Fractional Exhaled Nitric Oxide Nonsuppression Identifies Corticosteroid-Resistant Type 2 Signaling in Severe Asthma

To the Editor:

Recently, two *post hoc* analyses of clinical trials in moderate to severe asthma showed that fractional exhaled nitric oxide (Fe_{NO}) and the blood eosinophil count provide additive prognostic information on the occurrence of severe asthma attacks (1, 2). The effect is large, with a threefold increased risk in attacks seen in patients with Fe_{NO} ⩾50 ppb and blood eosinophils $\geqslant 0.3 \times 10^9$/L compared with those with a Fe_{NO} <25 ppb and blood eosinophils $<0.15 \times 10^9$/L (3). Importantly, this risk can be reduced with type 2 cytokine and alarmin-directed biologic agents (4–6). The additive, independent, and differentially modifiable risk associated with these biomarkers suggests that they identify different yet complementary aspects of type 2 airway inflammation.

Although raised Fe_{NO} classically identifies corticosteroid responsiveness, the advent of Fe_{NO} suppression testing for uncontrolled type 2–high asthma has proved that a third of patients have corticosteroid-resistant elevations in Fe_{NO}—and disease burden—despite objective evidence of treatment adherence (7, 8). Fe_{NO} nonsuppression provides a convenient model to control for nonadherence and independently study corticosteroid resistance in severe asthma.

We tested the hypothesis that Fe_{NO} and blood eosinophils relate differently to inflammation observed in the sputum (reflecting airway) and blood (reflecting systemic) compartments. An important feature of our approach was to study patients in whom we had a high degree of confidence in treatment adherence to high-dose inhaled corticosteroids and/or systemic corticosteroids.

Methods

Induced sputum eosinophils and sputum plus serum mediators were analyzed in a pooled cross-sectional analysis of patients with severe asthma and healthy control subjects.

We included patients with severe asthma who had sputum analyzed after a Fe_{NO} suppression test (8) or the RASP-UK (UK Refractory Asthma Stratification Programme) trial (NCT02717689) (9). Adherence was verified using different approaches. The Fe_{NO} suppression cohort underwent remotely monitored inhaled corticosteroids via a chipped inhaler and, if Fe_{NO} was suppressed by <42% by Day 7, a nurse-administered triamcinolone injection (8). The RASP-UK cohort underwent 8-weekly biomarker or clinically guided treatment advisories for 1 year (9) followed by a range of objective adherence measurements (prescription refills; cortisol and prednisolone blood concentrations if applicable; Fe_{NO} suppression testing if Fe_{NO} elevated) before being recruited for the associated bronchoscopy study (NCT02883530). Healthy control subjects were nonsmokers, reported no atopy or lung disease, and had normal lung function. All subjects provided written informed consent in ethically approved studies.

Patients and control subjects underwent same-day detailed clinical assessment, sputum induction, and phlebotomy when on maximum intensity treatment; only the Fe_{NO} suppression protocol included serum. Twenty-six sputum, serum, and clinical measurements were assessed (Table 1). Inflammatory proteins were measured in duplicates using multiplex electrochemiluminescent assays (Meso Scale Discovery) or single ELISAs (Cayman Chemical). Spearman correlations were computed between Fe_{NO}, blood eosinophils, and analytes, controlling for a false discovery rate <0.05. To translate significant correlations, Jonckheere-Terpstra ordinal trend tests were performed across Fe_{NO} (<25, 25 to <50, and ⩾50 ppb) and blood eosinophils (<0.15, 0.15 to <0.3, and $\geqslant 0.3 \times 10^9$/L) categories. Statistical analyses were performed using SPSS v27 with a two-sided α of 0.05.

Results

We included 74 patients with severe asthma and 10 healthy control subjects. Patients included from the Fe_{NO} suppression cohort ($n = 34$) and RASP-UK cohort ($n = 40$) were similar. Patients with asthma were 55% male, 74% atopic, and 85% nonsmokers. The mean (±SD) age was 53 ± 15 years; the mean Asthma Control Questionnaire score was 1.6 ± 1.2; the mean beclomethasone dipropionate-equivalent dose was 2,391 ± 1,084 μg/d; the mean post-bronchodilator FEV_1 was 85 ± 19% predicted; the mean FEV_1/FVC ratio was 70 ± 11%; and 53% were assessed on systemic corticosteroids. There were 60 sputum supernatants and 30 serum samples available for analysis in asthma.

We observed significant correlations between Fe_{NO} and sputum eosinophils, IL-4, IL-5, and IL-33, TSLP (thymic stromal lymphopoietin), eotaxin-3, TARC (thymus activation–regulated cytokine), and asthma attacks in the past year. Blood eosinophils correlated with serum IL-5 (Table 1). We observed no correlation between the Asthma Control Questionnaire score and the 26 analytes. Sputum eosinophils inversely correlated with lung function and closely mirrored the correlations observed with Fe_{NO} (Figure 1).

Fe_{NO} nonsuppression was associated with higher sputum eosinophils (fold difference in median values, Fe_{NO} <25 to ⩾50 ppb: 17-fold, P for trend = 0.001), IL-4 (7.6-fold, $P = 0.0006$), IL-5 (8.9-fold,

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Table 1. Analytes according to F_{ENO} and Blood Eos–based Stratification Strategies

Analyte (LLOD)*	F_{ENO} (*ppb*)				Blood Eos ($\times 10^9$/L)				Healthy Control Subjects
	<25 (*n* = 17)	25 to <50 (*n* = 30)	⩾50 (*n* = 27)	*r* (*P* Value)	<0.15 (*n* = 21)	0.15 to <0.30 (*n* = 22)	⩾0.30 (*n* = 31)	*r* (*P* Value)	(*n* = 10)
Biomarker									
F_{ENO}, ppb	16 (13–20)	39 (32–42)	83 (60–123)†	—	38 (23–55)	38 (26–74)†	45 (25–89)†	0.24 (0.04)	19 (11–28)
Blood Eos, $\times 10^9$/L	0.17 (0.1–0.54)	0.24 (0.1–0.35)	0.26 (0.19–0.55)†	0.24 (0.04)	0.09 (0.05–0.12)	0.23 (0.19–0.25)	0.54 (0.36–0.66)†	—	0.14 (0.09–0.18)
Sputum									
Eos, %	0.8 (0.4–5.3)	2.7 (1.1–17.8)†	12.8 (3.3–35.5)†	**0.51 (0.0002)**	2.7 (0.7–6.1)	5.1 (0.5–30.5)†	4.3 (1–21)†	NS	0.3 (0.3–0.4)
IL-4 (0.2)	0.1 (0.1–0.3)	0.4 (0.1–1.1)†	0.8 (0.2–1.2)†	**0.48 (<0.0001)**	0.3 (0.1–1)†	0.4 (0.1–0.9)	0.3 (0.1–1)†	NS	0.1 (0.1–0.1)
IL-5 (0.5)	1.2 (0.4–4.6)	4.6 (1.9–7.8)	10.9 (2.9–29.8)†	**0.47 (0.0002)**	2.3 (1.1–9.7)	5.3 (1.5–15.1)†	4.7 (1.8–10.8)†	NS	0.3 (0.2–2.7)
IL-13 (4.2)	6.4 (2.1–8.8)	7 (5.8–14.2)†	8.4 (6.4–13.9)†	0.26 (0.04)	7 (5.1–11.5)†	8.3 (4–12.5)†	7.6 (6–12.2)†	NS	2.1 (2.1–2.1)
IL-33 (0.6)	0.9 (0.3–1.3)	0.9 (0.3–2.1)†	1.7 (0.7–2.9)†	**0.35 (0.006)**	0.9 (0.3–1.9)	1.4 (0.5–2.6)†	1 (0.3–2.3)†	NS	0.3 (0.3–0.3)
TSLP (0.9)	2.4 (1–9.3)	6.4 (2.3–10.7)†	11.9 (5–20.7)†	**0.41 (0.001)**	4.9 (1.5–16.9)	9.1 (1.9–2.6)	7.1 (2.5–15)†	NS	0.9 (0.5–1.8)
Eotaxin-3 (4.2)	34 (2–71)	133 (23–369)†	353 (245–804)†	**0.55 (<0.0001)**	76 (23–264)	215 (9–418)†	191 (29–390)†	NS	2 (2–26)
TARC (0.4)	17 (9–89)	27 (18–77)	58 (38–301)†	**0.32 (0.02)**	35 (19–107)†	41 (9–101)	36 (17–88)	NS	6 (2–21)
LTE4 (7.8)	59 (23–114)	138 (42–465)†	133 (42–730)†	NS	64 (23–139)	94 (48–343)	163 (49–676)†	NS	7 (4–70)
PGD2 (19.5)	241 (173–384)	217 (119–354)	209 (135–439)	NS	213 (133–505)	219 (183–389)	222 (117–439)	NS	89 (43–200)
IFN-γ (0.3)	0.3 (0.2–0.5)	0.4 (0.2–1.8)	0.6 (0.2–1.5)	NS	0.5 (0.2–1.7)	0.4 (0.2–2.6)	0.3 (0.2–0.8)	NS	0.2 (0.2–2.1)
TNF (0.4)	1.5 (0.4–10.2)	2 (0.8–7.5)	3.3 (1.5–6.7)	NS	2.5 (1–6.7)	3.2 (0.5–8.5)	2 (0.7–8.6)	NS	2.9 (0.4–16.7)
Serum									
IL-4 (0.1)	0.1 (0.1–0.1)	0.1 (0.1–0.1)	0.1 (0.1–0.1)	NS	0.1 (0.1–0.1)	0.1 (0.1–0.1)	0.1 (0.1–0.1)	NS	0.1 (0.1–0.1)
IL-5 (0.4)	1.1 (1–1.2)†	0.6 (0.5–0.9)†	0.6 (0.4–1.6)†	NS	0.4 (0.4–0.6)	0.6 (0.6–1.6)†	0.8 (0.6–1.5)†	**0.41 (0.03)**	0.2 (0.2–0.4)
IL-13 (6.7)	3.3 (3.3–3.3)	3.3 (3.3–9.9)	3.3 (3.3–14.1)	NS	3.3 (3.3–3.3)	8.8 (3.3–13.3)	3.3 (3.3–10)	NS	9.2 (7.8–9.8)
IL-33 (0.4)	0.2 (0.2–0.3)	0.8 (0.2–0.8)	0.2 (0.2–0.8)	NS	0.2 (0.2–0.8)	0.4 (0.2–0.8)	0.2 (0.2–0.8)	NS	0.4 (0.4–0.4)
TSLP (0.5)	1.3 (1–2.1)	1.8 (0.6–2.5)	2.7 (1.8–4.5)†	NS	1.7 (1.2–3.6)	2.3 (1.9–4.4)†	1.8 (0.8–2.7)	NS	1.4 (0.9–1.5)
Eotaxin-3 (4.2)	9 (6–30)	18 (7–32)	16 (13–29)	NS	18 (14–31)	29 (12–34)†	13 (7–18)	NS	8 (4–13)
TARC (0.2)	427 (108–571)	252 (147–463)	226 (89–430	NS	314 (195–664)	190 (92–252)	344 (146–480)	NS	206 (124–285)
IFN-γ (0.3)	0.4 (0.4–0.5)	0.5 (0.2–0.7)	0.3 (0.2–1.2)	NS	0.3 (0.2–0.6)	0.7 (0.2–2.3)	0.2 (0.2–0.6)	NS	0.4 (0.2–0.7)
TNF (0.4)	1.8 (0.9–3.8)†	0.6 (0.2–2)	1.2 (0.2–4.2)†	NS	1.7 (0.2–2.3)†	0.6 (0.2–2)	0.9 (0.3–1.9)†	NS	0.2 (0.2–0.3)
Clinical									
ACQ-5 score	1.2 (0.5–1.8)	1.6 (0.2–2.2)	2 (0.8–3)	NS	1.6 (0.5–2.1)	1.7 (0.7–2.9)	1.2 (0.6–2.2)	NS	—
FEV_1, % predicted	88 (78–103)	85 (75–98)	81 (72–96)	NS	81 (77–94)	83 (74–97)	85 (76–99)	NS	—
FEV_1/FVC, %	72 (64–82)	68 (61–79)	72 (60–77)	NS	71 (62–82)	68 (61–77)	72 (61–80)	NS	—
Asthma attacks in the past year	1 (0–3)	1 (0–4)	3 (0–5)	**0.25 (0.03)**	1 (0–5)	1.5 (0–4)	1 (0–4)	NS	—

Definition of abbreviations: ACQ-5 = five-item Asthma Control Questionnaire; Eos = eosinophils; F_{ENO} = fractional exhaled nitric oxide; LLOD = lower limit of detection; LTE4 = leukotriene E4; NS = not significant; PGD2 = prostaglandin D2; TARC = thymus activation–regulated cytokine (CCL17); TNF = tumor necrosis factor; TSLP = thymic stromal lymphopoietin.

P values ⩾0.05 are not significant.

Data are presented as median (interquartile range) in pg/ml unless stated otherwise.

Spearman correlation coefficients (*r*) and associated *P* values are in bold if retained after controlling for a false discovery rate <0.05 across the 52 computed correlations.

*Cytokine levels that were not quantified were assigned the arbitrary value of 0.5 × the lower limit of detection to allow analysis.

†Adjusted *P* value <0.05 compared with healthy control subjects on Kruskal-Wallis test adjusted for six comparisons.

r \ *p*	FE_{NO}	Blood Eos	Sputum Eos	Sputum IL-4	Sputum IL-5	Sputum IL-13	Sputum IL-33	Sputum TSLP	Sputum Eotaxin-3	Sputum TARC	Serum IL-5	ACQ-5 score	FEV_1	FEV_1/FVC ratio	Asthma attacks (past yr)
FE_{NO}		0.04	**0.0002**	**<0.0001**	**0.0002**	0.04	**0.006**	**0.001**	**<0.0001**	**0.02**	ns	ns	ns	ns	**0.03**
Blood Eos	0.24		ns	ns	ns	ns	ns	ns	ns	ns	**0.03**	ns	ns	ns	ns
Sputum Eos	**0.51**	0.25		0.0005	<0.0001	0.02	ns	0.002	0.001	0.005	ns	ns	0.04	0.02	ns
Sputum IL-4	**0.48**	0.06	0.49		<0.0001	0.0004	<0.0001	<0.0001	<0.0001	<0.0001	ns	ns	ns	ns	ns
Sputum IL-5	**0.47**	0.14	0.55	0.71		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.006	ns	ns	0.01	ns
Sputum IL-13	0.26	0.05	0.33	0.44	0.60		<0.0001	<0.0001	<0.0001	<0.0001	ns	ns	ns	ns	ns
Sputum IL-33	**0.35**	0.03	0.25	0.81	0.65	0.48		<0.0001	<0.0001	<0.0001	ns	ns	ns	ns	ns
Sputum TSLP	**0.41**	0.14	0.44	0.65	0.84	0.57	0.61		<0.0001	<0.0001	ns	ns	ns	ns	0.049
Sputum Eotaxin-3	**0.55**	0.15	0.51	0.79	0.89	0.63	0.70	0.83		<0.0001	ns	ns	ns	ns	ns
Sputum TARC	**0.32**	0.02	0.42	0.63	0.83	0.51	0.56	0.85	0.70		0.04	ns	ns	0.04	ns
Serum IL-5	0.03	**0.41**	0.27	0.14	0.62	0.36	0.17	0.40	0.83	0.85		ns	ns	0.01	ns
ACQ-5 score	0.19	0.00	0.22	0.04	0.08	0.09	-0.11	0.07	0.06	0.13	0.00		ns	ns	ns
FEV_1	-0.17	0.04	-0.29	-0.16	-0.21	0.05	-0.06	-0.09	-0.14	-0.14	-0.06	-0.05		<0.0001	ns
FEV_1/FVC ratio	-0.14	0.01	-0.34	-0.18	-0.31	-0.07	-0.08	-0.23	-0.22	-0.27	-0.48	-0.03	0.69		ns
Asthma attacks	**0.25**	0.00	0.17	0.21	0.18	0.11	0.16	0.26	0.25	0.09	0.11	-0.01	-0.15	0.02	

r: 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0, -0.1, -0.2, -0.3, -0.4

Figure 1. Correlation matrix for FE_{NO}, blood Eos, and selected analytes in severe asthma. Bold Spearman coefficient of correlations (*r*) and *P* values were those retained after controlling for a false discovery rate <0.05 in the primary analysis (first two columns and rows); the rest of the matrix is exploratory. Asthma attacks are defined as acute events requiring ⩾3 days of systemic corticosteroids in the past year. ACQ-5 = five-item Asthma Control Questionnaire; Eos = eosinophils; FE_{NO} = fractional exhaled nitric oxide; ns = not significant ($P \geqslant 0.05$); TARC = thymus activation–regulated cytokine (CCL17); TSLP = thymic stromal lymphopoietin.

$P = 0.006$), IL-33 (1.8-fold, $P = 0.02$), TSLP (5-fold, $P = 0.002$), eotaxin-3 (10-fold, $P = 0.00003$), TARC (3.5-fold, $P = 0.005$), and asthma attacks in the past year (3-fold, $P = 0.03$). Greater blood eosinophils (<0.15 to ⩾0.3×10^9/L) was associated with higher serum IL-5 (1.9-fold, $P = 0.04$) (Table 1).

The highest FE_{NO} and blood eosinophil categories generally had greater sputum eosinophils, sputum/serum type 2 cytokine, and chemokine and alarmin levels than healthy control subjects (Table 1).

The directions of trends were consistent when removing systemic corticosteroid–treated patients or when separating the RASP-UK and FE_{NO} suppression cohorts. Exploratory multiple regression showed no additive effect for biomarkers to identify inflammation levels.

Discussion

We found that in severe asthma, FE_{NO} nonsuppression identifies increased airway type 2 cytokines (IL-4 and IL-5), chemokines (eotaxin-3 and TARC), alarmins (IL-33 and TSLP), and sputum eosinophilia. In contrast, blood eosinophils correlate with serum IL-5 and not with any assessed measure of airway inflammation. We base these conclusions on our cross-sectional study of patients with extremely high corticosteroid exposure and proven adherence.

Our results are consistent with the cross-sectional bronchial biopsy-based ADEPT study (10) but extend their findings by showing correlations between FE_{NO} and almost all of the assessed components of the airway type 2 immune response for a population with confirmed treatment adherence. The most striking finding of our study was the different relationship between FE_{NO}, blood eosinophils, and markers of airway and systemic type 2 inflammation. Our findings imply that FE_{NO} and blood eosinophils relate to different components and compartments of type 2 inflammation: FE_{NO} reflects airway type 2 activity and the chemotactic pull to the airways, whereas blood eosinophils reflect the systemic pool of available effector cells and circulating IL-5.

Our study has several limitations. Its cross-sectional design assessed correlation, not causality. The analysis of serum analytes was underpowered ($\beta = 0.43$ for $r = 0.40$ with critical $P < 0.041$), and we pooled two cohorts that used different approaches to confirm treatment adherence, although a sensitivity analysis analyzing both independently was supportive of our results. Unexpectedly, sputum IL-13 did not correlate with FE_{NO} after controlling for multiplicity of testing. This may reflect the complex dimeric receptor system signaling both IL-4/-13, a greater steroid-sensitivity of IL-13, and/or a slightly underpowered analysis.

To conclude, we found that FE_{NO} and blood eosinophils provide different and complementary mechanistic information in severe asthma. How airway signaling (reflected by FE_{NO}) and an increased systemic eosinophil pool (reflected by blood eosinophils) relate to the pathogenesis of asthma attacks and the response to treatment remains an important question. ■

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Selective Modulation of the Pulmonary Innate Immune Response Does Not Change Lung Microbiota in Healthy Mice

To the Editor:

Although long considered sterile, healthy lungs are now known to harbor diverse and dynamic low-abundance bacterial communities. Recent studies in humans (1) and animals (2) have revealed that lung immunity, even in health, is variable across individuals and correlated with variation in lung microbiota. Yet the causal relationships driving this correlation between lung microbiota and lung immunity remain undetermined. Does variation in lung microbiota propel variation in lung immunity activation? Or does variation in lung immunity create an altered respiratory microenvironment, resulting in altered lung bacterial communities?

A recent report in this journal by Wu and colleagues (3) demonstrated that direct modulation of murine lung microbiota results in rapid and persistent changes in lung immunity, conveying sustained protection from subsequent respiratory infection. These results reveal that the correlation between lung microbiota and lung immunity is, at least in part, attributable to the microbiome's influence on lung immunity. Yet, to our knowledge, the inverse

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