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MOLECULAR PHENOTYPING OF SEVERE ASTHMA USING PATTERN RECOGNITION OF BRONCHOALVEOLAR LAVAGE-DERIVED CYTOKINES

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Abstract

Background—Asthma is a heterogeneous clinical disorder. Methods for objective identification of disease subtypes will focus on clinical interventions and help identify causative pathways. Few studies have explored phenotypes at a molecular level.

Objective—We sought to discriminate asthma phenotypes based on cytokine profiles in bronchoalveolar lavage (BAL) samples from mild-moderate and severe asthmatics.

Methods—Twenty five cytokines were measured in BAL samples of 84 patients (41 severe, 43 mild-moderate) using bead-based multiplex immunoassays. The normalized data were subjected to statistical and informatics analysis.

Results—Four groups of asthmatic profiles could be identified on the basis of unsupervised analysis (hierarchical clustering) that were independent of treatment. One group, enriched in severe asthmatics, showed differences in BAL cellular content, reductions in baseline pulmonary function and enhanced response to methacholine provocation. Ten cytokines were identified that accurately predicted this group. Classification methods for predicting methacholine sensitivity were developed. The best model analysis predicted hyper-responders (HR) with 88% accuracy in 10 trials using a 10-fold cross validation. The cytokines that contributed to this model were IL-2, IL-4, and IL-5. Based on this classifier, three distinct HR Classes were identified that varied in BAL eosinophil count and PC₂₀ methacholine.

Conclusion—Cytokine expression patterns in BAL can be used to identify distinct types of asthma and identify distinct subsets of methacholine HR. Further biomarker discovery in BAL may be informative.

Keywords

Asthma; inflammation; cytokines; phenotypes; ELISA; hierarchical clustering; bioinformatics; class discovery

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways characterized by recurrent episodes of symptomatic airflow obstruction and various degrees of airways hyperreactivity to nonspecific stimuli (1). The recognition that this disease has a chronic inflammatory component has directed therapy towards early use of inhaled glucocorticoid therapy, typically producing significant reductions in inflammatory markers, and improvement in pulmonary function (1). However, there is a subset of patients (~5-7 %) with “severe”, or “refractory” asthma (2) that do not respond to glucocorticoids. These patients account for 40-50 % of the health costs of asthma, and incur significant morbidity and decrements in quality of life (3;4) .

Severe asthma is a heterogeneous disorder with distinct ages of onset (2), duration of disease, degree of airflow impairment, presence of modifying factors (GERD, sinusitis), and type of underlying inflammation (2;5;6). In this regard, phenotypic analysis of severe asthmatics prospectively enrolled in the Severe Asthma Research Program (SARP) has shown that severe asthmatics tend to be older and have a greater frequency of respiratory infection (sinusitis and/or pneumonia), suggesting that as a group, they have alterations in innate immune defenses (7). Additionally, at least some severe asthmatics have been characterized as having either neutrophil-predominant inflammation, or increased tissue eosinophils by endobronchial biopsy (8;9). These latter patients have been shown to have increased near-fatal events, especially those with early onset disease, and are associated with airway remodeling, indicated by increased sub-basement membrane thickening (5). However, others have found no clinical differences between the eosinophilic and noneosinophilic phenotypes (10). Together, these observations suggest that severe asthma is a pathologically heterogeneous disorder that still lacks an objective method for distinguishing clinically significant subtypes(6).

The findings that severe asthmatics have distinct inflammatory processes suggest that they may also express distinct airway cytokine profiles compared to those with responsive asthma. Here we investigate this hypothesis by examination of airway cytokine expression patterns in bronchoalveolar lavage (BAL) from a matched group of non-severe and severe asthmatics using bead-based multiplex cytokine arrays (luminex xMAP). The data were analyzed using both unsupervised and supervised classification methods. Accurate definition of asthmatic phenotypes based on molecular profiles may facilitate clinical investigation on the pathogenesis and treatment of asthma.

METHODS

Patients

In the SARP, enrollees are categorized as 1) healthy volunteer, 2) non-severe asthma, and 3) severe asthma, on the basis of a standardized manual of procedures (MOP) based on an NHLBI workshop (11;12). All enrollees have history, physical examination, spirometry, bronchodilator reversibility, allergy skin testing, and methacholine challenge testing. Healthy volunteers have normal lung function and negative methacholine challenge, no history of asthma, and no need for any routine medications. Non-severe asthmatics have lung function that can be normalized using standard doses of inhaled glucocorticoids, with

or without long-acting beta-agonists or leukotriene modifiers. Severe asthmatics are defined according to ATS consensus for refractory asthma(11). These patients are characterized by abnormal lung function in the face of aggressive standard inhaled glucocorticoid therapy and at least one additional control agent; they must in addition have at least 2 positive skin tests. Subjects performed spirometry before and after up to 8 puffs (90 µg/puff) of albuterol. The baseline FEV1 testing required a 4-6 hr withhold of short acting bronchodilators and a 10-12 hr hold for long acting bronchodilators. Hankinsen predicted values (with race correction) were utilized to obtain “percent predicted” values (13). Methacholine challenge was according to the SARP MOP. No testing was performed on subjects with <70% FEV₁. All studies were approved by the local institutional review boards and all subjects gave informed consent.

Bronchoalveolar lavage

Bronchoscopy and BAL were conducted according to the SARP MOP. Briefly, after topical anesthesia, bronchoscopy was performed. BAL was obtained using 2 aliquots of 50 ml each of 0.9% NaCl. Cells were separated by low speed centrifugation (400 X g, 20 min), and supernatants frozen for subsequent analysis.

BAL analysis

BAL and de-identified clinical information were obtained from the SARP for 84 randomly selected patients matched for age and gender (see Table I); by the SARP criteria, 43 were “non-severe” and 41 were “severe” asthmatics.

For each sample, 50 µL of BAL was clarified by high speed centrifugation (10,000 g for 3 min. at 4 °C). The supernatants were then analyzed for 25 human cytokines (BioSource 25-Plex panel). Duplicate samples and serial dilutions of the cytokine standards (50 µL) were incubated with anti human cytokine coated-beads in 96-well filtration plate (Millipore) for 30 min. In this assay, panels of colored microspheres conjugated with capture antibodies are bound to the sample (each capture antibody is conjugated with a uniquely colored microsphere). This panel includes IL-1, IL-1Ra, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, TNF-, IFN-, IFN-γ, GM-CSF, MIP-1, MIP-1, IP-10, MIG, Eotaxin, RANTES, and MCP-1. The plates were vacuum-washed 3 times with 100 µL of wash buffer and incubated with 25 µL of biotinylated antibody cocktail for 30 min. The immune reaction is then developed by adding 50 µL of streptavidin-phycoerythrin for 10 min followed by 3 washes. The samples are then resuspended in 100 µL of assay buffer and 100 beads of each cytokine are acquired and analyzed. For each cytokine, a standard curve is generated using recombinant proteins to estimate protein concentration in the unknown sample. In our hands, these assays have a sensitivity comparable to ELISA measurements, with a detection limit of 10-30 ng/L (depending on cytokine), low inter-assay variation (<10%), and with a dynamic range of up to 3 orders of magnitude.

Validation of IL-2 measurements were performed in 10 HR and 26 LR BAL using QuantiGlo human IL-2 chemiluminescent ELISA (R&D Systems) according to the manufacturer’s instruction (Supplemental Data, Fig. 4).

Data analysis

Chemokine concentrations were determined based on a simultaneously measured standard curve using a logistic curve fitting algorithm (Bio-Plex Manager 3.0 Software). The standard curve for each cytokine in this panel has a range between 2 to 5000 pg/ml. Sample data were used when duplicate measurements showed less than 10% difference. BAL fluid concentrations were analyzed as raw concentrations without normalization to total protein,

albumin, or other marker. This strategy is consistent with the recommendation of the Bronchoalveolar Lavage Cooperative Study Group (14).

Hierarchical clustering

The data were reduced from 25 to 18 features by removing those cytokines for which more than 50% of the data were undetectable. These cytokines were IL-1 β , IL-7, IL-10, IL-12, IL-13, IFN- α and GM-CSF. Unsupervised agglomerative (“bottoms up”) hierarchical clustering was performed on the 50 percentile normalized data using the unweighted paired group mean (UPGMA) using correlation as the similarity measure (Spotfire Decision Site 9.0). Cytokine values below the level of assay detection were replaced by values representing one tenth the lowest value measured on the standard curve.

Classifiers and Attribute Reduction

A C4.5 classifier(15) was applied on the Z-score transformed cytokine values using entropy for splitting and 10-fold cross validation (WEKA 3.5.6).

Statistical Analysis

ANOVA with multiple comparisons and Kruskal-Wallis tests were performed using SAS, version 9.1 (SAS, Inc., Cary, NC) and SPSS, Release 11.0.1 (SPSS, Inc., Chicago, IL). Shrunk centroid classification and feature reduction was performed using prediction analysis in microarray (PAM)(16).

RESULTS

The patients studied were 41 “severe” and 43 age and gender matched “non-severe” asthmatics enrolled by the SARP program whose characteristics are shown in Table I. There were no differences in the age of onset, gender distribution, serum IgE values or positive skin test to *Alternaria* between the two groups. Non-severe asthmatics had nearly normal FEV₁ (89% predicted) and FVC (99% predicted). Severe asthmatics had significant reductions in FEV₁ compared to nonsevere (72% vs 89%, $p<0.01$), a greater maximal FEV₁ reversal after albuterol inhalation (18% vs 12%, $p<0.05$), and a significantly greater number were taking oral glucocorticoids (41% vs 5%, $p<0.001$). The differences in baseline pulmonary function are representative of the severe asthmatic population (7), and the differences in treatment regimen are inherent in the classification of severe asthma by the ATS consensus criteria (12).

We initially focused on molecular profiling on the basis of cytokine measurements because these molecules mediate airway inflammation by recruiting leukocyte populations, affecting T_H1/T_H2 balance, and promoting smooth muscle cell proliferation. We were able to detect the expression of 18 cytokines in BAL (7 cytokine measurements were not detectable in the majority of patients and were excluded) and were further analyzed.

Identification of 4 asthma phenotypes

To reveal the natural underlying groupings, the cytokine concentration data were subjected to unsupervised agglomerative hierarchical clustering (Fig 1). Briefly, this method groups each subject based on mathematical similarities of BAL cytokine concentrations to the others (17). Initially, each subject is in its own cluster. At each step, the nearest two subjects (determined by Pearson’s correlation as the distance metric) are combined into a higher-level cluster. The iteration continues until all the subjects are grouped. Each row corresponds to a subject, and the individual cytokine values are shown in each column, with green being low expressing, red being high. From this analysis, four groups (G) labeled G1-G4 could be discerned displaying different patterns of cytokine expression. For example, G1

had high levels of IL-2, G2 had high levels of IL-1Ra, G3 had high levels of IP-10, and G4 had high levels of IL-2R and many other cytokines (Fig. 1).

To determine whether the patients within these groups represented biologically distinct subgroups of asthma, the clinical features of the four groups were compared with one another. We found that over 15 different variables were statistically different between these groups (Table II). Importantly, these included cellular features of BAL (pulmonary eosinophils, alveolar macrophages) and lung function measurements (values of lung function, FEV₁ response to bronchodilation and sensitivity to metacholine). To further determine how each group differed from one another, pairwise comparisons between the groups was performed using multiple comparisons in ANOVA (with Bonferroni correction). This analysis indicated that the patients in G1 had a significantly reduced FEV₁, FVC, and FEV₁ improvement after bronchodilator therapy compared to other groups (For the complete pairwise comparison, see Table II in the Supplemental Data).

Moreover, G1 was enriched in patients classified as “severe” by the ATS criteria (12), with 18 of the 30 patients (60%) assessed by SARP investigators as having “severe” asthma. G2, the group with the best preservation of lung function was enriched in non-severe asthmatics, with only 8 of the 13 (38%) being identified as “severe” by ATS criteria. These findings indicated that BAL cytokine patterns were informative of disease phenotypes as determined by non-overlapping clinical criteria.

Analysis of treatment effect on cytokine expression patterns

Because the subjects analyzed in this dataset had significant differences in glucocorticoid use, it was important to determine whether cytokine expression patterns were due to therapy or were reflective of the underlying disease process. For this purpose, data from subjects on inhaled glucocorticoids were analyzed and compared to those not taking glucocorticoids. We compared cytokine expression patterns using both descriptive statistics and unsupervised analyses. There were no significant between-group differences in the expression levels for the 18 cytokines analyzed (Supplemental data, Table I). Hierarchical clustering performed on both groups separately produced similar patterns as well (Fig. 2). From this comparison, we concluded that glucocorticoid medication did not significantly affect the cytokine expression patterns in these subjects with chronic stable asthma.

Identification of cytokines having greatest impact on Group 1 classification

Although clustering based on 18 cytokines identified phenotypically distinct subgroups, we sought to identify the cytokines that most contributed to the clustering result. For this purpose, a feature reduction technique using a robust linear discriminant method known as “shrunk centroids” was used(16). Centroids characterizes each class mathematically as a vector of its means (known as a ‘centroid’). Through a re-iterated process of training and cross validation, the number of features were reduced (shrunk) to those with the smallest variation within the class while still retaining classification accuracy. This identified a smaller set of cytokines that were most important in the decision process.

We therefore performed shrunk centroids to identify minimal features that differentiate patients in G1 from all the other asthma subtypes combined (G2,G3 and G4). This analysis identified 10 cytokines as being most important for identification of this severe group (Fig 3). The rank order of these cytokines (most informative to least) was IL-1Ra, MIP-1 α , MIG, IL-15, IL-2R, IP-10, IL-4, IL-6, MCP-1, and IL-2. Using this group of cytokines the subjects could be accurately clustered into the same groups (Supplemental data, Fig.4). Importantly, reducing this panel of discriminant cytokines further to 9 or 8 significantly increased the missclassification error (Supplemental Data, Fig. 1).

Classification model for hyper-responsiveness to methacholine

We next sought to identify cytokine patterns that best predicted airway hyper-responsiveness. For this purpose we defined “hyper-responders” (HR) using an objective measure, sensitivity to methacholine ($PC_{20} < 0.5$ mg/ml), a clinical feature identified by the unsupervised analysis (Table II). Using this established metric as an objective measure of hyper-responsiveness, 15 of the 67 for which methacholine measurements were performed were identified as HR and 52 (low responders, LR, Fig. 4). Pairwise comparison showed that only one analyte was significantly different between HR and LR (IL-2R, $p < 0.016$, Kruskal-Wallis Test). To determine whether combinations of the cytokines could distinguish HR from LR, the 18 cytokines were subjected to attribute reduction. This process identified IL-2, IL-4, IL-5, TNF α , MIG and RANTES as the most significant attributes. These cytokines were then used in a decision tree based learning method. The cytokine that best separates the HR from the LR is selected first, and the process is repeated. Ten-fold cross validation, a process dividing the data into random training and test sets, is performed to estimate classification error and to prune the tree to prevent over-fitting. Performing 10 trials using 10-fold cross validation resulted in the best model with an average accuracy of 88.1% (Fig. 5). The root node was IL-2 which produced a split identifying 5 HR (Class A). Two more HR classes were identified, with low IL-5 and low IL-4 (Class B), and the other, with high IL-5 (Class C).

The clinical demographics were compared for HR Class A, B and C. These groups differed significantly in BAL eosinophils and, interestingly, PC_{20} methacholine (Fig. 6). HR Class A had low BAL eosinophils and the lowest PC_{20} methacholine. These results indicated that decision tree separated three distinct HR sub-classes.

DISCUSSION

A current major challenge in the management of asthma is to accurately identify subtypes that differ in disease pathogenesis and response to therapy. Severe asthma is differentiated from mild-moderate disease by age of onset, duration of disease, degree of airflow impairment, cellular inflammation, presence of sinusitis and history pneumonia (2;7). However, these clinical features as well as noninvasive measurements of airway inflammation (sputum eosinophils and exhaled NO) have not resulted in a method for unambiguous separation of clinical phenotype, response to therapy or disease course (2;6;18). This experience indicates that a single discriminator that accurately separates different asthma types is not yet available. Recognizing that multiple discriminators may be necessary, we have conducted this study for two purposes. First, we sought to determine whether we could use informatics analysis to subgroup subjects based on patterns of BAL cytokine expression. This approach has the potential to identify distinct groups of asthmatics based on commonalities in inflammatory disease mechanisms. Second, we sought to determine whether clinically objective phenotypes could be predicted based on patterns of cytokine expression. Similar approaches have used mRNA expression patterns to separate different groups of responsive and unresponsive cancers. Using groupings based on mRNA expression profiles, patients can be identified using that are otherwise indistinguishable using conventional clinical staging criteria (16;19-21). However, whether this method for molecular phenotyping is generally applicable to other diseases, including asthma, has not been addressed. Although differences in mRNA expression patterns have been identified in asthmatics, not all mRNAs are translated. Therefore, our focus was on proteins (cytokines), which represent proximal mediators of the inflammatory component of asthma.

Our unsupervised hierarchical clustering analysis indicates that at least four phenotypically distinct subgroups of asthma can be identified. Recognizing that a potential confounding variable could be due to different treatment regimens, we have sought to determine whether

these groupings are significantly affected as a result of chronic glucocorticoid use. Reassuringly, similar groupings are identified in subjects not taking glucocorticoids (Fig. 2). We were particularly interested in the phenotype of G1, a group enriched in patients that meet the ATS consensus definition for severe asthma. This group is characterized by increased BMI, reduced FEV₁, reduced FVC, and enhanced sensitivity to methacholine. Importantly our findings indicate that no single cytokine value can be used to separate patients into these groups, but rather, an expression pattern consisting of a minimum of 10 distinct cytokines must be considered.

The biological roles of the chemokines that influence the G1 classification deserve some comment. Relative to the other groups, BAL samples from the subjects in the G1 group are characterized by reduced levels of IL-1 Receptor antagonist (IL-1Ra), MIP-1 α , MIG (and others, see Fig. 4) which are known to play various important roles in coordinating cellular trafficking and inflammation in the airways [see Ref (22)]. Reduction of IL-1Ra may be important because this protein competes for IL-1 α/β receptor binding blocking IL-1 α/β mediated inflammation. In subjects in G1, a reduction in IL-1Ra may result in enhanced IL-1 signaling, a signaling event that could result in neutrophilic inflammation, features characteristic of severe asthma (9). Similarly MIP-1 α (CCL3) is a CC chemokine expressed by macrophages and airway epithelial cells to induce chemotaxis of CD8 T-lymphocytes and eosinophils and whose expression is enhanced in the asthmatic lung (23). Again, here, reduction in MIP-1 α /CCL3 may result in neutrophil-predominant inflammation. Although MIP-1 is acutely inhibited by glucocorticoids, the level of MIP-1 in BAL from patients on glucocorticoid therapy is not statistically different from those not taking glucocorticoids (Table I, Supplemental Data). The IFN- γ inducible MIG(CXCL9) significantly reduces airway hyperresponsiveness and eosinophil accumulation in animal models of allergen challenge (24). MIG diminishes IL-4 and enhances IL-12 levels, directing activated T cells toward a T_H1 phenotype. The reduction in these cytokines in the G1 group relative to the less severe asthmatics therefore, are biologically plausible with our understanding of the pathogenesis of severe asthma.

Although most of the molecular classifiers that have been produced have been based mRNA expression patterns, we suggest that protein expression profiles may be more useful markers of disease than gene expression patterns. This may be particularly true when the proteins themselves play important roles in the underlying disease process, such as cytokines in asthma, and therefore represent *bone fide* biomarkers.

Previous work examining expression of selected cytokines have associations with asthma severity, however these associations are not strong and have not been widely replicated. For example, IL-8 was shown to be enhanced in severe asthmatics during an acute exacerbation and correlates with the number of neutrophils (25). Our study was conducted on stable asthmatics, and the processes involved in chronic inflammatory state may be different from those producing acute exacerbations. Other studies have shown increased IL-2 and -4 levels in severe asthmatics(26). Although we were unable to demonstrate a difference in BAL concentrations for IL-2 or -4 between severe and nonsevere asthmatics in our data set (Supplemental Data Fig. 4), these cytokines do contribute to a model predicting methacholine HR.

Our attempt to classify methacholine HR has yielded two important findings. First, methacholine HR can be accurately separated from LR based on cytokine profiles in BAL, and second, that the group of methacholine HR consists of at least three phenotypically distinct classes. HR Class A is a group characterized by low levels of IL-2, -4 and -5 and, as a group, contains those subjects with the lowest PC₂₀ methacholine responses in our study.

It will be interesting to extend this analysis to larger independent data sets to determine whether the subjects in the HR Classes have distinct clinical outcomes.

The feature reduction analysis has shown that at least 5 cytokines can be used to separate HR from LR in our dataset. It is also important to note that there are important differences in the groups of cytokines that identify G1 with those that identify HR. Specifically, IL-5, secreted into the BAL in response to allergen challenge, and a molecule important in eosinophil recruitment and survival, was identified as an important classification variable in HR, but not for G1. This observation may explain why single cytokine values alone may not be useful discriminators in asthma. Further exploration of these data are underway.

Our studies provide a first proof of concept that informative patterns of cytokines can be detected and interpreted in BAL from patients with asthma and may contribute to more objective classification of disease type. We interpret these findings to suggest that subjects with apparently similar clinical characteristics are in fact, composed of heterogeneous subtypes that can be further distinguished based on BAL cytokine profiles. However, before these findings can be applied to patient classification or management, a number of critical questions remain: what are the reproducibility of serial measurement of BAL cytokines? How stable are the individual phenotypes? Do these distinct subtypes will differ in clinical outcome or response to therapeutic interventions? Importantly, these findings and models will need to be tested on an independent study population. For these reasons, the findings of this study are not ready for translation into the clinic. Nevertheless, our findings indicate that important new diagnostic and prognostic information is available in airway fluids and indicate that future research in biomarker identification will be informative.

Clinical Implication

Definition of asthmatic phenotypes will aid in clinical investigation on the etiology and intervention for asthma.

Capsule summary

This study is the first to identify distinct phenotypes of asthma based on BAL cytokine patterns; when validated it will advance clinical investigation by accurately identifying different subtypes of asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BAL	bronchoalveolar lavage
ELISA	enzyme based immunosorbent assay
FEV₁	forced expiratory volume in 1 second

FVC	forced vital capacity
HR	hyper-responders
MOP	manual of procedures
UPGMA	unpaired group mean average clustering

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FIG 1. Hierarchical clustering of 18 cytokines. Shown is a heat map of clustering cytokine values. Each row is an individual patient. At left, dendrogram showing similarity of groups. Right, four major groups (G) are indicated by vertical bars (G1-G4).

FIG 2.

Treatment patterns as a result of glucocorticoid therapy. Patients were separately clustered based on glucocorticoid therapy at the time of BAL. Left, subjects taking glucocorticoids (inhaled or oral) vs right, subjects not on glucocorticoids. Note similar cytokine patterns are seen in both groups.

FIG 3.

Cytokine classifiers for G1. Shown is a rank ordered list of the 10 cytokines that minimize cross validation error for G1 asthmatics. Left, centroid of G1; right centroid of combined G2-G4 (threshold of 1.2). X axis, is deviation from the overall class centroid.

FIG 4. Identification of HR subjects. Shown is a frequency histogram of the 67 patients where PC₂₀ Methacholine sensitivity was measured. Patients with PC₂₀ methacholine response of <0.5 mg/ml were classified as HR.

FIG 5.

CART classification. C.4.5 decision tree was performed on the Z-Score normalized cytokine data. Shown is the most accurate model. For each node (rectangle) the classification and number of correctly grouped subjects is indicated. The identity of HR Classes A, B and C are indicated in upper right hand corner of each terminal leaf.

FIG 6. Pairwise comparison of methacholine HR Classes. Demographic variables of methacholine HR Class A, B and C were compared by ANOVA. Top panel, BAL eosinophils; bottom panel, PC₂₀ methacholine.

Table I

Patient Characteristics

Phenotype	Characteristic	No. Men 30 (36%)	No. Women 54 (64%)	All Subjects
<u>Not Severe (n=43)</u>		15 (34.9%)	28 (65.1%)	43 (100 %)
	Age of onset	11.6 ± 10.4	18.6 ± 11.5	16 ± 11.5
	Baseline FEV ₁ (l)	3.7 ± 1	2.8 ± 0.6	3.14 ± 1 [§]
	Baseline FEV ₁ (% pred.)	85.4 ± 18.1	90.2 ± 16.2	88.5 ± 16.8 [§]
	Baseline FVC (l)	5.3 ± 0.9	3.6 ± 0.7	4.2 ± 1.15 [§]
	Baseline FVC (% pred.)	97.7 ± 12.3	99.2 ± 15.7	98.6 ± 4.5 [§]
	MaxFEV ₁ Reversal	10.1 ± 5.8	12.3 ± 11.4	11.5 ± 9.8 [*]
	IgE Titer (Log)	2.22 ± 0.3	2.31 ± 0.49	2.28 ± 0.43
	Oral Glucocorticoid	7 %	4 %	5 % ^{§§}
	Reactive to Alternaria	43 %	16 %	26 %
<u>Severe (n=41)</u>		15 (36.6%)	26 (63.4%)	41 (100 %)
	Age of onset	10.2 ± 12.3	16.4 ± 15.4	14.1 ± 14.6
	Baseline FEV ₁ (l)	2.3 ± 0.91	2.2 ± 0.6	2.2 ± 0.8
	Baseline FEV ₁ (% pred.)	64.2 ± 16.7	76.1 ± 19.8	71.7 ± 19.4
	Baseline FVC (l)	3.41 ± 1	3.1 ± 0.74	3.2 ± 0.8
	Baseline FVC (% pred.)	79.7 ± 13.3	87.2 ± 18.3	84.5 ± 16.9
	MaxFEV ₁ Reversal	24.2 ± 20.7	14.7 ± 16	18.2 ± 18.2
	IgE Titer (Log)	2.48 ± 0.4	2.02 ± 0.5	2.19 ± 0.5
	Oral Glucocorticoid	33 %	46 %	41 %
	Reactive to Alternaria	36 %	19 %	26 %

*
p<0.05§
p<0.01§§
p< 0.001 (t test for All Subjects Severe vs Not Severe).

Table II

Between group differences in Groups (G)-1, 2, 3 and 4

Function	Characteristic	P- Value (ANOVA)
<u>General</u>	BMI	0.02
BAL Cellularity	Macrophages	0.05
	Lymphocytes	0.05
	Eosinophils	0.05
<u>Lung Function</u>		
(Baseline)	FEV ₁	<0.001
	FEV1pp	0.02
	FVC	<0.001
	FVCpp	0.03
	Max FEV ₁ / MPV	<0.001
	Max FVC / MPV	<0.001
	Max FVCpp / MPVLung	0.01
<u>Lung Function</u>		
(Post Treatment)	logPC20Methacholine	0.01
	FEV ₁ Reversal, percent	0.03
	FEV ₁ Albuterol reversal	<0.001