



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

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ABSTRACT Although interest in biomarkers in the nitrate–nitrite–NO pathway has recently increased, associations between nitrite (NO₂⁻) and nitrate (NO₃⁻), and asthma, allergic sensitisation and rhinitis remain unclear. The study aimed to evaluate the associations between NO₂/NO₃⁻ and exhaled fraction of nitric oxide (FeNO) levels with asthma, allergic sensitisation and rhinitis.

Plasma and exhaled breath condensate (EBC) NO₂⁻/NO₃⁻ and FeNO levels were measured in 523 adults of the French Epidemiological study on Genetics and Environment of Asthma. Allergic sensitisation was defined by a positive skin prick test for at least one aeroallergen. Subjects were classified as non-sensitised, sensitised and as having allergic rhinitis.

Plasma NO₂⁻/NO₃⁻ level was unrelated to any disease phenotypes. EBC NO₂⁻/NO₃⁻ level was unrelated to any asthma phenotypes. EBC NO₂⁻/NO₃⁻ and FeNO levels were correlated in sensitised subjects only ($r=0.21 \pm 0.10$, $p=0.01$). EBC NO₂⁻/NO₃⁻ and FeNO levels were higher in sensitised than in non-sensitised subjects (adjusted geometric mean (95% CI): 2.36 (1.96–2.84) versus 1.72 (1.38–2.14) $\mu\text{mol per mg}$ proteins, $p=0.008$; and 18.3 (16.7–20.0) versus 14.8 (13.3–16.5) ppb, $p=0.0006$, respectively), with gradual relationships from sensitised subjects to those with allergic rhinitis ($p<0.0001$).

Results suggest that EBC NO₂⁻/NO₃⁻ and FeNO levels may be considered as biological markers of intensity of allergic sensitisation and rhinitis.



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Introduction

The interest in measuring biological markers in exhaled breath condensate (EBC) in epidemiological studies of respiratory diseases has increased in the past 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]. NO metabolism is complex, and both NO measured by the exhaled fraction of NO (F_eNO) and NO-related compounds such as nitrites (NO_2^-) and nitrates (NO_3^-) are relevant biological markers that may help to better understand the pathophysiology of asthma and allergy [2].

F_eNO is the most studied of these, and it is commonly considered as a noninvasive indirect marker of airway inflammation [3]. Both epidemiological and clinical studies have shown increased level of F_eNO in children and adults with asthma, and positive associations between F_eNO and allergic sensitisation 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 F_eNO 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 F_eNO 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 compartmentalised production [19]. In this study, we compared the association between total NO_2^-/NO_3^- levels measured in two compartments (plasma and EBC) and F_eNO levels with asthma, allergic sensitisation and rhinitis among 523 adults from the EGEA study. We hypothesised 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, $n=2047$; <https://egeanet.vjf.inserm.fr/>). The protocol and descriptive characteristics have been described previously [20, 21]. A follow-up of the initial cohort was conducted between 2003 and 2007 [22]. 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 standardised 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 sensitisation, and available measurements of EBC NO_2^-/NO_3^- and F_eNO levels ($n=523$). Subjects included in the analyses were younger, more often reported 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 sensitisation, lung function tests, and eosinophil count (table S1 in supplementary data).

Ethical approval was obtained from the relevant institutional review board committees (Cochin Port-Royal Hospital and Necker-Enfants Malades Hospital, Paris, France). 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 sensitisation was defined by a positive skin prick test (SPT+) with a mean wheal diameter ≥ 3 mm than the negative control for at least one of 12 aeroallergens (indoor: cat, *Dermatophagoides pteronyssinus*, *Blattella germanica*; outdoor: olive, birch, *Parietaria judaica*, timothy grass, *Cupressus* and ragweed pollen; and moulds: *Aspergillus*, *Cladosporium herbarum* and *Alternaria tenuis*). Subjects were classified as sensitised 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-sensitised (no SPT+), sensitised 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 $\geq 5\%$. Details on other phenotypes are given in supplementary data.

Biological phenotypes

EBC was collected with an RTube™ (Respiratory Research Inc., Charlottesville, VA, USA) according to a standardised method. Briefly, the RTube was rinsed with deionised 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 manoeuvres (see supplementary data for more details).

Total nitrite–nitrate ($\text{NO}_2^-/\text{NO}_3^-$) levels were measured in plasma and EBC as previously described [23]. 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 FeNO were realised before other pulmonary function tests according to American Thoracic Society/European Respiratory Society recommendations (see supplementary data). The measurement was performed only in three of the five centres 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. FeNO level was measured at $50 \text{ mL}\cdot\text{s}^{-1}$ flow rate as previously described [22].

Statistical methods

Joint distribution of asthma, SPT+ and current rhinitis was shown with a Venn diagram (fig. 1). Total plasma and EBC $\text{NO}_2^-/\text{NO}_3^-$ and FeNO levels were \log_{10} -transformed as a result of their skewed distribution.

Also as part of the EGEA study, we previously reported that plasma $\text{NO}_2^-/\text{NO}_3^-$ level was increased with leafy vegetable consumption and decreased in smokers and with storage time, that EBC $\text{NO}_2^-/\text{NO}_3^-$ level was decreased in smokers and with exposure to ambient ozone concentration [23], and that FeNO level was associated with season of examination [22]. 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 $\text{NO}_2^-/\text{NO}_3^-$; 2) age, sex, smoking, ambient ozone concentration and centre for EBC $\text{NO}_2^-/\text{NO}_3^-$; and 3) age, sex, height, smoking and centre for FeNO . Since the ratio of higher oxides of nitrogen (NO_x , including NO_2^- and NO_3^-) to NO was reported to be more informative than each measurement alone by NGUYEN *et al.* [13], the $(\text{NO}_2^- + \text{NO}_3^-)/\text{NO}$ ratio (NO_x/NO ratio) has also been studied.

As inhaled corticosteroids (ICS) use can decrease FeNO levels, and as $\text{NO}_2^-/\text{NO}_3^-$ and FeNO are biological markers involved in the same pathway, association between ICS use and EBC $\text{NO}_2^-/\text{NO}_3^-$ 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 analysis.

Associations between total $\text{NO}_2^-/\text{NO}_3^-$ levels, FeNO levels, and the NO_x ratio $(\text{NO}_2^- + \text{NO}_3^-)/\text{NO}$ and asthma phenotypes, allergic sensitisation and current rhinitis were estimated with linear regression models. Parameter estimates were assessed by using generalised estimating equations (GEEs), 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).

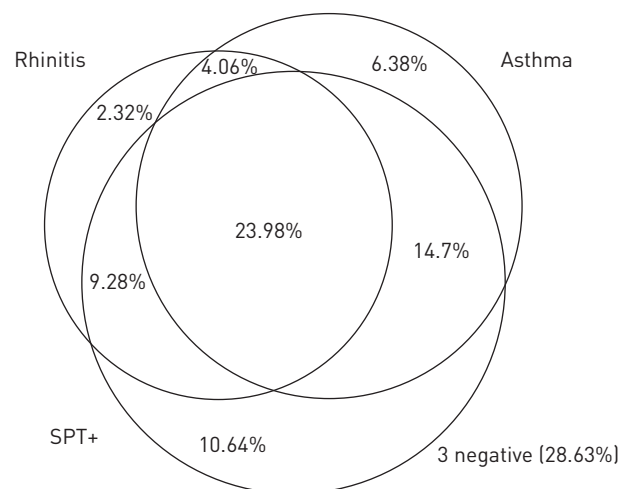


FIGURE 1 Concordance of ever asthma, allergic sensitisation (positive skin prick test, SPT+) and current rhinitis (proportional Venn diagram). Data on current rhinitis was missing for four participants without allergic sensitisation and asthma, one participant with asthma and one participant with allergic sensitisation ($n=517$).

TABLE 1 Characteristics of subjects according to asthma status

	All subjects	Subjects without asthma	Subjects with asthma	p-value
Subjects n	523	268	255	
Age years	39.9 ± 16.6	43.2 ± 15.9	36.5 ± 16.8	<0.0001
Sex female %	51.0	57.5	44.3	0.003
Body mass index kg·m⁻²	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[#]	1.17 ± 1.35 (517)	0.51 ± 0.81 (266)	1.86 ± 1.46 (251)	<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 per mm²) %			36.5	
FEV₁ % pred	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 past 12 months %	20.6	2.2	40.0	<0.0001
Inhaled corticosteroids past 3 months %	7.3	0.0	14.9	
SPT+ and current rhinitis				
SPT+ %	58.1	38.8	78.4	<0.0001
SPTQ (median number of SPT+)	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 (geometric means)				
EBC NO ₂ ⁻ /NO ₃ ⁻ μmol per 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)	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	39.1 [28.0–53.7] (510)	38.9 [26.3–56.4] (263)	39.3 [29.1–53.2] (247)	0.8
Eosinophil cells per mm ³	170 [100–280] (505)	145 [100–200] (256)	199 [100–300] (249)	<0.0001
IgE IU·mL ⁻¹	81.7 [28.8–226]	49.8 [19.4–128]	137 [59.0–332]	<0.0001

Data are presented as mean ± SD, unless otherwise stated. Medians and geometric means are shown with interquartile ranges. Values of n are present in italics when differing from total n. GINA: Global Initiative for Asthma; FEV₁: forced expiratory volume in 1 s; PD₂₀: provocative dose causing a 20% fall in FEV₁; SPT+: positive skin prick test, i.e. a mean wheal diameter ≥3 mm than the negative control for at least one of 12 aeroallergens; SPTQ: quantitative SPT score; EBC: exhaled breath condensate; FeNO: exhaled nitric oxide fraction; Ig: immunoglobulin. #: 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 FEV₁ <80% pred.

Results

The characteristics of the 523 adults according to their asthma status are summarised in table 1. As expected, subjects with asthma had significantly higher eosinophilia, lower FEV₁ % predicted, more often bronchial hyper-responsiveness (BHR), SPT+, and more often reported current rhinitis, than subjects without asthma. After adjustment for age, sex and smoking, the following associations between asthma and eosinophilia (OR 3.12, 95% CI 1.72–5.66), FEV₁ (mean ± SD 107.7 ± 10.5 versus 97.5 ± 11.0% pred), BHR (OR 4.18, 95% CI 2.66–6.57), SPT+ (OR 5.07, 95% CI 3.32–7.72) and current rhinitis (OR 4.62, 95% CI 3.11–6.85) were confirmed (all p < 0.0005). Eosinophil count, IgE and FeNO levels were significantly higher

TABLE 2 Pairwise association of exhaled breath condensate (EBC) $\text{NO}_2^-/\text{NO}_3^-$, exhaled nitric oxide fraction (FeNO) levels and eosinophil count in all subjects, and according to allergic sensitisation

	EBC $\text{NO}_2^-/\text{NO}_3^-$ level $\mu\text{mol per mg proteins}^\#$				FeNO level ppb^\ddagger			
	n	Estimate	SD	p-value	n	Estimate	SD	p-value
All subjects								
Eosinophil cells per mm^3	492	0.14	0.09	0.10	505	0.25	0.04	<0.0001
FeNO level ppb	510	0.14	0.08	0.10				
Non-sensitised subjects								
Eosinophil cells per mm^3	204	0.15	0.13	0.3	208	0.15	0.05	0.01
FeNO level ppb	215	-0.17	0.15	0.3				
Sensitised subjects								
Eosinophil cells per mm^3	288	0.15	0.11	0.2	297	0.31	0.06	<0.0001
FeNO level ppb	295	0.21	0.10	0.04				

[#]: adjusted for age, sex, smoking, ambient ozone concentration, asthma and centre; [‡]: adjusted for age, sex, smoking, height, asthma and centre (generalised estimating equation linear regression methods).

in subjects with asthma than in those without (all $p < 0.0001$). EBC $\text{NO}_2^-/\text{NO}_3^-$ level was unrelated to ICS use (data not shown, $p = 0.5$).

Pairwise association between EBC $\text{NO}_2^-/\text{NO}_3^-$, FeNO levels and blood eosinophil counts

EBC $\text{NO}_2^-/\text{NO}_3^-$ levels were unrelated to eosinophil counts, whereas FeNO levels were positively associated with eosinophils in all subjects, both in non-sensitised and sensitised subjects (table 2). In sensitised subjects, EBC $\text{NO}_2^-/\text{NO}_3^-$ was positively associated with FeNO levels.

The median (range) FeNO value in the population was 15.6 (2.4–99.0) ppb . Stratification according to this median value showed positive and significant association between EBC $\text{NO}_2^-/\text{NO}_3^-$ level and allergic sensitisation in subjects above the median only (2.66 (2.06–3.43) versus 1.64 (1.18–2.28) $\mu\text{mol per mg proteins}$, $p = 0.01$, and 2.03 (1.52–2.71) versus 1.76 (1.30–2.38) $\mu\text{mol per mg proteins}$, $p = 0.4$, in subjects above and below the median, respectively).

Plasma and EBC $\text{NO}_2^-/\text{NO}_3^-$, FeNO levels and asthma and asthma-related phenotypes

Both plasma and EBC $\text{NO}_2^-/\text{NO}_3^-$ levels were unrelated to ever asthma, current asthma, symptomatic score, and asthma control (data not shown, all $p > 0.3$). Furthermore, plasma $\text{NO}_2^-/\text{NO}_3^-$ level was unrelated to allergic sensitisation and current rhinitis.

Both plasma and EBC $\text{NO}_2^-/\text{NO}_3^-$ levels were not related to eosinophilic asthma nor to age at asthma onset (table 3). As expected, a positive and significant association was observed between FeNO level and eosinophilic asthma (table 3), but no other significant association was observed.

EBC $\text{NO}_2^-/\text{NO}_3^-$ and FeNO levels, NO_x/NO ratio and allergic sensitisation

A positive association at borderline significance was observed between EBC $\text{NO}_2^-/\text{NO}_3^-$ level and SPT+ (table 4). In a model adjusted for covariates, including asthma, EBC $\text{NO}_2^-/\text{NO}_3^-$ 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 $\text{NO}_2^-/\text{NO}_3^-$ level were observed with quantitative SPT score (SPTQ) (fig. S1 in supplementary data), and when subjects were classified in the following groups: no SPT+, SPT+ only, and both SPT+ and current rhinitis (table 4 and fig. 2). The median (range) EBC $\text{NO}_2^-/\text{NO}_3^-$ value in our population was 2.35 (1.14–4.9) $\mu\text{mol per mg proteins}$ (table 1). Stratification according to this median value showed positive and significant associations between subjects above the median value and 1) allergic sensitisation (OR 1.92, 95% CI 1.25–2.97, $p = 0.003$), 2) current rhinitis (OR 1.52, 95% CI 1.01–2.28, $p = 0.04$), and 3) SPT+ only or both SPT+ and current rhinitis versus no SPT+ (OR 1.64, 95% CI 1.01–2.66, $p = 0.04$, and OR 2.16, 95% CI 1.29–3.59, $p = 0.003$, respectively) in GEE regression models with adjustment for age, sex, smoking, ambient ozone concentration, asthma and centre.

Similarly to EBC $\text{NO}_2^-/\text{NO}_3^-$, FeNO level was also positively related to allergic sensitisation expressed as SPT+ (table 4), and gradually increased with SPTQ (fig. S1), and with allergic sensitisation and current rhinitis. FeNO was also significantly and positively associated with current rhinitis. The associations between

TABLE 3 Associations between exhaled nitric oxide fraction (F_{eNO}), total NO_2^-/NO_3^- and ratio levels in exhaled breath condensate (EBC) with eosinophilic asthma and age at onset

	EBC NO_2^-/NO_3^- level $\mu\text{mol per mg proteins}^{\#}$				F_{eNO} level ppb^{\ddagger}				$(NO_2^-+NO_3^-)/NO$ ratio level ⁺			
	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 [§]	94	17.8	15.3–20.7	0.7 [§]	94	0.25	0.14–0.35	0.4 [§]
>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 [§]	94	19.4	16.3–23.1	0.9 [§]	94	0.21	0.08–0.34	0.15 [§]
>16 years	56	1.59	1.02–2.00		56	18.4	14.6–23.3		56	0.17	0.01–0.34	

GM: geometric mean. [#]: adjusted for age, sex, smoking, ambient ozone concentration, asthma and centre; [†]: adjusted for age, sex, smoking, height, asthma and centre (generalised estimating equation regression methods); ⁺: adjusted for age, sex, smoking, asthma and centre; [§]: p-value for trend.

F_{eNO} levels and SPT+, and current rhinitis were confirmed when BMI instead of height was added as a covariate in the models (data not shown).

No significant association were observed between the $(NO_2^- + NO_3^-)/NO$ ratio (NO_x/NO ratio) and SPT+, current rhinitis or both (table 4 and fig. 2). When analysing SPT+ to indoor, outdoor or mould allergens separately, EBC NO_2^-/NO_3^- levels showed positive and significant associations with sensitisation to mould allergens, and F_{eNO} levels were positively associated to indoor allergens (table 4).

Discussion

The present study conducted on a large sample of adults with a precise phenotypic characterisation shows, for the first time, the similarities and differences for the associations of both F_{eNO} and EBC NO_2^-/NO_3^- levels with asthma, allergic sensitisation and rhinitis. Results showed higher EBC NO_2^-/NO_3^- and F_{eNO} levels in subjects with allergic sensitisation, with current rhinitis, and in particular when both are present. Only F_{eNO} levels were found to be higher with asthma. EBC NO_2^-/NO_3^- and F_{eNO} levels were positively associated in sensitised subjects only, and EBC NO_2^-/NO_3^- levels were found to be associated with allergic sensitisation in subjects with higher F_{eNO} levels only.

The selection of the 523 subjects included in the present analyses was driven first by the random availability of the F_{eNO} measurements in three of the five participating centres [22], 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 BHR, measured by a methacholine challenge test was quite high in subjects without asthma. A possible explanation is that some 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 epidemiological studies, ranging from 19.3 to 62.4%. Subjects included in the analyses had higher EBC NO_2^-/NO_3^- and F_{eNO} 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 compartmentalisation. In plasma NO_2^-/NO_3^- production derives from several sources, such as bacteria, enzymatic production and dietary sources [24]. In EBC ionised NO_3^- and NO_2^- (not volatile) may arise from NO after reaction with oxygen [25] or from activated immune cells present in the lining fluid of the lungs [26]. 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

TABLE 4 Associations between exhaled nitric oxide fraction (F_{ENO}), total NO₂⁻/NO₃⁻ and ratio levels in exhaled breath condensate (EBC) with allergic sensitisation

	EBC NO ₂ ⁻ /NO ₃ ⁻ level µmol per mg proteins [#]				F _{ENO} level ppb [†]				(NO ₂ ⁻ + NO ₃ ⁻)/NO ratio level [‡]			
	n	GM	95% CI	p-value	n	GM	95% CI	p-value	n	Mean	95% CI	p-value
Allergic sensitisation												
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	
SPT+	131	2.34	1.90-2.89	0.10 [§]	131	15.9	14.0-18.0	<0.0001 [§]	131	0.29	0.21-0.38	0.6 [§]
Both	172	2.47	2.07-2.94		172	18.4	16.5-20.5		172	0.32	0.25-0.39	
Model adjusted for centre only												
Allergic sensitisation												
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	
SPT+	131	2.12	1.70-2.64	0.04 [§]	131	17.5	15.6-19.8	<0.0001 [§]	131	0.24	0.15-0.34	0.4 [§]
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 sensitisation												
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 [§]	131	16.4	14.6-18.4	<0.0001 [§]	131	0.26	0.17-0.36	0.06 [§]
Both	166	2.50	2.01-3.12		172	19.7	17.7-21.9		172	0.32	0.23-0.40	
Allergic sensitisation (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 sensitisation (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 sensitisation (moulds 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

GM: geometric mean; SPT+: positive skin prick test, i.e. a mean wheal diameter ≥3 mm than the negative control for at least one of 12 aeroallergens. [#]: adjusted for age, sex, smoking, ambient ozone concentration, asthma and centre; [†]: adjusted for age, sex, smoking, height, asthma and centre (generalised estimating equation regression methods); [‡]: adjusted for age, sex, smoking, asthma and centre; [§]: p-value for trend.

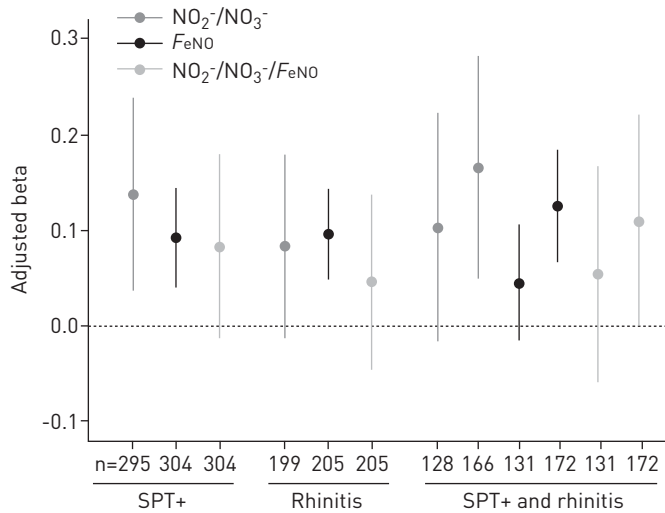


FIGURE 2 Associations between exhaled nitric oxide fraction ($FeNO$), total NO_2^-/NO_3^- and $(NO_2^- + NO_3^-)/NO$ ratio levels in exhaled breath condensate with allergic sensitisation, current rhinitis and both. Regression coefficients (beta) and 95% confidence intervals for associations between $FeNO$, total NO_2^-/NO_3^- levels, $(NO_2^- + NO_3^-)/NO$ ratio and allergic sensitisation, current rhinitis and both, estimated through generalised estimating equation 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 $FeNO$ level; age, sex, smoking, asthma and centre for $(NO_2^- + NO_3^-)/NO$ ratio. SPT+: positive skin prick test, *i.e.* a mean wheal diameter ≥ 3 mm than the negative control for at least one of 12 aeroallergens.

VILLANUEVA and GIULIVI [19], for whom the compartmentalised 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 compared to healthy nonsmoking subjects [17], healthy nonatopic 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 sensitisation 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 [27], 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 [28].

As reported in the literature [4, 29], positive associations between $FeNO$ level and asthma, allergic sensitisation, 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 $FeNO$ and exhaled breath condensate NO_2^-/NO_3^- levels with asthma, allergic sensitisation and current rhinitis. We found positive associations between EBC NO_2^-/NO_3^- and $FeNO$ levels in sensitised subjects, and between EBC NO_2^-/NO_3^- levels with allergic sensitisation in subjects with higher $FeNO$ levels. Consistently, we found that both EBC NO_2^-/NO_3^- and $FeNO$ levels increased with allergic sensitisation, with the number of SPT+, and that gradual relationships were observed between sensitised subjects only and those with both allergic sensitisation and rhinitis. An immediate practical utility could not be inferred from the results obtained in the framework of this epidemiological study, but taken together, our results suggest that EBC NO_2^-/NO_3^- and $FeNO$ levels may be considered as biological markers of intensity of allergic sensitisation 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 $FeNO$ levels rather than each alone, our results provided complementary interesting information. To go further, we also studied the association between the NO_x ($NO_2^- + NO_3^-$)/NO ratio and allergic sensitisation. Unfortunately, this ratio was not more informative than

considering the measurement of EBC $\text{NO}_2^-/\text{NO}_3^-$ alone. Contrary to our results, a ratio also including *S*-nitrosothiols, $(\text{NO}_2^- + \text{NO}_3^- + \textit{S}\text{-nitrosothiols})/\text{NO}$, 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 sensitisation 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 sensitisation. We suggest that exposure to allergens results in uptake and proceeding by dendritic cells inducing the development of Th2 cells in sensitised individuals. Recent evidence indicates that airway epithelium also plays an important role in the allergic airway response by the release of interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP) which activate dendritic cells, basophils, eosinophils and Th2 cells [30, 31]. 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-dependent pathway. NO and related compounds are produced by a wide variety of residential and inflammatory cells in the respiratory tract [32]. In response to allergens, both dendritic cells and airway epithelial cells are stimulated, and release various cytokines which activate dendritic cells, basophils, mast cells, eosinophils and Th2 cells, leading to eosinophil activation and proliferation [33]. We previously reported that *FeNO* level was positively associated with blood eosinophil counts [18], and there is *in vitro* evidence that human blood eosinophils produce NO and participate in the regulation of the NO pool in pulmonary tissues [34, 35]. Moreover, NO modulates the Th1/Th2 balance by favouring Th2 response and IL-5 production and thus recruiting eosinophils into the airways. Nevertheless, even if EBC $\text{NO}_2^-/\text{NO}_3^-$ 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 and GASTON [36], the complexity of the nitrate–nitrite–NO pathway provides evidence that more targeted biological markers are needed for inclusion in a global scheme to 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 $\text{NO}_2^-/\text{NO}_3^-$ and *FeNO* levels in EBC are associated with allergic sensitisation 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 *FeNO* and EBC $\text{NO}_2^-/\text{NO}_3^-$ may help to disentangle the associations between NO metabolism and asthma, allergic sensitisation and rhinitis.

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