ORIGINAL ARTICLE

A Severe Asthma Disease Signature from Gene Expression Profiling of Peripheral Blood from U-BIOPRED Cohorts

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Abstract

Rationale: Stratification of asthma at the molecular level, especially using accessible biospecimens, could greatly enable patient selection for targeted therapy.

Objectives: To determine the value of blood analysis to identify transcriptional differences between clinically defined asthma and nonasthma groups, identify potential patient subgroups based on gene expression, and explore biological pathways associated with identified differences.

Methods: Transcriptomic profiles were generated by microarray analysis of blood from 610 patients with asthma and control participants in the U-BIOPRED (Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes) study. Differentially expressed genes (DEGs) were identified by analysis of variance, including covariates for RNA quality, sex, and clinical site, and Ingenuity Pathway Analysis was applied. Patient subgroups based on DEGs were created by hierarchical clustering and topological data analysis.

Measurements and Main Results: A total of 1,693 genes were differentially expressed between patients with severe asthma and participants without asthma. The differences from participants without asthma in the nonsmoking severe asthma and mild/moderate asthma subgroups were significantly related (r = 0.76), with a larger effect size in the severe asthma group. The majority of, but not all, differences were explained by differences in circulating immune cell populations. Pathway analysis showed an increase in chemotaxis, migration, and myeloid cell trafficking in patients with severe asthma, decreased B-lymphocyte development and hematopoietic progenitor cells, and lymphoid organ hypoplasia. Cluster analysis of DEGs led to the creation of subgroups among the patients with severe asthma who differed in molecular responses to oral corticosteroids.

Conclusions: Blood gene expression differences between clinically defined subgroups of patients with asthma and individuals without asthma, as well as subgroups of patients with severe asthma defined by transcript profiles, show the value of blood analysis in stratifying patients with asthma and identifying molecular pathways for further study.

Clinical trial registered with www.clinicaltrials.gov (NCT01982162).

Keywords: biomarker; immune cell; microarray

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At a Glance Commentary

Scientific Knowledge on the Subject: Asthma is a heterogeneous disease that responds only partially to currently available therapies. Understanding heterogeneity in severe asthma at the molecular level and identifying biomarkers characterizing subgroups are essential to developing new, targeted therapies and to selecting patients most likely to respond to these therapies.

What This Study Adds to the Field: In this study, we detected marked differences in gene expression in blood cells between asthma and individuals without asthma and produced a severe asthma disease signature composed of nearly 1,700 genes. On the basis of gene expression, the population could be divided into two clusters: a cluster enriched for severe asthma (87%), which included users of oral corticosteroids and individuals with blood neutrophilia, and a second cluster enriched for mixed-severity asthma and individuals without asthma. This study shows the value of blood transcriptomics for the identification of asthma stratification biomarkers and for the unbiased identification of molecular pathways of interest. Follow-up studies are needed to further the present findings.

Asthma is a complex disease that presents in various clinical forms and degrees of severity (1). Although clinical severity is routinely used as an asthma classifier, it is not stable (2) and does not take into account the multiple underlying pathobiological processes. A substantial group of patients with severe asthma do not respond to existing therapies and have the greatest unmet needs (1, 3, 4). With the exception of patients treated with the anti-IgE antibody omalizumab, the majority are treated with the same drugs despite heterogeneous underlying pathobiological mechanisms (5). In their efforts to stratify patients, researchers have used mainly clinical and pathophysiological parameters to understand underlying mechanisms (5). Recognizing the limitations of this approach to asthma, investigators are making significant efforts to stratify patients with asthma using methods that bypass traditional clinical biases (6, 7).

Subgroups of patients with asthma have been identified using either mechanistic, hypothesis-driven approaches (e.g., [8]) or unbiased statistical analyses of clinical and pathophysiological characteristics (9, 10). In addition, molecular and genetic markers have been considered with the goal of understanding the pathobiological mechanisms underlying each subgroup (6). A subgroup of patients with mild/moderate asthma expresses high levels of some Th2-associated genes in airway epithelial brushings and is characterized by eosinophilic inflammation, atopy, and a good clinical response to inhaled corticosteroids (ICSs) (6). Although generally accepted biomarker(s) for the identification of T2 asthma do not exist yet, a biomarker set composed of blood eosinophil counts, fractional exhaled nitric oxide, and serum periostin levels is emerging as a predictor of corticosteroid response (11). In contrast, patients who lack some of the T2 features, the non-T2 asthma group, tend to be less responsive to ICSs (6). Understanding their disease, therefore, is important to developing effective treatments (12, 13).

The heterogeneity of severe asthma involves more than T2 gene expression (14, 15). The researchers in the U-BIOPRED (Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes) study of severe asthma (16) obtained omics data from bronchial biopsies, bronchial and nasal brushings, sputum, blood, and urine (17). In this article, we report a focused analysis of gene expression in blood.

Blood is an important medium through which inflammatory and immune cells, as well as systemic treatment, reach the lungs. Analysis of this compartment can provide insight into pathobiological pathways associated with disease severity or other clinically relevant features. We had two main objectives in this study. First, we sought to identify the major differences in blood transcript profiles and molecular pathways between study participants without asthma and those with asthma stratified as severe and mild/moderate (3), as well as whether this was affected by treatment with oral corticosteroids (OCSs). Our second aim was to use an unbiased approach to cluster patients on the basis of gene expression, independent of clinical parameters, hypothesizing that this would point to processes that transcend the standard clinical strata and management with current therapies. Some of the results of these studies were previously reported in the form of an abstract (18).

Methods

Study Population

U-BIOPRED is a multicenter prospective cohort study involving 16 clinical centers in 11 European countries. The adult part of the U-BIOPRED study consists of four cohorts (16): (*1*) severe asthma and nonsmoking (NSM) (n = 311), (*2*) severe asthma and smoking (SM) (n = 110), (*3*) mild/moderate asthma and NSM (n = 88), and (*4*) nonasthma and NSM (n = 101) (*see* METHODS section in the online supplement for more details).

Ethics Statement

The study was conducted in accordance with the principles expressed in the

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Declaration of Helsinki. It was approved by the institutional review boards of all the participating institutions and carried out in adherence to the standards set by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use and good clinical practice. All participants provided written informed consent. The study is registered with www.clinicaltrials.gov (NCT01982162).

Samples

Blood samples were collected from 606 study participants (309 nonsmoking patients with severe asthma, 110 smoking patients with severe asthma, 87 nonsmoking patients with mild/moderate asthma, and 100 nonsmoking individuals without asthma).

Microarray Analysis

RNA was isolated using the PAXgene Blood RNA kit (PreAnalytiX, Hombrechtikon, Switzerland) with on-column DNase treatment (QIAGEN, Valencia, CA). RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples with an RNA integrity number greater than or equal to 6 were processed for microarray as described elsewhere (19) and hybridized onto Affymetrix HT HG-U133+ PM arrays using a GeneTitan instrument according to Affymetrix technical protocols (Affymetrix, Santa Clara, CA). The microarray data are deposited in the Gene Expression Omnibus database under accession number GSE69683.

Data Analysis Sets

After RNA and microarray quality control and exclusion of samples owing to discrepancies with demographic data, the 498 samples available for analysis were randomized into training and validation sets (Table E1 in the online supplement).

Statistical Analysis

Data were normalized and $\log_2$ transformed prior to statistical analyses. Differentially expressed genes (DEGs) and gene signatures were identified using analysis of variance including covariates for RNA integrity number, clinical site, and sex. When age was examined as a covariate in the main model, there were no significant gene expression changes associated with age. Initial comparisons were made in a training set, and results were tested in a separate validation set (Table E1). Both sets were then combined for further analysis, including clustering. Volcano plots showed an area containing most null results (99.99%), referred to as the *patch of disbelief* (PoD). Findings outside the PoD with a local false discovery rate less than or equal to 0.05 were considered significant (20, 21). Correlations were found with differential cell counts as well as OCS use, and these variables were included as covariates in some analyses, as described in the online supplement.

To discover asthma subgroups without *a priori* hypotheses, unbiased hierarchical clustering was performed on standardized data using Euclidean distance and Ward's linkage method (*see* online supplement). Ingenuity Pathway Analysis (QIAGEN, Redwood City, CA) was used for functional analysis of DEG sets and predictors of upstream regulators.

Gene Correlation Network Analysis

Gene expression correlation matrices were created using subsets of genes as described in the text. Gene modules were identified using scaled correlation matrices (22).

Topological Data Analysis

The transcriptomic data were clustered by topological data analysis (TDA) (23–27). TDA provides geometric representation of the relationships between patient data and variables in high-dimensional data sets. TDA structures were generated using the Ayasdi Cure application (Ayasdi, Menlo Park, CA) with a norm correlation metric and two neighborhood lenses (resolution, 38 bins; gain, ×3.4; equalized). For further details of data acquisition and analysis, *see* the online supplement.

Results

Study Population

The demographic and clinical characteristics of the participants included in the gene expression analysis are shown in Table 1. Females were more highly represented in the severe asthma/nonsmoking cohort, and the median age in the mild/moderate asthma and nonasthma cohorts was lower. Approximately 40% of patients with severe asthma reported using OCSs. The following parameters were elevated in all the asthma cohorts compared with the nonasthma cohort: fractional exhaled nitric oxide, a marker of inflammation in the lung; IgE; white blood cell count; blood eosinophils; and neutrophils. The increase in white blood cell count was highest in patients with severe asthma using OCSs, as well as in smokers with severe asthma regardless of OCS use (Figure E1).

Severe Asthma Disease Signature

Compared with individuals without asthma, both the nonsmoking and smoking patients with severe asthma had markedly different gene expression profiles (Figures 1A and 1B). Both severe asthma cohorts had a larger number of DEGs and larger fold changes for some of these genes. Some of the DEGs were selective for particular immune cell subtypes, and major differences in expression were observed in genes associated with eosinophils and neutrophils (higher in the asthma groups) and B cells (lower in the asthma groups) (Table E2). There was good agreement between gene expression differences (severe asthma group vs. nonasthma group) discovered in the training set compared with the validation set ($r = 0.83$) (Figure E2).

By comparison, patients with mild/moderate asthma showed fewer differences from participants without asthma (Figure 1C). Most disease/nonasthma gene expression ratios in the severe and mild/moderate asthma groups trended in the same direction, even in cases where the differences were not statistically significant. Although the differences relative to participants without asthma were greater in the severe asthma group than in the mild/moderate asthma group, there was a good correlation between the groups ($r = 0.76$) (Figure E3).

A severe asthma disease signature (SADS) was developed using data from the combined data set. The collection of probe sets outside the PoD from the severe asthma versus nonasthma comparison was designated the SADS; it contained 2,695 probe sets corresponding to 1,693 genes. The top DEGs in severe asthma ranked by significance are shown in Table 2, and the full list is provided in Table E3.

Gene expression differences between the patients with severe asthma and individuals without asthma could be due either to differences in cell populations or to different activation states of the cells. To

Table 1. Demographic Characteristics

Characteristic	Severe Asthma/Nonsmoking (*n* = 246)	Severe Asthma/Smoking (*n* = 88)	Mild/Moderate Asthma/Nonsmoking (*n* = 77)	Nonasthmatic/ Nonsmoking (*n* = 87)
Sex				
Male, n (%)	85 (34.6)	45 (51.1)	40 (51.9)	53 (60.9)
Female, n (%)	161 (65.4)	43 (48.9)	37 (48.1)	34 (39.1)
Age, yr*	53 (43–62)	55 (48–61)	39 (27–55)	37 (27–49)
White race, %	87.4	95.5	93.5	92.0
OCS dose normalized to prednisolone, mg*†	10 (5.8–16.3)	10 (7.5–18.8)	NA	NA
Atopy, positive/negative/unavailable	180/47/19	54/27/7	68/8/1	32/44/11
FEV_1, % predicted*	67 (50–84)	65 (53–75)	92 (77–102)	103 (94–110)
F_{ENO}, ppb*	26 (15.5–48.9)	25 (11.5–48)	25.5 (18.4–45.4)	19 (13.8–26.8)
IgE, IU/ml*	112 (44–317)	140 (70–378)	102 (53–244)	27 (9–68)
Blood eosinophils, $10^3/\mu l$*	0.20 (0.10–0.40)	0.23 (0.12–0.40)	0.20 (0.10–0.30)	0.10 (0.10–0.20)
Blood neutrophils, $10^3/\mu l$*	4.7 (3.5–6.1)	4.8 (3.8–6.6)	3.3 (2.7–4.5)	3.0 (2.4–3.9)
Blood lymphocytes, $10^3/\mu l$*	1.9 (1.5–2.4)	2.1 (1.6–2.6)	1.7 (1.5–2.1)	1.7 (1.4–2.2)
Total white blood cells, $10^3/\mu l$*	7.8 (6.2–9.4)	7.9 (6.7–9.8)	5.8 (5.1–7.2)	5.3 (4.7–6.4)

Definition of abbreviations: F_{ENO} = fractional exhaled nitric oxide; NA = not applicable; OCS = oral corticosteroid.
*Median (interquartile range).
†Proportions of 40% and 37.5% of patients used OCSs in the severe asthma/nonsmoking and severe asthma/smoking cohorts, respectively.

control for cell-count effects, the statistical analysis was repeated with total white blood cell counts, monocyte percentages, lymphocytes, neutrophils, and eosinophils as covariates in the analysis. This reduced the number of probe sets with differential expression from 2,695 to 268 (Figure E4 and Table E4).

Forty percent of the patients with severe asthma were receiving OCS maintenance treatment, which can have an effect on transcriptional regulation. The steroid-inducible gene *FKBP5* was easily detected in the blood of the U-BIOPRED participants (Figure E5), but levels were elevated only in a subset of patients with asthma taking OCSs, whereas ICSs did not appear to have an effect on blood *FKBP5* transcript levels (Figure E5).

When only patients with severe asthma who were not taking OCSs were compared with control participants without asthma, the signature was reduced to 877 probe sets (Table E5), of which 774 were shared with the SADS. Thus, about 30% of the SADS was not due to OCS use. A direct comparison of patients with severe asthma who were using OCSs with those not using OCSs showed distinct differences between the groups (Figure E6). There were 1,442 differentially expressed probe sets corresponding to 893 genes. B-cell–associated genes had lower expression among OCS users, and a small number of eosinophil-associated genes had higher expression in this group.

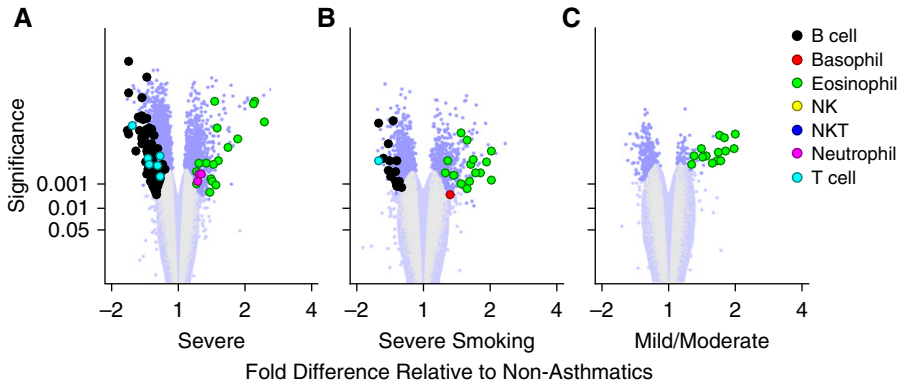

Figure 1. Comparison of nonsmoking patients with mild/moderate asthma as well as nonsmoking and smoking patients with severe asthma with nonsmoking individuals without asthma. (*A*) Nonsmoking patients with severe asthma. (*B*) Smoking patients with severe asthma. (*C*) Patients with mild/moderate asthma. A positive fold difference indicates higher expression in patients with asthma than in individuals without asthma, and a negative fold difference indicates lower expression. Differentially expressed genes that were not assigned a cell type (*purple*) are ubiquitously expressed in immune cells. *Light purple shaded areas* represent patches of disbelief (nonsignificant differences). NK = natural killer cells; NKT = natural killer T cells.

Hierarchical Clustering of the SADS

Hierarchical clustering using the full data set and the probe sets in the SADS showed two major patient clusters (Figure 2A). One of the patient clusters contained more than 87% of patients with severe asthma (severe asthma–enriched cluster [SA-EC]), whereas the other contained approximately 58% of patients with severe asthma (mixed cluster [MC]) (Table 3). About 90% and 86% of the nonasthma and mild/moderate asthma groups, respectively, were assigned to the MC.

To test the robustness of this clustering, we generated severe asthma versus nonasthma signatures separately in the training set and the validation set. For both data sets, their respective signatures generated two main clusters, one corresponding to the MC and the other to the SA-EC. As in the full data set, more than 87% of participants in the SA-EC were from the severe asthma cohort. The MC contained about 88% and 84% individuals without asthma and patients with mild/moderate asthma, respectively.

The robustness of the clustering into SA-EC and MC was tested further by using

Table 2. Genes with Most Significant Differential Expression between Patients with Severe Asthma and Individuals without Asthma

Higher Expression in Severe Asthma				Lower Expression in Severe Asthma			
Gene Symbol	**Fold Difference**	***P* Value**	**IFDR**	**Gene Symbol**	**Fold Difference**	***P* Value**	**IFDR**
DEFA4	2.58	7.13E-10	0.00	*TCL1A*	−1.71	9.51E-07	0.00
OLFM4	2.43	2.27E-07	0.00	*EBF1*	−1.69	4.56E-09	0.00
CEACAM8	2.40	2.17E-09	0.00	*TSPAN13*	−1.69	1.91E-12	0.00
LTF	2.20	5.79E-09	0.00	*IGK@*	−1.68	1.31E-09	0.00
MMP8	2.18	9.98E-09	0.00	*LRRN3*	−1.66	4.83E-07	0.00
BPI	2.03	3.57E-09	0.00	*CUX2*	−1.49	2.63E-09	0.00
LCN2	2.03	5.45E-09	0.00	*AFF3*	−1.47	8.43E-08	0.00
CRISP3	1.98	8.07E-09	0.00	*BLNK*	−1.46	2.89E-09	0.00
RNASE3	1.98	2.65E-08	0.00	*AKAP2*	−1.44	1.96E-08	0.00
CEACAM6	1.97	8.44E-09	0.00	*STRBP*	−1.43	1.83E-09	0.00
CCL23	1.86	3.52E-06	0.00	*IL6ST*	−1.42	2.30E-09	0.00
HP	1.75	2.52E-09	0.00	*MAN1C1*	−1.40	1.59E-08	0.00
DEFA1	1.72	3.04E-10	0.00	*FAM129C*	−1.39	3.69E-08	0.00
MS4A3	1.69	1.09E-06	0.00	*TCF4*	−1.39	5.04E-11	0.00
CTSG	1.68	5.90E-07	0.00	*TTN*	−1.38	5.81E-09	0.00
CD24	1.62	5.34E-08	0.00	*CCR6*	−1.37	3.00E-09	0.00
ANXA3	1.60	2.33E-08	0.00	*RAB11FIP3*	−1.37	1.20E-09	0.00
SLPI	1.60	2.16E-09	0.00	*BCL11A*	−1.36	2.99E-09	0.00
CAMP	1.58	6.95E-09	0.00	*GPM6B*	−1.36	8.25E-10	0.00
RNASE2	1.56	1.57E-07	0.00	*RAB30*	−1.35	7.20E-09	0.00
TCN1	1.52	7.10E-08	0.00	*PDE7A*	−1.34	1.41E-10	0.00
F5	1.47	1.21E-10	0.00	*SLFNL1*	−1.31	6.97E-09	0.00
CEBPE	1.46	6.86E-09	0.00	*CCDC50*	−1.29	3.15E-09	0.00
GAPT	1.36	4.56E-10	0.00	*ENAM*	−1.26	3.80E-09	0.00
PNPLA1	1.35	1.40E-08	0.00	*LUC7L*	−1.25	9.86E-10	0.00
STXBP5	1.31	1.04E-08	0.00	*NSUN6*	−1.25	2.79E-09	0.00
ACSL1	1.30	6.85E-09	0.00	*TGIF2*	−1.25	7.67E-10	0.00
ALAS1	1.21	1.46E-09	0.00	*ZBTB20*	−1.25	1.17E-10	0.00
AMPD3	1.19	8.84E-10	0.00	*CBFA2T2*	−1.22	1.30E-09	0.00
MYD88	1.18	1.70E-10	0.00	*KIAA0355*	−1.20	3.76E-10	0.00

Definition of abbreviation: IFDR = local false discovery rate.

the signature gene list generated from the training set to cluster the validation set and vice versa. There was a significant overlap between this and the original classification ($P = 3e\text{-}10$ by Fisher's exact test). The signature gene list from the validation set was then used to cluster the training set, again with significant overlap ($P = 2e\text{-}5$).

TDA of the SADS

TDA clustering was first performed on the training and validation gene expression sets and then on the full data set, thereby creating three TDA networks. TDA reproduced the two clusters identified by hierarchical clustering, with good separation of the SA-EC and MC clusters. (Figure 2B). In all three networks, the participants identified as members of the SA-EC (presented as *red nodes* in Figure 2B) localized to the top part of the structure, whereas the MC participants (presented as *blue nodes* in Figure 2B) localized to the bottom part. There was little mixing of the SA-EC and MC and edges throughout the structures, supporting the classification by hierarchical clustering.

Differences between Asthma Clusters

The proportion of OCS users was greater in the SA-EC: 60 of 101 (2 participants had no information on OCS use) patients with severe asthma in the SA-EC used OCSs (59%; 95% confidence interval, 50–69%) versus 39 of 131 in the MC (30%; 95% confidence interval, 22–38%). There were also differences in blood cell counts between the clusters, with SA-EC having more total white cells and neutrophils but fewer lymphocytes (Figure E7).

When overlaid onto the TDA structure, participants with high neutrophil counts, defined as greater than 60% neutrophils in blood, were distributed similarly to patients from the SA-EC (Figure 2B, *right*). In the MC, lymphocyte counts were elevated in the OCS users compared with nonusers, whereas in the SA-EC, neutrophil counts were elevated in OCS users and eosinophil counts trended lower ($P = 0.07$ by analysis of variance). No other clinical or pathophysiological variables were clearly associated with the MC and SA-EC.

Pathway Analysis

Notable pathways upregulated in peripheral blood of patients with severe asthma compared with individuals without asthma were chemotaxis, mobilization, migration, and infiltration of myeloid cells (Table 4). Gene sets similar to those affecting myeloid cells were involved in functions associated with decreased viral and bacterial infections. In the peripheral blood of patients with severe asthma, there also was a decrease in pathways related to the abundance of B lymphocytes and hematopoietic progenitor cells, B-cell development, and hypoplasia of lymphoid organs.

Table 5 shows the five top upstream activators (positive activation scores) and inhibitors (negative activation scores). The upstream regulators included cytokines,

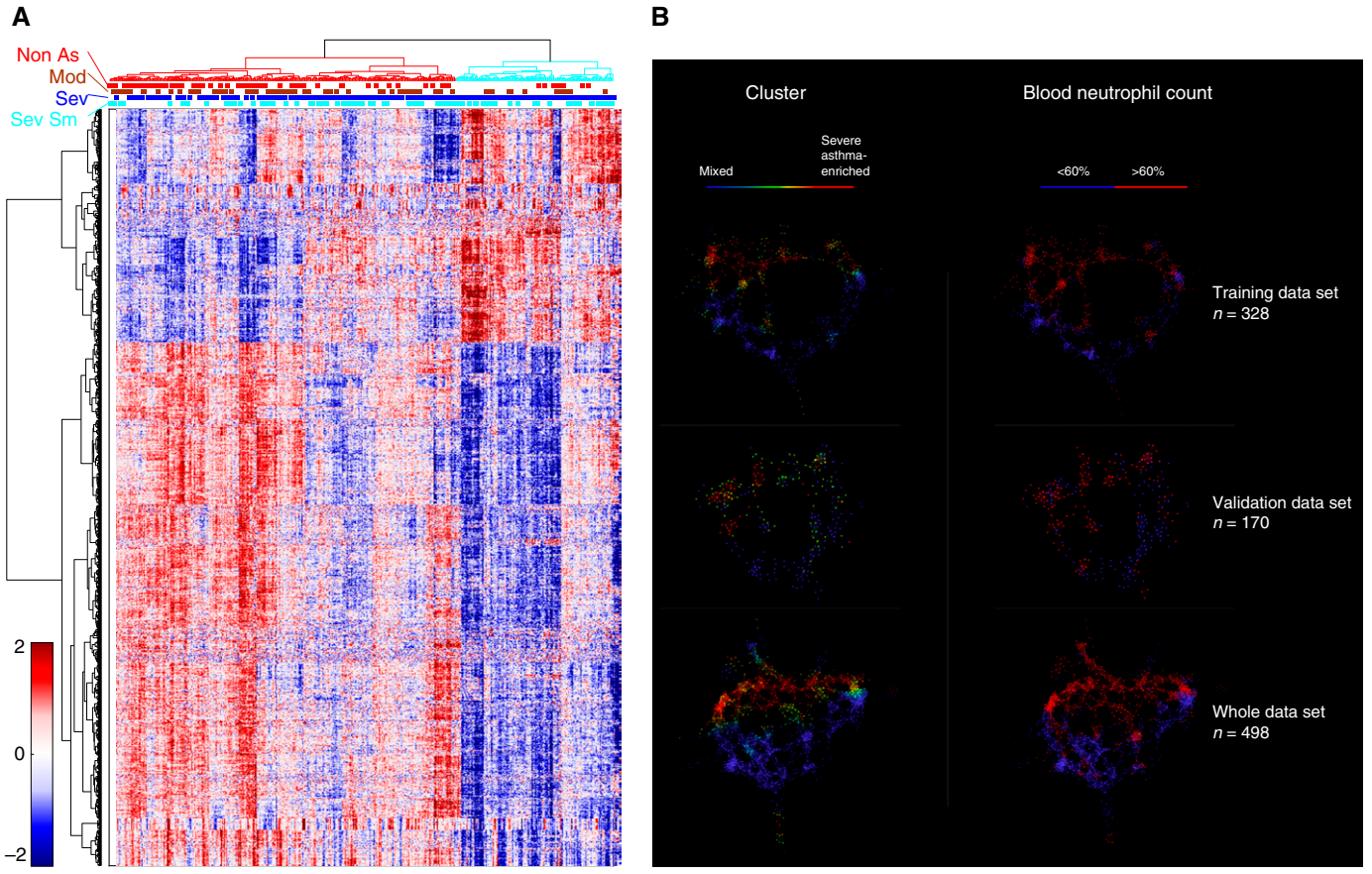

Figure 2. (*A*) Two-dimensional clustering of the severe asthma disease signature. The heat map was generated using all the samples in the study and the genes that were differentially expressed between individuals without asthma and patients with severe asthma. *Turquoise branches* represent class enriched in severe asthma; *red branches* represent mixed class containing 90% of the participants without asthma and 86% of the patients with mild/moderate asthma. *Blue squares* represent nonsmoking patients with severe asthma; *turquoise squares* represent smoking patients with severe asthma; *brown squares* represent patients with mild/moderate asthma; *red squares* represent individuals without asthma. (*B*) Topological data analysis (TDA). (*Left*) The distribution of classes derived from hierarchical clustering of the severe asthma disease signature in a network created by TDA using data from the training set (*top*; n = 328), the validation set (*middle*; n = 170), and the whole data set (*bottom*; n = 498). *Red* and *blue nodes* represent participants identified by hierarchical clustering as being within the severe asthma–enriched cluster and the mixed cluster, respectively. (*Right*) The distribution of study participants with high neutrophil cell counts in TDA structures. *Red nodes* represent those with blood neutrophil cell counts greater than 60%, and *blue nodes* represent those with blood neutrophil cell counts less than 60%. Metric: norm correlation. Lenses: neighborhood lens 1 (resolution, 38 bins; gain, ×3.4), neighborhood lens 2 (resolution, 38 bins; gain, ×3.4). Mod = patients with moderate asthma; Non As = individuals without asthma; Sev = patients with severe asthma; Sev Sm = smoking patients with severe asthma.

enzymes, transcription factors, and fluticasone. Because OCS use was a clinical variable and there were apparent differences in the effect of OCS use in the SA-EC and the MC, we looked for effects of OCS use on gene expression by cluster. In the MC, there were no gene expression differences between OCS users and nonusers, whereas extensive differences were present in the SA-EC. Pathway analysis of the DEGs between OCS users and nonusers in the SA-EC showed that these genes are known corticosteroid targets. The top three predicted upstream regulators were dexamethasone, fluticasone, and prednisolone (data not shown).

Discussion

To our knowledge, this is the first comprehensive study of whole-genome expression in circulating cells in asthma, a complex respiratory disease in which the contribution of circulating inflammatory cells is poorly understood. We also believe it is the first study to explore the value of transcriptomic analysis of blood cells for asthma stratification. It shows that gene expression does not follow a standard clinical classification that is routinely used in clinical practice and for drug development. Prespecified statistical analyses were first applied to data from asthma cohorts defined by the U-BIOPRED consensus group (3) and to data from participants without asthma. Marked differences between individuals without asthma and patients with severe asthma, as well as fewer differences between patients with mild/moderate asthma and participants without asthma, suggest a continuum of pathobiology from mild/moderate to severe disease. We then applied unbiased clustering, the principal objective of U-BIOPRED, which stratified the study participants into two main clusters: an SA-EC, which consisted mostly of patients with severe asthma, and an MC, consisting of about 58% patients with

Table 3. Representation of the Four Cohorts in the Two Transcript Classes

Cohort	Cluster Classification, n (%)	
	Mixed Cluster	Severe Asthma–enriched Cluster
Individuals without asthma, NSM	78 (22.8)	9 (5.8)
Patients with mild/moderate asthma, NSM	66 (19.3)	11 (7.1)
Patients with severe asthma, NSM	143 (41.8)	103 (66.0)
Patients with severe asthma, SM	55 (16.1)	33 (21.2)
Total	342 (100)	156 (100)

Definition of abbreviations: NSM = nonsmokers; SM = smokers.

severe asthma. More than 85% of the enrolled participants without asthma and patients with mild/moderate asthma were in the MC.

In the pairwise comparisons between the clinically defined nonasthma and severe asthma cohorts, several DEGs expressed in specific immune cells stood out. Genes associated with B lymphocytes, including *TCL1A*, *EBF1*, *TSPAN13*, *IGK@*, *BLNK*, and *FAM129C*, and genes associated with T cells (*IL6ST*, *LRRN3*, and *MAN1C1*) ([28, 29] and unpublished Amgen data) had lower expression in patients with severe asthma. Other downregulated genes in patients with severe asthma (*AKAP2*, *STRBP*, *TCF4*, *BCL11A*, *RAB30*, and *CCDC50*) have been reported as most highly expressed in B lymphocytes but are also present in other cell types ([28, 29] and unpublished Amgen data). Genes associated mainly with granulocytes with some contributions from T cells (e.g., *DEF4A*, *OFLM4*, *CEACAM8*, *LTF*, *CCL23*, or *BPI*) ([28, 29] and unpublished Amgen data) had higher expression in patients with severe asthma. Recently, signatures of eosinophilic and granulocytic inflammatory signals in whole blood were found to be associated with lower asthma control (30). Of the six triggering receptors expressed on myeloid cells-1/LPS signaling genes, *CCL23*, *OLIG1*, and *OLIG2* were contained in the SADS.

The number of DEGs was reduced by about 90% when blood cell counts were included as covariates. The DEGs that persisted may indicate altered cell function, although we cannot exclude the possibility that additional cell types for which we did not have frequency data contributed to the SADS. After we adjusted for cell counts, genes associated with B lymphocytes and Ingenuity Pathway Analysis functions such as lymphoid organ hypoplasia and decrease in B-lymphocyte development remained significant. Whereas these differences were clearly detectable in the severe asthma group, they were reduced or absent in the mild/moderate asthma group. This observation is consistent with the notion that effects on B lymphocytes are due either to the severity of the disease itself or to OCS treatment in patients with severe asthma. Indeed, our analysis of OCS users with severe asthma and OCS nonusers showed a considerable number of DEGs. About 10% of the DEGs with lower expression in OCS users were B-cell selective. However, in this comparison, we cannot rule out contributions from disease, because OCS users by definition have more severe disease than OCS nonusers. Furthermore, the number of probe sets in a signature derived from a comparison of patients with severe asthma not using OCSs with individuals without asthma was considerably less than the SADS. As in the SADS, B- and T-cell–selective genes had lower expression in the asthma group, and eosinophil-selective genes had higher expression. In addition to the absence of OCS effects, better asthma control and, therefore, less severe disease and the smaller sample size likely contributed to this observation.

Table 4. Functional Ingenuity Pathway Analysis

Summary of Functions	Total Genes
Increased chemotaxis, recruitment and infiltration of myeloid cells such as neutrophils, increased angiogenesis, and arthritis	448
Increase in protein metabolism and decrease in cancer	347
Decreased quantity and differentiation of lymphocytes and mononuclear leukocytes	293
Decreased viral infection	282
Increased apoptosis and decreased cell survival	219
Increased migration of mononuclear leukocytes and killing of bacteria; decrease in infections, including fungal infections	211
Hypoplasia of lymphoid organs and decreased quantity of hematopoietic progenitor cells, increase in quantity of double-negative T cells	93
Decreased development of pre-B, pro-B, and B lymphocytes	72
Increased aggregation and coagulation of blood cells	54

Consistent with the observed gene expression differences, the top upstream inhibitors included *PAX5*, *TCF3*, and *MYC*, all of which are transcriptional regulators with roles in B- and T-cell development, early B-cell differentiation, or lymphoid carcinogenesis (31, 32). The top upstream activators included genes involved in activation of granulocyte production, differentiation, and function (upstream regulators *CSF3*, *NOS2*, and *CEBPE*) (33–35). The identification of the topical ICS fluticasone as an upstream activator confirmed the presence of steroid effects on gene expression. *TGM2* is a cross-linking enzyme with a role in cell adhesion, wound healing, proliferation, and cellular motility (36). Its expression is increased in inflammatory and allergic conditions, and there is evidence for a role of *TGM2* in allergic asthma (37, 38). It is also the implicated autoantigen in celiac disease (39).

An important consideration for the observations made in this study is whether they reflect clinical severity of asthma or treatment. Severity is defined by symptoms, lung physiology, and the various types and doses of medications used for symptom control (5). Of these three elements, treatment with OCSs potentially has the greatest impact on gene expression of circulating cells. OCS is therefore the most important confounder because of greater systemic bioavailability than ICSs, which have a predominantly local antiinflammatory effect (40). Furthermore,

Table 5. Ingenuity Pathway Analysis of Top Upstream Regulators and Inhibitors

Upstream Regulator	Molecule Type	*P* Value of Overlap	Activation *z*-Score
CSF3	Cytokine	9.18E-07	4.47
TGM2	Enzyme	4.09E-08	4.08
NOS2	Enzyme	1.00E+00	3.52
Fluticasone	Chemical drug	1.19E-05	3.38
CEBPE	Transcription regulator	4.58E-04	3.11
PAX5	Transcription regulator	2.94E-06	−2.76
TCF3	Transcription regulator	1.41E-01	−2.80
Immunoglobulin	Complex	1.10E-05	−3.29
MKL2	Transcription regulator	1.73E-03	−3.32
MYC	Transcription regulator	1.15E-04	−4.83

the inflammatory milieu within the lungs may influence gene expression in blood cells, partly because many of them recirculate and partly because of systemic mediator signals from the lungs. Corticosteroids inhibit neutrophil apoptosis and contribute to neutrophil activation (41–43), and they are likely to account, at least partly, for the observed blood neutrophilia. Corticosteroids also reduce eosinophil longevity (44). Furthermore, glucocorticoids induce cell death in lymphoid cells and are included in chemotherapy regimens for lymphoid malignancies (45). However, the doses used in asthma therapy may not be sufficient to kill lymphoid cells, and no reduction in lymphoid cell count was observed. Therefore, corticosteroid effects on lymphocyte function are probably more relevant.

Corticosteroids act through specific receptors, including the nuclear receptor subfamily 3, group C (NR3C), which function as ligand-dependent transcriptional regulators (46). In the absence of robust published data exploring the effects of OCS on blood cell gene expression in a human population *in vivo*, *FKBP5* expression was used to gauge the effects of asthma therapy. *FKBP5* is an NR3C chaperone, is highly expressed in the blood of both individuals without and with asthma, and is itself corticosteroid inducible (47). With the exception of one patient with asthma, all the OCS users with elevated *FKBP5* levels were in the SA-EC. The segregation of patients with elevated *FKBP5* levels into the SA-EC, especially the nonsmokers, suggests that disease severity is associated with high *FKBP5* expression. Whether this is due to underlying disease mechanisms or to treatment with OCSs is unclear. Whether the high variability within each patient group reflects interindividual differences in the ability to respond to corticosteroids, possibly owing to *NR3C* or *FKBP5* genetic variation (48), the presence of oxidative/nitrosative stress (*NOS2* above), or the lack of adherence to asthma treatment, requires further analysis.

This study creates a solid basis for further assessment of the clinical value of the observed DEGs. The two classes of patients distinguished by blood transcript profiling did not align in a simple way with clinical characteristics, so the clinical applicability has yet to be defined. Gene sets identifying the two clusters can be developed on the basis of data from this study and can be tested prospectively in observational and intervention studies. Observational studies would serve to validate the findings of this study, whereas intervention studies could provide insight into appropriate courses of treatment for the two classes of patients.

Blood eosinophilia is now widely used as a stratification biomarker of so-called T2-type asthma (49–52). Because the reporting of eosinophil counts can vary between laboratories in multicenter studies, the expression of several eosinophil-selective genes (*ALOX15*, *ADORA3*, or *CCL23*) found in this study to correlate with eosinophil counts could be used for a more consistent measure of eosinophil numbers. However, blood transcript analysis cannot be used to stratify patients on the basis of other genes associated with T2 asthma: *POSTN*, *CLCA1*, and *SERPINB2*. These T2 genes were shown to be elevated in lung epithelial cells and in steroid-naive asthma (53) and to be responsive to IL-13 and corticosteroids (53, 54), but only *SERPINB2* transcripts were detectable in blood in the present study, likely derived from myeloid cells. Furthermore, this transcript was not elevated in patients with asthma, and there was no indication of downregulation in patients taking maintenance OCSs. Thus, these genes appear to play a role in T2-high or T2-low classification only in lung samples and not in blood.

One limitation of this study is that a number of clinical outcomes used in U-BIOPRED relied on self-reporting by the participants, which is subject to greater inaccuracy than measurements of physiological or pathobiological biomarkers. For proper interpretation of blood transcript profiling data, it is important to have reliable information on OCS use. Nonadherence to treatment by patients with severe asthma is estimated to be 30 to 70% (55). In U-BIOPRED, participants were required to have been under follow-up by a respiratory physician for at least 6 months, while their asthma control was optimized and medication adherence assessed using the Medication Adherence Report Scale (16). The average Medication Adherence Report Scale score among nonsmoking patients with severe asthma was 22.44, suggesting good adherence to treatment. However, self-reported adherence tends to overestimate actual adherence to treatment (56). Furthermore, some patients with severe asthma show relative corticosteroid insensitivity (57), which was not assessed in this study. The SA-EC cluster of asthma included patients not taking OCSs, but they still had expression profiles that were more similar to those of patients with severe asthma taking OCSs in the SA-EC than the patients with severe asthma in the MC cluster.

In summary, this study provides convincing evidence of differential gene expression in the blood of patients with asthma. Our study shows, for the first time to our knowledge, major differences in the activity of circulating cells that do not follow the currently applied clinical classification based on severity of asthma. The findings presented here are hypothesis generating, and clinical studies are needed to determine the utility of stratifying markers derived from this study. Follow-up biological studies may lead to new insights into asthma disease mechanisms and may open new avenues for therapeutic intervention. ■

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