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Exacerbation risk in severe asthma is stratified by inflammatory phenotype using longitudinal measures of sputum eosinophils

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Running Title: Airway inflammatory profiles in difficult asthma

Key words: Severe asthma, biomarkers, longitudinal study, asthma exacerbation

ABSTRACT

Background: Airway inflammatory phenotyping is increasingly applied to subjects with asthma. However its relationship to clinical outcomes in difficult asthma is incompletely elucidated.

Objective: The goal of our study was to determine the relationship between exacerbation rates and phenotypes of difficult asthma based on longitudinal measures of sputum eosinophils and neutrophils.

Methods: Subjects in the longitudinal observational study from 2 tertiary care centers that completed 1 year of observation and provided at least 3 sputum samples were classified by inflammatory phenotypes using previously established thresholds. Kaplan-Meier curves, univariable and multivariable Cox Proportional Hazards models were used to determine the association between inflammatory phenotypes and exacerbation rate.

Results: During the study, 115 exacerbations occurred in 73 severe asthmatic subjects. Subjects with the persistently eosinophilic phenotype had a significantly shorter time to first exacerbation and greater risk for exacerbation over a 1-year period than those with the non-eosinophilic phenotype based on univariable and multivariable Cox Proportional Hazards model (Hazard Ratio [HR], 3.24; 95% confidence interval [CI], 1.35-7.72; adjusted HR 3.90; 95% CI 1.34-11.36). No significant differences in time to first exacerbation or exacerbation risk over a 1-year period were found among the neutrophilic phenotypes.

Conclusions: The persistent eosinophilic phenotype is associated with increased exacerbation risk compared to the non-eosinophilic phenotype in severe asthma. No

differences in time to first exacerbation or exacerbation risk over a 1-year period were detected among neutrophilic phenotypes.

INTRODUCTION

Difficult asthma is a heterogeneous disease characterized by poor clinical control, frequent exacerbations and uncontrolled airway inflammation despite inhaled corticosteroids ¹.

Disease monitoring and management of asthma guided by inflammatory markers are promising strategies ² and sputum eosinophil measurement has recently been incorporated into Canadian guidelines for difficult asthma management ³. Eosinophilic inflammation is associated with impaired lung function and a recent meta-analysis suggests that treatment based on sputum eosinophil counts reduces exacerbation rates ². Given the socioeconomic costs of exacerbations of asthma the identification of subjects at risk is of paramount importance.

Most studies of sputum inflammatory cells in severe asthma have used cross-sectional analysis and several studies have found sputum eosinophils were significantly correlated with asthma severity ⁴, inversely correlated with asthma control ^{5,6} or associated with increased rate of exacerbation ⁷. Severe asthmatics have been found to have higher airway neutrophil levels than mild-to-moderate asthmatics ^{8,9}. However, it has been suggested that non-eosinophilic asthma represents a milder phenotype than eosinophilic asthma ¹⁰. A cross-sectional study of severe asthmatics by Wenzel et al ¹¹ found non-eosinophilic asthma associated with fewer exacerbations requiring intubation and normal subepithelial layer thickness compared to eosinophilic asthmatics.

Longitudinal studies of inflammatory biomarkers in severe asthma and their relationship to exacerbation rates and disease activity are lacking. While some studies have concluded that sputum inflammatory patterns are fairly stable and reproducible over time¹²⁻¹⁴, this has been challenged by more recent studies of severe asthma^{15,16}. In a previous longitudinal analysis of difficult asthmatics¹⁶, we observed large within-subject variations in sputum inflammatory cell percentages over a 1-year period suggesting that repeated measures of inflammatory cells over time would be necessary to establish reliable inflammatory phenotypes. Thus, we conducted a prospective study of severe asthmatics during the course of 1 year to determine relationships between inflammatory phenotypes based on longitudinal measures of airway biomarkers and exacerbation rates. We hypothesized that asthmatic subjects with persistently elevated eosinophils would have higher rates of exacerbation than non-eosinophilic asthmatics in our cohort. As neutrophils are potent sources of proteases and oxidant species, we hypothesized that subjects with persistent neutrophilia would have higher rates of exacerbation than those without neutrophilia.

METHODS

Study design

In this prospective study we recruited severe asthmatics between the ages of 18-80 years from 2 specialized respiratory clinics (Montreal Chest Institute of McGill University Health Centre and Sacré Coeur Hospital) over a 13-year period (2000-2013). Subjects were seen monthly for clinical visits consisting of focused history and physical examination, asthma control questionnaire (ACQ), spirometry, induced sputum cell counts and exhaled nitric oxide (F_ENO) measurements. Data from a subset of the subjects in this study has been included in previous publications¹⁶⁻¹⁸.

Subjects

Asthma was defined according to the American Thoracic Society (ATS) criteria¹⁹. Subjects were considered as having severe asthma if, on enrollment, they fulfilled 1 major criterion and 2 minor criteria as previously defined by the ATS workshop on difficult-to-treat asthma²⁰. Adherence to medications was assessed in both groups for 1 month prior to study enrollment using a compliance counter attached to a metered dose inhaler containing fluticasone (250µg/puff) and salmeterol (25µg/puff). If subjects took fewer than 70% of the prescribed doses of 2 inhalations twice a day they were excluded from the study. Subjects who had smoked more than 10 pack-years, were current smokers or who were known to have other pulmonary diseases, including chronic obstructive pulmonary disease (COPD) were excluded. Subjects that produced fewer than 3 sputum samples over 12 months were excluded from the study. The study was approved by institutional ethics committees. All subjects provided signed informed consent.

Exacerbations

Exacerbations during the study period were defined as a change in symptoms that required an addition of oral corticosteroid for at least 5 days for patients not on oral maintenance corticosteroid, or an increase in oral corticosteroid of at least 10 mg per day for a minimum of 5 days for steroid-dependent subjects, at the discretion of the subject's chest physician. Exacerbation rates were measured through visits to the asthma clinic or emergency department and through telephone calls over the 1-year study period in the event that the subject missed a scheduled appointment.

Measurements/Procedures

Symptom severity was graded according to the Juniper Asthma Control Questionnaire (ACQ)

²¹. Spirometry was performed according to ATS standards. Allergy skin prick tests with commercial extracts from common allergens were performed using the modified skin prick method and results were regarded as positive if the wheal was >3 mm. Subjects were regarded as atopic if they had 1 or more positive allergy tests. Fraction of exhaled nitric oxide (F_ENO) analysis was performed according to ATS guidelines ²². An expiratory flow rate between 0.315 L/s and 0.385 L/s was used during collection of exhaled gas. Sputum was induced using inhalation of increasing concentrations (3%, 4%, and 5%) of hypertonic saline over 7 minutes delivered by an ultrasonic nebulizer supervised by 1 of 2 research nurses as previously described by Pin *et al* ²³. Sputum was processed and examined at either Montreal Chest Institute or Sacré Coeur Hospital by 1 of 4 trained technicians as previously described by Pizzichini *et al.* ¹². The same sputum induction, F_ENO, and spirometry protocols were used throughout the course of the study.

Subjects were classified into 1 of 3 eosinophil phenotypes using a sputum eosinophil threshold of > 2% as previously defined by McGrath *et al.* ²⁴ and Hastie *et al* ²⁵. Persistent eosinophilia was defined as sputum eosinophils >2% on >90% of sputum analyses. Non-eosinophilic asthma was defined as sputum eosinophils ≤2% on > 90% of sputum analyses. Intermittent eosinophilia was defined as sputum eosinophils > 2% on ≥10% and ≤ 90% of sputum analyses.

Subjects were also classified into 1 of 3 neutrophil phenotypes using the neutrophil threshold above the upper limit of normal, > 61 % as previously defined by Simpson *et al* ²⁶. Persistent neutrophilia was defined by sputum neutrophils > 61% on > 90% of sputum analysis. Non-neutrophilic asthma was defined as sputum neutrophils ≤61% on > 90% of

sputum analysis. Intermittent neutrophilia was defined as sputum neutrophils $> 61\%$ on $\geq 10\%$ and $\leq 90\%$ of analyses.

Statistical analysis

The Shapiro-Wilk test was used to detect the presence of non-normality. For data with non-normal distributions, descriptive statistics were expressed as median and interquartile range (IQR), otherwise mean and standard error of the mean (SEM) were used to summarize the clinical characteristics and demographic data of the entire cohort and the eosinophilic and neutrophilic phenotypes. Differences in normally distributed continuous variables (age, pre-BD FEV₁(% predicted), and FEV₁/FVC,) and non-normally distributed continuous variables (age of onset, BMI, ACQ score, sputum neutrophils (%), sputum eosinophils (%), F_ENO, beclomethasone or equivalent dose) were compared between eosinophilic phenotypes and between neutrophilic phenotypes using one-way analysis of variance (ANOVA) and Kruskal-Wallis test, respectively. Differences in categorical and discrete variables (sex, atopic status, smoking history, exacerbation data, use of prednisone at any time over 1 year, use of omalizumab over 1 year, collection of F_ENO and sputum cells) between eosinophilic phenotypes and between neutrophilic phenotypes were tested using Fisher's exact test.

Kaplan-Meier (KM) curves were used to compare times to first exacerbation among eosinophilic phenotypes and neutrophilic phenotypes. Cox Proportional Hazards (CPH) regression for recurrent events using the Andersen-Gill model was used to calculate the hazard ratios (HRs) and confidence intervals (CIs). The Andersen-Gill model accounts for potential correlation between multiple exacerbations from the same subject. Age was split into 5 classes (20-34, 34-44, 45-54, and ≥ 65 years) and was adjusted for KM curves and Cox models using the direct adjustment method described by Therneau *et al*²⁷. Potential

confounders were sex, atopic status, baseline BMI, previous smoking history, and site of sputum analysis. Potential confounders were considered actual confounders if their inclusion in the multivariable model changed the unadjusted HR by $\geq 10\%$. Schoenfeld residuals and log-log plots were generated to confirm that the proportional hazards assumption was not violated. Statistical analysis using the R statistical software (version 2.15.2) was used for all analysis. KM curves and hazard models were performed using the *survival* package.

For additional analysis of exacerbation and sputum inflammatory cells without the use of predefined thresholds, regression models of count outcomes were fit. Due to inverse correlation between sputum eosinophils (%) and neutrophils (%), these predictor variables were modeled separately (**Supplemental Figure 1**). The dependent variable was the number of exacerbations over a 1-year period; the predictor variables were either median value of eosinophils (%) over 1-year period per subject or median value of neutrophils (%) over 1-year period per subject, and the significant confounder variable smoking history. Six different types of regression models were tested: Poisson (P), negative binomial (NB) using the *MASS* package, hurdle Poisson (HP), hurdle NB (HNB), zero-inflated Poisson (ZIP), and zero-inflated negative binomial (ZINB), using the *pscl* package. Models were compared using Vuong's non-nested hypothesis test and Aikake Information Criteria (AIC) using the *bbmle* package. The regression model with the lowest AIC value was selected for further analysis for eosinophils (%) and neutrophils (%) separately. A *p*-value of 0.05 was considered to indicate statistical significance, and 95 percent confidence intervals are provided when relevant.

RESULTS

Subject characteristics

One hundred and twenty five asthmatic subjects were enrolled and 73 severe asthmatics met study criteria and were followed for 1 year with repeated assessment of induced sputum at monthly intervals producing a median of 9.0 samples per subject (range 3-13, IQR 4).

Baseline characteristics are shown in **Table 1**.

Exacerbations

One hundred and fifteen exacerbations occurred over the 1-year study period. The median number of exacerbations over the 1-year period was 1 exacerbation per patient (IQR 2).

Twenty four of the 73 subjects did not have any exacerbations during the 1-year period.

Figure 1 shows the distribution of exacerbations per patient per month and the median sputum eosinophils (%) categorized by the eosinophilic phenotypes. **Figure 2** shows the distribution of exacerbations per patient per month and the median sputum neutrophils (%) categorized by the neutrophilic phenotypes.

Sputum samples

The median percentage of sputum eosinophils per sample was 3.25% (interquartile range [IQR] 12.2, **Table 1**). The percentage of all sputum samples with eosinophils >2% was 55.1%. The median percentage of sputum neutrophils per sample was 54.6% (IQR 56.6,

Table 1). The percentage of all sputum samples with neutrophils >61% was 41.6%. There were no significant differences in the number of sputum samples analyzed among eosinophilic phenotypes ($p=0.895$) or neutrophilic phenotypes ($p=0.682$) (**Table 2A Table 2B**). Unobtainable or missing sputum samples were 31.1% of total sputum samples versus 11.2% of total F_{ENO} samples that were missing for these subjects. No significant correlations were found between F_{ENO} and sputum eosinophils (Pearson $r = -0.05$) or sputum neutrophils (Pearson $r = 0.01$). The percentage of missed visits was 1.3%. There were no

significant differences in the percentage of missed visits among eosinophilic phenotypes ($p=0.721$) or neutrophilic phenotypes ($p=0.874$).

Clinical characteristics of eosinophilic and neutrophilic phenotypes

Eosinophilic phenotypes. No differences in age, asthma age of onset, sex, BMI, atopic status, or previous smoking history were detected among the 3 eosinophilic phenotypes (persistently eosinophilic [$n=11$], intermittently eosinophilic [$n=52$] and non-eosinophilic [$n=10$] phenotypes) (**Table 2A**). Persistently and intermittently eosinophilic subjects had a significantly higher ACQ score (2.0 vs 1.6 vs 1.4, $p<0.001$), $FEV_1(\%)$ (61.5 vs 67.8 vs 53.9, $p<0.001$) and $FEV_1/FVC(\%)$ (63.9 vs 67.3 vs 55.7, $p<0.001$) compared to non-eosinophilic subjects. Persistently eosinophilic subjects had the highest mean sputum eosinophil (%) levels and the lowest mean neutrophil (%) levels among the 3 groups. Mean levels of $F_{E}NO$ in intermittently eosinophilic subjects were significantly lower than in persistently and non-eosinophilic subjects (21.0 vs 26.7 vs 26.0 ppb, $p=0.003$). Non-eosinophilic subjects received significantly lower doses of inhaled corticosteroid than intermittently or persistently eosinophilic subjects (1000.0 vs 1500.0 vs 1600.0 $\mu\text{g/day}$ beclamethasone or equivalents, $p<0.001$). There were no differences in the proportion of subjects receiving prednisone or omalizumab in over the 1-year study period among the three groups.

Neutrophilic phenotypes No differences in age, asthma age of onset, sex, previous smoking history, BMI, atopic status, or ACQ scores were found among the 3 neutrophilic phenotypes (persistently neutrophilic [$n=6$], intermittently neutrophilic [$n=57$], and non-neutrophilic [$n=10$] phenotypes) (**Table 2B**). Persistently neutrophilic subjects had the lowest pre-bronchodilator $FEV_1(\%)$ (51.2 vs 65.6 vs 69.1, $p<0.001$) and $FEV_1/FVC(\%)$ (55.9 vs 66.2 vs 66.8, $p<0.001$) and the highest mean sputum neutrophil (%) level and the lowest mean sputum eosinophil (%) level among the 3 groups. Non-neutrophilic subjects had the lowest $F_{E}NO$ levels (16.6 vs 32.7 vs 30.7 ppb, $p=0.005$). There were no differences between the 3

groups in dosages of inhaled corticosteroid or proportion of patients receiving prednisone or omalizumab over the 1-year study period.

Relationship between rate of exacerbation and inflammatory phenotype

Eosinophilic phenotypes The persistently eosinophilic phenotype was associated with increased risk of exacerbation over the 1-year study period compared to the non-eosinophilic phenotype using Fisher's exact test (2.0 vs 1.0 vs 0.5 exacerbations per subject, $p=0.001$) and using univariable and multivariable Cox Proportional Hazards (Andersen-Gill) model. The unadjusted Hazard Ratio (HR) for exacerbation risk over a 1-year period calculated by the Andersen-Gill model was 3.24 (95% Confidence Interval [CI] 1.35-7.72) for persistently eosinophilic subjects. For intermittently eosinophilic subjects, no significant difference in risk compared to non-eosinophilic subjects was detected using the Andersen-Gill model (HR 1.65 [95% CI 0.73-3.71]). (**Table 3**). **Figure 3** presents the age adjusted Kaplan-Meier (KM) curves for the time to first exacerbation stratified by eosinophil phenotype. As can be seen by the KM curves, the relationship between time to first exacerbation and eosinophil status was significant ($p=0.023$), with persistently eosinophilic subjects having the earliest time to first exacerbation. Never smokers were found to have increased risk of exacerbation versus subjects with a history of smoking (HR 2.08; 95% CI 1.28-3.41) based on the univariable Cox Proportional Hazards model (**Table 3**). The significant association between increased exacerbation risk and persistent eosinophilia was preserved after adjustment for the confounding variables previous smoking history and direct age adjustment of the multivariable model (adjusted HR 3.90, 95% CI 1.34-11.36) (**Table 4A**). Patient sex, atopic status, BMI, and site of sputum analysis were not found to be important confounding variables. The intermittently eosinophilic phenotype was not found to have significantly higher risk of exacerbation compared to non-eosinophilic subjects based on the multivariable model.

Neutrophilic phenotypes Significant differences in the proportion of exacerbations between the 3 neutrophilic phenotypes were found using Fisher's exact test ($p = 0.012$, **Table 2B**).

Univariable analysis found the persistently neutrophilic phenotype had a lower rate of exacerbation compared to the non-neutrophilic phenotype (HR 0.36; 95% CI 0.15-0.88) (**Table 3**), however this was not preserved after adjustment for the confounding variables (previous smoking history and direct age adjustment) using multivariable Cox Proportional Hazard models (adjusted HR 0.46; 95% CI 0.18-1.23) (**Table 4B**). Kaplan-Meier curves found no significant differences in time to first exacerbation between the neutrophilic phenotypes ($p = 0.785$) (**Figure 4**). Patient sex, atopic status, BMI, and site of sputum analysis were not found to be important confounding variables.

Regression models for number of exacerbations.

Six regression count models were fit and evaluated: Poisson (P) and negative binomial (NB) as well as their extensions using Zero-inflated (ZIP and ZINB) and Hurdle models (HP and HNB). Hurdle Poisson model was top-ranked for modeling exacerbation counts for eosinophils and for neutrophils based on minimizing Akaike's Information Criteria (**Supplemental Table 2A,B and 3A,B**).

Hurdle Poisson model using sputum eosinophils (%) as predictor variable.

The binary component of the Hurdle Poisson model found that one unit increase in sputum eosinophils (%) increased the odds ratio (OR) of having 1 or more exacerbations by 1.12 (95% CI, 1.02-1.23, $p=0.041$) (**Table 5A**). Smoking history was non-significant (OR 1.59 [95% CI, 0.64- 3.98]). For the truncated count component of the Hurdle Poisson model, among those who have 1 or more exacerbations, exacerbation relative risk (RR) was increased by 2.8 (95% CI 1.50 - 5.18) for never-smokers. There was no significant relationship between RR of exacerbation and eosinophils (%) among subjects with more than 1 exacerbation.

Hurdle Poisson model using sputum neutrophils (%) as predictor

For both the binary and count components of the Hurdle Poisson model, no significant relationship between exacerbations and neutrophils (%) was detected. The truncated count component found exacerbation RR increased by 2.74 (95% CI, 1.48-5.09) for non-smokers with 1 or more exacerbations (**Table 5B**).

Comparison of inflammatory profiles between ex-smokers and never smokers

No differences in the proportion of ex-smokers were detected between neutrophilic or eosinophilic phenotypes (**Table 2A and B**), nor between the median percentages of sputum eosinophils or neutrophils (**Supplemental table 1**).

DISCUSSION

We conducted a prospective study of severe asthmatics followed for a 1-year period at 2 tertiary care centers to determine whether inflammatory phenotypes based on longitudinal measures of biomarkers could be used to stratify exacerbation rates. To classify subjects based on robust inflammatory phenotypes, we collected monthly measures of sputum inflammatory cells in addition to $F_{E}NO$ and the standard spirometric and clinical data. A recent study of difficult asthmatics has concluded that repeated measures of biomarkers are required to obtain dependable phenotypes¹⁵. Indeed, results of our previous longitudinal study¹⁶ and those of McGrath *et al*²⁴ have also demonstrated a significant variability in sputum biomarker longitudinally, resulting in a significant proportion of subjects shifting groups when relying on cross-sectional classification of phenotypes.

The original finding of this study is that longitudinal measures of sputum eosinophils but not neutrophils can be used to stratify time to first exacerbation and risk of asthma exacerbation over a 1-year period in severe asthma. Persistent eosinophilic asthmatics were

found to have higher rates of exacerbation over a 1-year period compared to non-eosinophilic asthmatics based on univariable and multivariable Cox Proportional Hazards models. Our prospective observational study directly examined the relationships between subjects classified by longitudinal airway biomarkers and exacerbation rate as we sought to determine the “natural course” of asthma outcomes by measuring airway biomarkers in response to the traditional evidence-based titration of therapy using clinical parameters (spirometry, clinical findings and ACQ scores)²⁸. We did not include pre-specified therapeutic interventions based on biomarkers, in contrast to the “sputum management” approach that used sputum eosinophil thresholds to guide inhaled corticosteroid dosage², as applied in a recent study by Newby *et al*²⁹ which found no differences in rate of exacerbation between 3 eosinophilic phenotypes of severe asthma.

In another longitudinal analysis of difficult asthma, Kupczyk *et al*⁷ characterized frequent exacerbators among severe and moderate asthmatics and found that the majority of frequent exacerbators had either high eosinophils and low neutrophils or high eosinophils and high neutrophils based on a single sputum analysis. While the median rate of exacerbation in our cohort was very similar to the severe asthmatics in their study, there were some differences. Their study found smoking history was the only factor significantly associated with frequent exacerbation (3 or more exacerbations per year). Our analysis found that subjects with no smoking history had higher risk of exacerbation compared to ex-smokers, however this was no longer significant after adjusting for age and eosinophilic phenotype. Several previous studies have found smoking history associated with sputum inflammatory profiles in asthma, with some studies finding ex-smokers having fewer sputum eosinophils than never smokers^{30,31}, whereas a cross-sectional study of severe asthma comparing ex-smokers with never smokers found higher neutrophils but no differences in eosinophils³². We did not detect any differences in the inflammatory profiles of our severe asthma cohort

based on smoking history suggesting that other factors may be driving differences in exacerbation risk between these two groups. There have been several studies identifying a non-eosinophilic subgroup of asthmatics that are hypothesized to be a distinct asthma phenotype^{8,13,24} possibly driven by innate immune activation³³. In comparison to eosinophilic subjects in our cohort, the non-eosinophilic subjects had a lower FEV₁/FEV(%) and FEV₁(%). Wenzel et al¹¹ found FEV₁(%) marginally lower in non-eosinophilic versus eosinophilic subjects in a cross-section analysis of severe asthma. Sputum neutrophils in our severe asthmatic cohort, with the notable exception of the non-neutrophilic phenotype, were higher than normal subjects³⁴. The non-neutrophilic phenotype had the lowest median sputum neutrophil percentage and the highest median eosinophil percentage among the neutrophilic phenotypes. This finding reflects the negative correlation between proportions of sputum neutrophils and eosinophils, a relationship noted by previous investigators³⁵. It remains unclear whether this represents technical artifact since cells are expressed as percentages and therefore reciprocal relationships may be expected or a biological association between these inflammatory cells. We did not have sputum inflammatory cell count data available and therefore could not compare use of cell percentages with cell counts.

While no differences in exacerbation rate were detected among the neutrophilic phenotypes, non-eosinophilic asthmatics were found to have the lowest rates of exacerbation based on the univariable and multivariable model suggesting that non-eosinophilic asthma as defined in this study may signify a distinct pathobiological subtype of difficult asthma. It remains a possibility that subjects classified as non-eosinophilic in our study were previously eosinophilic asthmatics that became non-eosinophilic due to greater responsiveness to inhaled corticosteroid therapy compared to subjects classified as persistently eosinophilic in our study. Indeed, persistently eosinophilic asthmatics received a significantly larger dose of inhaled corticosteroid than non-eosinophilic subjects over the 1-year study period.

Persistence of airway eosinophilia despite inhaled corticosteroids has been observed in previous studies and has been explained by a variety of hypotheses including under-treatment with inhaled corticosteroids, glucocorticoid resistance of T lymphocytes^{36,37}, inaccessibility of inhaled steroid “particles” to small airways and poor technique or adherence. The latter seems an unlikely explanation in our study because, although adherence was not “strictly” measured during the study period, we did objectively monitor compliance for a 1-month period prior to enrollment into the study and excluded subjects that were non-compliant. Furthermore, poor compliance has been observed across all levels of severity¹ and is not specific to severe asthmatics.

Induced sputum analysis was chosen over endobronchial biopsy in this study for several reasons. It is technically more difficult to perform and analyze repeated biopsies than induced sputum. Furthermore, induced sputum is felt to be a better representation of inflammation within the airway as it samples both the large and small airways, unlike endobronchial biopsies, which only sample a small portion of tissue from the proximal airways³⁸. While sputum eosinophils were found to have a better correlation to asthma clinical control than airway wall tissue eosinophil counts in a cross-sectional analysis³⁸, we cannot exclude the possibility that longitudinal examination of tissue from proximal airways has greater association with asthma exacerbation. Induced sputum collection for airway inflammatory cells was technically more difficult to collect than F_ENO in our study which was reflected in the higher proportion of missing sputum samples compared to F_ENO.

We found no clear trends between median levels of F_ENO and sputum eosinophilia or neutrophilia. Several studies have found a dissociation between changes in sputum eosinophils and F_ENO and an inhaled corticosteroid dose-response relationship for F_ENO levels but not for sputum eosinophils³⁸⁻⁴⁰. This has lead some researchers to conclude that F_ENO levels do not necessarily reflect airway eosinophilia in severe steroid-dependent

asthma^{2,41,42}. Rather, it is hypothesized that iNOS from airway epithelium (the dominant source of exhaled NO⁴³) is effectively suppressed in response to corticosteroid whereas eosinophils and other aspects of airway dysfunction are incompletely treated⁴¹.

This study examined phenotyping based on longitudinal measures of either sputum eosinophils or neutrophils using cutpoint criteria from the literature. While potential limitations related to the use of cutpoints have been described⁴⁴, we chose to apply cutpoints derived from previous studies as they provide easy interpretation for clinicians and potential for use as clinical prediction rules. Our findings were supported by regression modeling of the number of exacerbations and sputum inflammatory cells which demonstrated increased odds of 1 or more exacerbation(s) with increasing sputum eosinophils (%) but not sputum neutrophils (%). It will be necessary to further validate the application of this cutpoint-based risk stratification method with another cohort of severe asthmatics in the future. Whether classification of asthma phenotypes as applied here results in more effective therapeutic management for exacerbation risk reduction in severe asthmatics will also be worth investigating.

The strengths of our study are its prospective design with careful patient selection and frequent clinical visits and measurements of airway biomarkers over a 1-year period in a well characterized cohort of severe asthmatics. This timeframe may not be clinically practical, however it serves as a “gold standard”¹³ with which future investigations using fewer visits and measurements may be compared.

In summary, longitudinal measures of sputum eosinophils to classify severe asthmatics into eosinophilic phenotypes enabled stratification of time to first exacerbation using Kaplan-Meier curves and exacerbation risk over a 1-year period based on univariable and multivariable Cox Proportional Hazards model. Future studies to delineate the molecular pathobiology of eosinophil phenotypes and therapeutic trials applying classification of

eosinophil phenotypes using longitudinal measures of eosinophils in severe asthma are warranted.

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Statement of interest

No authors have any conflicts of interest to declare

FIGURE CAPTIONS

Figure 1 Median sputum eosinophils by monthly visit categorized by eosinophil phenotype (top). Distribution of exacerbations by monthly visit categorized by eosinophil phenotype (bottom).

Figure 2 Median sputum neutrophils by monthly visit categorized by neutrophil phenotype (top). Distribution of exacerbations by monthly visit categorized by neutrophil phenotype (bottom).

Figure 3. Age adjusted Kaplan-Meier analysis for time to first exacerbation by eosinophilic phenotypes.

Figure 4 Age adjusted Kaplan-Meier analysis for time to first exacerbation by neutrophilic phenotypes.

Supplemental Figure 1: Inverse relationship between sputum neutrophils (%) and sputum eosinophils (%). *N* = 654 sputum samples collected over a 1-year period from 73 severe asthmatics.

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Table 1. Demographic data and clinical characteristics of the severe asthma cohort [mean values and standard error of the mean (SEM), unless stated differently].

[§] Number of exacerbations and median number and interquartile range (IQR) of exacerbations per subject in each group over the 1-year study period.

	Severe asthma cohort
Number of subjects (<i>n</i>)	73
Number of exacerbations [<i>n</i> , median (IQR)] [§]	115 [1 (2)]
Age (years)	49.7 (1.5)
Age of asthma onset (years) [median (IQR)]	20 (32)
Women (% , <i>n</i>)	43.8 (32)
BMI	28.3 (5.7)
Ex-smoker (% , <i>n</i>)	31.5 (23)
Atopy (% , <i>n</i>)	75.3 (55)
Pre-BD FEV ₁ (% predicted)	60.5 (26.5)
FEV ₁ /FVC (%)	65.0 (1.5)
ACQ score	2.0 (0.1)
Induced sputum and F_ENO	
Number of sputum samples per patient [median(IQR)]	9.0 (4.0)
Sputum neutrophils (%) [median (IQR)]	54.6 (56.6)
Sputum eosinophils (%) [median (IQR)]	3.25 (12.2)
Number of F _E NO samples per patient [median(IQR)]	12 (2)
F _E NO (ppb) [median (IQR)]	16.6 (32.3)
Asthma treatment	
Beclomethasone or equivalent dose (µg/day), [median (IQR)]	1200.0 (600.0)
Prednisone use at baseline (% subjects, <i>n</i>)	32.5 (38)
Omalizumab use at baseline (% subjects, <i>n</i>)	0 (0)

Table 2A – Clinical characteristics among eosinophilic phenotypes (mean values and standard error of the mean [SEM], unless stated differently).

§ Number of exacerbations and median number and interquartile range (IQR) of exacerbations per subject in each group over the 1-year study period.

	Non-eosinophilic	Intermittently eosinophilic	Persistently eosinophilic	p-value
Clinical parameters				
Subjects (<i>n</i>)	10	52	11	
Age (years)	48.1(1.3)	50.4(0.5)	48.2(0.9)	0.802
Age of onset (years) [median (IQR)]	5 (11.0)	21 (30.5)	30 (42.0)	0.126
Women (% , <i>n</i>)	30.0 (3)	46.2 (24)	45.5 (5)	0.703
Ex-smoker (% , <i>n</i>)	30.0 (3)	34.6 (18)	18.2 (2)	0.621
Atopy (% , <i>n</i>)	90.0 (9)	73.1 (38)	72.7 (8)	0.292
BMI [median (IQR)]	28.7 (3.6)	28.4 (5.7)	25.9 (9.1)	0.642
Pre-BD FEV ₁ (% predicted)	53.9 (1.5)	67.8 (0.8)	61.5 (1.5)	< 0.001
FEV ₁ /FVC (%)	57.7 (1.0)	67.3 (0.5)	63.9 (0.7)	< 0.001
ACQ score [median (IQR)]	1.4 (1.0)	1.6 (1.4)	2.0 (1.4)	< 0.001
Number of exacerbations [<i>n</i> , median (IQR)] §	9 [0.5(1.75)]	76 [1.0(2.0)]	30 [2.0(3.0)]	0.001
Induced sputum and F_ENO				
Number of sputum samples per patient [median (IQR)]	10.0 (2.0)	9.0(3.0)	8.0(5.5)	0.895
Sputum neutrophils (%) [median (IQR)]	71.6 (41.3)	53.5 (53.0)	27.9 (41.7)	< 0.001
Sputum eosinophils (%) [median (IQR)]	0.0(1.0)	3.0(7.7)	21.7(36.0)	< 0.001
Number of F _E NO samples per patient [median (IQR)]	12.5 (1.0)	12.0 (2.3)	12.0 (2.0)	0.880
F _E NO (ppb) [median (IQR)]	26.0 (44.4)	21.0 (27.0)	26.7 (33.6)	0.003
Asthma treatment				
Beclomethasone or equivalent dose (µg/day),[median(IQR)]	1000.0 (400.0)	1500.0 (800.0)	1600.0 (800.0)	< 0.001
Prednisone any time in 1 year (% subjects, <i>n</i>)	30.0 (3)	50.0 (26)	81.8 (9)	0.06
Omalizumab any time in 1 year (% subjects, <i>n</i>)	10.0 (1)	11.5 (6)	9.1 (1)	1

Table 2B – Clinical characteristics among neutrophilic phenotypes [mean values and standard error of the mean [SEM], unless stated differently).

§ Number of exacerbations and median number and interquartile range (IQR) of exacerbations per subject in each group over the 1-year study period.

	Non-neutrophilic	Intermittently neutrophilic	Persistently neutrophilic	p-value
Clinical parameters				
Subjects (<i>n</i>)	10	57	6	
Age (years)	50.0 (1.1)	48.7 (0.5)	59.5 (0.8)	0.147
Age of onset (years) [median (IQR)]	34.0 (26.0)	16.0 (28.0)	24.5 (34.0)	0.050
Women (% , <i>n</i>)	60.0 (6)	40.4 (23)	50.0 (3)	0.493
Ex-smoker (% , <i>n</i>)	40.0 (4)	28.1 (16)	50.0 (3)	0.434
Atopy (% , <i>n</i>)	50.0 (5)	77.2 (44)	100.0 (6)	0.120
BMI [median (IQR)]	26.3(5.9)	28.7(5.5)	26.2(15.0)	0.632
Pre-BD FEV ₁ (% predicted)	69.1(2.1)	65.6(0.7)	51.2(2.6)	< 0.001
FEV ₁ /FVC (%)	66.8 (1.1)	66.2 (0.5)	55.9 (1.7)	< 0.001
ACQ score [median (IQR)]	2.0 (1.6)	1.6(1.4)	1.9(1.0)	0.090
Number of exacerbations [<i>n</i> , median (IQR)] [§]	26 [2.0 (3.0)]	83 [1.0 (2.0)]	6 [1.0 (1.5)]	0.012
Induced sputum and F_ENO				
Number of sputum samples per patient [median (IQR)]	10.0 (5.3)	9.0 (3.0)	10.5 (2.5)	0.682
Sputum neutrophils (%) [median (IQR)]	18.3 (21.2)	53.0 (49.2)	83.3 (14.5)	<0.001
Sputum eosinophils (%) [median (IQR)]	19.0 (44.8)	2.5 (7.4)	2.0 (5.1)	<0.001
Number of F _E NO samples per patient [median (IQR)]	13.0 (1.8)	12.0 (3.0)	13.0 (0.0)	0.858
F _E NO (ppb) [median (IQR)]	30.7(2.5)	32.7(1.1)	16.6 (4.3)	0.005
Asthma treatment				
Beclomethasone or equivalent dose (µg/day), [median (IQR)]	1000.0 (800.0)	1400.0 (800.0)	1400.0 (1000.0)	0.014
Prednisone any time in 1 year (% subjects, <i>n</i>)	80.0 (8)	45.6 (26)	66.7 (4)	0.088
Omalizumab any time in 1 year (% subjects, <i>n</i>)	0 (0)	35.0 (8)	0 (0)	0.64

Table 3 – Univariable Cox Proportional Hazards (Andersen-Gill) Models to determine risk factors for exacerbation among severe asthmatics. The number of subjects (*n*) in each age category is shown.

Factor	Severe asthmatics HR (95% CI)
Age	
20-34 yr (<i>n</i> =10)	1.00(reference)
35-44 yr (<i>n</i> = 15)	2.99(1.01-8.28)
45-54 yr (<i>n</i> = 17)	2.22(0.83-5.91)
55-64 yr (<i>n</i> = 24)	1.87(0.70-5.01)
≥65 yr (<i>n</i> = 7)	1.25(0.37-4.25)
Sex	
Female	1.00(reference)
Male	1.05(0.63-1.76)
Smoking history	
Ex-smoker	1.00(reference)
Never smoker	2.08(1.28-3.41)
BMI at baseline	1.00(0.95-1.05)
Atopy	
Non-atopic	1.00(reference)
Atopic	0.86(0.49-1.53)
Eosinophilic phenotype	
Non-eosinophilic	1.00(reference)
Intermittently eosinophilic	1.65(0.73-3.71)
Persistently eosinophilic	3.24(1.35-7.72)
Neutrophilic phenotype	
Non-neutrophilic	1.00(reference)
Intermittently neutrophilic	0.54 (0.28-1.02)
Persistently neutrophilic	0.36 (0.15-0.88)

Table 4A – Multivariable Cox Proportional Hazards (Andersen-Gill) model to determine risk of exacerbation for eosinophilic phenotypes adjusted by confounding variables with direct age adjustment.

Factor	Whole study group, adjusted HR (95% CI)
Eosinophilic status	
Non-eosinophilic	1.00(reference)
Intermittently eosinophilic	2.45(0.89-6.74)
Persistently eosinophilic	3.90(1.34-11.36)
Smoking history	
Ex-smoker	1.00(reference)
Never smoker	1.91(1.01-3.62)

Table 4B – Multivariable Cox Proportional Hazards (Andersen-Gill) model to determine risk of exacerbation for neutrophilic phenotypes adjusted by confounding variables with direct age adjustment.

Factor	Whole study group, adjusted HR (95% CI)
Neutrophilic status	
Non-neutrophilic	1.00(reference)
Intermittently neutrophilic	0.55(0.27-1.16)
Persistently neutrophilic	0.46(0.18-1.23)
Smoking history	
Ex-smoker	1.00(reference)
Never smoker	2.19(1.06-4.51)

Table 5A. Hurdle Poisson regression model for number of exacerbations and median sputum eosinophils (%) per subject over a 1-year period among severe asthmatics.

* Statistically significant at 0.05 level.

Factor	Binary Odds Ratio (95% CI)	Count Relative risk (95% CI)
Intercept	0.82 (0.35-1.90)	0.80 (0.44-1.46)
Sputum eosinophils (%)	1.12 (1.02-1.23)*	1.01 (1.00-1.02)
Smoking history	1.59 (0.64-3.98)	2.79 (1.50-5.18)*

Table 5B. Hurdle Poisson regression model for the number of exacerbations and median neutrophils (%) per subject over a 1-year period among severe asthmatics.

* Statistically significant at 0.05 level.

Factor	Binary Odds Ratio (95% CI)	Count Relative risk (95% CI)
Intercept	2.06 (0.67-6.31)	1.30 (0.65-2.59)
Sputum neutrophils (%)	0.99 (0.98-1.01)	0.99 (0.98-1.00)
Smoking history	1.51 (0.63-3.61)	2.74 (1.48-5.09)*

Figure 1

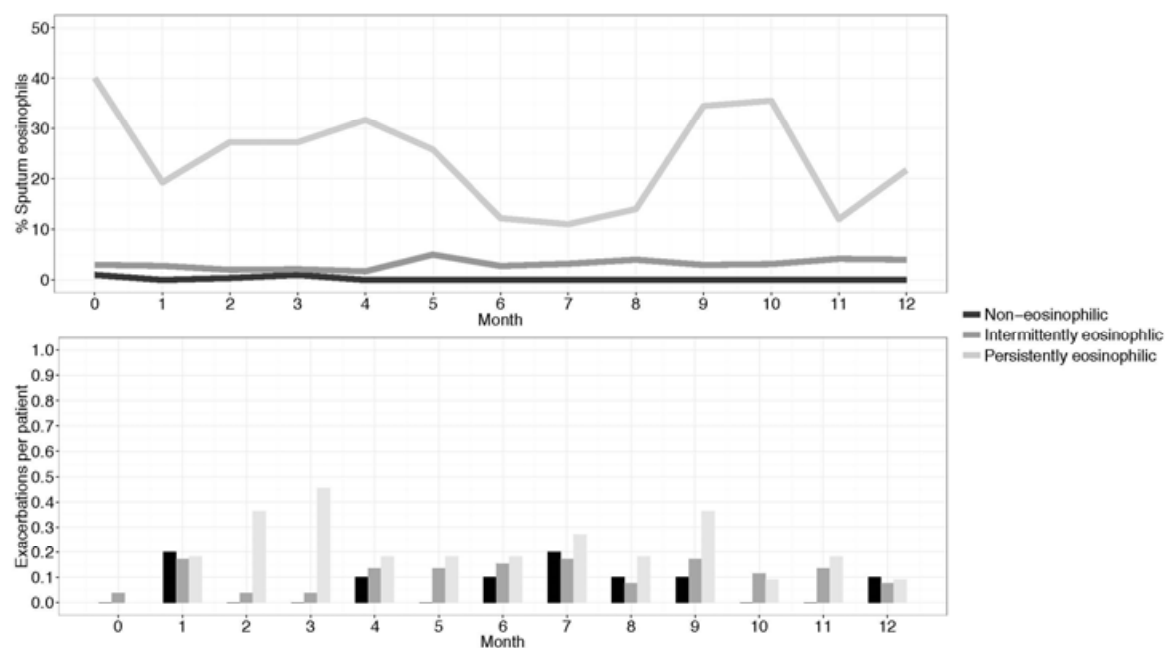


Figure 2

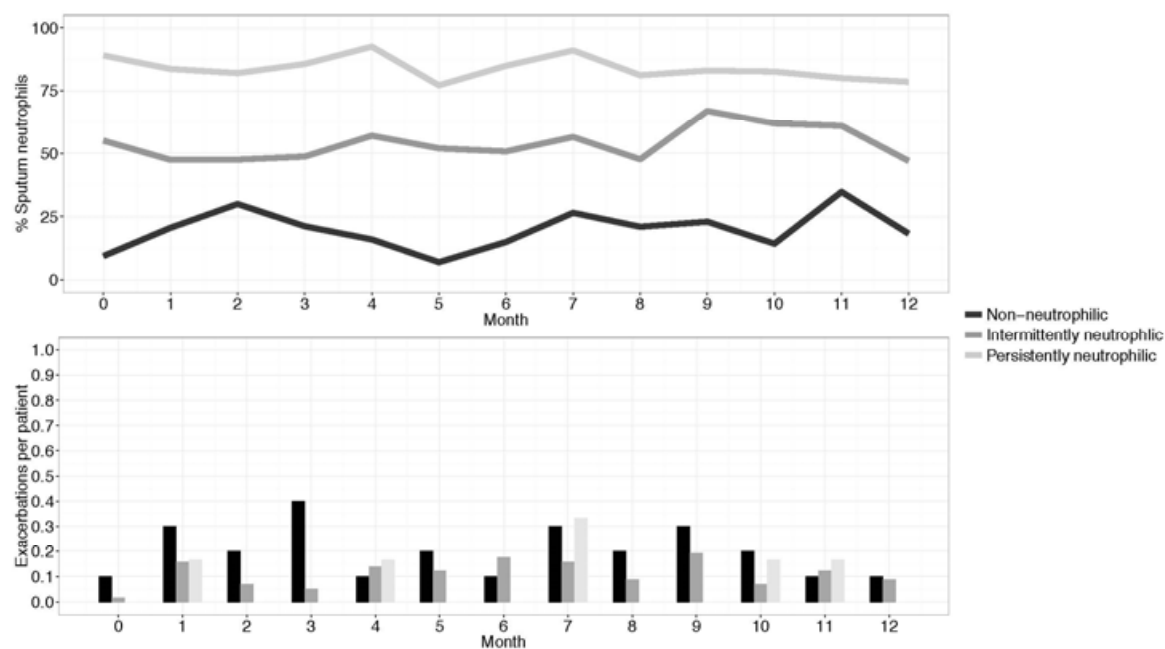


Figure 3

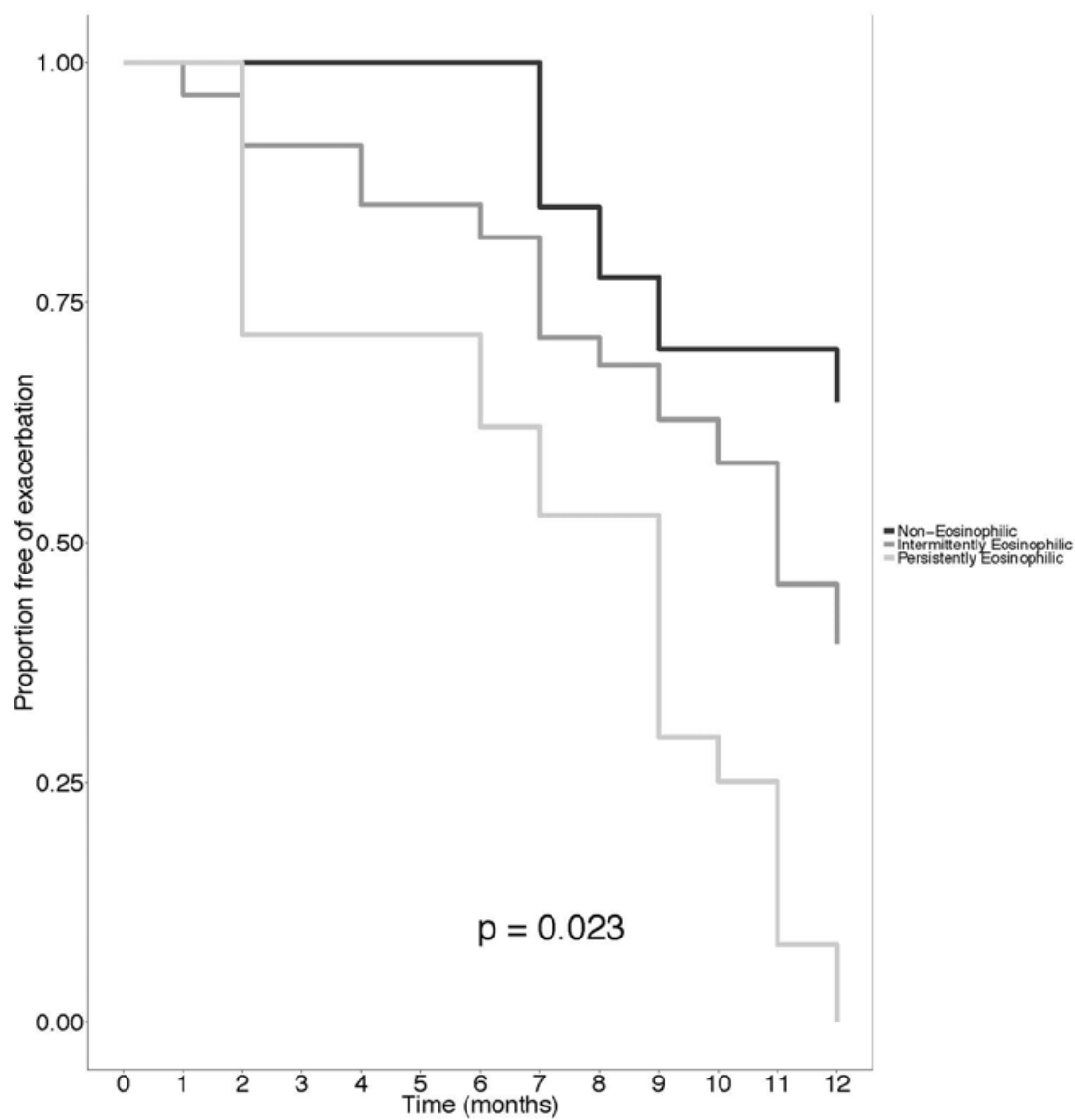


Figure 4

