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Circulating neutrophil and eosinophil extracellular traps are markers of severe asthma

To the Editor,

Asthma is a chronic inflammatory airway disease in which neutrophils and eosinophils are major players (1,2). Eosinophilic airway infiltration is a hallmark of type 2 inflammation that affects about 50% of adult asthma patients, while neutrophilic infiltration defines a subgroup of patients with severe and steroid-refractory asthma (2). Both cells participate in airway epithelium damage by releasing cytotoxic proteins, reactive oxygen species, and possibly, extracellular traps. Neutrophil and eosinophil extracellular traps (NETs and EETs) are web-like DNA filaments with anti-infectious and pro-inflammatory properties, whose involvement in the pathophysiology of asthma seems likely. In mouse models, NET inhibition was protective against rhinovirus-induced asthma exacerbations in a dust mite allergic asthma model (3), and DNase I therapy improved airway resistance and reduced NET accumulation in another model of ovalbumin-induced asthma (4). In humans, NETs and EETs have been observed in lung biopsies of asthma patients, and a link between cell-free DNA in sputum and asthma severity has been recently suggested (5,6). However, cell-free DNA can have multiple origins and is not considered a specific marker of netosis (7). Moreover, relevance of NETs in serum and BALF of asthma patients has not been evaluated so far. Since DNA-granular protein complexes are considered the best markers of NET remnants to date (7), we measured the concentration of myeloperoxidase (MPO)-DNA and eosinophil major basic protein (EMBP)-DNA complexes in serum and bronchoalveolar lavage fluid (BALF) from patients included in the French national COBRA cohort (8). To establish the clinical relevance of EETs and NETs in human asthma, we correlated these levels with clinical data.

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Clinical characteristics of the 217 asthma patients are shown in Table 1. BALF samples from 51 patients were also analyzed. Systemic and alveolar inflammation was evaluated by multiplex cytokine measurement. MPO-DNA and EMBP-DNA complexes were measured by ELISA. (See online supplement for detailed methods).

Patients with severe asthma had higher circulating NET level than patients with either mild or moderate asthma (GINA score 4 vs 1-3) or healthy controls ($p=0.035$ and $p=0.020$, respectively; Fig. 1a). NET level was also significantly increased in patients with poor or partial versus good control ($p=0.0047$ and $p=0.011$, respectively; Fig. 1b). Patients with high circulating NET level were significantly younger and had earlier asthma onset than those with low NET level (Table 1, $p=0.004$ and $p=0.032$, respectively). These results were confirmed by logistic regression analysis showing NET level as an independent predictor of poor control even after adjustment for age, age of asthma onset, smoking status, inhaled corticosteroid (ICS) dose and sex (OR= 2.43, $p=0.013$; Supplemental Table 1). In contrast to NET level, circulating EET level was not linked to asthma severity (Fig. 1c) but was associated with asthma control ($p=0.039$; Fig. 1d), increased exacerbation rate, and ex-smoker status (Table 1, $p=0.041$ and $p=0.0053$, respectively). However, the association with control was much weaker with EET than NET level and did not reach statistical significance on multivariate analysis (Supplemental Table 1).

In BALF, we could readily measure NETs but not EETs, even though 53% of patients with available BALF samples had alveolar eosinophil count $>1.2\%$. This could be related to inhaled corticosteroid therapy, since corticosteroids were shown to downregulate eosinophil activation (9), or reflect a specific subpopulation of eosinophils with low EET release capacity. Alveolar NET presence was closely associated with airway neutrophil count in BAL ($p=0.0068$) but not with other cell counts (Fig. 1e). In addition, NET level in BALF and serum were not correlated, which suggests that NET production in blood and lung are independent processes. In contrast to circulating NETs, alveolar NET level was significantly higher in patients with mild/moderate asthma as compared with both healthy controls and patients with severe asthma (Fig. 1f, $p=0.025$ and $p=0.043$, respectively). Furthermore, BALF NET levels were not associated with asthma control (Fig. 1g).

Because NETs have well-known anti-infectious properties, we investigated whether the increase in BALF NET concentration was also associated with subclinical pulmonary infection. BALF NET level, along with BALF neutrophil count, was significantly higher when bronchial aspirate cultures were positive for microorganisms (mostly *Candida albicans* and *Aspergillus species*; $p=0.039$ and $p=0.04$, respectively; Fig. 1h-i). Thus, airway neutrophil infiltration and NET release are independent of clinical asthma severity and are associated with subclinical microbial colonization.

To assess if NETs could be triggered by systemic or local inflammation, we assessed a panel of inflammatory mediators in serum and BALF samples. Overall, NET and EET levels in serum did not correlate positively with any mediator concentration (Supplemental Fig. 1a). In contrast, cytokine and NET levels were strongly correlated in BALF (Supplemental Fig. 1b). NET level was significantly correlated with the concentration of CCL11, CCL26, IL-4, IL-8, IL-1 β , IL-6, CCL2, IL-3, IL-7 and IL-33 (all $p < 0.002$). The concentrations of these cytokines were also higher in BALF from patients with subclinical infection, 5 of them reaching statistical significance (IL-7: $p = 0.005$; IL-8: $p = 0.02$; CCL2: $p = 0.008$; CCL11: $p = 0.002$; CCL26: $p = 0.009$, Supplemental Fig. 1c-g). Hence, we found no link between ETosis and markers of systemic inflammation in patient serum, but NETs in BALF were associated with the expression of several pro-inflammatory cytokines, likely caused by a sustained inflammatory response to subclinical infection. Lastly, despite granular protein-DNA complexes being recognized as specific markers for netosis (7), we can't formally exclude that part of the complexes were formed by neutrophil activation outside netosis (10). However, the presence of NETs in asthma patients bronchi (11), and the increased susceptibility of their circulating neutrophils to NETosis (12) both argue in favor of a netotic origin of these complexes. In conclusion, this study highlights a different impact of ETs on asthma features according to their nature and localization. Circulating NET level, which reflects neutrophil activation, appears to be a better marker of asthma severity and poor control than alveolar NET level, which seems impacted by bacterial colonization. Absence of EET in the BAL remains intriguing and may reflect differences in eosinophils activation and/or phenotype. Future studies are needed to confirm these results and their clinical relevance for patient follow-up and characterization of asthma phenotypes.

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CONFLICTS OF INTEREST

Pr. Taillé reports grants and personal fees from Sanofi, grants and personal fees from GSK, grants and personal fees from AstraZeneca, grants and personal fees from Novartis, outside this work. Dr. Létuvé reports grants from Astra Zeneca and grants from GSK, outside this work. Dr. NEUKIRCH reports personal fees from Novartis, personal fees from Astrazeneca, personal fees from ALK Abello, personal fees from Stallergènes, personal fees from Mylan, personal fees from Menarini, outside this work. Pr. Aubier reports grants from AStraZeneca, grants from GSK, grants from Boston Scientific, grants and personal fees from Chiesi, outside this work.

FIGURE LEGENDS

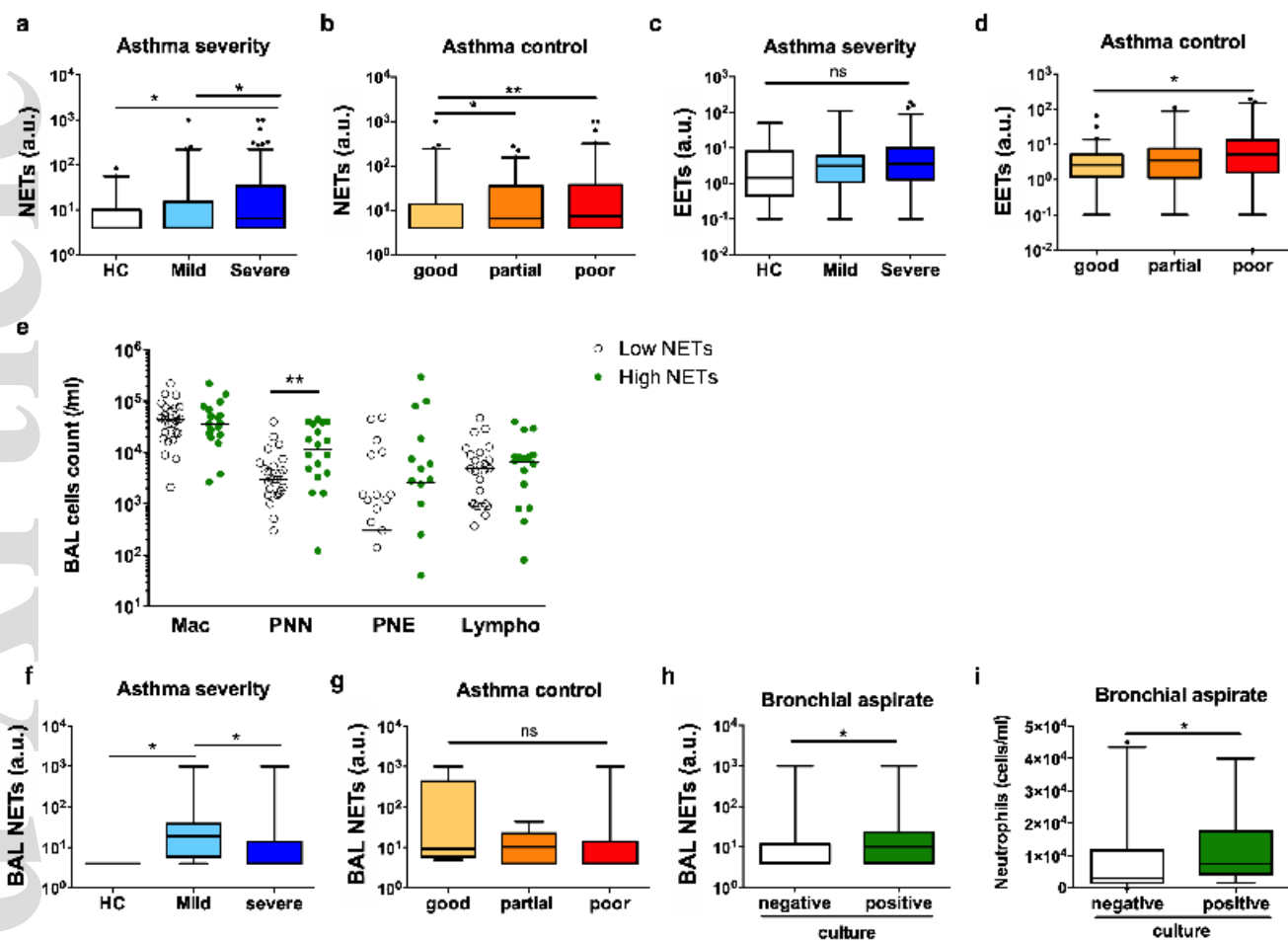
Fig. 1. Circulating and alveolar neutrophil and eosinophil extracellular trap (NET and EET) levels by biological and clinical features of asthma

(a-b) Circulating NET levels (DNA-MPO) by asthma severity and asthma control level. (c-d) Circulating EET levels (DNA-EMBP) by asthma severity and asthma control level. (e) BALF cell counts of macrophages (Mac), neutrophils (PNN), eosinophils (PNE) and lymphocytes (Lymph) by BALF NET levels. (f) Comparison of BALF NET level between healthy controls, patients with mild/moderate asthma or severe asthma. (g) Comparison of BALF NET level by asthma control level. (h-i) Comparison of BALF NET level and BALF neutrophil count by positivity of bronchial aspirate culture. Data are median (horizontal line), box edges are Q1-Q3 and whiskers are 5-95th percentile. * $p < 0.05$, ** $p < 0.001$.

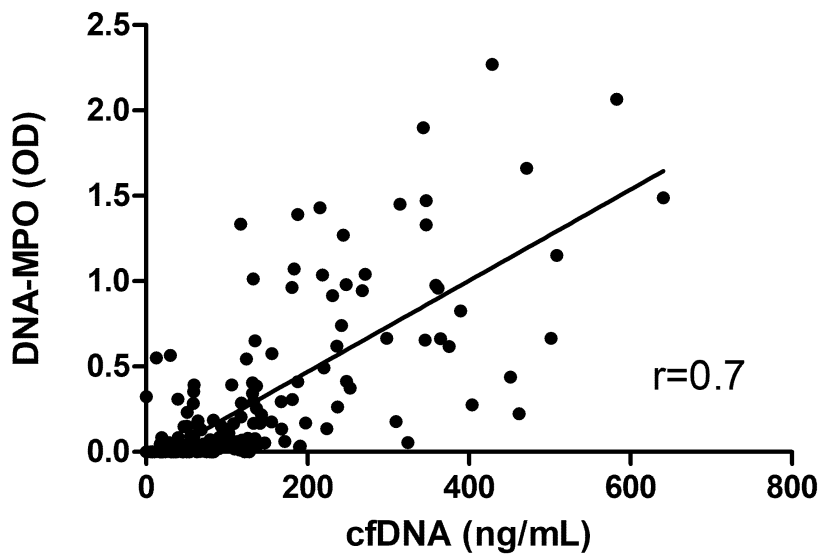
Table 1: Demographic, clinical and laboratory features of the cohort with asthma and their association with NETs and EETs level

	Full cohort N=217	High NET level N=108	Low NET level N=109	p-value	High EET level N=108	Low EET level N=109	p-value
Age, mean (SD)	50.2 (13.6)	48 (12)	53 (14)	0.004	49.5 (12.6)	51 (14)	0.23
Sex M/F	75/142	45/63	30/79	0.046	42/66	33/76	0.25
Atopy, n (%)	130 (60)	64 (59)	66 (61)	0.89	60 (55)	70 (64)	0.21
Age of asthma onset, median (Q1, Q3)	21 (5,40)	18 (4,40)	27 (7,43)	0.032	20 (5,40)	24 (7,40)	0.46
Baseline FEV1% predicted, median (Q1, Q3)	77 (59,93)	75 (59,91)	82 (57,94)	0.53	76 (60,93)	77 (56,92)	0.73
Max FEV1% predicted, median (Q1, Q3)	85 (64,97)	79 (61,96)	89 (70,99)	0.058	82 (63,98)	89 (66,97)	0.51
ΔFEV1 ml/s (Max FEV1 – Baseline FEV1), median (Q1,Q3)	130 (50,255)	100 (40,180)	170 (80,270)	0.056	120 (30,210)	130 (70,270)	0.21
FEV1/FVC%, median (Q1, Q3)	67 (57,76)	66 (54,76)	67 (58,76)	0.41	69 (57,76)	65 (55,75)	0.27
Mild and moderate, n (%) /severe, n (%)	59 (27) /158 (73)	23(21) / 85(79)	36 (33) / 73(67)	0.067	28(26) / 80(74)	31(28) / 78(72)	0.76
Asthma control, uncontrolled, n (%) /fully controlled, n (%)	151(70) / 65(30)	86(80) / 22(20)	65(60) / 43(40)	0.0029	79(73) / 29(27)	72(67) / 36(33)	0.37
Number of exacerbations in the last 6 months, median (Q1, Q3)	0 (0,3)	1 (0,3)	0 (0,3)	0.14	1 (0,3)	0 (0,3)	0.041
BMI, kg/m ² , median (Q1, Q3)	26 (23,31)	26 (23,31)	26 (23,31)	0.89	26 (23,30)	26 (23,32)	0.28
Smoking, ex/never/current (n)	71/134/12	40/65/3	31/69/9	0.11	46/58/4	25/76/8	0.0053
Pack years, median (Q1, Q3)	15 (6,26)	10 (5,20)	17 (6,30)	0.088	15 (8,25)	15 (6,30)	0.90
ICS use, yes/no	207/10	103/5	104/5	1.00	102/6	105/4	0.75
ICS dose (µg daily BDP equivalent), median (Q1, Q3)	2000 (1000,2000)	2000 (1000,4000)	2000 (1000,2000)	0.15	2000 (1000,4000)	2000 (1000,2000)	0.42
OCS use, yes/no	37/180	20/88	17/92	0.72	14/94	23/86	0.15
OCS dose (mg daily) median (Q1, Q3)	20 (10,40)	20 (10,38)	20 (10,54)	0.73	20 (10,40)	15 (10,42)	0.64
Blood eosinophil count (mm ⁻³), median (Q1, Q3)	230 (128,430)	230 (85,395)	230 (140,470)	0.27	230 (83,425)	230 (130,433)	0.51
Blood neutrophil count (mm ⁻³), median (Q1, Q3)	4520 (3500,6480)	4910 (3860,7280)	4420 (3158,6058)	0.0085	4865 (3605,7443)	4420 (3315,6115)	0.064
Total serum IgE level (UI/ml), median (Q1,Q3)	184 (52,584)	140 (55,569)	204 (50,707)	0.38	118 (49,581)	212 (72,586)	0.20

FEV1, forced expiratory volume; FVC, forced vital capacity; BMI, Body mass index; ICS, inhaled corticosteroids; BDP, beclomethasone dipropionate; OCS, oral corticosteroids. The median value of the cohort was used as cut-off to define high and low circulating NET and EET level groups.



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Correlation between cell-free DNA and DNA-MPO complexes

DNA-MPO and cell-free DNA were measured in serum of asthma patients. Cell-free DNA was quantified by PicoGreen Quant-iT dsDNA Assay kit (Thermo Fisher Scientific) after DNA extraction from serum using QIAamp Ultrasens Virus kit (Qiagen) according to manufacturer's instruction. DNA-MPO were measured by an in-house ELISA as described in the Supplementary methods. Correlation coefficient (Spearman) was 0.73 ($p<0.0001$)

Supplementary Methods

Patients

We studied 217 patients with asthma from the longitudinal follow-up cohort COhort of BRonchial obstruction and Asthma (COBRA). The cohort was approved by the regional ethics committee (no. 09-11962) and all participants gave their written informed consent. Among these 217 patients, 51 had a BALF sample available. The majority of the BALF samples (67%) were taken at the same day than the blood, and within a few weeks within a few weeks for all others. For 50 of these 51 patients, standard microbiology culture results were available for a bronchial aspirate performed concomitantly to the bronchoalveolar lavage. Serum and BALF samples were obtained outside exacerbations. Asthma control was established by the Global Initiative for Asthma (GINA) guidelines as follows: 1) good asthma control was considered with all of the following features: no more than 2 daytime symptoms per week in the last month, no nocturnal symptoms (woken up because of asthma or by an attack of shortness of breath) in the last month, use of short-acting β_2 -agonist inhalers ≤ 2 /week and no activity limitation due to asthma in the last month; 2) partial asthma control was considered with absence of 1 or 2 of these features; and (3) poor asthma control was considered with absence of ≥ 3 of these features. Exacerbations were defined by asthma symptom onset or worsening >2 days requiring oral corticosteroids. Severe asthma was defined by a GINA score of 4. Atopy was defined as a positive skin prick test result or IgE sensitization to a common aeroallergen (specific IgE > 0.35 kUI/L). Samples from an existing biobank of healthy blood donors were used to determine normal range of NETs and EETs. To investigate links between level of circulating NETs and EETs to other clinical and/or biological phenomena, the cohort was divided into two groups by median NET or ET concentration because no biologically relevant cutoff has been described so far.

NET and EET measurement

NET level was quantified by measuring myeloperoxidase (MPO)-DNA complexes in serum and BALF samples with an in-house capture ELISA previously described (Kessenbrock Nature Med 2009) using mouse anti-MPO from Merck-Millipore for coating. Sample concentrations were interpolated from a standard curve and are expressed in arbitrary units (a.u.). The detection range was 4-1000 a.u. Normal NET range in serum was established with samples from 39 healthy blood donors. EET level was quantified by measuring EMBP-DNA complexes in serum and BALF samples with a similar capture ELISA as above. We used 10 μ g/mL mouse anti-human EMBP antibody (sc-59164, Clinisciences, Nanterre, France) for coating, and results are expressed in arbitrary units (a.u.) applying a fixed conversion scale from optical density (OD) values (0.1 OD unit = 10 a.u.). The detection range was 0-300 a.u. Normal EET range in serum was established with samples from 30 healthy blood donors.

Multiplex cytokine measurement

Cytokines and other inflammatory molecules were quantified in serum and BALF samples by using customized bead arrays (Procartaplex, Life Technology, Saint Aubin, France) to measure B-cell activating factor (BAFF), CCL11, CCL2, CCL22, CCL26, CX3CL1, granulocyte-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, interferon γ , IL-1 β , IL-10, IL-12p70, IL-13, IL-17A, IL-2R, IL-23, IL-3, IL-33, IL-4, IL-5, IL-6, IL-7, IL-8, and tumor necrosis factor α . All assays were performed according to the manufacturer's instructions except for reaction volume, which was reduced to 25 μ L using DropArray 96 plates (Clinisciences, Nanterre, France). All assays were read on a BioPlex system (Biorad, Marnes-la-coquette, France). The cytokines below detection levels in more than 75% of patients were removed from the analysis.

Statistical analysis

Data were analyzed with Prism 7.0 (Graphpad Software Inc.) and Genesis 1.7.6 (Institute for Genomics and Bioinformatics, Graz University). Because this was an observational study without an *a priori* hypothesis, statistical power could not be calculated before the analysis. Differences between groups were assessed by Mann-Whitney or Kruskal-Wallis test, followed by Dunn's post-test when suitable. For correlation analysis, Spearman's correlation coefficients were calculated. When appropriate, Fisher's exact test or chi-square test was used to compare proportions. Hierarchical clustering was applied with the Euclidian distance and average linkage clustering method. We used logistic regression models to investigate the association between asthma control and severity, and circulating NET level. In multiple regression approaches, variables with $p \leq 0.20$ comparing high and low NET groups (divided by median NET level) were considered potential confounders [age, age of asthma onset (continuous), smoking status (ex/never/always), inhaled corticosteroids (ICS) dose (high: ≥ 2000 μ g; medium: 1000-2000 μ g; low: <1000 μ g) and sex]. No adjustment on OC use was possible because of a strong imbalance in asthma control level for this variable, and no adjustment on neutrophil counts was possible because of a mediation link between neutrophil and NETs. Logistic regression analyses were made using SAS v9.3 (SAS Inst. Inc., Cary, NC, USA). Results were considered statistically significant at $p < 0.05$. Bonferroni correction was applied to control for multiple comparison where appropriate.

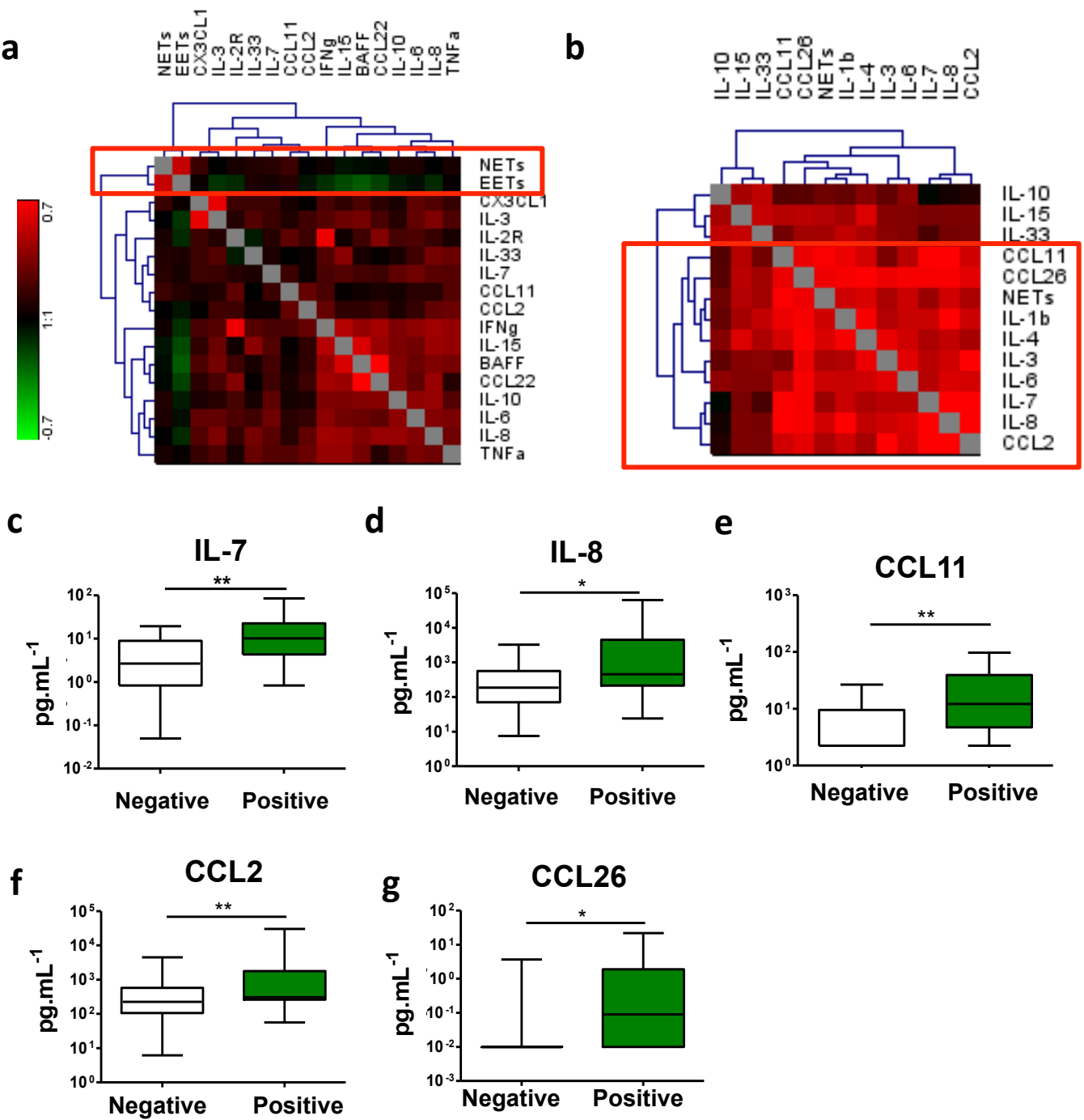
Supplemental Table 1: Logistic regression analysis of association between NET and EET levels and asthma outcomes

High <i>versus</i> low NET level				
	OR (95% CI)	p	aOR [95% CI]	p
Uncontrolled asthma* (vs good asthma control)	2.59 [1.41-4.74]	0.002	2.43 [1.21-4.89]	0.013
Severe asthma (vs mild-moderate asthma)	1.82 [0.99-3.35]	0.054	1.43 [0.59-3.48]	0.43
High <i>versus</i> low EET level				
	OR (95% CI)	p	aOR [95% CI]	p
Uncontrolled asthma* (vs good asthma control)	1.36 [0.76-2.44]	0.30	1.16 [0.59-2.29]	0.66
Severe asthma (vs mild-moderate asthma)	1.14 [0.62-2.07]	0.68	0.77 [0.32-1.86]	0.56

Data are crude odds ratio (OR) and 95% confidence intervals (CIs) (left) and adjusted OR (aOR) (right): adjusted for age, sex, age of asthma onset, smoking status and inhaled corticosteroids dose. *Uncontrolled asthma corresponds to poor or partial control.

Supplementary Table 2 : Smoking status and medications by NET level in BALF

	High BAL NET level N=24	Low BAL NET level N=27	p-value
Smoking, ex/never/current (n)	8/16/0	9/17/1	1.0
Pack years, median (Q1, Q3)	10 [3.5-22.5]	10 [10-20]	0.58
ICS use, yes/no	23/1	24/3	0.61
ICS dose (μ g daily BDP equivalent), median (Q1, Q3)	2000 [1500-4000]	1800 [1000-2000]	0.11
OCS use, yes/no	5/19	8/19	0.47
OCS dose (mg daily) median (Q1, Q3)	20 [20-20]	25 [10-50]	0.56



Suppl Fig. 1 Expression of inflammatory markers in serum and BALF of asthma patients
(a,b) Hierarchical clustering of the correlation matrix of cytokines, NETs and EETs concentrations in serum (a) and BALF (b). Intensity of color is proportional to Spearman correlation coefficient. The red line highlights the cluster containing NETs and EETs. (c-g): BALF concentration of cytokines according to microbiological culture result. Data in c-g are median (horizontal line), box edges are Q1-Q3 and whiskers are 5-95th percentiles. *p<0.05; **p<0.005.