Elevated serum IgE, OCS-dependence, and IL-17/22 expression in highly neutrophilic asthma

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Online supplementary data

Supplementary Text

Methods

Patients

In this observational cross-over study, bronchial biopsies were obtained from asthmatic patients referred to the tertiary level Asthma Unit of the University Hospital San Luigi Gonzaga (University of Turin, Italy). The present study comprises 58 patients from the previous study and 12 additional new patients recruited later with the same protocol. Asthma was identified and treated according to the Global Initiative for Asthma and ERS/ATS criteria [2, 3]. Clinical data and patient medical history were obtained at recruitment, when blood hematology and pulmonary function testing with bronchoreversibility were also performed. Spirometry and lung volumes were assessed with a body plethysmograph (Vmax Encore 62, Carefusion, Germany) before and 15 min after the administration of 400 mcg of albuterol. Smoking history was defined as ≥10 pack-years, based on previous data [4]. Patients were excluded if they presented evidence of asthma-COPD overlap (ACO) [5], based on DLCO and KCO % predicted values (<80 % pred. and <100 % pred., respectively) or imaging findings (emphysema detected at CT scan). Chronic sinusitis was defined according to guidelines [6, 7]. Atopy was assessed by serum IgE levels and skin prick tests to common allergens. Polysensitivity was defined as a positive prick test for ≥2 allergens. Fractional exhaled NO (FeNO) was measured as previously described [1]. Pulmonary function testing was repeated during the week preceding the biopsy procedure. Bronchial biopsies (n=8/patient) were obtained from lobar, segmental, and sub-segmental bifurcations during flexible bronchoscopy [8] with patients in stable conditions (no exacerbations and stable treatment regimen in the previous 6

weeks) and in conformity with the local Ethics Committee Guidelines. Written informed consent was obtained from each participant. The study conformed to the Declaration of Helsinki and was approved by the local Ethics Committees (A.O.U. San Luigi Hospital: protocols 1759/2008 and 14871/2009).

Phenotype definitions

Based on the density of neutrophils and eosinophils in their bronchial mucosa, asthmatic patients were divided into 4 groups: isolated eosinophilic (≥12.45 eosinophils/mm^2 and <47.17 neutrophils/mm^2), isolated neutrophilic (≥47.17 neutrophils/mm^2 and <12.45 eosinophils/mm^2), mixed (≥12.45 eosinophils/mm2 and ≥47.17 neutrophils/mm^2), and paucigranulocytic (<12.45 eosinophils/mm^2 and <47.17 neutrophils/mm^2). These cutoffs were used as they were shown to differentiate asthmatic patients from controls with a good specificity and sensitivity in a previous study from our laboratory [1]. Neutrophilic patients were defined as those having ≥47.17 neutrophils/mm^2 independently of concomitant bronchial eosinophilia (mixed granulocytic and isolated neutrophilic together), while non-neutrophilic patients were defined as those with <47.17 neutrophils/mm^2 (isolated eosinophilic and paucigranulocytic). Neutrophilic patients were further divided into 2 groups, based on the median value of bronchial neutrophil count (94.34 neutrophils/mm^2), with the aim to discriminate the clinical, functional and biological parameters most likely to be influenced by the magnitude of bronchial neutrophilia in neutrophilic asthma. Patients were thus described as highly neutrophilic (≥94.34 neutrophils/mm^2) or intermediate neutrophilic (≥47.17.and <94.34 neutrophils/mm^2).

Immunohistochemistry, immunofluorescence and confocal microscopy analysis

Immunostaining was performed on 6 µm thick frozen sections using antibodies directed at the following proteins in adequate concentrations: eosinophil cationic protein (ECP, rabbit polyclonal bs-8615R, Bioss, USA) for eosinophils, neutrophil elastase for neutrophils (mouse monoclonal, clone NP57, Dako, Denmark), CD4 for T helper lymphocytes (mouse monoclonal, clone 4B12, Dako, Denmark), CD8 for cytotoxic T cells (mouse monoclonal, clone C8/144B, Dako, Denmark), tryptase for mast cells (mouse monoclonal, clone G3, EMD Millipore Corporation, CA, USA), CD68 for macrophages (mouse monoclonal Ab-3, clone KP1, Thermo Fisher Scientific, UK), IL-17A (goat polyclonal, AF317NA, R&D Systems, USA), IL-17F (goat polyclonal, AF1335NA, R&D Systems, USA), IL-21 (goat SC17649, Santa Cruz Biotechnology, USA), IL-22 (goat polyclonal AF782, R&D Systems, USA), and IL-23 (goat polyclonal, SC21079, Santa Cruz Biotechnology, USA). Biotinylated secondary antibodies were then applied; avidin-biotin complex and alkaline phosphatase were used as detection systems (PK6100 and AK5000, Vector Laboratories, UK) with Fast Red and DAB as substrates (Sigma Aldrich, USA). For each marker studied, two to four biopsies per patient were stained (median: 3) in duplicate. The mean count of each subject was used for further analysis.

For confocal microscopy analysis, sections were fixed in 4% paraformaldehyde for 10 minutes, washed with phosphate buffered saline (PBS) and incubated 1 hour, at room temperature (RT), with PBS containing 1% normal goat serum (NGS) and 0.1% Triton X-100. The following primary antibodies were used: rabbit anti-human CD68 (diluted 1:100, Santa Cruz Biotechnology, USA) and mouse anti-human neutrophil elastase (clone NP57, diluted 1:100, Dako, Denmark). After washing with PBS, the preparations were incubated for 1 hour with the appropriate secondary Alexa Fluor 488- or Alexa Fluor 568-conjugated antibodies (Invitrogen S.R.L., Milan, Italy) diluted in PBS. Alexa Fluor 568-tagged anti-CD68 were visualized in red and Alexa Fluor 488-tagged anti-neutrophil elastase in green. Images were analyzed by using a laser-scanning microscope ZEISS LSM 800 at a final magnification of 63×. The Zeiss ZEN2 software package was used for acquisition, storage, and analysis. Final results, expressed as percentage of co-localization, were

calculated as the average of the cell counts performed in each section. Co-localized pixels representing co-expression of NE and CD68 were displayed in yellow in the merge images. [11].

Histomorphometry

Histomorphometry was performed by a single operator blinded to the subjects' ID. Inter-observer agreement between the operator that performed the counts in the present study and the one who performed the counts in the previous study was tested on 5 randomly selected slides for each marker already studied. Mean bias per biopsy ranged from 0 to 6 cells/mm^2 lamina propria, and was proportional to the total number of positively stained cells (lowest for eosinophils and greatest for macrophages). This was judged as an acceptable bias and justifies using the same cutoff used in the previous publication [1] to classify subjects as neutrophilic and eosinophilic. All counts used in the present study were those performed by the new operator on previously attained and archived slides. Whenever old slides were judged as not suitable for assessment, new cuts were obtained.

Cells laying within the lamina propria (100 micron beneath the basal membrane) staining positive for each of the markers studied (ECP, neutrophil elastase, CD4, CD8, IL-17A, IL-17F, IL-21, IL-22, and IL-23) and with a clearly identifiable nucleus were counted and the results expressed as positive cells/mm^2 of lamina propria. A minimum of 3 high power fields (40x) was assessed for each section where the epithelium, basal membrane and lamina propria were clearly identifiable and the tissue structure and architecture were preserved. All good quality fields were assessed in each section (median: 5 fields/section; range 3-14). Lamina propria was defined by the widest possible zone of maximum 100-μm depth beneath the reticular basement membrane, excluding bronchoalveolar lymphoid tissue, airway smooth muscle and damaged tissue. Cell density was expressed as the mean of the counts performed.

Statistical analysis

Predictors were selected among clinical/functional parameters with the aim to identify non-invasive, low-cost and readily-available markers. The choice was based on results of t-tests and Chi-squared tests or because of their physiological meaning. When two or more predictors were significantly related, only one of them was maintained in the model. Values of ΔFEV_1 and ΔFVC were divided into 4 classes each, based on the median and 25-75% interquartile range (IQR) values. Reference values were those within the 1st IQR (min-25% quartile) for ΔFEV_1 and those within the 2nd IQR for ΔFVC. This allowed to compare patients with absent (1st IQR) vs. mild (2nd IQR) ΔFVC, as well as patients with mild (2nd IQR) vs. greater (3rd and 4th IQR) ΔFVC.

Results

Double staining/confocal microscopy for NE^+ with $CD68^+$ cells in the bronchial mucosa of asthmatics

In order to confirm the specificity of our NE count for neutrophils, we performed double immunofluorescence with specific antibodies for neutrophil (NE) and macrophages (CD68). We performed this double staining on five biopsies from five different patients, and results showed that in bronchial mucosa the co-localization of $NE^+/CD68^+$ cells was 3± 2.3 % (Figure S4).

Predictors of bronchial neutrophilia

Smoking, ICS dose and the lung function parameters tested (FVC% and FEV_1/FVC) were not significant predictors of neutrophilia based on our model, after adjusting for the other factors. Of note, we did not study the interaction among these parameters due to the low number of subjects included in the analysis. Each parameter was assessed as an independent predictor of bronchial neutrophilia. This revealed ΔFEV_1 and ΔFVC as independent predictors of bronchial neutrophilia, after adjusting for smoking, ICS dose, and airway obstruction (FVC% and FEV_1/FVC). In detail, compared to patients with $\Delta FEV_1 < 220$ ml, the odds of bronchial neutrophilia was reduced by almost 30-fold in patients with $\Delta FEV_1 > 280$ ml. Neutrophilia was even less likely to occur in patients with values of $\Delta FEV_1 > 390$ ml. Overall, this means that the likelihood of bronchial neutrophilia increases with decreasing ΔFEV_1. These results were obtained from patients with reversible asthma and hence may not apply to persistent asthma.

Detailed results of our models are reported in **Table S7**.

Table S1. Clinical and functional parameters of patients according to their bronchial inflammatory phenotype.

	Isolated Eosinophilic	Isolated Neutrophilic	Mixed Granulocytic	Paucigranulocytic	*P value*
N (%)	28 (40)	2 (3)	36 (51)	4 (6)	-
Severe asthma cases, n (%)	9 (32)	1 (50)	22 (61)	2 (50)	*0.15*
Age, y	49 (45, 58)	65 (65, 66)	49 (43, 62)	54 (20, 69)	*0.40*
Sex, M/F	17/11	1/1	15/21	1/3	*0.36*
Asthma onset, y	28 (14, 40)	26 (25, 27)	28 (14, 33)	16 (2, 40)	*0.68*
Late onset asthma (≥ 18 y), n (%)	18 (64)	2 (100)	22 (61)	2 (50)	*0.67*
Asthma duration, y	18 (10, 30)	39 (38, 41)	22 (16, 31)	28 (18, 48)	*0.36*
Smokers (≥10 pack-years)[§], n (%)	10 (36)	1 (50)	6 (17)	1 (25)	*0.30*
Atopy, n (%)	16 (57)	1 (50)	24 (67)	3 (75)	*0.80*
Serum IgE (KUI/L)	106 (53, 172)	135 (24, 246)	132 (49, 187)	135 (65, 2109)	*0.83*
Polisensitivity (>1 allergen), n (%)	14 (50)	1 (50)	20 (55)	3 (75)	*0.84*
Sensitization to perennial allergens, n (%)	9 (32)	1 (50)	15 (42)	3 (75)	*0.41*
Sensitization to Mycophyta, n (%)	3 (11)	0 (0)	6 (17)	0 (0)	*0.69*
Sinusitis, n (%)	15 (54)	2 (100)	21 (58)	0 (0)	*0.14*
BMI, kg/m2	25.9 (24.0, 27.9)	24.8 (19.5, 28.1)	25.4 (24.1, 26.5)	27.4 (27.4, 32.7)	*0.40*
FEV1, % pred.	82 (79, 90)	68 (40, 96)	77 (71, 83)	85 (75, 104)	*0.43*
FVC, % pred.	96 (90, 101)	96 (76, 116)	81 (55, 96)	104 (53, 114)	*0.12*
ΔFEV1 post β2 agonist (mL)	**360 (260, 420)***	**315 (200, 430)**	**245 (210, 300)**	**320 (230, 630)**	***0.04***
ΔFVC post β2 agonist (mL)	280 (220, 360)	345 (120, 570)	315 (220, 430)	210 (90, 330)	*0.63*
RV, % pred.	102 (100, 139)	154 (118, 191)	117 (104, 145)	93 (84, 153)	*0.15*
FRC, % pred.	97 (87, 116)	161 (137, 186)	105 (100, 119)	99 (74, 107)	*0.06*
FEV1/FVC, %	66 (62, 76)	53 (42, 64)	64 (57, 70)	66 (51, 83)	*0.33*
RV/TLC, %	35 (32, 41)	48 (39, 58)	37 (34, 44)	31 (24, 51)	*0.24*

FeNO, ppb	29 (18, 37)	16 (15, 17)	20 (15, 33)	36 (28, 104)	*0.20*
Exacerbations per year	1 (0,1)	3 (0, 6)	1 (1, 2)	1.5 (0, 3)	*0.44*
Frequent exacerbators (>1/year), n (%)	7 (25)	1 (33)	18 (47)	2 (50)	*0.38*
Beclomethasone HFA equivalent daily dose, µg	**160 (100, 400)****	**400 (200, 400)**	**400 (200, 480)**	**280 (150, 480)**	***0.01***
OCS, n (%)	1 (3)	1 (50)	6 (17)	0(0)	*0.25*
Blood neutrophils, cells/mL	3370 (2750, 3910)	3255 (3070, 3440)	3520 (3080, 3860)	3675 (2980, 6240)	*0.83*
Blood neutrophils, %	53 (50, 58)	51 (49, 53)	51 (48, 54)	58 (25, 63)	*0.26*
Blood eosinophils, cells/mL	250 (190, 310)	500 (180, 820)	200 (130, 370)	355 (180, 760)	*0.58*
Blood eosinophils, %	3 (3, 5)	8 (3, 13)	3 (2, 5)	5 (2, 11)	*0.53*

Continuous variables are presented as median (95% CI of the median). P values according to Kruskal-Wallis and Dunn's post-tests or Fisher exact/Chi-squared tests. Values in bold represent significant differences. [§]: Smokers are intended as current and ex-smokers with a smoking history ≥10 pack-years. *: different from Mixed (p=0.04). **: different from Mixed (p=0.01).

Table S2. Patients' inflammatory and biological parameters.

	Isolated Eosinophilic	Isolated Neutrophilic	Mixed	Paucigranulocytic	*P value*
ECP$^+$ cells/mm^2 lamina propria	**33 (19, 43)**	**4 (0, 10)**	**38 (24, 47)**	**0 (0, 10)*‡**	***0.0009***
NE$^+$ cells/mm^2 lamina propria	**24 (19, 33)****	**90 (68, 113)**	**94 (80, 113)**	**17 (13, 19)****	***<0.0001***
CD4$^+$ cells/mm^2 lamina propria	19 (9, 28)	23 (14, 31)	28 (19, 38)	7 (2, 19)	*0.09*
CD8$^+$ cells/mm^2 lamina propria	13 (5, 24)	9 (5, 13)	23 (14, 28)	13 (4, 28)	*0.05*
CD68$^+$ cells/mm^2 lamina propria	277 (159, 381)	248 (200, 297)	207 (159, 394)	173 (67, 442)	*0.82*
Tryptase$^+$ cells/mm^2 lamina propria	68 (44, 135)	13 (12, 14)	35 (19, 91)	115 (34, 130)	*0.11*
IL-17A$^+$ cells/mm^2 lamina propria	9 (5, 24)	52 (47, 58)	19 (11, 24)	14 (2, 62)	*0.08*
IL-17F$^+$ cells/mm^2 lamina propria	10 (7, 24)#	27 (4, 52)	21 (13, 33)	24 (4, 38)	*0.11*
IL-21$^+$ cells/mm^2 lamina propria	18 (14, 24)	11 (11, 11)	19 (12, 28)	7 (4, 9)	*0.22*
IL-22$^+$ cells/mm^2 lamina propria	13 (9, 24)	53 (53, 53)	19 (13, 33)	31 (9, 52)	*0.11*
IL-23$^+$ cells/mm^2 lamina propria	19 (9, 25)	14 (14, 14)	15 (7, 19)	7 (5, 9)	*0.45*

Continuous variables are presented as median (95% CI of the median). P values are based on Kruskal-Wallis and Dunn's post-tests. Values in bold represent statistically significant differences. *: significantly different from Mixed (p=0.004). ‡: significantly different from Eos (p=0.01). **: significantly different from Mixed (p<=0.0006). #: significantly different from Mixed (p=0.04).

Table S3. Clinical and functional parameters differentiating neutrophilic vs. non-neutrophilic asthma in non-smokers.

	Non Neutrophilic (<47.17 cells/mm^2)	Neutrophilic (≥47.17 cells/mm^2)	Difference between the medians (95% CI difference)	*P value*
N (%)	21 (40)	31 (60)	-	-
Severe asthma cases, n (%)	**3 (14)**	**18 (58)**	**-**	***0.002***
Age, y	46 (38, 53)	47 (41, 62)	-	*0.27*
Sex, M/F	8/13	12/19	-	*1.00*
Asthma onset, y	24 (5, 37)	26 (13, 33)	2 (-7, 13)	*0.60*
Late onset asthma (≥ 18 y), n (%)	11 (52)	18 (58)	-	*0.78*
Asthma duration, y	18 (6, 33)	22 (16, 31)	4 (-7, 13)	*0.57*
Smokers (≥10 pack-years)[§], n (%)	-	-	-	-
Atopy, n (%)	16 (76)	23 (74)	-	*1.00*
Serum IgE (KUI/L)	100 (57, 228)	137 (58, 223)	37 (-79, 79)	*0.87*
Polisensitivity (>1 allergen), n (%)	15 (71)	19 (61)	-	*0.38*
Sensitization to perennial allergens, n (%)	11 (52)	15 (48)	-	*1.00*
Sensitization to Mycophyta, n (%)	3 (14)	6 (19)	-	*0.72*
Sinusitis, n (%)	8 (42)**	20 (65)	-	*0.15*
BMI, kg/m2	25.8 (20.8, 27.5)	25.2 (23.8, 26.6)	-0.6 (-2.7, 3.2)	*0.86*
FEV1, % pred.	84 (79, 96)	79 (71, 85)	-5 (-16, 2)	*0.11*
FVC, % pred.	**100 (95, 104)**	**85 (55, 97)**	**-14 (-41, -3)**	***0.01***
ΔFEV1 post β2 agonist (mL)	**410 (240, 630)**	**240 (210, 300)**	**-170 (-310, -40)**	***0.0007***
ΔFVC post β2 agonist (mL)	330 (170, 520)	270 (210, 410)	-60 (-150, 100)	*0.89*
RV, % pred.	101 (95, 137)	115 (100, 137)	14 (-4, 26)	*0.19*
FRC, % pred.	99 (87, 116)	105 (95, 119)	6 (-6, 21)	*0.28*
TLC, % pred.				
FEV1/FVC, %	**70 (62, 81)**	**64 (57, 72)**	**-6 (-16, -1)**	***0.02***
RV/TLC, %	34 (29, 37)	37 (33, 42)	3 (-1, 10)	*0.10*

FeNO, ppb	35 (28, 62)	21 (17, 34)	-14 (-24, 2)	*0.10*
Exacerbations per year	1 (0, 2)	1 (1, 3)	0 (0, 2)	*0.08*
Frequent exacerbators (>1/year), n (%)	6 (29)	15 (48)	-	*0.25*
ICS daily dose, µg	**160 (100, 200)**	**400 (200, 740)**	**240 (50, 380)**	***0.0003***
OCS, n (%)	0 (0)	6 (19)	-	*0.07*
Blood neutrophils, cells/mL	3220 (2670, 3830)	3360 (3070, 3810)	140 (-460, 610)	*0.69*
Blood neutrophils, %	54 (50, 59)	50 (48, 56)	-3 (-7, 1)	*0.12*
Blood eosinophils, cells/mL	270 (180, 400)	270 (130, 400)	0 (-120, 120)	*0.80*
Blood eosinophils, %	4 (3, 6)	4 (2, 5)	0 (-2, 1)	*0.59*

Continuous variables are presented as median (95% CI of the median). P values according to Mann-Whitney or Fisher exact/Chi-squared tests. Values in bold represent statistically significant differences between the groups. Red fields: parameters for which the difference between groups lost statistical significance when smokers were removed. Blue fields: parameters for which the difference between groups gained statistical significance smokers were excluded from the analysis. [§]: Smokers are intended as current and ex-smokers with a smoking history ≥10 pack-years. **: information missing for 2 patients.

Table S4. Inflammatory and biological parameters differentiating neutrophilic vs. non-neutrophilic asthma in non-smokers.

	Non Neutrophilic (<47,17 cells/mm^2)	Neutrophilic (≥47,17 cells/mm^2)	Difference of the medians (95% CI difference)	*P value*
N (%)	32 (46)	38 (54)	-	-
ECP$^+$ cells/mm^2 lamina propria	28 (19, 42)	38 (24, 52)	9 (-4, 19)	*0.16*
NE$^+$ cells/mm^2 lamina propria	**19 (14, 33)**	**94 (80, 113)**	**75 (57, 94)**	***<0.0001***
CD4$^+$ cells/mm^2 lamina propria	**15 (5, 28)**	**28 (19, 42)**	**13 (2, 24)**	***0.01***
CD8$^+$ cells/mm^2 lamina propria	**10 (4, 19)**	**23 (13, 28)**	**12 (2, 18)**	***0.01***
CD68$^+$ cells/mm^2 lamina propria	268 (106, 442)	221 (163, 410)	-47 (-160, 110)	*0.65*
Tryptase$^+$ cells/mm^2 lamina propria	56 (34, 130)	28 (13, 67)	-28 (-54, 3)	*0.09*
IL-17A$^+$ cells/mm^2 lamina propria	12 (5, 28)	16 (11, 24)	3 (-5, 9)	*0.39*
IL-17F$^+$ cells/mm^2 lamina propria	**10 (7, 26)**	**23 (15, 33)**	**12 (1, 20)**	***0.02***
IL-21$^+$ cells/mm^2 lamina propria	19 (11, 38)	19 (13, 27)	0 (-9, 8)	*0.84*
IL-22$^+$ cells/mm^2 lamina propria	19 (9, 26)	21 (16, 34)	2 (-3, 17)	*0.18*
IL-23$^+$ cells/mm^2 lamina propria	14 (6, 24)	14 (7, 19)	0 (-6, 9)	*0.91*

Variables are presented as median (95% CI of the median). P values are based on Mann-Whitney or Fisher exact/Chi-squared tests. Values in bold represent statistically significant differences between the groups. Red fields: parameters for which the difference between groups lost statistical significance when smokers were removed. Blue fields: parameters for which the difference between groups gained statistical significance when smokers were excluded from the analysis.

Table S5. Clinical and functional parameters of asthmatics in high vs intermediate bronchial neutrophilia (smokers included).

	High Neutrophilia (≥94.34 cells/mm^2)	Intermediate Neutrophilia (≥47.17 and <94.34 cells/mm^2)	Difference between the means (95% CI difference)	*P value*
N (%)	21 (55)	17 (45)	-	-
Severe asthma cases, n (%)	15 (71)	8 (47)	-	*0.18*
Age, y	52 (43, 64)	49 (41, 65)	-3 (-12, 6)	*0.60*
Sex, M/F	8/13	8/9	-	*0.74*
Asthma onset, y	27 (8, 35)	25 (13, 35)	-2 (-13, 11)	*0.90*
Late onset asthma (≥ 18 y), n (%)	13 (62)	11 (65)	-	*1.00*
Asthma duration, y	24 (17, 38)	21 (11, 36)	-3 (-15, 8)	*0.74*
Smokers (≥10 pack-years)§, n (%)	2 (9)	5 (29)	-	*0.21*
Atopy, n (%)	16 (76)	9 (53)	-	*0.18*
Serum IgE (KUI/L)	**146 (77, 245)**	**49 (11, 156)**	**-97 (-186, -14)**	***0.02***
Polisensitivity (>1 allergen), n (%)	15 (94)	6 (67)	-	*0.12*
Sensitization to perennial allergens, n (%)	**12 (57)**	**4 (23)**	**-**	***0.05***
Sensitization to Mycophyta, n (%)	4 (19)	2 (12)	-	*0.67*
Sinusitis, n (%)	14 (67)	9 (53)	-	*0.51*
BMI, kg/m2	25.2 (23.7, 26.6)	25.0 (22.9, 28.4)	-0.2 (-2.1, 3.1)	*0.72*
FEV1, % pred.	73 (68, 83)	81 (74, 92)	7 (-5, 17)	*0.25*
FVC, % pred.	55 (52, 96)	89 (57, 101)	34 (-7, 38)	*0.21*
ΔFEV1 post β2 agonist (mL)	230 (210, 270)	270 (220, 360)	40 (-20, 110)	*0.29*
ΔFVC post β2 agonist (mL)	240 (212, 449)	400 (306, 583)	160 (-30, 290)	*0.19*
RV, % pred.	118 (99, 163)	117 (97, 156)	-0.5 (-23, 23)	*0.81*
FRC, % pred.	110 (92, 135)	104 (95, 133)	-6 (-17, 17)	*0.94*
FEV1/FVC, %	60 (53, 68)	66 (57, 72)	6 (-3, 13)	*0.19*
RV/TLC, %	39 (34, 58)	36 (32, 42)	-3 (-13, 4)	*0.36*
FeNO, ppb	20 (17, 27)	15 (12, 47)	-5 (-10, 24)	*0.19*
Exacerbations per year	**2 (1, 6)**	**1 (0, 1)**	**-1 (-3, 1)**	***0.001***

Frequent exacerbators (>1/year), n (%)	**14 (67)**	**3 (18)**	**-**	***0.003***
Beclomethasone HFA equivalent daily dose, µg	480 (200, 800)	200 (200, 480)	-280 (-280, 80)	*0.32*
OCS, n (%)	**7 (33)**	**0 (0)**	**-**	***0.01***
Blood neutrophils, cells/mL	3560 (3070, 3950)	3360 (2640, 3860)	-200 (-1300, 390)	*0.30*
Blood neutrophils, %	50 (48, 56)	50 (43, 53)	0 (-7, 3)	*0.51*
Blood eosinophils, cells/mL	270 (130, 430)	150 (110, 370)	-120 (-200, 50)	*0.36*
Blood eosinophils, %	4 (2, 5)	2 (2, 7)	-1 (-2, 1)	*0.66*

Continuous variables are presented as median (95% CI of the median). P values are based on Mann-Whitney or Fisher exact/Chi-squared tests. Values in bold represent statistically significant differences between the groups. [§]: Smokers are intended as current and ex-smokers with a smoking history ≥10 pack-years.

Table S6. Inflammatory and biological parameters of asthmatics in high vs. intermediate bronchial neutrophilia (smokers included).

	High Neutrophilia (≥94.34 cells/mm^2)	Intermediate Neutrophilia (≥47.17 and <94.34 cells/mm^2)	Difference between the means (95% CI difference)	*P value*
N (%)	21 (55)	17 (45)	-	-
ECP$^+$ cells/mm^2 lamina propria	34 (22, 52)	38 (23, 52)	4 (-16, 14)	*0.86*
NE$^+$ cells/mm^2 lamina propria	**113 (104, 132)**	**68 (57, 80)**	**-45 (-66, -38)**	***<0.0001***
CD4$^+$ cells/mm^2 lamina propria	**33 (23, 57)**	**19 (7, 34)**	**-14 (-28, 0)**	***0.03***
CD8$^+$ cells/mm^2 lamina propria	23 (14, 38)	19 (5, 33)	-4 (-16, 7)	*0.32*
CD68$^+$ cells/mm^2 lamina propria	224 (115, 413)	200 (145, 394)	-24 (-112, 102)	*0.92*
Tryptase$^+$ cells/mm^2 lamina propria	23 (6, 86)	48 (14, 113)	25 (-13, 69)	*0.31*
IL-17A$^+$ cells/mm^2 lamina propria	19 (15, 31)	13 (5, 38)	-6 (-15, 4)	*0.16*
IL-17F$^+$ cells/mm^2 lamina propria	**33 (23, 47)**	**10 (6, 17)**	**-23 (-33, -10)**	***0.0001***
IL-21$^+$ cells/mm^2 lamina propria	19 (11, 33)	17 (7, 35)	-2 (-11, 9)	*0.71*
IL-22$^+$ cells/mm^2 lamina propria	28 (19, 47)	13 (9, 35)	-15 (-23, 0)	*0.11*
IL-23$^+$ cells/mm^2 lamina propria	14 (8, 23)	9 (5, 19)	-5 (-9, 6)	*0.91*

Variables are presented as median (95% CI of the median). P values based on Mann-Whitney test. Values in bold represent statistically significant differences between the groups. Blue field = parameters for which the difference between the groups becomes statistically significant when smokers are excluded from the analysis.

Table S7. Predictors of bronchial neutrophilia (≥47.17 cells/mm^2).

	OR	95% CI OR	*P value*
Non-smokers (<10 pack-year)	1 (REF)	-	-
Smokers (≥10 pack-year)	2.09	0.36; 11.79	*0.40*
ICS dose	1.00	0.99; 1.00	*0.89*
FVC % pred.	0.95	0.91; 1.00	*0.07*
FEV_1/FVC	227.88	0.05; >999	*0.21*
ΔFEV_1			*0.05*
ΔFEV_1 <220 ml	1 (REF)	-	-
ΔFEV_1 220-280 ml	0.21	0.01; 1.81	*0.18*
ΔFEV_1 280-390 ml	**0.03**	**0.001; 0.418**	***0.02***
ΔFEV_1 >390 ml	**0.01**	**0.0003; 0.244**	***0.01***
ΔFVC			*0.06*
ΔFVC < 210 ml	9.72	0.90; 171.4	*0.08*
ΔFVC 210-305 ml	1 (REF)	-	-
ΔFVC 305-450 ml	5.93	0.53; 103.5	*0.17*
ΔFVC >450 ml	**182.92**	**6.95; 1537.60**	***0.006***

Values in bold represent significant predictors of bronchial neutrophilia (≥47.17 cells/mm^2). SE: standard error. 95% CI: 95% confidence interval. OR: odds ratio. ICS: inhaled corticosteroids. FEV_1: forced expiratory volume in 1 second. FVC: forced vital capacity. ΔFEV_1: bronchodilator induced change in FEV_1 expressed in ml. ΔFVC: bronchodilator induced change in FVC expressed in ml.

Supplementary figures

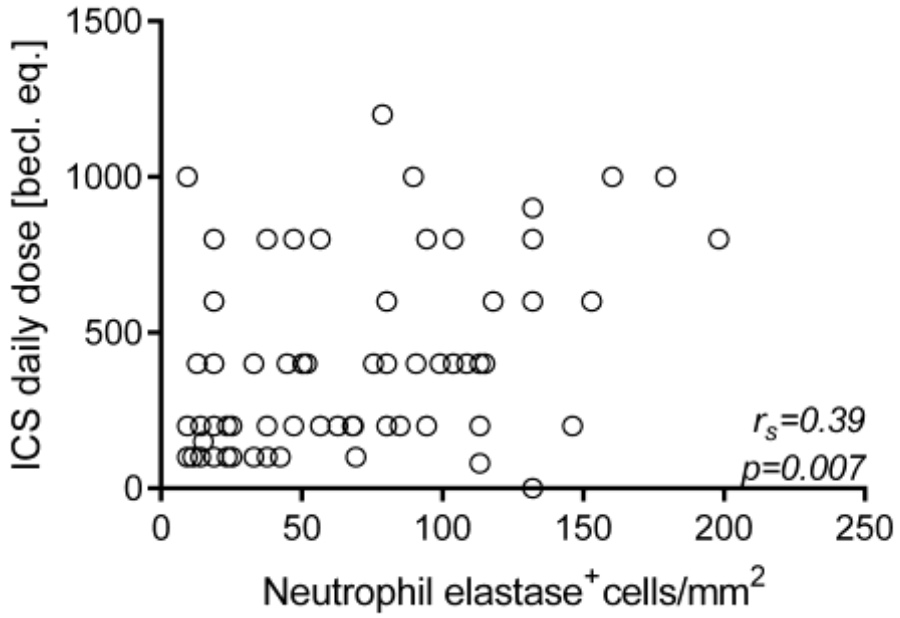

Figure S1. Correlations between bronchial neutrophilia and ICS dose in bronchial tissue in all asthmatic patients (n=70). Statistical analysis was performed by Spearman's rank test. Significance was set at $p < 0.05$.

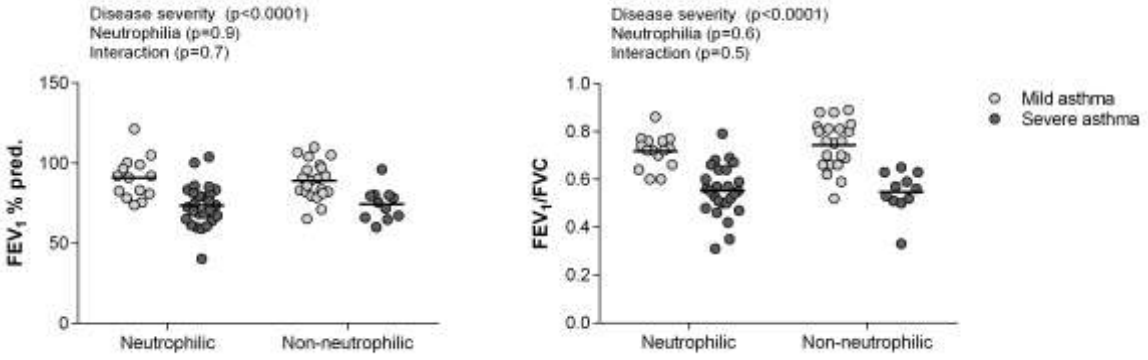

Figure S2. Markers of airflow obstruction in asthmatic patients of our cohort stratified by bronchial neutrophilia and disease severity.

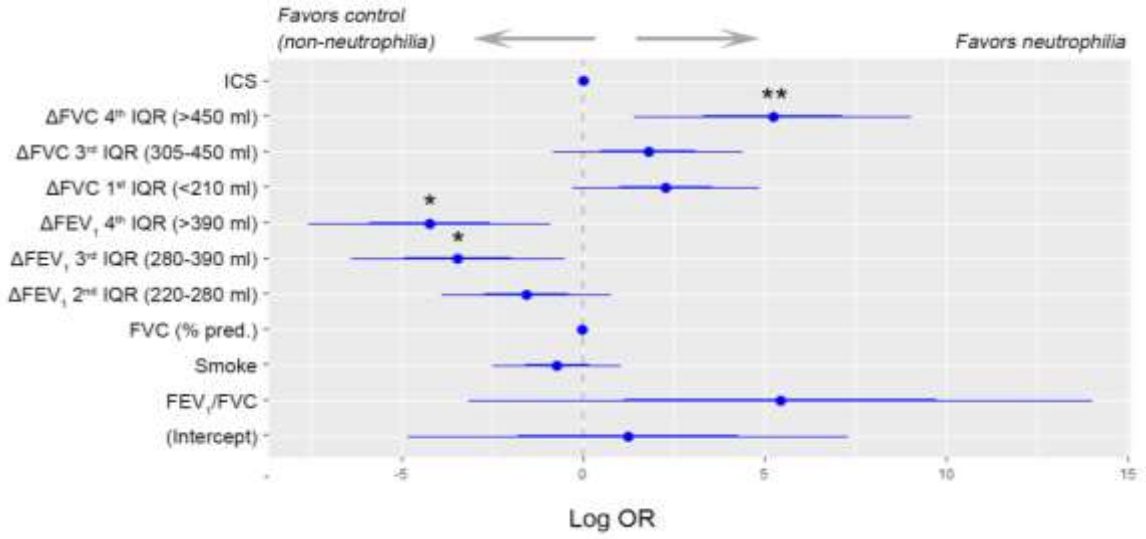

Figure S3. Forest plot indicating predictors of bronchial neutrophilia in asthmatic patients. IQR: interquartile interval range. OR: odds ratio. *: $p<0.05$. **: $p<0.01$.

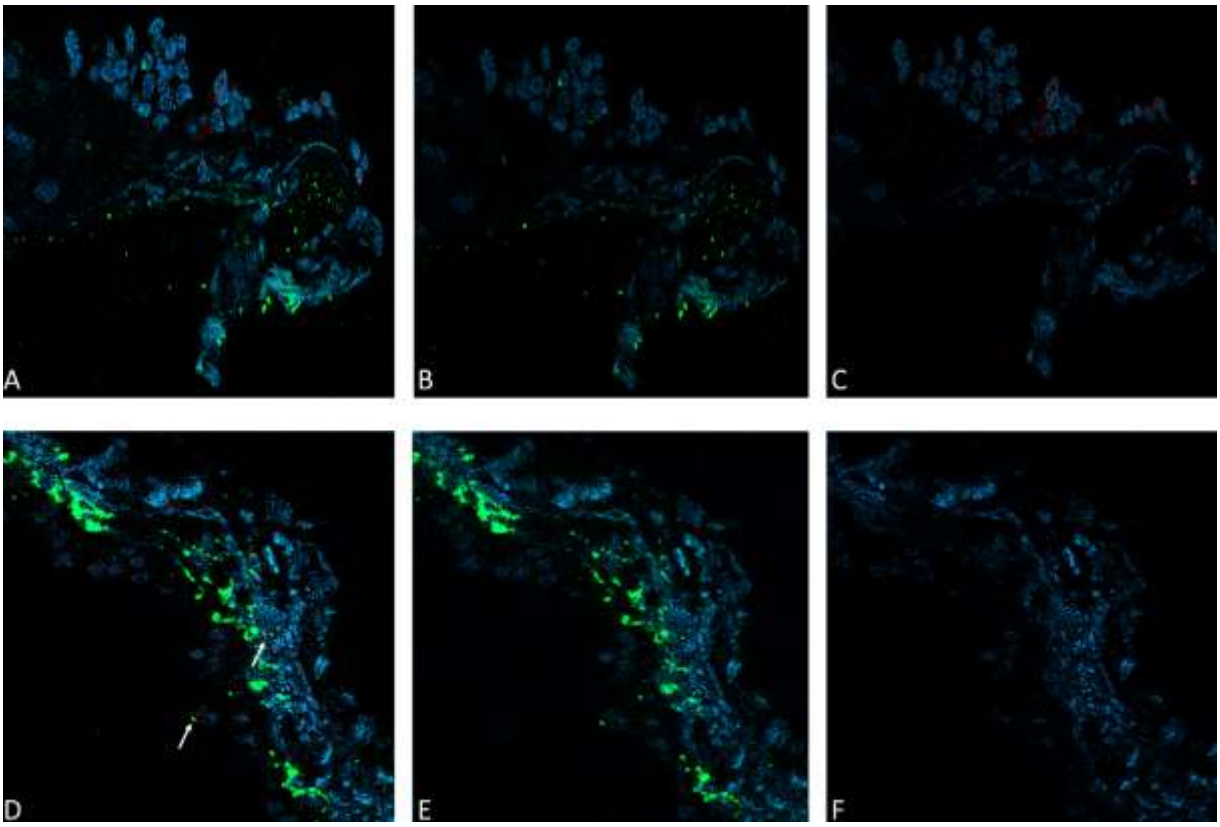

Figure S4. Confocal images of double immunofluorescence for macrophages (CD68) and neutrophil elastase (NE) expression in the bronchial biopsies of two asthmatic patients. Alexa Fluor 488-green staining represents NE, Alexa Fluor 568-red staining represents CD68 (panel A-F) whereas co-localized pixels are displayed in yellow (denoted by arrows).

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