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Conflict of interest

None.

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.

Objective: To evaluate the associations between NO_2^-/NO_3^- and exhaled fraction of nitric oxide (FE_{NO}) levels with asthma, allergic sensitization and rhinitis.

Methods: Plasma and exhaled breath condensate (EBC) NO_2^-/NO_3^- and FE_{NO} levels were measured in 523 adults of the French Epidemiological study on Genetics and Environment of Asthma. Allergic sensitization was defined by a positive skin prick test for at least one aeroallergen. Subjects were classified as non-sensitized, sensitized and as having allergic 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±0.10, 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).

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

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

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_{NO}) and NO-related compounds such as nitrites (NO_2^-) and nitrates (NO_3^-) are relevant biological markers that may help to better understand the patho-physiology of asthma and allergy [2].

FE_{NO} is the most studied one, and it is commonly considered as a non-invasive indirect marker of airway inflammation [3]. Both epidemiological and clinical studies in adults showed increased level of FE_{NO} in children and adults with asthma, and positive associations between FE_{NO} and allergic sensitization are consistent over the studies, regardless of rhinitis or asthma [4]. Studies on associations between NO_2^- and NO_3^- levels with asthma, allergy or rhinitis have led to more conflicting results both in adults and children [5–17]. Until now, none of these studies has simultaneously performed measurements of EBC NO_2^-/NO_3^- and FE_{NO} 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_2^-/NO_3^- and FE_{NO} levels were found to be correlated in subjects without asthma [18].

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.

Methods

Study design

Data used for the analyses were collected in the framework of the 12-year follow-up of EGEA. EGEA is a French cohort study based on an initial group of asthma cases and their first-degree relatives, and controls (first survey, n=2047) [20]. The protocol and descriptive characteristics have been described previously [21,22]. A follow-up of the initial cohort was conducted between 2003 and 2007 [23]. Among the alive cohort (n=2002), 92% (n=1845) completed a short self-administered questionnaire and among them 1601 had a complete examination. All subjects responded to a questionnaire based on international standardized tools to diagnose asthma and to determine respiratory and allergic symptoms, treatments, and environmental exposures. The present cross-sectional analysis includes those who were adults at the second survey (≥ 16 years old, n=1570 adults) with available data on asthma, current rhinitis, allergic sensitization, and available measurements of exhaled breath condensate NO_2^-/NO_3^- and F_{ENO} levels (n=523). Subjects included in the analyses were younger, reported more often ever asthma and current rhinitis, and had higher levels of NO_2-/NO_3- and Immunoglobulin E (IgE) than those not included in the analyses (n=1047). The two groups were similar for sex, smoking, current asthma status, allergic sensitization, lung function tests, 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.

Respiratory phenotypes

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 if they were recruited as asthmatic cases at the first survey.

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 (indoor: cat, *Dermatophagoides pteronyssinus*, *Blattela germanica*, outdoor: olive, birch, *Parieteria judaica*, timothy grass, *Cupressus and* ragweed pollen, and molds: *Aspergillus*, *Cladosporium herbarum*, *Alternaria tenuis*). Subjects were classified as sensitized if they have one or more SPT+. 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).

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

Biological phenotypes

Exhaled breath condensate (EBC) was collected with an RTube™ 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].

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.

In the same study, we previously reported that plasma NO_2^-/NO_3^- level was increased with leafy vegetable consumption and decreased in smokers and with storage time, that EBC NO_2^-/NO_3^- level was decreased in smokers and with exposure to ambient ozone concentration [24], and that FE_{NO} level was associated with season of examination [23]. Furthermore, storage time and season of examination varied with centre. Therefore estimates 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 NO_2^-/NO_3^-, and 3) age, sex, height, smoking and centre for FE_{NO}. Since the ratio of higher oxides of nitrogen (HiNOx including NO_2^- and NO_3^-) to NO was reported be more informative than each measurement alone by Nguyen *et al.* [13], the $(NO_2^- + NO_3^-)/NO$ ratio (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_2^-/NO_3^- levels, FE_{NO} levels, and the NOx ratio (NO_2^- + NO_3^-)/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 α=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).

Results

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

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

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

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.

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_2^-/NO_3^- value in our population was 2.25 µmol/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 adjustement for age, sex,
206 smoking, ambient ozone concentration, asthma and centre.
207 Similarly to EBC NO_2^-/NO_3^-, FE_{NO} 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. FE_{NO} was also significantly and
210 positively associated with current rhinitis. The associations between FE_{NO} 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 $(NO_2^- + NO_3^-)/NO$ ratio (NOx/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 NO_2^-/NO_3^- levels showed positive and
216 significant associations with sensitization to molds allergens, and FE_{NO} levels were positively
217 associated to indoor allergens (Table 4).
218

Discussion

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

The selection of the 523 subjects included in the present analyses was driven first by the random availability of the FE_{NO} measurements in three of the five participating centres [23], and secondly by the availability of the other variables of interest. Definition of asthma case is very precise in our study since asthmatic cases were recruited in chest clinics, and a procedure was set up to include true asthmatics, leading to a very limited risk of false positives. Prevalence of bronchial hyper-responsiveness, measured by a methacholine challenge test was quite high in subjects without asthma. A possible explanation is that part of the subjects without asthma are first degree relatives of asthma cases. Nevertheless, this result is consistent with the relatively considerable number of asymptomatic subjects with BHR reported in cross-sectional epidemiologic studies, ranging from 19.3 to 62.4%. Subjects included in the analyses had higher EBC $NO_2^-/NO_3^-$and FE_{NO} levels than non-selected subjects. Other limitations of the present study were those commonly related to cross-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 NO_2^-/NO_3^- production derives from several sources, such as bacteria, enzymatic production and dietary sources [25]. In EBC ionized NO_3^- and NO_2^- (not volatile) may arise from NO after reaction with oxygen [26] or from activated immune cells present in the lining fluid of the lungs [27]. Overall, the specificities of the NO metabolism in plasma and in EBC may partly explain the lack of association with any clinical phenotypes in plasma. Our results are consistent with the hypotheses of Villanueva and Giulivi [19], for whom the compartmentalized production of NO better explains its different functions and roles in pathophysiology.

No association was found between total NO_2^-/NO_3^- level in EBC and asthma phenotypes, as previously reported in other studies [8,14]. Contrary to our results, other studies have reported total NO_2^-/NO_3^- level in EBC to be elevated in subjects with asthma as compared to healthy non-smoking subjects [17], healthy non-atopic controls [5], or controls [9]. These conflicting 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 for comparisons and other differences such as those related to phenotypes definition. Beside, information regarding allergic sensitization was not available or subjects were defined as asthmatics if they had both asthma and allergy, suggesting that the increase in NO_2^-/NO_3^- level could be more related to allergy than to asthma. Furthermore, none of these previous studies have expressed the NO_2^-/NO_3^- level divided by the amount of proteins. As reported by Gessner and Wirtz [28], the measurement of total protein in EBC is important to confirm that protein and peptide markers are comparable between studies. They should always be 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].

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

study reported for the first time similarities and differences for the associations of both FE_{NO} and exhaled breath condensate NO_2^-/NO_3^- levels with asthma, allergic sensitization and current rhinitis. We found positive associations between EBC NO_2^-/NO_3^- and FE_{NO} levels in sensitized subjects, and between EBC NO_2^-/NO_3^- levels with allergic sensitization in subjects with higher FE_{NO} levels. Consistently, we found that both EBC NO_2^-/NO_3^- and FE_{NO} levels increased with allergic sensitization, with the number of SPT+, and that gradual relationships were observed between sensitized subjects only and those with both allergic sensitization and rhinitis. An immediate practical utility could not be inferred from the results obtained in the 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 sensitization and rhinitis. Longitudinal studies are also needed to better understand the role of these biomarkers, in line with the idea that part of the "allergic march" involves oxidative and nitrosative processes.

By considering together EBC NO_2^-/NO_3^- and FE_{NO} levels rather than each alone, our results provided complementary interesting information. To go further, we also studied the association between the $NOx(NO_2^- + NO_3^-)/NO$ ratio and allergic sensitization. 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 ($NO_2^- + NO_3^-$ + S-nitrosothiols)/NO was found to better evaluate inflammation in a case-control study on asthma [13] than the measurement of each oxide of nitrogen alone. This discrepancy in the results may be partly explained by the lack of measurement of S-nitrosothiols in our study, by the fact that we studied allergic sensitization rather than inflammation, and/or by differences 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.

The results reported in this study highlight the complexity of NO metabolism. Initially considered completely inert, it is now apparent that nitrate and nitrite are physiologically recycled in blood and tissues to form NO and other bioactive nitrogen oxides [2]. They may be viewed as storage pools for NO-like bioactivity, thereby complementing the NO synthase (NOS)-dependent pathway. NO and related compounds are produced by a wide variety of residential and inflammatory cells in the respiratory tract[33]. In response to allergens, both dendritic cells (DCs) and airway epithelial cells are stimulated, and release various cytokines which activate DCs, basophils, mast cells, eosinophils and Th2 cells, leading to eosinophil activation and proliferation [34]. We previously reported that FE_{NO} level was positively associated with blood eosinophil counts [18], and there are in vitro evidences that human blood eosinophils produce NO and participate in the regulation of the NO pool in pulmonary tissues [35,36]. Moreover, NO modulates the Th1/Th2 balance by favoring Th2 response and IL-5 production and thus recruiting eosinophils into the airways. Nevertheless, even if EBC NO_2^-/NO_3^- level can be viewed as a potential biological marker of allergy in our study, its specific role remains unknown, and mechanistic studies are required. As suggested through the results of the present study, and as reported by Erzurum *et al.* [37], the complexity of the nitrate-nitrite-NO pathway provide evidence that more targeted biological markers are needed 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.

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 sensitization and rhinitis. The role of the nitrate-nitrite-NO pathway in the "allergic march" need to be further investigated in longitudinal studies. However, contrary to what has been 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_2^-/NO_3^- may be helpful for disentangle the associations between NO metabolism and asthma, allergic sensitization and rhinitis.

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Figure legends

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

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).

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.

Regression coefficients (Beta) and 95%CI for associations between Fe_{NO}, total NO_2^-/NO_3^- levels, $(NO_2^- + NO_3^-)/NO$ ratio and allergic sensitization, current rhinitis and both, estimated through GEE linear regression methods, and adjusted for covariates: age, sex, smoking, ambient ozone concentration, asthma and centre for NO_2^-/NO_3^- level; age, sex, smoking, height, asthma and centre for Fe_{NO} level; age, sex, smoking, asthma and centre for $(NO_2^- + NO_3^-)/NO$ ratio.

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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 ± 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/m², mean ± SD	23.7 ± 3.9	23.8 ± 3.8	23.5 ± 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	
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 ± SD	1.17 ± 1.35	0.51 ± 0.81	1.86 ± 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/mm²), %	-	-	36.5	-
FEV_1 % predicted, mean ± SD	103.4 ± 17.2	108.5 ± 16.6	98.0 ± 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_2^-/NO_3^-, µmol/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^-, µM	*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^3	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 ≥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.

Table 2. Pair-wise association of EBC NO_2-NO_3 , FeNO levels and eosinophil count in all subjects, and according to allergic sensitization

	EBC NO_2^-/NO_3^- level, µmol/mg proteins [a]				**Fe_{NO} level, ppb [b]**			
	N	Estimate	SD	p-value	N	Estimate	SD	p-value
All subjects								
Eosinophils, cells/mm^3	492	0.14	0.09	0.10	505	**0.25**	0.04	<0.0001
Fe_{NO} level, ppb	510	0.14	0.08	0.10				
Non-sensitized subjects								
Eosinophils, cells/mm^3	204	0.15	0.13	0.3	208	**0.15**	0.05	0.01
Fe_{NO} level, ppb	215	-0.17	0.15	0.3				
Sensitized subjects								
Eosinophils, cells/mm^3	288	0.15	0.11	0.2	297	**0.31**	0.06	<0.0001
Fe_{NO} level, ppb	295	**0.21**	0.10	0.04				

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

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

		EBC NO_2^-/NO_3^- level, µmol/mg proteins [a]				**Fe_{NO} level, ppb [b]**				**$(NO_2^- + NO_3^-)/NO$ ratio level [c]**			
		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 [d]	94	17.8	15.3;20.7	0.7 [d]	94	0.25	0.14;0.35	0.4 [d]
	>16 years	56	1.99	1.38;2.87		56	16.8	13.6;20.8		56	0.26	0.11;0.41	
Model adjusted for all covariates													
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 [d]	94	19.4	16.3;23.1	0.9 [d]	94	0.21	0.08;0.34	0.15 [d]
	>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_2^-/NO_3^-: nitrite/nitrate.

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

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

		EBC NO_2^-/NO_3^- level, µmol/mg proteins [a]				**Fe_{NO} level, ppb [b]**				**$(NO_2^- + NO_3^-)/NO$ ratio level [c]**			
		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,		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 [d]	131	15.9	14.0;18.0	<0.0001 [d]	131	0.29	0.21;0.38	0.6 [d]
	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 centre only													
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,		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 [d]	131	17.5	15.6;19.8	<0.0001 [d]	131	0.24	0.15;0.34	0.4 [d]
	SPT+	172	2.37	1.94;2.89		172	20.7	18.6;23.0		172	0.29	0.20;0.37	
	Both												

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,		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 [d]	131	0.26	0.17;0.36	0.06 [d]
	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 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_2^-/NO_3^-: nitrite/nitrate.
[a]: adjusted for age, sex, smoking, ambient ozone concentration, asthma and centre; [b]: adjusted for age, sex, smoking, height, asthma and centre (GEE regression methods); [c]: adjusted for age, sex, smoking, asthma and centre; [d]: p value for trend.

Figure 1

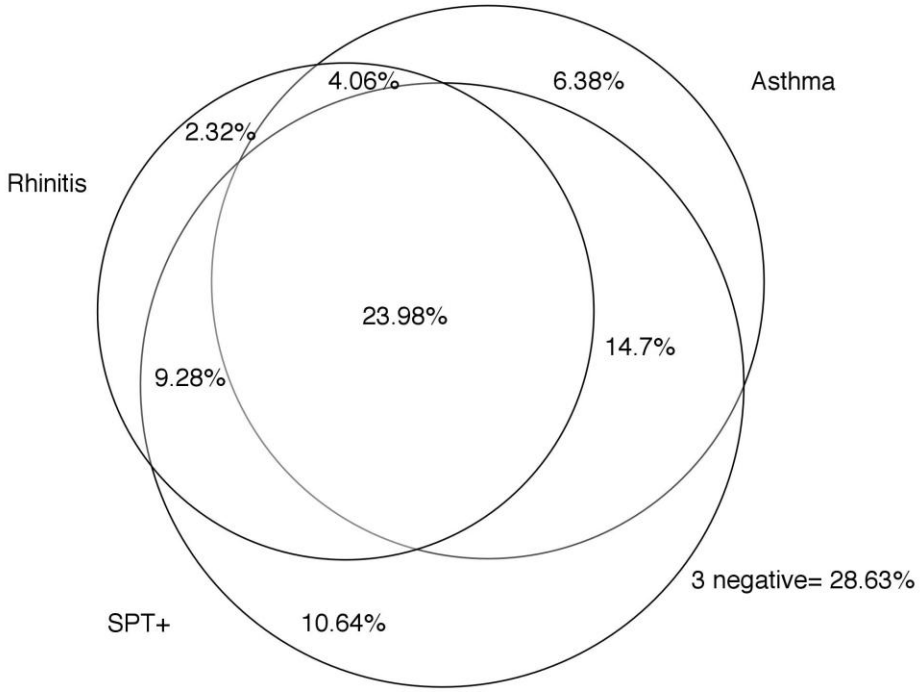
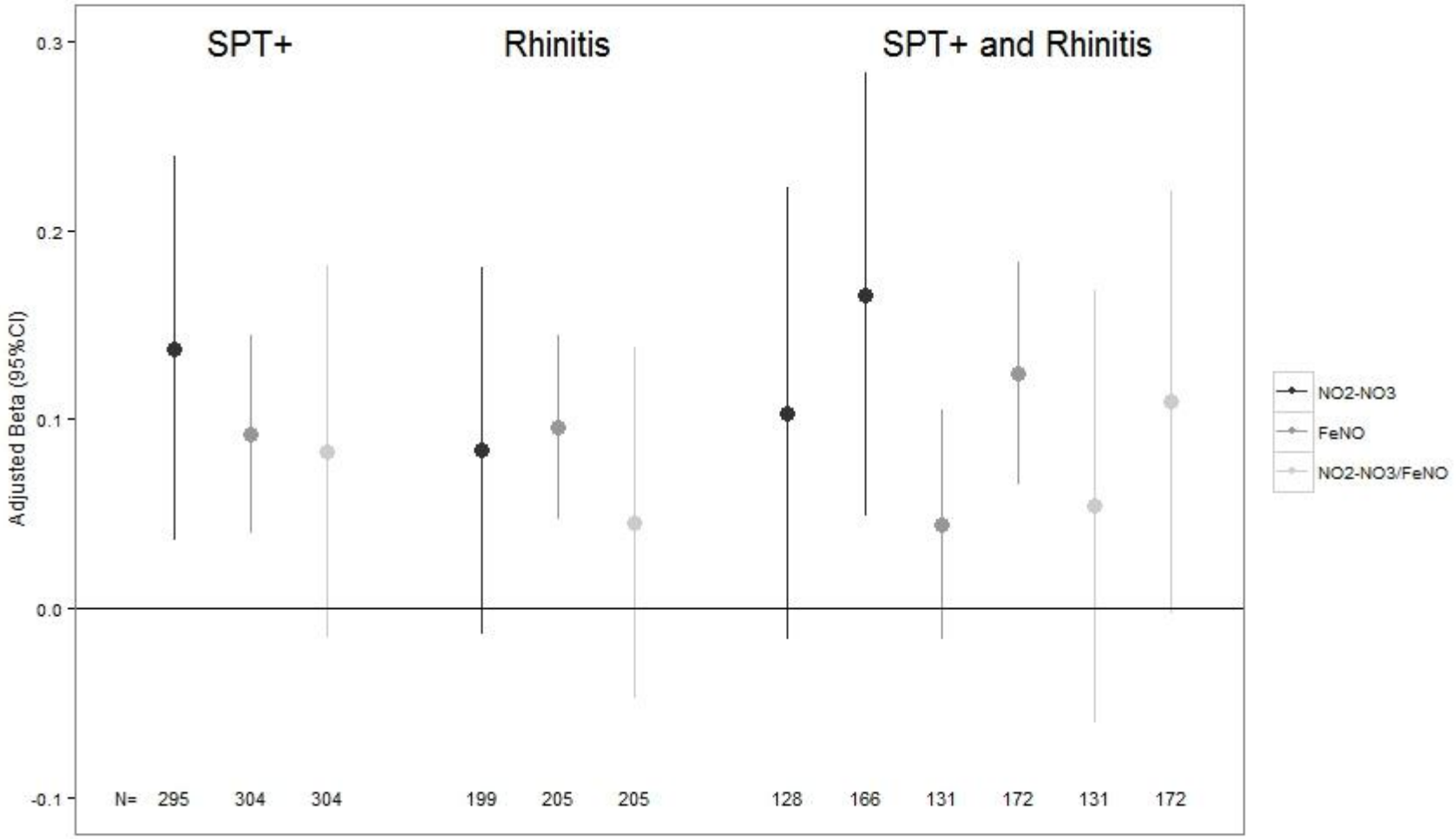

Asthma
Rhinitis
SPT+
4.06%
6.38%
2.32%
23.98%
14.7%
9.28%
10.64%
3 negative= 28.63%

Fig 2


SPT+
Rhinitis
SPT+ and Rhinitis
Adjusted Beta (95%CI)
0.3
0.2
0.1
0.0
-0.1
N= 295 304 304 199 205 205 128 166 131 172 131 172
NO2-NO3
FeNO
NO2-NO3/FeNO