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

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

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**Abstract** (249/250)

Information on the clinical traits associated to bronchial neutrophilia in asthma is scant, preventing its recognition and adequate treatment. We aimed to assess clinical, functional and biological features of neutrophilic asthma and identify possible predictors of bronchial neutrophilia.

The inflammatory phenotype of 70 mild-to-severe asthmatics was studied cross-sectionally on the basis of eosinophilic/neutrophilic counts in their bronchial *lamina propria*. Patients were classified as neutrophilic or non-neutrophilic. Neutrophilic asthmatics (neutrophil count cutoff: 47.17 neutrophils/$mm^2$; range: 47.17-198.11 neutrophils/$mm^2$; median: 94.34 neutrophils/$mm^2$) were further classified as high (≥94.34 neutrophils/$mm^2$) or intermediate (≥47.17 and <94.34 neutrophils/$mm^2$). The effect of smoking ≥10 pack-years was also assessed.

Neutrophilic asthmatics (n=38; 36 mixed eosinophilic/neutrophilic) had greater disease severity, FRC, ICS dose and exacerbations, and decreased FVC% and $FEV_1$ reversibility than non-neutrophilic asthmatics (n=32; 28 eosinophilic, 4 paucigranulocytic). Neutrophilic asthmatics had similar eosinophil counts, increased bronchial $CD8^+$, IL17-$F^+$, IL-$22^+$ cells, and decreased mast cells compared to non-neutrophilic asthmatics. $FEV_1$ and FVC reversibility were independent predictors of bronchial neutrophilia in our cohort. High neutrophilic patients (n=21) had increased serum IgE levels, sensitivity to perennial allergens, exacerbation rate, OCS-dependence, $CD4^+$ and IL-$17F^+$ cells in their bronchial mucosa. Excluding smokers revealed also increased IL-$17A^+$ and IL-$22^+$ cells in highly neutrophilic patients.

We provide new evidence linking the presence of high bronchial neutrophilia in asthma to an adaptive immune response associated with allergy (IgE) and IL-17/IL-22 cytokine expression. High bronchial neutrophilia may discriminate a new endotype of asthma. Further research is warranted on the relationship between bronchoreversibility test and bronchial neutrophilia.

**“Take home” message:**

Asthma with high bronchial neutrophilia is associated with increased serum IgE, perennial allergy, and enhanced IL-17 expression . This new endotype is associated clinically with an increased exacerbation rate in the last year.

**Keywords:**

Asthma, biopsy, bronchi, neutrophil, exacerbations, bronchoreversibility, air-trapping, IL-17, allergy, immunohistochemistry.

**Abbreviations:**

AUC: area under the curve; FeNO: fractional exhaled nitric oxide; FRC: functional residual capacity; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; GINA: Global initiative for asthma; ICS: inhaled corticosteroids; IgE: immunoglobulin E; IL: interleukin; IQR: interquartile range; NLRP: Nod-like receptor protein; ROC: receiver operating characteristic; RV: residual volume; TLC: total lung capacity.

## Introduction

Asthma is a complex heterogeneous disorder with a variety of cellular and molecular inflammatory mechanisms. The use of unbiased statistical approaches to classify asthma patients based on their clinical and biological features has recently helped to identify subgroups of patients more likely to respond to targeted therapy. Neutrophilia in asthma is associated with aging and oxidative stress, increased disease severity and exacerbations, non-atopy, cigarette smoking exposure, persistent airflow limitation, reduced reversibility, steroid insensitivity and with comorbidities such as rhinosinusitis, sleep disturbances and gastro-esophageal reflux disease [1].

In spite of the growing interest in neutrophilic inflammation, asthma is still dichotomously defined as eosinophilic or non-eosinophilic [2]. This classification disregards the fact that neutrophilic inflammation may coexist with eosinophilic inflammation in a subset of patients displaying the so-called mixed inflammatory phenotype [3]. This phenotype, at least when observed in sputum samples, is characterized by greater severity [4, 5]. Sputum neutrophilia is found in up to 28% of patients [6], while only 3-8% have mixed granulocytic inflammation [6-8]. Data from bronchial biopsy samples [9], however, suggest a different inflammatory phenotype distribution, but whether bronchial neutrophilia has any significance in asthma remains unclear.

The paucity of information on neutrophilic asthma is partly due to the lack of biomarkers of bronchial/sputum neutrophilia recognized for use in clinical settings [1], which slows down the clinical trials of existing targeted therapies and the development of new ones for this phenotype of the disease. Hence, identifying reliable markers of bronchial neutrophilia in asthma is essential. Age and functional residual capacity (FRC) were previously identified as significant predictors of sputum neutrophilia >76% [7], Recent evidences suggest that assessing airway inflammation based on sputum may be misleading however, especially in paucigranulocytic asthma [10]. For this reason, we choose to define asthma inflammatory phenotypes based on bronchial inflammation using biopsy samples. This study was set up 1) to assess the clinical, functional and biological

differences in mild-to-severe asthmatics with or without bronchial neutrophilia, based on cutoffs previously shown to differentiate patients vs. controls [11], and 2) to identify significant predictors of bronchial neutrophilia in our sample. Moreover, we aimed to identify among the parameters studied those that were related to (or influenced by) the magnitude of bronchial neutrophilia. To do this, neutrophilic patients were divided in 2 groups using the median value of cell counts as cutoff (≥94.34 neutrophils/$mm^2$). As this cutoff was greater than those previously related to severe (≥50.31 neutrophils/$mm^2$) and exacerbation-prone asthma (EPA, ≥2 exacerbations/year; ≥72.68 neutrophils/$mm^2$) in a subset of the patients included in the current study [11], we expect our results on highly neutrophilic patients to help expanding our knowledge on the pathobiology (endo/pheno-typing) of neutrophilic EPA.

Of note, this study addresses novel questions compared to the one previously published by our group [11]. Specifically: 1) it investigates the implications of high bronchial neutrophilia in asthma, 2) it dissects the effects of smoking on the neutrophilic component of bronchial inflammation, and 3) it provides new data in support of a sub-endotype of neutrophilic asthma characterized by an intimate relationship between neutrophils-allergy-IL-17 pathway, a pathologic pathway associated to EPA.

## Methods

Patients were recruited as previously described [11]. More details are available online. The inflammatory phenotypes of asthma patients were initially described using bronchial eosinophil (12.45 cells/mm$^2$) and neutrophil (47.17 cells/mm$^2$) cutoffs previously shown to differentiate controls from asthmatics.[11] Patients were then classified as neutrophilic or non-neutrophilic (≥47.17 vs. <47.17 neutrophils/mm$^2$), irrespective of the concomitant bronchial eosinophil content. Lastly, neutrophilic patients were described as highly (≥94.34 neutrophils/mm$^2$) or intermediate neutrophilic (≥47.17 and <94.34 neutrophils/mm$^2$), based on the median neutrophil cell count in this group.

### *Statistical analysis*

Differences among the 4 inflammatory phenotypes described were assessed using Kruskal-Wallis and Dunn's post-test (mixed inflammatory phenotype used as control). Differences in neutrophilic vs. non-neutrophilic and high vs. intermediate neutrophilic asthma were studied with Mann-Whitney or unpaired t-test, depending on data distribution, including and excluding smokers (≥10 pack-year). **Figure 1** details the study design. We used Chi-squared and Fisher's exact test to compare proportions between groups. The effect of neutrophilia and disease severity were studied with two-way ANOVA and Sidak's multiple comparison post-tests. Correlations were performed using Pearson or Spearman tests, depending on data distribution (assessed with D'Agostino and Pearson omnibus normality test).

Logistic regression analysis on all asthma patients assessed the association between the binary outcome bronchial neutrophilia (yes/no; cutoff: ≥47.17 cells/mm$^2$) and the predictors: ICS dose, smoking history (≥10 vs. <10 pack-year), FVC%, $FEV_1$/FVC, Δ$FEV_1$ and ΔFVC. Statistical significance was set at $p \leq 0.05$. More details are reported online.

## Results

### *Inflammatory phenotypes*

Clinical details and bronchial inflammatory cell counts of the 70 patients studied are presented in **Supplementary tables S1-S2**. Our sample was composed mainly of mixed granulocytic (n=36, 51%) and isolated eosinophilic (n=28, 40%) asthmatics, with a small minority of isolated neutrophilic (n=2, 3%) and paucigranulocytic (n=4, 6%) patients (**Figure 2**).

### *Neutrophilic asthma*

Neutrophilic (isolated neutrophilic and mixed granulocytic) asthma was observed in 38/70 (54%) patients, most of whom (36/38, 95%) had concomitant bronchial eosinophilia. Clinical/functional and biological differences between neutrophilic and non-neutrophilic asthma are reported, respectively, in **Tables 1** and **2**. Overall, neutrophilic asthma was associated with an increased frequency of severe disease (p=0.03), more exacerbations in the previous 12 months (p=0.02), a decreased $FEV_1$ response to bronchodilators ($\Delta FEV_1$) (p=0.004), and with functional markers of airway closure/air-trapping (decreased FVC%, p=0.03; increased FRC%, p=0.04) compared to non-neutrophilic asthma. ICS dose was more than 2-fold greater in neutrophilic than non-neutrophilic asthma (p=0.001), and the frequency of patients on OCS for ≥6 months/year tended to be increased in presence of bronchial neutrophilia (p=0.06). Stratifying patients on both bronchial neutrophilia and disease severity showed however that ICS dose was associated with disease severity (p<0.0001) but not with bronchial neutrophilia (p=0.08, **Figure 3C**). Similarly, patients on OCS for ≥6 months/year were significantly increased among severe vs. mild neutrophilic asthmatics (7/23 vs. 0/15; p=0.03) but not in neutrophilic vs. non neutrophilic severe asthmatics (7/23 vs. 1/10; p=0.22). According to this sub-analysis, FVC reversibility (ΔFVC) was greater in neutrophilic than non-neutrophilic patients in severe asthmatics only (p=0.008, **Figure 3A**), in spite of similar values of

airway obstruction ($FEV_1$% and $FEV_1$/FVC, **Supplementary figure S1**). Values of ΔFVC >315 ml discriminated severe (but not mild) neutrophilic vs. non-neutrophilic patients with 70% sensitivity and 78% specificity (AUC=0.72; p=0.05; 3.13 likelihood ratio, **Figure 3G**). In contrast, $\Delta FEV_1$<340 ml discriminated only mild neutrophilic vs. non-neutrophilic patients with 77% sensitivity and 76% specificity (AUC=0.08; p=0.005; 3.27 likelihood ratio, **Figure 3H**). In light of the ΔFVC results, we investigated more in depth the presence and degree of air-trapping in our cohort. Severe neutrophilic asthmatics had the highest numerical value of RV% and the highest proportion of air-trappers (defined as patients with RV≥130%, **Figure 3D**), but differences were not statistically significant. When mild and severe neutrophilic patients were pooled, the proportion of air-trappers was similar to that observed in non-neutrophilic patients (p=0.6, **Figure 3E**). Among air-trappers, those with bronchial neutrophilia tended to have higher values of RV% (p=0.08, **Figure 3F**). Although RV/TLC did not reach statistical significance, it tended to be higher in neutrophilic than in non-neutrophilic patients (p=0.06).

In their bronchial biopsies, neutrophilic patients had more $CD8^+$, IL17-$F^+$, and IL-$22^+$ cells and less mast cells in the bronchial lamina propria compared to non-neutrophilic patients. A trend was also evident towards higher numbers of $CD4^+$ and IL-$17A^+$ cells in neutrophilic compared to non-neutrophilic patients (**Table 2**). Excluding paucigranulocytic patients from the non-neutrophilic group left the medians of all parameters unchanged (data not shown).

The proportion of smokers was not different between neutrophilic and non-neutrophilic patients in our study (p=0.17). However, as bronchial neutrophilia has been strongly linked with smoking [12], we repeated the analysis excluding current and ex-smokers (≥10 pack-years). Results are summarized in **Supplementary tables S3-S4**. Briefly, in this reduced sample (n=52; 31 neutrophilic, 21 non-neutrophilic), we confirmed that neutrophilic patients were more frequently affected by severe asthma (p=0.002), with increased airflow limitation (p=0.02), airway closure/air-trapping (p=0.01), decreased $\Delta FEV_1$ (p=0.0007) and higher ICS dose (p=0.0004). Excluding

smokers, the differences previously observed between neutrophilic and non-neutrophilic asthma concerning number of exacerbations in the previous year, FRC%, and number of IL-22$^+$ cells in the bronchial submucosal were lost, but the number of CD4$^+$ cells in the lamina propria became significantly greater in neutrophilic asthma (p=0.01).

*Highly neutrophilic asthma*

Among neutrophilic asthmatics, those with high bronchial neutrophilia were 21/38 (55%, **Figure 4A-C**). Clinical and functional parameters of high vs. intermediate neutrophilic asthmatics are reported in **Supplementary table S5**. Asthmatics with high bronchial neutrophilia had increased serum IgE (p=0.02), sensitization to perennial allergens (p=0.05), exacerbations (p=0.001) and patients on OCS for ≥6 months/year (p=0.01) compared to those with intermediate bronchial neutrophilia (**Figure 4D-G**). The bronchial eosinophil count was similar in the two groups (p=0.99, **Supplementary table S6**). Asthmatic patients with high bronchial neutrophilia differed from those with intermediate bronchial neutrophilia as regards the 3-fold increase in the number of IL-17F$^+$ cells in their submucosa (p=0.0001) and the greater number of CD4$^+$ cells p=0.03, **Supplementary table S6**).

After excluding neutrophilic patients with a smoking history ≥10 pack-years, asthmatics with high bronchial neutrophilia still had increased serum IgE levels (p=0.01) and exacerbations (p=0.006) than asthmatics with intermediate neutrophilia. Sensitivity to perennial allergens and patients on OCS still tended to be increased in high than intermediate neutrophilic patients (p=0.06). Excluding smokers confirmed the increased number of CD4$^+$ and IL-17F$^+$ cells in high bronchial neutrophilia (**Figure 5A-F**), and also revealed increased number of IL-17A$^+$ and IL-22$^+$ cells in this group (**Figure 5G-L**).

*Correlations*

Among all neutrophilic patients, significant correlations were observed between bronchial neutrophil counts and both the number of exacerbations in the last 12 months and serum IgE levels, but not ICS dose (**Figure 6A-C**). Bronchial neutrophilia also correlated with $CD4^+$, $IL22^+$ and $IL\text{-}17F^+$ cells (**Figure 6D-F**). Bronchial neutrophilia negatively correlated with bronchial mast cell counts ($r_s$=-0.27; p=0.04). A negative correlation was also observed between bronchial mast cell and $IL\text{-}17A^+$ cell counts ($r_s$=-0.34; p=0.01). In these patients, a significant correlation was observed also between IgE and $IL\text{-}17F^+$ cells (**Figure 6G**). IgE also correlated with blood eosinophilia expressed both as absolute cell count and as percentage (**Figure 6H-I**).

*Predictors of bronchial neutrophilia*

Lung function reversibility parameters were the only predictors associated with bronchial neutrophilia (≥47.17 cells/$mm^2$) among those studied (**Supplementary figure S2**). In detail, compared to patients with $\Delta FEV_1$ <220 ml (1$^{st}$ IQR), those with values ≥280 ml (3$^{rd}$ and 4$^{th}$ IQR) had a significantly lower probability of having bronchial neutrophilia (p=0.01). Concerning ΔFVC, the probability of having bronchial neutrophilia was similar in patients with values <210 ml (1$^{st}$ IQR) or 210-305 ml (2$^{nd}$ IQR). Instead, patients with values ≥450 ml (4$^{th}$ IQR) had a significantly greater probability of having bronchial neutrophilia compared to those with 210-305 ml (2$^{nd}$ IQ; p=0.006). Further details are available online.

**Discussion**

Asthma inflammatory phenotypes are commonly defined on the basis of sputum cell counts. Among adult asthmatics of all severities, about 20-30% have sputum neutrophilia [6, 7], mostly in the absence of concomitant eosinophilia. The proportion of patients with sputum neutrophilia increases in severe asthma [5]. In our study on bronchial biopsies, we observed bronchial neutrophilia in 54% of mild-to-severe patients, and this percentage further rose to 68% when only severe patients were considered. Excluding smokers, 60% of our patients were neutrophilic, indicating that neutrophils are key-players in asthma pathogenesis and merits investigation. Whether sputum cell counts reflect bronchial wall inflammation remains an open question. While it seems to be the case for eosinophils, available data suggest that sputum neutrophilia cannot predict bronchial neutrophilic inflammation [13, 14], underlining the need for alternative non-invasive and reliable markers for bronchial neutrophilia.

The paucity of information regarding the clinical traits associated to bronchial neutrophilia in asthma prevents its recognition, the implementation of adequate treatment and the possibility to improve disease control [1]. Our data reveal that bronchial neutrophilia is associated with severe asthma and characterized by reduced $FEV_1$ reversibility ($\Delta FEV_1$), prominent airway closure/air-trapping and pulmonary hyperinflation (lower FVC%, higher FRC%, borderline difference noticed also for RV/TLC). Due to the close relationship among smoking, neutrophilia, and permanent airway obstruction (with airflow limitation and air-trapping) [12], we repeated our analysis excluding smokers. This revealed that pulmonary hyperinflation likely results from smoking (or smoking-related neutrophilia). It confirmed low FVC% and $\Delta FEV_1$ as clinical traits observed with bronchial neutrophilia, its association with the severe form of the disease, and revealed a significantly greater airflow limitation (reduced $FEV_1/FVC$) in non-smoking neutrophilic vs. non-neutrophilic patients. Overall, bronchial neutrophilia characterizes patients with a more severe smoking-independent form of asthma that affects all levels of the bronchial tree and manifests as

enhanced obstruction and reduced reversibility, in agreement with previous studies [15, 16]. In the light of previous data showing asthma severity as a critical determinant of the bronchodilator-induced reversibility response [17], we investigated more in depth the combined effect of bronchial neutrophilia and disease severity on bronchoreversibility parameters. Our findings show the existence of significant interactions between bronchial neutrophilia and disease severity, with distinct patterns of bronchoreversibility in mild and severe asthmatics. Mild asthmatics have low ΔFVC irrespective of their bronchial neutrophilia, likely indicating the absence of air-trapping/airway closure, whilst $\Delta FEV_1$ is reduced only in presence of bronchial neutrophilia in this group. Given the similar ICS doses in neutrophilic and non-neutrophilic mild asthmatics (and of similar values of $FEV_1$% and $FEV_1$/FVC indicating similar degrees of obstruction), the reduced $FEV_1$ reversibility observed with neutrophilia could be the expression of irreversible airway remodeling or of steroid-resistance on this subset of patients. Differently, severe asthmatics had all low $\Delta FEV_1$, confirming the reduced reversibility of obstruction in these patients, whilst ΔFVC increased in neutrophilic asthmatics (with similar $FEV_1$% and $FEV_1$/FVC). To a further level, $\Delta FEV_1$ <340 ml and ΔFVC >315 ml were identified as significant discriminants of bronchial neutrophilia in mild and severe asthmatics, respectively. This is in line with recent studies reporting that FVC reversibility increases with the level of airflow obstruction [18, 19], and that patients with FVC reversibility (either alone or co-existing with $FEV_1$ reversibility) have worse lung function and poorer asthma control than patients with $FEV_1$ reversibility only [20]. Further studies will have to ascertain our findings, but the available evidence suggests that both $FEV_1$ and FVC reversibility merit attention during the clinical assessment of asthma patients.

The role of neutrophils in asthma is unclear. Our data suggest that when their number in the bronchi exceeds that of healthy patients there is an effect on lung function, but no worsening of the effect with further increases of neutrophils. Stepping up from intermediate to high bronchial neutrophilia was instead associated with increased serum IgE, sensitization to perennial allergens, OCS

treatment, and exacerbations, although mast cell number remained unchanged. Patients with high bronchial neutrophilia had also increased airway $CD4^+$ and IL-$17F^+$ cells, and values of neutrophil counts, serum IgE, IL-$17F^+$ and $CD4^+$ cells strongly correlated, indicating a possible role of neutrophils and IL-17 in allergic mechanisms in this disease phenotype, in accordance with dual Th-2/Th-17 immune response [21, 22]. Smoke exposure might influence bronchial neutrophils, serum IgE and Th-17 cytokine levels [23, 24]. Repeating the analysis excluding smokers confirmed our data however, revealing also an increased number of cells expressing IL-17A and IL-22, two Th-17 effector cytokines [25], in asthmatic airways with high neutrophilia. In this context, previous studies support serum IgE and neutrophils interplay in asthma [26], possibly carrying to a phenotype characterized by atopy and increased exacerbations [27], in line with our observations. IgE exposure induces passive sensitization of airway smooth muscle cells, enhancing its contractile response *in vitro* [28] and possibly favoring exacerbations *in vivo*. Overall, these findings support the notion that a shift from a predominantly Th2 mast cell-mediated to a predominantly IL-17 and neutrophil-mediated allergic process may occur in EPA patients.

The IL-17 pathway has been associated with severe and neutrophilic asthma, with the frequent exacerbator asthma phenotype [11, 25, 29], and, recently, also with atopic asthma [27, 30]. While T(h)-2 and T(h)-17 are generally viewed as being mutually exclusive immune pathways, significant interactions have been shown between Th-17 and Th-2 responses, with dual Th2/Th17 T-cells found in blood and bronchoalveolar lavage of stable allergic and severe asthmatics [21, 22]. Data from a murine model suggest dual Th2/Th17 T-cells (able to induce IgE secretion from B cells in vitro) are increased in the lungs during the chronic asthma phase and may be responsible for disease exacerbation through the recruitment of neutrophils and eosinophils [22]. Accordingly, bronchial neutrophilia was observed concomitantly with bronchial eosinophilia in our patients, except for 2 cases. The rise in neutrophils and eosinophils was not correlated, however, possibly because bronchial eosinophils decrease following corticosteroid treatment in asthma, while the effect is dampened for neutrophils [31, 32]. Alternatively, the corticosteroid-induced increase in neutrophil

phagocytic activity [33] might explain the more effective removal of eosinophils from the bronchial tissue. Taken together, our data suggest that in a subset of neutrophilic asthmatics, allergic mechanisms deviate from a classical T(h)2 to an alternative T(h)17 or dual T(h)2/T(h)-17-mediated immune response and are associated with increased exacerbation frequency. The molecular mechanisms by which this paradigm shift occurs and neutrophils contribute to the allergic response warrant further attention.

Data on the effect of corticosteroids on bronchial neutrophils are discordant. Although the majority of studies on bronchial biopsies show a decrease or a steady state of neutrophils after corticosteroid treatment [32, 34-37], some report an increase [38]. Of note, however, lymphocytes were always decreased or remained unchanged in these studies and in other sputum studies where ICS-induced neutrophilia was observed [39]. In our study, instead, we noted a concomitant increase of bronchial neutrophils and $CD4^+$ cells, in association with elevated activity of the IL-17 pathway. Moreover, bronchial neutrophilia did not correlate with ICS dose in neutrophilic asthma. In vitro, IL-17 cytokines induces steroid-resistance in peripheral blood mononuclear cells and in bronchial epithelial cells [40, 41]. IL-17 also induces the secretion of IL-8, a potent neutrophil chemoattractant, in bronchial epithelial cells [42]. All-together, these data suggest that bronchial neutrophilia in asthma is likely the expression of a deviated/altered adaptive response [43], rather than a side effect of ICS treatment.

In summary, our results contribute to a more precise characterization of the role of airway neutrophilia in asthma, highlighting its association with lung function alterations such as increased airflow limitation, airway closure/air-trapping, and altered reversibility patterns. We provide enticing preliminary evidence in support of a new endotype of asthma related to high bronchial neutrophilia, serum IgE and markers of the IL-17 pathway, and characterized clinically by increased sensitivity to perennial allergens, OCS-dependence, and exacerbations.

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**References**

1. Panettieri RA, Jr. Neutrophilic and Pauci-immune Phenotypes in Severe Asthma. *Immunol Allergy Clin North Am* 2016; 36(3): 569-579.

2. Carr TF, Zeki AA, Kraft M. Eosinophilic and Non-Eosinophilic Asthma. *Am J Respir Crit Care Med* 2017.

3. Fahy JV. Eosinophilic and neutrophilic inflammation in asthma: insights from clinical studies. *Proc Am Thorac Soc* 2009; 6(3): 256-259.

4. Loza MJ, Adcock I, Auffray C, *et al.* Longitudinally Stable, Clinically Defined Clusters of Patients with Asthma Independently Identified in the ADEPT and U-BIOPRED Asthma Studies. *Ann Am Thorac Soc* 2016; 13 Suppl 1: S102-103.

5. Moore WC, Hastie AT, Li X, *et al.* Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol* 2014; 133(6): 1557-1563 e1555.

6. Simpson JL, Scott R, Boyle MJ, *et al.* Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirology* 2006; 11(1): 54-61.

7. Schleich FN, Manise M, Sele J, *et al.* Distribution of sputum cellular phenotype in a large asthma cohort: predicting factors for eosinophilic vs neutrophilic inflammation. *BMC Pulm Med* 2013; 13: 11.

8. Douwes J, Gibson P, Pekkanen J, *et al.* Non-eosinophilic asthma: importance and possible mechanisms. *Thorax* 2002; 57(7): 643-648.

9. Wenzel SE, Szefler SJ, Leung DY, *et al.* Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med* 1997; 156(3 Pt 1): 737-743.

10. Demarche S, Schleich F, Henket M, *et al.* Detailed analysis of sputum and systemic inflammation in asthma phenotypes: are paucigranulocytic asthmatics really non-inflammatory? *BMC Pulm Med* 2016; 16: 46.

11. Ricciardolo FLM, Sorbello V, Folino A, *et al.* Identification of IL-17F/frequent exacerbator endotype in asthma. *J Allergy Clin Immunol* 2017; 140(2): 395-406.

12. Thomson NC. Asthma and smoking-induced airway disease without spirometric COPD. *Eur Respir J* 2017; 49(5).

13. Arron JR, Choy DF, Laviolette M, *et al.* Disconnect between sputum neutrophils and other measures of airway inflammation in asthma. *Eur Respir J* 2014; 43(2): 627-629.

14. Berry M, Morgan A, Shaw DE, *et al.* Pathological features and inhaled corticosteroid response of eosinophilic and non-eosinophilic asthma. *Thorax* 2007; 62(12): 1043-1049.

15. Woodruff PG, Khashayar R, Lazarus SC, *et al.* Relationship between airway inflammation, hyperresponsiveness, and obstruction in asthma. *J Allergy Clin Immunol* 2001; 108(5): 753-758.

16. Shaw DE, Berry MA, Hargadon B, *et al.* Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest* 2007; 132(6): 1871-1875.

17. Sorkness RL, Bleecker ER, Busse WW, *et al.* Lung function in adults with stable but severe asthma: air trapping and incomplete reversal of obstruction with bronchodilation. *J Appl Physiol (1985)* 2008; 104(2): 394-403.

18. Quanjer PH, Ruppel GL, Langhammer A, *et al.* Bronchodilator Response in FVC Is Larger and More Relevant Than in FEV1 in Severe Airflow Obstruction. *Chest* 2017; 151(5): 1088-1098.

19. McCartney CT, Weis MN, Ruppel GL, *et al.* Residual Volume and Total Lung Capacity to Assess Reversibility in Obstructive Lung Disease. *Respir Care* 2016; 61(11): 1505-1512.

20. Simpson AJ, Fowler SJ, Group UBS. Reclassification of Bronchodilator Reversibility in the U-BIOPRED Adult Asthma Cohort Using z Scores. *Chest* 2018; 153(4): 1070-1072.

21. Irvin C, Zafar I, Good J, *et al.* Increased frequency of dual-positive TH2/TH17 cells in bronchoalveolar lavage fluid characterizes a population of patients with severe asthma. *J Allergy Clin Immunol* 2014; 134(5): 1175-1186 e1177.

22. Wang YH, Voo KS, Liu B, *et al.* A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J Exp Med* 2010; 207(11): 2479-2491.

23. Oryszczyn MP, Annesi-Maesano I, Charpin D, *et al.* Relationships of active and passive smoking to total IgE in adults of the Epidemiological Study of the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and Atopy (EGEA). *Am J Respir Crit Care Med* 2000; 161(4 Pt 1): 1241-1246.

24. Chang Y, Al-Alwan L, Alshakfa S, *et al.* Upregulation of IL-17A/F from human lung tissue explants with cigarette smoke exposure: implications for COPD. *Respir Res* 2014; 15: 145.

25. Newcomb DC, Peebles RS, Jr. Th17-mediated inflammation in asthma. *Curr Opin Immunol* 2013; 25(6): 755-760.

26. Alphonse MP, Saffar AS, Shan L, *et al.* Regulation of the high affinity IgE receptor (Fc epsilonRI) in human neutrophils: role of seasonal allergen exposure and Th-2 cytokines. *PLoS One* 2008; 3(4): e1921.

27. Manise M, Holtappels G, Van Crombruggen K, *et al.* Sputum IgE and cytokines in asthma: relationship with sputum cellular profile. *PLoS One* 2013; 8(3): e58388.

28. Redhu NS, Gounni AS. The high affinity IgE receptor (FcepsilonRI) expression and function in airway smooth muscle. *Pulm Pharmacol Ther* 2013; 26(1): 86-94.

29. Chakir J, Shannon J, Molet S, *et al.* Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003; 111(6): 1293-1298.

30. Sorbello V, Ciprandi G, Di Stefano A, *et al.* Nasal IL-17F is related to bronchial IL-17F/neutrophilia and exacerbations in stable atopic severe asthma. *Allergy* 2015; 70(2): 236-240.

31. Djukanovic R, Wilson JW, Britten KM, *et al.* Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am Rev Respir Dis* 1992; 145(3): 669-674.

32. Nguyen LT, Lim S, Oates T, *et al.* Increase in airway neutrophils after oral but not inhaled corticosteroid therapy in mild asthma. *Respir Med* 2005; 99(2): 200-207.

33. Brooks CR, Van Dalen CJ, Harding E, *et al.* Effects of treatment changes on asthma phenotype prevalence and airway neutrophil function. *BMC Pulm Med* 2017; 17(1): 169.

34. de Kluijver J, Schrumpf JA, Evertse CE, *et al.* Bronchial matrix and inflammation respond to inhaled steroids despite ongoing allergen exposure in asthma. *Clin Exp Allergy* 2005; 35(10): 1361-1369.

35. Foley SC, Hamid Q. Images in allergy and immunology: neutrophils in asthma. *J Allergy Clin Immunol* 2007; 119(5): 1282-1286.

36. Ward C, Pais M, Bish R, *et al.* Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. *Thorax* 2002; 57(4): 309-316.

37. Fukakusa M, Bergeron C, Tulic MK, *et al.* Oral corticosteroids decrease eosinophil and CC chemokine expression but increase neutrophil, IL-8, and IFN-gamma-inducible protein 10 expression in asthmatic airway mucosa. *J Allergy Clin Immunol* 2005; 115(2): 280-286.

38. Hauber HP, Gotfried M, Newman K, *et al.* Effect of HFA-flunisolide on peripheral lung inflammation in asthma. *J Allergy Clin Immunol* 2003; 112(1): 58-63.

39. Cowan DC, Cowan JO, Palmay R, *et al.* Effects of steroid therapy on inflammatory cell subtypes in asthma. *Thorax* 2010; 65(5): 384-390.

40. Vazquez-Tello A, Halwani R, Hamid Q, *et al.* Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J Clin Immunol* 2013; 33(2): 466-478.

41. Zijlstra GJ, Ten Hacken NH, Hoffmann RF, *et al.* Interleukin-17A induces glucocorticoid insensitivity in human bronchial epithelial cells. *Eur Respir J* 2012; 39(2): 439-445.

42. Jones CE, Chan K. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am J Respir Cell Mol Biol* 2002; 26(6): 748-753.

43. Allen JE, Sutherland TE, Ruckerl D. IL-17 and neutrophils: unexpected players in the type 2 immune response. *Curr Opin Immunol* 2015; 34: 99-106.

**Online supplementary material**

Supplementary text. Methods: patients, inflammatory phenotype definition, immunohistochemistry, histomorphometry and statistical analysis. Results: predictors of bronchial neutrophilia.

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

Table S2. Patients' inflammatory and biological parameters.

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

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

Table S5. Clinical and functional parameters high and intermediate neutrophilic asthma (smokers included).

Table S6. Inflammatory and biological parameters in high and intermediate neutrophilic asthma (smokers included).

Table S7. Predictors of bronchial neutrophilia ($\geq$47.17 cells/mm$^2$).

Figure S1. Markers of airflow obstruction in asthmatic patients of our cohort stratified by bronchial neutrophilia and disease severity (smokers included).

Figure S2. Forest plot indicating predictors of bronchial neutrophilia in asthmatic patients.

**Table 1.** Clinical and functional parameters of neutrophilic vs. non-neutrophilic asthma patients (smokers included).

| | Non Neutrophilic (<47.17 cells/mm$^2$) | Neutrophilic (≥47.17 cells/mm$^2$) | Difference between the medians (95% CI difference) | *P value* |
|---|---|---|---|---|
| N (%) | 32 (46) | 38 (54) | - | - |
| **Severe asthma cases, n (%)** | **11 (34)** | **23 (60)** | **-** | ***0.03*** |
| Age, y | 49 (45, 58) | 48 (44, 62) | -1 (-5, 9) | *0.64* |
| Sex, M/F | 18/14 | 16/22 | - | *0.34* |
| Asthma onset, y | 28 (10, 40) | 26 (14, 31) | -2 (-10, 7) | *0.81* |
| Late onset asthma (≥ 18 y), n (%) | 20 (62) | 24 (63) | - | *1.00* |
| Asthma duration, y | 19 (13, 30) | 23 (17, 34) | 3 (-4, 11) | *0.35* |
| Smokers (≥10 pack-year)*, n (%) | 11 (34) | 7 (18) | - | *0.17* |
| Atopy, n (%) | 19 (59) | 25 (66) | - | *0.62* |
| Serum IgE (KUI/L) | 108 (57, 163) | 132 (49, 187) | 24 (-54, 53) | *0.77* |
| Polisensitivity (>1 allergen), n (%) | 17 (53) | 21 (55) | - | *0.68* |
| Sensitization to perennial allergens, n (%) | 12 (37) | 16 (42) | - | *0.81* |
| Sensitization to Mycophyta, n (%) | 3 (9) | 6 (16) | - | *0.49* |
| Sinusitis, n (%) | 15 (50)** | 23 (60) | - | *0.62* |
| BMI, kg/m2 | 26.9 (24.8, 27.9) | 25.2 (24.1, 26.3) | -1.8 (-2.9, 1.1) | *0.35* |
| FEV1, % pred. | 82 (78, 91) | 77 (71, 83) | -5 (-13, 1) | *0.11* |
| **FVC, % pred.** | **98 (90, 102)** | **81 (55, 96)** | **-16 (-34, -1)** | ***0.03*** |
| **ΔFEV1 post β2 agonist (mL)** | **355 (260, 420)** | **245 (210, 300)** | **-110 (-150, -20)** | ***0.004*** |
| ΔFVC post β2 agonist (mL) | 280 (220, 350) | 290 (210, 430) | 10 (-60, 170) | *0.15* |
| RV, % pred. | 102 (97, 139) | 118 (104, 148) | 16 (-3, 24) | *0.11* |
| **FRC, % pred.** | **97 (87, 110)** | **107 (101, 122)** | **10 (1, 24)** | ***0.04*** |
| FEV1/FVC, % | 66 (59, 76) | 64 (57, 69) | -2 (-12, 1) | *0.08* |
| RV/TLC, % | 34 (32, 38) | 38 (34, 44) | 3 (-1, 8) | *0.06* |
| FeNO, ppb | 30 (21, 37) | 19 (15, 29) | -11 (-16, 3) | *0.14* |
| **Exacerbations per year** | **1 (0, 1)** | **1 (1, 2)** | **0 (0, 1)** | ***0.02*** |
| Frequent exacerbators (>1/year), n (%) | 9 (28) | 17 (45) | - | *0.21* |

| **ICS daily dose, µg** | **160 (100, 400)** | **360 (200, 640)** | **200 (40, 300)** | ***0.001*** |
|---|---|---|---|---|
| OCS, n (%) | 1 (3) | 7 (18) | - | *0.06* |
| Blood neutrophils, cells/mL | 3435 (2910, 3910) | 3480 (3080, 3810) | 45 (-560, 481) | *0.95* |
| Blood neutrophils, % | 54 (50, 59) | 50 (48, 53) | -3 (-7, 0) | *0.10* |
| Blood eosinophils, cells/mL | 250 (180, 350) | 200 (130, 370) | -50 (-110, 70) | *0.71* |
| Blood eosinophils, % | 3 (3, 5) | 3 (3, 5) | -1 (-1, 1) | *0.51* |

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. * Smokers are intended as current and ex-smokers with a smoking history ≥10 pack-years. **: information missing for 2 patients.

**Table 2.** Inflammatory and biological parameters of neutrophilic vs. non-neutrophilic asthma patients (smokers included).

| | 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 | 24 (19, 42) | 38 (24, 44) | 13 (-5, 16) | *0.25* |
| **NE$^+$ cells/mm$^2$ lamina propria** | **24 (19, 33)** | **94 (80, 113)** | **71 (57, 85)** | ***<0.0001*** |
| CD4$^+$ cells/mm$^2$ lamina propria | 19 (9, 28) | 28 (19, 35) | 9 (0, 19) | *0.07* |
| **CD8$^+$ cells/mm$^2$ lamina propria** | **13 (5, 24)** | **23 (14, 28)** | **10 (1, 15)** | ***0.02*** |
| CD68$^+$ cells/mm$^2$ lamina propria | 260 (159, 374) | 209 (163, 297) | -51 (-131, 71) | *0.55* |
| **Tryptase$^+$ cells/mm$^2$ lamina propria** | **74 (44, 130)** | **29 (14, 87)** | **-45 (-60, 0)** | ***0.04*** |
| IL-17A$^+$ cells/mm$^2$ lamina propria | 9 (5, 19) | 19 (11, 24) | 9 (0, 13) | *0.06* |
| **IL-17F$^+$ cells/mm$^2$ lamina propria** | **13 (8, 24)** | **21 (13, 33)** | **8 (1, 17)** | ***0.03*** |
| IL-21$^+$ cells/mm$^2$ lamina propria | 17 (9, 24) | 19 (12, 27) | 1 (-5, 9) | *0.57* |
| **IL-22$^+$ cells/mm$^2$ lamina propria** | **13 (9, 25)** | **19 (13, 32)** | **6 (0, 15)** | ***0.03*** |
| IL-23$^+$ cells/mm$^2$ lamina propria | 16 (9, 24) | 14 (9, 19) | -2 (-6, 7) | *0.72* |

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.

**Figure captions**

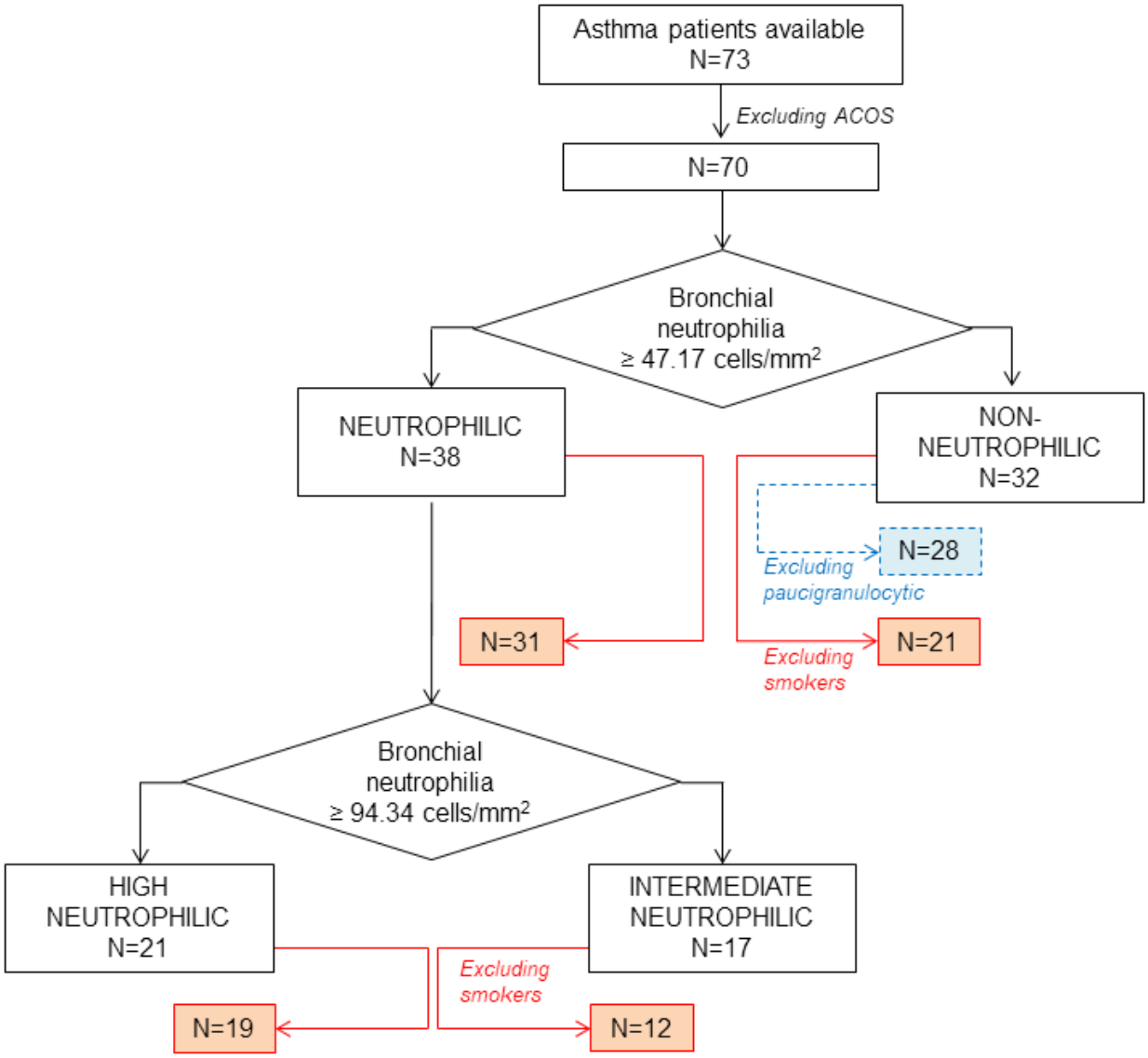


**Figure 1.** Study design.

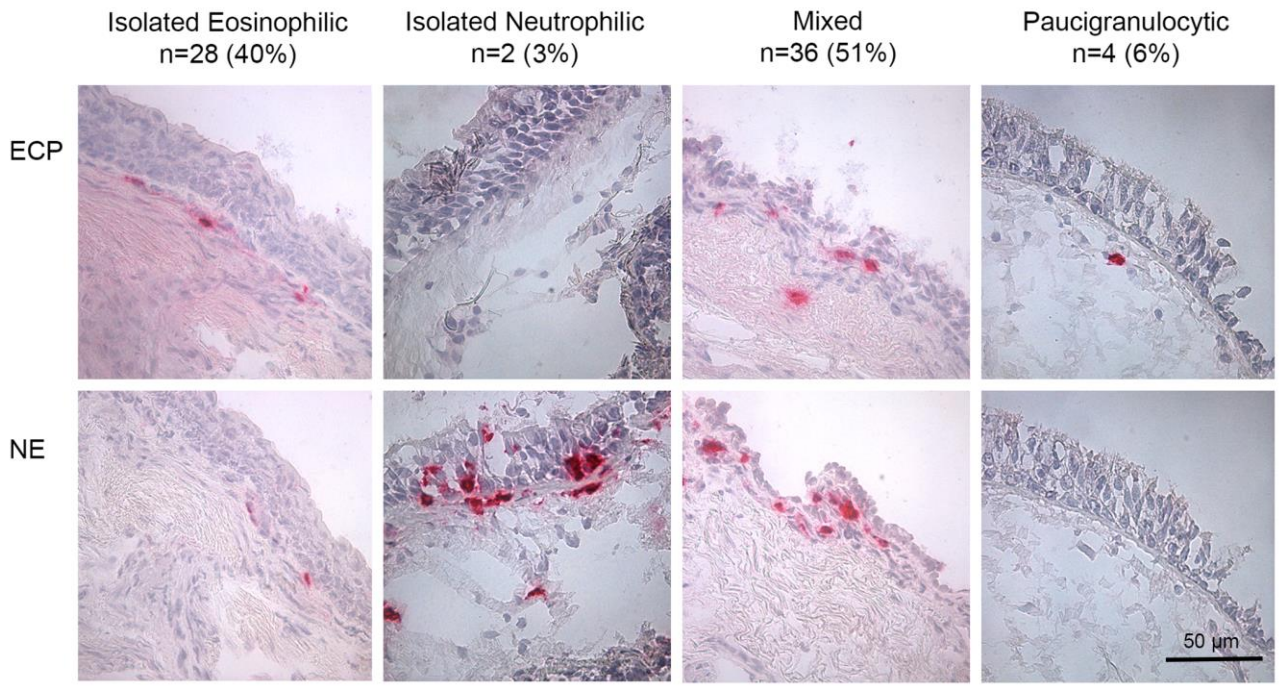


**Figure 2.** Distribution and representative images of the asthma inflammatory phenotypes studied, showing cells staining positive for eosinophil cationic protein (ECP, upper panels) and for neutrophil elastase (NE, lower panels).

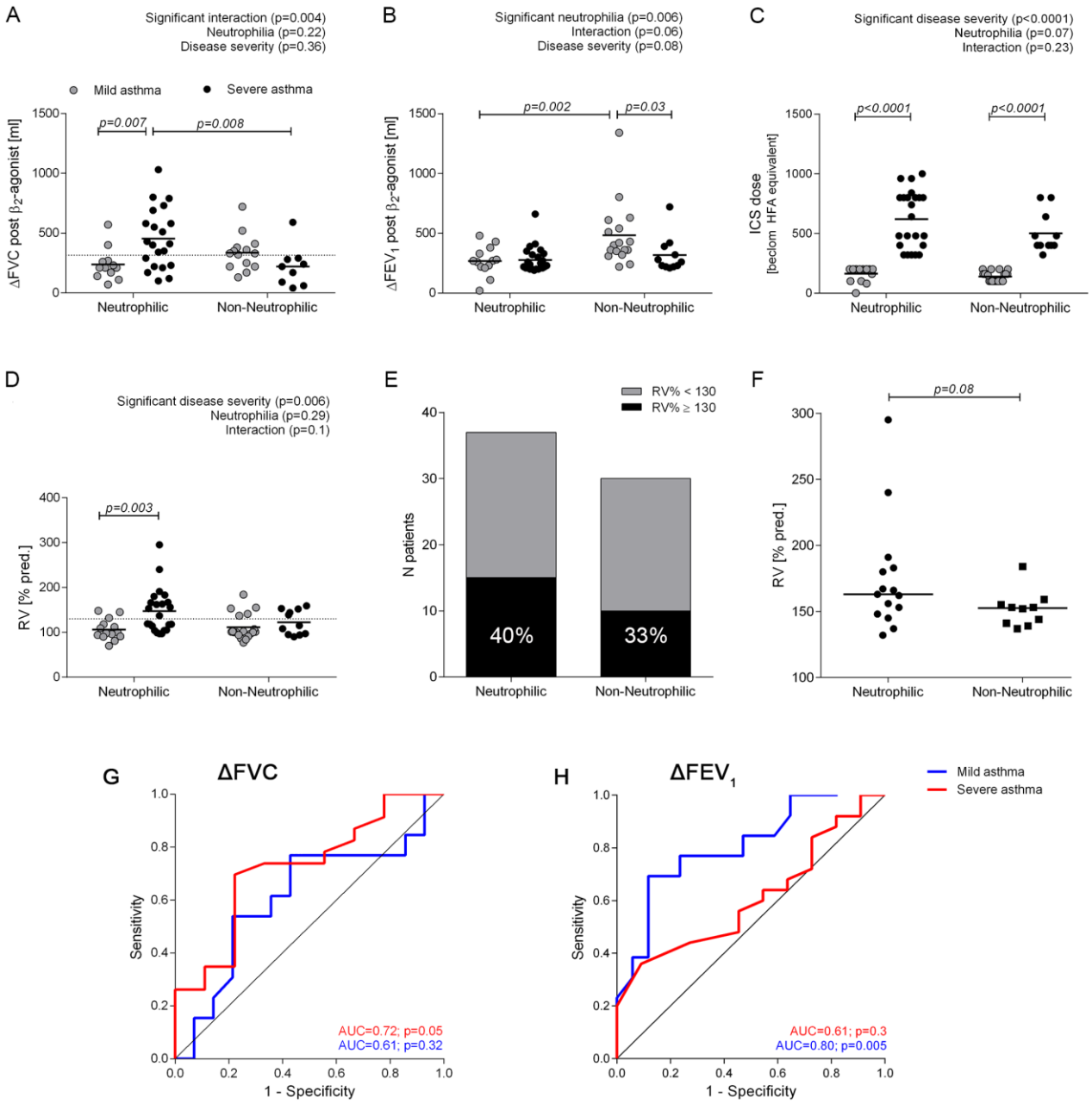


**Figure 3.** Effect of bronchial neutrophilia and disease severity on bronchodilator-induced ΔFVC (A) and $\Delta FEV_1$ (B), on ICS (C) and on basal RV% (D-F). Panel F: only patients with RV ≥ 130% (air-trappers) are included in the analysis. Panels G-H show the ROC curves for optimal cutoff points at which ΔFVC (G) and $\Delta FEV_1$ (H) discriminate mild (blue) or severe (red) asthmatic patients with bronchial neutrophilia. Smokers were included.

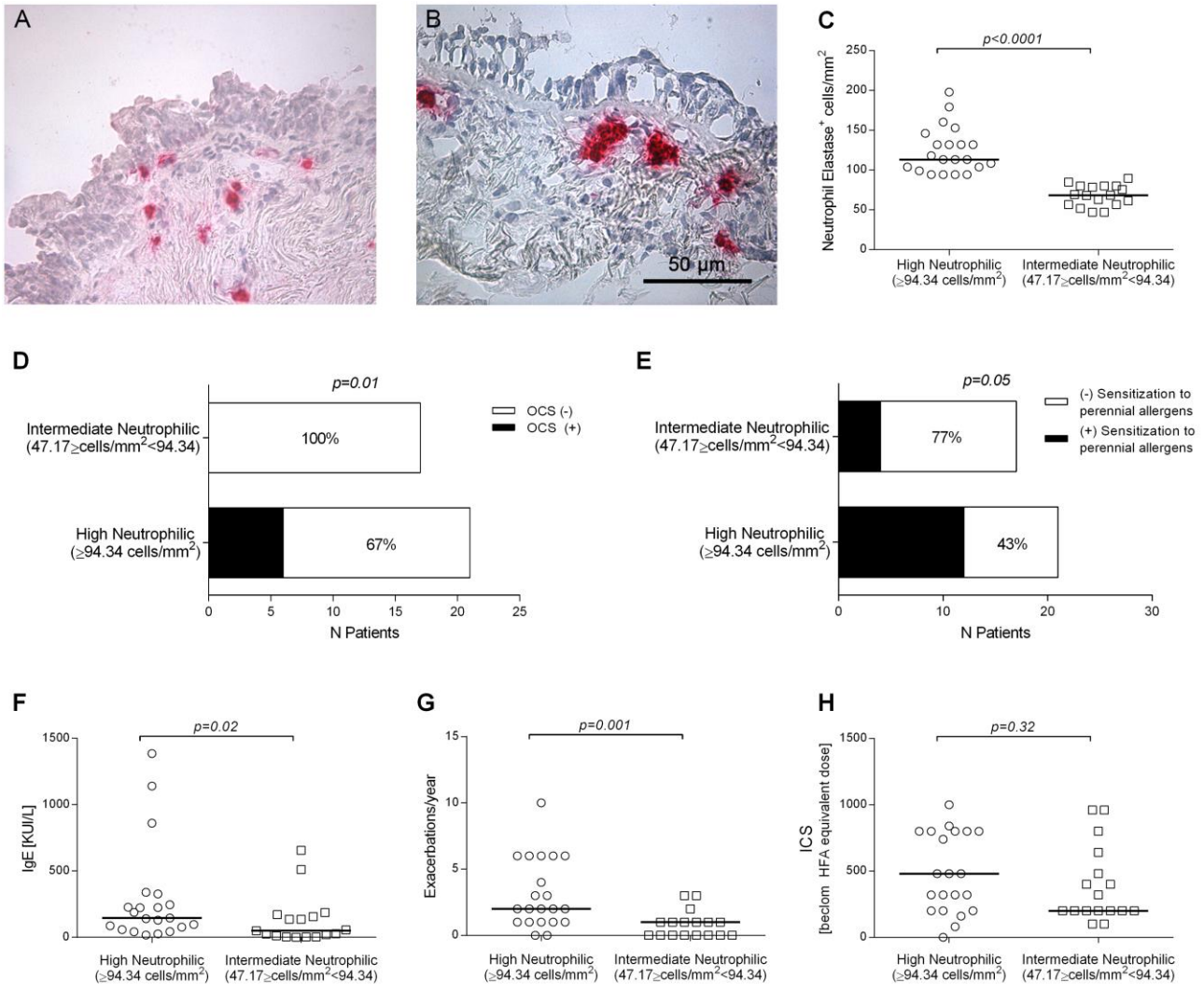


**Figure 4.** Representative images of high (A) and intermediate (B) bronchial neutrophilia, and relative counts (C). Panels D-H: Clinical parameters associated with high bronchial neutrophilia in asthma patients.

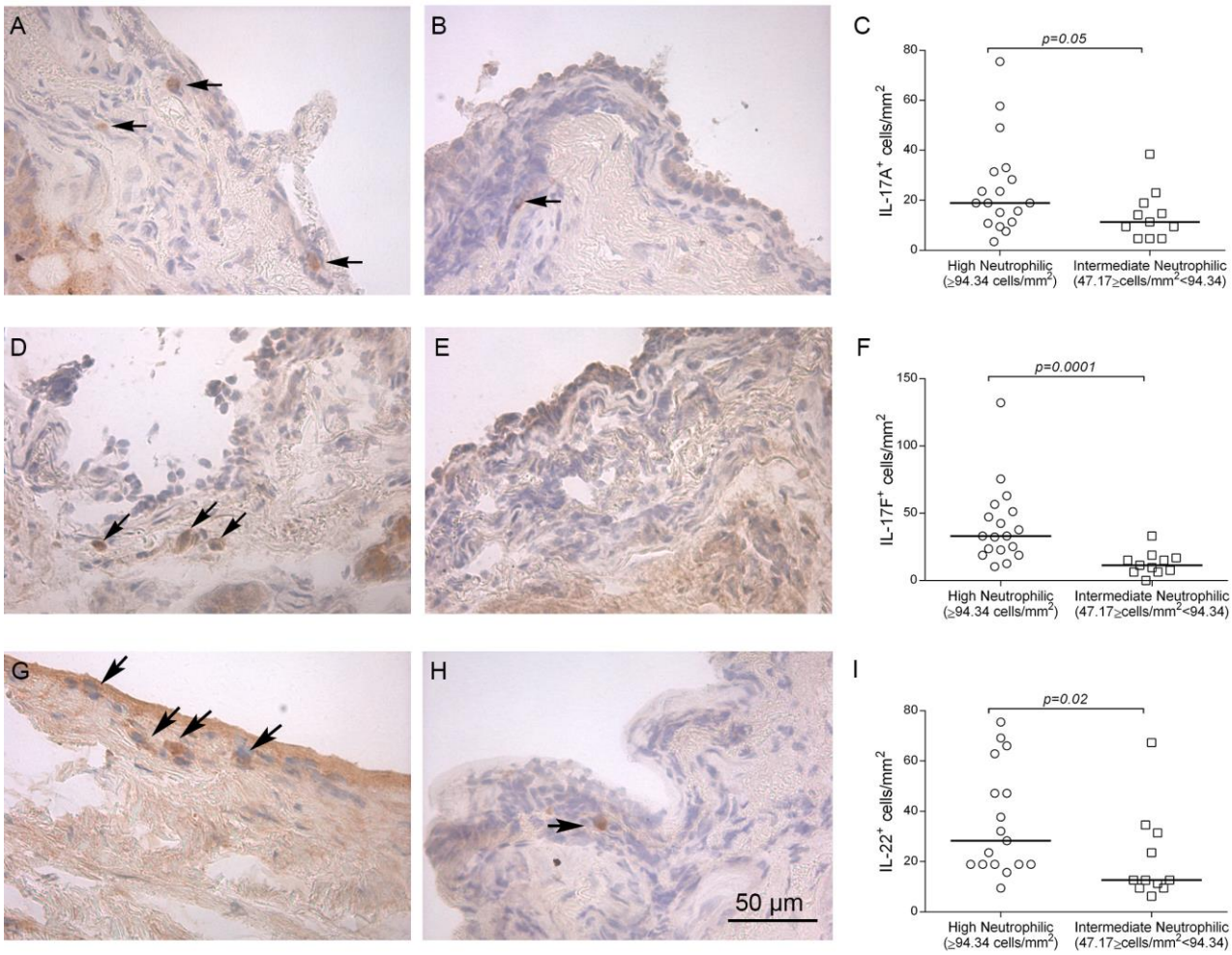


**Figure 5.** Th-17 signature in neutrophilic asthma (smokers excluded). Representative images and bronchial count values of asthmatics with high (A, D, G, J) and intermediate (B, E, H, K) neutrophilia, immunostained for CD4 (A-C), IL-17F (D-F), IL-17A (G-I) and IL-22 (J-L).

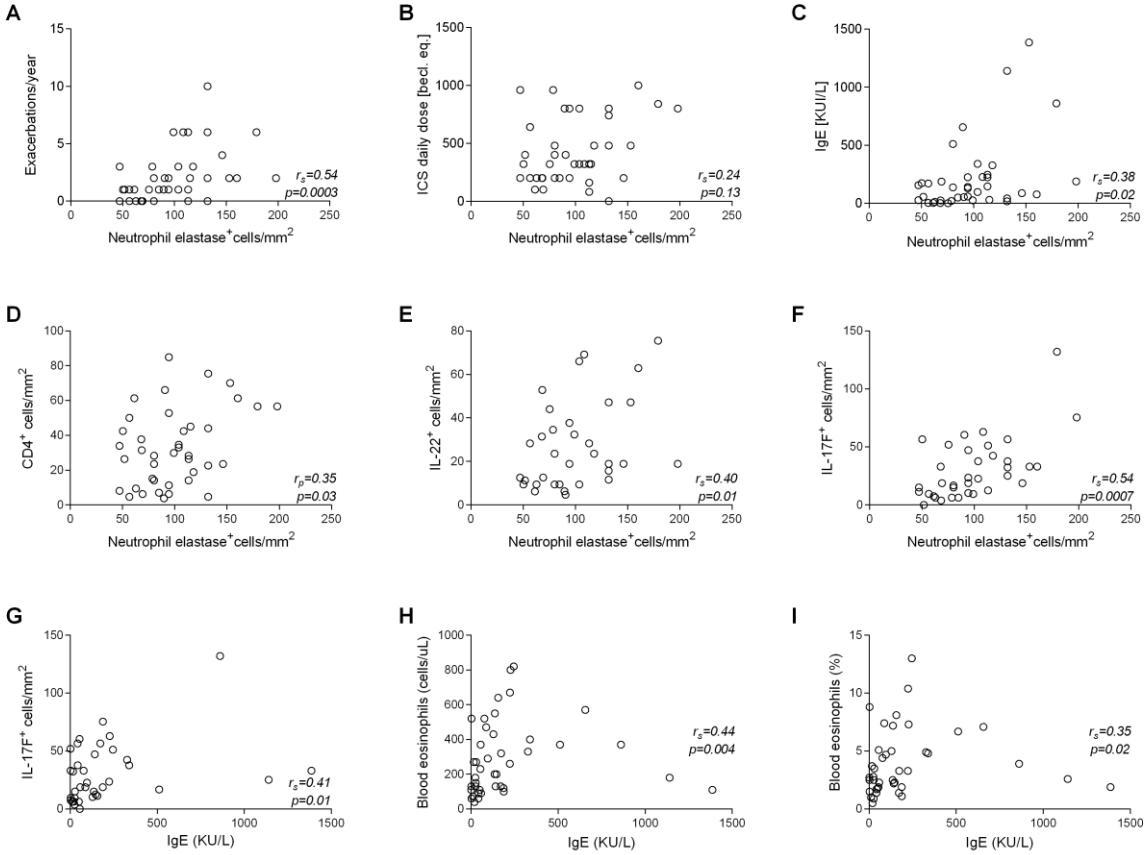


**Figure 6.** Neutrophilic patients only (n=41). Correlations between bronchial neutrophilia and number of exacerbations in the last year (A), ICS dose (B), serum IgE levels (C), $CD4^+$, (D) $IL\text{-}22^+$ (E), and $IL\text{-}17F^+$ (F) cells in bronchial tissue. Panels G-I show the correlations between serum IgE levels and $IL\text{-}17F^+$ cells and blood eosinophilia. $r_p$= Pearson correlation coefficient; $r_s$= Spearman correlation coefficient.

**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 bronchial biopsies of all except 3 patients included in a previous study [1] were also included in our analysis. Thus, the present study comprises 58 patients from the previous study and 12 additional 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 history were obtained at recruitment, when blood hematology and pulmonary function test with bronchoreversibility were also performed. Spirometry and lung volumes were assessed with a body plethysmograph (Vmax Encore 62, Carefusion, Germany) before and 15 minutes 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 positive prick test for ≥2 allergens. Fractional exhaled NO (FeNO) was measured as previously described [1]. Pulmonary function test 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 for the last 6

weeks) and in conformity with the local Ethics Committee Guidelines. Written informed consent was obtained from each subject. The study conformed to the Declaration of Helsinki and 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 paper 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 in 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*

Immunostaining was performed on 6 mm 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, United Kingdom) 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.

*Histomorphometry*

Histomorphometry was performed by a single operator blinded to the subject ID. Inter-observer agreement between the operator that performed the counts in the present study and the one that 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. Cellular 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 $\Delta FEV_1$ 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 $\Delta FEV_1$ 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

*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 $\Delta FEV_1$ 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 is reduced by almost 30-fold in patients with $\Delta FEV_1 > 280$ ml. Neutrophilia is even less likely to occur in patients with values of $\Delta FEV_1$ are > 390 ml. Overall, this means that the likelihood of bronchial neutrophilia increases with decreasing $\Delta FEV_1$. Of note, these results were obtained from patients with reversible asthma and need to be interpreted accordingly, as they might 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-year)[§], 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, 320)**** | **260 (200, 320)** | **360 (200, 640)** | **280 (150, 480)** | ***0.02*** |
| 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-year)[§], 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)** | **320 (200, 740)** | **160 (50, 340)** | ***0.0004*** |
| 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 the groups has lost statistical significance due to removing smokers. Blue fields: parameters for which the difference between the groups has gained statistical significance by excluding smokers 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 the groups has lost statistical significance due to removing smokers. Blue fields: parameters for which the difference between the groups has gained statistical significance by excluding smokers from the analysis.

**Table S5.** Clinical and functional parameters of asthmatics with high and 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-year)[§], 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 with high and 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* |
| Δ$FEV_1$ | | | *0.05* |
| Δ$FEV_1$ <220 ml | 1 (REF) | - | - |
| Δ$FEV_1$ 220-280 ml | 0.21 | 0.01; 1.81 | *0.18* |
| **Δ$FEV_1$ 280-390 ml** | **0.03** | **0.001; 0.418** | ***0.02*** |
| **Δ$FEV_1$ >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. Δ$FEV_1$: bronchodilator

induced change in $FEV_1$ expressed in ml. ΔFVC: bronchodilator induced change in FVC expressed in ml.

## Supplementary figures

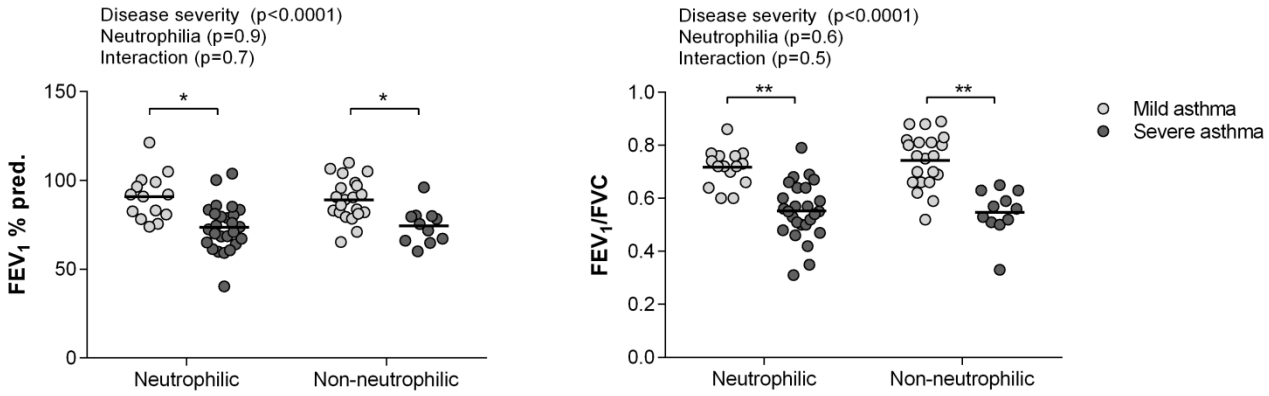


**Figure S1.** Markers of airflow obstruction in asthmatic patients of our cohort stratified by bronchial neutrophilia and disease severity.

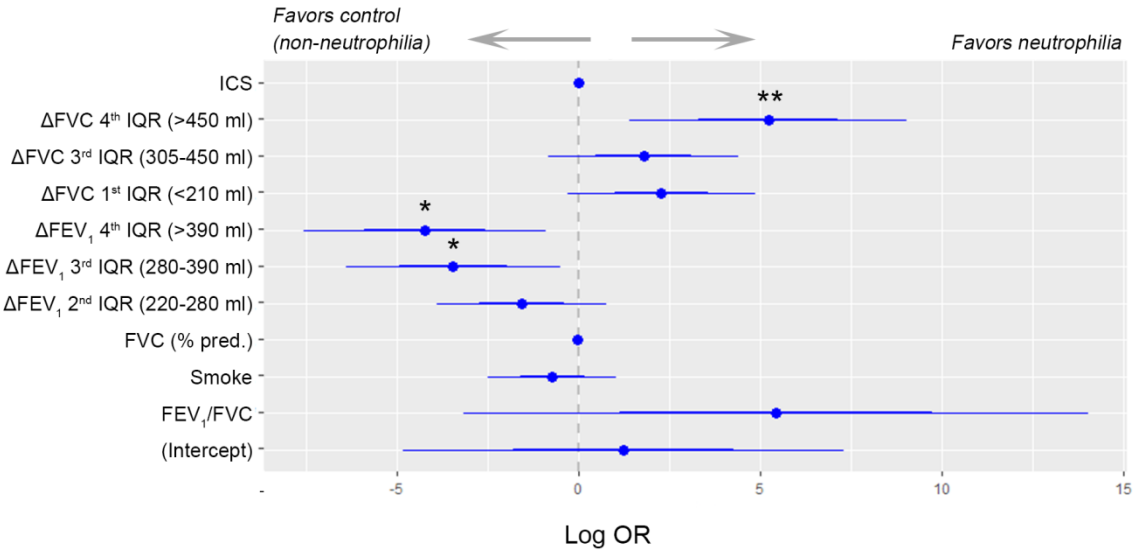


**Figure S2.** Forest plot indicating predictors of bronchial neutrophilia in asthmatic patients. IQR: interquartile interval range. OR: odds ratio. *: $p<0.05$. **: $p<0.01$.

**References**

1. Ricciardolo FLM, Sorbello V, Folino A, *et al.* Identification of IL-17F/frequent exacerbator endotype in asthma. *J Allergy Clin Immunol* 2017; 140(2): 395-406.
2. Chung KF, Wenzel SE, Brozek JL, *et al.* International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014; 43(2): 343-373.
3. GINA Guidelines (2018), Global Strategy for Asthma Management and Prevention. [cited 11/23/2017]; Available from:
4. Hancox RJ, Gray AR, Poulton R, *et al.* The Effect of Cigarette Smoking on Lung Function in Young Adults with Asthma. *Am J Respir Crit Care Med* 2016; 194(3): 276-284.
5. Tommola M, Ilmarinen P, Tuomisto LE, *et al.* Differences between asthma-COPD overlap syndrome and adult-onset asthma. *Eur Respir J* 2017; 49(5).
6. Bousquet J, Khaltaev N, Cruz AA, *et al.* Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008; 63 Suppl 86: 8-160.
7. Fokkens WJ, Lund VJ, Mullol J, *et al.* EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012; 50(1): 1-12.
8. Ricciardolo FL, Sabatini F, Sorbello V, *et al.* Expression of vascular remodelling markers in relation to bradykinin receptors in asthma and COPD. *Thorax* 2013; 68(9): 803-811.