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Airway and Serum Biochemical Correlates of Refractory Neutrophilic Asthma

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Biochemical Correlates of Refractory Asthma

Refractory Vs. Non-refractory Asthma

Parameters that Attained Significance

- ↑ Age
- ↑ Subclinical Infection
- ↑ BAL neutrophils and lymphocytes
- ↑ Lipocalin-2, serum amyloid A, CXCL7, IL8, thrombospondin-1, IL1 β and IL6
- ↓ IL18, IL22

Title: Airway and Serum Biochemical Correlates of Refractory Neutrophilic Asthma

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Abstract

Background: Despite the progress in diagnosis and management of asthma, many patients have poorly controlled or refractory asthma. The mechanism of this refractory asthma is not well understood.

Objective: Explore the relationship between neutrophils and other biomarkers of refractory asthma.

Method: Sixty subjects with refractory asthma (RA), 30 with non-refractory asthma (NRA) and 20 healthy subjects were enrolled. We performed a comprehensive characterization of these study subjects, which included laboratory and pulmonary function studies, chest CT, and bronchoscopy with bronchoalveolar lavage. We analyzed BAL and serum for a total of 244 biomolecules by multiplex assay and correlated them with the clinical and other laboratory parameters.

Results: RA was significantly different from NRA with regard to pulmonary function indices, bronchial basement membrane thickness, and BAL neutrophils and lymphocytes but not eosinophils. BAL neutrophils negatively and positively correlated with the forced vital capacity and age, respectively. Of the 244 biomolecules studied, 52 and 14 biomolecules from BAL and serum, respectively, were significantly different among the study groups. Thirteen of these 52 molecules correlated with BAL neutrophils. BAL from 40% of RA patients was positive for a pathogenic microbe. Infection-negative neutrophilic RA was associated with an increase in select biomarkers of inflammation in the serum suggesting the presence of systemic inflammation.

Conclusions: RA was associated with increased number of neutrophils and proneutrophilic biomolecules in the airways. Subclinical infection was present in 40% of RA patients, which likely contributed to neutrophilic inflammation. A subgroup of non-infected neutrophilic RA was associated with systemic inflammation.

Clinical Implications

This study identifies airway neutrophils and proneutrophilic biomolecules as a significant contributor to refractory asthma. A subgroup of neutrophilic asthma was associated with a subclinical infection. This group is likely to benefit from specific anti-microbial therapy. Neutrophilic asthma without a subclinical infection showed signs of systemic inflammation. Therapies targeting proneutrophilic biomolecules and/or autoinflammation are likely to control asthma in this subgroup.

Capsule summary

Through a comprehensive analysis of bronchoalveolar lavage and blood this paper identified neutrophils and proneutrophilic biomolecules as strong correlates of refractory asthma.

Key words: Refractory asthma, neutrophilic asthma, bronchoalveolar lavage, cytokines, infection

Abbreviations: BAL: bronchoalveolar lavage; Inf: infection; MMP: matrix metalloproteinase; OCS: oral corticosteroid; RA: refractory asthma; NRA: non-refractory asthma

Introduction

Majority of asthma patients respond well to controller medications such as inhaled corticosteroids and long-acting β -agonists. However, a subset of patients fails to respond to multiple controller medications including omalizumab. Their asthma remains poorly controlled. This group of patients is labeled as having refractory asthma (RA) or severe asthma (1, 2, and 3). These patients experience a high level of morbidity due to the disease. Many of these patients require chronic systemic steroid therapy, which results in severe side effects and adds to the morbidity. Because of heavy utilization of health care services (ER and hospital) RA patients accounts for a disproportionately higher health care cost (2). It is estimated that about 3–10% of patients suffer from severe refractory asthma (2-5). The treatment options for refractory asthma are limited, primarily due to the lack of understanding of its mechanisms.

Asthma is a heterogeneous disease. Based upon clinical and laboratory findings asthma has been variably classified as atopic vs. non-atopic, eosinophilic vs. neutrophilic vs. non-eosinophilic/non-neutrophilic, obesity-associated asthma (reviewed in ref. 3), asthma/COPD overlap syndrome (6), Th2-high (7), Th2/Th17-high (8), and Th2-low (7, 8), etc. Severe and refractory asthma can occur within each category of patients. It is unclear if a single or multiple mechanisms are operative in severe refractory asthma. Previous studies have identified a number of factors in severe asthma. Heightened activation of type 1 (Th1, the interferon pathway) (9), type 2 (Th2 cells, type 2 CD8 T cells) (7, 10), innate response (11) and airway neutrophilia (12) have all been implicated in refractory asthma. Type 3 cytokines—IL17A and IL17F are increased in asthma (8, 13). However, they were not associated with Th17 in human asthma nor were they associated with neutrophilic asthma (13-15), which is unlike what has been reported in the mouse model of asthma (16). Increased epithelial injury, smooth muscle hyperplasia and hypertrophy, and airway remodeling are some of the recognized intrinsic factors of refractory asthma (1, 2). The external factors associated with refractory asthma include heightened exposure to sensitizing allergens (17, 18), air pollution (19, 20) and infections (21-23). Heightened levels of select cytokines/ inflammatory mediators were associated with many of the foregoing subgroups of refractory asthma (23, 24).

Despite the progress in the field there is a need to examine the precise molecular mechanism of heterogeneity of refractory asthma that allows development of targeted therapeutic interventions. To address this matter, we studied 60 patients with refractory asthma (3) and compared them with 30 non-refractory asthma patients and 20 healthy controls. Bronchoalveolar lavage (BAL) and serum were analyzed for 244 biomolecules, which were then correlated with the clinical and laboratory features of asthma. The latter included pulmonary function, PC20 for methacholine, allergic sensitivity, fractional exhaled nitric oxide (FENO), body mass index, blood eosinophils, total IgE, BAL inflammatory cells (eosinophils, neutrophils, lymphocytes and macrophages), indices of airway tissue inflammation (quantitative histologic evaluation of bronchial tissue) and basement thickness, and the result of microbiological studies with BAL, tissue biopsy and airway brushing. The focus of this manuscript is on the neutrophilic phenotype of RA.

Materials and Methods

Human subjects: The RA study subjects were recruited from the outpatient clinics of National Jewish Health. NRA and healthy controls were recruited from the community. The study protocol for bronchoscopy and BAL was approved by the Institutional Review Board (IRB: HS#2639). A written informed consent was obtained from the study subjects. Patients were allowed to continue their routine medication. Asthma patients had a comprehensive phenotypic and laboratory work-up. The latter included full PFT, methacholine test (if FEV1 was above 60% of predicted), chest CT, fractional exhaled nitric oxide (FENO), blood tests for eosinophils, total IgE and IgE antibody (when appropriate), bronchoscopy with BAL, airway brushing and endobronchial biopsy (Online Repository Figure E1A).

Bronchoscopy, bronchoalveolar lavage, brushing, biopsy and other laboratory work-up: The foregoing procedures were performed as described previously (3). BAL was processed within an hour. Cells were isolated by centrifugation. Supernatant fluid was aliquoted into small samples and frozen at -80°C. Total BAL cells and differential counts, a PCR-based respiratory viral panel (12 different respiratory viruses) with BAL cells and bronchial brushing, a PCR-based test for *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* with airway biopsies, brushes and BAL cells, BAL culture in triplicates for bacteria, *Mycobacterium tuberculosis* and non-tuberculous mycobacteria, and a quantitative analysis of airway tissue inflammatory cells including CD117+ mast cells (by immunohistochemical staining) were performed in the NJH clinical lab (ADx-Advanced Diagnostic Lab, NJH).

Multiplex biomolecule analyses of BAL and serum: Aliquots of BAL and serum were analyzed for a total 244 biomolecules (Human Discovery MAP 250+, v2) by Myriad RBM, Inc. (<https://rbm.myriad.com>). A list of the biomolecules analyzed by this method is presented in the online repository table 1.

Pathway analyses: We performed pathway analyses for the 36 biomolecules that distinguished RA from NRA. We used multiple software at the Enrichnet.org website: the Gene Ontology (GO) for biological process and molecular function, Reactome, and KEGG.

Statistical Analyses: Comparison between the study groups was done by Mann-Whitney U test. Comparison among multiple study groups was performed by Kruskal Wallis test followed by Dunn's test in order to correct for multiple comparisons. In addition, we performed Benjamini-Hochberg test for multiple comparisons, which corrects for false discovery. The false discovery rate for this test was set at 0.1. Pearson correlation coefficient was used to calculate correlation coefficient. Statistical analyses were performed by the software Prism (GraphPad.com).

Results

Study populations: We recruited 20 healthy controls, 30 subjects with well controlled asthma and 60 subjects with refractory asthma as defined by American Thoracic Society definition (25). The NRA patients did not meet the criteria for RA as their asthma was well controlled by inhaled corticosteroids and other controllers. Thirty six percent of RA patients (22 of 60) were on systemic steroids (10-60 mg daily). Asthmatic patients with a history of current or past smoking were excluded from the study. The demographic details of the study population are shown in Table 1.

Clinical and phenotypical characteristics: Healthy controls and NRA patients were younger than RA patients (Table 1). BMI was not significantly different among the study groups. RA patients were more atopic (88%) than NRA patients (66%) and healthy controls (55%) as judged by skin test positivity or by the presence of IgE antibody against an environmental allergen. Pulmonary function indices (FVC and FEV1) were significantly lower and reversibility was significantly higher in RA as compared to NRA. There was no difference in PC20 for methacholine between RA and NRA. FENO was not different among the three study groups. Blood eosinophils and total IgE were increased in asthma (RA and NRA) but were not different between RA and NRA. Neither BAL nor airway tissue eosinophils showed any significant difference between NRA and RA groups. In contrast, BAL and airway tissue neutrophils, and BAL lymphocytes were increased in RA. Surprisingly, tissue CD117+ cells, which mostly represent mast cells, were decreased in RA. Subepithelial basement membrane thickness, a sign of airway remodeling, was significantly increased in asthma and the greatest thickness occurring in RA.

Biochemical analyses of BAL: Of the 244 biomolecules assayed by the Human DiscoveryMAPs v2 multiplex assay, 66 were below the detection limit (Online repository Figure E1B). One hundred and one biomolecules did not show any significant difference among the study groups—healthy controls, NRA and RA. Fifty two biomolecules were significantly different either in multi-group comparison or in comparison between RA and NRA (Table 2). Thirty seven of these 52 biomolecules distinguished RA from NRA (Table 2 and Online

repository Figure E1C). Since BAL neutrophils were one of the cell types that distinguished RA from NRA, we focused on neutrophil-associated biomolecules for this study. We identified 17 neutrophil-associated biomolecules that were significantly elevated in RA when compared with NRA and healthy controls (Table-3). Of these 17 biomolecules 13 showed a significant positive correlation with the BAL neutrophil count (Table-4). Four biomolecules did not show any significant correlation. It is noteworthy that only one of these 13 biomolecules, CCL3 (MIP1 β), correlated with BAL eosinophils. We classified these biomolecules based upon their published cellular sources (online repository table 2). This classification suggests that innate immune and airway tissue cells are the primary source of refractory asthma-associated proneutrophilic biomolecules. This also implies that activation of innate immune and airway tissue cells significantly contribute to refractory asthma.

Biochemical analyses of serum: Of the 244 biomolecules assayed 29 were undetectable. One hundred and seventy two biomolecules did not show any difference among the study groups. Fourteen biomolecules were significantly different among all 3 study groups and also between NRA and RA (Table 5). Of these 14 biomolecules 6 of them were also detected in BAL and were different among the study groups. They include ficolin-3, MMP7, thrombospondin-1, apolipoprotein E, human epididymal protein-4 and neuropilin-1. Next, we examined the correlation of these serum biomolecules with BAL inflammatory cells (online repository table 3). Five biomolecules correlated with increased BAL neutrophils: Growth and differentiation factor-15 (GDF-15), human epididymal protein-4, MMP7, tetranectin, and von Willebrand factor. None of these biomolecules correlated with BAL eosinophils or lymphocytes.

Microbial analyses of BAL: BAL fluid from each patient was cultured in triplicates to detect pathogenic microbial organisms. The results were presented as negative or normal respiratory flora, or positive for a pathogenic strain. A total of 24 of 60 RA patients were positive for bacteria and/or a virus. The following bacterial strains were identified in 22 RA patients: methicillin-sensitive *Staphylococcus aureus* (7 patients), methicillin-resistant *Staphylococcus aureus* (1 patient), *Pseudomonas aeruginosa* (5 patients), *Hemophilus*

influenza (1 patient), Hemophilus, not influenza (subspecies not identified, 2 patients), Hemophilus parainfluenza (1 patient), beta hemolytic Streptococci (not group A) (1 patient), alpha hemolytic Streptococci (1 patient), Streptococcus pneumonia (1 patient), Moraxella catarrhalis (1 patient), Serratia marcescens (1 patient) and Capnocytophaga species (1 patient). Note that some patients had more than 1 strain of bacteria. Two patients were positive for viruses only (1 with respiratory syncytial virus and 1 with coronavirus OC43). Four patients with a bacterial isolate were also positive for viruses (3 with rhinovirus and 1 with parainfluenza virus 3). It should be noted that these infections were subclinical in nature as the patients did not report symptoms of acute infection (fever, coryza, pharyngitis, or myalgia). All patients had chest CT. A summary of chest CT findings from patients, who were positive for infection, is shown in online repository table 4. One patient with pseudomonas had consolidation; one patient with Serratia marcescens had infiltrates and 3 patients had ground glass opacity. The rest of the patients did not have any sign of acute infection.

Effect of infection on clinical and laboratory parameters of RA: Next, we examined the effect of infection on cellular and molecular parameters in RA patients. As anticipated, infection increased the frequency of neutrophils more than that of eosinophils in the airways when compared to NRA (Online repository figure E1B). This was associated with a dramatic increase in the neutrophilic granular protein myeloperoxidase (MPO) (Online repository figure E1A) and a number of pro-neutrophilic biomolecules, especially MMP9, thrombospondin-1, IL8, IL1 β , serum amyloid A (SAA), IL6, lipocalin-2, NAP2 and 6Ckine (Online repository figure E1B). Although the foregoing changes were statistically significant for the groups, not all infection+ RA patients had increased BAL neutrophil counts. Of the 24 infection+ RA patients, 15 had elevated neutrophil frequency ($\geq 3\%$, threshold based upon the normal range for the clinical lab) in BAL and 9 patients did not. Ten of these 15 infection+ neutrophilic asthmatic patients had elevated ($\geq 1\%$) eosinophils in BAL. Conversely, 4 of the 9 infection+ but non-neutrophilic patients had elevated eosinophils in BAL.

We compared the BAL neutrophil frequency (%) and absolute neutrophil counts in blood among infection+ and infection- RA and NRA patients. A total of 22 RA patients (10 with infection+ RA and 12 with infection- RA)

were on systemic steroids, which could have a direct effect on blood and BAL neutrophil counts. For this reason we analyzed data with and without the inclusion of patients on oral corticosteroids (OCS). BAL neutrophils were elevated in both infection+ and infection- RA patients regardless of their OCS usage compared to normal controls (Figure 1A). Blood neutrophils were also increased in both infection+ and infection- RA when the data was analyzed for the entire group (Figure 1B). However, this increase was lost in the blood but not BAL (Figure 1C&D) in the infection- RA group when the patients on OCS were excluded from the analysis. The results suggest that blood neutrophilia in infection- RA patients was due to OCS. Strikingly, the absolute neutrophil number remained increased in the infection+ RA group even when the patients on OCS were excluded from the analysis. The results indicate that an increase in absolute neutrophil count could serve as a blood biomarker for airway infection in RA patients. The results also suggest that blood neutrophilia in this subgroup was due to airway infection (Figure 1C).

Asthma is a type 2 immune response disease and eosinophils play an important role. As noted earlier, the median BAL eosinophil number did not increase significantly in RA as compared to NRA. Since infection contributed to RA in a significant number of patients, we reasoned that the former might have affected the eosinophilic influx by favoring neutrophilic influx. For this reason we analyzed BAL eosinophils as well as neutrophils separately in infection+ and infection- RA patients and compared them with NRA patients. The median BAL eosinophil count was 1, 1 and 1 in NRA, Infection+ and infection- RA, respectively. On the other hand the median BAL neutrophil count was 2.2, 8 and 3.2 in NRA, Infection+ and infection- RA, respectively, and difference among the groups was statistically significant ($P=0.02$).

Serum biomolecules that identify neutrophilic asthma in non-infected patients: The subclinical infection was the most plausible explanation for increased airway neutrophils in our infection+ RA patients. The cause of neutrophilic asthma in infection- RA patients was of major interest to us. To this goal we compared serum proneutrophilic biomolecules between infection- and infection+ RA patients (Figure 2). IL1 β , IL6, IL8, CXCL1, and CCL3 were increased in the serum from infection- RA patients as compared to infection+ RA and

NRA patients. Interestingly, the BAL concentrations of these molecules were not increased in the infection-RA patients when compared to infection+ RA patients. The results raised the possibility of a systemic inflammatory process contributing to airway neutrophilia in infection-RA. Next, we compared the levels of C-reactive protein (CRP), soluble ST2 (serum stimulation 2, also known as suppressor of tumorigenicity 2) and GDF-15 (growth and differentiation factor 15), three molecules associated with atherosclerosis, cardiovascular diseases and systemic inflammatory diseases (27, 28). Indeed, the levels of all three molecules were increased in infection- but not in infection+ neutrophilic asthma patients (Figure 3). ESR was similar in both groups. We were concerned about a potential role for inhaled corticosteroids in airway neutrophilia. To this goal we compared inhaled corticosteroid treatment of NRA and RA patients (online repository Table 5). All RA patients were on high dose inhaled steroids and LABA. In contrast, some NRA patients were on low to medium dose inhaled steroids. Thus, it is possible that this difference in the dose of inhaled steroids contributed to airway neutrophilia.

Clinical correlations: Next, we examined the clinical correlation of the airway inflammatory cells and biomolecules. There was a positive correlation ($r=0.49$, $P<0.001$) between the age of the patient and the BAL PMN count. Age also positively correlated with FVC ($r=0.52$, $P<0.05$) and BAL IL1 β ($r=0.67$, $P<0.001$) but not with other proneutrophilic biomolecules including IL6 ($r=0.40$) and IL8 ($r=0.18$). BAL PMN but not BAL eosinophils, lymphocytes or blood eosinophils negatively correlated with FVC% (Online Repository Table 6). CD117+ cells, which largely but not exclusively represent mast cells, did not correlate with any BAL biomolecules. Two BAL biomolecules correlated with two pulmonary function indices. Myoglobin negatively correlated with FVC% whereas prostasin negatively correlated with reversibility and positively with FEV1%. We performed pathway analyses with the 36 BAL biomolecules that distinguished RA from NRA. We compared the pathway results using the Gene Ontology (GO) for biological process and molecular function, Reactome, and KEGG (not shown). The GO analysis identified the most meaningful pathways. The top ones included neutrophil chemotaxis, chemokine receptor signaling, fibroblast growth factor stimulation and cellular response to IL1 (Online Repository Table 7).

Discussion

We showed that RA was associated with an increase in neutrophils and lymphocytes in the airways. As mentioned previously, we specifically focused on neutrophilic asthma in this manuscript. There was a concurrent increase in a multitude of biomolecules that are known to be associated with innate immune and airway tissue response. We identified 51 biomolecules that were increased in asthma and 36 of them distinguished RA from NRA. Sixteen biomolecules correlated with neutrophilic asthma. Vast majority of these biomolecules are known to be produced by macrophages and airway epithelial cells, which indirectly points to their role in neutrophilic asthma. A pathway analysis identified neutrophilic influx as an important biological process in RA. Multiple previous studies demonstrated a similar role for neutrophilic inflammation in severe asthma. Our RA group resembles cluster 5 (neutrophilic) of the SARP cohort (29). In a subsequent study of SARP patients increased neutrophil, and mixed neutrophil and eosinophil counts in the sputum (cluster C & D) were associated with severity of asthma (30). Our RA patients are similar to cluster H (mixed neutrophilic/eosinophilic) of the multinational study by Hinks et al (31). This cluster had severe asthma, which was associated with increased IL6, IL8, MMP3 and MMP8 in the sputum.

One of the weaknesses of this study is that RA patients were significantly older than NRA patients. RA is more prevalent in older patients (4, 5), which led to this unintended recruitment outcome. Increased age is associated with sputum neutrophilia even in non-asthmatic subjects (32). Thus, increased age could affect the quality and quantity of the mediators that are involved in neutrophilic asthma. We used a commercially available multiplex assay service for measurement of biomolecules. Multiplex assays are less sensitive than other highly sensitive assays (e.g. ELISA). This perhaps explains why we did not detect the typical type 2 (e.g. IL4, IL5, and IL13) or type 3 (IL17) cytokines. However, the scope of biomolecules detected by this assay is an advantage.

One outstanding issue related to the heightened production of proneutrophilic biomolecules is what induces their production in RA. We observed subclinical infection (both bacterial and viral) in one third of the RA patients. This subclinical infection could stimulate the production of the proneutrophilic biomolecules. We used standard microbiological cultures to detect bacteria. The sensitivity of these cultures is relatively low when

compared to DNA-based technologies. It is possible that the new technologies will identify additional patients with subclinical infections, which could explain elevated airway neutrophils in the currently classified “infection- patients” (infection negative patients).

The persistence of infection at a subclinical level in a subgroup of patients raises concerns for a defective immune response. This inability to clear infection could be primary or secondary to ongoing treatment with steroids (inhaled steroids in all patients and systemic steroids in 36% of RA patients). The potential role of inhaled steroids in subclinical infection cannot be addressed at this time as their discontinuation in RA will be considered unethical. We detected decreased levels of IL18 and IL22 in RA patients. Low levels of IL18 were previously reported in sputum from asthmatic patients and in patients during rhinovirus infection (33, 34). IL18 and IL22 are important for defense against infection (35, 36). Their decrease could impair clearance of infection. Macrophage scavenging of infected and dead cells is important for resolution of inflammation. Axl receptor tyrosine kinase is specifically expressed on airway macrophages (37). We observed decreased expression of Axl receptor in BAL, which suggested a decreased expression on macrophages and consequently, an impaired scavenger function. The latter could promote persistence of infection. Thrombospondin-1 is a multifunctional and pleiotropic protein, which is increased in RA. It was recently reported to inhibit bactericidal activity of neutrophils and delay the clearance of lung infection (38). Thus, we have identified a number of biomolecules that could promote persistence of subclinical infection in RA.

Systemic glucocorticoids are known to induce blood and tissue neutrophilia (39). Thirty six percent of RA patients was on systemic steroids. Our analysis of patients, who were not on OCS suggested that systemic steroids contributed to blood but not airway neutrophilia in infection- RA patients. Our understanding of the role of inhaled steroids in airway neutrophilia is incomplete. A previous study showed that only systemic but not inhaled steroids were associated with tissue neutrophilia (39). However, this study was done with a small number of patients and the result showed a trend for increased airway neutrophils with inhaled steroids. Both NRA and RA patients were on inhaled steroids in our study. However, unlike RA patients, not all NRA patients

were on maximal doses of inhaled steroids. Thus, it is possible that higher doses of inhaled steroids in RA patients contributed, to some extent, to airway neutrophilia. Environmental toxicants frequently induce cytokines/chemokines that can elicit low level neutrophilic inflammation (40). There could be a cumulative effect during aging. The Th17 immune response induces neutrophilic inflammation in animal models (16). Our assay did not detect IL17, likely due to low sensitivity of the assay. However, the assay detected increased levels of multiple proneutrophilic biomolecules including serum amyloid A (SAA), NAP2, thrombospondin-1, IL6, IL8, C3, CCL3, CCL20, EGF and RAGE. All except the last three significantly correlated with BAL neutrophils. SAA induces G-CSF and stimulates neutrophil generation (41). It also induces Th17 (42). IL6 is an inducer of Th17 (43) and a marker of systemic inflammation (44). NAP2, IL8, C3, and CCL3 directly induce neutrophilic inflammation (45-48). C3 also induces IL1, IL8 and other cytokines/chemokines, which could indirectly affect neutrophilic inflammation (48). Some of our findings on biomolecules are in agreement with many previous studies. Similar to our finding, IL1 β and IL6 discriminated severe from moderate asthma in a SARP (Severe Asthma Research Program of NHLBI) study (25). IL8 has previously been reported to be increased in sputum (49, 50) and BAL (51) from patients with refractory and neutrophilic asthma.

Although the existence of neutrophilic asthma in the absence of infection has been known for many years, its mechanism is poorly understood. Our study identified a unique subgroup of infection- neutrophilic RA, which was selectively associated with increased markers of inflammation in the serum. These markers included IL1 β , IL6, IL8, CXCL1, and CCL3. The concomitant increase in CRP in the same patients suggested a systemic inflammatory process akin to what has been observed in atherosclerotic cardiovascular diseases (52) and autoimmune inflammatory diseases (53). Remarkably, this subgroup of patients also had elevated two additional cardiovascular risk factors— GDF15 (54) and soluble ST2 (IL33 receptor) (52). We recognize that the latter is a non-specific biomarker of systemic inflammation. Four of 36 (11%) infection- neutrophilic RA and 5 of 24 (21%) infection+ neutrophilic RA patients had a history of cardiovascular disease. Therefore, the frequency of existing cardiovascular disease did not explain the systemic (serum) increase in these inflammatory cytokines. We also did not find any correlation of these inflammatory cytokines with other common comorbidities—

diabetes and autoimmune disorders. There are multiple known mechanisms for induction of IL1, IL6, IL8, CXCL1 and CCL3. This constellation of inflammatory cytokines also constitutes the senescence associated secretory product (SASP) (55). Senescence is a biological process that defends against DNA-damage induced carcinogenesis but, by producing multiple cytokines, contributes to systemic inflammation (56). Recently, GDF15 has been shown to induce cellular senescence (57). The correlation of neutrophilic asthma with age also raises the possibility that senescence contributed to the increased proneutrophilic cytokine profile in the serum. Additional studies will be needed to establish the relationship between cellular senescence and predisposition to neutrophilic refractory asthma.

It should be noted that 37% of infection+ RA patients did not have neutrophilia in BAL, which further underscores the notion that this was a subclinical infection. Nearly half of them had elevated eosinophils in BAL suggesting a type 2 immuno-dominant asthma. Type 2 cytokines inhibit IL8 and other proneutrophilic cytokines (58), which could explain lack of neutrophilia. However, the absence of neutrophilia in non-eosinophilic infection+ RA patients suggests a defective immune response or perhaps an active immune evasion by the microbial organism.

Of the 14 serum biomolecules that were different among the study groups only 5 biomolecules overlapped with those from the BAL. However, the pattern of change was different from that in BAL. Four of these biomolecules showed a reduced expression in NRA but not in RA when compared to healthy controls. The reason for this reduced expression only in NRA but not RA is not clear. Ficolin-3 is the only biomolecule in the serum that distinguished RA from NRA. Ficolin-3 is a recognition molecule of the lectin pathway of the complement system. Its deficiency is associated with increased infection (59).

If subclinical infection is one of the mechanisms of RA in a subgroup of patients, its importance can be tested through targeted and bacterial strain specific antibiotic therapy. We are aware that antibiotic therapy of RA patients produced mixed results in the past (60, 61). The failure of antibiotic therapy in some studies could be due to inappropriate selection of the study patients (e.g. inclusion of patients who did not have any infection)

and use of an inappropriate antibiotic (3). Note that we detected both Gram+ and Gram- bacteria as well as viruses in RA patients. The results underscore the importance of the selection of an appropriate antibiotic (targeting Gram+ vs Gram- bacteria) and its application to a properly identified target patient population. If a low level systemic inflammation is indeed the cause of airway neutrophilia in infection- RA, this hypothesis can be tested using therapeutic agents targeting molecules such as IL1, IL6 and IL8. Some of these therapeutic agents are already in use in rheumatologic conditions.

The number of CD117+ cells representing mostly mast cells was reduced in RA. Reduced number of tryptase+ cells representing mast cells in severe asthma was previously reported in a SARP study (62). This was thought to be due to the loss of tryptase as a consequence of mast cell degranulation. However, unlike tryptase, CD117 is a cell surface marker, not a granular protein, and is not expected to be affected by mast cell degranulation. It is possible that the reduced CD117+ cell number is a consequence of the underlying disease process.

Figure legends

Figure 1. Comparison of blood and BAL neutrophils among infection+ and infection- refractory asthma patients (RA) and non-refractory asthma (NRA) patients including and excluding RA patients on orals corticosteroids (OCS).

Figure 2. Comparison of biomolecule levels between serum and BAL among infection+ (Inf+) RA, infection- (Inf-) RA and NRA patients.

Figure 3. Comparison of systemic inflammatory markers in the serum from infection+ (Inf+) RA, infection- (Inf-) RA and NRA.

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Table 1. Demographic, Clinical and Laboratory Features of the Study Groups

Variable	Healthy Controls (N=20)		Non-refractory Asthma (N=30)		Refractory Asthma (N=60)		P for all groups	P Non-refractory vs refractory
	Mean	Median	Mean	Median	Mean	Median		
Age	33.6±2	31	38±2	35.5	54.5±1	57	<0.0001	<0.0001
F/M	13/7		19/11		36/24			
BMI	29.7±1.2	29.8	28±1.1	27.4	29.7±0.7	29.4	0.12	0.051
Atopy	50%		66%		88%			
FVC (L)	4.1±0.1	4.3	3.6±0.1	3.5	2.7±0.1	2.5	<0.0001	<0.0001
FEV1 (L)	3.3±0.1	3.4	2.6±0.1	2.7	1.8±0.09	1.6	<0.0001	<0.0001
FVC (%)	101±2.9	97.5	92±2.5	88	71±2	71	<0.0001	<0.0001
FEV1 (%)	96.5±2.6	95	78±3	74	60±2.5	55	<0.0001	<0.0001
Reversibility (%)	5.2±0.5	5.5	17.6±2.1	17	22±2.5	16	<0.0001	<0.0001
PC20 for methacholine	25±0	25	2.5±0.5 (N=29 of 30)	1	3.4±1.1 (N=23 of 60)	1.5	<0.0001	0.19
FENO	23±3	21	35±3	28.7	43.8±6	22.5	0.17	0.55
IgE (kU/L)	47±9	33	333±199	106	406±149	116	<0.001	0.6
Blood Eos/uL	150±21	100	283±28	300	352±43	300	<0.0001	0.4
Blood PMN K/uL	4.0±0.2	3.6	3.6±0.3	3.2	6.5±0.4	5.1	0.0001	0.0001
BAL WBC (x10 ⁶)	10.2±0.8	9.4	8.7±0.7	8.7	15.8±2.7	8	0.6	0.5
BAL Eos % (total x 10 ⁶)	0.48 ±0.1 (0.04±0.01)	0.2 (0.022)	1.4±0.2 (0.11±0.02)	1 (0.079)	2.6±0.6 (0.32±0.09)	1 (0.042)	0.07 (0.19)	0.3 (0.6)
BAL PMN % (total x 10 ⁶)	1.9±0.2 (0.19±0.02)	1.8 (0.16)	2.5±0.4 (0.22±0.05)	2 (0.15)	10.9±2.4 (3.12±1.8)	4.5 (0.40)	0.002 (0.003)	0.01 (0.004)
BAL Lymph % (total x10 ⁶)	6±0.7 (0.61±0.08)	5.2 (0.45)	6.4±1.1 (0.55±0.12)	4.6 (0.30)	11.3±1.3 (1.43±0.27)	8 (0.60)	0.08 (0.2)	0.03 (0.1)
BAL macrophages % (total x10 ⁶)	91.5±0.9 (9.42±0.7)	92.8 (8.8)	89.7±1.2 (7.87±0.6)	91.8 (7.6)	75.2±2.8 (10.8±1.7)	82.5 (6.3)	0.001 (0.3)	0.006 (0.7)
Biopsy Eos/HPF	1.2±0.6	0	6.1±4.3	0	10.2±3.4	0	0.6	0.4
Biopsy PMN/HPF	0.7±0.1	0.5	0.4±0.2	0	2.3±2	0	0.0001	0.09
Biopsy CD117+ cells	3.8±1.3	1	4.9±1	3	1.5±0.3	0	0.002	0.001
BM thickness	6.4±0.6	6	7.5±0.6	7.2	8.8±0.4	8	0.01	0.04
Serum vit D	28.4±3	26.4	37.5±2.6	34.7	36.5±2.8	34.6	0.056	0.4

Table 2. Biomolecules that are statistically different among the study groups

Biomolecules	Healthy (N=20)	Non-refractory (N=40)	<i>P</i> vs Healthy	Refractory (N=60)	<i>P</i> vs healthy	<i>P</i> vs non-refractory	<i>P</i> for multigroup analyses
1. Alpha-1 anti-trypsin* (µg/ml)	0.4	0.7	NS	1.5	0.009	0.01	0.005
2. Angiogenin (ng/ml)	0.63	0.6	NS	0.85	0.002	0.003	0.007
3. Apolipoprotein C III (µg/ml)	0.01	0.018	NS	0.027	0.02	NS	NS
4. Apolipoprotein E (ng/ml)	0.1	0.95	0.008	1.5	0.001	NS	0.001
5. Axl receptor (ng/ml)	1.2	0.6	<0.001	0.39	0.001	NS	0.004
6. C3 (ng/ml)	0.34	0.33	NS	0.66	0.04	0.03	0.03
7. CCL3 (MIP1β) (pg/ml)	11	12	NS	23	0.007	0.002	0.0001
8. CCL5(RANTES) (pg/ml)	6.8	7.2	NS	12	0.009	0.03	0.01
9. CCL20 (MIP3α) (pg/ml)	19	16	NS	31	NS	0.02	NS
10. CCL21 (pg/ml)	3.8	4	NS	5.1	NS	0.01	0.02
11. CXCL9 (MIG) (pg/ml)	69	79	NS	138	NS	0.02	NS
12. EGF (pg/ml)	1.1	1.3	NS	2.6	NS	0.01	0.03
13. Ferritin (ng/ml)	6.1	5.5	NS	9.8	0.01	0.03	0.01
14. Ficolin-3 (ng/ml)	8.2	6.5	NS	11	0.03	0.001	0.002
15. Human Epididymal protein-4 (pM)	5000	3820	NS	4980	NS	0.01	0.03
16. IL1β (pg/ml)	0	0	NS	0	0.07	0.01	0.01
17. IL6 (pg/ml)	0	0	0.01	0	NS	0.005	0.003
18. IL8 (pg/ml)	11	11	NS	24	0.01	0.002	0.0005
19. IL18 (pg/ml)	120	34	0.03	14	0.007	0.003	0.001
20. IL22 (pg/ml)	170	100	0.001	59	<0.001	0.001	<0.001
21. Kallikrein-7 (pg/ml)	0	0	NS	145	NS	0.006	0.002
22. Lipocalin-2 (ng/ml)	65	82	NS	111	0.05	0.04	0.04
23. MIF (pg/ml)	0.39	1.5	0.0007	1.3	0.0016	NS	0.004
24. MMP3 (ng/ml)	0	0	NS	0.01	<0.001	<0.001	<0.001
25. MMP7 (ng/ml)	0.9	0.6	0.001	1.4	NS	0.04	0.04
26. MMP9, total (ng/ml)	2.9	4.3	NS	20	<0.001	<0.001	<0.001
27. Myeloperoxidase (ng/ml)	51	46	NS	133	0.0002	<0.0001	<0.0001
28. Myoglobin (ng/ml)	0.57	0.94	0.03	0.91	0.04	NS	NS
29. NAP2 (pg/ml)	0.41	0.79	NS	1.7	0.01	0.02	0.001
30. Neuropilin-1 (pg/ml)	980	560	0.001	580	<0.001	NS	0.001
31. Osteoprotegerin (pM)	0	0	NS	0	NS	0.006	0.02
32. PAI-1 (pg/ml)	42	41	NS	11	<0.001	<0.001	<0.001
33. Pepsinogen I (ng/ml)	0.064	0.06	NS	0.093	0.007	<0.001	0.001
34. Pigment epithelium-derived factor (ng/ml)	3.2	6.3	NS	11	0.02	0.006	0.005

Biomolecules	Healthy (N=20)	Non-refractory (N=40)	<i>P vs Healthy</i>	Refractory (N=60)	<i>P vs healthy</i>	<i>P vs non-refractory</i>	<i>P for multigroup analyses</i>
35. Progranulin (ng/ml)	13	5.2	<i>0.01</i>	4	<i>0.03</i>	<i>NS</i>	<i>0.007</i>
36. Prostatin (ng/ml)	520	309	<i>0.01</i>	278	<i>0.001</i>	<i>NS</i>	<i>0.005</i>
37. RAGE	0.3	1	<i>NS</i>	6.5	<i><0.001</i>	<i><0.001</i>	<i><0.001</i>
38. sCD25 (pg/ml)	69	39	<i>NS</i>	29	<i>0.003</i>	<i>0.01</i>	<i>0.001</i>
39. sCD40 (ng/ml)	0.093	0.056	<i>0.008</i>	0.05	<i>0.002</i>	<i>NS</i>	<i>0.03</i>
40. Serum Amyloid A (ng/ml)	0.51	0.57	<i>NS</i>	2	<i>0.0002</i>	<i>0.001</i>	<i>0.003</i>
41. sICAM (pg/ml)	30	19	<i>0.04</i>	16	<i>0.004</i>	<i>NS</i>	<i>0.02</i>
42. sIL1 receptor (pg/ml)	65	26	<i>0.01</i>	22	<i>0.003</i>	<i>NS</i>	<i>0.008</i>
43. Sortilin (ng/ml)	0	0.05	<i>NS</i>	0.12	<i>0.01</i>	<i>0.01</i>	<i>0.008</i>
44. SP-D* (ng/ml)	50	39	<i>NS</i>	35.5	<i>0.01</i>	<i>NS</i>	<i>0.04</i>
45. Thrombospondin-1 (ng/ml)	0	1.4	<i>NS</i>	4.4	<i><0.001</i>	<i><0.001</i>	<i><0.001</i>
46. TIMP-1 (ng/ml)	1.3	1.8	<i>NS</i>	3.1	<i>0.004</i>	<i>0.003</i>	<i>0.001</i>
47. TNFSF12 (pg/ml)	50	30	<i>NS</i>	20	<i>0.006</i>	<i>0.01</i>	<i>0.001</i>
48. TNFSF13 (pg/ml)	18	4.3	<i>0.002</i>	1.3	<i>0.001</i>	<i>NS</i>	<i>0.001</i>
49. Trefoil factor-3 (µg/ml)	0.1	0.22	<i>NS</i>	0.42	<i>0.004</i>	<i>0.004</i>	<i>0.006</i>
50. VEGF (pg/ml)	364	292	<i>NS</i>	177	<i>0.001</i>	<i>NS</i>	<i>0.008</i>
51. Vit D-binding protein* (µg/ml)	0.34	0.15	<i>0.002</i>	0.2	<i>0.01</i>	<i>NS</i>	<i>0.008</i>
52. vWF (µg/ml)	0.004	0.008	<i>NS</i>	0.012	<i>0.003</i>	<i>0.002</i>	<i>0.009</i>

*Steroid-sensitive

NS: not significant

Statistical results are Italicized

Table 3. Neutrophil-associated biomolecules that are statistically different among the study groups

Biomolecules	Healthy (N=20)	Non-refractory (N=40)	<i>P vs Healthy</i>	Refractory (N=60)	<i>P vs healthy</i>	<i>P vs non-refractory</i>	<i>P for multigroup analyses</i>
1. C3 (ng/ml)	0.34	0.33	<i>NS</i>	0.66	<i>0.06</i>	<i>0.03</i>	<i>0.03</i>
2. CCL3 (MIP1 β) (pg/ml)	11	12	<i>NS</i>	23	<i>0.007</i>	<i>0.002</i>	<i>0.0001</i>
3. CCL20 (MIP3 α) (pg/ml)	19	16	<i>NS</i>	31	<i>NS</i>	<i>0.02</i>	<i>0.05</i>
4. EGF (pg/ml)	1.1	1.3	<i>NS</i>	2.6	<i>NS</i>	<i>0.01</i>	<i>0.03</i>
5. Ficolin-3 (ng/ml)	8.2	6.5	<i>NS</i>	11	<i>0.03</i>	<i>0.001</i>	<i>0.002</i>
6. IL1 β (pg/ml)	0	0	<i>NS</i>	0	<i>0.07</i>	<i>0.01</i>	<i>0.01</i>
7. IL6 (pg/ml)	0	0	<i>0.01</i>	0	<i>NS</i>	<i>0.005</i>	<i>0.003</i>
8. IL8 (pg/ml)	11	11	<i>NS</i>	24	<i>0.01</i>	<i>0.002</i>	<i>0.0005</i>
9. Lipocalin-2 (ng/ml)	65	82	<i>NS</i>	111	<i>0.05</i>	<i>0.04</i>	<i>0.04</i>
10. MMP7 (ng/ml)	0.68	0.6	<i>NS</i>	1.6	<i>0.002</i>	<i>0.0003</i>	<i>0.0003</i>
11. MMP9, total (ng/ml)	2.9	4.3	<i>NS</i>	20	<i><0.001</i>	<i><0.001</i>	<i><0.001</i>
12. Myeloperoxidase (ng/ml)	51	46	<i>NS</i>	133	<i>0.0002</i>	<i><0.0001</i>	<i><0.0001</i>
13. NAP2 (pg/ml)	0.41	0.79	<i>NS</i>	1.7	<i>0.01</i>	<i>0.02</i>	<i>0.001</i>
14. RAGE	0.3	1	<i>NS</i>	6.5	<i><0.001</i>	<i><0.001</i>	<i><0.001</i>
15. Serum Amyloid A (ng/ml)	0.51	0.57	<i>NS</i>	2	<i>0.0002</i>	<i>0.001</i>	<i>0.003</i>
16. Thrombospondin-1 (ng/ml)	0	1.4	<i>NS</i>	4.4	<i><0.001</i>	<i><0.001</i>	<i><0.001</i>
17. TIMP-1 (ng/ml)	1.3	1.8	<i>NS</i>	3.1	<i>0.004</i>	<i>0.003</i>	<i>0.001</i>

NS: not significant

Statistical results are Italicized

Table 4. Correlation of BAL biomolecules with inflammatory and other biological processes

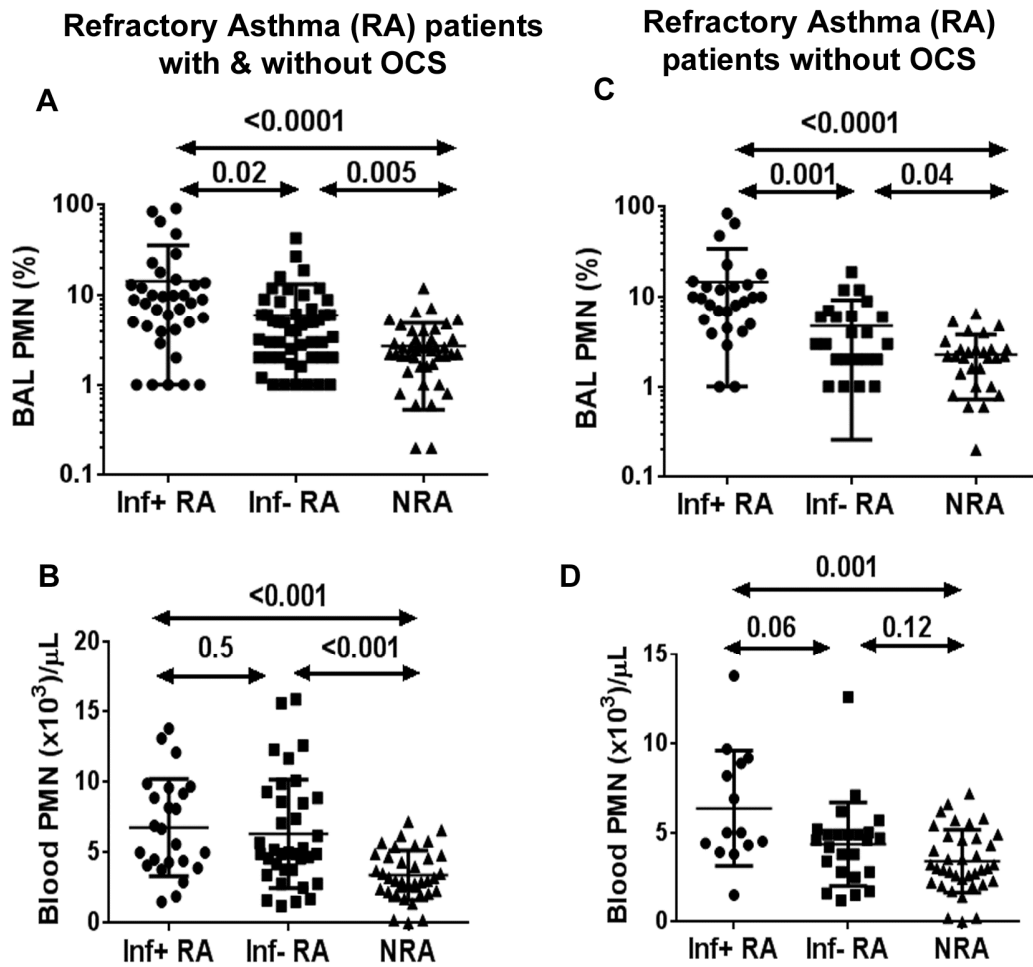
Biomolecules	Correlation with BAL neutrophils (r)	P	Correlation with BAL eosinophils (r)	P	Correlation with BAL lymphocytes (r)	P
Group 1. Biomolecules correlated with neutrophils (11)						
1. Lipocalin-2	0.86	<0.001	0.13	NS	-0.15	NS
2. Serum Amyloid A	0.70	<0.001	0.25	NS	0.002	NS
3. CXCL7 (NAP2)	0.65	<0.001	0.07	NS	0.002	NS
4. IL8	0.62	<0.001	-0.04	NS	0.06	NS
5. <i>Thrombospondin-1</i>	0.60	<0.001	0.06	NS	0.15	NS
6. IL1 β	0.58	<0.001	0.05	NS	0.03	NS
7. <i>Ficolin-3</i>	0.57	0.001	0.18	NS	0.32	0.03
8. IL6	0.52	<0.001	0.07	NS	0.17	NS
9. PAI	0.46	0.003	-0.02	NS	-0.02	NS
10. MMP7	0.41	0.01	0.12	NS	0.16	NS
11. MMP9, total	0.37	0.003	0.05	NS	-0.07	NS
Group 2. Biomolecules correlated with multiple inflammatory cells (2)						
1. C3	0.48	<0.001	0.11	NS	0.45	0.002
2. CCL3 (MIP1 β)	0.36	0.006	0.41	0.001	0.19	NS
Group 3. Known proneutrophilic biomolecules not correlated with neutrophils (4)						
1. CCL20	0.12	NS	0.09	NS	0.05	NS
2. EGF	-0.01	NS	0.13	NS	0.01	NS
3. RAGE	-0.01	NS	0.13	NS	0.08	NS
4. TIMP-1	0.21	NS	0.23	NS	0.36	NS

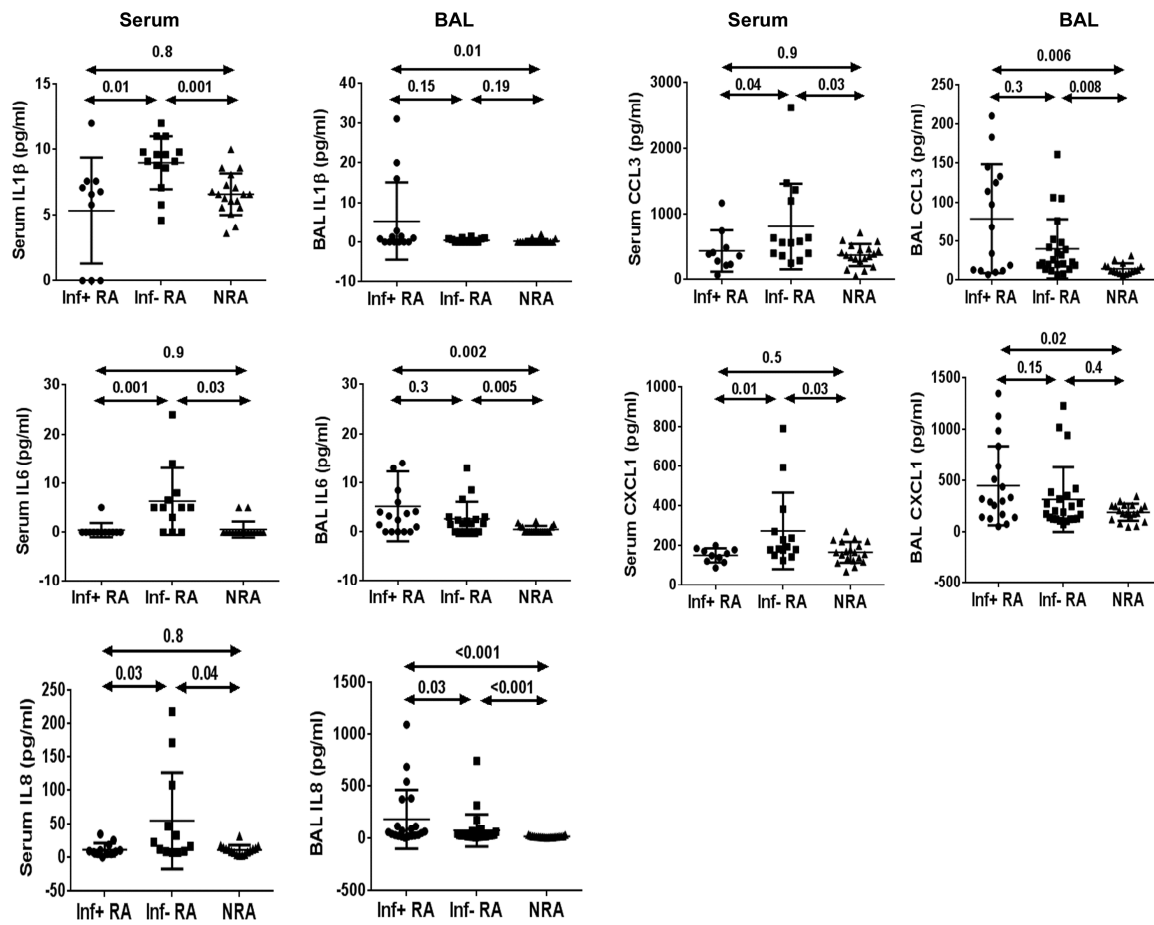
Italicized biomolecules are also changed in the serum

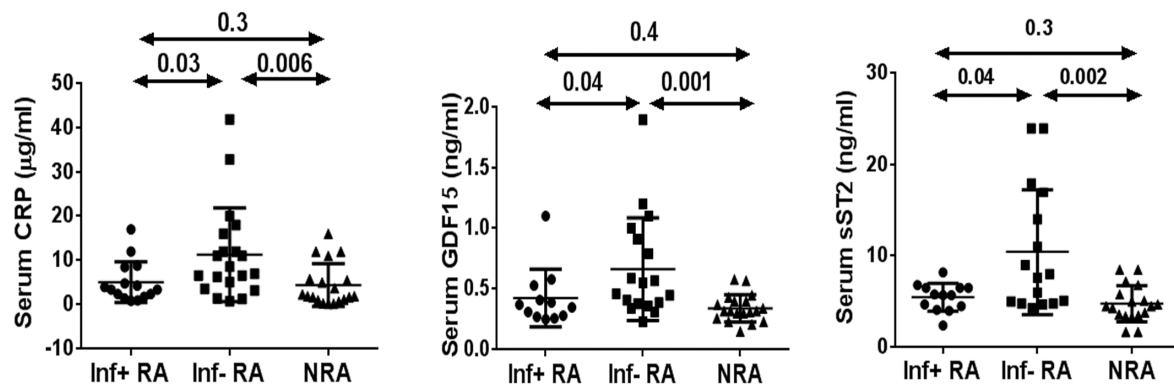
Table 5. Serum biomolecules that are statistically different among the study groups

Biomolecules (total 14)	Healthy (N=20)	Non-refractory (N=40)	P vs Healthy	Refractory (N=60)	P vs healthy	P vs non-refractory	P for multigroup analyses
<i>Proinflammatory (7)</i>							
1. CCL18 (PARC) (ng/ml)	105	97	NS	146	0.006	0.0006	0.0006
2. Cathepsin D	569	480	0.02	585	NS	<0.0001	0.004
3. <i>Ficolin-3</i> (μ g/ml)	21.5	21	NS	29	NS	0.002	0.002
4. GDF-15 (ng/ml)	0.33	0.32	NS	0.40	NS	0.02	0.03
5. Thrombin-activable Fibrinolysis (μ g/ml)	11.8	8.6	0.008	11	NS	0.005	0.005
6. von Willebrand Factor (μ g/ml)	109.5	61	0.08	120	NS	<0.0001	0.0002
7. YKL-40 (ng/ml)	40	27	NS	42	NS	0.003	0.008
<i>Remodeling/Regeneration/Angiogenesis (4)</i>							
1. <i>MMP7</i> (ng/ml)	5.7	4.1	0.03	5.1	NS	0.002	0.009
2. Tenascin-C (ng/ml)	493	400	NS	733	0.009	0.003	0.002
3. Tetranectin (μ g/ml)	22	20	NS	18	0.002	0.02	0.005
4. <i>Thrombospondin-1</i> (μ g/ml)	14.7	12	0.01	14	NS	0.001	0.004
<i>Anti-inflammatory (3)</i>							
1. <i>Apolipoprotein E</i> (ng/ml)	65	56	NS	65	NS	0.01	0.07
2. <i>Human Epididymal protein-4</i> (pM)	690	555	0.07	787	NS	0.002	0.005
3. <i>Neuropilin-1</i> (pg/ml)	248	177	0.005	214	0.003	0.006	0.003

Italicized variables were also altered in BAL samples







Legends for the Online Repository Figures

Online Repository Figure 1. Study design and an overview of the results. A: A schematic presentation of the study design. B: The inserted table summarizes the number of biomolecules studied, not detected, changed and unchanged in BAL and serum from the study subjects. C: The figure shows the number of biomolecules in the BAL that were significantly ($P < 0.05$) different among the study populations.

Online Repository Figure 2. Fold increase in biomolecules in refractory asthma as compared to non-refractory asthma (A & B). Refractory asthma patients were subgrouped as having infection (infection+, black bars) or no infection (infection-, grey bars). The results in Figure A were separated from the results in Figure B because of the higher level of increase (see labels in X-axis).

Online Repository Table 1 List of Biomolecules

Analytes	Units	Myriad RBM LDD	Myriad RBM LLOQ
6Ckine	pg/mL	8.2	15
Adiponectin	ug/mL	0.000086	0.00030
Agouti-Related Protein (AgRP)	pg/mL	24	23
Aldose Reductase	ng/mL	0.41	0.67
Alpha-1-Antichymotrypsin (AACT)	ug/mL	0.70	1.4
Alpha-1-Antitrypsin (AAT)	mg/mL	0.000000038	0.000000082
Alpha-1-Microglobulin (A1Micro)	ug/mL	0.000014	0.000025
Alpha-2-Macroglobulin (A2Macro)	mg/mL	0.000098	0.000087
Alpha-Fetoprotein (AFP)	ng/mL	0.014	0.11
Amphiregulin (AR)	pg/mL	131	199
Angiogenin	ng/mL	0.016	0.0081
Angiopoietin-2 (ANG-2)	ng/mL	0.048	0.048
Angiotensin-Converting Enzyme (ACE)	ng/mL	0.019	0.035
Angiotensinogen	ng/mL	0.051	0.092
Apolipoprotein(a) (Lp(a))	ug/mL	0.00020	0.00091
Apolipoprotein A-I (Apo A-I)	mg/mL	0.000000016	0.00000006
Apolipoprotein A-II (Apo A-II)	ng/mL	0.000011	0.000014
Apolipoprotein A-IV (Apo A-IV)	ug/mL	0.075	0.16
Apolipoprotein B (Apo B)	ug/mL	0.00044	0.0021
Apolipoprotein C-I (Apo C-I)	ng/mL	0.0000062	0.000022
Apolipoprotein C-III (Apo C-III)	ug/mL	0.0000024	0.000011
Apolipoprotein D (Apo D)	ug/mL	0.020	0.037
Apolipoprotein E (Apo E)	ug/mL	0.00089	0.0024
Apolipoprotein H (Apo H)	ug/mL	0.000015	0.000062
AXL Receptor Tyrosine Kinase (AXL)	ng/mL	0.0058	0.010
B cell-activating factor (BAFF)	pg/mL	1.5	3.7
B Lymphocyte Chemoattractant (BLC)	pg/mL	9.8	12
Beta-2-Microglobulin (B2M)	ug/mL	0.000045	0.000051
Betacellulin (BTC)	pg/mL	19	27
Brain-Derived Neurotrophic Factor (BDNF)	ng/mL	0.0046	0.0072
C-Peptide	ng/mL	0.0041	0.0030
C-Reactive Protein (CRP)	ug/mL	0.0000034	0.0000078
Calbindin	ng/mL	0.53	0.90
Cancer Antigen 125 (CA-125)	U/mL	0.45	0.76
Cancer Antigen 15-3 (CA-15-3)	U/mL	0.055	0.090
Cancer Antigen 19-9 (CA-19-9)	U/mL	2.1	1.4
Cancer Antigen 72-4 (CA 72-4)	U/mL	6.5	4.0
Carcinoembryonic Antigen (CEA)	ng/mL	0.028	0.037
Cathepsin D	ng/mL	0.23	0.18
CD5 Antigen-like (CD5L)	ng/mL	0.0056	0.0081
CD 40 antigen (CD40)	ng/mL	0.0036	0.0031
CD40 Ligand (CD40-L)	ng/mL	0.0037	0.0033
Cellular Fibronectin (cFib)	ug/mL	0.0089	0.042
Chemokine CC-4 (HCC-4)	ng/mL	0.0066	0.0095

Analytes	Units	Myriad RBM LDD	Myriad RBM LLOQ
Chromogranin-A (CgA)	ng/mL	5.0	2.6
Ciliary Neurotrophic Factor (CNTF)	pg/mL	4.5	19
Clusterin (CLU)	ug/mL	0.0013	0.0024
Collagen IV	ng/mL	1.2	1.9
Complement C3 (C3)	mg/mL	0.000000016	0.0000001
Complement Factor H – Related Protein 1 (CFHR1)	ug/mL	0.00076	0.0011
Cortisol (Cortisol)	ng/mL	1.2	2.0
Creatine Kinase-MB (CK-MB)	ng/mL	0.035	0.070
Cystatin-C	ng/mL	0.011	0.0075
E-Selectin	ng/mL	0.033	0.041
EN-RAGE	ng/mL	0.0036	0.0076
Endoglin	ng/mL	0.0023	0.0041
Endostatin	ng/mL	0.014	0.054
Eotaxin-1	pg/mL	1.9	29
Eotaxin-2	pg/mL	4.7	7.6
Eotaxin-3	pg/mL	16	31
Epidermal Growth Factor (EGF)	pg/mL	0.78	5.8
Epidermal Growth Factor Receptor (EGFR)	ng/mL	0.031	0.058
Epiregulin (EPR)	pg/mL	3.7	14
Epithelial cell adhesion molecule (EpCam)	pg/mL	16	80
Epithelial-Derived Neutrophil-Activating Protein 78 (ENA-78)	ng/mL	0.0030	0.017
Ezrin	ng/mL	2.5	3.6
Factor VII	ng/mL	1.2	0.77
Fas Ligand (FasL)	pg/mL	2.6	5.3
FASLG Receptor (FAS)	ng/mL	0.47	1.2
Fatty Acid-Binding Protein, adipocyte (FABP, adipocyte)	ng/mL	0.023	0.049
Fatty Acid-Binding Protein, heart (FABP, heart)	ng/mL	1.0	3.0
Fatty Acid-Binding Protein, liver (FABP, liver)	ng/mL	2.8	4.8
Ferritin (FRTN)	ng/mL	0.013	0.021
Fetuin-A	ug/mL	0.00034	0.00062
Fibrinogen	mg/mL	0.00000022	0.00000024
Fibroblast Growth Factor 4 (FGF-4)	pg/mL	123	87
Fibroblast Growth Factor basic (FGF-basic)	pg/mL	5.9	6.9
Fibulin-1C (Fib-1C)	ug/mL	0.00015	0.00015
Follicle-Stimulating Hormone (FSH)	mIU/mL	0.11	0.13
Galectin-3	ng/mL	0.071	0.074
Gelsolin	ug/mL	0.00034	0.00072
Glucagon	pg/mL	31	64
Glucagon-like Peptide 1, active (GLP-1 active)	pg/mL	1.4	2.6
Glucagon-like Peptide 1, total (GLP-1 total)	pg/mL	0.89	1.1
Glucose-6-phosphate Isomerase (G6PI)	ng/mL	0.062	0.12
Glutathione S-Transferase alpha (GST-alpha)	ng/mL	0.29	0.59
Glutathione S-Transferase Mu 1 (GST-M1)	ng/mL	0.58	1.3
Granulocyte Colony-Stimulating Factor (G-CSF)	pg/mL	0.73	1.1

Analytes	Units	Myriad RBM LDD	Myriad RBM LLOQ
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	pg/mL	3.2	18
Growth Hormone (GH)	ng/mL	0.0054	0.030
Growth-Regulated alpha protein (GRO-alpha)	pg/mL	0.36	0.94
Haptoglobin	mg/mL	0.00000012	0.00000032
HE4	pM	16	14
Heat Shock Protein 60 (HSP-60)	ng/mL	5.3	7.4
Heparin-Binding EGF-Like Growth Factor (HB-EGF)	pg/mL	9.2	43
Hepatocyte Growth Factor (HGF)	ng/mL	0.33	0.20
Hepatocyte Growth Factor receptor (HGF receptor)	ng/mL	0.055	0.079
Hepsin	pg/mL	15	14
Human Chorionic Gonadotropin beta (hCG)	mIU/mL	0.24	0.45
Human Epidermal Growth Factor Receptor 2 (HER-2)	ng/mL	0.0029	0.0060
Immunoglobulin A (IgA)	mg/mL	0.000000035	0.00000028
Immunoglobulin E (IgE)	U/mL	0.43	3.6
Immunoglobulin M (IgM)	mg/mL	0.00000036	0.00000047
Insulin	uIU/mL	0.0035	0.043
Insulin-like Growth Factor-Binding Protein 1 (IGFBP-1)	ng/mL	0.11	0.19
Insulin-like Growth Factor-Binding Protein 2 (IGFBP-2)	ng/mL	0.57	0.48
Insulin-like Growth Factor-Binding Protein 3 (IGFBP-3)	ng/mL	1.2	3.2
Insulin-like Growth Factor Binding Protein 4 (IGFBP4)	ng/mL	0.19	0.30
Insulin-like Growth Factor Binding Protein 5 (IGFBP5)	ng/mL	1.3	1.9
Insulin-like Growth Factor Binding Protein 6 (IGFBP6)	ng/mL	0.65	0.77
Intercellular Adhesion Molecule 1 (ICAM-1)	ng/mL	0.35	0.26
Interferon gamma (IFN-gamma)	pg/mL	0.49	0.30
Interferon gamma Induced Protein 10 (IP-10)	pg/mL	2.9	25
Interferon-inducible T-cell alpha chemoattractant (ITAC)	pg/mL	4.0	4.2
Interleukin-1 alpha (IL-1 alpha)	ng/mL	0.00030	0.00040
Interleukin-1 beta (IL-1 beta)	pg/mL	0.30	0.57
Interleukin-1 receptor antagonist (IL-1ra)	pg/mL	9.9	19
Interleukin-2 (IL-2)	pg/mL	1.0	1.7
Interleukin-2 receptor alpha (IL-2 receptor alpha)	pg/mL	25	18
Interleukin-3 (IL-3)	ng/mL	0.0011	0.0032
Interleukin-4 (IL-4)	pg/mL	4.4	5.9
Interleukin-5 (IL-5)	pg/mL	1.0	2.6
Interleukin-6 (IL-6)	pg/mL	0.84	2.2
Interleukin-6 receptor (IL-6r)	ng/mL	0.0027	0.0054
Interleukin-6 receptor subunit beta (IL-6R beta)	ng/mL	0.023	0.049
Interleukin-7 (IL-7)	pg/mL	2.1	1.8
Interleukin-8 (IL-8)	pg/mL	0.63	0.79
Interleukin-10 (IL-10)	pg/mL	1.1	1.4
Interleukin-12 Subunit p40 (IL-12p40)	ng/mL	0.016	0.055
Interleukin-12 Subunit p70 (IL-12p70)	pg/mL	5.9	9.7
Interleukin-13 (IL-13)	pg/mL	0.63	1.2

Analytes	Units	Myriad RBM LDD	Myriad RBM LLOQ
Interleukin-15 (IL-15)	ng/mL	0.12	0.079
Interleukin-16 (IL-16)	pg/mL	4.3	17
Interleukin-17 (IL-17)	pg/mL	1.2	0.99
Interleukin-18 (IL-18)	pg/mL	3.7	8.3
Interleukin-23 (IL-23)	ng/mL	0.12	0.080
Kallikrein 5	ng/mL	0.045	0.14
Kallikrein-7 (KLK-7)	pg/mL	90	67
Kidney Injury Molecule-1 (KIM-1)	ng/mL	0.0071	0.0030
Lactoylglutathione lyase (LGL)	ng/mL	0.039	0.18
Latency-Associated Peptide of Transforming Growth Factor beta 1 (LAP TGF-b1)	ng/mL	0.0092	0.015
Lectin-Like Oxidized LDL Receptor 1 (LOX-1)	ng/mL	0.16	0.18
Leptin	ng/mL	0.011	0.027
Luteinizing Hormone (LH)	mIU/mL	0.042	0.15
Macrophage Colony-Stimulating Factor 1 (M-CSF)	ng/mL	0.035	0.053
Macrophage-Derived Chemokine (MDC)	pg/mL	1.2	3.7
Macrophage Inflammatory Protein-1 alpha (MIP-1 alpha)	pg/mL	8.2	8.4
Macrophage Inflammatory Protein-1 beta (MIP-1 beta)	pg/mL	4.0	6.2
Macrophage Inflammatory Protein-3 alpha (MIP-3 alpha)	pg/mL	2.5	6.0
Macrophage inflammatory protein 3 beta (MIP-3 beta)	pg/mL	3.2	7.8
Macrophage Migration Inhibitory Factor (MIF)	ng/mL	0.0015	0.0020
Macrophage-Stimulating Protein (MSP)	ng/mL	0.037	0.051
Malondialdehyde-Modified Low-Density Lipoprotein (MDA-LDL)	ng/mL	25	49
Maspin	pg/mL	466	353
Matrix Metalloproteinase-1 (MMP-1)	ng/mL	0.12	0.18
Matrix Metalloproteinase-2 (MMP-2)	ng/mL	1.3	0.87
Matrix Metalloproteinase-3 (MMP-3)	ng/mL	0.0066	0.012
Matrix Metalloproteinase-7 (MMP-7)	ng/mL	0.017	0.0088
Matrix Metalloproteinase-9 (MMP-9)	ng/mL	5.0	7.9
Matrix Metalloproteinase-9, total (MMP-9, total)	ng/mL	0.26	0.34
Matrix Metalloproteinase-10 (MMP-10)	ng/mL	0.0027	0.0054
Mesothelin (MSLN)	nM	0.32	0.31
MHC class I chain-related protein A (MICA)	pg/mL	9.2	15
Monocyte Chemotactic Protein 1 (MCP-1)	pg/mL	4.6	9.0
Monocyte Chemotactic Protein 2 (MCP-2)	pg/mL	1.1	1.3
Monocyte Chemotactic Protein 3 (MCP-3)	pg/mL	0.45	1.9
Monocyte Chemotactic Protein 4 (MCP-4)	pg/mL	131	89
Monokine Induced by Gamma Interferon (MIG)	pg/mL	10	22
Myeloid Progenitor Inhibitory Factor 1 (MPIF-1)	ng/mL	0.0052	0.028
Myeloperoxidase (MPO)	ng/mL	3.2	3.7
Myoglobin	ng/mL	0.011	0.040
N-terminal prohormone of brain natriuretic peptide (NT proBNP)	pg/mL	11	15
Nerve Growth Factor beta (NGF-beta)	ng/mL	0.0099	0.016

Analytes	Units	Myriad RBM LDD	Myriad RBM LLOQ
Neuron-Specific Enolase (NSE)	ng/mL	0.030	0.035
Neuronal Cell Adhesion Molecule (Nr-CAM)	ng/mL	0.044	0.040
Neuropilin-1	ng/mL	0.00077	0.0017
Neutrophil Gelatinase-Associated Lipocalin (NGAL)	ng/mL	0.013	0.058
Osteopontin	ng/mL	0.30	0.62
Osteoprotegerin (OPG)	pM	0.075	0.17
Pancreatic Polypeptide (PPP)	pg/mL	0.11	0.96
Pepsinogen I (PGI)	ng/mL	0.029	0.029
Peptide YY (PYY)	pg/mL	31	26
Phosphoserine Aminotransferase (PSAT)	ng/mL	0.067	0.12
Placenta Growth Factor (PLGF)	pg/mL	3.7	11
Plasminogen Activator Inhibitor 1 (PAI-1)	ng/mL	0.0079	0.016
Platelet-Derived Growth Factor BB (PDGF-BB)	pg/mL	31	144
Progesterone	ng/mL	0.45	0.65
Proinsulin, Intact	pM	1.4	0.73
Proinsulin, Total	pM	7.9	6.7
Prolactin (PRL)	ng/mL	0.013	0.017
Prostasin	ng/mL	0.28	0.33
Prostate-Specific Antigen, Free (PSA-f)	ng/mL	0.00053	0.0027
Protein S100-A4 (S100-A4)	ng/mL	1.0	1.9
Pulmonary and Activation-Regulated Chemokine (PARC)	ng/mL	1.2	1.2
Receptor for advanced glycosylation end products (RAGE)	ng/mL	0.034	0.069
Receptor tyrosine-protein kinase erbB-3 (ErbB3)	ng/mL	0.0086	0.0065
Resistin	ng/mL	0.0012	0.0052
S100 calcium-binding protein B (S100-B)	ng/mL	0.076	0.099
Serotransferrin (Transferrin)	mg/dl	0.0000069	0.000047
Serum Amyloid P-Component (SAP)	ug/mL	0.000011	0.000014
Sex Hormone-Binding Globulin (SHBG)	nmol/L	0.00038	0.0010
Sortilin	ng/mL	0.046	0.044
Squamous Cell Carcinoma Antigen-1 (SCCA-1)	ng/mL	0.053	0.22
Stem Cell Factor (SCF)	pg/mL	18	23
Stromal cell-derived factor-1 (SDF-1)	pg/mL	8.7	13
Superoxide Dismutase 1, soluble (SOD-1)	ng/mL	0.032	0.024
T-Cell-Specific Protein RANTES (RANTES)	ng/mL	0.00018	0.0010
T Lymphocyte-Secreted Protein I-309 (I-309)	pg/mL	7.0	11
Tamm-Horsfall Urinary Glycoprotein (THP)	ug/mL	0.000045	0.00011
Tenascin-C (TN-C)	ng/mL	5.7	9.7
Testosterone, Total	ng/mL	0.061	0.10
Tetranectin	ug/mL	0.00093	0.0025
Thrombomodulin (TM)	ng/mL	0.017	0.049
Thrombospondin-1	ng/mL	0.12	0.35
Thyroglobulin (TG)	ng/mL	1.8	2.8
Thyroid-Stimulating Hormone (TSH)	uIU/mL	0.0036	0.0077
Thyroxine-Binding Globulin (TBG)	ug/mL	0.000019	0.000035
Tissue Inhibitor of Metalloproteinases 1 (TIMP-1)	ng/mL	0.010	0.016

Analytes	Units	Myriad RBM LDD	Myriad RBM LLOQ
Tissue type Plasminogen activator (tPA)	ng/mL	0.0075	0.089
TNF-Related Apoptosis-Inducing Ligand Receptor 3 (TRAIL-R3)	ng/mL	0.034	0.19
Transforming Growth Factor alpha (TGF-alpha)	pg/mL	4.6	17
Transforming Growth Factor beta-3 (TGF-beta-3)	pg/mL	4.2	21
Transthyretin (TTR)	mg/dl	0.00000095	0.000004
Trefoil Factor 3 (TFF3)	ug/mL	0.00005	0.00010
Tumor Necrosis Factor alpha (TNF-alpha)	pg/mL	2.9	4.5
Tumor Necrosis Factor beta (TNF-beta)	pg/mL	2.3	1.9
Tumor Necrosis Factor Receptor I (TNF RI)	pg/mL	5.9	7.6
Tumor necrosis factor receptor 2 (TNFR2)	ng/mL	0.0011	0.0057
Tyrosine kinase with Ig and EGF homology domains 2 (TIE-2)	ng/mL	0.021	0.036
Urokinase-type Plasminogen Activator (uPA)	pg/mL	8.1	23
Urokinase-type plasminogen activator receptor (uPAR)	ng/mL	0.034	0.059
Vascular Cell Adhesion Molecule-1 (VCAM-1)	ng/mL	0.0090	0.057
Vascular Endothelial Growth Factor (VEGF)	pg/mL	12	6.8
Vascular endothelial growth factor B (VEGF-B)	ng/mL	2.7	2.3
Vascular Endothelial Growth Factor C (VEGF-C)	ng/mL	0.19	1.1
Vascular endothelial growth factor D (VEGF-D)	pg/mL	100	100
Vascular Endothelial Growth Factor Receptor 1 (VEGFR-1)	pg/mL	29	91
Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2)	ng/mL	0.0074	0.12
Vascular endothelial growth factor receptor 3 (VEGFR-3)	ng/mL	0.26	1.1
Vitamin D-Binding Protein (VDBP)	ug/mL	0.00002	0.000028
Vitamin K-Dependent Protein S (VKDPS)	ug/mL	0.000021	0.000022
Vitronectin	ug/mL	0.013	0.029
von Willebrand Factor (vWF)	ug/mL	0.0016	0.0034
YKL-40	ng/mL	0.0040	0.0073

LDD (Least Detectable Dose)-determined as the mean + 3 standard deviations of 20 blank readings. Results below the LDD will be more variable than results above the LDD.

LLOQ (Lower Limit of Quantitation)-lowest concentration of an analyte in a sample that can be reliably detected and at which the total error meets the laboratory's requirements for accuracy. MRBM's requirement for accuracy is the concentration of an analyte at which the coefficient of variation of replicate standard samples is 30%.

ND-Not Detected.

Online Repository Table 2. Predominant cellular sources of the biomolecules

Biomolecule	Cellular Source	Biomolecule	Cellular source
Innate immune cell-derived biomolecules		Mixed cell derived biomolecules	
1. C3	M, DC	1. IL1 β	Most cell types
2. CCL3 (MIP1 beta)	M	2. IL6	M, DC, Ep
3. CXCL7 (NAP2)	P, M	3. IL8	Ep, M, En, F, SM
4. Ficolin-3	M	4. Lipocalin-2	N, Ep
5. MMP9, total	N	5. MMP7	N, Ep
		6. PAI	M, Ma, Ep
Airway tissue-derived biomolecules		7. Serum Amyloid A	M, Ep
1. Thrombospondin-1	F		

DC: dendritic cell; Ep: epithelial cell; En: endothelial cell; F: fibroblast; M: macrophage/monocyte; Ma: mast cell; N: neutrophil; P: platelet; SM: smooth muscle

Online Repository Table 3. Correlation of serum biomolecules with inflammatory cells

Variables	Correlation with BAL neutrophils (r)	P	Correlation with BAL eosinophils (r)	P	Correlation with BAL lymphocytes (r)	P
Serum biomolecules associated with neutrophils (5)						
1. GDF-15	0.44	0.003	-0.1	NS	-0.18	NS
2. <i>Human Epididymal protein-4</i>	0.43	0.004	0.29	0.05	-0.05	NS
3. MMP7	0.35	0.02	-0.06	NS	-0.25	NS
4. Tetranectin	-0.35	0.02	0.02	NS	0.06	NS
5. von Willebrand Factor	0.34	0.02	0.08	NS	0.03	NS

Online Repository Table 4. Chest CT findings in Infection+ refractory asthmatic patients

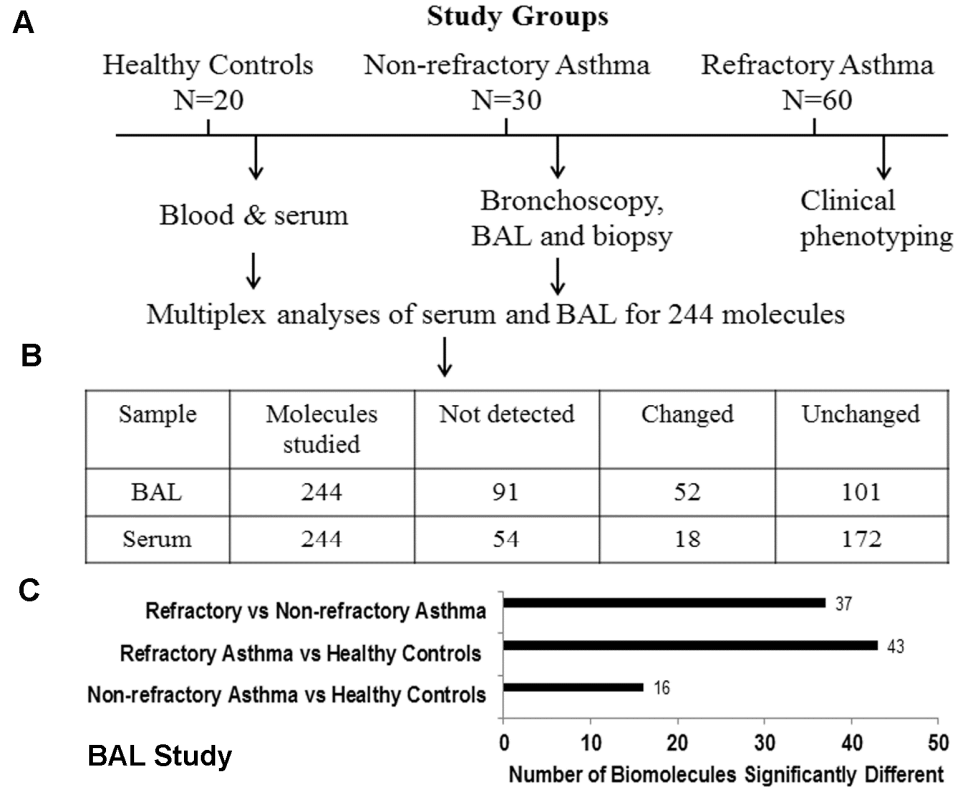
Identifier	Description of chest CT findings
001R	Bronchial Wall Thickening
003R	Bronchiolitis, Consistent with Aspiration, Ground Glass, Consistent with Asthma, Consistent with Reflux, Esophageal Thickening
004R	Bronchial Wall Thickening, Consistent with Asthma, Bronchiectasis, Infiltrates, Hiatal Hernia, Esophageal Thickening, Consistent with Reflux, air trapping
005R	Bronchial Wall Thickening, Consistent with Aspiration, Consistent with Asthma, Tracheobronchomalacia, Pulmonary Hypertension, Consistent with Reflux, Esophageal Thickening, Hiatal Hernia
006R	Bronchial Wall Thickening, Consistent with Aspiration, Consistent with Asthma, Dysmotility, Consistent with Reflux
008R	Tracheobronchomalacia, Bronchial Wall Thickening, Consistent with Asthma, Consistent with Aspiration, Dysmotility, Consistent with Reflux
011R	Bronchial Wall Thickening, Bronchiectasis, Air Trapping, Hiatal Hernia, Dysmotility
015R	Hiatal Hernia, Esophageal Thickening, Bronchial Wall Thickening, Air Trapping, Consistent with Reflux
012R	Bronchiectasis, Bronchial Wall Thickening, Consistent with Asthma, Scarring, Hiatal Hernia, Dysmotility
020R	Bronchial Wall Thickening; Ground Glass; Esophageal Thickening; Hiatal Hernia; Dysmotility; Consistent with Reflux
021R	Bronchial Wall Thickening; Consistent with Asthma; Consistent with Aspiration; Hiatal Hernia; Consistent with Reflux; Esophageal Thickening
030R	Bronchial Wall Thickening, Hiatal Hernia, Esophageal Thickening
033R	Hiatal Hernia, Bronchiectasis, Consistent with Aspiration, Bronchial Wall Thickening, Air Trapping,
035R	Bronchial Wall Thickening, Consistent with Asthma, Esophageal Thickening, Dysmotility, Consistent with Reflux, Air Trapping, Tracheobronchomalacia
018R	Air Trapping, Bronchial Wall Thickening, Consolidation, Hiatal Hernia, Esophageal Thickening, Bronchiectasis, Consistent with Reflux
047R	Tracheobronchomalacia; Consistent with Reflux; Consistent with Aspiration; Consistent with Asthma; Bronchial Wall Thickening; Air Trapping; Esophageal Thickening
048R	Bronchial Wall Thickening, Atelectasis
049R	Hiatal Hernia; Bronchial Wall Thickening; Scarring; Granuloma; Air Trapping; Tracheobronchomalacia
053R	Bronchial Wall Thickening, Atelectasis, Air Trapping, Consistent with Asthma
055R	Bronchial Wall Thickening, Scarring, Ground Glass, Consistent with Reflux, Hiatal Hernia, Consistent with Asthma, Consistent with Aspiration
061R	Bronchial Wall Thickening, Atelectasis, Scarring, Air Trapping, Consistent with Asthma
062R	Esophageal Thickening, Bronchial Wall Thickening, Atelectasis, Scarring, Air Trapping, Consistent with Reflux, Dysmotility, Consistent with Aspiration
063R	Esophageal Thickening, Bronchial Wall Thickening, Air Trapping, Consistent with Asthma
068R	Bronchial Wall Thickening, Atelectasis, Scarring, Air Trapping, Tracheobronchomalacia, Dysmotility

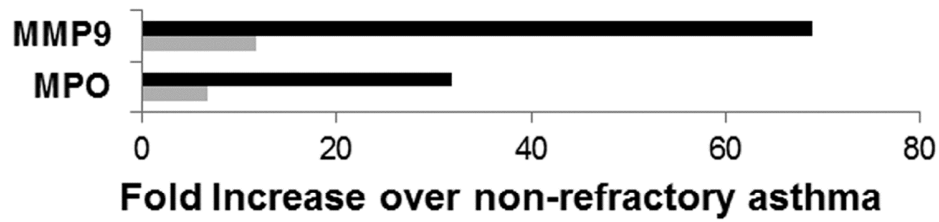
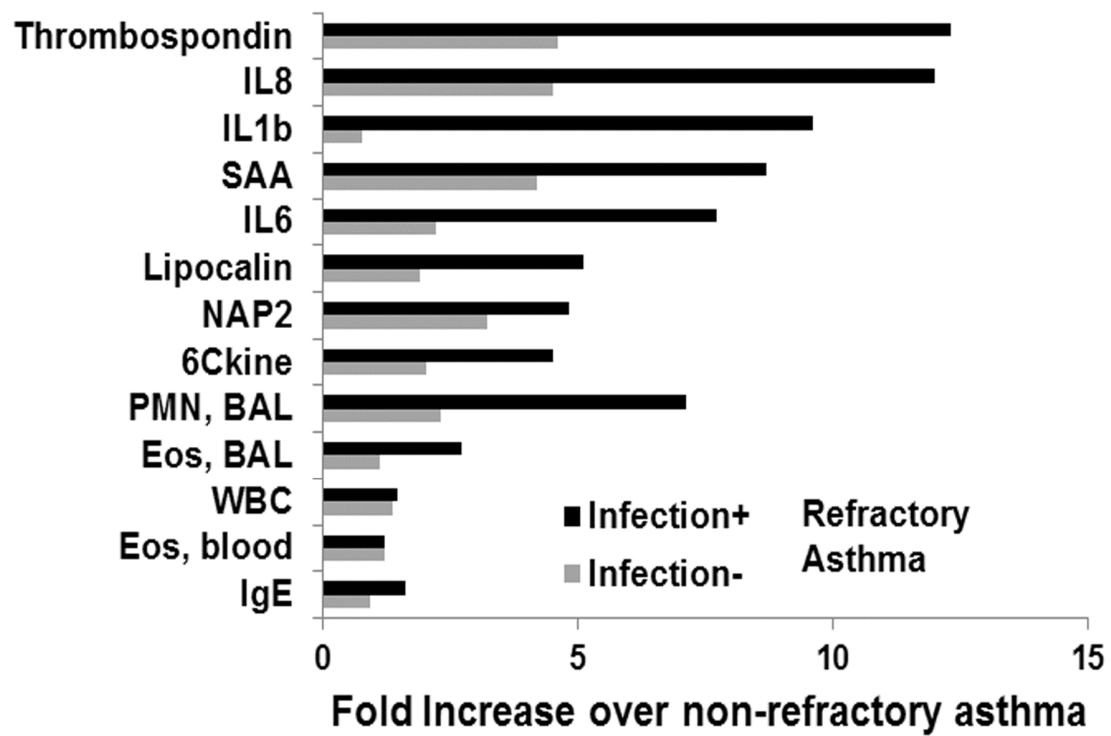
Online Repository Table 6. Correlations among the clinical and laboratory variables

Variables	Correlation FEV1 % (r)	P	Correlation with FVC% (r)	P	Correlation with Reversibility (r)	P
Inflammatory cells						
PMN, BAL	-0.16	NS	-0.32	0.01	0.15	NS
Eos, BAL	-0.23	NS	-0.20	NS	0.15	NS
Lymphocytes, BAL	0.03	NS	0.05	NS	-0.15	NS
Eos, blood	0.02	NS	0.04	NS	0.06	NS
BAL biomolecules						
Myoglobin	-0.24	0.05	-0.27	0.03	0.12	NS
Prostasin	0.27	0.03	0.15	NS	-0.27	0.04

Online repository table 7. Gene Ontology (Biological Process) Pathway Analysis

"Annotation (pathway/process)"	"XD-score"	"Fisher q-value"	"Gene set size"	"Pathway size"	"Overlap size"
"GO:0090023~positive regulation of neutrophil chemotaxis"	0.5976409	0.58840	1	15	1
"GO:0044344~cellular response to fibroblast growth factor stimulus"	0.5976409	0.58840	1	15	1
"GO:0050930~induction of positive chemotaxis"	0.5270527	0.58840	1	17	1
"GO:0048566~embryonic digestive tract development"	0.4476409	0.58840	1	20	1
"GO:0031623~receptor internalization"	0.3190695	0.58840	1	28	1
"GO:0071347~cellular response to interleukin-1"	0.2976409	0.58840	1	30	1



A**B**

Online repository Table 5 List of Medications

Patient ID	Asthma Medications (ICS, LABA, oral steroid and SABA)	Oral steroid	Medication by pharmacological class	Others
002NRA	Advair 250/50 bid, xopenex prn	No		
003NRA	Symbicort 160/4.5 2 puffs bid, albuterol prn	No		
004NRA	Proventil prn, Advair HFA 230/12 bid	No		
005NRA	Alvesco 80mcg qd, Albuterol prn	No		
007NRA	Alvesco 80mcg 2 puffs qd, Albuterol MDI prn	No		
008NRA	Xopenex prn, Advair 100/50	No		
009NRA	Advair 250/50 BID	No		
010NRA	Flovent 220 bid, albuterol prn	No		
011NRA	Dulera 100/5 bid, albuterol prn	No		
012NRA	QVAR 80 BID, albuterol PRN	No		
013NRA	Flovent 44 2 puffs BID	No		
014NRA	Alvesco 160; Xopenex prn	No		
015NRA	QVAR 80 2 puffs/ day	No		
016NRA	QVAR 80 2 puffs/BID; albuterol PRN	No		
017NRA	Flovent 220 BID	No		
018NRA	Flovent 220 BID, albuterol prn	No		
022NRA	Dulera 100/5 BID	No		
024NRA	QVAR 2 puffs BID	No		
026NRA	Advair 100/50 BID	No		
027NRA	Alvesco 160 2 puffs/day; Xopenex prn	No		
028NRA	Flovent 110 2 puffs/day, albuterol prn	No		
029NRA	Advair 250/50 BID; ProAir PRN	No		
030NRA	Advair 250/50 BID; albuterol once/week	No		
036NRA	QVAR 80 2 puffs/day; albuterol PRN	No		
039NRA	Pulmicort 180mcg/day, ProAir PRN	No		
040NRA	Dulera 100/50 2 puffs BID; ventolin PRN	No		
042NRA	QVAR 80 2 puffs/day; proventil PRN	No		
044NRA	Advair 100/50 BID	No		
046NRA	Advair 100/50, Proventil PRN	No		
049NRA	QVAR 80 BID	No		
001R	Advair 500/50 bid, monthly prednisone tapers, QVAR 160mcg BID	Yes		
002R	Spiriva 18mcg daily, Symbicort 160/4.5 2x bid, albuterol prn, Singulair 10mg daily, QVAR 80mcg 4x bid	No	LAMA, ICS, LTRA	
003R	Dulera 200/5.0 2x bid, fluticasone 220 2x bid, Xopenex prn	No	ICS	
004R	Prednisone 40mg/day; Symbicort 160 2x bid; Combivent 2 puffs prn; DuoNeb prn	Yes	LAMA	
005R	Advair HFA 230/21 2 puffs BID; albuterol PRN, prednisone, Singulair 10mg qd, Proventil	Yes	LTRA	
006R	Dulera 200 two puffs BID; albuterol neb prn, Acapella device	No		
007R	40mg prednisone daily, Advair 500/50 BID, Singulair 10mg daily	Yes	LTRA	
008R	Symbicort 160/4.5 2 puffs bid; QVAR 4 puffs bid; Proventil prn	No	ICS	
009R	Advair 500/50 BID, QVAR 80 2x BID, Singulair 10mg	No	ICS, LTRA	

010R	Advair 250/50 bid; Singulair 10mg daily; ProAir prn	No	LTRA	
011R	Advair 250/50 bid, Ventolin 2x bid	No		
012R	Advair 500/50 bid, Singulair 10mg qd, Spiriva once daily, Xopenex neb prn	No	LAMA, LTRA	
013R	Advair 500/50 bid, Flovent 110 twice bid, Singulair 10mg/day, Ventolin prn	No	ICS, LTRA	
015R	Advair HFA 230/21 2 puffs BID, prednisone 60mg qd, Ventolin prn, Combivent 18-103 prn	Yes	LAMA	
016R	Xolair, Symbicort 160/4.5 two puffs bid, Spiriva qd, theophylline 900mg qd, albuterol neb prn	No	LAMA, theophylline, Xolair	
017R	Symbicort 160/4.5 2 puffs BID; Alvesco 160 2 puffs BID; Xopenex prn	No	ICS	
018R	Zyflo CR 1200 mg bid; Advair HFA 230/21 two puffs bid	No	Zileuton	
020R	Prednisone 60mg qd; Singulair 10mg qd; Advair 500/50 bid	Yes	LTRA	
021R	Albuterol HFA PRN; Asmanex 220 mcg bid; prednisone 15mg qd; Serevent bid; Zyflo 600mg qd	Yes	Zileuton	
022R	Dulera 200/5.0 2 puffs BID, Pulmicort 180mcg 2 puffs BID, Singulair 10mg qd, Ventolin prn	No	ICS, LTRA	
023R	Advair 500/50 bid, Ventolin two puffs every 4 hours, DuoNeb prn	No		
024R	Theophylline 300mg 3x/day; albuterol neb prn; Ventolin HFA prn; Symbicort 160-4.5 mcg two puffs BID; Spiriva handihaler 18mcg qd; Alvesco 160mcg two puffs BID; Accolate 20mg bid; prednisone 2mg/day	Yes	LAMA, LTRA, Theophylline,	
025R	Advair 500/50 BID, ProAir HFA prn	No		
026R	Singulair 10mg, Daliresp 500mg, Advair 500/50 bid; Alvesco 160 bid; prednisone taper prn on monthly basis, Proventil MDI prn; albuterol neb prn; Xolair	Yes	LTRA, roflumilast, Xolair	
028R	Prednisone 20mg BID; Symbicort 160/4.5 two puffs BID & QVAR 80mcg 2-4 puffs BID; albuterol HFA or neb PRN	Yes		
029R	20mg Prednisone; theophylline 300mg 3x/day; Spiriva nebs prn every 2-4 hours; albuterol every 2-4 hours; Zyflo 600mg BID; Advair 230/21 two puffs BID; Xolair 150mg every 2 months	Yes	LAMA, LTRA, theophylline, Xolair, zileuton	
030R	ProAir HFA; prednisone taper ongoing; Dulera 200-5 2 puffs BID	Yes		
031R	30mg Prednisone; albuterol/ipatropium neb BID; Dulera 200/5 2 puffs BID; Albuterol prn; Xolair	Yes	LAMA, Xolair	
032R	Advair 230/21 two puffs BID	No		
033R	Symbicort 160/4.5 two puffs BID; DuoNeb	No		
034R	Advair 500/50 BID; ProAir 2 puffs PRN; QVAR 80 three puffs BID	No	ICS	
035R	Spiriva 18mcg daily; Flovent HFA 220mcg two puffs BID; Ventolin and Xopenex prn	No	LAMA	
036R	Advair 500/50 with QVAR 80 two puffs BID	No		
037R	10mg Prednisone/day; Advair 500/50 BID; Pro-Air prn	Yes		
038R	Albuterol HFA prn; Proventil HFA 108 prn; Pulmicort 180 2 puffs BID; QVAR 2 puffs BID	No	ICS	
039R	Symbicort 160/4.5 two puffs BID; QVAR two puffs BID; ProAir and albuterol neb PRN; Zyrtec 10 mg	No	ICS	
041R	Albuterol neb PRN; Albuterol HFA PRN; Dulera AERO 2 puffs BID; Prednisone 10mg every other day	Yes		
042R	5mg/day Prednisone, Advair 500/50 BID; Spiriva 18mcg/day;	Yes	LAMA	

	albuterol neb daily prn			
043R	Symbicort 160 two puffs BID; Flovent 110 two puffs once/day; Xopenex neb BID; Spiriva qd	No	ICS, LAMA	
046R	Symbicort 160/4.5 two puffs BID; prednisone 20mg every other day	Yes		
047R	Ipratropium-Albuterol for neb prn; Advair HFA AERO	No	LAMA	
048R	Albuterol prn; Dulera 200-5 two puffs BID	No		
049R	Advair 500/50 BID; Prednisone 10mg 3x/week; albuterol prn; Xolair; ProAir HFA 2 puffs every 4-6 hours	Yes	Xolair	
050R	Dulera 100/5 two puffs BID; QVAR 80 two puffs BID; Zflo 1200mg BID; prednisone 20mg qd; Nasal Saline washes	Yes		
051R	Advair 500/50 BID	No		
052R	Dulera 200/5 2 puffs BID; Ventolin prn; Zyrtec 10-20mg; Nasonex prn; Albuterol neb prn	No		
053R	Advair HFA 230 two puffs BID; fluticasone nasal spray, theophylline 300 BID, albuterol prn	No		
054R	Symbicort 160/4.5 two puffs BID; QVAR 80mcg 3 puffs BID; montelukast 10mg; Allegra 180mg; Fluticasone 50 mcg; Ventolin PRN; NSW	No		
055R	albuterol nebs/MDI PRN; Brovana 15mcg neb BID; budesonide 1000mg neb BID; Pulmicort; Omeprazole 40mg BID; ranitidine	No		
056R	albuterol neb TID; Symbicort 160/4.5 two puffs BID, Singulair 10 mg; saline nasal rinse with budesonide 0.5 mg twice a day;	No		
057R	Albuterol nebulizer treatment every 4 hours; Dulera 200/5 two puffs BID; Asmanex to 2 puffs BID, ProAir prn; Spiriva daily; Flonase; Singulair;	No		
058R	Dulera 200-5MCG/ACT two puffs BID; ProAir inhaler prn; Singulair 10MG; Zyrtec; Zetonna; QVAR 2 puffs BID, Nexium 40mg	No	Xolair, Zetonna	
059R	Prednisone 10mg/day, ProAir HFA prn; Dulera 200/5 2p BID; DuoNeb prn	Yes		
060R	Xopenex and Atrovent nebulizers p.r.n.; Symbicort 160 two puffs b.i.d.	No		
061R	Singulair; Albuterol nebulizer PRN; Advair 500/50 BID, Cetirizine prn, Flonase	No		
062R	Advair 500 mcg BID, albuterol MDI/neb	No		
063R	Alvesco, methylprednisolone 4mg qd, Prilosec, Qnasl, Spiriva, Symbicort 160, Theophylline 300mg BID, Ventolin PRN, Zyrtec, Nasacort, Patanase nasal solution	Yes	Theophylline	
066R	Pulmicort 180 mcg two puffs BID, Symbicort 160/4.5 two puffs BID, theophylline, DuoNeb	No	Theophylline	
067R	QVAR 80 mcg 2 puffs twice daily; Symbicort 160/4.5 two puffs twice daily; albuterol nebs prn; Singulair 10 mg daily;	No	Flonase prn; NSW prn	Lipitor, Lisinopril
068R	Albuterol inhaler/neb prn, Symbicort 160 two puffs BID, QVAR 80 two puffs BID, Zyrtec	Yes		

NRA: Refractory asthma

R: refractory asthma