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Background: Patients with type-2 (T2) cytokine-low severe asthma often have persistent symptoms despite suppression of T2 inflammation with corticosteroids.

Objectives: We sought to analyze whole blood transcriptome from 738 samples in T2-biomarker-high/-low patients with severe asthma to relate transcriptomic signatures to T2 biomarkers and asthma symptom scores.

Methods: Bulk RNA-seq data were generated for blood samples (baseline, week 24, week 48) from 301 participants recruited to a randomized clinical trial of corticosteroid optimization in severe asthma. Unsupervised clustering, differential gene expression analysis, and pathway analysis were performed. Patients were grouped by T2-biomarker status and symptoms. Associations between clinical characteristics and differentially expressed genes (DEGs) associated with biomarker and symptom levels were investigated.

Results: Unsupervised clustering identified 2 clusters; cluster 2 patients were blood eosinophil-low/symptom-high and more likely to be receiving oral corticosteroids (OCSs). Differential gene expression analysis of these clusters, with and without stratification for OCSs, identified 2960 and 4162 DEGs, respectively. Six hundred twenty-seven of 2960 genes remained after adjusting for OCSs by subtracting OCS signature genes. Pathway analysis identified dolichyl-diphosphooligosaccharide biosynthesis and assembly of RNA polymerase I complex as significantly enriched pathways. No stable DEGs were associated with high symptoms in T2-biomarker-low patients, but numerous associated with elevated T2 biomarkers, including 15 that were upregulated at all time points irrespective of symptom level.

Conclusions: OCSs have a considerable effect on whole blood transcriptome. Differential gene expression analysis demonstrates a clear T2-biomarker transcriptomic signature, but no signature was found in association with T2-biomarker-low patients, including those with a high symptom burden. (J Allergy Clin Immunol 2023;152:876-86.)

Key words: *Severe asthma, whole blood transcriptome, biomarker, T2-low, T2-cytokine, oral corticosteroids*

Multiple new therapies target the type-2 (T2) cytokine-driven eosinophilic inflammation observed in more than 90% of patients with severe asthma.[1,2] However, many patients continue to have uncontrolled symptoms despite suppression of T2 inflammation, resulting in overtreatment particularly with corticosteroids (CSs).[1-4] The mechanism of uncontrolled persistent symptoms in T2-biomarker-low patients appears to be multifactorial and heterogeneous, involving extrapulmonary factors, such as

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Abbreviations used
ACQ-7: 7-item Asthma Control Questionnaire
CS: Corticosteroid
DEG: Differentially expressed gene
FC: Fold change
FDR: False-discovery rate
FENO: Fractional exhaled nitric oxide
IPA: Ingenuity pathway analysis
OCS: Oral corticosteroid
T2: Type 2

obesity, airway structural change with fixed airflow limitation, and infection-related pathways, but other potential non-T2 inflammatory mechanisms may also be involved.[3,4]

In a recent study, 2 strategies were investigated for adjusting CS therapy in patients with severe asthma: T2-biomarker adjustment of CS treatment versus adjustment using a symptom/risk-based algorithm based on asthma symptoms and recent asthma exacerbation history[2]; this cohort was enriched for T2-biomarker-low participants to explore the impact of CS reduction in T2-biomarker-low participants with severe asthma and biological samples were obtained as part of the study protocol.[2] In this study, blood transcriptomic samples were analyzed from the T2-biomarker-low population with severe asthma to identify transcriptomic signatures associated with uncontrolled T2-biomarker-low severe asthma.

METHODS

Study design and participants

Between January 8, 2016, and July 12, 2018, a total of 301 participants were enrolled into the study (see Fig E1 in this article's Online Repository at www.jacionline.org). The study design and clinical outcomes were reported previously.[2] In brief, a randomized, controlled, single-blind (study participant), multicenter, parallel-group 48-week clinical trial was conducted in patients with severe asthma (Global Initiative for Asthma steps 4 and 5)[5] and a fractional exhaled nitric oxide (FENO) level of less than 45 parts per billion to enrich for a T2-biomarker-low population within the study cohort; full inclusion and exclusion criteria and study Consort diagram (Fig E1) can be found in the Appendix. The study compared biomarker-based adjustment of CS therapy (using a composite index of T2 biomarkers—blood eosinophil count, serum periostin, and FENO) to adjustments using an algorithm based on asthma symptoms, lung function, and recent exacerbation history (standard care arm).[2] The protocol was reviewed and approved by the Office for Research Ethics Northern Ireland (NI0158) and obtained local National Health Service Research and Development approval for study sites. This study was conducted in accordance with the principles of the Declaration of Helsinki, and all patients provided written informed consent for study participation including biosampling before enrollment. Conduct of the trial was monitored by an independent trial steering committee. The trial was registered on ClinicalTrials.gov (NCT02717689).

Following randomization, patients attended the clinic every 8 weeks for review of their asthma control and treatment, and software provided treatment advice based on a predefined study algorithm. Blood samples for whole blood transcriptomic analysis were obtained at baseline, week 24 (visit 3), and week 48 (visit 6, study end). Venous blood samples were mixed immediately with the blood RNA stabilization buffer in the PAXgene blood RNA tube (Qiagen, #762615) and incubated at room temperature for 2 hours before storing at −80C until processing.

Blood RNA isolation and quality control

The PAXgene samples were thawed for 2 hours at room temperature and, after mixing (inverting ×10 gently), each 5-mL sample was subjected to RNA isolation using the MagMAX RNA isolation kit for stabilized blood tubes by following the manufacture's manual instruction (AM1837, ThermoFisher Scientific, Waltham, Mass). The crude RNA pellet was resuspended after washing with nuclease-free water and digested with protease (55°C for 10 minutes in a thermomixer at 1000 rpm). After pelleting the cell debris (16,000*g*, 3 minutes), the RNA was mixed and magnetically captured by MagMAX RNA-binding beads. The beads were washed (×2) and treated with Turbo DNase (10 minutes at room temperature). After rebinding to the beads, the RNA samples were quantified by Nanodrop 8000 spectrophotometer (ThermoFisher Scientific, ND-8000-GL) and the integrity of the RNA samples was determined using a 4200 Tapestation system (Agilent, G2991BA), respectively.

RNA-seq library construction and quality control

Isolated RNA (100 ng) was used to prepare total stranded RNA-seq libraries using a modified KAPA RNA HyperPrep kit protocol with RiboErase (HMR) Globin (KR1520, KAPA Biosystems, Wilmington, Mass). After the removal of ribosomal and globin RNA, Dnase1 digestion, and KAPAPure bead purification, the reactions of fragmentation and priming were followed by reverse transcription for the first-strand cDNA synthesis (Superscript II, Invitrogen, Waltham, Mass). An incorporation method using dUTP was applied for the second-strand synthesis along with completion of the step of A-Tailing. Ligation of index adaptors was performed sequentially followed by the cleanup step with KAPAPure bead for generation of cDNA libraries. Non–dUTP-containing strands of the cDNA library were PCR-amplified exclusively with proofreading Taq polymerase for library enrichment and introduction of strand specificity and sample index. Enriched cDNA libraries were cleaned (AMPure XP, #A63881, Beckman Coulter, Indianapolis, Ind), analyzed (Agilent Bioanalyser), and quantified (Quant-iTTM Pico-Green assays, Life Technologies, Carlsbad, Calif) before being loaded onto a HiSeq platform (Illumina, San Diego, Calif).

Quality control of RNA-seq data

RNA-seq data were generated as 150-bp paired-end reads with a minimum coverage of 45 million reads; most of the reads were of Q-30 or above. After passing the data through GEAR 3.0 RNA-seq quality control pipeline, 738 samples demonstrated acceptable RNA-seq data quality with no significant quality concerns regarding RNA-seq mapping (including 3'-bias, read redundancy, and mapping rate). In addition, principal-component analysis was performed to ensure there were no technical outliers.

Statistical analysis

Clinical and demographic details are presented as means (SD), medians [interquartile range], or counts (%) as appropriate. Univariate comparisons were made using the *t* test (normally distributed

variables), Mann-Whitney *U* test (non-normally distributed variables), and chi-square test (categorical variables). Descriptive analyses were conducted using STATA 16 (StataCorp, College Station, Tex).

R package DESeq2 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) was used for analyzing RNA-seq data.[6] Batch effect and cell counts, including total white blood cells, monocytes, lymphocytes, neutrophils, and eosinophils (when not used as a grouping criterion), were corrected for in the analysis. For cell counts, zero values were imputed as 50% of the observed minimum of non-zero values. A multifactor design in DESeq2 accounting for differences between samples while estimating effect due to the condition was applied to analyze longitudinal data collected from the same patient at different time points. Significant differentially expressed genes (DEGs) were defined by false discovery rate (FDR) less than 0.05.

For the unsupervised cluster analysis, a sample-to-sample distance matrix was generated using DESeq2 based on a normalized RNA-seq count matrix with covariates (batch and cell counts) accounted for. Hierarchical clustering was then applied to the distance matrix to identify patient subgroups. Sample outliers and genes expressed in less than 10% of the samples were excluded from this analysis. An orthogonal approach using K-means clustering as implemented in R package "stats" was applied to confirm the stability of the clusters.

Pathway analysis

Significant DEGs were used as the input for ingenuity pathway analysis (IPA, June 2021)[7] to identify overrepresented canonical pathways. Core analysis using Ingenuity Knowledge Base (gene only) as the reference set was performed. *P* values were calculated using a 1-tailed Fisher exact test, reflecting the likelihood that the overlap between the input and a given gene set was due to random chance. Benjamini-Hochberg correction was used to correct for multiple testing, and significant pathways were defined by FDR less than 0.05.

Gene set enrichment analysis was performed for a subset of comparisons to understand whether a curated set of genes was statistically significant, and to identify pathways enriched for upregulated and downregulated genes.[8,9] All genes detected in RNA-seq were ranked on the basis of a metric score calculated as "signed fold change (FC) $\times -\log_{10}$(FDR)." The ranked gene list was then supplied to the gene set enrichment analysis preranked tool to obtain a normalized enrichment score and an FDR for each gene set. Hallmark (H), curated (C2), and immunologic signature (C7) gene sets from MSigDB were included in this analysis. Gene sets with FDR less than 0.05 were considered significant.

Derivation of oral CS transcriptomic signature

Thirty-five participants switched from not being on oral corticosteroids (OCSs) to being on OCSs between 2 consecutive time points (baseline-week 24 or week 24-week 48). Pairwise comparison of the second time point (time point 2) to the first time point (time point 1) yielded 5638 DEGs (set 1), including 30 DEGs with $|\log_2(FC)|$ greater than or equal to 1 (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). Fourteen patients received decreased OCS dose from baseline to week 24. Pairwise comparison of baseline versus week 24 identified 1619 DEGs (set 2), including 53 DEGs with $|\log_2FC|$ greater than or equal to 1 (Fig E4, *B*). Genes highly regulated by OCS were defined as the union of set 1 and set 2, herein referred to as the "OCS signature" (n = 6393). Of note, pairwise comparison in patients who switched their OCS usage status from "on OCS" to "not on OCS" from time point 1 to time point 2 was not performed because of insufficient numbers (n = 7).[10]

Patient subgroups based on biomarker and symptom levels

Patients were assigned into the following 4 subgroups at each study time point (baseline, week 24, and week 48) based on their composite biomarker measurements of blood eosinophil and FENO, and symptom levels (defined by 7-item Asthma Control Questionnaire, ACQ-7) at the time of sampling: (a) T2-biomarker-low, symptom-high: eosinophil less than or equal to 150, and FENO less than or equal to 20, and ACQ-7 score greater than 1.5; (b) T2-biomarker-low, symptom-low: eosinophil less than or equal to 150, and FENO less than or equal to 20, and ACQ-7 score less than or equal to 1.5; (c) T2-biomarker-high, symptom-high: eosinophil greater than or equal to 300, and FENO greater than or equal to 35, and ACQ-7 score greater than 1.5; (d) T2-biomarker-high, symptom-low: eosinophil greater than or equal to 300, and FENO greater than or equal to 35, and ACQ-7 score less than or equal to 1.5.

RESULTS

Seven hundred thirty-eight samples from 289 (289 of 301) participants met the required RNA quality threshold (277 at baseline, 234 at week 24, and 227 at week 48) and were available for bulk RNA-seq analysis. Baseline demographics, medical history, comorbidities, lung function, and CS treatment are summarized in Table E1 (in the Online Repository available at www.jacionline.org). The analysis workflow design is presented in Fig E2 (in the Online Repository available at www.jacionline.org).

Unsupervised cluster analysis of transcriptomic signatures

Unsupervised clustering of baseline patient samples identified 2 major clusters (see Methods, and Fig E3, *A*, in this article's Online Repository at www.jacionline.org). Repeated analysis using only the top 5000 genes with the highest variance (Fig E3, *B*) and an orthogonal approach using K-means clustering (Fig E3, *C*) confirmed the cluster assignment of 95% and 93% of the subjects, respectively. Univariate analyses of clinical and demographic features associated with the 2 clusters are presented in Table I, highlighting that patients in cluster 2 tended to be eosinophil-low/symptom-high and have a significantly higher rate of OCS use ($P < .0001$). Even when restricting to those who were on OCSs, cluster 2 patients tended to be treated with a higher dose of OCS ($P = .001$).

Differential gene expression analysis between cluster 1 (227 participants) and cluster 2 (40 participants) demonstrated 4162 DEGs (2057 upregulated and 2105 downregulated in cluster 1). Among all DEGs, 93 genes have a $|\log_2FC|$ greater than or equal to 1, with 46 upregulated and 47 downregulated in cluster 1. Pathway analysis (IPA) and gene set enrichment analysis for DEGs between cluster 1 and cluster 2 are presented in the Online Repository (see Table E2 and Table E3 in this article's Online Repository at www.jacionline.org).

TABLE I. Univariate analysis of clinical and demographic features associated with the 2 major transcriptomic clusters

Variable	Cluster 1	Cluster 2	*P* value
No. of patients; N = 267	227	40	
Age at inclusion (y); N = 267	55.4 (13.1)	58.7 (13.2)	.1402
Age at asthma diagnosis (y); N = 267	25.7 (20.5)	32.2 (20.1)	.0620
Sex; N = 267			.6214
Female	144 (63.4)	27 (67.5)	
Male	83 (36.6)	13 (32.5)	
Ethnicity; N = 267			.9259
White	209 (92.1)	37 (92.5)	
Non-White	18 (7.9)	3 (7.5)	
BMI (kg/m^2); N = 266	31.4 (7.4)	31.9 (6.9)	.6833
Smoking status; N = 267			.117
Never smoked	174 (76.7)	26 (65.0)	
Ex-smoker	53 (23.3)	14 (35.0)	
Atopic disease; N = 266	157 (69.5)	25 (62.5)	.3821
Hospital admissions for asthma in last year; N = 267	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.2297
A&E visits in the last year; N = 267	0.0 (0.0, 0.0)	0.0 (0.0, 0.5)	.4822
GP visits for asthma in the last year; N = 267	1.0 (0.0, 3.0)	0.0 (0.0, 2.5)	.4726
Rescue courses of oral steroids in the last year; N = 267	2.0 (1.0, 4.0)	3.0 (1.0, 4.0)	.2416
Prior admission for asthma to a high dependency or intensive care unit; N = 267	41 (18.1)	11 (27.5)	.1646
No. of prior admissions for asthma to a high dependency or intensive care unit; N = 51	1.0 (1.0, 1.5)	2.0 (1.0, 5.0)	.0437
Ever been ventilated; N = 51	17 (41.5)	5 (50.0)	.625
History of rhinitis; N = 267	163 (71.8)	22 (55.0)	.0336
History of eczema; N = 267	72 (31.7)	13 (32.5)	.922
History of nasal polyps; N = 267	54 (23.8)	13 (32.5)	.2413
Prior nasal surgery; N = 267	53 (23.3)	12 (30.0)	.3661
History of esophageal reflux; N = 267	122 (53.7)	32 (80.0)	.0019
History of aspirin sensitivity; N = 267	33 (14.5)	9 (22.5)	.2022
Depression/anxiety; N = 267	64 (28.2)	14 (35.0)	.3828
Hypertension; N = 267	65 (28.6)	16 (40.0)	.1494
Osteoporosis/osteopenia; N = 267	45 (19.8)	14 (35.0)	.0329
Osteoarthritis; N = 267	58 (25.6)	14 (35.0)	.2143
Hypercholesterolemia; N = 267	36 (15.9)	11 (27.5)	.0747
Diabetes; N = 267	27 (11.9)	2 (5.0)	.1963
Cataracts; N = 267	20 (8.8)	9 (22.5)	.0103
Obstructive sleep apnea; N = 267	13 (5.7)	4 (10.0)	.3075
Ischemic heart disease; N = 267	8 (3.5)	4 (10.0)	.0683
Peptic ulcer; N = 267	2 (0.9)	5 (12.5)	<.0001
Stroke; N = 267	4 (1.8)	0 (0.0)	.3976
Chronic kidney disease; N = 267	3 (1.3)	4 (10.0)	.0015
Glaucoma; N = 267	3 (1.3)	1 (2.5)	.5716
Myocardial infarction; N = 267	2 (0.9)	1 (2.5)	.3704
FEV_1 (L); N = 267	2.2 (0.8)	2.1 (0.6)	.5073
% Predicted FEV_1; N = 267	76.6 (19.2)	78.1 (15.3)	.6389
FVC (L); N = 267	3.3 (0.9)	3.2 (0.7)	.4197
% Predicted FVC; N = 267	92.2 (16.3)	92.6 (12.1)	.8872
FEV_1/FVC; N = 267	0.66 (0.12)	0.66 (0.09)	.7244
PEFR (L/min); N = 265	383.7 (127.2)	360.7 (114.7)	.2862
ACQ-7 score; N = 267	1.9 (1.1)	2.3 (1.2)	.0253
AQL total score; N = 260	5.0 (1.4)	4.4 (1.3)	.0082
Sputum eosinophils (%); N = 107	1.7 (0.4, 8.6)	1.3 (0.3, 18.4)	.8863
Sputum neutrophils (%); N = 107	59.5 (28.3, 78.4)	51.6 (32.0, 75.0)	.6746
Sputum lymphocytes (%); N = 107	0.4 (0.0, 1.5)	0.3 (0.0, 0.8)	.2987
Macrophage sputum (%); N = 107	22.5 (9.0, 40.7)	26.9 (22.8, 52.8)	.188
FENO (ppb); N = 267	21 (13, 29)	19 (12, 33)	.5992
Blood eosinophils (10^9/L); N = 267	0.24 (0.14, 0.35)	0.08 (0.04, 0.21)	<.0001
Periostin (ng/mL); N = 266	52.8 (16.0)	56.4 (18.7)	.2102
OCS user; N = 98 of 267	61 (26.9)	37 (92.5)	<.0001
OCS dose (mg); N = 98	8 (5, 10)	10 (8, 15)	.001
ICS dose (BDP μg equivalent); N = 267	2215 (653)	2441 (1005)	.0658

A&E, Accident & Emergency; *AQL*, Asthma Quality of Life questionnaire (Juniper); *BDP*, beclomethasone dipropionate; *GP*, general practitioner; *ICS*, inhaled corticosteroid; *PEFR*, peak expiratory flow rate; *ppb*, parts per billion.

Adjustment for OCS exposure

Pairwise comparison of longitudinal data from individuals who received CS treatment adjustments during the study, including decreased dosage of inhaled CS and increased or decreased OCS dose, revealed strong gene signatures associated with OCS use, but not inhaled CS (Fig E4).

Given that OCS use was significantly different between the 2 clusters, and that OCS use strongly influences the transcriptomic profile, 2 approaches were implemented to adjust for the effects of OCS exposure. First, univariate analysis was restricted to participants on OCSs. Comparison of clinical and demographic features associated with the 2 clusters is presented in Table E4 (in the Online Repository available at www.jacionline.org) and is consistent with the analysis without restriction on OCS usage, with patients in cluster 2 having lower blood eosinophil counts and treated with a higher OCS dose. Differential gene expression between cluster 1 on OCSs (61 participants) and cluster 2 on OCSs (37 participants) demonstrated 2960 DEGs (1449 upregulated and 1511 downregulated in cluster 2). Among all DEGs, 64 genes have a $|\log_2 FC|$ greater than or equal to 1, with 16 upregulated and 48 downregulated in cluster 2. Notably, 2520 of the DEGs (85%) overlap with DEGs from the comparison without OCS-use stratification.

Second, an OCS blood transcriptomic signature was derived by comparing the blood transcriptome of individual patients before and after switching their OCS usage status (see Methods and Table E5 in this article's Online Repository at www.jacionline.org). The OCS signature (n = 6393) was subtracted from the 2960 DEGs yielded from the comparison of cluster 1 and cluster 2 samples after stratifying for individuals who were on OCSs at baseline. Six hundred twenty-seven genes remained after removing OCS-regulated genes (see Online Repository File 1 in this article's Online Repository at www.jacionline.org), including 44 genes with $|\log_2 FC|$ greater than or equal to 1. Pathway analysis using IPA identified 2 significantly enriched pathways among this gene set (n = 627), which are dolichyl-diphosphooligosaccharide biosynthesis (FDR = 0.04) and assembly of RNA polymerase I complex (FDR = 0.05) (see Table E6 in this article's Online Repository at www.jacionline.org).

Analysis of symptom-high/T2-biomarker-low patients with severe asthma

Patients were assigned into 4 groups on the basis of their biomarker and symptom levels at each study time point to examine the transcriptomic signature in symptom-high/T2-biomarker-low patients (see Table E7 in this article's Online Repository at www.jacionline.org). Comparison of clinical characteristics between patient groups at baseline is presented in Table II.

Because OCS treatment has a major effect on blood transcriptome, the proportion of patients on OCS was examined at each time point, in all patient subgroups defined by biomarker profile and symptom score as described above. A significantly imbalanced rate of OCS usage was observed in only 3 of the 12 comparisons (see Table E8 in this article's Online Repository at www.jacionline.org), and so stratified analysis by OCS usage was performed only for these comparisons.

Analysis of the transcriptomic data at baseline showed that the comparison of T2-biomarker-high versus T2-biomarker-low patients, irrespective of their symptom burden, yielded numerous significant DEGs, including 127 that were shared by both comparisons (Fig 1 and panel *A* of Fig 2); among the shared DEGs, 64 have a $|\log_2 FC|$ greater than or equal to 1 in both tests. Despite small numbers, stratified analysis on individuals not on OCSs (n = 11 and 18 for T2-biomarker-high and -low participants, respectively) at baseline was able to confirm 86 of the 127 shared DEGs. In contrast, the comparison of symptom-high versus symptom-low patients, irrespective of the biomarker level, yielded almost no DEGs (Fig 2, *B* and *C*), suggesting that differences at the transcriptomic level are mainly driven by differences in T2 biology expressed by biomarkers (blood eosinophils and FENO), rather than symptom burden. The analysis was repeated at week 24 and week 48 to assess gene signature stability. At week 24, similar to the result at baseline, comparison of T2-biomarker-high versus T2-biomarker-low patients yielded numerous significant DEGs irrespective of the symptom level (including 44 that are shared by both comparisons, among which 22 have a $|\log_2 FC| \geq 1$ in both tests). Almost no significant DEGs were identified when comparing symptom-high versus symptom-low patients irrespective of biomarker levels (see Fig E5 in this article's Online Repository at www.jacionline.org).

At week 48 (close-out visit), comparison of T2-biomarker-high versus T2-biomarker-low patients, irrespective of the symptom levels, once again yielded many significant DEGs, with 21 shared by both comparisons, including 15 that have a $|\log_2 FC|$ greater than or equal to 1 in both tests. Consistent with previous time points, the comparison of samples that are symptom-high versus symptom-low in T2-biomarker-high participants yielded almost no DEGs. However, at this time point, analysis of T2-biomarker-low participants resulted in hundreds of significant DEGs in symptom-high versus symptom-low patients, though the magnitude (FC) was small for all DEGs (Fig 3).

To determine whether the DEGs at the week-48 visit in these T2-biomarker- low/symptom-high patients (compared with those with good asthma control) were driven by the increased rate of patients on OCS treatment, the analysis was repeated, after removing individuals who were on OCSs at close-out visit and subsequently almost all DEGs became nonsignificant, suggesting that the difference observed is largely attributable to OCS usage. To further investigate the effect of OCS treatment, we overlapped the 220 DEGs associated with the OCS gene signature (n = 6393) and found that 105 of 220 (48%) DEGs were genes highly regulated by OCS. In addition, pathway analysis of the remaining DEGs after removing genes overlapping with the OCS signature identified no significantly enriched pathways. In all, our analyses suggest that OCS use is a likely driver of transcriptomic differences observed at close-out visit.

DEGs were compared across all time points and 15 genes were found to be consistently upregulated in T2-biomarker-high patients over time regardless of their symptom level (Fig 4).

DISCUSSION

Understanding the pathophysiology and developing targeted therapies for patients with a persistent and significant symptom burden, despite maximum asthma therapies, remains a major unmet need in severe asthma. To better investigate the molecular mechanisms underlying T2-biomarker-low severe asthma, we curated a selected cohort enriched for T2-biomarker-low individuals to specifically explore mechanisms for persistent symptoms in this important patient population. This study demonstrates that, in patients with severe asthma, although there is a clear transcriptomic-level change associated with elevated T2 biomarkers, there is no

TABLE II. Clinical and demographic features in patients at baseline based on composite measurements of T2 biomarkers (blood eosinophils and FENO) and symptom score (ACQ) levels at time of sampling (see Methods for patient group definitions)

Variable	Biomarker-high, Symptom-high	Biomarker-high, Symptom-low	Biomarker-low, Symptom-high	Biomarker-low, Symptom-low	*P* value
No. of patients; N = 92	14	11	45	22	
Age at inclusion (y); N = 92	55.6 (13.2)	45.6 (17.9)	53.2 (12.4)	53.0 (13.8)	.3008
Age at asthma diagnosis (y); N = 92	26.9 (17.5)	29.0 (23.4)	22.9 (19.7)	26.2 (17.8)	.7536
Sex; N = 92					.6950
Female	8 (57.1)	6 (54.5)	29 (64.4)	16 (72.7)	
Male	6 (42.9)	5 (45.5)	16 (35.6)	6 (27.3)	
Ethnicity; N = 92					.4738
White	12 (85.7)	9 (81.8)	42 (93.3)	21 (95.5)	
Non-White	2 (14.3)	2 (18.2)	3 (6.7)	1 (4.5)	
BMI (kg/m^2); N = 92	29.1 (5.5)	26.4 (3.7)	32.2 (6.3)	30.6 (5.6)	.0223
Smoking status; N = 92					.1051
Never smoked	9 (64.3)	6 (54.5)	38 (84.4)	18 (81.8)	
Ex-smoker	5 (35.7)	5 (45.5)	7 (15.6)	4 (18.2)	
Atopic disease; N = 92	8 (57.1)	8 (72.7)	32 (71.1)	15 (68.2)	.7841
Hospital admissions for asthma in last year; N = 92	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 1.0)	.6544
A&E visits in last year; N = 92	0.0 (0.0, 1.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.6276
GP visits for asthma in the last year; N = 92	0.0 (0.0, 4.0)	0.0 (0.0, 1.0)	3.0 (1.0, 4.0)	0.5 (0.0, 3.0)	.0094
Rescue courses of oral steroids in the last year; N = 92	3 (1, 5)	1 (0, 3)	3 (1, 4)	2 (1, 3)	.1639
Prior admission for asthma to a high dependency or intensive care unit; N = 92	1 (7.1)	1 (9.1)	17 (37.8)	3 (13.6)	.0231
Number of prior admissions for asthma to a high dependency or intensive care unit; N = 21	1.0 (1.0, 1.0)	0.0 (0.0, 0.0)	1.0 (1.0, 3.0)	1.0 (1.0, 5.0)	.4390
Ever been ventilated; N = 21	1 (100.0)	0 (0.0)	8 (50.0)	0 (0.0)	.1979
History of rhinitis; N = 92	10 (71.4)	7 (63.6)	30 (66.7)	17 (77.3)	.8009
History of eczema; N = 92	5 (35.7)	1 (9.1)	15 (33.3)	7 (31.8)	.4319
History of nasal polyps; N = 92	5 (35.7)	6 (54.5)	5 (11.1)	1 (4.5)	.0008
Prior nasal surgery; N = 92	6 (42.9)	6 (54.5)	3 (6.7)	1 (4.5)	<.0001
History of esophageal reflux; N = 92	8 (57.1)	6 (54.5)	35 (77.8)	10 (45.5)	.0523
History of aspirin sensitivity; N = 92	3 (21.4)	2 (18.2)	10 (22.2)	4 (18.2)	.9783
Depression/anxiety; N = 92	5 (35.7)	1 (9.1)	20 (44.4)	3 (13.6)	.0242
Hypertension; N = 92	4 (28.6)	0 (0.0)	16 (35.6)	4 (18.2)	.0809
Osteoporosis/osteopenia; N = 92	4 (28.6)	0 (0.0)	16 (35.6)	4 (18.2)	.0809
Osteoarthritis; N = 92	1 (7.1)	0 (0.0)	14 (31.1)	6 (27.3)	.0655
Hypercholesterolemia; N = 92	3 (21.4)	0 (0.0)	8 (17.8)	3 (13.6)	.4463
Diabetes; N = 92	1 (7.1)	1 (9.1)	10 (22.2)	2 (9.1)	.3368
Cataracts; N = 92	1 (7.1)	1 (9.1)	6 (13.3)	1 (4.5)	.6955
Obstructive sleep apnea; N = 92	0 (0.0)	0 (0.0)	3 (6.7)	1 (4.5)	.6324
Ischemic heart disease; N = 92	2 (14.3)	0 (0.0)	1 (2.2)	2 (9.1)	.2365
Peptic ulcer; N = 92	0 (0.0)	0 (0.0)	1 (2.2)	1 (4.5)	.7679
Stroke; N = 92	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)	.1309
Chronic kidney disease; N = 92	0 (0.0)	0 (0.0)	2 (4.4)	0 (0.0)	.5448
Glaucoma; N = 92	0 (0.0)	0 (0.0)	1 (2.2)	0 (0.0)	.7877
Myocardial infarction; N = 92	1 (7.1)	0 (0.0)	1 (2.2)	0 (0.0)	.5013
FEV_1 (L); N = 92	1.8 (0.8)	2.8 (0.7)	2.1 (0.7)	2.7 (0.8)	.0002
% Predicted FEV_1; N = 92	61.5 (15.4)	87.1 (10.5)	71.5 (19.0)	91.3 (15.0)	<.0001
FVC (L); N = 92	2.9 (1.0)	4.0 (1.1)	3.1 (0.9)	3.6 (0.8)	.0038
% Predicted FVC; N = 92	80.1 (13.3)	101.3 (10.6)	85.1 (16.1)	98.8 (13.2)	<.0001
FEV_1/FVC; N = 92	0.61 (0.14)	0.70 (0.05)	0.67 (0.13)	0.74 (0.10)	.0168
PEFR (L/min); N = 90	329.6 (141.9)	480.3 (137.2)	351.6 (128.4)	452.7 (129.4)	.0017
ACQ-7 score; N = 92	2.8 (1.1)	0.7 (0.4)	2.7 (0.7)	0.9 (0.4)	<.0001
AQL total score; N = 88	4.1 (1.3)	6.4 (0.6)	4.1 (1.1)	5.7 (0.8)	<.0001
Sputum eosinophils (%); N = 32	71.8 (71.8, 71.8)	10.4 (5.0, 55.8)	0.3 (0.0, 1.2)	0.5 (0.0, 5.4)	.0017
Sputum neutrophils (%); N = 32	2.1 (2.1, 2.1)	19.6 (11.4, 42.1)	73.5 (47.2, 89.0)	54.3 (35.5, 87.6)	.0133
Sputum lymphocytes (%); N = 32	6.5 (6.5, 6.5)	0.5 (0.3, 1.9)	0.5 (0.0, 1.0)	0.0 (0.0, 0.0)	.0563
Macrophage sputum (%); N = 32	15.5 (15.5, 15.5)	36.8 (24.4, 57.8)	24.6 (8.3, 44.0)	44.3 (7.0, 50.8)	.6799
FENO (ppb); N = 92	48 (39, 53)	38 (36, 44)	12 (8, 15)	13 (11, 18)	<.0001
Blood eosinophils (10^9/L); N = 92	0.56 (0.36, 0.72)	0.74 (0.54, 1.03)	0.07 (0.04, 0.12)	0.10 (0.06, 0.12)	<.0001
Periostin (ng/mL); N = 92	62.7 (16.3)	73.3 (24.8)	47.2 (12.6)	43.8 (14.0)	<.0001
OCS user; N = 38 of 92	3 (21.4)	2 (18.2)	27 (60.0)	6 (27.3)	.0047
OCS dose (mg); N = 38	5 (5, 10)	6 (5, 7)	10 (10, 15)	9 (5, 10)	.0625
ICS dose (BDP μg equivalent); N = 92	2386 (762)	2309 (723)	2338 (799)	2027 (442)	.3480

A&E, Accident & Emergency; *AQL*, Asthma Quality of Life questionnaire (Juniper); *BDP*, beclomethasone dipropionate; *GP*, general practitioner; *ICS*, inhaled corticosteroid; *PEFR*, peak expiratory flow rate; *ppb*, parts per billion.

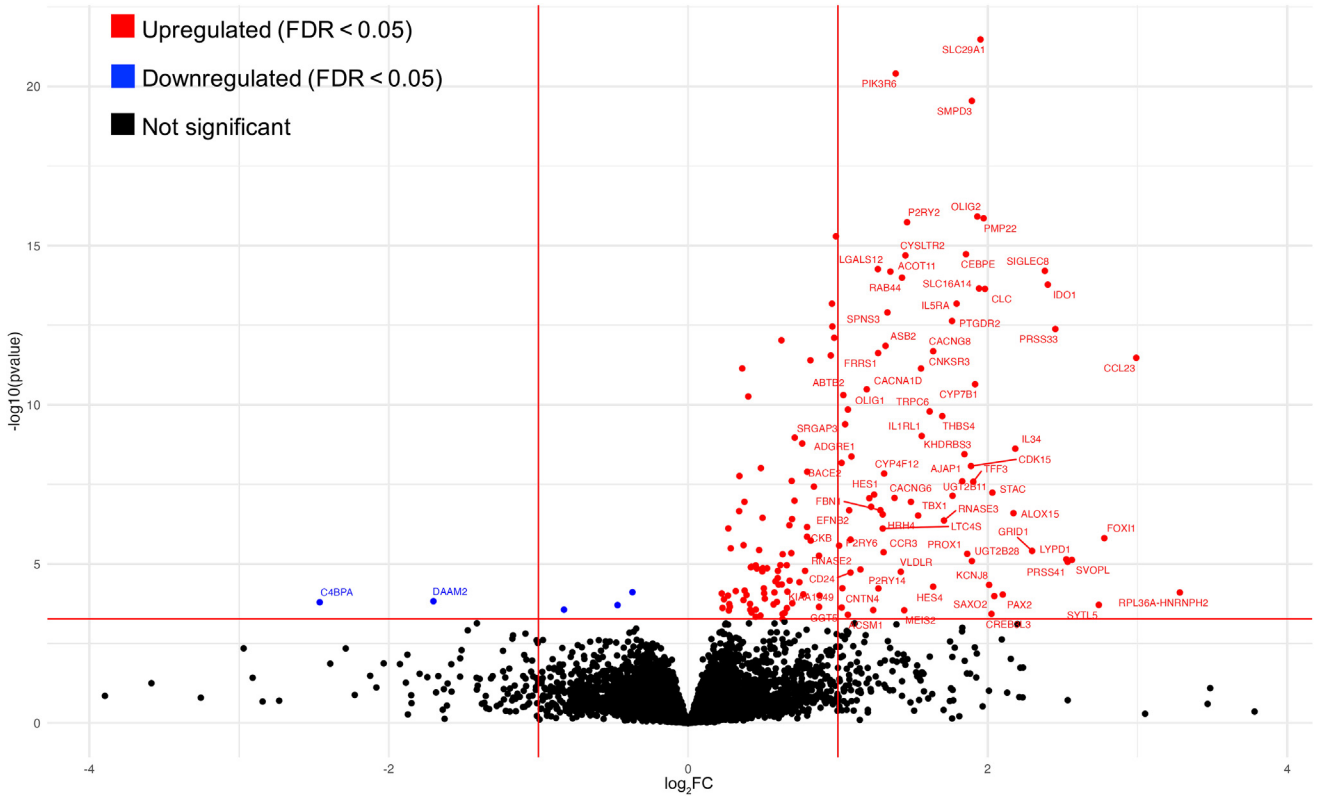

FIG 1. Volcano plots of DEGs in patient groups defined by composite biomarker measurements of T2 biomarkers (blood eosinophils and FENO) and symptoms (ACQ-7 score) at baseline: Biomarker-high (n = 14) vs -low (n = 45) among symptom-high patients. Genes not significant in the test are in black. For significant DEGs (FDR < 0.05), upregulated and downregulated genes are denoted in red and blue, respectively. The red vertical lines denote $\log_2$FC equals −1 and 1, respectively. The red horizontal lines denote $-\log_{10}$(*P*-value corresponding to FDR = .05).

clear signature associated with a highly symptomatic, type-2 biomarker-low population. The absence of signature in this symptom-high group does not support evidence for activation of an alternative systemic inflammatory pathway in this group and is consistent with multiple negative trials of therapies targeting putative non-T2 pathways including IL-17, IL-23, CXC-chemokine-receptor-2, and TNF-α in uncontrolled asthma.[11-14]

Initial cluster analysis identified a cluster (cluster 2) with suppressed eosinophil count and high symptom score, with higher rate and dose of OCS treatment. Differential gene expression analysis between cluster 1 and cluster 2 demonstrated 4162 DEGs. Pathways found to be enriched among the DEGs are broadly associated with innate and adaptive immune responses as well as metabolic reprogramming (Table E2). These pathways are consistent with those identified from prior blood transcriptomics in patient populations with severe asthma.[15] The strong overlap between pathways implicated generally in severe asthma and those implicated in our analysis of cluster 2 further illustrates the lack of a fundamentally distinctive signature for patients with severe asthma characterized by the T2-biomarker-low phenotype. CSs have been implicated as regulators of the severe asthma blood gene signature.[15] Further analysis demonstrated that only 627 of 2960 residual DEGs remained after the subtraction of the OCS signature, confirming that the substantial majority of these DEGs were driven by OCS exposure. This demonstrates the importance of accounting for background treatment exposure in patients with severe asthma and other diseases, where treatment has broad effects on the blood transcriptomic signature. The clinical readout from the UK Medical Research Council Refractory Asthma Stratification Program biomarker study demonstrated that in patients with uncontrolled asthma (ACQ-7 score > 1.5), the CS dose could be reduced safely using a T2-biomarker–directed algorithm, without worsening asthma control or increased exacerbation rate.[2] Taken together, this suggests that in patients expressing low type-2 biomarkers with high symptom burden, the transcriptomic signature is driven by excessive OCS treatment, and further that this can be reduced safely using T2-biomarker–directed adjustments.

Two approaches were adopted to adjust for the background "OCS signature" to further explore novel potential pathways in severe asthma. First, restriction of the cluster analysis to those patients who were on OCSs. Clinical and demographic features associated with these 2 clusters again demonstrated that patients in cluster 2 tended to have lower blood eosinophil counts and were treated with higher OCS doses. Notably, of the 2960 DEGs in this OCS-treated population, 2520 genes (85%) overlap with DEGs from the comparison without OCS usage stratification, suggesting that there could be residual treatment effect from OCS dosage difference between the 2 groups as well as other underlying drivers unrelated to the use of OCSs.

CSs regulate gene expression through glucocorticoid receptors. The binding sites of glucocorticoid receptors have been shown to be highly tissue- and cell type–specific[16] and consistent with this, limited overlap was observed when comparing OCS signatures identified in A549 cells,[17] a human lung epithelial carcinoma cell line, and blood cell lines from different pathological samples (eg, patients with chronic obstructive pulmonary disease[18] and

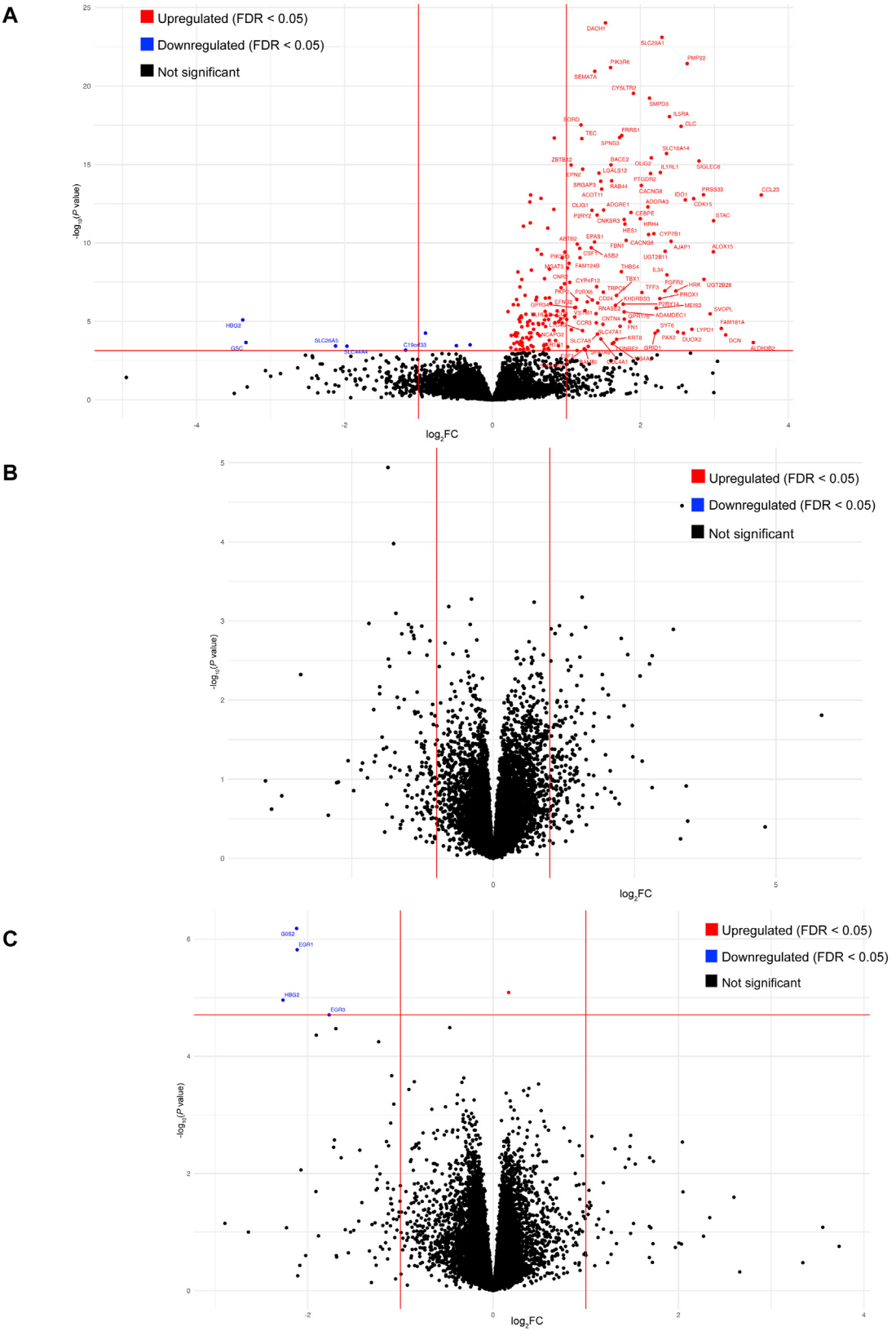

FIG 2. Volcano plots of DEGs in patient groups defined by composite biomarker measurements of T2 biomarkers (blood eosinophils and FENO) and symptoms (ACQ-7 score) at baseline: **A,** Biomarker-high (n = 11) vs -low (n = 22) among symptom-low patients. **B,** Symptom-high (n = 14) vs -low (n = 11) among biomarker-high patients. **C,** Symptom-high (n = 45) vs -low (n = 22) among biomarker-low patients. Genes not significant in the test are in black. For significant DEGs (FDR < 0.05), upregulated and downregulated genes are denoted in red and blue, respectively. The red vertical lines denote $\log_2$FC equals −1 and 1, respectively. The red horizontal lines denote $-\log_{10}$(*P*-value corresponding to FDR = .05).

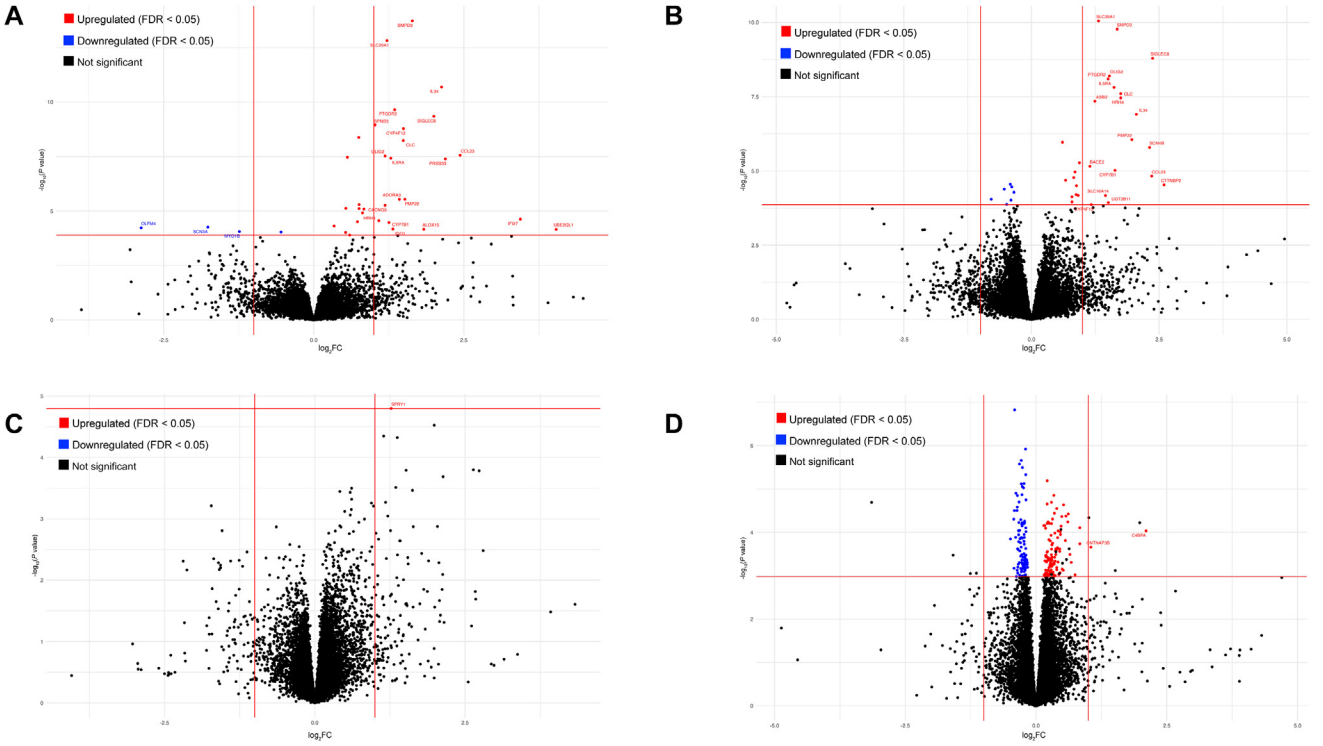

FIG 3. Volcano plots of DEGs in patient groups defined by composite biomarker measurements of T2 biomarkers (blood eosinophils and FENO) and symptoms (ACQ-7 score) at week 48 (close-out visit): **A,** Biomarker-high (n = 17) vs -low (n = 26) among symptom-high patients. **B,** Biomarker-high (n = 10) vs -low (n = 19) among symptom-low patients. **C,** Symptom-high (n = 17) vs -low (n = 10) among biomarker-high patients. **D,** Symptom-high (n = 26) vs -low (n = 19) among biomarker-low patients. Genes not significant in the test are in black. For significant DEGs (FDR < 0.05), upregulated and downregulated genes are denoted in red and blue, respectively. The red vertical lines denote $\log_2$FC equals −1 and 1, respectively. The red horizontal lines denote $-\log_{10}$(*P*-value corresponding to FDR = .05).

healthy volunteers[19]). Given the lack of gene expression signature for OCSs in the blood transcriptome for asthma, an OCS signature was derived, which can be used to adjust for the background transcriptomic changes due to OCS use. The OCS signature, as defined here, encompassed 100% and 61% of the OCS blood transcriptomic signatures derived from healthy volunteers and patients with chronic obstructive pulmonary disease, respectively.

The "OCS signature" was subtracted from the 2960 DEGs yielded from comparison of the cluster 1 and cluster 2 samples in the OCS-treated population to fully account for OCS treatment effect, demonstrating 627 genes remained after removing all OCS-regulated genes. One caveat of this approach is the potential incidental removal of disease pathogenic pathways due to their overlap with the OCS signature. However, any such pathways are likely to be CS responsive and are unlikely to contribute to the persistently high symptom burden in these T2-biomarker-low heavily OCS-treated patients. Pathway analysis using IPA identified that 2 significantly enriched pathways among the gene set remained (n = 627), which are dolichyl-diphosphooligosaccharide biosynthesis (FDR = 0.04) and assembly of RNA polymerase I complex (FDR = 0.05). Interestingly, a meta-analysis across 8 studies exploring gene expression in the airway epithelium in patients with asthma compared with nonasthmatic controls found dolichyl-diphosphooligosaccharide biosynthesis as one of the top pathways identified by pathway enrichment.[20] Further investigation is required to fully understand how dolichyl-diphosphooligosaccharide biosynthesis contributes to the difference in clinical characteristics observed between the cluster 1 and cluster 2 participants and the role of the assembly of RNA polymerase I complex pathway in severe asthma.

The T2-biomarker-low/symptom-high patient group was compared with the T2-biomarker-low/symptom-low patient group, and even though ACQ-7 score is one of the grouping criteria applied, it is still worth noting that there was a striking quantitative difference in the ACQ-7 scores of these 2 groups (2.7 vs 0.9, $P < .0001$). The T2-biomarker-low/symptom-high patients had a significantly higher proportion of patients on OCSs, which is consistent with our hypothesis that these patients have persistent symptoms despite suppression of T2 pathways, and their symptoms are not responsive to CSs. In addition, significantly lower lung function and greater airflow obstruction were observed in patients who are T2-biomarker-low/symptom-high despite them being T2-biomarker-low. Therefore, we suspect the difference in lung function is one of the factors that lead to worse symptoms in some patients. Obesity is one of the common causes for reduced lung volume and capacity[21]; however, differences in body mass index between the 2 groups were not statistically significant ($P = .31$), suggesting that other factors (eg, esophageal reflux and depression) are driving this observation.

In the T2-biomarker-high patients, DEGs were compared across all time points and 15 genes were consistently upregulated in T2-biomarker-high patients over time irrespective of their symptom level. Among these genes, CLC,[22-25] SIGLEC-8,[26] and IL5RA[27,28] are well-known signatures and have previously been associated with eosinophilic and mast cell/basophil biology.[22-28] This supports our patient grouping approach to identifying a population where eosinophilic inflammation was relevant. It also supports our conclusion that when controlling for exposure to OCSs in the T2-biomarker-low patients, there was no specific gene signature in peripheral

Gene
SLC29A1
PIK3R6
SMPD3
OLIG2
SORD
SIGLEC8
CLC
IL5RA
SPNS3
PTGDR2
ASB2
CCL23
CACNA1D
CYP4F12
HRH4

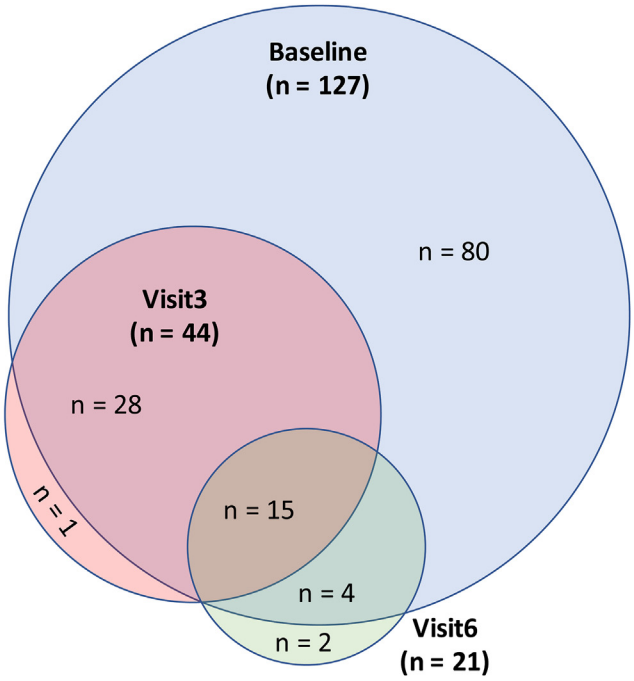

FIG 4. Genes stably upregulated in T2-biomarker-high patients at all time points irrespective of symptom status. A total of 127, 44, and 21 genes were upregulated in T2-biomarker-high patients regardless of symptom status at baseline (blue), visit3 (red), and visit6 (green), respectively. Among these genes, 15 are shared by all 3 time points (detailed in the table).

blood associated with a high symptom burden. The overall sample size was small, but this study actively enriched for the T2-low severe asthma phenotype and we believe it is unlikely that a T2-biomarker-low signature was not detected because of the limited sample size. However, we recognize the potential existence of a local inflammatory process in the lung that confers a novel gene expression signature in the airway associated with patient symptom burden, but this is beyond the scope of the current study.

One limitation of this study is that, given our unique cohort curation criteria to enrich for a T2-biomarker-low population, we are not able to validate our findings due to the lack of an equivalent replication cohort and our findings should be considered exploratory. In addition, our study cohort is predominantly composed of individuals of European descent (92.4%); therefore, conclusions from this work cannot be generalized to other ethnic groups until the result is replicated in a more ethnically diverse population.

In summary, this novel transcriptomic data from a population with severe asthma has shown that, after controlling for OCS therapy, there are distinct genetic signatures seen in those patients with evidence of T2 biology as identified by blood eosinophils and FENO. However, this work did not identify any such gene signatures associated with T2-biomarker-low participants, irrespective of symptom burden and after controlling for exposure to OCSs. Further investment in understanding factors driving symptomology in this patient group is required, including looking at noninflammatory and extrathoracic mechanisms.

DISCLOSURE STATEMENT

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Disclosure of potential conflict of interest: X. Zeng, J. Qing, C.-M. Li, J. Lu, T. Yamawaki, B. V. Lugt, Y.-H. Hsu, and H. Hsu are all employees of Amgen. L. G. Heaney has received grant funding and has taken part in advisory boards and given lectures at meetings supported by Hoffmann-La Roche–Genentech and Aerocrine, has taken part in asthma clinical trials sponsored by Hoffmann-La Roche–Genentech, for which his institution was remunerated, and is Academic Lead for the Medical Research Council Stratified Medicine UK Consortium in Severe Asthma, which involves industrial partnerships with a number of pharmaceutical companies, including Hoffman-La Roche and Aerocrine. R. Djukanovic reports receiving fees for lectures at symposia organized by Novartis, AstraZeneca, and Teva, consultation for Teva and Novartis as a member of advisory boards, and participation in a scientific discussion about asthma organized by GSG; is a cofounder and consultant, and has shares in Synairgen, a University of Southampton spin-out company; and has given lectures at symposia organized by pharmaceutical companies and has consulted companies as a member of advisory boards and received nonfinancial support from GlaxoSmithKline, Chiesi, Novartis, and Napp Pharmaceuticals. A. Woodcock reports personal fees from GlaxoSmithKline, Novartis, and Chiesi, other fees from Reacta Biotech, and other services from Axalbion and the Medicines Evaluation Unit. J. R. Arron was an employee of Genentech, holding stock and stock options in the Roche Group, and is a named inventor on patents pending relating to diagnosis and treatment of asthma. D. F. Choy is an employee and holds stocks and options in Genetech, is a member of the Roche Group, and is an inventor of planned or filed patents related to the diagnosis and treatment of respiratory diseases. P. Bradding has received grant funding from Genentech, acts as a consultant for Roche and GlaxoSmithKline, and has received speaker fees from AstraZeneca and support to attend conferences from GlaxoSmithKline. C. E. Brightling has received grants or

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Clinical implications: Absence of any transcriptomic signature in our highly symptomatic, T2-low participants with severe asthma is consistent with there not being an alternative systemic inflammatory pathway in these patients.

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