

Alveolar Nitric Oxide and Peripheral Oxygen Saturation in Frequent Exacerbators with Asthma: A Pilot Study

Andrea E. Sprio^{a, b} Francesca Bertolini^a Rossana Fucà^a Stefano Levra^a
Vitina Carriero^a Marieann Högman^c Fabio L.M. Ricciardolo^a

^aDepartment of Clinical and Biological Sciences, University of Turin, Rare Lung Disease Unit and Severe Asthma Centre, San Luigi Gonzaga University Hospital, Turin, Italy; ^bDepartment of Research, ASOMI College of Sciences, Marsa, Malta; ^cDepartment of Medical Sciences, Respiratory, Allergy and Sleep Research, Uppsala University, Uppsala, Sweden

Keywords

Asthma exacerbation · F_ENO · C_ANO · Oxygen saturation

Abstract

Introduction: Asthmatics can experience recurrent exacerbations (AEs), irrespectively of asthma severity. Airway inflammatory monitoring could be fundamental to optimize the asthma management. The present study evaluated whether exhaled NO concentrations in proximal and distal respiratory compartments are different in AE-prone patients in combination with T2 blood biomarkers and resting oxygen saturation (SpO₂). **Methods:** In this observational cross-sectional study, 91 mild-to-severe asthmatics were enrolled. Clinical characteristics, blood and lung function parameters including SpO₂, and F_ENO were evaluated. On 50 randomly selected patients, C_ANO and J_{aw}NO were also analyzed. Then, patients were stratified in frequent exacerbators (FEs) or non-frequent exacerbators (nFEs), according to AE frequency in the previous year (phase I). Chart data were re-evaluated through a 12-month follow-up period post exhaled NO measurement to detect occurrence of novel AE (phase II). **Results:** FE asthmatics had poorer asthma control and required higher therapeutic intensity ($p < 0.05$). F_ENO, C_ANO, and J_{aw}NO were similar between FE and nFE. FE exhibited higher total serum IgE levels and residual volume values but

reduced SpO₂ than nFE ($p < 0.05$); SpO₂ < 96.5% characterized the FE patient (odds ratio = 2.94). In phase II, C_ANO was higher in the group with novel AE at 1-month post-NO measurement ($p < 0.05$), but not afterward. A higher prevalence of C_ANO > 6 ppb was detected in asthmatics who developed AE within 1 month, suggesting its potential clinical use as biomarker in predicting near-future AE (RR = 11.20). **Conclusion:** AE-susceptible asthmatics are characterized by air trapping and distal airway inflammation in conjunction with lower oxygen saturation. C_ANO and SpO₂ could exert specific roles, respectively, in predicting AE and monitoring FE asthmatics.

© 2021 S. Karger AG, Basel

Introduction

Asthma is defined as a heterogeneous disease associated with chronic airway inflammation, variable respiratory symptoms, and reversible airflow obstruction. Clinically, asthma is generally well-controlled by inhaled corticosteroid (ICS) treatment, but some patients experience recurrent asthma exacerbations (AEs), irrespectively of asthma severity [1]. Notwithstanding, increased AE fre-

A.E.S. and F.B. contributed equally.
Edited by: D.Y. Wang, Singapore.

quency has been associated with severe asthma [2], which requires treatment with oral corticosteroid (OCS). When at least 2 AEs per year occurred, patients are classifiable as frequent exacerbator (FE) phenotype and are demanding in terms of asthma management [3].

Inflammatory monitoring of the airways could be fundamental to optimize the asthmatic patient's management [4, 5] because symptoms do not always reflect the inflammatory processes. The measurement of the exhaled nitric oxide ($F_{E}NO$) produced in the airways is an indicator of corticosteroid-sensitive airway inflammation due to the negative modulation of the inducible nitric oxide synthase (iNOS) expression in the bronchial mucosa as one of the multiple effects of corticosteroids in asthma [6]. Thus, $F_{E}NO$ is used to monitor adherence, and predict the response to ICSs [7]. Increased $F_{E}NO$ levels are associated with a higher prevalence of uncontrolled and/or severe asthma [8–10]. Nevertheless, more specific information can be achieved from the NO exchange dynamics [11]. In fact, NO exchange can be compartmentalized into the steady-state alveolar NO concentration ($C_{A}NO$) and the NO flux from the proximal airway compartment ($J_{aw}NO$), which gives similar information as $F_{E}NO$ at the flow of 50 mL/s ($F_{E}NO_{50}$) [11, 12]. As $C_{A}NO$ may represent the degree of distal airway inflammation, the persistence of high $C_{A}NO$ in patients treated with ICSs could reflect the incapacity of these drugs to reach distal airways [8, 13]. Moreover, $C_{A}NO$ has been reported to increase in patients with severe and poor controlled asthma [14], and in COPD subjects with reduced peripheral oxygen saturation as adaptation to dyspnea [15].

We hypothesized that different levels of NO exchange could be detected among patients prone to AE. We aimed to compare NO exchange in FE (≥ 2 AE/year) and non-frequent exacerbators (nFEs, < 2 AE/year) in combination with T2 blood biomarkers and resting oxygen saturation (SpO_2) as a marker of gas exchange, thus investigating the diagnostic role of NO exchange and SpO_2 in identifying patients prone to AE.

Materials and Methods

Patients and Study Design

This observational cross-sectional study evaluated a series of 91 mild-to-severe asthmatics enrolled at the “Severe Asthma Centre” of the San Luigi Gonzaga University Hospital (Orbassano, Torino, Italy) between November 2018 and June 2020. The study was approved by the San Luigi Gonzaga University Hospital Review Board (Prot. no. 4478/2017). All participants provided written in-

formed consent. Inclusion criteria were adult age and asthma diagnosis confirmed according to Global Initiative for Asthma (GINA) and ERS/ATS guidelines [16, 17], whereas exclusion criteria were active smoking habit, smoking cessation earlier than 1 year, and any comorbidities that could interfere with the interpretations of results.

Collection of Clinical, Functional, and Blood Parameters

The collected data included age, gender, BMI, smoking history, allergen sensitization, comorbidities, hematological examination, pulmonary function tests, AEs, asthma control and severity (GINA steps) [17], asthma medications (ICS and OCS, long-acting beta-agonists [LABA], long-acting muscarinic antagonists [LAMA], omalizumab [anti-IgE], mepolizumab [anti-IL-5]), and exhaled nitric oxide.

Pulmonary function was assessed by spirometry, performed before and 15 min after albuterol administration (400 μ g), and lung volumes using body plethysmograph (Vmax[®] Encore 62, Carefusion, Germany). The resting SpO_2 was measured by pulse oximetry (HS10A, Hunan Accurate Bio-Medical Technology, China).

Blood neutrophil and eosinophil counts were performed using a Cell-Dyn Sapphire (Abbott, Rome, Italy) from routinely performed hematological examinations. For patients treated with mepolizumab only, circulating eosinophils data referred to counts pre- or post-mepolizumab treatment, respectively, for phase I or phase II analyses.

Asthma control was evaluated through the ACT questionnaire [18, 19]: asthma was considered well control for ACT score ≥ 25 , partly control for ACT score between 20 and 24, and poor control for ACT score ≤ 19 . The asthma treatment strategy was done and graded following GINA guidelines (box 7: GINA steps) [17]. Allergic patients were detected by positive skin prick tests and serum total IgE levels [20]. Patients sensitized to ≥ 2 allergens have been considered as poly-sensitized [21].

Measurement of Exhaled Nitric Oxide

Exhaled NO was analyzed before spirometry and plethysmography with $F_{E}NO+$ instrument (Medisoftware, Sorinnes, Belgium) according to the manufacturer's instruction. Briefly, $F_{E}NO_{50}$ was detected in every patient at the flow 50 mL/s. Deeper analyses were conducted on 50 randomly selected patients. For them, additional flows of 100 and 150 mL/s were used to evaluate the alveolar ($C_{A}NO$, ppb) and bronchial ($J_{aw}NO$, nL/s) origin of NO, in accordance to Tsoukias and George two-compartment model [11]. All NO measurements were conducted at least in duplicate, and results mediated.

Patients' Stratification

Patients were stratified based on their frequency of AE in the previous year (AE/y) (phase I). We considered only severe AE, which are defined as those requiring systemic corticosteroids burst for more than 3 consecutive days [16]. Patients who had AE/y < 2 have been classified as nFE ($n = 55$), whereas the term FE ($n = 36$) defined the patients who experienced AE/y ≥ 2 [3, 22, 23]. All analyses were performed on patients in stable disease conditions.

Analyzing data available in medical records, patients were retrospectively followed to detect onset of novel AE (phase II). Thereby, patients were re-grouped as exacerbators (EXA) and as non-exacerbators (nEXA) considering as time points 1, 3, 6, and 12 months after measurement of exhaled nitric oxide.

Table 1. Demographic and clinical characteristics of patients

	All patients (n = 91)	Phase I of the study			Phase II of the study		
		nFE (n = 55)	FE (n = 36)	p value	nEXA (n = 79)	EXA (n = 12)	p value
Age, year	56±15	56±16	56±14	0.892	56±15	59±15	0.476
Gender (female)	57/91 (63%)	37/55 (67%)	20/36 (56%)	0.364	52/79 (65.8%)	5/12 (41.7%)	0.197
BMI, kg/m ²	27±6	27±6	28±6	0.397	27±6	28±4	0.938
Age at asthma onset	34±17	36±19	31±15	0.209	34±17	32±18	0.593
Asthma duration, years	24±17	22±17	27±18	0.171	23±17	28±18	0.438
Allergy	60/91 (66%)	37/55 (67%)	23/36 (64%)	0.915	53/79 (67.1%)	7/12 (58.3%)	0.788
Polysensitization	51/60 (85%)	29/37 (78%)	22/23 (96%)	0.147	45/53 (84.9%)	6/7 (85.7%)	0.612
Perennial allergen sensitization	48/60 (79%)	31/37 (84%)	17/23 (74%)	0.550	44/53 (83.0%)	4/7 (57.1%)	0.269
Seasonal allergen sensitization	52/60 (87%)	30/37 (81%)	22/23 (96%)	0.221	45/53 (84.9%)	7/7 (100%)	0.608
Smoking history (PY)	7.2±13.9	8.4±16.0	5.4±9.9	0.844	7.6±14.6	4.4±8.0	0.454
Past smokers (PY>10)	23/91 (25%)	15/55 (27%)	8/36 (22%)	0.768	20/79 (25.3%)	3/12 (25.0%)	0.739
White blood cells, cell/μL	7,288±1,755	7,220±1,631	7,379±1,929	0.702	7,164±1,740	7,528±1,604	0.499
Blood neutrophils, cells/μL	4,311±1,504	4,095±1,507	4,621±1,469	0.142	4,129±1,439	4,753±1,679	0.193
Blood eosinophils, cells/μL	399±272 ⁰	373±251 ⁰	437±300 ⁰	0.435	267±216 [§]	312±166 [§]	0.500
Total IgE, U/mL [†]	175±153	145±138	296±270	0.022	176±149	262±324	0.221

Phase I, patients stratified based on history of exacerbation in the previous 12 months; Phase II, patients stratified based on retrospective analysis of exacerbation 1 month later the measurements of C_ANO and SpO₂. nFE, non-frequent exacerbator phenotype; FE, frequent exacerbator phenotype; nEXA, patient without new asthma exacerbation within 1 month from NO measurement; EXA, patient with at least one new asthma exacerbation within 1 month from NO measurement; PY, pack-year; SpO₂, oxygen saturation. ⁰ Pre-mepolizumab counts. [§] Post-mepolizumab counts. [†] Data without outliers; p value <0.050 are reported in bold.

Statistical Analysis

D'Agostino & Pearson test was used to evaluate normality distribution. The nonparametric Mann-Whitney U-test compared differences in continue variables with exception of SpO₂ (unpaired T-Test). In the text, these variables were referred as median and 25th, 75th percentile (F_{ENO50}, C_ANO, and J_{aw}NO) or mean ± SD (SpO₂). In figure, boxplots represent data from the first to third quartiles, whiskers are drawn according to the Tukey method; “+” indicates the mean value. Fisher F test was employed to compare categorical variables. For each analysis, outliers were identified with the ROUT method [24] and excluded, if present. Associations between T2, biological and chemical markers with lung function parameters were conducted with bivariate analyses (linear regression and Spearman's rank correlation coefficient ρ). Interconnections among markers were evaluated by correlation matrix and then by bivariate analysis. Kernel density estimation was used to recognize patterns correlate to NO. In the violin plot, dotted lines represent data from the first to third quartiles, dashed line represents median value, and continuous line represents the cutoff values. Receiver operating characteristic (ROC) curves were used to determine optimal cutoff value of SpO₂. Odds ratio (OR) and relative risk (RR) was used to estimate the probability to have high AE frequency or the risk to incur into AE after up to 12 months from estimation of exhaled NO parameters, respectively. AE-free survival (fraction of patients who did not experienced AE) was assessed by means of Kaplan-Meier curves. Log-rank and Gehan-Breslow-Wilcoxon tests (for early events) were used to compare Kaplan-Meier estimates between groups. Results were considered statistically significant for p < 0.05. Statistical evaluations were performed with GraphPad Prism 9.0.2 (GraphPad Software, San Diego, CA, USA).

Results

Demographic and Clinical Characteristics

Demographic and clinical data of the asthmatic patients are reported in Table 1. Briefly, FE and nFE were comparable for demographic data and the history of smoking habit. Allergy frequency and type of sensitization were comparable, but total serum IgE levels were higher in FE groups (p < 0.05). Finally, counts of white blood cells, and in particular neutrophils and eosinophils, were similar between FE and nFE patients. After the regrouping in EXA and nEXA at 1-month post-NO measurement, no differences were detected in both demographic and clinical variables (shown in Table 1), whereas only few differences in allergy prevalence were detected at 6-month time point (shown in online suppl. Table 1; see www.karger.com/doi/10.1159/000518320 for all online suppl. material).

Asthma Control and Treatment

FE patients are characterized by lower ACT score (p < 0.05) and are affected by higher asthma severity than nFE (p < 0.01, GINA treatment step distribution), thereby they received higher impact treatment (shown in Table 2). FE patients are administered with higher ICS doses (p < 0.001) and more frequently are treated with OCS for at least 6 month/year (p < 0.05), LABA (p < 0.01), and biologics (p <

Table 2. Asthma control and treatment in the asthmatic population

	All patients (n = 91)	Phase I of the study			Phase II of the study		
		nFE (n = 55)	FE (n = 36)	p value	nEXA (n = 79)	EXA (n = 12)	p value
ACT score	20±4	21±3	19±4	0.027	21±3	15±4	<0.001
GINA treatment steps 1–2 (mild asthma)	15/91 (16%)	14/55 (26%)	1/36 (3%)	0.010	14/79 (17.7%)	1/12 (8.3%)	0.690
GINA treatment steps 3–4 (moderate asthma)	38/91 (42%)	26/55 (47%)	12/36 (33%)	0.271	30/79 (38.0%)	8/12 (66.7%)	0.118
GINA treatment step 5 (severe asthma)	38/91 (42%)	15/55 (27%)	23/36 (64%)	0.001	35/79 (44.3%)	3/12 (25.0%)	0.342
ICS/day (fluticasone propionate, µg)	464±326	375±308	601±309	<0.001	460±332	490±294	0.775
OCS (≥6 months/year)	7/91 (8%)	1/55 (2%)	6/36 (17%)	0.028	5/79 (6.3%)	2/12 (16.7%)	0.502
LABA	75/91 (82%)	40/55 (73%)	35/36 (97%)	0.007	64/79 (81.0%)	11/12 (91.7%)	0.620
LAMA	18/91 (20%)	9/55 (16%)	9/36 (25%)	0.458	16/79 (20.2%)	2/12 (16.7%)	0.922
Biologics	25/91 (28%)	10/55 (18%)	15/36 (42%)	0.027	25/79 (30.4%)	1/12 (8.3%)	0.212
Omalizumab	10/91 (11%)	6/55 (11%)	4/36 (11%)	0.755	10/79 (12.7%)	0/12 (0.0%)	0.417
Mepolizumab	15/91 (16%)	4/55 (7%)	11/36 (31%)	0.008	14/79 (17.7%)	1/12 (8.3%)	0.690

p values <0.050 are reported in bold. Phase I, patients stratified based on history of exacerbation in the previous 12 months; Phase II, patients stratified based on retrospective analysis of exacerbation 1 month later the measurements of C_ANO and SpO₂. nFE, non-frequent exacerbator phenotype; FE, frequent exacerbator phenotype; nEXA, patient without new asthma exacerbation within 1 month from NO measurement; EXA, patient with at least one new asthma exacerbation within 1 month from NO measurement; ACT, asthma control test; GINA, Global Initiative for Asthma; ICS, inhaled corticosteroid; OCS, oral corticosteroid; LABA, long-acting beta-agonists; LAMA, long-acting muscarinic antagonists; SpO₂, oxygen saturation.

Table 3. Spirometry and plethysmography parameters in the asthmatic population

	All patients (n = 91)	Phase I of the study			Phase II of the study		
		nFE (n = 55)	FE (n = 36)	p value	nEXA (n = 79)	EXA (n = 12)	p value
FVC, %pred.	101±19	103±18	98±21	0.280	102±19	94±21	0.175
PB FVC, %pred.	105±19	107±18	103±21	0.399	105±20	106±19	0.917
PB FVC, mL change	199±243	178±212	232±287	0.414	160±163	438±462	0.002
FEV ₁ , %pred.	83±23	86±21	80±27	0.244	85±22	71±27	0.053
PB FEV ₁ , %pred.	90±22	92±19	87±26	0.353	91±22	88±20	0.783
PB FEV ₁ , mL change	264±202	268±174	258±244	0.297	240±153	411±375	0.018
FEV ₁ /FVC, %	67±11	68±10	66±13	0.519	68±11	61±10	0.049
PB FEV ₁ /FVC, %	70±11	70±10	69±13	0.736	70±12	68±7	0.656
RV, %pred.	126±39	119±33	137±45	0.047	124±38	140±46	0.251
TLC, %pred.	107±16	106±15	108±16	0.523	107±15	107±19	0.934
RV/TLC, %	43±12	42±12	46±12	0.174	42±12	48±8	0.171
FRC, %pred.	111±23	114±23	105±24	0.315	110±24	117±17	0.700

p values <0.050 are reported in bold. Phase I, patients stratified based on history of exacerbation in the previous 12 months; Phase II, patients stratified based on retrospective analysis of exacerbation 1 month later the measurements of C_ANO and SpO₂. nFE, non-frequent exacerbator phenotype; FE, frequent exacerbator phenotype; nEXA, patient without new asthma exacerbation within 1 month from NO measurement; EXA, patient with at least one new asthma exacerbation within 1 month from NO measurement; PB, post-bronchodilation; SpO₂, oxygen saturation.

0.05). In particular, the use of omalizumab was similar between the groups, whereas mepolizumab use was more common in FE ($p < 0.01$). After a 1-month period, we detected those patients who further exacerbated (EXA) had the lowest ACT score (shown in Table 2, $p < 0.001$ vs. nEXA). This pattern was also conserved at all the different time points (shown in online suppl. Table 2). Further, EXA

patients at 12-month used more LABA and were less commonly classified in GINA step-1 than nEXA ($p < 0.05$).

Lung Functional Parameters

Baseline spirometry parameters (FVC, FEV₁, and FEV₁/FVC) were comparable between FE and nFE asthmatics (shown in Table 3). In all groups, the administration of

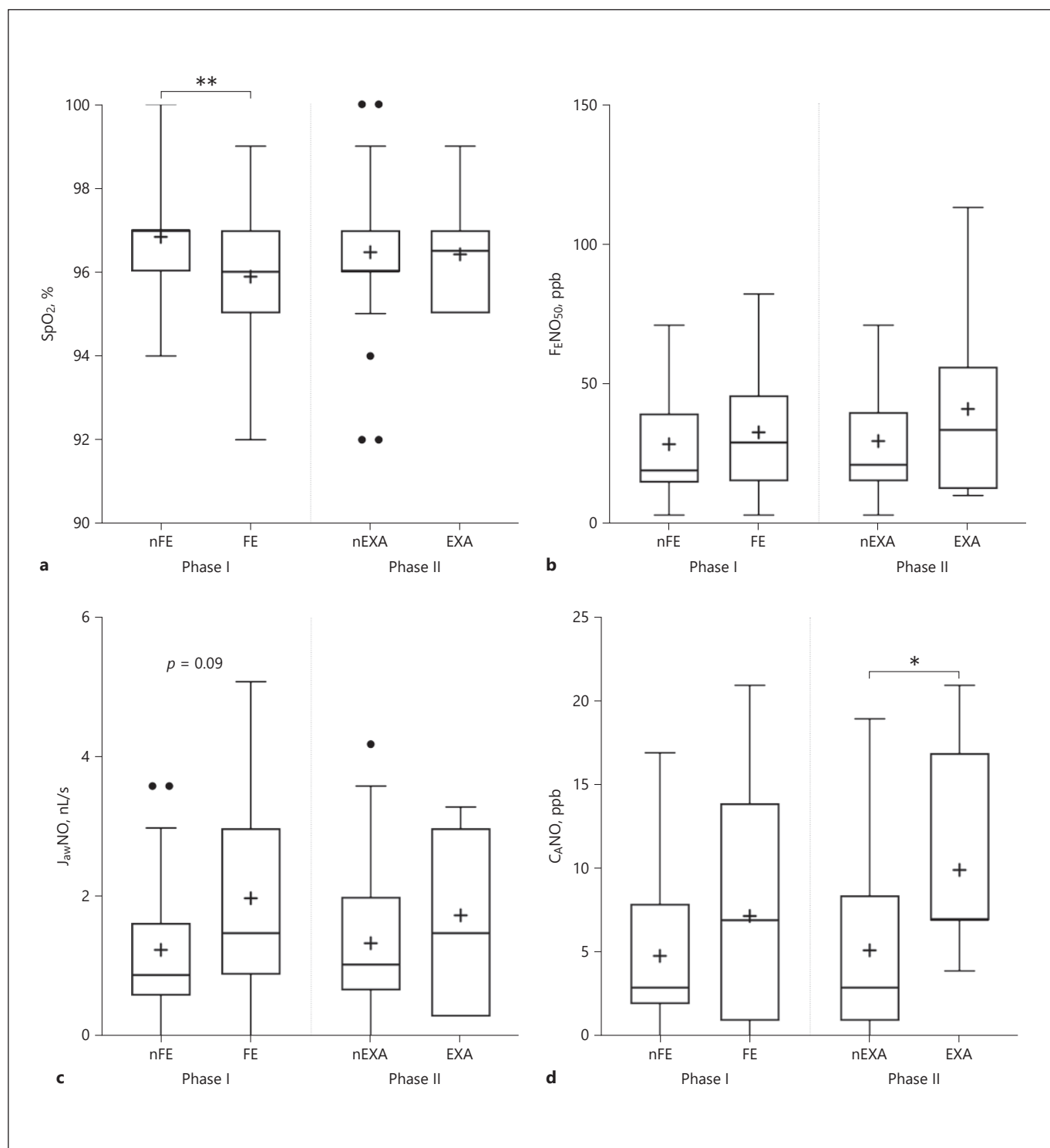


Fig. 1. Characterization of peripheral oxygen saturation (**a**, SpO_2) and airway NO exchange (**b**, $F_E NO_{50}$; **c**, $J_{AW} NO$; and **d**, $C_A NO$) in patients stratified according to occurrences of AE; * $p < 0.05$; ** $p < 0.01$. AE, asthmatic exacerbation; FE, frequent exacerbators; nFE, non-frequent exacerbators; EXA, asthmatics with novel exacerbations after 1 month; nEXA, asthmatics without novel exacerbations after 1 month; SpO_2 , oxygen saturation.

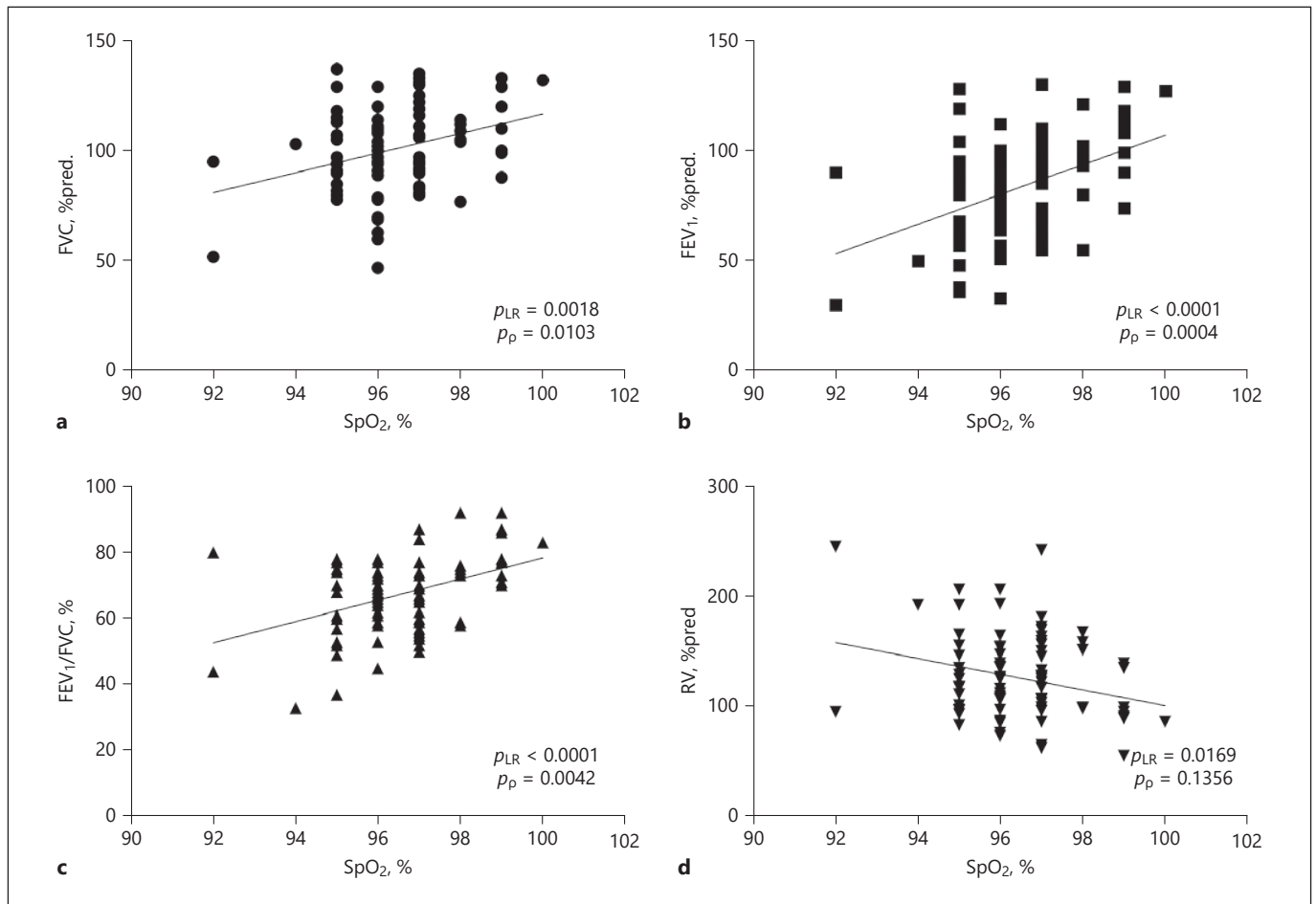


Fig. 2. Linear regressions between peripheral oxygen saturation and FVC %pred (a); FEV₁ %pred (b); FEV₁/FVC % (c); and RV %pred (d). p_{LR} , p value of linear regression; p_p , p value of correlation.

bronchodilator mediated similar FVC and FEV₁ changes (mL). Thus, post-bronchodilation (PB)-FVC, PB-FEV₁, and PB-FEV₁/FVC were similar between the 2 groups.

FE patients had higher RV (RV %pred.) than nFE ($p < 0.05$). Other plethysmography parameters (TLC, RV/TLC, and FRC) were otherwise comparable.

EXA patients had higher airway obstruction (FEV₁/FVC) than nEXA asthmatics at 1 month ($p < 0.05$) but demonstrated higher FVC ($p < 0.01$) and FEV₁ ($p < 0.05$) reversibility (shown in Table 3). Otherwise, no differences in terms of lung function were detected afterward between EXA and nEXA, except for FEV₁ and RV/TLC at 6 months ($p < 0.05$, online suppl. Table 3).

NO Exchange and Peripheral Oxygen Saturation

Peripheral SpO₂ was lower in FE ($96 \pm 1.4\%$) than nFE ($97 \pm 1.4\%$, $p < 0.01$, Fig. 1a). Meanwhile, concen-

trations of F_ENO₅₀ were statistically similar between the 2 groups (shown in Fig. 1b), although slightly higher in FE. In fact, F_ENO₅₀ was 29 (15, 46) ppb for FE and 19 (14, 40) ppb for nFE. The deeper analysis of NO exchange conducted in a subpopulation revealed that C_ANO and J_{aw}NO were 7 (1, 14) ppb and 1.5 (0.9, 3.0) nL/s in the FE group compared to 3 (2, 8) ppb and 0.9 (0.6, 1.7) nL/s (although at higher ICS dose) in the nFE group (shown in Fig. 1c, d).

At 1 month of the follow-up (phase II), no differences in terms of SpO₂, F_ENO₅₀, and J_{aw}NO, have been detected between EXA asthmatics ($96 \pm 1.3\%$, 34 [12, 56] ppb, and 1.5 [0.3, 3.0] nL/s, respectively) and nEXA asthmatics ($96 \pm 1.5\%$, 21 [15, 40] ppb, and 1.0 [0.7, 2.0] nL/s, respectively). Otherwise, at 1 month of the follow-up, C_ANO was significantly higher in EXA, 7.0 (7.0, 17.0) ppb, than in nEXA, 3.0 (1.0, 8.5) ppb ($p < 0.05$),

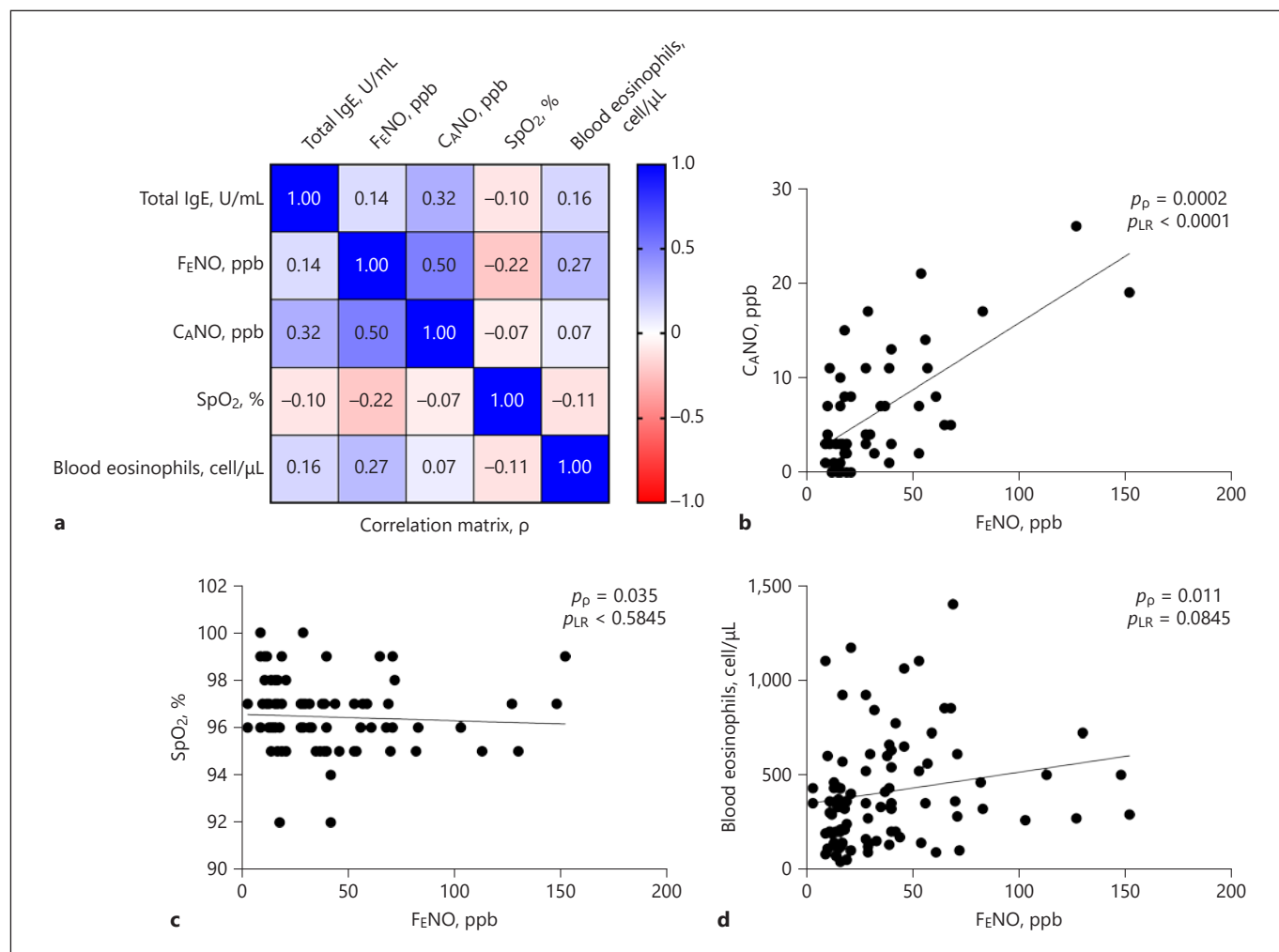


Fig. 3. ρ values of correlation matrix among T2, biological, and chemical biomarkers (a), and linear regressions between FeNO₅₀ and CA NO (b); SpO₂ (c); and blood eosinophils (d). p_{LR} , p value of linear regression; p_p , p value of correlation. SpO₂, oxygen saturation.

as shown in Figure 1d. At 3, 6, and 12 months of the follow-up, the data were similar between the 2 groups (data not shown).

Correlation and Linear Regression Models

Among the main T2 (serum IgE, FeNO, and blood eosinophils), biological (CA NO), and chemical (SpO₂) biomarkers, only peripheral oxygen saturation demonstrated causal and linear dependence with lung functional parameters (Fig. 2). In particular, SpO₂ had positive linearity with FVC (slope 4.42, 95% CI 1.70–7.15, $p < 0.01$), FEV₁ (slope 6.71, 95% CI 3.54–9.87, $p < 0.001$), and FEV₁/FVC (slope 3.19, 95% CI 1.65–4.74, $p < 0.001$), and negative linearity with RV (slope -7.11, 95% CI -12.91 to -1.31, $p < 0.05$). However, weak bivariate correlations

were detected only with spirometry parameters (FVC $\rho = 0.28$, $p < 0.05$; FEV₁ $\rho = 0.37$, $p < 0.001$; and FEV₁/FVC $\rho = 0.30$, $p < 0.01$), being SpO₂ and RV not directly related ($\rho = -0.17$, $p = 0.13$).

Further, to evaluate whether biomarkers are interconnected among them, a correlation matrix was performed (Fig. 3a). Statistically significant correlations were detected only between FeNO and CA NO (Fig. 3b, $p < 0.001$), SpO₂ (Fig. 3c, $p < 0.05$), and blood eosinophils (Fig. 3d, $p < 0.05$). In our population, FeNO correlations were moderate (vs. CA NO) or weak (vs. SpO₂ and blood eosinophils) (Fig. 3a), and causal relationship was detected only between FeNO and CA NO (slope 0.14, 95% CI 0.09–0.19, $p < 0.001$, Fig. 3b).

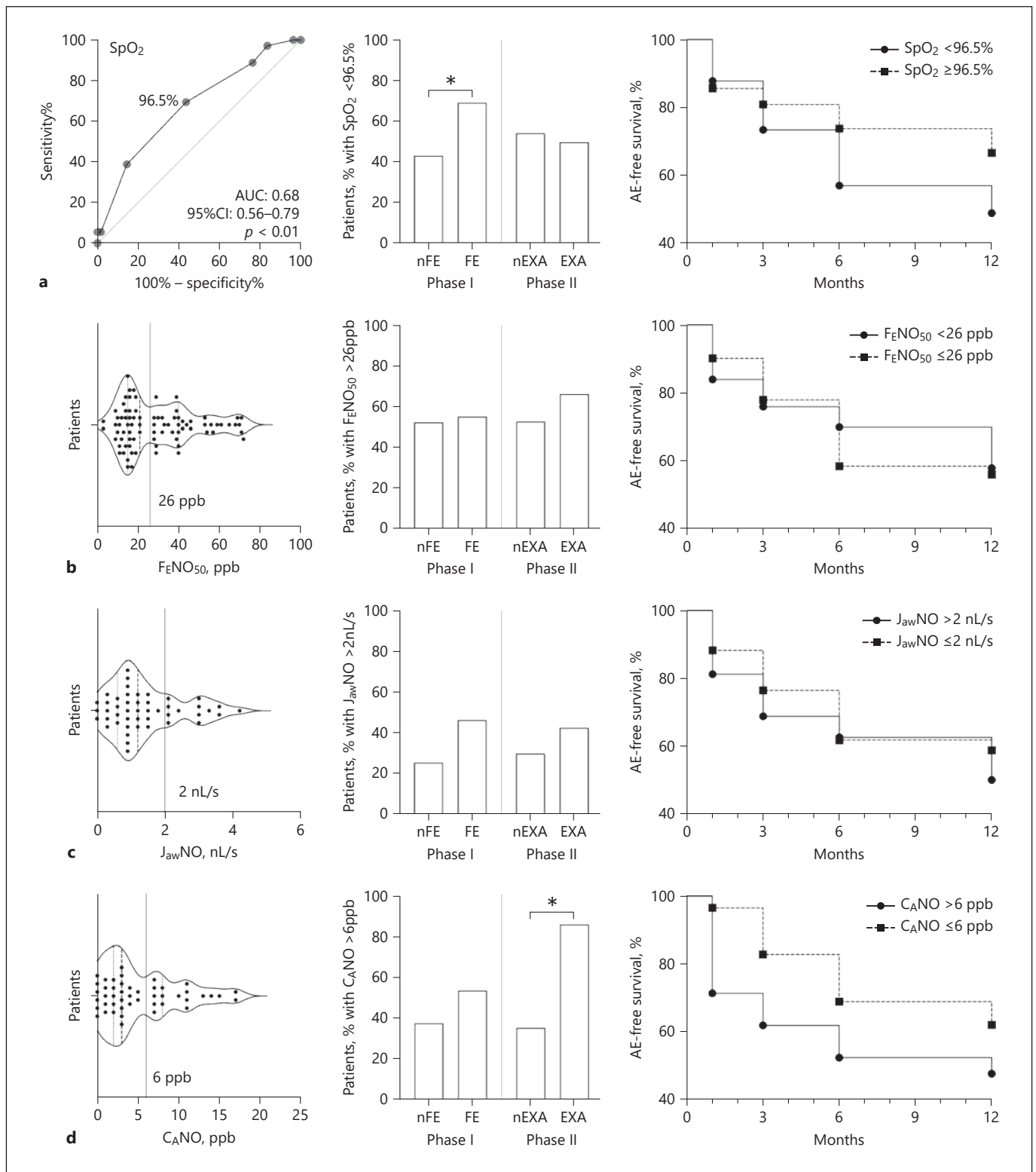


Fig. 4. Predictive role of peripheral oxygen saturation and airway NO exchange. Cutoff selection (left), correspondent patient frequencies (center), and Kaplan-Meier estimates for AE-free survival (fraction of patients without AE) (right) of SpO_2 (a); $F_{ENO_{50}}$ (b); J_{awNO} (c); and C_{ANO} (d). * $p < 0.05$. AE, asthmatic exacerbation; SpO_2 , oxygen saturation.

Risk Assessment

The predictive role of SpO₂ and NO exchange to discriminate patients prone to AE has been explored. Possible cutoffs were estimated through ROC curve analysis or kernel density estimation (shown in Fig. 4, left column). Values of SpO₂ <96.5% discriminated FE patients (Fig. 4a, OR = 2.94, 95% CI 1.23–7.18), whereas C_ANO >6 ppb predicted occurrence of AE within 1 month (Fig. 4d, central column, RR = 11.20, 95% CI 1.45–132.2), with significant results ($p < 0.05$). The prevalence of patients with C_ANO >6 ppb was higher in EXA ($p < 0.05$) and that with SpO₂ <96.5% was higher in FE ($p < 0.05$). Otherwise, cutoffs of F_ENO₅₀ and J_{aw}NO did not achieve adequate discrimination power. Finally, Kaplan-Meier estimates for occurrences of AE (Fig. 4, right column) did not reveal significant differences in the 12 months follow-up.

Discussion/Conclusion

In this study, we analyzed 91 asthmatic patients: demographic and clinical characteristics, lung function parameters including SpO₂, and concentration of T2-inflammation biomarkers (blood eosinophils, serum IgE, and F_ENO₅₀) were evaluated for each patient; otherwise, the alveolar (C_ANO) and bronchial (J_{aw}NO) origins of exhaled NO were analyzed on a randomly selected subgroup, only.

In the first phase of our study, patients were classified as nFEs or FEs based on the AE/y frequency [22, 23]. We showed that FE asthmatics exhibited higher serum total IgE and peripheral airway obstruction (air trapping as determined by higher RV) but reduced SpO₂ than nFE confirming a role of a systemic T2 biomarker and distal airway dysfunction in conjunction with reduced oxygen saturation in the FE phenotype [23]. Further, FE had lower ACT score and required higher impact therapy than nFE asthmatics, while other T2 inflammation markers (blood eosinophils and F_ENO₅₀) were comparable. The latter evidence indicate that the FE phenotype is characterized by poor control asthma with a higher drug consumption [23]. Finally, in the second phase of the study, we re-classified patients as EXA or nEXA, according with the occurrence of novel AE up to 12 months after the NO measurement, to detect the potential role of NO exchange in predict AE in the near future.

In literature, high blood eosinophilia has been correlated with high asthma morbidity, in terms of need for access to the emergency room [25, 26] because of exacerbation and severe acute events [27], while elevated F_ENO (>300% pred.) is an AE predictor at 1 year [28]. Further, concomitant presence of high blood eosinophilia (≥ 300

cell/ μ L) and high F_ENO values (≥ 20 –25 ppb) have been correlated with loss of asthma control [25, 26]. In our population, no statistical difference has been detected in terms of blood eosinophils (either pre- or post mepolizumab treatment) and F_ENO₅₀. Thus, blood eosinophils and F_ENO₅₀ appeared not involved in the features of the FE phenotype, nor seemed able to predict future AE likely because of different inclusion criteria and sample size limitations of the pilot cohort.

The tendency of higher levels of NO (F_ENO₅₀, but also J_{aw}NO and C_ANO) in FE than nFE groups could potentially be influenced by the higher doses of ICS regimen in the FE group (fluticasone propionate average: 601 μ g/day) compared to the nFE group (fluticasone propionate average: 375 μ g/day), thus affecting iNOS expression. Higher concentrations of F_ENO₅₀ are indeed present in highly exacerbating asthmatic patients, but F_ENO₅₀ can be elevated even in those who not exacerbated. To corroborate this hypothesis, both FE and nFE demonstrated higher F_ENO₅₀ compared to healthy individuals at the same age-group [29] gives indication of an airway inflammation. Deepening this aspect, we detected that the J_{aw}NO was not to a larger extent increased compared to the healthy individuals, but the estimation of C_ANO was higher in FE group of asthmatics compared to the healthy individuals [29], indicating the presence of active inflammation in the most distal airways and alveoli [30]. Furthermore, in our population, C_ANO and F_ENO₅₀ were positively correlated with causal effects. In this point of view, C_ANO should be considered as a surrogate biomarker of F_ENO₅₀ in the detection of future AE. In fact, elevated C_ANO can be likely the result of a possible mechanism for resistance or lacking peripheral distribution of ICS in the distal airways. This concept was supported by the presence of high C_ANO and RV levels in FE patients who required higher doses of ICS or OCS to achieve control of their symptoms. Indeed, we detected a higher C_ANO concentration and poor asthma control, at the time of F_ENO/C_ANO measurements, in asthmatics prone to near-future exacerbation (EXA at 1-month post-NO measurement). These patients were characterized by higher air-flow obstruction but had also higher airway relaxant response after bronchodilator administration (Δ FEV₁ and Δ FVC). Further, the greater prevalence of C_ANO >6 ppb in EXA group suggested its potential clinical use as a biomarker in predicting near-future AEs (RR = 11.20).

In literature, the role of IgE in AE development is highly debated. In fact, potential roles of IgE in the pathogenesis of AE have been highlighted through impairment of virus recognition and IFN α production [1, 31], and by direct airway smooth muscle cells activation [32]. How-

ever, other study demonstrated a non-causal effect [33] or even an inverse relationship [34]. Here, higher concentrations of total serum IgE were detected in FE compared to nFE, but no further differences or correlations have been demonstrated, thus endorsing the hypothesis that IgE could have only a limited impact on the risk of developing AE.

Moreover, the FE phenotype showed airway functional abnormalities, even at disease stable phase [23], and our FE patients showed, in particular, a higher RV. It could be a cause of impaired gas exchange and, therefore, of the reduced SpO₂ detected in the FE group of asthmatic population. In fact, we detected an inverse linearity between SpO₂ and RV (though the 2 variables appeared not directly correlated), and a direct causal and linear dependence among SpO₂ and spirometry parameters (FVC, FEV₁ and FEV₁/FVC). In our knowledge, this is the first time in which decrease in peripheral oxygen saturation have been correlated in asthma patients with progressive improvement in airflow obstruction, further confirming the axiom “the greater the airway obstruction the worse the gas exchange” [35]. This finding is in line with the cut-point detected in the general population (asymptomatic adults) by Witting and Scharf that consider SpO₂ <97% as a limit to detect abnormal pulmonary function and to yield a 5.9 OR for asthma [36]. Thus, SpO₂ can be considered as a chemical marker of gas exchange because it is easily accessible and values <96.5% can be able to discriminate potentially FE patients (OR = 2.94).

Despite the limitation of a small sample size due to the pilot nature of the study, our findings support the presence of active distal NO-related airway inflammation in conjunction with lower oxygen saturation in AE-susceptible asthmatics suggesting a clinically relevant role of both C_ANO and SpO₂. These markers could exert specific roles in predicting near-future AE (C_ANO) and monitoring FE asthmatic patients (SpO₂). Nevertheless,

further studies are required to validate the measurement of C_ANO and SpO₂ in asthmatics with frequent exacerbations and/or prone to exacerbate.

Statement of Ethics

This study was approved by the local Ethics Committees (San Luigi Gonzaga University Hospital: protocol 4478/2017) and conducted in conformity to the Declaration of Helsinki between November 2018 and June 2020. All participants provided written informed consent.

Conflict of Interest Statement

F.L.M.R. reports grants, personal fees and other from Astra-Zeneca, Chiesi, GSK and Novartis, personal fees from Sanofi, all outside the submitted work.

Funding Sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contributions

Substantial contributions to the conception or design of the work: F.L.M.R. Substantial contributions to the acquisition, analysis, or interpretation of data for the work: A.E.S., F.B., R.F., S.L., V.C., M.H. Drafting the work or revising it critically for important intellectual content: A.E.S., F.B. Final approval of the version to be published: A.E.S., F.B., R.F., S.L., V.C., M.H., F.L.M.R. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: F.L.M.R.

Data Availability Statement

The data that support the findings of this study are available upon request to the corresponding author.

References

- Castillo JR, Peters SP, Busse WW. Asthma exacerbations: pathogenesis, prevention, and treatment. *J Allergy Clin Immunol Pract*. 2017 Jul–Aug;5(4):918–27.
- Bloom CI, Nissen F, Douglas IJ, Smeeth L, Cullinan P, Quint JK. Exacerbation risk and characterisation of the UK's asthma population from infants to old age. *Thorax*. 2018 Apr;73(4):313–20.
- Kupczyk M, ten Brinke A, Sterk PJ, Bel EH, Papi A, Chanez P, et al. Frequent exacerbators: a distinct phenotype of severe asthma. *Clin Exp Allergy*. 2014 Feb;44(2):212–21.
- Ciprandi G, Tosca MA, Silvestri M, Ricciardolo FLM. Inflammatory biomarkers for asthma endotyping and consequent personalized therapy. *Expert Rev Clin Immunol*. 2017 Jul;13(7):715–21.
- Ciprandi G, Marseglia GL, Ricciardolo FLM, Tosca MA. Pragmatic markers in the management of asthma: a real-world-based approach. *Children*. 2020 May 18;7(5):48.
- Ricciardolo FLM, Silkoff PE. Perspectives on exhaled nitric oxide. *J Breath Res*. 2017 Sep 27;11(4):047104.
- Price DB, Buhl R, Chan A, Freeman D, Gardner E, Godley C, et al. Fractional exhaled nitric oxide as a predictor of response to inhaled corticosteroids in patients with non-specific respiratory symptoms and insignificant bronchodilator reversibility: a randomised controlled trial. *Lancet Respir Med*. 2018 Jan;6(1):29–39.
- Ricciardolo FL. Revisiting the role of exhaled nitric oxide in asthma. *Curr Opin Pulm Med*. 2014 Jan;20(1):53–9.

- 9 Ricciardolo FL, Sorbello V, Ciprandi G. FeNO as biomarker for asthma phenotyping and management. *Allergy Asthma Proc.* 2015 Jan–Feb;36(1):e1–8.
- 10 Menzies-Gow A, Mansur AH, Brightling CE. Clinical utility of fractional exhaled nitric oxide in severe asthma management. *Eur Respir J.* 2020 Mar;55(3):1901633.
- 11 Tsoukias NM, George SC. A two-compartment model of pulmonary nitric oxide exchange dynamics. *J Appl Physiol* (1985). 1998 Aug;85(2):653–66.
- 12 Gelb AF, Barnes PJ, George SC, Ricciardolo FL, DiMaria G, Zamel N. Review of exhaled nitric oxide in chronic obstructive pulmonary disease. *J Breath Res.* 2012 Dec;6(4):047101.
- 13 Lehtimäki L, Kankaanranta H, Saarelainen S, Turjanmaa V, Moilanen E. Inhaled fluticasone decreases bronchial but not alveolar nitric oxide output in asthma. *Eur Respir J.* 2001 Oct;18(4):635–9.
- 14 Puckett JL, Taylor RW, Leu SY, Guijon OL, Aledia AS, Galant SP, et al. Clinical patterns in asthma based on proximal and distal airway nitric oxide categories. *Respir Res.* 2010 Apr 28;11:47.
- 15 Högman M, Thornadsson A, Bröms K, Jansson C, Lisspers K, Stållberg B, et al. Higher alveolar nitric oxide in COPD is related to poorer physical capacity and lower oxygen saturation after physical testing. *Eur Respir J.* 2019 Aug;54(2):1900263.
- 16 Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J.* 2014 Feb;43(2):343–73.
- 17 Global Initiative for Asthma. *Global strategy for asthma management and prevention*; 2020. Available from: www.ginasthma.org.
- 18 Nathan RA, Sorkness CA, Kosinski M, Schatz M, Li JT, Marcus P, et al. Development of the asthma control test: a survey for assessing asthma control. *J Allergy Clin Immunol.* 2004;113(1):59–65.
- 19 Meltzer EO, Schatz M, Nathan R, Garris C, Stanford RH, Kosinski M. Reliability, validity, and responsiveness of the Rhinitis Control Assessment Test in patients with rhinitis. *J Allergy Clin Immunol.* 2013;131(2):379–86.
- 20 Muraro A, Roberts G, Halken S, Agache I, Angier E, Fernandez-Rivas M, et al. EAACI guidelines on allergen immunotherapy: executive statement. *Allergy.* 2018 Apr;73(4):739–43.
- 21 Hellings PW, Fokkens WJ, Bachert C, Akdis CA, Bieber T, Agache I, et al. Positioning the principles of precision medicine in care pathways for allergic rhinitis and chronic rhinosinusitis: a EUFOREA-ARIA-EPOS-AIRWAYS ICP statement. *Allergy.* 2017 Sep;72(9):1297–305.
- 22 Carriero V, Bertolini F, Sprio AE, Bullone M, Ciprandi G, Ricciardolo FLM. High levels of plasma fibrinogen could predict frequent asthma exacerbations. *J Allergy Clin Immunol Pract.* 2020 Mar 7;8(7):2392–5.e7.
- 23 Sprio AE, Carriero V, Levra S, Botto C, Bertolini F, Di Stefano A, et al. Clinical characterization of the frequent exacerbator phenotype in asthma. *J Clin Med.* 2020;9(7):2226.
- 24 Motulsky HJ, Brown RE. Detecting outliers when fitting data with nonlinear regression: a new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics.* 2006;7:123.
- 25 Malinovschi A, Janson C, Borres M, Alving K. Simultaneously increased fraction of exhaled nitric oxide levels and blood eosinophil counts relate to increased asthma morbidity. *J Allergy Clin Immunol.* 2016 Nov;138(5):1301–e2.
- 26 Mogensen I, Alving K, Jacinto T, Fonseca J, Janson C, Malinovschi A. Simultaneously elevated FeNO and blood eosinophils relate to asthma morbidity in asthmatics from NHANES 2007–12. *Clin Exp Allergy.* 2018 Aug;48(8):935–43.
- 27 Price DB, Rigazio A, Campbell JD, Bleeker ER, Corrigan CJ, Thomas M, et al. Blood eosinophil count and prospective annual asthma disease burden: a UK cohort study. *Lancet Respir Med.* 2015 Nov;3(11):849–58.
- 28 Zeiger RS, Schatz M, Zhang F, Crawford WW, Kaplan MS, Roth RM, et al. Elevated exhaled nitric oxide is a clinical indicator of future uncontrolled asthma in asthmatic patients on inhaled corticosteroids. *J Allergy Clin Immunol.* 2011 Aug;128(2):412–4.
- 29 Högman M, Thornadsson A, Liv P, Hua-Huy T, Dinh-Xuan AT, Tufvesson E, et al. Effects of growth and aging on the reference values of pulmonary nitric oxide dynamics in healthy subjects. *J Breath Res.* 2017 Sep 13;11(4):047103.
- 30 Lehtimäki L, Kankaanranta H, Saarelainen S, Turjanmaa V, Moilanen E. Increased alveolar nitric oxide concentration in asthmatic patients with nocturnal symptoms. *Eur Respir J.* 2002 Oct;20(4):841–5.
- 31 Dunican EM, Fahy JV. The role of Type 2 inflammation in the pathogenesis of asthma exacerbations. *Ann Am Thorac Soc.* 2015 Nov;12(Suppl 2):S144–9.
- 32 Matucci A, Vultaggio A, Maggi E, Kasujee I. Is IgE or eosinophils the key player in allergic asthma pathogenesis? Are we asking the right question? *Respir Res.* 2018 Jun 8;19(1):113.
- 33 Jackson DJ, Humbert M, Hirsch I, Newbold P, Garcia Gil E. Ability of serum IgE concentration to predict exacerbation risk and benralizumab efficacy for patients with severe eosinophilic asthma. *Adv Ther.* 2020 Feb;37(2):718–29.
- 34 Denlinger LC, Phillips BR, Ramratnam S, Ross K, Bhakta NR, Cardet JC, et al. Inflammatory and comorbid features of patients with severe asthma and frequent exacerbations. *Am J Respir Crit Care Med.* 2017 Feb 1;195(3):302–13.
- 35 Rodriguez-Roisin R. Acute severe asthma: pathophysiology and pathobiology of gas exchange abnormalities. *Eur Respir J.* 1997 Jun;10(6):1359–71.
- 36 Witting MD, Scharf SM. Diagnostic room-air pulse oximetry: effects of smoking, race, and sex. *Am J Emerg Med.* 2008 Feb;26(2):131–6.

eTable 1: Demographic and clinical characteristics of patients stratified for presence of asthma exacerbations after NO measurement

Follow-up post NO measurement (Phase II)	3 Months			6 Months			12 Months		
	nEXA (n=70)	EXA (n=21)	P	nEXA (n=59)	EXA (n=32)	P	nEXA (n=52)	EXA (n=39)	P
Age (year)	56±15	58±16	0.527	55±15	59±14	0.203	55±16	58±14	0.288
Gender (Female)	44/70 (62.9%)	13/21 (61.9%)	0.859	36/59 (61.0%)	21/32 (65.6%)	0.836	31/52 (59.6%)	26/39 (66.7%)	0.639
BMI (kg/m ²)	27±6	28±5	0.905	27±5	29±6	0.061	27±6	28±6	0.181
Age at asthma onset	35±17	31±19	0.387	35±18	31±17	0.321	35±18	33±16	0.694
Asthma duration (years)	22±16	29±20	0.129	21±17	29±17	0.050	22±17	26±17	0.320
Allergy	47/70 (67.1%)	13/21 (61.9%)	0.856	44/59 (74.6%)	16/32 (50.0%)	0.033	39/52 (75.0%)	21/39 (53.8%)	0.060
Polysensitization	40/47 (85.1%)	11/13 (84.6%)	0.693	37/44 (84.1%)	14/16 (87.5%)	0.935	33/39 (84.6%)	18/21 (85.7%)	0.791
Perennial allergen sensitization	38/47 (80.8%)	10/13 (76.9%)	0.938	35/44 (79.6%)	13/16 (81.2%)	0.827	32/39 (82.0%)	16/21 (76.2%)	0.839
Seasonal allergen sensitization	40/47 (85.1%)	12/13 (92.3%)	0.830	37/44 (84.1%)	15/16 (93.8%)	0.587	33/39 (84.6%)	19/21 (90.5%)	0.811
Smoking history (PY)	8.2±15.3	3.8±6.9	0.200	7.5±14.5	6.8±12.9	0.810	7.9±15.1	6.3±12.2	0.576
Past smokers (PY>10)	19/70 (27.1%)	4/21 (19.0%)	0.644	15/59 (25.4%)	8/32 (25.0%)	0.835	14/52 (26.9%)	9/39 (23.1%)	0.862
White Blood Cells (cell/μL)	7164±1744	7371±1664	0.634	7176±1610	7279±1906	0.791	7117±1632	7330±1827	0.572
Blood Neutrophils (cells/μL)	4140±1406	4439±1701	0.433	4016±1340	4562±1660	0.104	4043±1375	4424±1589	0.243
Blood Eosinophils (cells/μL) §	277±219	226±161	0.321	277±228	243±165	0.461	256±162	277±255	0.632
Total IgE (U/mL) †	199±166	108±108	0.059	176±134	195±206	0.668	166±132	203±197	0.397

nEXA: patient without new asthma exacerbation after NO measurement; EXA: patient with at least one new asthma exacerbation after NO measurement; PY: pack-year; § post-Mepolizumab counts; † data without outliers; *=p<0.05 vs correspondent nEXA; P-value <0.050 are reported in bold.

eTable 2: Asthma control and treatment in the asthmatic population stratified for presence of asthma exacerbations after NO measurement

Follow-up post NO measurement (Phase II)	3 Months			6 Months			12 Months		
	nEXA (n=70)	EXA (n=21)	P	nEXA (n=59)	EXA (n=32)	P	nEXA (n=52)	EXA (n=39)	P
ACT score	21±3	17±4	<0.001	22±3	18±4	<0.001	22±3	19±4	<0.001
GINA treatment steps 1-2 (mild asthma)	14/70 (20.0%)	1/21 (4.8%)	0.188	13/59 (22.0%)	2/32 (6.2%)	0.101	13/52 (25.0%)	2/39 (5.1%)	0.025
GINA treatment steps 3-4 (moderate asthma)	26/70 (37.1%)	12/21 (57.1%)	0.168	24/59 (40.7%)	14/32 (43.8%)	0.951	21/52 (40.4%)	17/39 (43.6%)	0.927
GINA treatment step 5 (severe asthma)	30/70 (42.9%)	8/21 (38.1%)	0.892	22/59 (37.3%)	16/32 (50.0%)	0.341	18/52 (34.6%)	20/39 (51.3%)	0.167
ICS/day (Fluticasone propionate, µg)	450±341	512±271	0.449	415±313	555±336	0.051	406±323	542±319	0.049
OCS (≥6 months/year)	5/70 (7.1%)	2/21 (9.5%)	0.914	4/59 (6.8%)	3/32 (9.4%)	0.975	2/52 (3.8%)	5/39 (12.8%)	0.233
LABA	55/70 (78.6%)	20/21 (95.2%)	0.152	45/59 (76.3%)	30/32 (93.8%)	0.071	38/52 (73.1%)	37/39 (94.9%)	0.015
LAMA	13/70 (18.6%)	5/21 (23.8%)	0.829	11/59 (18.6%)	7/32 (21.9%)	0.925	9/52 (17.3%)	9/39 (23.1%)	0.676
Biologics	21/70 (30.0%)	4/21 (19.0%)	0.479	17/59 (28.8%)	8/32 (25.0%)	0.886	15/52 (28.8%)	10/39 (25.6%)	0.919
Omalizumab	9/70 (12.9%)	1/21 (4.8%)	0.521	7/59 (11.9%)	3/32 (9.4%)	0.991	7/52 (13.5%)	3/39 (7.7%)	0.595
Mepolizumab	12/70 (17.1%)	3/21 (14.3%)	0.979	10/59 (17.0%)	5/32 (15.6%)	0.894	8/52 (15.4%)	7/39 (18.0%)	0.967

nEXA: patient without new asthma exacerbation after NO measurement; EXA: patient with at least one new asthma exacerbation after NO measurement; ACT: asthma control test; GINA: Global Initiative for Asthma; ICS: inhaled corticosteroids; OCS: oral corticosteroids; LABA: long-acting beta-agonists; LAMA: long-acting muscarinic antagonists; *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$ vs correspondent nEXA; P-value <0.050 are reported in bold.

eTable 3: Spirometry and plethysmography parameters in the asthmatic population stratified for presence of asthma exacerbations after NO measurement

Follow-up post NO measurement (Phase II)	3 Months			6 Months			12 Months		
	nEXA (n=70)	EXA (n=21)	P	nEXA (n=59)	EXA (n=32)	P	nEXA (n=52)	EXA (n=39)	P
FVC (%pred.)	102±19	98±21	0.432	104±19	96±20	0.098	103±19	99±19	0.306
PB FVC (%pred.)	106±17	103±25	0.618	107±15	102±24	0.208	107±16	103±23	0.349
PB FVC (mL change)	169±171	290±385	0.106	164±180	259±320	0.158	155±183	254±298	0.128
FEV ₁ (%pred.)	85±22	78±26	0.252	87±21	76±26	0.039	86±22	79±25	0.129
PB FEV ₁ (%pred.)	91±21	88±26	0.674	92±19	88±27	0.414	91±19	89±26	0.716
PB FEV ₁ (mL change)	261±152	273±320	0.834	276±160	244±260	0.548	277±167	267±243	0.544
FEV ₁ /FVC (%)	68±11	64±11	0.276	69±10	64±12	0.056	68±11	65±12	0.170
PB FEV ₁ /FVC (%)	70±12	70±8	0.812	70±12	69±11	0.614	70±12	69±11	0.556
RV (%pred.)	123±40	136±36	0.228	122±39	134±38	0.149	123±41	131±37	0.387
TLC (%pred.)	106±15	108±17	0.634	107±15	106±17	0.732	108±16	106±16	0.612
RV/TLC (%)	42±13	47±9	0.149	41±13	47±9	0.036	41±14	46±10	0.076
FRC (%pred.)	111±25	109±12	0.847	109±25	113±22	0.721	110±26	112±21	0.845

nEXA: patient without new asthma exacerbation after NO measurement; EXA: patient with at least one new asthma exacerbation after NO measurement; PB: post-bronchodilation; P-value <0.050 are reported in bold.