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Exhaled breath NOx levels in a middle-aged adults population-based study: reference values and association with the smoking status

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Competing interests

The authors declare that they have no competing interests.

Abstract

Background: Biomarkers in exhaled breath condensate (EBC) are potentially sensitive indicators of early biochemical changes in airways following exposure to pneumotoxic substances, particularly in susceptible subjects. NOx are the stable end products of the nitrite–nitrate–NO oxidative stress pathway and can be used to monitor airway inflammatory diseases, especially in asthma. Nevertheless, population-based surveys are needed to better interpret EBC NOx levels in clinical studies. The aim of this study was to establish reference values of EBC NOx in a large group of middle-aged, healthy adults of a sample of the general population with particular focus on the smoking status. **Methods:** The EBC NOx levels were analysed from 2872 subjects among the ELISABET population-based cross sectional study including a representative sample of men and women aged from 40 to 66 years olds conducted in northern France, which included comprehensive questionnaires by interview and spirometry data. Healthy participants were defined as participants with no self-reported respiratory disease. **Results:** For the healthy subjects (n=1251), the median NOx concentration (IQR) was equal to 7.2µM (3.12) and concentrations of NOx in EBC did not differ significantly according to smoking status. The upper fifth percentile (95%) (ULN) of NOx concentrations among healthy subjects was equal to 13.6µM, ranging from 12.7µM (smokers) to 14.4µM (ex smokers). Among subjects with EBC NOx values higher than the ULN and compared with subjects that had EBC NOx values lower than the ULN, we found a significant higher proportion of subjects with current asthma (10.5% vs 6.5%) or with chronic bronchitis symptoms (7.6% vs 3.3%). **Conclusion:** This population-based study has provided the distribution and the upper limit reference value of a nitrosative stress biomarker (NOx) in EBC of middle aged, healthy adults. EBC NOx levels were not associated with smoking status.

Keywords

Exhaled breath condensate, Nitrosative stress, Nitric oxides, Reference value, Upper limit value

Abbreviations

ALF: airway lining fluid
ANOVA: analysis of variance
BMI: body mass index
CI: confidence interval
EBC: Exhaled Breath Condensate
ELF: Epithelial Lining Fluid
FEF25-75: forced expiratory flow between 25 and 75% of FVC
FeNO: fraction of exhaled nitric oxyde
FEV1: forced expiratory volume in 1s
FVC: Forced vital capacity
GLI 2012: Global Lung Function Initiative reference equations 2012
ICC: intraclass correlation coefficient
ICS: inhaled corticosteroids
lnNOx: natural logarithm of NOx
LOD: Limit of detection
NO: Nitric Oxide
NOx: Nitric Oxides (nitrites and nitrates)
NO_2: nitrites
NO_3: nitrates
py: pack-years
ULN: Upper limit of normal

Introduction

Exhaled Breath Condensate (EBC) collection is a non-invasive method of studying the composition of fluid in the airway lining (ALF) [1–3]. The analysis of pulmonary biomarkers in EBC provides a useful approach to the pathophysiology of pulmonary disease [4]. Biomarkers in EBC also have potential as sensitive indicators of early biochemical changes in the airways of subjects exposed to pneumotoxic substances, in either environmental or occupational exposure scenarios [5, 6], and particularly for susceptible subjects with pulmonary diseases [7].

Investigations of exhaled breath have increased in recent years, principally through the study of biomarkers of pulmonary inflammation and oxidative stress or markers of environmental exposure (eg, metals, minerals). The first formal recommendations from 2005 were later clarified in 2012, and provide a roadmap to avoid potential pitfalls and standardize the collection and analysis of EBC to facilitate clinical and epidemiological studies [8, 9]. Recommendations for research on EBC include identification of reference values of the different inflammatory biomarkers in healthy subjects, studies on the reproducibility of measurements and studies to clarify the relationships of biomarkers in EBC with symptoms and lung function [9, 10].

Among the biomarkers analysed in EBC are nitrites (NO_2) and nitrates (NO_3), collectively referred to as NOx. They are indirect, stable indicators of nitric oxide (NO) enzymatic synthesis from L-arginine, involved in the nitrosative pathway, also called the nitrite-nitrate-NO pathway. These biomarkers are involved in several physiological functions including neuromodulation, regulation of vascular tone and immune responses, and also in cardiovascular and airway diseases [1, 11–13]. Many clinical studies have been conducted on the fraction of exhaled NO (FeNO) [11, 14]. NO is highly reactive in solution and is rapidly converted to nitrite and nitrate more stable (NOx). NO concentrations in the airway can therefore be evaluated by measurement of both nitrate and nitrite, which are the ultimate metabolites of NO. As Moshage et al. reported that measurement of nitrite only was less

useful because nitrate is more stable than nitrite and present at concentrations 5-10 times higher than that of nitrite, it appears important to measure NOx [15].

Literature on EBC analysis suggests that measurement of NOx in EBC can be used to monitor inflammatory diseases of the airway, especially asthma for which higher levels of NOx may be associated with disease severity and eosinophilic inflammation [1, 4, 7, 16–19]. Indeed, nitrite oxydes could be physiologically recycled in blood and tissues to form NO and other bioactive oxides [11]. NO and related compounds are associated with blood eosinophil counts on the one hand because human blood eosinophils produce NO and participate in the regulation of the NO pool in pulmonary tissues, and on the other hand because NO favors type 2 inflammation [18, 20]. Recent studies have demonstrated that cytokines produced during this Th2 inflammatory response (i.e. interleukins 4, 5 and 13) can induce eosinophilopoiesis [21]. NOx analysis by colorimetric method is relatively inexpensive and thus commonly assayed in studies [22]. Nevertheless, as studies on EBC analysis have generally involved a limited number of patients, results should be interpreted with caution and larger studies are required to clarify their significance [23]. NOx levels in EBC should be compared with predicted values and upper limits of normal (ULN) that are appropriate for the individual being tested. However, few studies have established the ranges of NOx in EBC in large populations [9]. Therefore, the aim of our study was to establish reference values of EBC NOx in a large group of middle-aged, healthy adults of a sample of the general population with particular focus on the smoking status.

Methods

Study design

The participants were men and women (aged from 40 to 66 years at time of examination) enrolled in the 2011-2013 ELISABET (Enquête Littoral Souffle Air Biologie Environnement), a population-based, cross-sectional study in France [24]. All the participants had been resident in a city of the northern France (urban area of Lille or Dunkerque) for at least the preceding 5 years. The non-inclusion criteria were as follows: pregnant women, persons deprived of liberty, inability to consent or not benefiting from social insurance. The participants were selected from electoral rolls by random sampling (stratified for gender and age). All participants (aged from 40 to 65 at time of sampling) were recruited between January 2011 and November 2013. Each selected participant received a letter asking him/her to contact the coordinating team and make an appointment for face-to-face interviews and data collection. In the absence of a reply, participants were contacted by telephone. Data were collected at home (rarely during a consultation in a healthcare establishment). A detailed questionnaire was administered by a trained, registered nurse. EBC collection, lung function tests and blood sampling were performed in a single visit.

The study protocol was approved by the local investigational review board (Trial registration: Local investigational review board: CPP Nord Ouest IV, reference number: 2010-A00065-34; ClinicalTrials.gov identifier: NCT02490553, retrospectively registered in 2015), in compliance with the French legislation on biomedical research. All participants provided their informed, written consent to participation in the study.

General characteristics

A detailed questionnaire was administered by a trained nurse. Current asthma was defined by (a "yes" answer in response to the question "have you ever been diagnosed with asthma by a physician?" AND self-reported wheezing in the previous 12 months) OR use of asthma medications

(including adrenergic, glucocorticoid or anti-cholinergic drugs). Atopy was defined as self-reported symptoms of allergic rhinitis or hayfever in the previous 12 months OR eczema OR anti-allergic medication (including anti-histaminic drug) OR a previous positive skin prick test (51% of atopic subjects) OR allergen desensitization therapy. Allergic asthma and non-allergic asthma were then defined as asthma with or without atopy, respectively.

Respiratory symptoms included chronic cough, defined as a cough persisting for longer than 3 months each year; chronic bronchitis, defined as a cough and sputum production persisting for longer than 3 months each year; wheezing and dyspnea, evaluated by the modified Medical Research Council (mMRC) dyspnoea scale consisting of five statements that describe almost the entire range of dyspnea from none (Grade 0) to almost complete incapacity (Grade 4) [25] - dyspnea was defined in our study as grade 3 (ie. stop for breath after walking about 100 yards or after a few minutes on level ground) and 4 (ie. too breathless to leave the house or breathless when dressing).

Healthy participants were defined as participants with no reported respiratory disease (including asthma, chronic bronchitis or respiratory infection in the previous 4 weeks), no respiratory symptoms (including wheezing, chronic cough or dyspnea), no asthma medication use, no atopy and no airflow obstruction.

Smoking status was qualified as smokers (at least one cigarette per day for a year), ex smokers (smoking cessation for more than 3 months) or never smokers. Tobacco consumption was estimated in pack-years (py).

Exhaled breath condensate (EBC) collection and analyse

Fasting EBC were collected by RTube© (Respiratory Research, Inc., Charlottesville, VA, USA) and before smoking. Subjects wore nose clips to avoid nasal contamination. Collection was completed after 15 minutes of tidal breathing. EBC were immediately aliquoted into cryotubes and kept at -20°C

during for a few hours until they were conveyed to the final storage site where they were stored at -80°C until analyzed.

NOx concentrations were assayed in EBC by the Griess reaction (Griess Reagent Kit, Invitrogen, Molecular Probes), in duplicate, using spectrophotometric detection (Apollo, LB912, Berthold Technologies) after reduction of nitrate. The concentration measured represented the sum of nitrite and nitrate initially present in the EBC. We used an enzymatic technique in tubes, with nitrate reductase (Sigma, St. Quentin Fallavier, France) for the reduction stage. The limit of detection (LOD) was 0.7µM. As our published and unpublished studies on long-term conservation showed excellent NOx stability in EBC, the analyses were performed between 6 and 40 months after the collection [7].

Lung function

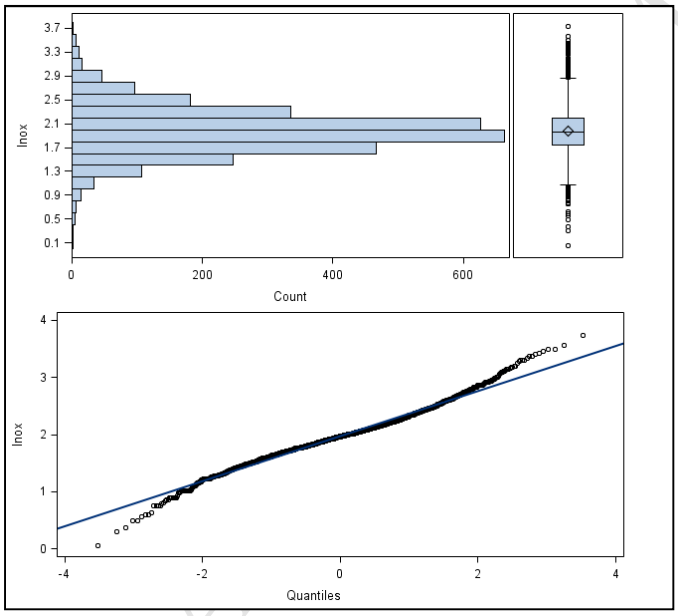
Spirometry data, including forced vital capacity (FVC), forced expiratory volume in 1s (FEV_1), FEV_1/FVC and forced expiratory flow between 25 and 75% of FVC (FEF_{25-75}) were measured using weekly-calibrated, Micro 6000 devices (Medisoft, Belgium), according to the 2005 ATS/ERS guideline[26], and were validated by a trained specialist. Spirometric indices were adjusted for age and sex using the recent GLI (Global Lung Function Initiative) reference equations 2012 [27]. Measured values were converted to z-scores which describe how many standard deviations a measured value differs from the predicted value, independent of sex, age and height. No reversibility test was performed prior to spirometric examinations. Airflow obstruction was defined by a z-score of FEV_1/FVC ratio ≤ -1.64.

Statistical analyses

The agreement between the two sets of NOx measurements in EBC was evaluated by calculating the intraclass correlation coefficient (ICC). Excellent agreement for the ICC was defined as ranging from 1.00 to 0.91, good from 0.90 to 0.71, moderate from 0.70 to 0.51, slight from 0.50 to 0.31, and poor from 0.30 to 0.00 (Fermanian 1984).

Numeric tests of normality (Kolmogorov-Smirnov test) for the NOx values in EBC, didn't showed neither a normal nor lognormal distribution of these data. As n is larger than 2 000 in our study, we added a qualitative component to assessing the data distribution in the form of a quartile/quartile, or 'QQ' plot [28]. The QQ-plot of NOx values after log transformation indicated that the original data were log-normally distributed (Figure 1). Statistical tests were therefore performed on log-transformed NOx values. When concentrations of NOx in EBC were below the LOD, they were assigned a value of 0.5 LOD [29]. ANOVA test for normally distributed continuous variables or the Kruskal Wallis or Wilcoxon test for non-normally distributed continuous variables were used to test for differences between groups and post-hoc comparisons were determined with Bonferroni correction. Analysis of contingency tables was performed using the chi-squared test or Fisher's exact test.

Figure 1: QQ-plot of NOx values after log transformation



The upper limit of normal (ULN) of NOx was defined as the upper fifth percentile (95%) of NOx levels among healthy subjects (i.e. participants with no self-reported respiratory disease, respiratory symptoms, asthma medication use, atopy or airflow obstruction). For the assessment of significant predictors of the natural logarithm (lnNOx) of NOx in healhy subjects, generalized linear model (GLM)

was applied with age, gender, BMI, height, usual dietary intake of vegetables, usual dietary intake of fruits, FVC (z-score), FEV1 (z-score), FEV1/FVC (z-scores), FEF25–75 (z-score), blood neutrophil and eosinophil count as explanatory variable. Characteristics of subjects below and above a NOx threshold were compared using Student's tests or Fisher's exact tests.

Results

Among participant contacted, 52.3% (3276/6265) accepted to participate (cooperation rate). The participation rate was lower in young men compared with women and older men [24]. A total of 3276 subjects were included in the ELISABET French population based study. NOx analysis was performed in duplicate for 3 087 patients. Among them, 215 had incomplete or not validated data. A total of 2 872 were included in our study.

The detection rate of NOx in EBC was 99.9%. The agreement between the two sets of NOx measurements was excellent, according to Fermanian's classification, with an ICC [95%CI] equal to 0.98 [0.97; 0.98].

Tables 1 and 2 respectively describe the general characteristics of the overall population and in healthy subjects taking into account smoking status. In the overall population (Table 1), the mean (SD) age and BMI of the population were respectively 53 years (7.0) and 27.1 (5.15) with differences between smokers, never smokers and ex smokers for these two parameters ($p<0.0001$). The proportion of smokers was lower among healthy participants (13%) than the rest of the population (23%). The proportion of current non-allergic asthma, respectively at 2.6%, 0.8% and 1.9% for smokers, never smokers and ex smokers, was higher in smokers ($p=0.008$, data not show) as was the proportion of subjects with respiratory symptoms. The proportion of atopic subjects was not different according to the smoking status. Z-scores of FVC, FEV_1, were lower among current smokers. LnNOx did not differ significantly according to smoking status ($p=0.65$). There is no relationship between lnNOx and tobacco consumption (pack-years) in smokers ($p=0.772$) or in ex-smokers ($p=0.670$). The same trends were found in healthy subjects (Table 2) except for the absence of difference between never smokers and ex smokers for z-scores of FEV_1/FVC ratio and FEF_{25-75} and between smokers and ex smokers for z-scores of FEV1/FVC ratio.

Table 1: Characteristics of the study population (overall and as a function of smoking status)

Variable	All n=2872	Smokers n=538 (19%)	Never smokers n=1441 (50%)	Ex smokers n=893 (31%)	p[a]
Age (years)[b]	53 (7.2)	51 (6.8)	53 (7.3)	54(7.1)	**<0.0001** [$,§,£]
Gender, n(%) males	1373 (47.8)	299 (55.6)	516 (35.8)	558 (62.5)	**<0.0001** [$,§,£]
BMI (kg/m^2) [b]	27.1 (5.16)	26.3 (4.98)	27.0 (5.24)	27.8 (5.05)	**<0.0001** [$,§,£]
Educational level, n(%)					**0.0001**
≤ 7 years of full-time education	292 (10.2)	44 (8.2)	161 (11.2)	87 (9.7)	
12 years of full-time education	1500 (52.2)	334 (62.1)	711 (49.3)	455 (50.9)	
15 years of full-time education	537 (18.7)	83 (15.4)	282 (19.6)	172 (19.3)	
≥17 years of full-time education	543 (18.9)	77 (14.3)	287 (19.9)	179 (20.0)	
Tobacco consumption (pack-years)[b]	8.9 (14.77)	22.6 (18.17)	-	15.4 (14.94)	**<0.0001**[**]
Tobacco consumption (pack-years), n(%)					**<0.0001**
0 pack-years	1441 (51.1)	-	1441 (100.0)	-	
<10 pack-years	540 (19.1)	138 (25.8)	-	402 (47.3)	
From 10 to 20 pack-years	386 (13.7)	153 (28.6)	-	233 (27.4)	
From 20 to 30 pack-years	211 (7.5)	106 (19.8)	-	105 (12.4)	
> 30 pack-years	246 (8.7)	137 (25.7)	-	109 (12.8)	
Current asthma, n(%)	194 (6.8)	50 (9.3)	84 (5.83)	60 (6.72)	**0.0227**
Adrenergic inhalants, n(%)	96 (3.3)	26 (4.8)	37 (2.6)	33 (3.7)	**0.0347**
Glucocorticoid inhalants, n(%)	44 (1.5)	12 (2.2)	17 (1.2)	15 (1.7)	0.2171
Symptoms					
Chronic cough, n (%)	266 (9.3)	99 (18.4)	92 (6.4)	75 (8.4)	**<0.0001**
Chronic bronchitis symptoms, n (%)	102 (3.5)	44 (8.2)	26 (1.8)	32 (3.6)	**<0.0001**
Wheezing, n(%)	461 (16.1)	183 (34.1)	154 (10.7)	124 (13.9)	**<0.0001**
Dyspnea, (MRC ≥2) n(%)	229 (8.0)	62 (11.5)	108 (7.5)	59 (6.6)	**0.0025**
Atopy, n(%)	935 (32.6)	168 (31.2)	488 (33.9)	279 (31.2)	0.4874
Allergic rhinitis, n(%)	625 (21.8)	114 (21.2)	321 (22.3)	190 (21.3)	0.7982
Respiratory infection in the previous 4 weeks, n(%)	265 (9.2)	80 (14.9)	114 (7.9)	71 (7.9)	**<0.0001**
Usual dietary intake of vegetables (frequency/day)[b]	1.2 (0.69)	1.0 (0.65)	1.3 (0.69)	1.3 (0.68)	**<0.0001**
Usual dietary intake of fruits (frequency/day)[b]	1.1 (0.85)	0.8 (0.75)	1.3 (0.85)	1.2 (0.85)	**<0.0001**
Airflow obstruction, n(%)	306 (10.6)	108 (20.1)	111 (7.7)	87 (9.7)	**<0.0001**
FVC (z-score) [b]	0.08 (1.039)	-0.14 (1.120)	0.13 (1.012)	0.13 (1.015)	**<0.0001** [$,§]
FEV_1 (z-score)[b]	-0.19 (1.141)	-0.63 (1.213)	-0.06 (1.074)	-0.15 (1.140)	**<0.0001** [$,§]
FEV_1/FVC (z-scores)[b]	-0.49 (0.960)	-0.84 (1.074)	-0.36 (0.890)	-0.50 (0.933)	**<0.0001** [$,§,£]
FEF_{25-75} (z-score) [b]	0.01 (1.061)	-0.45 (1.140)	0.18 (0.985)	0.01 (1.052)	**<0.0001** [$,§,£]
Neutrophil count (cell/mm3)[b]	3579 (1285.1)	4391 (1639.1)	3338 (1110.9)	3480 (1099.3)	**<0.0001** [$,§,£]
Eosinophil count (cell/mm3)[b]	190 (120.8)	234 (135.7)	173 (111.7)	191 (118.7)	**<0.0001** [$,§,£]
NOx in EBC (µM)[c]		7.2 (1.51)	7.1 (1.54)	7.3 (1.52)	0.6490

BMI: Body mass index; NOx: Nitric Oxides; EBC: Exhaled Breath Condensate; FVC: Forced vital capacity; FEV_1: forced expiratory volume in 1s; FEF25-75: forced expiratory flow between 25 and 75% of FVC

[a] For normally distributed continuous variables: ANOVA test. For non-normally distributed continuous variables: Kruskal Wallis. For categorical variables: X^2 or Fisher's exact test.

Post hoc comparisons (Bonferroni): [$] Smokers vs Never smokers; [§] Smokers vs Ex smokers; [£] Ex smokers vs Never smokers

[**] Non-parametric Wilcoxon test between Smockers and Ex smockers

[b] mean (SD)

[c] geometric mean (geometric SD)

Table 2: Characteristics of the healthy subjects (overall and as a function of smoking status)

Variable	All n=1251	Smokers n=167 (13%)	Never smokers n=683 (55%)	Ex smokers n=401 (32%)	p[a]
Age (years)[b]	53 (7.1)	51 (6.7)	53 (7.1)	54 (6.9)	**<0.0001**[$,§]
Gender, n(%) males	614 (49.1)	101 (60.5)	261 (38.2)	252 (62.8)	**<0.0001**[$,£]
BMI (kg/m^2) [b]	26.8 (4.82)	26.8 (4.49)	26.4 (4.62)	27.5 (4.80)	**0.0011**[£]
Educational level, n(%)					0.1062
≤ 7 years of full-time education	107 (8.5)	11 (6.6)	67 (9.8)	29 (7.2)	
12 years of full-time education	653 (52.2)	103 (61.7)	351 (51.4)	199 (49.6)	
15 years of full-time education	237 (18.9)	27 (16.2)	126 (18.4)	84 (20.9)	
≥17 years of full-time education	254 (20.3)	26 (15.6)	139 (20.3)	89 (22.2)	
Tobacco consumption (pack-years)[b]	6.9 (12.23)	18.8 (15.81)	-	14.2 (13.23)	**0.0004**[**]
Tobacco consumption (pack-years), n(%)					**0.0002**
0 pack-years	683 (55.6)	-	683 (55.6)	-	
<10 pack-years	241 (19.6)	55 (33.3)	-	186 (48.8)	
From 10 to 20 pack-years	164 (13.3)	50 (30.3)	-	114 (29.9)	
From 20 to 30 pack-years	72 (5.9)	34 (20.6)	-	38 (10.0)	
> 30 pack-years	69 (5.6)	26 (15.8)	-	43 (11.3)	
Usual dietary intake of vegetables (frequency/day) [b]	1.3 (0.69)	1.0 (0.63)	1.3 (0.70)	1.3 (0.67)	**<0.0001**[$,§]
Usual dietary intake of fruits (frequency/day) [b]	1.2 (0.83)	0.9 (0.75)	1.2 (0.84)	1.2 (0.83)	**<0.0001**[$,§]
FVC (z-score) [b]	0.18 (1.021)	-0.10 (1.048)	0.23 (1.018)	0.22 (0.998)	**0.0005**[$,§]
FEV_1 (z-score) [b]	0.06 (1.026)	-0.30 (0.985)	0.14 (1.011)	0.08 (1.140)	**<0.0001**[$,§]
FEV_1/FVC (z-score) [b]	-0.25 (0.704)	-0.37 (0.694)	-0.20 (0.686)	-0.28 (0.732)	**0.0100**[$]
FEF_{25-75} (z-score) [b]	0.30 (0.842)	0.03 (0.812)	0.38 (0.818)	0.26 (0.871)	**<0.0001**[$,§]
Neutrophil count (cell/mm3) [b]	3488 (1222.4)	4331 (1539.6)	3311 (1132.9)	3452 (1081.1)	**<0.0001**[$,§,£]
Eosinophil count (cell/mm3) [b]	173 (105.2)	233 (124.4)	155 (93.4)	180 (106.5)	**<0.0001**[$,§,£]
NOx in EBC (µM) [c]		7.2 (1.45)	7.0 (1.52)	7.2 (1.48)	0.3325

BMI: Body mass index; NOx: Nitric Oxides; EBC: Exhaled Breath Condensate; FVC: Forced vital capacity; FEV_1: forced expiratory volume in 1s; FEF25-75: forced expiratory flow between 25 and 75% of FVC

[a] For normally distributed continuous variables: ANOVA test. For non-normally distributed continuous variables: Kruskal Wallis. For categorical variables: X^2 or Fisher's exact test.

[**] Non-parametric Wilcoxon test between Smockers and Ex smockers

Post hoc comparisons (Bonferroni): [$] Smokers vs Never smokers; [§] Smokers vs Ex smokers; [£] Ex smokers vs Never smokers

[b] mean(SD)

[c] geometric mean (geometric SD)

Table 3 specifically describes the distribution according to the smoking status of the NOx for the subgroup of healthy subjects (n=1251). The median NOx concentration (IQR) was equal to 7.2µM (3.24) and the ULN was equal to 13.6µM, ranging from 12.7µM in smokers to 14.4µM for ex smokers.

Table 3: Distribution of NOx levels in EBC in the 1251 healthy participants

	Geometric mean	Geometric SD	IQR	Percentiles						
				2.5th	5th	25th	50th	75th	95th	97.5th
Healthy	7.1	1.49	3.12	3.39	3.82	5.64	7.17	8.76	13.60	17.12
Smokers, n=167 (13%)	7.2	1.45	3.55	3.42	3.63	5.75	7.10	9.30	12.94	15.49
Never smokers, n=683 (55%)	7.0	1.52	2.97	3.39	3.82	5.70	7.10	8.67	13.07	15.64
Ex smokers, n=401 (32%)	7.2	1.48	3.12	3.42	3.74	5.64	7.31	8.76	14.44	18.54

NOx: Nitric oxides; EBC: Exhaled breath condensate

The NOx levels are expressed in µM; IQR: Interquartile range

In our total population, 172 subjects (6%) had EBC NOx values higher than the ULN. Among these subjects and compared with subjects that had EBC NOx values lower than the ULN, we found a significant higher proportions of subjects with current asthma (10.5% vs 6.5%) or with chronic bronchitis symptoms (7.6% vs 3.3%) and similar proportions of subjects that reported atopy, dyspnea, wheezing symptoms or allergic rhinitis (Table 4). We also showed that NOx levels in EBC

were significantly higher in current asthma than those of healthy subjects (median[IQR], 7.8µM [4.1] versus 7.1µM [3.2]; p=0.004). Among subjects that have NOx levels higher than the ULN, we did not find relationship between NOx levels and spirometry indices.

Table 4: Characteristics of participants with EBC NOx level below and above the 95% reference value in healthy participants

Variable	NOx<13.6µM (n=2700)	NOx>13.6µM (n=172)	p*
Atopy, n(%)	879 (33.4)	56 (33.3)	0.9838
Current asthma, n(%)	176 (6.5)	18 (10.5)	**0.0460**
Symptoms			
Chronic bronchitis symptoms, n(%)	89 (3.3)	13 (7.6)	**0.0034**
Wheezing, n(%)	428 (15.9)	33 (19.2)	0.2490
Dyspnea. (mMRC ≥2), n(%)	213 (7.9)	16 (9.3)	0.5070
Allergic rhinitis n(%)	588 (21.8)	37 (21.5)	0.9346
Airflow obstruction, n(%)	286 (10.6)	20 (11.6)	0.6696

NOx: Nitric oxides concentration in EBC (µM); mMRC: modified Medical Research Council dyspnoea scale
*** Khi-2 test or Fisher's exact test**

Discussion

To the best of our knowledge this is the first study to provide reference values of NOx levels in EBC from a general, middle-aged population. Our results give the distributions of NOx levels in EBC from the subset of 1251 healthy subjects. The upper limit of normal (ULN) of NOx concentrations in EBC from this latter group was equal to 13.6 µM. Finally, concentrations of NOx did not differ significantly according to smoking status.

The present study had strengths and limitations. One of the strengths was the use of a large enough general population sample size to specify a strong ULN. The study's main limitation was self-reporting of respiratory symptoms even if it was a questionnaire by interview with a trained nurse; similarly, the definition of atopy is weak because it is not confirmed by performing cutaneous sensitivity tests even if a positive prick test was self-reported in 51% of our atopic subjects. In France these tests are rarely authorised by the ethics committee in population-based studies which limits the characterisation of atopy. Healthy subjects had already been described in a previous study by the absence of abnormalities in lung function tests even if the absence of respiratory diseases was defined by their self-reported symptoms or diseases [30].

The detection rate of NOx in EBC and the agreement between the two sets of NOx measurements, according to Fermanian's classification, were excellent and builds upon our previous study in which we developed methods for collecting and analyzing NOx in EBC [7]. We had previously shown a good long-term stability (93%) for the NOx measurements, and NOx assays performed on three consecutive days (short-term stability) remained consistently stable (98%). The intra-assay and intra-subject reproducibility of the NOx assay in the EBC of 10 controls were also assessed, and results were similar to these reported by other researchers [31, 32].

We compared our results with those of studies using the same NOx assay technique (which is the most commonly used). Our levels of NOx in the EBC of healthy subject (median=7.2µM and IQR=3.12)

were similar with the healthy controls of Ueno *et al* study (mean=6.59µM and SEM=0.47; n=58), the Pirozzi *et al* study (mean=7.6µM and SD=16.5; n=9), the Tomasiak-Lozowska *et al* study (mean = 9.02µM and SD=3.33) and the Radauceanu *et al* study (median= 8.46µM and IQR=7.01; n=16) [6, 33–35].

Other studies, in order to reduce the dilution factor effect in collection of EBC, had chosen to normalise NOx levels to protein or tyrosine [18, 36]. It has already been shown that EBC protein or tyrosine levels can be influenced by respiratory diseases [37–39]. We did not use these correcting factors because they have not yet been validated in consensual international recommendations [8, 40–42]. Consequently, it's difficult to compare our results with those of the latter cited studies.

Our results show that NOx levels in EBC were significantly higher in current asthma than those of healthy subjects (median [IQR]=7.8µM [4.1] versus 7.1µM [3.2]; p=0.004). These results were in agreement with previous studies on smaller samples. Indeed, for Ganas and coll in 2001, the EBC NO_2/NO_3 level in expired breath condensate was higher in 50 patients with stable asthma than in 10 normal non-atopic subjects (1.08, 95%CI 0.86-1.3µM vs 0.6; 95%CI 0.46-0.8). In 2006, Ratnawati and coll showed that EBC NOx level in 62 children with asthma (mean 8.4µM,CI 7.5–9.4) was significantly elevated when compared with 16 normal (4.8µM, CI 3.4–6.2, P=0.0007) and 14 atopic children (6.5µM, CI 4.0–9.1, P=0.02). In their study, Ueno et coll found in 2008 that EBC NOx level for the group of 55 asthmatics with disease severity ranging from mild intermittent to severe persistent (18.01 ± 0.91µmol/L) were significantly higher than that for the control group of 58 non-smoking healthy subjects (6.59 ± 0.47µmol/L), the group of 7 asymptomatic smokers (5.11±0.32µmol/L), and the group of 9 subjects with common cold (n=97.92 ± 1.12µmol/L). Similarly, Chérot-Kornobis and coll found in 2011 that EBC NOx level was significantly higher in the group of 23 asthmatics (geometric mean [IQR] 14.4µM [10.4 - 19.7]) than in 23 controls (9.9µM [7.5 - 15.0]). In 2012, Tomasiak-Lozowska and coll showed that compared with 19 healthy subjects, EBC from 91 asthmatics had significantly higher levels of NOx (healthy subjects: 9.02 ± 3.33 µM ; steroid-naïve

asthmatics: 12.46 ± 5.34, p = 0.04; ICS-treated stable asthmatics: 13.56 ± 5.88, p = 0.003, and ICS-treated unstable asthmatics: 15.45 ± 7.91, p < 0.001). More recently in 2013, Raulf-Heimsoth and coll found that EBC NOx level was significantly higher in29 subjects with ongoing asthmatic symptoms than in subjects without symptoms (p=0.027) [7, 33, 35, 43–45].

The elevated NOx levels in EBC in subjects with current asthma confirmed that nitric oxide (NO) plays a biological role in the inflammatory process. Indeed, nitric oxide production involve pro-inflammatory and oxidative response that generate nitric oxide products such as nitrite and nitrate by reacting free radicals of NO with oxygen [35, 39, 43]. Nevertheless, this difference is weak. This may be due to recruitment in general population suggesting a selection of patients with mild asthma since the severity of the asthma can modulate the results [35, 46, 47]. In this study, it was not possible to assess the severity of asthma. The design of the survey did not include a specific questionnaire on the severity or control of asthma, only the notion of old or current asthma and respiratory symptomatology. However, the prevalence of current asthma in our population is close to that of the latest national surveys (6 to 7% in adults) [48].

We found no relationship between NOx level in EBC and airway obstruction determined from spirometric indices adjusted for age and sex using the recent GLI reference equations 2012. We chose these equations because a recent study from our laboratory demonstrated that the GLI 2012 equations provided a good fit for a French population [49]. Using different equations, Tomasiak-Lozowska et al also reported a lack of correlations between NOx levels in EBC and FEV1 [35]. The concordance of our results with this study is perhaps unsurprising since NOx levels in EBC are considered biomarkers of inflammatory processes that may be pre-clinical, while FEV_1 better reflects large airway obstruction and may be less sensitive to early-stage airway inflammatory conditions. Moreover, our results show that the proportion of subjects with active asthma or symptoms of chronic bronchitis is superior when the NOx values are above the ULN than when they are below the

ULN whereas this difference does not exist for obstruction of airways (Table 4). This may confirm the inflammatory origin of the NOx increase in EBC independently of airway obstruction.

Concentrations of NOx did not differ significantly according to smoking status, even if exogenous compounds like oxidants from cigarette smoke could be a source of NOx. Indeed, smoking is a classic challenge exposure for airway oxidative stress [30] and the fact NOx values are not higher in smokers could be explained by the induction of protective anti-oxidants like S-nitrosothiols such as nitrosoglutathione (GSNO) in the airway surface liquid and/or inhibition of the airway reductase (GSNO-R) which degrades GSNO. Chronic oxidative stress play a key role in enhancing the inflammation through the activation of mitogen-activated protein kinases and redox-sensitive transcription factors such as nuclear factor kappa B and activator protein-1 (AP-1) which regulate the genes for pro-inflammatory mediators and protective antioxidant genes [50, 51]. In their study, Balind and colleagues showed temporarily increased NOx in EBC after smoking, but there was no difference in the baseline levels of NOx between smokers and never smokers [52]. In our study, the acute and transient effects of tobacco were not studied because EBC were collected before smoking.

Conclusion

In conclusion, this epidemiological study has defined distribution and reference values of a nitrosative stress biomarker (NOx) in EBC of middle aged-adults. We found this nitrosative stress biomarker to be not associated with smoking status. These results will facilitate the interpretation of variations in EBC NOx levels, and provide quality, baseline data for the use of this non-invasive technique in the context of epidemiological studies on respiratory diseases or effects of exposure to toxic inhaled environmental contaminants.

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Authors contributions: LD, PA, RM and AS initiated the ELISABET survey and generated research funds. LD led and coordinated the project. LD and JG were responsible for patients' inclusion, follow-up and adjudication of outcomes. NCK coordinate NOx analysis in Exhaled Breath Condensate. JLE and NCK did the statistical analyses and interpreted the results. NCK, SH, VdB and JLE wrote the manuscript. All authors read and approved the final version.

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Highlights

The median (IQR) NOx level in EBC of 1251 healthy subjects was equal to 7.2µM (3.12)

Among healthy subjects, the upper fifth percentile of NOx in EBC was equal to 13.6µM

Concentrations of NOx in EBC did not differ according to smoking status

There is a higher proportion of asthmatics with EBC NOx above the ULN than below

Declarations and Conflict of Interest Statement

Ethics approval and consent to participate

The study protocol was approved by the local investigational review board (CPP Nord Ouest IV, reference number: 2010-A00065-34; ClinicalTrials.gov identifier: NCT02490553), in compliance with the French legislation on biomedical research. All participants provided their informed, written consent to participation in the study.

Availability of data and materials

The data that support the findings of this study are available from Dr. Luc Dauchet but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Dr. Luc Dauchet.

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

LD, PA, RM and AS initiated the ELISABET survey and generated research funds. LD led and coordinated the project. LD and JG were responsible for patients' inclusion, follow-up and adjudication of outcomes. NCK coordinate NOx analysis in Exhaled Breath Condensate. JLE and NCK did the statistical analyses and interpreted the results. NCK, SH, VdB and JLE wrote the manuscript. All authors read and approved the final version.

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