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# Analyses of Asthma Severity Phenotypes and Inflammatory Proteins in Subjects Stratified by Sputum Granulocytes

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## Abstract

**Background—**Patients with severe asthma have increased granulocytes in their sputum compared to patients with mild to moderate asthma.

**Objective—**We hypothesized that inflammatory granulocytes in sputum may identify specific asthma severity phenotypes and are associated with different patterns of inflammatory proteins in sputum supernatants.

**Methods—**This hypothesis was tested in 242 asthmatics enrolled in the Severe Asthma Research Program who provided sputum samples for cell count, differential cell determinations, cell lysates for Western blot, and supernatant analyses by inflammatory protein microarrays and ELISAs. ANOVA and multiple linear regression models tested mediator associations.

**Results—**Stratified by sputum granulocytes, < or ≥2%eosinophils and < or ≥40%neutrophils, subjects with both increased eosinophils and neutrophils had the lowest lung function, increased symptoms and healthcare utilization. Subjects with elevated eosinophils with or without increased neutrophils had significantly increased FeNO, serum eosinophils and greater frequency of daily β-agonist use. Microarray data, stratified by granulocytes revealed 25–28 inflammatory proteins increased >2-fold in sputa with ≥40% neutrophils. Microarray analyses stratified by severity of asthma, identified 6–9 proteins increased >2-fold in sputa in subjects with severe asthma compared to nonsevere asthma. ELISA data, stratified by sputum granulocytes, showed significant increases in BDNF, IL-1β, and MIP-3α/CCL20 for those with ≥40%neutrophils; these mediators demonstrated positive associations with neutrophil counts.

**Conclusion—**Combined increased sputum eosinophils and neutrophils identified asthmatics with the lowest lung function and worse asthma control, increased symptoms and healthcare requirements. Inflammatory protein analyses of sputum supernatants found novel mediators increased in asthmatics, predominantly associated with increased sputum neutrophils.

### Keywords

asthma phenotypes; protein microarrays; BDNF; CXCL13; TNFSF14; CCL20; CCL18

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This article has supplemental data for the online repository

## INTRODUCTION

Eosinophils and neutrophils have each been separately observed in sputum from severe, poorly controlled or persistent asthma[1–7]. Severe asthma, as well as milder asthma, is therefore heterogenous and comprised of subgroups with potentially different underlying inflammatory pathologies. Subjects may be stratified according to sputum granulocytes to determine whether inflammatory differences are associated with asthma severity phenotypes. In the past asthmatics with both elevated eosinophils and elevated neutrophils were assigned to either the group with high eosinophils[8] or to the group with high neutrophils[9]. This approach potentially obscures distinquishing features for mixed granulocytic inflammation. One study evaluated these subjects as a separate group but found no significant differences in clinical characteristics between the groups except for age[10]. The small number of subjects with mixed granulocytes may have reduced differentiation from other phenotypes. We hypothesized that asthmatic subjects with both granulocytes increased in sputum represent a separate phenotypic group different from those with either only increased eosinophils, only increased neutrophils, or neither granulocyte increased.

In addition, only a few reports have investigated protein constituents of sputum, despite recognition that mucosal leukocytic infiltration is regulated by inflammatory mediator release. Earlier reports have measured limited numbers of proteins present in sputum, primarily those predicted for a single cell type, such as eosinophil cationic protein or IL-8, and compared these to normal controls[11–14]. We hypothesized that the patterns of infiltrating leukocytes in sputum are determined by differences in the inflammatory proteins contributing to asthmatic phenotypes.

To test these hypotheses, comprehensively characterized severe and nonsevere subjects with asthma enrolled in the Severe Asthma Research Program (SARP) provided sputa and were stratified on the basis of granulocyte percents. A subset of sputa were screened with a commercial protein microarray assessing 120 inflammatory proteins to identify mediators differing between subjects characterized by both granulocytes increased, eosinophils increased, neutrophils increased, or neither granulocyte increased. Selected proteins were confirmed by standard ELISA in sputum supernatants from the larger group of asthmatics. Mediator levels were additionally examined for association with specific cell counts, and clinical characteristics. Sputum cell lysates were analyzed by Western blotting for presence of specific mediators.

## METHODS

Characterization of severe and not severe asthma was performed according to the SARP protocol15. Non-smoking subjects (<5 packyears) met ATS criteria for diagnosis of asthma, and provided informed consent approved by the institutional review board. Comprehensive evaluation included spirometry, bronchodilator reversibility and bronchial responsiveness, assessment of atopy, collection of blood, exhaled NO, sputum induction, and administered questionnaire that characterized asthma symptoms, quality of life, medications and healthcare utilization [15] (online repository).

### Subjects

SARP subjects with severe (N=48, as defined by the ATS Workshop on Refractory Asthma16) and nonsevere asthma (N=194, asthmatics not meeting “severe” criteria), enrolled at Wake Forest University (WFU), had sputum induction, or, if safety criteria were not met, a spontaneous sputum collected. WFU collected a majority of the sputum samples and not all SARP sites participated in the sputum protocol. Demographics and clinical characteristics for

the subjects in the sputum subset matched those previously reported for SARP subjects from all sites 15 (Table E1 online repository).

### Sputum Induction and Processing

The sputum induction method was adopted from the Asthma Clinical Research Network 17. Sputum was processed immediately; cell cytospins were stained for differential count of leukocytes, bronchial epithelial and squamous cells; and aliquots of sputum supernatant were stored at −80°C. The sputum cell differential counts from 175 subjects were adequate for further analyses[17]. Cell lysates were examined by Western blotting for presence of specific proteins (online repository).

### Inflammation Microarrays and ELISAs

Inflammation protein microarray analyses (RayBiotech) were performed on sputum supernatants from subjects with asthma (N=12 nonsevere, noinhaled corticosteroid [ICS] treatment, N=12 nonsevere, ICS-treated, and N=12 severe, high dose ICS treatment) diluted to 1 mg total protein (online repository). Densities of duplicate reactions were averaged and normalized to controls on each membrane. Proteins identified from analyses based on corticosteroid use, granulocyte%, or asthma severity, were investigated by specific ELISA (R & D Systems, except PARC/CCL18 and Eotaxin 2 from RayBiotech).

### Statistics

Microarray density data were $\log_2$ transformed and analyzed by Significance Analysis of Microarrays (SAM 18). A score>1, false discovery rate of less than 5% and a greater than 2-fold change were the criteria for significance. The SAM program is designed to address a chance identification problem for larger datasets than the 120 protein microarray 18. Demographic and ELISA data are presented as means ± standard deviations, standard errors, or medians (25%–75% quartiles). Measures not meeting Kolmogorov-Smirnov test for normal distribution, were transformed to log, or square root values. A zero in cell differentials or ELISA assays was replaced with a value half of the lowest observed before log transformation19. Continuous variables were tested by ANOVA, or by student's t-test, if parametric; or by Kruskal-Wallis and Mann-Whitney, if non-parametric (SAS 9.2, or Sigmastat 2.03). Initial analyses with a significant difference were further explored by post-hoc pairwise analyses (Tukey). Categorical variables were analyzed using Chi-square tests. Multivariate linear regression models examined mediators for association with sputum cells, age and clinical characteristics. Bonferroni correction was applied to variables with a p value <0.05 to determine significance.

## RESULTS

### Subject Stratification Based on Sputum Inflammation %Eosinophils and %Neutrophils

There were no significant differences in sputum cell percentages for severe compared to nonsevere subjects (Table E2, online repository), and therefore, these were combined for stratification by granulocytes: <2%Eos + <40%Neu, <2%Eos + ≥ 40%Neu, ≥2%Eos + <40% Neu, and ≥2%Eos + ≥40%Neu (Table I). The group with both increased Eos and increased Neu had lowest lung function ($FEV_1$%predicted, $FEV_1$/FVC ratio, change $FEV_1$%predicted [maximum-baseline]), highest frequency of daily wheeze, and most frequent healthcare utilization (Table I). Subjects with ≥2%Eos and <40%Neu had the highest FeNO ($p<0.001$). Subjects with ≥40%Neu, with or without ≥2%Eos, were significantly older, but there was no difference in age of asthma onset. No differences were observed for + skin test frequency or number. Greater frequency of positive response to "daily use of β-agonist", and daily wheeze were associated with ≥2%Eos, with or without ≥40%Neu; whereas, a greater frequency of

positive response to ">1 urgent healthcare visit in the past year" was associated with ≥40% Neu, with or without ≥2%Eos. While only >1 urgent healthcare visit in the past year reached significance, other measures of healthcare utilization were higher in the combined increased granulocyte group than the other groups.

### Stratification of Microarray Data based on Sputum %Eosinophils and %Neutrophils

Microarray data were analyzed based on sputum Eos and Neu of groups divided by granulocyte %. Corticosteroid effects[20, 21] were considered by first comparing nonsevere without corticosteroid treatment to nonsevere with corticosteroid treatment. Only MCP3 was significantly reduced >2fold in the corticosteroid treatment group. Nevertheless to control for corticosteroid effects, only ICS-treated subjects were examined in the comparison of subjects with primarily elevated eosinophils (N=6) to subjects with primarily elevated neutrophils (N=7). Twenty-five proteins were increased >2-fold in the group with elevated Neu (Table II comparison 1). For subjects with elevated eosinophils (N=6) compared to subjects with both granulocytes elevated (N=8), 30 proteins were significantly increased >2-fold for those subjects with both granulocytes elevated (Table II, comparison 2). Eight new proteins were observed in this comparison, and 22 of the increased proteins were previously observed in comparison 1. Subjects with elevated Neu (N=7) were also compared to those with both granulocytes elevated (N=8), but showed no protein differences despite differing %Eos (not shown).

Microarray data stratified by subjects with <2%Eos compared to ≥2%Eos, without considering %Neu, showed just two proteins, IL-11 (2.2X) and IL-2Ra (2.3X), significantly increased >2-fold (false discovery rate =0%) in those subjects with ≥2%Eos (not shown). In contrast, microarray data stratified by subjects with <40%Neu compared to ≥40%Neu without considering %Eos, had 28 proteins significantly increased in those subjects with ≥40%Neu (Table II comparison 3). The three comparisons identified 19 proteins increased in common (highlighted). Limited supernatant fluid available required reducing the large number of potential biomarkers for specific ELISA confirmation. Therefore, microarray data were alternatively stratified by asthma severity.

### Stratification of Microarray Data based on Asthma Severity

Comparisons of microarray data for nonsevere asthma subjects without and with inhaled corticosteroid treatment (N=12 and N=12, respectively) to severe asthma subjects (N=12) included: 1) all nonsevere with severe asthmatics, 2) nonsevere subjects receiving corticosteroids with severe subjects, and 3) nonsevere asthmatics receiving corticosteroids, and$FEV_1$%predicted >80% with severe subjects. These 3 comparisons yielded 9, 6, and 33 proteins, respectively, with significant increases >2fold in sputa of severe subjects. Three proteins matched in all 3 comparisons (highlighted in Table E3): Brain-derived neutrophic factor (BDNF), B-lymphocyte chemoattractant (BLC/CXCL13), and epidermal growth factor (EGF). The comparison of nonsevere with mild disease to severe asthmatics found 25 proteins significantly increased >2-fold in the severe subjects which were not observed in the other 2 comparisons (Table E4, online repository). Based on the microarray results, proteins were selected for ELISA assay on the larger panel of sputa available (N=175). IL-13 and IFNγ were also examined although identified in only one microarray comparison (Comparison 1, Table II).

### ELISAs on Selected Inflammatory Mediators Stratified by Sputum %Eosinophils and % Neutrophils

ELISA data stratified into the 4 groups based on sputum granulocytes, < or ≥ 2%Eos + < or ≥40%Neu, revealed significant increases in mediators for those subjects with ≥40% Neu (Table III). BDNF, IL-1β, and macrophage inflammatory protein 3α/MIP-3 α/CCL20 were

significantly increased in sputa containing ≥40%Neu, either with <2%Eos or with ≥2%Eos. Only Eotaxin 2 showed an increase associated with ≥2%Eos, which was enhanced in combination with ≥40%Neu, but did not reach significance after correction.

### Associations of Mediators with Leukocytes and Clinical Characteristics

Associations of each inflammatory mediator's concentration with the actual count of specific cell types occurring in the sputum sample were investigated (Table IV). Age is positively associated with %Neu[22] and therefore included as an independent variable. Only Neu were observed to have positive associations with those mediators showing significance. Despite positive association of Neu with age and with several mediators, age independently showed associations with only LIGHT/TNFSF14 and PARC/CCL18 which were not significant.

Inflammatory mediators were also examined for association with clinical characteristics including spirometric measures, FeNO, number of positive skin tests, and IgE levels (online repository Table E6). IL-8 and PARC/CCL18 had negative associations with baseline FVC% predicted, and LIGHT/TNFSF14 had a negative association with baseline $FEV_1$%predicted. TNFα had a positive association with FeNO. Neither IgE nor number of positive skin tests showed associations with any mediator.

### Western Blots of Sputum Cell Lysates Probed for Mediator Presence

Sputum cell lysates were analyzed by western blots for BDNF and IL-1β. Mean densities for both BDNF and IL-1β in lysates from subjects with combined elevated Eos+Neu were higher than in those with elevated Eos, elevated Neu or neither granulocyte elevated, but did not reach significance (Figure E2 of online repository).

## DISCUSSION

Sputum represents the best available, non-invasive assessment of bronchial inflammation in asthma and reflects underlying pathology caused by infiltrating cells and soluble mediators. Instead of comparing sputum cellular and biochemical components between normals and asthmatic subjects, our objective was to assess whether comprehensive analysis of induced sputum over a spectrum of asthma severity improves our understanding of the factors that characterize different asthma phenotypes. At present definitions of asthma severity are based primarily on the recommendations of asthma guidelines and on consensus statements. Unfortunately, these definitions may not rely on evidence based science for classification of asthma severity. Currently, the NHLBI SARP network is investigating new approaches to classify asthma severity, including new imaging techniques, unbiased cluster methods[23] similar to these reported by Haldar et al[24] and the assessment based on sputum granulocytes described here. Our hypothesis that sputum inflammatory granulocytes identify phenotypic subgroups of differing pathology and clinical characteristics was examined in a unique, well-characterized SARP population including patients with and without corticosteroid treatment.

Other investigators have observed that asthma, including severe asthma, contains subgroups categorized by sputum granulocytes, eosinophilic or neutrophilic, or "non-eosinophilic"[25, 26], and further subdivided in a 4-way stratification based on both eosinophils and neutrophils[10]. However, this latter report observed only age differences for a small number of subjects having mixed granulocytes. In contrast, our larger groups stratified by granulocyte % s showed significant differences in lung function, asthma control, healthcare utilization and symptoms. Although most of the soluble mediators examined did not differ between the elevated Eos + elevated Neu group compared to the elevated Neu group, Eotaxin 2 and clinical characteristics, support a distinction between these 2 groups. Phenotypic characteristics associated with the 4 asthma groups revealed that combined increased sputum eosinophils and

neutrophils defined the most severe patients with lowest lung function measures, worse asthma control, greatest symptoms and use of healthcare resources.

Inflammatory mediators in sputum have been reported for specific mediators, predicted by increases in specific cell types such as ECP for eosinophils or IL-8 for neutrophils[12, 26, 27]. Additional mediators reported in sputum for asthma include vascular endothelial growth factor, basic fibroblast growth factor, elastase, GMCSF, IFN-γ, IL-4, IL-5, IL-13, endostatin, MIP3α/CCL20, metalloproteinase 9, tissue-inhibitor metalloproteinase 1, PARC, RANTES and α1-antitrypsin[12, 28–33]. However, most increased inflammatory proteins are reported for subjects with asthma compared to normals, instead of within asthma severity subgroups where proteins may reflect important mechanisms determining disease severity.

We used a more comprehensive approach with a focused 120 inflammatory protein microarray to identify mediators in sputum supernatants from severe and nonsevere asthmatics. A recent report[28] used a similar protein microarray approach, but examined fewer proteins and only compared nine asthmatics to atopic and normal controls. PARC, GRO-α and Eotaxin-2 were elevated in sputum from subjects with asthma, but subgroups of asthma were not investigated[28].

A 4-group stratification of our microarray data by sputum %eosinophils and %neutrophils identified significant increases in nineteen inflammatory mediators in sputa with ≥40%Neu. ELISA data confirmed the significant increases in sputa with ≥40%Neu, either with <2%Eos or with ≥2%Eos, for BDNF, IL-1β, and MIP-3α/CCL20. Eotaxin 2 increases in ELISA data were observed with increased Eos, but did not reach significance. Nevertheless, higher levels of Eotaxin 2 were observed for sputum having combined increased Eos and increased Neu. Regression analyses showed significant positive associations of mediators with Neu counts, and less robust positive or negative associations with Eos counts.

The positive association of neutrophils with BDNF, IL-1β and MIP3α raises the question whether neutrophils produce or are elicited by the actions of these proteins. Immunohistology in mouse models and isolated human peripheral monocytes identified cellular sources for BDNF as bronchial epithelium, activated T cells, and macrophages[34–35]. Likewise, IL-1β is synthesized by multiple cells, including macrophages, epithelial cells and neutrophils[36–38]. Both BDNF and IL-1β levels in our sputum cell lysates were higher for combined increased Eos+Neu but not significantly, suggesting contribution from other cells. Cell sources for MIP3α include airway epithelium, monocytes, eosinophils and neutrophils; release is either constitutive or in response to LPS, particulates, allergens, or TNFα, IL1β, IL-4, or IL-13[33, 39–40]. Thus, any one or a combination of sputum cell types may contribute to the levels of MIP3α observed, including neutrophils.

The results demonstrate that mediators previously less well recognized for involvement in asthmatic inflammation may be identified by this approach. BDNF, BLC/CXCL13 and EGF were found to be significantly increased by microarray analysis of sputum from subjects with severe compared to nonsevere asthma groups. ELISA results on the larger panel of sputum supernatants showed that higher levels of these mediators occurred in the severe group, but did not reach significance (online repository).

BDNF has been implicated in bronchial hyperresponsiveness and inflammation[41] but not specifically connected to asthma severity. Allergic asthmatics have higher levels of BDNF, promoting eosinophil survival, in bronchoalveolar lavage fluid after segmental allergen challenge[35, 42]. Both epithelial cells and monocytes release BDNF in response to TNFα, IL1β, and IL-6[35, 43–44].

BLC/CXCL13 has not previously been associated with asthma severity. CXCL13 upregulation in STAT6 deficient mice after repeated allergen challenge is associated with neutrophilic inflammation[45]. Similarly, we observed positive association of CXCL13 with neutrophils. Monocytes and mature macrophages treated with lipopolysaccharide secrete CXCL13[46]. IL-6 signaling results in accumulation of B cell follicles in the lung expressing CXCL13[47]. IL-6 was increased in microarrays for sputa with elevated Neu.

EGF stimulates IL-8 release and enhances TNFα-induced IL8 release from epithelium, and is thereby linked to neutrophilic inflammation in severe asthma[48–49]. There is evidence of increased EGF receptor in bronchial biopsies from severe compared to mild asthma[48], but EGF either is similar [50] or increased[51], in 2 studies examining small numbers of asthmatics compared to nonasthmatic controls. Our study confirms association of EGF with neutrophils and higher amounts in subjects with severe asthma, although not significantly.

Paired microarray comparisons of dialyzed to autologous undialyzed samples, and of protease inhibitor-treated supernatants to autologous untreated supernatants indicated minimal consistent alteration of sputum proteins due either to dithiothreitol use or proteolysis detected by microarrays. Moreover, known amounts of standards for each ELISA generally displayed full recovery when assessed in normal sputum supernatants or buffer solution containing dithiothreitol (online repository).

The processing method employing whole sputum potentially contributes variable amounts of saliva but is counteracted by a two step collection[52]. In fact, %squamous cells did not differ for any stratification. Observed significant association of mediators in the supernatant with actual cell counts further supports that variable amounts of saliva had little dilutional effect on mediators.

Woodruff et al.21 reported that age, sex, ethnicity and use of inhaled corticosteroids were important confounders of cellular inflammation in asthma. This contrasts with the report of Thomas et al. [22], which demonstrated age significantly affected %neutrophils but sex did not, in a normal population. We confirm a positive association between age and %Neu in asthmatics. Inclusion of age into our models examining mediator association with specific sputum cell types showed associations of age with LIGHT/TNFSF14 and PARC/CCL18 (neither significant) but otherwise had little effect on Neu association with mediators. Subject stratification by sputum granulocytes did not show a difference in age of asthma onset; although subjects with greater %neutrophils were older and therefore, possibly had longer duration of asthma.

We did not find any significant effect for race or for corticosteroid use on sputum cell counts or on mediator levels. The Caucasian and African American proportions in our population (53–77% and 23–45%, respectively) are slightly different than those reported by Woodruff [21] (68% Caucasian, and 12% African American), however, lack of an effect for race/ethnicity in our data cannot be attributed to insufficient numbers of minority subjects.

Certain mediators identified, IL-1β, IL-8 and TNFα, are usually associated with innate immunity and a $T_H1$ response, rather than the $T_H2$ mediators, IL-4, IL-5, and IL-13, typical of allergic asthma. This finding is consistent with the heterogeneity of clinical asthma that may be influenced by different pathologic mechanisms. However, we did observe increased MIP3α/CCL20, recently reported as a critical link between TRAIL and the activation of $T_H2$ cells in allergic airway disease[33]. TNFα, which we found increased in microarrays, induces increased MIP3a/CCL20 secretion from primary bronchial epithelium[33], indicating overlap of $T_H1$ and $T_H2$ inflammatory pathways. It is interesting that Kikuchi et al.[53] reported both IL-8 and neutrophils were necessary to promote greatest trans-basement membrane migration of

eosinophils. The group of subjects, with both elevated sputum Eos and Neu, had equally high, if not the highest levels of mediators, supporting this observation.

In conclusion, stratification of asthmatics by sputum %Eos and %Neu demonstrated that combined increased granulocytes identify those asthmatics with lowest lung function, increased frequency of symptoms and healthcare utilization. Protein microarray screening of sputa identified novel proteins that have been less well recognized for participation in asthma, and suggested a $T_H1$ component to inflammation in more severe asthma. The levels of these mediators were generally higher in subjects with severe asthma, but showed stronger association with neutrophils than with eosinophils. These approaches delineating cellular and biochemical proteomics provides better understanding of the pathogenesis of asthma subphenotypes and may lead to development of biomarkers differentiating heterogeneity in asthma.

**Key Messages**

- Four-way stratification of subjects, based on high or low %eosinophils and high or low %neutrophils in sputum, identified an association of subjects characterized by both high %eosinophils and high %neutrophils with the lowest lung function, worse asthma control, and increased use of healthcare resources.
- Protein microarray data revealed novel proteins increased in sputum with both increased neutrophils and eosinophils, suggesting the importance of combined granulocyte effects on asthma severity.

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## Abbreviations

| | |
|---|---|
| BDNF | brain derived neurotrophic factor |
| BLC | B-lymphocyte chemoattractant/CXCL13 |
| BMP | bone morphogenic protein |
| EGF | epidermal growth factor |
| Eos | eosinophils |
| ICS | inhaled corticosteroids |
| IL | interleukin |
| MIP3α | macrophage inflammatory protein 3α/CCL20 |
| Neu | neutrophils |
| PARC | pulmonary and regulated chemokine/CCL18 |
| SARP | Severe Asthma Research Program (of NHLBI) |
| TNFSF14 | Tumor necrosis factor superfamily factor 14/LIGHT |

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**Table I**

Demographics of Subjects Stratified by Sputum %Eos+%Neu. Results from ANOVA are presented as mean+SEM; results from Kruskal-Wallis analysis of variance are presented as median (25%–75%), p values meeting Bonferroni correction are in bold font:

| **Categories:** | | **<2%Eos <40%Neu** | **<2%Eos ≥40%Neu** | **≥2%Eos <40%Neu** | **≥2%Eos ≥40%Neu** | **P value** |
|---|---|---|---|---|---|---|
| **N=175** | | 63 (36%) | 50 (29%) | 42 (24%) | 20 (11%) | |
| **Gender** | %Female | 81% | 76% | 74% | 60% | 0.30 |
| **Age (yr)** | | 33±1.6 | 42±1.9 | 34±1.8 | 40±2.1 | **<0.001** |
| **Age Onset** | | 10 (4–24) | 13 (5–26) | 13.5 (2–23) | 7.5 (3–30) | 0.92 |
| **Duration** | | 17±1.6 | 25±2.1 | 19±1.9 | 23±2.5 | 0.014 |
| **Lung Function:** | Baseline $FEV_1$ % Predict. | 86.5±2.0 | 80.4±2.3 | 76.6±2.8 | 66.6±4 | **<0.001** |
| | Baseline FVC% Predict. | 93.8±1.8 | 87.0±2.0 | 90.5±2.7 | 81.7±3.7 | 0.01 |
| | FEV/FVC | 0.77±0.01 | 0.75±0.01 | 0.70±0.02 | 0.66±0.03 | **<0.001** |
| | Max $FEV_1$% Predict. | 95.4±1.8 | 88.8±1.9 | 90.3±2.6 | 80.8±4.2 | 0.003 |
| | %Rev 2puff β agonist | 9.89±10 | 7.98±5.8 | 14.5±12.3 | 15.8±15.5 | 0.005 |
| | Change $FEV_1$%pred (max-baseline) | 9.1±6.5 | 8.3±6.4 | 13.7±10.3 | 14.7±10.1 | **0.002** |
| | $LogPC_{20}$ * | 0.47±0.1<br>N=55 | 0.41±0.1<br>N=46 | −0.013±0.1<br>N=34 | ;0.23±0.3<br>N=14 | 0.006 |
| | FeNO | 22.9±1.1 | 20.0±1.1 | 49.0±1.1 | 30.9±1.2 | **<0.001** |
| | Freq + skin test | 83% | 78% | 86% | 89% | 0.66 |
| | No. + skin tests | 4 (2–6.8) | 3 (1–5.3) | 4 (2–7.3) | 4 (2–7) | 0.59 |
| | Serum eos | 0.20 (0.1–3.3) | 0.20 (0.1–2.5) | 0.30 (0.2–2.5) | 0.30 (0.2–2.1) | **0.001** |
| | IgE | 126±1.2 | 78±1.3 | 191±1.3 | 100±1.3 | 0.06 |
| **Asthma Control & Healthcare Utilization**†: | daily use β-agonist | 24% | 28% | 60% | 55% | **<0.001** |
| | Ever ER visits | 57% | 70% | 69% | 80% | 0.21 |
| | >1 urgent HC visit in past year | 22% | 40% | 19% | 70% | **<0.001** |
| | Symptoms worse if reduce CS | 49% | 39% | 38% | 70% | 0.07 |
| | >3 oral CS bursts/yr | 17% | 12% | 9% | 30% | 0.17 |
| | Ever spent night in Hospital | 25% | 40% | 38% | 40% | 0.07 |
| | Ever ICU admitted | 6% | 8% | 21% | 25% | 0.009 |
| **Symptoms**†: | >daily wheeze | 13% | 14% | 26% | 50% | **0.002** |

* Not all subjects met safety criteria for methacholine challenge.

† % of category answering "yes"

## Table II

Three Comparisons of Inflammatory Protein Microarray Data in Subjects (all treated with corticosteroids) Stratified by Sputum Eosinophils (Eos) and Neutrophils (Neu). Comparison 1 examines differences in mediators between elevated Eos+low Neu and elevated Neu+low Eos; comparison 2 examines differences between elevated Eos+low Neu and elevated Eos+elevated Neu; comparison 3 examines differences between low Neu and elevated Neu without considering Eos. Highlighted proteins were observed to meet criteria in all 3 comparisons.

| Protein | 1. ≥2%Eos+ <40%Neu (N=6) vs <2%Eos+ ≥40%Neu (N=7) | | 2. ≥2% Eos+ <40%Neu (N=6) vs ≥2% Eos + ≥40% Neu (N=8) | | 3. <40% Neu (N=9) vs ≥40% Neu (N=15) | |
|---|---|---|---|---|---|---|
| | False positive rate=0–1% | | False positive rate=0% | | False positive rate=0–1% | |
| | Criteria Failure* | Increase for ≥40% Neu Fold change | Criteria Failure* | Increase for ≥40% Neu Fold change | Criteria Failure* | Increase for ≥40% Neu Fold change |
| BDNF | *a* | | | 3.3 | | 2.2 |
| BLC | *a* | | | 3.2 | *a* | |
| BMP-4 | | 3.3 | | 3.8 | | 2.4 |
| BMP-6 | | 5.5 | | 6.2 | | 3.2 |
| CK b8-1 | *a, b* | | *b* | | | 2.2 |
| EGF | | 2.9 | | 3.4 | | 2.0 |
| Eotaxin-2 | | 3.0 | | 5.3 | | 2.1 |
| Eotaxin-3 | | 3.6 | | 3.6 | | 2.7 |
| FGF-7 | | 2.2 | | 2.5 | *b* | |
| GCP-2 | | 4.0 | | 3.2 | | 2.7 |
| GDNF | *a* | | | 2.5 | *a, b* | |
| GM-CSF | *a, b* | | | 3.8 | *b* | |
| HGF | *a* | | ns | | | 3.1 |
| I-309 | *a* | | | 2.3 | *b* | |
| IFN-γ | | 2.6 | *a* | | *a, b* | |
| IGFBP-1 | | 2.6 | | 2.7 | | 2.1 |
| IGFBP-2 | | 11.7 | | 16.1 | | 7.7 |
| IGF-1 | | 3.1 | | 3.3 | *b* | |
| IL-1β | | 9.0 | | 8.4 | | 6.8 |
| IL-2 | | 4.1 | | 2.9 | | 2.1 |
| IL-4 | | 5.9 | | 4.2 | | 2.2 |
| IL-6 | | 3.1 | | 3.5 | | 3.2 |

| Protein | 1. ≥2%Eos+ <40%Neu (N=6) vs <2%Eos+ ≥40%Neu (N=7) | | 2. ≥2% Eos+ <40%Neu (N=6) vs ≥2% Eos + ≥40% Neu (N=8) | | 3. <40% Neu (N=9) vs ≥40% Neu (N=15) | |
|---|---|---|---|---|---|---|
| | False positive rate=0–1% | | False positive rate=0% | | False positive rate=0–1% | |
| | Criteria Failure* | Increase for ≥40% Neu Fold change | Criteria Failure* | Increase for ≥40% Neu Fold change | Criteria Failure* | Increase for ≥40% Neu Fold change |
| **IL-10** | | **2.7** | | **3.8** | | **2.1** |
| **IL-13** | | **2.8** | *a* | | *a* | |
| **IL-15** | | **2.1** | | **3.5** | | **2.4** |
| **LIGHT** | | **2.1** | | **3.3** | | **2.8** |
| **MCP-1** | *a* | | | **2.7** | | **2.4** |
| **MCP-2** | | **3.8** | | **2.5** | *a* | |
| **MDC** | *a, b* | | | **3.0** | *b* | |
| **MIP-1δ** | | **3.9** | | **3.6** | | **2.6** |
| **MIP-3α** | | **3.0** | | **4.8** | | **3.0** |
| **NT-3** | | **2.4** | *a, b* | | | **2.3** |
| **SCF** | | **2.4** | | **2.6** | | **2.1** |
| **SDF-1** | *a* | | | **3.3** | *a, b* | |
| **TARC** | *a* | | *a, b* | | | **2.4** |
| **TIMP-2** | ns | | ns | | | **2.1** |
| **TNF-α** | | **4.8** | | **5.5** | | **2.8** |
| **TRAIL R3** | ns | | ns | | | **2.5** |
| **uPAR** | ns | | ns | | | **3.3** |

*ns = not significant (q>5%);

*a*scored <1;

*b*fold increase <2.

**Table III**

Sputum Supernatant Mediator Levels Stratified by Sputum %Eos and %Neu. P values meeting Bonferroni correction are in bold font.

| | <2%Eos+ <40%Neu | <2%Eos+ ≥40%Neu | ≥2%Eos+ <40%Neu | ≥2%Eos+ ≥40%Neu | P value For 4 grps* |
|---|---|---|---|---|---|
| **BDNF pg/ml** | 9.5 (6–14.5) | 18.4 (11.2–29) | 15.4 (8–26.5) | 20 (14–39) | **<0.001** |
| **BLC/CXCL13 pg/ml** | 110±29 | 223±48 | 94±20 | 142±49 | 0.005 |
| **BMP4 pg/ml** | 4.9±1.5 | 2.8±0.6 | 1.8±0.3 | 3.7±1.1 | 0.33 |
| **EGF pg/ml** | 159 (108–205) | 190 (116–263) | 171 (90–212) | 199 (145–256) | 0.13 |
| **Eotaxin 2/CCL24 pg/ml** | 0.88 (0.01–2.38) | 1.13 (0.01–6.78) | 2.39 (0.38–4.63) | 8.90 (2.5–14.1) | 0.022 |
| **IFNγ pg/ml** | 77 (37–149) | 47 (2–128) | 108 (49–175) | 91 (23–125) | 0.11 |
| **IL-1β pg/ml** | 104±14 | 224±56 | 76±12 | 228±65 | **<0.001** |
| **IL-8 ng/ml** | 1.5±0.1 | 2.1±0.2 | 1.6±0.2 | 1.9±0.2 | 0.017 |
| **IL-13 pg/ml** | 91±18 | 74±9.9 | 64±12 | 81±18 | 0.55 |
| **LIGHT/TNFSF14 pg/ml** | 36 (12–69) | 92 (36–156) | 33 (13–108) | 58 (24–184) | 0.021 |
| **MIP-3 α/CCL20 pg/ml** | 390±41 | 781±85 | 341±52 | 668±121 | **<0.001** |
| **PARC/CCL18 pg/ml** | 6.7 (0.4–19) | 12.7 (4.7–59) | 8.3 (1.7–22) | 11 (4.6–40) | 0.037 |
| **TNF α pg/ml** | 0.3 (0.01–1.24) | 1.1 (0.01–2.6) | 0.39 (0.01–1.9) | 0.7 (0.01–8.1) | 0.44 |

*ANOVA (mean±SEM) or Kruskal-Wallis (median [25–75% quartiles])

**Table IV**

Multiple Linear Regression Analyses for Mediators Significantly Associated with Specific Sputum Cells and Age. The overall R and p values for each mediator are reported with the coefficient and individual p values for specific cell types or age necessary to predict a linear model for the mediator. P values meeting Bonferroni correction are in bold font:

| Cytokine or Growth Factor | R & P value | Br. Epith. Cell Count* | Mac/Mono Count* | Lym Count* | Neu Count* | Eos Count* | Age* |
|---|---|---|---|---|---|---|---|
| **BDNF** | 0.51<br>**<0.001** | 0.414<br>0.032 | | | 0.769<br>**<0.001** | 0.238<br>0.064 | |
| **BLC/CXCL13** | 0.388<br>0.008 | | | | 0.265<br>**0.003** | −0.131<br>0.065 | |
| **BMP4** | 0.393<br>0.122 | | | | | −0.197<br>0.0167 | |
| **EGF** | 0.508<br>**<0.001** | | | 32.6<br>0.014 | 26.1<br>0.02 | | |
| **Eotaxin2** | 0.27<br>0.29 | | | | | 0.28<br>0.02 | |
| **IL-1β** | 0.486<br>**<0.001** | −0.12<br>0.06 | | | 0.174<br>0.016 | | |
| **IL-8** | 0.585<br>**<0.001** | | 0.569<br>0.033 | | 0.382<br><0.005 | | |
| **IL-13** | 0.171<br>0.86 | | | | | | |
| **IFNγ** | 0.324<br>0.136 | 19.21<br>0.08 | | | | | |
| **LIGHT/TNFSF14** | 0.651<br>**<0.001** | | 3.76<br>0.014 | | 2.22<br>0.007 | | 0.097<br>0.018 |
| **MIP3α/CCL20** | 0.621<br>**<0.001** | | | | 5.59<br>**<0.001** | −2.24<br>0.014 | |
| **PARC/CCL18** | 0.553<br>**<0.001** | | | | 0.338<br>0.006 | 0.184<br>0.025 | 0.016<br>0.026 |
| **TNF α** | 0.567<br>**<0.001** | | 0.613<br>0.062 | 0.458<br>0.007 | | | |

*Values under Cell type are regression coefficient and p value for that specific cell. Blank indicates that the cell type or age were not found to be necessary for linear model of association with the cytokine.