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

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23 **Word count:** 3173.

24 **Conflict of interest**

25 None.

26

27 **Abstract**

28 **Background:** Although interest in biomarkers in the nitrate-nitrite-NO pathway has recently  
29 increased, associations between nitrite ( $\text{NO}_2^-$ ), nitrate ( $\text{NO}_3^-$ ) and asthma, allergic  
30 sensitization and rhinitis remain unclear.

31 **Objective:** To evaluate the associations between  $\text{NO}_2^-/\text{NO}_3^-$  and exhaled fraction of nitric  
32 oxide ( $\text{FeNO}$ ) levels with asthma, allergic sensitization and rhinitis.

33 **Methods:** Plasma and exhaled breath condensate (EBC)  $\text{NO}_2^-/\text{NO}_3^-$  and  $\text{FeNO}$  levels were  
34 measured in 523 adults of the French Epidemiological study on Genetics and Environment of  
35 Asthma. Allergic sensitization was defined by a positive skin prick test for at least one  
36 aeroallergen. Subjects were classified as non-sensitized, sensitized and as having allergic  
37 rhinitis.

38 **Results:** Plasma  $\text{NO}_2^-/\text{NO}_3^-$  level was unrelated to any disease phenotypes. EBC  $\text{NO}_2^-/\text{NO}_3^-$   
39 level was unrelated to any asthma phenotypes. EBC  $\text{NO}_2^-/\text{NO}_3^-$  and  $\text{FeNO}$  levels were  
40 correlated in sensitized subjects only ( $r=0.21\pm 0.10$ ,  $p=0.01$ ). EBC  $\text{NO}_2^-/\text{NO}_3^-$  and  $\text{FeNO}$  levels  
41 were higher in sensitized than in non-sensitized subjects (adjusted GM (95%CI): 2.36 (1.96;  
42 2.84) vs. 1.72 (1.38; 2.14)  $\mu\text{mol}/\text{mg}$  proteins,  $p=0.008$ ; and 18.3 (16.7; 20.0) vs. 14.8 (13.3;  
43 16.5) ppb,  $p=0.0006$  respectively), with gradual relationships from sensitized subjects to those  
44 with allergic rhinitis ( $p<0.0001$ ).

45 **Conclusion:** Results suggest that EBC  $\text{NO}_2^-/\text{NO}_3^-$  and  $\text{FeNO}$  levels may be considered as  
46 biological markers of intensity of allergic sensitization and rhinitis.

47

48 **Key words:** asthma, allergic sensitization, rhinitis, adults, NO metabolism, exhaled breath  
49 condensate, total nitrite/nitrate, exhaled fraction of NO.  
50

## 51 **Introduction**

52 The interest in measuring biological markers in exhaled breath condensate (EBC) in  
53 epidemiological studies on respiratory diseases has increased in the last years. Among the  
54 pathways involved in the pathophysiology of asthma, the metabolism of nitric oxide (NO)  
55 also called the nitrate-nitrite-NO pathway has taken a growing place in this research field [1].  
56 The NO metabolism is complex, and both NO measured by the exhaled fraction of NO ( $FE_{NO}$ )  
57 and NO-related compounds such as nitrites ( $NO_2^-$ ) and nitrates ( $NO_3^-$ ) are relevant biological  
58 markers that may help to better understand the patho-physiology of asthma and allergy [2].  
59  $FE_{NO}$  is the most studied one, and it is commonly considered as a non-invasive indirect marker  
60 of airway inflammation [3]. Both epidemiological and clinical studies in adults showed  
61 increased level of  $FE_{NO}$  in children and adults with asthma, and positive associations between  
62  $FE_{NO}$  and allergic sensitization are consistent over the studies, regardless of rhinitis or asthma  
63 [4]. Studies on associations between  $NO_2^-$  and  $NO_3^-$  levels with asthma, allergy or rhinitis  
64 have led to more conflicting results both in adults and children [5–17]. Until now, none of  
65 these studies has simultaneously performed measurements of EBC  $NO_2^-/NO_3^-$  and  $FE_{NO}$   
66 levels in the same subjects. Recently, in a large number of adults from the French  
67 Epidemiological study on Genetics and Environment of Asthma (EGEA), EBC  $NO_2^-/NO_3^-$   
68 and  $FE_{NO}$  levels were found to be correlated in subjects without asthma [18].  
69 Nitric oxide has different functions and roles in pathophysiology, which may be better  
70 explained by considering its compartmentalized production [19]. In this study, we compared  
71 the association between total  $NO_2^-/NO_3^-$  levels measured in two compartments (plasma and  
72 exhaled breath condensate) and  $FE_{NO}$  levels with asthma, allergic sensitization and rhinitis  
73 among 523 adults from the EGEA study. We hypothesized that the associations will be  
74 different depending on the compartments, biomarkers and outcomes studied.

75

76 **Methods**

77 *Study design*

78 Data used for the analyses were collected in the framework of the 12-year follow-up of  
79 EGEA. EGEA is a French cohort study based on an initial group of asthma cases and their  
80 first-degree relatives, and controls (first survey, n=2047) [20]. The protocol and descriptive  
81 characteristics have been described previously [21,22]. A follow-up of the initial cohort was  
82 conducted between 2003 and 2007 [23]. Among the alive cohort (n=2002), 92% (n=1845)  
83 completed a short self-administered questionnaire and among them 1601 had a complete  
84 examination. All subjects responded to a questionnaire based on international standardized  
85 tools to diagnose asthma and to determine respiratory and allergic symptoms, treatments, and  
86 environmental exposures. The present cross-sectional analysis includes those who were adults  
87 at the second survey ( $\geq 16$  years old, n=1570 adults) with available data on asthma, current  
88 rhinitis, allergic sensitization, and available measurements of exhaled breath condensate  
89  $\text{NO}_2^-/\text{NO}_3^-$  and  $\text{F}_{\text{ENO}}$  levels (n=523). Subjects included in the analyses were younger,  
90 reported more often ever asthma and current rhinitis, and had higher levels of  $\text{NO}_2^-/\text{NO}_3^-$  and  
91 Immunoglobulin E (IgE) than those not included in the analyses (n=1047). The two groups  
92 were similar for sex, smoking, current asthma status, allergic sensitization, lung function tests,  
93 and eosinophil (EOS) count (see Table 1 in supplementary data).

94 Ethical approval was obtained from the relevant institutional review board committees  
95 (Cochin Port-Royal Hospital and Necker-Enfants Malades Hospital, Paris). Written informed  
96 consent was signed by all participants.

97

98 ***Respiratory phenotypes***

99 Subjects with ever asthma were defined by a positive answer to either: “*Have you ever had*  
100 *attacks of breathlessness at rest with wheezing?*”, or “*Have you ever had asthma attacks?*”, or  
101 if they were recruited as asthmatic cases at the first survey.

102 Allergic sensitization was defined by a positive skin prick test (SPT+) with a mean wheal  
103 diameter  $\geq 3$ mm than the negative control for at least one of 12 aeroallergens (indoor: cat,  
104 *Dermatophagoides pteronyssinus*, *Blattella germanica*, outdoor: olive, birch, *Parietaria*  
105 *judaica*, timothy grass, *Cupressus* and ragweed pollen, and molds: *Aspergillus*, *Cladosporium*  
106 *herbarum*, *Alternaria tenuis*). Subjects were classified as sensitized if they have one or more  
107 SPT+. Current rhinitis was defined by a positive answer to one of the two questions: “*Have*  
108 *you ever had rhinitis?*” or “*Have you ever had hay fever?*” and a positive answer to “*have*  
109 *you had sneezing problems or a runny nose in the past 12 months?*” Allergic rhinitis was  
110 defined as having both current rhinitis and one or more SPT+. Subjects were also classified in  
111 three groups as non sensitized (no SPT+), sensitized only (having one or more SPT+ and no  
112 current rhinitis) and as having allergic rhinitis (one or more SPT+ and current rhinitis).

113 Eosinophilia was defined as eosinophil count  $\geq 5\%$ . Details on other phenotypes are given in  
114 supplementary data.

115

116 ***Biological phenotypes***

117 Exhaled breath condensate (EBC) was collected with an RTube™ according a standardized  
118 method. Briefly, the RTube (TM) was rinsed with deionized water and dried thoroughly.

119 Participants breathed orally at tidal volumes into a mouthpiece attached to a cold condenser (-  
120 20°C). They were seated comfortably with a headrest. All headrests and back seats were tilted  
121 slightly to avoid any saliva contamination during breathing maneuvers (see supplementary  
122 data for more details).

123 Total nitrite-nitrate ( $\text{NO}_2^-/\text{NO}_3^-$ ) levels were measured in plasma and EBC as previously  
124 described [24]. All measurements were done in duplicate. Analytical intra-run imprecision  
125 was below 3%. Measurements with a coefficient of variation >15% and extreme outliers  
126 ( $n=7$ ) were excluded from the analyses (see supplementary data for more details).  
127 Measurements of  $\text{F}_{\text{ENO}}$  were realized before other pulmonary function tests according to  
128 ATS/ERS recommendations (see supplementary data). The measurement was performed only  
129 in 3 of the 5 centers involved in the EGEA study, which explained in a large part the attrition  
130 on numbers of subjects included in the analysis compared to the total number.  $\text{F}_{\text{ENO}}$  level was  
131 measured at 50mL/s flow rate as previously described [23].

132

### 133 ***Statistical Methods***

134 Joint distribution of asthma, SPT+ and current rhinitis was shown with a Venn diagram  
135 (Figure 1). Total plasma and EBC  $\text{NO}_2^-/\text{NO}_3^-$  and  $\text{F}_{\text{ENO}}$  levels were log10-transformed as a  
136 result of their skewed distribution.

137 In the same study, we previously reported that plasma  $\text{NO}_2^-/\text{NO}_3^-$  level was increased with  
138 leafy vegetable consumption and decreased in smokers and with storage time, that EBC  
139  $\text{NO}_2^-/\text{NO}_3^-$  level was decreased in smokers and with exposure to ambient ozone  
140 concentration [24], and that  $\text{F}_{\text{ENO}}$  level was associated with season of examination [23].  
141 Furthermore, storage time and season of examination varied with centre. Therefore estimates  
142 were adjusted for 1) age, sex, smoking, leafy vegetable consumption and centre for plasma  
143  $\text{NO}_2^-/\text{NO}_3^-$ , 2) age, sex, smoking, ambient ozone concentration and centre for EBC  
144  $\text{NO}_2^-/\text{NO}_3^-$ , and 3) age, sex, height, smoking and centre for  $\text{F}_{\text{ENO}}$ . Since the ratio of higher  
145 oxides of nitrogen (HiNOx including  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ) to NO was reported be more  
146 informative than each measurement alone by Nguyen *et al.* [13], the  $(\text{NO}_2^- + \text{NO}_3^-)/\text{NO}$  ratio  
147 (NOx/NO ratio) has also been studied.



148 As inhaled corticosteroids (ICS) use can decrease  $FE_{NO}$  levels, and as  $NO_2^-/NO_3^-$  and  $FE_{NO}$   
149 are biological markers involved in the same pathway, association between ICS use and EBC  
150  $NO_2^-/NO_3^-$  level was studied. Since increased body mass index (BMI)/obesity has been  
151 associated with lower  $FE_{NO}$  level, estimates were also adjusted for BMI as a sensitivity  
152 analyses.

153 Associations between total  $NO_2^-/NO_3^-$  levels,  $FE_{NO}$  levels, and the  $NO_x$  ratio ( $NO_2^- +$   
154  $NO_3^-$ )/ $NO$  and asthma phenotypes, allergic sensitization and current rhinitis were estimated  
155 with linear regression models. Parameter estimates were assessed by using generalized  
156 estimating equations, with an exchangeable working correlation to account for the potential  
157 clustering within families (SAS MIXED procedure). The level of statistical significance was  
158 set at  $\alpha=0.05$ . Two-sided P values were reported for all association estimates. All analyses  
159 were conducted using SAS software, version 9.3 (SAS Institute, Inc., Cary, NC, USA).

160

## 161 **Results**

162 The characteristics of the 523 adults according to their asthma status are summarized in Table  
163 1. As expected, subjects with asthma had significantly higher eosinophilia, lower  $FEV_1\%$   
164 predicted, more often bronchial hyper-responsiveness (BHR), SPT+, and reported more often  
165 current rhinitis than subjects without asthma. After adjustment for age, sex and smoking, the  
166 following associations between asthma and eosinophilia (odds ratio (OR) 3.12, 95%  
167 confidence interval (CI, 1.72-5.66),  $FEV_1\%$  predicted (mean  $\pm$  SD:  $107.7 \pm 10.5$  vs.  $97.5 \pm$   
168  $11.0$ ), BHR (OR, 4.18; 2.66-6.57), SPT+ (OR, 5.07; 3.32-7.72) and current rhinitis (OR, 4.62;  
169 3.11-6.85) were confirmed (all  $P<0.0005$ ). EOS count, IgE and  $FE_{NO}$  levels were significantly  
170 higher in subjects with asthma than in those without (all  $P<0.0001$ ). EBC  $NO_2^-/NO_3^-$  level  
171 was unrelated to ICS use (data not shown, P value=0.5).

172

173 ***Pairwise association between EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>, FE<sub>NO</sub> levels and blood eosinophil counts***

174 EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels were unrelated with EOS, whereas FE<sub>NO</sub> levels were positively  
175 associated with EOS in all subjects, both in non-sensitized and sensitized subjects (Table 2).

176 In sensitized subjects, EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> was positively associated with FE<sub>NO</sub> levels.

177 The median FE<sub>NO</sub> value in the population was 15.6 ppb (range 2.4 to 99.0 ppb). Stratification  
178 according to this median value showed positive and significant association between EBC  
179 NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> level and allergic sensitization in subjects above the median only (2.66 (2.06-  
180 3.43) vs. 1.64 (1.18-2.28), P=0.01 and 2.03 (1.52-2.71) vs. 1.76 (1.30-2.38), P=0.4 in subjects  
181 above and below the median respectively).

182

183 ***Plasma and EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>, FE<sub>NO</sub> levels and asthma and asthma-related phenotypes***

184 Both plasma and EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels were unrelated to ever asthma, current asthma,  
185 symptomatic score, and asthma control (data not shown, all P values>0.3). Furthermore,  
186 plasma NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> level was unrelated to allergic sensitization and current rhinitis.

187 Both plasma and EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels were unrelated to eosinophilic asthma nor to age at  
188 asthma onset (Table 3). As expected, a positive and significant association was observed  
189 between FE<sub>NO</sub> level and eosinophilic asthma (Table 3) but no other significant association was  
190 observed.

191

192 ***EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> and FE<sub>NO</sub> levels, NO<sub>x</sub>/NO ratio and allergic sensitization***

193 A positive association at borderline significance was observed between EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> level  
194 and SPT+ (see Table 4). In a model adjusted for covariates, including asthma, EBC  
195 NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> level was positively and significantly associated with SPT+, and a positive  
196 association at borderline significance was observed with current rhinitis. Furthermore,  
197 positive and gradual increases in EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> level were observed with SPTQ (see Figure

198 1 in supplementary data), and when subjects were classified in the following groups: no  
199 SPT+, SPT+ only, and both SPT+ and current rhinitis (see Table 4 and Figure 2). The median  
200 EBC  $\text{NO}_2^-/\text{NO}_3^-$  value in our population was 2.25  $\mu\text{mol}/\text{mg}$  proteins (range 1.12 to 4.98,  
201 Table 1). Stratification according to this median value showed positive and significant  
202 associations between subjects above the median value and 1) allergic sensitization (OR, 1.92;  
203 1.25-2.97,  $p=0.003$ ), 2) current rhinitis (OR, 1.52; 1.01-2.28,  $p=0.04$ ), and 3) SPT+ only or  
204 both SPT+ and current rhinitis versus no SPT+ (OR, 1.64; 1.01-2.66,  $p=0.04$ , and 2.16; 1.29-  
205 3.59,  $p=0.003$  respectively) in GEE regression models with adjustment for age, sex,  
206 smoking, ambient ozone concentration, asthma and centre.

207 Similarly to EBC  $\text{NO}_2^-/\text{NO}_3^-$ ,  $\text{F}_{\text{ENO}}$  level was also positively related to allergic sensitization  
208 expressed as SPT+ (Table 4), and gradually increased with SPTQ (see Figure online for  
209 details), and with allergic sensitization and current rhinitis.  $\text{F}_{\text{ENO}}$  was also significantly and  
210 positively associated with current rhinitis. The associations between  $\text{F}_{\text{ENO}}$  levels and SPT+,  
211 and current rhinitis were confirmed when BMI instead of height was added as covariate in the  
212 models (data not shown).

213 No significant association were observed between the  $(\text{NO}_2^- + \text{NO}_3^-)/\text{NO}$  ratio ( $\text{NO}_x/\text{NO}$   
214 ratio) and SPT+, current rhinitis or both (Table 4 and Figure 2). When analysing SPT+ to  
215 indoor, outdoor or molds allergens separately, EBC  $\text{NO}_2^-/\text{NO}_3^-$  levels showed positive and  
216 significant associations with sensitization to molds allergens, and  $\text{F}_{\text{ENO}}$  levels were positively  
217 associated to indoor allergens (Table 4).

218

219 **Discussion**

220 The present study conducted on a large sample of adults with a precise phenotypic  
221 characterization shows for the first time the similarities and differences for the associations of  
222 both  $FE_{NO}$  and exhaled breath condensate  $NO_2^-/NO_3^-$  levels with asthma, allergic  
223 sensitization and rhinitis. Results showed higher EBC  $NO_2^-/NO_3^-$  and  $FE_{NO}$  levels in subjects  
224 with allergic sensitization, with current rhinitis, and in particular when both are present. Only  
225  $FE_{NO}$  levels were found to be higher with asthma. EBC  $NO_2^-/NO_3^-$  and  $FE_{NO}$  levels were  
226 positively associated in sensitized subjects only, and EBC  $NO_2^-/NO_3^-$  levels were found to be  
227 associated with allergic sensitization in subjects with higher  $FE_{NO}$  levels only.

228 The selection of the 523 subjects included in the present analyses was driven first by the  
229 random availability of the  $FE_{NO}$  measurements in three of the five participating centres [23],  
230 and secondly by the availability of the other variables of interest. Definition of asthma case is  
231 very precise in our study since asthmatic cases were recruited in chest clinics, and a procedure  
232 was set up to include true asthmatics, leading to a very limited risk of false positives.  
233 Prevalence of bronchial hyper-responsiveness, measured by a methacholine challenge test was  
234 quite high in subjects without asthma. A possible explanation is that part of the subjects  
235 without asthma are first degree relatives of asthma cases. Nevertheless, this result is  
236 consistent with the relatively considerable number of asymptomatic subjects with BHR  
237 reported in cross-sectional epidemiologic studies, ranging from 19.3 to 62.4%. Subjects  
238 included in the analyses had higher EBC  $NO_2^-/NO_3^-$  and  $FE_{NO}$  levels than non-selected  
239 subjects. Other limitations of the present study were those commonly related to cross-  
240 sectional analyses of the data.

241 We reported no association between  $NO_2^-/NO_3^-$  level measured in plasma and any disease  
242 phenotypes. We previously reported that plasma and EBC  $NO_2^-/NO_3^-$  levels were not  
243 correlated [18]. The metabolism of NO is complex, and the production of  $NO_2^-/NO_3^-$  in

244 plasma differs from that in EBC due to their compartmentalization. In plasma  $\text{NO}_2^-/\text{NO}_3^-$   
245 production derives from several sources, such as bacteria, enzymatic production and dietary  
246 sources [25]. In EBC ionized  $\text{NO}_3^-$  and  $\text{NO}_2^-$  (not volatile) may arise from NO after reaction  
247 with oxygen [26] or from activated immune cells present in the lining fluid of the lungs [27].  
248 Overall, the specificities of the NO metabolism in plasma and in EBC may partly explain the  
249 lack of association with any clinical phenotypes in plasma. Our results are consistent with the  
250 hypotheses of Villanueva and Giulivi [19], for whom the compartmentalized production of  
251 NO better explains its different functions and roles in pathophysiology.  
252 No association was found between total  $\text{NO}_2^-/\text{NO}_3^-$  level in EBC and asthma phenotypes, as  
253 previously reported in other studies [8,14]. Contrary to our results, other studies have reported  
254 total  $\text{NO}_2^-/\text{NO}_3^-$  level in EBC to be elevated in subjects with asthma as compared to healthy  
255 non-smoking subjects [17], healthy non-atopic controls [5], or controls [9]. These conflicting  
256 results may be due to the very small number of subjects included in these studies, the various  
257 methodologies used for measuring  $\text{NO}_2^-$  and  $\text{NO}_3^-$  levels, the choice of the reference group  
258 for comparisons and other differences such as those related to phenotypes definition. Beside,  
259 information regarding allergic sensitization was not available or subjects were defined as  
260 asthmatics if they had both asthma and allergy, suggesting that the increase in  $\text{NO}_2^-/\text{NO}_3^-$   
261 level could be more related to allergy than to asthma. Furthermore, none of these previous  
262 studies have expressed the  $\text{NO}_2^-/\text{NO}_3^-$  level divided by the amount of proteins. As reported by  
263 Gessner and Wirtz [28], the measurement of total protein in EBC is important to confirm that  
264 protein and peptide markers are comparable between studies. They should always be  
265 performed in addition to specific markers investigated, and we previously found that  
266  $\text{NO}_2^-/\text{NO}_3^-$  level in EBC was positively related to protein concentration in our study [29].  
267 As reported in the literature [4,30], positive associations between  $\text{FE}_{\text{NO}}$  level and asthma,  
268 allergic sensitization, and current rhinitis were found in this study. To our knowledge, our

269 study reported for the first time similarities and differences for the associations of both  $F_{E_{NO}}$   
270 and exhaled breath condensate  $NO_2^-/NO_3^-$  levels with asthma, allergic sensitization and  
271 current rhinitis. We found positive associations between EBC  $NO_2^-/NO_3^-$  and  $F_{E_{NO}}$  levels in  
272 sensitized subjects, and between EBC  $NO_2^-/NO_3^-$  levels with allergic sensitization in subjects  
273 with higher  $F_{E_{NO}}$  levels. Consistently, we found that both EBC  $NO_2^-/NO_3^-$  and  $F_{E_{NO}}$  levels  
274 increased with allergic sensitization, with the number of SPT+, and that gradual relationships  
275 were observed between sensitized subjects only and those with both allergic sensitization and  
276 rhinitis. An immediate practical utility could not be inferred from the results obtained in the  
277 framework of this epidemiologic study; but taken together, our results suggest that EBC  
278  $NO_2^-/NO_3^-$  and  $F_{E_{NO}}$  levels may be considered as biological markers of intensity of allergic  
279 sensitization and rhinitis. Longitudinal studies are also needed to better understand the role of  
280 these biomarkers, in line with the idea that part of the "allergic march" involves oxidative and  
281 nitrosative processes.

282 By considering together EBC  $NO_2^-/NO_3^-$  and  $F_{E_{NO}}$  levels rather than each alone, our results  
283 provided complementary interesting information. To go further, we also studied the  
284 association between the  $NO_x(NO_2^- + NO_3^-)/NO$  ratio and allergic sensitization.  
285 Unfortunately, this ratio was not more informative than considering the measurement of EBC  
286  $NO_2^-/NO_3^-$  alone. Contrary to our results, a ratio including also S-nitrosothiols ( $NO_2^- + NO_3^-$   
287 + S-nitrosothiols)/NO was found to better evaluate inflammation in a case-control study on  
288 asthma [13] than the measurement of each oxide of nitrogen alone. This discrepancy in the  
289 results may be partly explained by the lack of measurement of S-nitrosothiols in our study, by  
290 the fact that we studied allergic sensitization rather than inflammation, and/or by differences  
291 in study designs.

292 Overall, even if our results need to be replicated, they may suggest a role of the nitrate-nitrite-  
293 NO pathway in allergic sensitization. We suggest that exposure to allergens results in uptake

294 and proceeding by dendritic cells inducing the development of Th2 cells in sensitized  
295 individuals. Recent evidence indicates that airway epithelium also plays an important role in  
296 the allergic airway response by the release of IL-25, IL-33 and TSLP which activate dendritic  
297 cells, basophils, eosinophils and Th2 cells [31,32]. TSLP, IL-25 and IL-33 promote  
298 eosinophilia in airway mucosa by inducing IL-5 production. Eosinophilic airway  
299 inflammation may increase the NO concentration and subsequently produces the formation of  
300  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and reactive nitrogen species in EBC.

301 The results reported in this study highlight the complexity of NO metabolism. Initially  
302 considered completely inert, it is now apparent that nitrate and nitrite are physiologically  
303 recycled in blood and tissues to form NO and other bioactive nitrogen oxides [2]. They may  
304 be viewed as storage pools for NO-like bioactivity, thereby complementing the NO synthase  
305 (NOS)-dependent pathway. NO and related compounds are produced by a wide variety of  
306 residential and inflammatory cells in the respiratory tract[33]. In response to allergens, both  
307 dendritic cells (DCs) and airway epithelial cells are stimulated, and release various cytokines  
308 which activate DCs, basophils, mast cells, eosinophils and Th2 cells, leading to eosinophil  
309 activation and proliferation [34]. We previously reported that  $\text{FE}_{\text{NO}}$  level was positively  
310 associated with blood eosinophil counts [18], and there are in vitro evidences that human  
311 blood eosinophils produce NO and participate in the regulation of the NO pool in pulmonary  
312 tissues [35,36]. Moreover, NO modulates the Th1/Th2 balance by favoring Th2 response and  
313 IL-5 production and thus recruiting eosinophils into the airways. Nevertheless, even if EBC  
314  $\text{NO}_2^-/\text{NO}_3^-$  level can be viewed as a potential biological marker of allergy in our study, its  
315 specific role remains unknown, and mechanistic studies are required. As suggested through  
316 the results of the present study, and as reported by Erzurum *et al.* [37], the complexity of the  
317 nitrate-nitrite-NO pathway provide evidence that more targeted biological markers are needed  
318 to put them into a global scheme that help us to identify a type of response or phenotype for a

319 given patient, requiring the integration of multiple factors in a system biology approach.  
320 Further studies are also warranted to better investigate the associations we observed in this  
321 epidemiological study, and the potential for a practical utility of our findings.  
322 In conclusion, we report for the first time in a large epidemiological study that both total  
323  $\text{NO}_2^-/\text{NO}_3^-$  and  $\text{F}_{\text{ENO}}$  levels in exhaled breath condensate are associated with allergic  
324 sensitization and rhinitis. The role of the nitrate-nitrite-NO pathway in the "allergic march"  
325 need to be further investigated in longitudinal studies. However, contrary to what has been  
326 shown with  $\text{F}_{\text{ENO}}$ , we did not find an association of this biomarker with clinical phenotypes  
327 of asthma. Studying both exhaled fraction of NO and EBC  $\text{NO}_2^-/\text{NO}_3^-$  may be helpful for  
328 disentangle the associations between NO metabolism and asthma, allergic sensitization and  
329 rhinitis.  
330



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339

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360

361

362 **Figure legends**

363 **Figure 1. Concordance of ever asthma, allergic sensitization (SPT+) and current rhinitis**  
364 **(Proportional Venn Diagram).**

365 Data on current rhinitis was missing for four participants without allergic sensitization and  
366 asthma, one participant with asthma and one participant with allergic sensitization (n=517).

367

368 **Figure 2. Associations between Fe<sub>NO</sub>, total NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> and (NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup>)/NO ratio levels**  
369 **in exhaled breath condensate with allergic sensitization, current rhinitis and both.**

370 Regression coefficients (Beta) and 95%CI for associations between Fe<sub>NO</sub>, total NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>  
371 levels, (NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup>)/NO ratio and allergic sensitization, current rhinitis and both, estimated  
372 through GEE linear regression methods, and adjusted for covariates: age, sex, smoking,  
373 ambient ozone concentration, asthma and centre for NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> level; age, sex, smoking,  
374 height, asthma and centre for Fe<sub>NO</sub> level; age, sex, smoking, asthma and centre for (NO<sub>2</sub><sup>-</sup> +  
375 NO<sub>3</sub><sup>-</sup>)/NO ratio.

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485

**Table 1.** Characteristics of subjects according to asthma status

	All subjects N=523	Subjects without asthma N=268	Subjects with asthma N=255	P value
Age, year, mean $\pm$ SD	39.9 $\pm$ 16.6	43.2 $\pm$ 15.9	36.5 $\pm$ 16.8	<0.0001
Sex, women, %	51.0	57.5	44.3	0.003
Body mass index (BMI), kg/m <sup>2</sup> , mean $\pm$ SD	23.7 $\pm$ 3.9	23.8 $\pm$ 3.8	23.5 $\pm$ 4.0	0.4
Smoking habits, %				
Never smokers	51.6	49.6	53.7	
Ex-smokers	25.3	28.0	22.4	0.3
Current smokers	23.1	22.4	23.9	
<b>Asthma</b>				
Ever asthma, %	48.7	-	100.0	-
Current asthma, %	40.5	-	83.1	-
Age at asthma onset, %			237	
[0-4] years	-	-	36.7	
]4-16] years	-	-	39.7	-
>16 years	-	-	23.6	
Symptomatic score, <i>n</i>	517	266	251	
mean $\pm$ SD	1.17 $\pm$ 1.35	0.51 $\pm$ 0.81	1.86 $\pm$ 1.46	<0.0001
Asthma control, GINA 2006, %				

Controlled	20.6		42.3	
Partly controlled	10.7	-	22.0	-
uncontrolled	8.2		16.9	
Eosinophilia (cells>5%), %	12.4	6.7	18.4	<0.0001
Eosinophilic asthma ( $\geq 250$ cells/mm <sup>2</sup> ), %	-	-	36.5	-
FEV <sub>1</sub> % predicted, mean $\pm$ SD	103.4 $\pm$ 17.2	108.5 $\pm$ 16.6	98.0 $\pm$ 17.5	<0.0001
Methacholine test*, PD20 $\leq$ 4 mg, %	45.4	28.8	65.1	<0.0001
Inhaled corticosteroids, last 12 months, %	20.6	2.2	40.0	<0.0001
Inhaled corticosteroids, last 3 months, %	7.3	0.0	14.9	-
<b>SPT+ and current rhinitis</b>				
SPT+, %	58.1	38.8	78.4	<0.0001
SPTQ, number of SPT+, median [Q1-Q3]	1 [0-3]	0 [0-1]	2 [1-4]	<0.0001
Current rhinitis, %	39.2	22.3	56.9	<0.0001
SPT+ and Current rhinitis, %	32.9	18.0	48.6	<0.0001
<b>Biological phenotypes, GM [Q1-Q3]</b>				
EBC NO <sub>2</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup> , $\mu$ mol/mg proteins	2.25 [1.12-4.98]	2.33 [1.15-4.81]	2.16 [1.13-5.11]	0.5
FeNO, ppb	15.1 [10.0-23.0]	13.2 [9.00-18.8]	17.5 [11.5-29.6]	<0.0001
EBC (NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> )/NO ratio, median [Q1-Q3]	0.30 [0.04-0.60]	0.34 [0.06-0.61]	0.26 [0.04-0.58]	0.16
Plasma NO <sub>2</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup> , $\mu$ M	510	263	247	
	39.1 [28.0-53.7]	38.9 [26.3-56.4]	39.3 [29.1-53.2]	0.8
Eosinophils, <i>n</i>	505	256	249	

cells/mm <sup>3</sup>	170 [100-280]	145 [100-200]	199 [100-300]	<0.0001
IgE, IU/ml	81.7 [28.8-226]	49.8 [19.4-128]	137 [59.0-332]	<0.0001

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SPT+: a mean wheal diameter  $\geq 3$ mm than the negative control for at least one of 12 aeroallergens.

The symptomatic score is based on the number of asthma symptoms (wheeze and breathlessness, woken with chest tightness, woken by attack of shortness of breath, attack of shortness of breath at rest, attack of shortness of breath after exercise).

\*Methacholine challenge test was not performed if baseline FEV<sub>1</sub> <80% predicted. GM= geometric mean, Q1-Q3=first and third quartile.

**Table 2.** Pair-wise association of EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>, FeNO levels and eosinophil count in all subjects, and according to allergic sensitization

	EBC NO <sub>2</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup> level, μmol/mg proteins <sup>a</sup>				FeNO level, ppb <sup>b</sup>			
	N	Estimate	SD	p-value	N	Estimate	SD	p-value
<b>All subjects</b>								
Eosinophils, cells/mm <sup>3</sup>	492	0.14	0.09	0.10	505	<b>0.25</b>	0.04	<0.0001
FeNO level, ppb	510	0.14	0.08	0.10				
<b>Non-sensitized subjects</b>								
Eosinophils, cells/mm <sup>3</sup>	204	0.15	0.13	0.3	208	<b>0.15</b>	0.05	0.01
FeNO level, ppb	215	-0.17	0.15	0.3				
<b>Sensitized subjects</b>								
Eosinophils, cells/mm <sup>3</sup>	288	0.15	0.11	0.2	297	<b>0.31</b>	0.06	<0.0001
FeNO level, ppb	295	<b>0.21</b>	0.10	0.04				

Estimates are adjusted for <sup>a</sup>: age, sex, smoking, ambient ozone concentration, asthma and centre; <sup>b</sup>: age, sex, smoking, height, asthma and centre (GEE linear regression methods).

**Table 3.** Associations between Fe<sub>NO</sub>, total NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> and ratio levels in exhaled breath condensate with eosinophilic asthma and age at onset

	EBC NO <sub>2</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup> level, μmol/mg proteins <sup>a</sup>				Fe <sub>NO</sub> level, ppb <sup>b</sup>				(NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> )/NO ratio level <sup>c</sup>			
	N	GM	95%CI	p-value	N	GM	95%CI	p-value	N	mean	95%CI	p-value
Eosinophilic asthma, No	162	1.97	1.61;2.41		162	15.4	13.7;17.2		162	0.25	0.16;0.34	
Yes	93	2.54	1.98;3.26	0.12	93	22.0	19.0;25.5	0.0002	93	0.30	0.22;0.39	0.4
Age at onset, [0-4] years	87	2.49	1.93;3.21		87	17.7	15.1;20.6		87	0.33	0.22;0.43	
]4-16] years	94	2.09	1.60;2.75	0.3 <sup>d</sup>	94	17.8	15.3;20.7	0.7 <sup>d</sup>	94	0.25	0.14;0.35	0.4 <sup>d</sup>
>16 years	56	1.99	1.38;2.87		56	16.8	13.6;20.8		56	0.26	0.11;0.41	
<b>Model adjusted for all covariates</b>												
Eosinophilic asthma, No	157	1.79	1.35;2.37		162	16.8	14.6;19.4		162	0.20	0.09;0.32	
Yes	92	2.30	1.66;3.18	0.13	93	23.9	20.2;28.3	<0.0001	93	0.27	0.14;0.40	0.3
Age at onset, [0-4] years	84	2.26	1.60;3.20		87	18.8	15.6;22.6		87	0.30	0.17;0.43	
]4-16] years	93	1.91	1.37;2.67	0.15 <sup>d</sup>	94	19.4	16.3;23.1	0.9 <sup>d</sup>	94	0.21	0.08;0.34	0.15 <sup>d</sup>
>16 years	56	1.59	1.02;2.00		56	18.4	14.6;23.3		56	0.17	0.01;0.34	

Results are expressed as GM or mean (and 95%CI).

Abbreviations: GM: geometric mean; CI: Confidence Interval; EBC: Exhaled Breath Condensate; NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>: nitrite/nitrate.

<sup>a</sup>: adjusted for age, sex, smoking, ambient ozone concentration, asthma and centre; <sup>b</sup>: adjusted for age, sex, smoking, height, asthma and centre (GEE regression methods); <sup>c</sup>: adjusted for age, sex, smoking, asthma and centre; <sup>d</sup>: p-value for trend.

**Table 4.** Associations between Fe<sub>NO</sub>, total NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> and ratio levels in exhaled breath condensate with allergic sensitization

	EBC NO <sub>2</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup> level, μmol/mg proteins <sup>a</sup>				Fe <sub>NO</sub> level, ppb <sup>b</sup>				(NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> )/NO ratio level <sup>c</sup>			
	N	GM	95%CI	p-value	N	GM	95%CI	p-value	N	mean	95%CI	p-value
Allergic sensitization, No	219	2.03	1.73;2.38		219	12.6	11.6;13.6		219	0.29	11.6;13.6	
Yes	304	2.41	2.11;2.76	0.10	304	17.3	15.9;18.8	<0.0001	304	0.31	15.9;18.8	0.7
Current rhinitis No	312	2.20	1.93;2.51		312	13.8	12.8;14.8		312	0.30	0.25;0.36	
Yes	205	2.30	1.94;2.72	0.7	205	17.7	16.1;19.6	<0.0001	205	0.29	0.22;0.36	0.8
SPT+ and Current rhinitis, No	219	2.03	1.73;2.38		219	12.6	11.6;13.6		219	0.29	0.22;0.36	
Yes	131	2.34	1.90;2.89	0.10 <sup>d</sup>	131	15.9	14.0;18.0	<0.0001 <sup>d</sup>	131	0.29	0.21;0.38	0.6 <sup>d</sup>
SPT+ Both	172	2.47	2.07;2.94		172	18.4	16.5;20.5		172	0.32	0.25;0.39	
<b>Model adjusted for centre only</b>												
Allergic sensitization, No	219	1.87	1.55; 2.26		219	14.2	12.8; 15.7		219	0.25	0.17;0.33	
Yes	304	2.26	1.92; 2.66	0.06	304	19.4	17.7; 21.1	<0.0001	304	0.27	0.20;0.34	0.6
Current rhinitis No	312	1.97	1.66;2.33		312	15.2	13.9;16.7		312	0.25	0.18;0.32	
Yes	205	2.26	1.87;2.72	0.19	205	20.1	18.2;22.2	<0.0001	205	0.27	0.19;0.35	0.7
SPT+ and Current rhinitis, No	219	1.87	1.55;2.26		219	14.2	12.8;15.7		219	0.25	0.17;0.33	
Yes	131	2.12	1.70;2.64	0.04 <sup>d</sup>	131	17.5	15.6;19.8	<0.0001 <sup>d</sup>	131	0.24	0.15;0.34	0.4 <sup>d</sup>
SPT+ Both	172	2.37	1.94;2.89		172	20.7	18.6;23.0		172	0.29	0.20;0.37	

**Model adjusted for all covariates**

Allergic sensitization, No	215	1.72	1.38; 2.14		219	14.8	13.3; 16.5		219	0.21	0.13; 0.30	
Yes	295	2.36	1.96; 2.84	0.008	304	18.3	16.7; 20.0	0.0006	304	0.29	0.22; 0.36	0.10
Current rhinitis No	305	1.90	1.57; 2.31		312	15.4	14.0; 16.9		312	0.24	0.16; 0.31	
Yes	199	2.31	1.87; 2.84	0.09	205	19.2	17.4; 21.2	0.0001	205	0.28	0.20; 0.36	0.3
SPT+ and Current rhinitis, No	215	1.71	1.37; 2.13		219	14.8	13.3; 16.5		219	0.21	0.12; 0.29	
SPT+	128	2.17	1.70; 2.76	0.005 <sup>d</sup>	131	16.4	14.6; 18.4	<0.0001 <sup>d</sup>	131	0.26	0.17; 0.36	0.06 <sup>d</sup>
Both	166	2.50	2.01; 3.12		172	19.7	17.7; 21.9		172	0.32	0.23; 0.40	
Allergic sensitization (Indoor only)												
No	215	1.66	1.28; 2.16		219	14.2	12.7; 15.9		219	0.21	0.10; 0.31	
Yes	76	2.03	1.47; 2.81	0.2	80	17.1	14.8; 19.7	0.02	80	0.26	0.13; 0.40	0.4
Allergic sensitization (Outdoor only)												
No	215	1.66	1.28; 2.15		219	14.6	13.2; 16.3		219	0.20	0.09; 0.31	
Yes	45	1.78	1.21; 2.62	0.7	46	17.2	14.6; 20.2	0.06	46	0.20	0.03; 0.36	0.9
Allergic sensitization (Molds only)												
No	217	1.58	1.20; 2.06		219	14.5	13.0; 16.2		219	0.18	0.07; 0.30	
Yes	7	3.96	1.69; 9.29	0.04	7	21.2	14.6; 30.6	0.06	7	0.49	0.11; 0.87	0.12

Results are expressed as GM or mean (and 95% CI).

Abbreviations: GM: geometric mean; CI: Confidence Interval; EBC: Exhaled Breath Condensate; NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>: nitrite/nitrate.

<sup>a</sup>: adjusted for age, sex, smoking, ambient ozone concentration, asthma and centre; <sup>b</sup>: adjusted for age, sex, smoking, height, asthma and centre (GEE regression methods); <sup>c</sup>: adjusted for age, sex, smoking, asthma and centre; <sup>d</sup>: p value for trend.



**Figure 1**

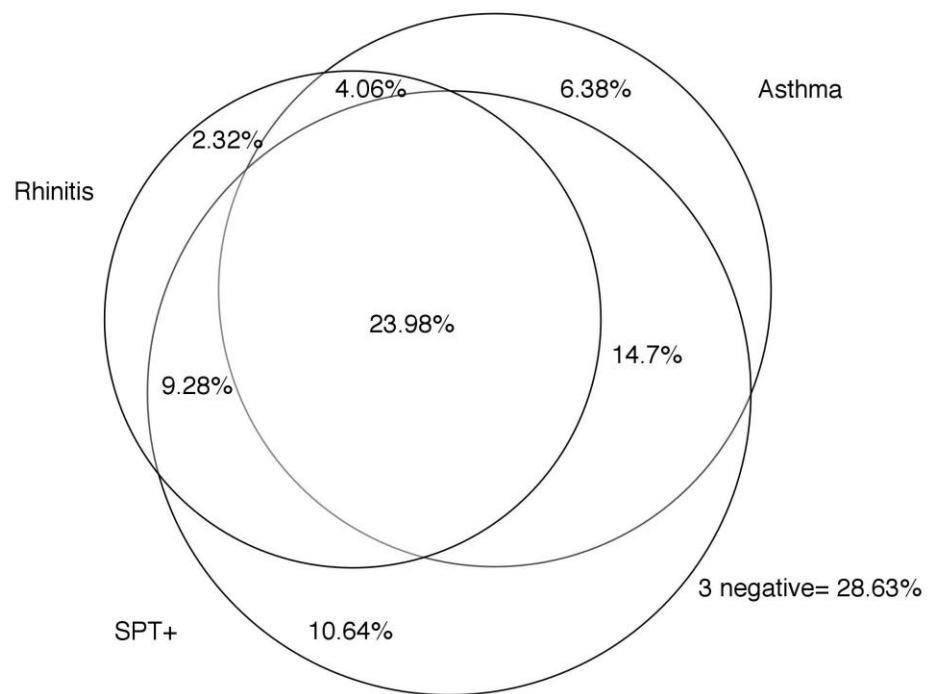


Fig 2

