

# Exhaled nitric oxide, nitrite/nitrate levels, allergy, rhinitis and asthma in the EGEA study.

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

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- 25 None.
- 26
- 27 Abstract

**Background:** Although interest in biomarkers in the nitrate-nitrite-NO pathway has recently increased, associations between nitrite  $(NO_2^-)$ , nitrate  $(NO_3^-)$  and asthma, allergic sensitization and rhinitis remain unclear.

31 **Objective:** To evaluate the associations between  $NO_2^{-}/NO_3^{-}$  and exhaled fraction of nitric 32 oxide (FE<sub>NO</sub>) levels with asthma, allergic sensitization and rhinitis.

33 **Methods:** Plasma and exhaled breath condensate (EBC)  $NO_2^{-}/NO_3^{-}$  and  $FE_{NO}$  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.

**Results:** Plasma  $NO_2^{-}/NO_3^{-}$  level was unrelated to any disease phenotypes. EBC  $NO_2^{-}/NO_3^{-}$ level was unrelated to any asthma phenotypes. EBC  $NO_2^{-}/NO_3^{-}$  and  $FE_{NO}$  levels were correlated in sensitized subjects only ( $r=0.21\pm0.10$ , p=0.01). EBC  $NO_2^{-}/NO_3^{-}$  and  $FE_{NO}$  levels were higher in sensitized than in non-sensitized subjects (adjusted GM (95%CI): 2.36 (1.96; 2.84) *vs*.1.72 (1.38; 2.14) µmol/mg proteins, p=0.008; and 18.3 (16.7; 20.0) *vs*.14.8 (13.3; 16.5) ppb, p=0.0006 respectively), with gradual relationships from sensitized subjects to those with allergic rhinitis (p<0.0001).

45 **Conclusion**: Results suggest that EBC  $NO_2^{-}/NO_3^{-}$  and FeNO levels may be considered as 46 biological markers of intensity of allergic sensitization and rhinitis.

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

#### 51 Introduction

The interest in measuring biological markers in exhaled breath condensate (EBC) in epidemiological studies on respiratory diseases has increased in the last years. Among the pathways involved in the pathophysiology of asthma, the metabolism of nitric oxide (NO) also called the nitrate-nitrite-NO pathway has taken a growing place in this research field [1]. The NO metabolism is complex, and both NO measured by the exhaled fraction of NO (FE<sub>NO</sub>) and NO-related compounds such as nitrites (NO<sub>2</sub><sup>-</sup>) and nitrates (NO<sub>3</sub><sup>-</sup>) are relevant biological markers that may help to better understand the patho-physiology of asthma and allergy [2].

59 FE<sub>NO</sub> 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 increased level of FE<sub>NO</sub> in children and adults with asthma, and positive associations between 61 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<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> and FE<sub>NO</sub> 66 levels in the same subjects. Recently, in a large number of adults from the French Epidemiological study on Genetics and Environment of Asthma (EGEA), EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> 67 and FE<sub>NO</sub> levels were found to be correlated in subjects without asthma [18]. 68

Nitric oxide has different functions and roles in pathophysiology, which may be better explained by considering its compartmentalized production [19]. In this study, we compared the association between total  $NO_2^{-}/NO_3^{-}$  levels measured in two compartments (plasma and exhaled breath condensate) and  $FE_{NO}$  levels with asthma, allergic sensitization and rhinitis among 523 adults from the EGEA study. We hypothesized that the associations will be different depending on the compartments, biomarkers and outcomes studied.

#### 76 Methods

#### 77 Study design

Data used for the analyses were collected in the framework of the 12-year follow-up of 78 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  $NO_2^{-}/NO_3^{-}$  and  $FE_{NO}$  levels (n=523). Subjects included in the analyses were younger, 90 reported more often ever asthma and current rhinitis, and had higher levels of NO<sub>2</sub>-/NO<sub>3</sub>- 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).

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

#### 98 **Respiratory phenotypes**

99 Subjects with ever asthma were defined by a positive answer to either: "*Have you ever had 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  $\geq$ 3mm than the negative control for at least one of 12 aeroallergens (indoor: cat, 104 Dermatophagoides pteronyssinus, Blattela germanica, outdoor: olive, birch, Parieteria 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 you ever had rhinitis?" or " Have you ever had hay fever?" and a positive answer to "have 108 109 vou 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).

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

115

#### 116 Biological phenotypes

Exhaled breath condensate (EBC) was collected with an RTube<sup>™</sup> according a standardized method. 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 (see supplementary data for more details). Total nitrite-nitrate  $(NO_2^{-}/NO_3^{-})$  levels were measured in plasma and EBC as previously described [24]. 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 (see supplementary data for more details).

Measurements of  $FE_{NO}$  were realized before other pulmonary function tests according to ATS/ERS recommendations (see supplementary data). The measurement was performed only in 3 of the 5 centers involved in the EGEA study, which explained in a large part the attrition on numbers of subjects included in the analysis compared to the total number.  $FE_{NO}$  level was measured at 50mL/s flow rate as previously described [23].

132

#### 133 Statistical Methods

Joint distribution of asthma, SPT+ and current rhinitis was shown with a Venn diagram (Figure 1). Total plasma and EBC  $NO_2^{-}/NO_3^{-}$  and  $FE_{NO}$  levels were log10-transformed as a result of their skewed distribution.

137 In the same study, we previously reported that plasma NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> level was increased with 138 leafy vegetable consumption and decreased in smokers and with storage time, that EBC 139 NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> level was decreased in smokers and with exposure to ambient ozone 140 concentration [24], and that FE<sub>NO</sub> 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  $NO_2^{-}/NO_3^{-}$ , 2) age, sex, smoking, ambient ozone concentration and centre for EBC 143  $NO_2^{-}/NO_3^{-}$ , and 3) age, sex, height, smoking and centre for FE<sub>NO</sub>. Since the ratio of higher 144 oxides of nitrogen (HiNOx including  $NO_2^-$  and  $NO_3^-$ ) to NO was reported be more 145 informative than each measurement alone by Nguyen *et al.* [13], the  $(NO_2^- + NO_3^-)/NO$  ratio 146 147 (NOx/NO ratio) has also been studied.

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

Associations between total NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels, FE<sub>NO</sub> levels, and the NOx ratio (NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup>)/NO and asthma phenotypes, allergic sensitization and current rhinitis were estimated with linear regression models. Parameter estimates were assessed by using generalized estimating equations, with an exchangeable working correlation to account for the potential clustering within families (SAS MIXED procedure). The level of statistical significance was set at  $\alpha$ =0.05. Two-sided P values were reported for all association estimates. All analyses 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<sub>1</sub>% 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<sub>NO</sub> 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).

#### 173 Pairwise association between EBC $NO_2^{-}/NO_3^{-}$ , $FE_{NO}$ levels and blood eosinophil counts

174 EBC  $NO_2^{-}/NO_3^{-}$  levels were unrelated with EOS, whereas  $FE_{NO}$  levels were positively 175 associated with EOS in all subjects, both in non-sensitized and sensitized subjects (Table 2). 176 In sensitized subjects, EBC  $NO_2^{-}/NO_3^{-}$  was positively associated with  $FE_{NO}$  levels.

The median  $FE_{NO}$  value in the population was 15.6 ppb (range 2.4 to 99.0 ppb). Stratification according to this median value showed positive and significant association between EBC  $NO_2^{-}/NO_3^{-}$  level and allergic sensitization in subjects above the median only (2.66 (2.06-3.43) *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 above and below the median respectively).

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#### 183 Plasma and EBC $NO_2^{-}/NO_3^{-}$ , $FE_{NO}$ levels and asthma and asthma-related phenotypes

Both plasma and EBC  $NO_2^{-}/NO_3^{-}$  levels were unrelated to ever asthma, current asthma, symptomatic score, and asthma control (data not shown, all P values>0.3). Furthermore, plasma  $NO_2^{-}/NO_3^{-}$  level was unrelated to allergic sensitization and current rhinitis.

Both plasma and EBC  $NO_2^{-}/NO_3^{-}$  levels were unrelated to eosinophilic asthma nor to age at asthma onset (Table 3). As expected, a positive and significant association was observed between  $FE_{NO}$  level and eosinophilic asthma (Table 3) but no other significant association was observed.

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#### 192 **EBC** $NO_2^{-}/NO_3^{-}$ and $FE_{NO}$ levels, NOx/NO ratio and allergic sensitization

A positive association at borderline significance was observed between EBC  $NO_2^{-}/NO_3^{-}$  level and SPT+ (see Table 4). In a model adjusted for covariates, including asthma, EBC  $NO_2^{-}/NO_3^{-}$  level was positively and significantly associated with SPT+, and a positive association at borderline significance was observed with current rhinitis. Furthermore, positive and gradual increases in EBC  $NO_2^{-}/NO_3^{-}$  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 NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> value in our population was 2.25 µmol/mg proteins (range 1.12 to 4.98, Table 1). Stratification according to this median value showed positive and significant 201 202 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 adjustement for age, sex, smoking, ambient ozone concentration, asthma and centre. 206

Similarly to EBC  $NO_2^{-}/NO_3^{-}$ ,  $FE_{NO}$  level was also positively related to allergic sensitization expressed as SPT+ (Table 4), and gradually increased with SPTQ (see Figure online for details), and with allergic sensitization and current rhinitis.  $FE_{NO}$  was also significantly and positively associated with current rhinitis. The associations between  $FE_{NO}$  levels and SPT+, and current rhinitis were confirmed when BMI instead of height was added as covariate in the models (data not shown).

No significant association were observed between the  $(NO_2^- + NO_3^-)/NO$  ratio (NOx/NOratio) and SPT+, current rhinitis or both (Table 4 and Figure 2). When analysing SPT+ to indoor, outdoor or molds allergens separately, EBC  $NO_2^-/NO_3^-$  levels showed positive and significant associations with sensitization to molds allergens, and FE<sub>NO</sub> levels were positively associated to indoor allergens (Table 4).

#### 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 sensitization and rhinitis. Results showed higher EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>and FE<sub>NO</sub> levels in subjects 223 224 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<sub>2</sub>/NO<sub>3</sub> and FE<sub>NO</sub> levels were 226 positively associated in sensitized subjects only, and EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> levels were found to be 227 associated with allergic sensitization in subjects with higher FE<sub>NO</sub> 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<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> and FE<sub>NO</sub> levels than non-selected 239 subjects. Other limitations of the present study were those commonly related to cross-240 sectional analyses of the data.

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

plasma differs from that in EBC due to their compartmentalization. In plasma NO2-/NO3-244 production derives from several sources, such as bacteria, enzymatic production and dietary 245 246 sources [25]. In EBC ionized NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> (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 NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> 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 NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> 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 methodologies used for measuring  $NO_2^-$  and  $NO_3^-$  levels, the choice of the reference group 257 258 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 NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> 261 level could be more related to allergy than to asthma. Furthermore, none of these previous 262 studies have expressed the  $NO_2^{-}/NO_3^{-}$  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  $NO_2^{-}/NO_3^{-}$  level in EBC was positively related to protein concentration in our study [29]. 266

As reported in the literature [4,30], positive associations between  $FE_{NO}$  level and asthma, 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 FENO and exhaled breath condensate  $NO_2^{-}/NO_3^{-}$  levels with asthma, allergic sensitization and 270 current rhinitis. We found positive associations between EBC  $NO_2^{-}/NO_3^{-}$  and  $FE_{NO}$  levels in 271 sensitized subjects, and between EBC  $NO_2^{-}/NO_3^{-}$  levels with allergic sensitization in subjects 272 with higher  $FE_{NO}$  levels. Consistently, we found that both EBC  $NO_2^{-}/NO_3^{-}$  and  $FE_{NO}$  levels 273 274 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  $NO_2^{-}/NO_3^{-}$  and  $FE_{NO}$  levels may be considered as biological markers of intensity of allergic 278 279 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  $FE_{NO}$  levels rather than each alone, our results 283 provided complementary interesting information. To go further, we also studied the association between the  $NOx(NO_2^- + NO_3^-)/NO$  ratio and allergic sensitization. 284 285 Unfortunately, this ratio was not more informative than considering the measurement of EBC  $NO_2^{-}/NO_3^{-}$  alone. Contrary to our results, a ratio including also S-nitrosothiols ( $NO_2^{-} + NO_3^{-}$ 286 287 + 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.

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

and proceeding by dendritic cells inducing the development of Th2 cells in sensitized individuals. Recent evidence indicates that airway epithelium also plays an important role in the allergic airway response by the release of IL-25, IL-33 and TSLP which activate dendritic cells, basophils, eosinophils and Th2 cells [31,32]. TSLP, IL-25 and IL-33 promote eosinophilia in airway mucosa by inducing IL-5 production. Eosinophilic airway inflammation may increase the NO concentration and subsequently produces the formation of  $NO_2^-$ ,  $NO_3^-$  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 FE<sub>NO</sub> 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  $NO_2^{-}/NO_3^{-}$  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 given patient, requiring the integration of multiple factors in a system biology approach.
Further studies are also warranted to better investigate the associations we observed in this
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  $NO_2^{-}/NO_3^{-}$  and  $FE_{NO}$  levels in exhaled breath condensate are associated with allergic 323 324 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 FENO, we did not find an association of this biomarker with clinical phenotypes of asthma. Studying both exhaled fraction of NO and EBC NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> may be helpful for 327 328 disentangle the associations between NO metabolism and asthma, allergic sensitization and 329 rhinitis.

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339

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360

362 **Figure legends** 

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

365 Data on current rhinitis was missing for four participants without allergic sensitization and

asthma, one participant with asthma and one participant with allergic sensitization (n=517).

367

# Figure 2. Associations between $Fe_{NO}$ , total $NO_2^-/NO_3^-$ and $(NO_2^- + NO_3^-)/NO$ ratio levels in exhaled breath condensate with allergic sensitization, current rhinitis and both.

370 Regression coefficients (Beta) and 95%CI for associations between  $Fe_{NO}$ , total  $NO_2^{-}/NO_3^{-}$ 

371 levels,  $(NO_2^- + NO_3^-)/NO$  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  $NO_2^-/NO_3^-$  level; age, sex, smoking, 374 height, asthma and centre for Fe<sub>NO</sub> level; age, sex, smoking, asthma and centre for  $(NO_2^- +$ 375  $NO_3^-)/NO$  ratio.

376

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<b>Table 1.</b> Characteristics of subjects according to asthma st
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	All subjects N=523	Subjects without asthma N=268	Subjects with asthma N=255	P value
Age, year, mean $\pm$ SD	39.9 ± 16.6	43.2 ± 15.9	36.5 ± 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	$23.7\pm3.9$	$23.8\pm3.8$	$23.5\pm4.0$	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	
Asthma				
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, n	517	266	251	
mean $\pm$ SD	$1.17 \pm 1.35$	$0.51\pm0.81$	$1.86 \pm 1.46$	< 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 (≥250 cells/mm <sup>2</sup> ), %	-	-	36.5	-
FEV <sub>1</sub> % predicted, mean $\pm$ SD	$103.4\pm17.2$	$108.5\pm16.6$	$98.0 \pm 17.5$	< 0.0001
Methacholine test*, PD20≤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	-
SPT+ and current rhinitis				
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
Biological phenotypes, GM [Q1-Q3]				
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 $(NO_2^- + NO_3^-)/NO$ 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 $NO_2^{-}/NO_3^{-}$ , $\mu M$	510	263	247	
	39.1 [28.0-53.7]	38.9 [26.3-56.4]	39.3 [29.1-53.2]	0.8
Eosinophils, n	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

SPT+: a mean wheal diameter  $\geq$ 3mm 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  $FEV_1 < 80\%$  predicted. GM= geometric mean, Q1-Q3=first and third quartile.

	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>			
	Ν	Estimat	e SD	p-value	Ν	Estimate	SD	p-value		
All subjects										
Eosinophils, cells/mm <sup>3</sup>	492	0.1	4 0.09	0.10	505	0.25	0.04	< 0.0001		
Fe <sub>NO</sub> level, ppb	510	0.14	4 0.08	0.10						
Non-sensitized subjects										
Eosinophils, cells/mm <sup>3</sup>	204	0.1	5 0.13	0.3	208	0.15	0.05	0.01		
Fe <sub>NO</sub> level, ppb	215	-0.1	7 0.15	0.3						
Sensitized subjects										
Eosinophils, cells/mm <sup>3</sup>	288	0.1	5 0.11	0.2	297	0.31	0.06	< 0.0001		
Fe <sub>NO</sub> level, ppb	295	0.2	<b>1</b> 0.10	0.04						

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

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).

	EBC $NO_2^{-}/NO_3^{-}$ level,					Fe <sub>NO</sub> level, ppb <sup>b</sup>				(NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> )/NO			
	µmol/mg proteins <sup>a</sup>									ratio level <sup>c</sup>			
		Ν	GM	95%CI	p-value	Ν	GM	95%CI	p-value	Ν	mean	95%CI	p-value
Eosinophilic as	sthma, 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	
Model adjuste	ed for all covaria	ites											
Eosinophilic as	sthma, 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	

**Table 3.** Associations between  $Fe_{NO}$ , total  $NO_2^{-}/NO_3^{-}$  and ratio levels in exhaled breath condensate with eosinophilic asthma and age at onset

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

Abbreviations: GM: geometric mean; CI: Confidence Interval; EBC: Exhaled Breath Condensate;  $NO_2^-/NO_3^-$ : 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.

		EBC	NO <sub>2</sub> <sup>-</sup> /N	NO <sub>3</sub> <sup>–</sup> level,	Fe <sub>NO</sub> level, ppb <sup>b</sup>					(NO <sub>2</sub> <sup>-</sup> + NO <sub>3</sub> <sup>-</sup> )/NO			
		μmol	/mg pro	oteins <sup>a</sup>						ratio level <sup>c</sup>			
		Ν	GM	95%CI	p-value	Ν	GM	95%CI	p-value	Ν	mean	95%CI	p-value
Allergic sensitization	on,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 r	hinitis,	219	2.03	1.73;2.38		219	12.6	11.6;13.6		219	0.29	0.22;0.36	
	No	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+	172	2.47	2.07;2.94		172	18.4	16.5;20.5		172	0.32	0.25;0.39	
Both													
Model adjusted for	r centre onl	у											
Allergic sensitization	on,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,		219	1.87	1.55;2.26		219	14.2	12.8;15.7		219	0.25	0.17;0.33	
	No	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+	172	2.37	1.94;2.89		172	20.7	18.6;23.0		172	0.29	0.20;0.37	
	Both												

**Table 4.** Associations between  $Fe_{NO}$ , total  $NO_2^{-}/NO_3^{-}$  and ratio levels in exhaled breath condensate with allergic sensitization

#### Model adjusted for all covariates

Allergic sensitization	n,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 rh	initis,	215	1.71	1.37;2.13		219	14.8	13.3;16.5		219	0.21	0.12;0.29	
	No	128	2.17	1.70;2.76	$0.005^{\ d}$	131	16.4	14.6;18.4	<0.0001 <sup>d</sup>	131	0.26	0.17;0.36	0.06 <sup>d</sup>
	SPT+	166	2.50	2.01;3.12		172	19.7	17.7;21.9		172	0.32	0.23;0.40	
	Both												
Allergic sensitization (Indoor o		ly)											
	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	n (Outdoor o	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 o		ly)											
	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;  $NO_2^{-}/NO_3^{-}$ : 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.







