 Grenoble, France
⁷Univ Joseph Fourier, Grenoble, F-38000, France
⁸Pédiatrie, CHU de Grenoble, 38043 Grenoble, France

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=26271141114). 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% naphthylethylene-dimine and 1 sulfanilamide in 5% H$_3$PO$_4$) 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$_2$O. 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 FE$_{NO}$ at the 50mL/s flow rate on each of the 3 analyzers were combined. Participants with only 1 FE$_{NO}$ measurement or poor calibration were excluded from the analyses (5%).
References


Figure. S1. Associations between total nitrite-nitrate (NO$_2^-$/NO$_3^-$) levels (FIG1A) and FeNO 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 (FeNO). 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>
<thead>
<tr>
<th></th>
<th>Subjects included in the present analyses (n=523)</th>
<th>Non selected subjects (n=1047)</th>
<th>All subjects (n=1570)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>percent</td>
<td>n</td>
</tr>
<tr>
<td>Age, year, mean (SD)</td>
<td>523</td>
<td>39.9 (16.6)</td>
<td>1047</td>
</tr>
<tr>
<td>Sex, women</td>
<td>267</td>
<td>51</td>
<td>528</td>
</tr>
<tr>
<td>Smoking habits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smokers</td>
<td>270</td>
<td>51.6</td>
<td>509</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>132</td>
<td>25.3</td>
<td>290</td>
</tr>
<tr>
<td>Current smokers</td>
<td>121</td>
<td>23.1</td>
<td>240</td>
</tr>
<tr>
<td>Asthma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever asthma</td>
<td>255</td>
<td>48.7</td>
<td>428</td>
</tr>
<tr>
<td>Current asthma</td>
<td>212</td>
<td>40.5</td>
<td>389</td>
</tr>
<tr>
<td>FEV₁ % predicted, n, mean (SD)</td>
<td>523</td>
<td>103.4 (17.2)</td>
<td>864</td>
</tr>
<tr>
<td>Methacholine test*, PD20≤4 mg, %</td>
<td>377</td>
<td>45.4</td>
<td>489</td>
</tr>
<tr>
<td>SPT+#</td>
<td>304</td>
<td>58.1</td>
<td>428</td>
</tr>
<tr>
<td>SPTQ, median [Q1-Q3]</td>
<td>523</td>
<td>1 [0-3]</td>
<td>780</td>
</tr>
<tr>
<td>Current rhinitis</td>
<td>205</td>
<td>39.2</td>
<td>348</td>
</tr>
<tr>
<td>FeNO, ppb, Gmean [Q1-Q3]</td>
<td>523</td>
<td>15.1 [10.0-23.0]</td>
<td>186</td>
</tr>
<tr>
<td>EBC NO₂-NO₃, µmol/mg, GM [Q1-Q3]</td>
<td>523</td>
<td>2.25 [1.12-4.98]</td>
<td>449</td>
</tr>
<tr>
<td>Plasma NO₂-NO₃, µM, GM [Q1-Q3]</td>
<td>510</td>
<td>39.1 [28.0-53.7]</td>
<td>683</td>
</tr>
<tr>
<td>IgE, IU/ml, GM [Q1-Q3]</td>
<td>523</td>
<td>81.7 [28.8-226]</td>
<td>873</td>
</tr>
</tbody>
</table>

**Table S1.** Comparison of main characteristics between subjects included in the present study, and non selected subjects and all subjects
SPTQ: number of positive skin prick tests; GM= geometric mean, Q1-Q3= first and third quartile.
*Methacholine challenge test was not performed if baseline FEV₁ <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

- **Left Panel**: 
  - Title: Log10 NO2⁻/NO3⁻/protein (μmol/mg)
  - Data points: N=219, 95, 67, 142
  - P for trend = 0.016

- **Right Panel**: 
  - Title: Log10 FeNO (ml/s)
  - Data points: N=219, 95, 67, 142
  - P for trend < 0.001