ORIGINAL ARTICLE

Gene Expression Correlated with Severe Asthma Characteristics Reveals Heterogeneous Mechanisms of Severe Disease

Brian D. Modena[1,2], Eugene R. Bleecker[3], William W. Busse[4], Serpil C. Erzurum[5], Benjamin M. Gaston[6,7], Nizar N. Jarjour[4], Deborah A. Meyers[3], Jadranka Milosevic[1], John R. Tedrow[1], Wei Wu[8], Naftali Kaminski[9], and Sally E. Wenzel[1]

[1]Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh Asthma Institute at UPMC, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; [2]Scripps Translational Science Institute, The Scripps Research Institute, La Jolla, California; [3]Center for Genomics and Personalized Medicine Research, Wake Forest School of Medicine, Winston-Salem, North Carolina; [4]Division of Allergy, Pulmonary, and Critical Care Medicine, University of Wisconsin, Madison, Wisconsin; [5]Department of Pathobiology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio; [6]Division of Pediatric Pulmonary, Allergy and Immunology, Case Western Reserve University, Cleveland, Ohio; [7]Rainbow Babies and Children's Hospital, Cleveland, Ohio; [8]Lane Center for Computational Biology School of Computer Science, Carnegie Mellon University, Pittsburgh, Pennsylvania; and [9]Pulmonary, Critical Care and Sleep Medicine, Yale School of Medicine, New Haven, Connecticut

Abstract

Rationale: Severe asthma (SA) is a heterogeneous disease with multiple molecular mechanisms. Gene expression studies of bronchial epithelial cells in individuals with asthma have provided biological insight and underscored possible mechanistic differences between individuals.

Objectives: Identify networks of genes reflective of underlying biological processes that define SA.

Methods: Airway epithelial cell gene expression from 155 subjects with asthma and healthy control subjects in the Severe Asthma Research Program was analyzed by weighted gene coexpression network analysis to identify gene networks and profiles associated with SA and its specific characteristics (i.e., pulmonary function tests, quality of life scores, urgent healthcare use, and steroid use), which potentially identified underlying biological processes. A linear model analysis confirmed these findings while adjusting for potential confounders.

Measurements and Main Results: Weighted gene coexpression network analysis constructed 64 gene network modules, including modules corresponding to T1 and T2 inflammation, neuronal function, cilia, epithelial growth, and repair mechanisms. Although no network selectively identified SA, genes in modules linked to epithelial growth and repair and neuronal function were markedly decreased in SA. Several hub genes of the epithelial growth and repair module were found located at the 17q12–21 locus, near a well-known asthma susceptibility locus. T2 genes increased with severity in those treated with corticosteroids but were also elevated in untreated, mild-to-moderate disease compared with healthy control subjects. T1 inflammation, especially when associated with increased T2 gene expression, was elevated in a subgroup of younger patients with SA.

Conclusions: In this hypothesis-generating analysis, gene expression networks in relation to asthma severity provided potentially new insight into biological mechanisms associated with the development of SA and its phenotypes.

Keywords: severe asthma; gene expression; bronchial epithelial cells; networks; mechanisms

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Correspondence and requests for reprints should be addressed to Sally E. Wenzel, M.D., Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine, University of Pittsburgh Asthma Institute at UPMC, NW 931 Montefiore, 3459 Fifth Avenue, Pittsburgh, PA 15213. E-mail: wenzelse@upmc.edu

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At a Glance Commentary

Scientific Knowledge on this Subject: Recent gene expression studies of human bronchial epithelial cells in individuals with asthma have provided biological insight and underscored possible mechanistic differences among individuals by demonstrating that up-regulated expression of three coexpressed IL-13/T2 signature genes are associated with atopy, eosinophilic airway inflammation, and a positive response to inhaled corticosteroids in corticosteroid-naive individuals with mild asthma. Notwithstanding these findings, gene expression profiles and differences in underlying molecular mechanisms in patients with severe asthma remains unknown.

What This Study Adds to the Field: Airway epithelial cell gene expression from 155 subjects with asthma and healthy control subjects in the Severe Asthma Research Program was analyzed by weighted gene coexpression network analysis and LIMMA to identify gene networks and gene profiles associated with severe asthma and its specific characteristics (i.e., pulmonary function tests, quality of life scores, urgent healthcare use, and steroid use), potentially also revealing underlying biological processes. In this hypothesis-generating analysis, gene expression networks in relation to asthma severity provided new insight into biological mechanisms associated with the development of severe asthma and its phenotypes.

Severe asthma (SA), a clinically heterogeneous disease, accounts for 50% of its disease-associated costs, although includes <10% of all asthma (1, 2). Gene expression and genetic variation studies both indicate that asthma is a polygenic disease with multiple molecular roots (3, 4). Gene expression studies of freshly brushed human bronchial epithelial cells (HBECs) have begun to identify different molecular mechanisms that separate asthmatic phenotypes. In a group of mild, corticosteroid-naive patients and healthy control (HC) subjects, up-regulated expression of three coexpressed IL-13/T2 signature genes in HBECs were associated with atopy, eosinophilic airway inflammation, and a positive response to inhaled corticosteroids (ICSs) (5, 6). In a later study that analyzed HBECs from 155 subjects with asthma and HC subjects in the NHLBI-sponsored Severe Asthma Research Program (SARP), hypothesis-free clustering associated gene expression with the inflammatory biomarker fractional exhaled nitric oxide (F_{ENO}) to identify five phenotypes of asthma, each with unique gene expression profiles, biological pathways, and clinical characteristics (4). Notwithstanding these findings, differences in underlying molecular mechanisms among individuals with asthma remain poorly understood.

Publically available gene expression data, stored in databases such as the Gene Expression Omnibus (GEO), contain vast amounts of data that have not yet been fully utilized (7, 8). As data analysis techniques evolve, previously analyzed data sets should be open to new and complementary analyses. Weighted gene coexpression network analysis (WGCNA) is a newer methodology that can construct gene coexpression networks, or groups of similarly behaving genes with a common biological relationship or function (9, 10). WGCNA has been successfully used on sputum cell gene expression profiles to show that T1-like/cytotoxic and IFN-γ signaling pathways are decreased in children with asthma with deficits in baseline lung function compared with children with asthma with normal lung function (11).

In this follow-up analysis of previously reported HBEC microarray gene expression obtained through the SARP network (4), WGCNA was used to associate coexpressed gene networks with extensive demographic and inflammatory participant traits, including American Thoracic Society (ATS)-defined asthma severity, age, body mass index (BMI), race, F_{ENO}, history of chronic rhinosinusitis (CRS), total Juniper Asthma Quality of Life Questionnaire (AQLQ) scores, FEV_1% predicted, emergency department visit or hospitalization in the last year (ED/hosp), use of ICS and oral corticosteroids (OCS). The biological function was determined for gene networks, and networks that significantly associated with asthma severity and its specific characteristic, including FEV_1% predicted, AQLQ, ED/hosp, steroid use, were highlighted. Because the relationship between gene expression and clinical traits may potentially be confounded by batch effects and numerous, nonrelevant sample traits, a linear model software (LIMMA, R/Bioconductor) was used to confirm these findings while adjusting for potential confounders (e.g., use of corticosteroids). T2 and T1 inflammatory networks identified in the primary data set were validated through a coexpression analysis on an external data set downloaded from the GEO, consisting of 43 HC subjects and 62 subjects with asthma with mild-to-moderate disease who were not on ICS (12).

Methods

Study Population

Bronchial brushing samples and matching demographic data from 155 participants were obtained from 2009 to 2011 as part of SARP. Detailed clinical characteristics of the SARP cohort is found in a previous publication (4), and gene expression of the SARP and external cohorts are available online (GEO database; http://www.ncbi.nlm.nih.gov/geo; accession numbers: GSE63142,GSE67472).

WGCNA and LIMMA Analyses

Microarray data was normalized using cyclic local regression. Median values were used for probes with identical RefSeq numbers, resulting in a filtered list of 30,889 probes. A full explanation of the WGCNA is in the online supplement. In summary, all expressed genes were input into WGCNA, which identified gene coexpression networks (modules) or similarly behaving genes. WGCNA was used to correlate or associate highly coexpressed genes (modules) with all demographic data (*see* the online supplement). WGCNA uses the terminology "cor" to report both associations and correlations with categorical and numeric values, respectively. Ingenuity pathway analysis and the PANTHER (Protein Analysis through Evolutionary Relationships) Classification System were used to assign biological functions of modules identified.

Using WGCNA, gene expression was correlated with sample trait values (gene significance [GS]) and plotted against module membership (MM). MM is a measurement of how close a gene's expression tracks with the eigenvalue of

each module, or in other words, how "tight" a gene clusters with other genes within its respective module. A gene with a high GS and high MM is found to be both significantly associated and/or correlated with the sample trait, and to track closely with central elements of a module, respectively. These genes are possibly key components to biological processes that associate strongly with the sample trait.

Because the relationship between gene expression and clinical traits may potentially be confounded by batch effects and other, nonrelevant sample traits, LIMMA was used to confirm these findings while adjusting for potential confounders (e.g., use of corticosteroids) in a linear model. LIMMA is an R/Bioconductor package designed for analyzing data from gene expression experiments with a variety of experimental conditions (13, 14).

Results

Demographic Data

Demographic and phenotypic data have been previously reported (4). The basic demographics are summarized in Table 1. A total of 129 of the 155 participants had asthma. Approximately one-third of patients (n = 51) met the ATS 2000 definition of SA, and 63% of these patients were using OCS. Twenty-eight participants (18%) did not have Fe_{NO} values, and 17 (11%) had incomplete or missing total AQLQ scores. All other sample traits used had less than 10% missing data.

Construction of Gene Coexpression Modules and Correlation with Clinical Variables Using WGCNA

Using the default parameter settings and all expressed genes (n = 30,889), 64 gene modules were identified (*see* the online supplement). WGCNA was then used to correlate each module with all clinical traits available in our participant database, calculating a *P* value significance for each module–trait correlation calculated. After an initial screening for strong correlations between all modules and available sample traits (*see* heat map images in the online supplement), the final analysis was based on the module–trait correlations with the highest significance and interest. The biological plausibility of correlating modules with clinical traits is demonstrated by an extremely high correlation of tightly coexpressed, Y-linked genes (module: lightpink4/Sex) with male sex (cor = 0.93; $P = 7 \times 10^{-83}$).

The clinical traits that most correlated (based on *P* value significance) with one or more modules were BMI, race, history of CRS, Fe_{NO}, asthma severity, AQLQ scores, FEV_1% predicted, ED/hosp, and use of ICS and OCS, represented by the heat map of Figure 1. FVC% predicted was correlated with the same modules as FEV_1% predicted, but had a lower significance.

Modules Up-regulated with Severe Asthma

Four modules positively correlated with disease severity: magenta (cor = 0.35; $P = 7 \times 10^{-6}$), red (cor = 0.35; $P = 1 \times 10^{-5}$), thistle1 (cor = 0.3; $P = 2 \times 10^{-4}$), and mediumpurple3 (cor = 0.27; $P = 7 \times 10^{-4}$) (Figure 1). The magenta module, designated "mitosis," contained genes that encode proteins related to mitosis and cell division (e.g., *PTTG1*, *BIRC5*, *NCAPG*, *CDCA2*, and *FANCI*). Gene enrichment analysis using Ingenuity Pathway Analysis (IPA) reflected "mitotic roles of polo-like kinase" and "cell cycle: G2/M DNA damage checkpoint regulation" as the top two signaling pathways ($P = 9.6 \times 10^{-11}$; $P = 0.04 \times 10^{-9}$). Magenta demonstrated a negative correlation with AQLQ scores (cor = −0.39; $P = 5 \times 10^{-7}$) (note: a lower AQLQ relates to increased symptoms) and FEV_1% predicted (cor = −0.33; $P = 3 \times 10^{-5}$), and the highest positive association with use of ICS (cor = 0.35; $P = 9 \times 10^{-6}$) and OCS (cor = 0.3; $P = 2 \times 10^{-4}$).

The red module (n = 982), designated "T2," contained all the same genes previously described with airway T2 inflammation (5, 12). Red/T2 expression was associated with race (cor = 0.23; $P = 0.004$), correlated strongly with Fe_{NO} (cor = 0.47; $P = 1 \times 10^{-7}$), and BMI (cor = 0.35; $P = 7 \times 10^{-6}$), and was positively associated with use of ICS (cor = 0.25; $P = 0.002$). Red/T2 gene expression, as measured by the geometric mean of all red genes, was greater in African Americans than those of European ancestry (857 ± 19 vs. 801 ± 11; $P = 0.006$). Red/T2 had a modest negative correlation with FEV_1% predicted (cor = −0.33; $P = 3 \times 10^{-5}$) and the highest negative correlation with AQLQ (cor = −0.42; $P = 6 \times 10^{-8}$).

Thistle1, designated "histone," was a small module (n = 89) with mostly histone or histone-related proteins (e.g., top hubs: *HIST1H2BB*, *HIST1H2BF*, *HIST1H2BE*,

Table 1. Summary of Clinical Characteristics of the Severe Asthma Research Program Cohort

	HCs	Mild-Mod No ICS	Mild + ICS	Mod + ICS	Severe	*P* Value	Significant Intergroup Comparison*
Age, yr, mean ± SE	33 ± 2	28 ± 2	36 ± 2	36 ± 3	45 ± 2	<0.0001	SA > HC, SA > Mild No ICS
Sex, M/F	12/14	13/24	6/16	8/11	16/35	0.61	No intergroup difference
Race, W/AA/O	18/5/3	21/10/5	14/5/2	8/10/1	31/14/3	0.43	No intergroup difference
BMI, kg/m^2, median (IQR)	24 (22–30)	28 (23–32)	28 (25–34)	29 (25–32)	32 (26–37)	0.006	SA > HC
FEV_1% pred, mean ± SD	94 ± 3	85 ± 3	92 ± 3	68 ± 4	56 ± 2	<0.0001	HC > SA, Mild No ICS > SA, Mild + ICS > SA, HC > Mod + ICS, Mild No ICS > Mod + ICS, Mild + ICS > Mod + ICS
Fe_{NO}, ppb, median (IQR)	20 (13–37)	37 (19–60)	21 (13–39)	39 (22–59)	37 (21–63)	0.043	No intergroup difference

Definition of abbreviations: AA = African American; BMI = body mass index; Fe_{NO} = fractional exhaled nitric oxide; HC = healthy control; ICS = inhaled corticosteroids; IQR = interquartile range; Mod = moderate; O = other; SA = severe asthma; W = white.
A full description of demographics is found in a preceding publication involving this dataset (4).
*All significant intergroup differences listed achieved a Bonferroni corrected *P* value of $P \leq 0.005$.

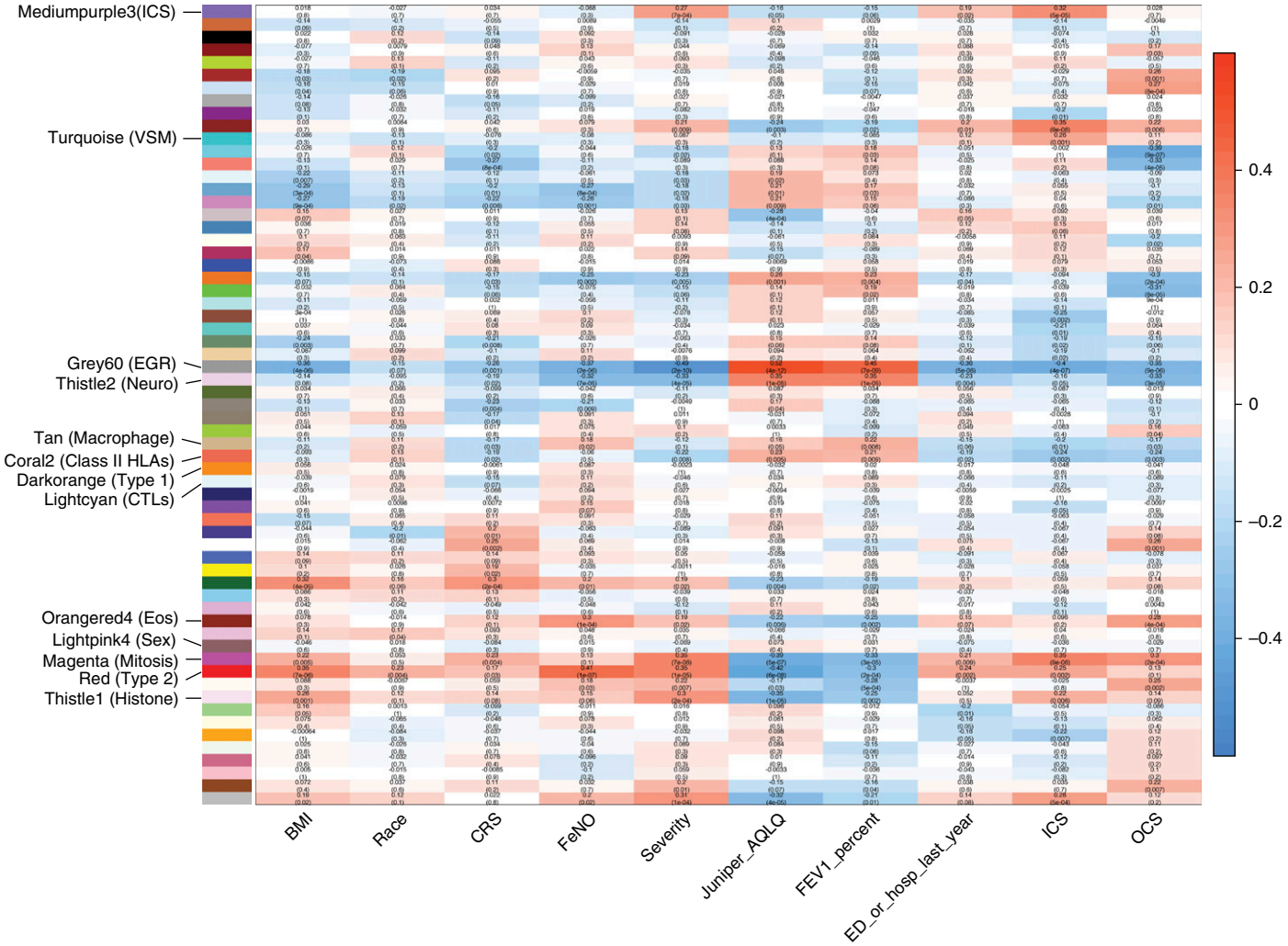

Figure 1. Weighted gene coexpression network analysis heat map. Using the default parameter settings and all expressed genes (n = 30,889), 64 gene modules were identified using weighted gene coexpression network analysis that were correlated with body mass index (BMI), race, history of chronic rhinosinusitis (CRS), fractional exhaled nitric oxide (Fe_{NO}), American Thoracic Society–defined asthma severity classes, total Juniper Asthma Quality of Life Questionnaire (AQLQ) scores, FEV_1% predicted, emergency department visit or hospitalization in the past year (ED_or_hosp_last_year), and use of inhaled (ICS) and oral (OCS) corticosteroids. Positive correlations are *red*, and negative correlations are *blue*. Putative biological functions of significant modules, identified by gene enrichment tools and literature review, are given on the *left graph margin*. CTLs = cytotoxic T lymphocytes; EGR = epithelial growth and repair; Eos = eosinophils; HLA = human leukocyte antigen; Neuro = neuronal function and development; VSM = vascular smooth muscle.

HIST1H2BN, and *HIST1H2BH*). Thistle1 was negatively correlated with AQLQ (cor = −0.35; $P = 1 \times 10^{-5}$). Finally, mediumpurple3, designated "ICS," was another small module (n = 137) with genes induced by glucocorticoids [e.g., top hub genes: *FKBP5* (15), *ZBTB16* (16), *PHACTR3*, *TFEB*, and *KLF9* (17); and others including *KLF11* (18), *KLF15* (19), and *ERBB4* (20, 21)]. Mediumpurple3/ICS was up-regulated with ICS use (cor = 0.32; $P = 5 \times 10^{-5}$), but, surprisingly, not OCS (cor = 0.028; $P = 0.7$).

Of the four gene modules, magenta/mitosis and red/T2 were the most associated with disease severity, ED/hosp, and use of ICS and OCS. They were also the most negatively correlated with FEV_1% predicted and AQLQ. Magenta/mitosis gene expression increased with each step of disease severity: HCs less than mild-to-moderate asthma not treated with ICS (mild-mod no ICS) less than mild-to-moderate asthma treated with ICS (mild-mod + ICS) less than moderate (mod + ICS) less than SA. In contrast, red/T2 expression was equally elevated in mild-mod no ICS compared with mod + ICS and SA (Figures 2A and 2B). T2 expression was lowest in mild-mod + ICS, although it did not reach statistical significance in comparison to mild-mod no ICS, mod + ICS ($P = 0.048$), and SA ($P = 0.036$) after a multiple comparison correction.

Using WGCNA to calculate GS versus MM, we found that red and magenta genes most significantly associated with SA characteristic traits (GS) were also the most important (central) elements of modules (MM), as demonstrated by the upper right genes in the plots of Figure 3. As demonstrated by the positive slopes in Figure 3, the GS of red and magenta modules as a group correlated strongly with membership (MM) for AQLQ (cor = 0.43; $P = 8 \times 10^{-33}$ and cor = 0.47; $P = 4.1 \times 10^{-55}$ for magenta and

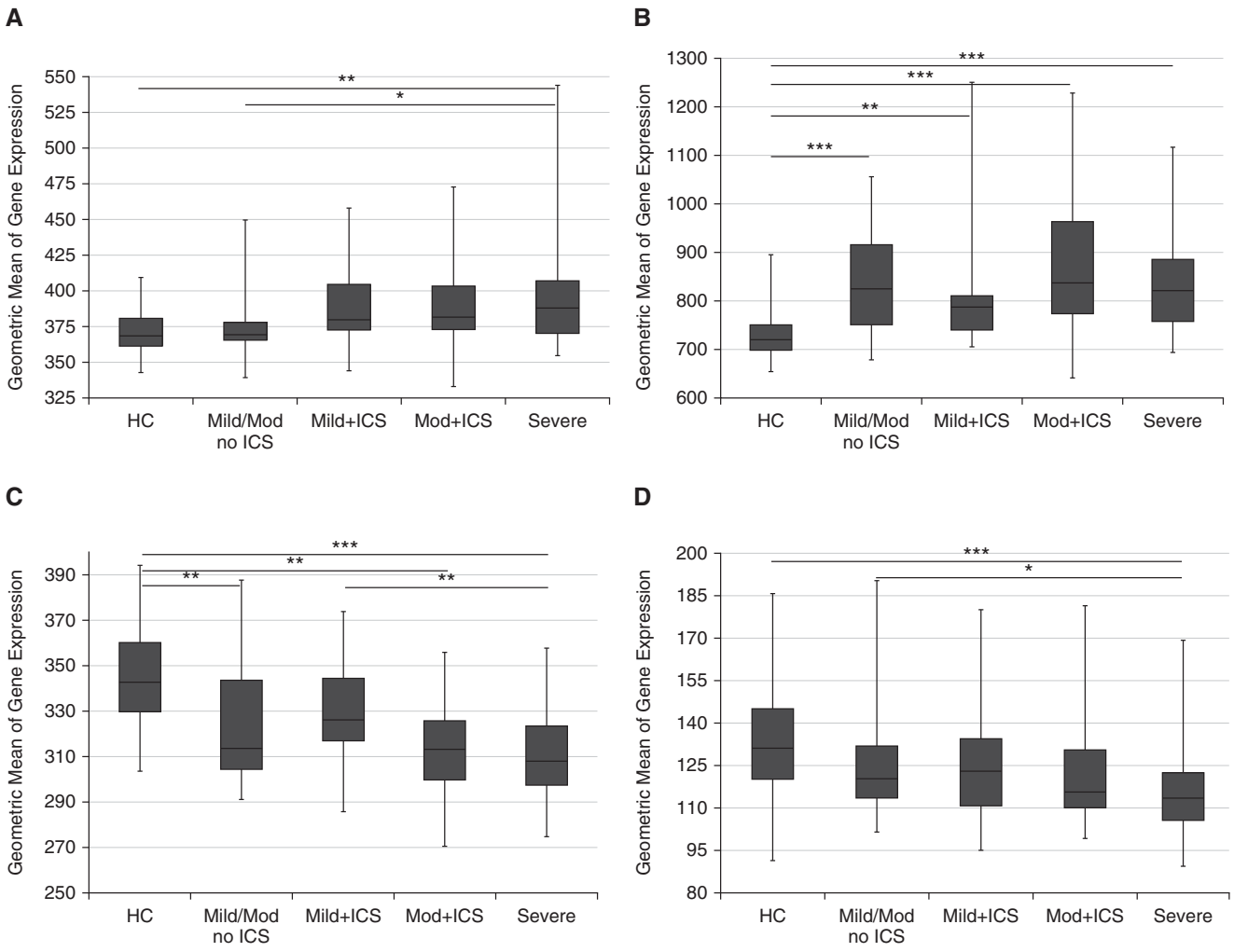

Figure 2. Magenta/mitosis, red/T2, grey60/epithelial growth and repair (EGR), and thistle2/neuro gene expression in relationship to asthma severity. The geometric means (including ranges and interquartile ranges) of (*A*) magenta/mitosis, (*B*) red/T2, (*C*) grey60/EGR, and (*D*) thistle2/neuro genes were measured according to asthma severity. These classes included healthy controls (HC), subjects with mild-to-moderate asthma not on inhaled corticosteroids (Mild/Mod no ICS), patients with mild-to-moderate asthma on inhaled corticosteroids (Mild + ICS), subjects with moderate asthma on ICS (Mod + ICS), and subjects with severe asthma (Severe). Significant differences are defined as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

red, respectively), FEV_1% predicted (cor = 0.41; $P = 1 \times 10^{-29}$; cor = 0.3; $P = 7.2 \times 10^{-22}$), and ED/hosp (cor = 0.24; $P = 1.3 \times 10^{-10}$; cor = 0.24; $P = 2.5 \times 10^{-14}$).

Modules Down-regulated with Severe Asthma

Two modules were negatively correlated with disease severity: grey60 (cor = −0.49; $P = 2 \times 10^{-10}$) and thistle2 (cor = −0.33; $P = 4 \times 10^{-5}$) (Figure 1). Grey60, designated "epithelial growth and repair (EGR)," contained cell surface growth receptors (*ERBB2*, *HPN*), cell integrity enzymes (*TGM7*), and remodeling enzymes (*MMP16*). Grey60/EGR demonstrated a high negative correlation with BMI (cor = −0.36; $P = 4 \times 10^{-6}$) and F_{ENO} (cor = −0.37; $P = 2 \times 10^{-6}$). Grey60/EGR had the highest positive correlation with AQLQ (cor = 0.52; $P = 4 \times 10^{-12}$), FEV_1% predicted (cor = 0.45; $P = 7 \times 10^{-9}$), and the highest negative association with CRS (cor = −0.26; $P = 0.001$), ED/hosp (cor = 0.36; $P = 5 \times 10^{-6}$), and use of ICS (cor = −0.4; $P = 4 \times 10^{-7}$) and OCS (cor = −0.35; $P = 9 \times 10^{-6}$).

The thistle2 module, designated "neuro," included 699 genes related to neuronal function and/or development (e.g., top hubs: *DDC*, *SCGN*, *SYT1*, *SCG5*, and *BEX1*). Thistle2/neuro was positively correlated with AQLQ (cor = 0.35; $P = 1 \times 10^{-5}$) and FEV_1% predicted (cor = 0.35; $P = 1 \times 10^{-5}$). It was negatively correlated with F_{ENO} (cor = −0.32; $P = 7 \times 10^{-5}$) and use of OCS (cor = −0.33; $P = 3 \times 10^{-5}$).

Both grey60/EGR and thistle2/Neuro gene expression decreased with each step of disease severity: HCs more than mild-mod no ICS more than mod + ICS more than SA (Figures 2C and 2d). Both modules were down-regulated in association with use of ICS and OCS. Finally, as demonstrated by the positive slopes in Figure 4, the GS of grey60/EGR and thistle2/Neuro modules as a group correlated strongly with membership (MM) for AQLQ (cor = 0.73; $P = 4.1 \times 10^{-80}$; cor = 0.57; $P = 8 \times 10^{-9}$), FEV_1% predicted (cor = 0.62; $P = 1.1 \times 10^{-51}$; cor = 0.61; $P = 8.6 \times 10^{-11}$), and ED/hosp (cor = 0.6; $P = 1.1 \times 10^{-47}$; cor = 0.46; $P = 3.5 \times 10^{-6}$). In comparison to magenta/mitosis and red/T2 modules, grey60 and thistle2 GS versus MM

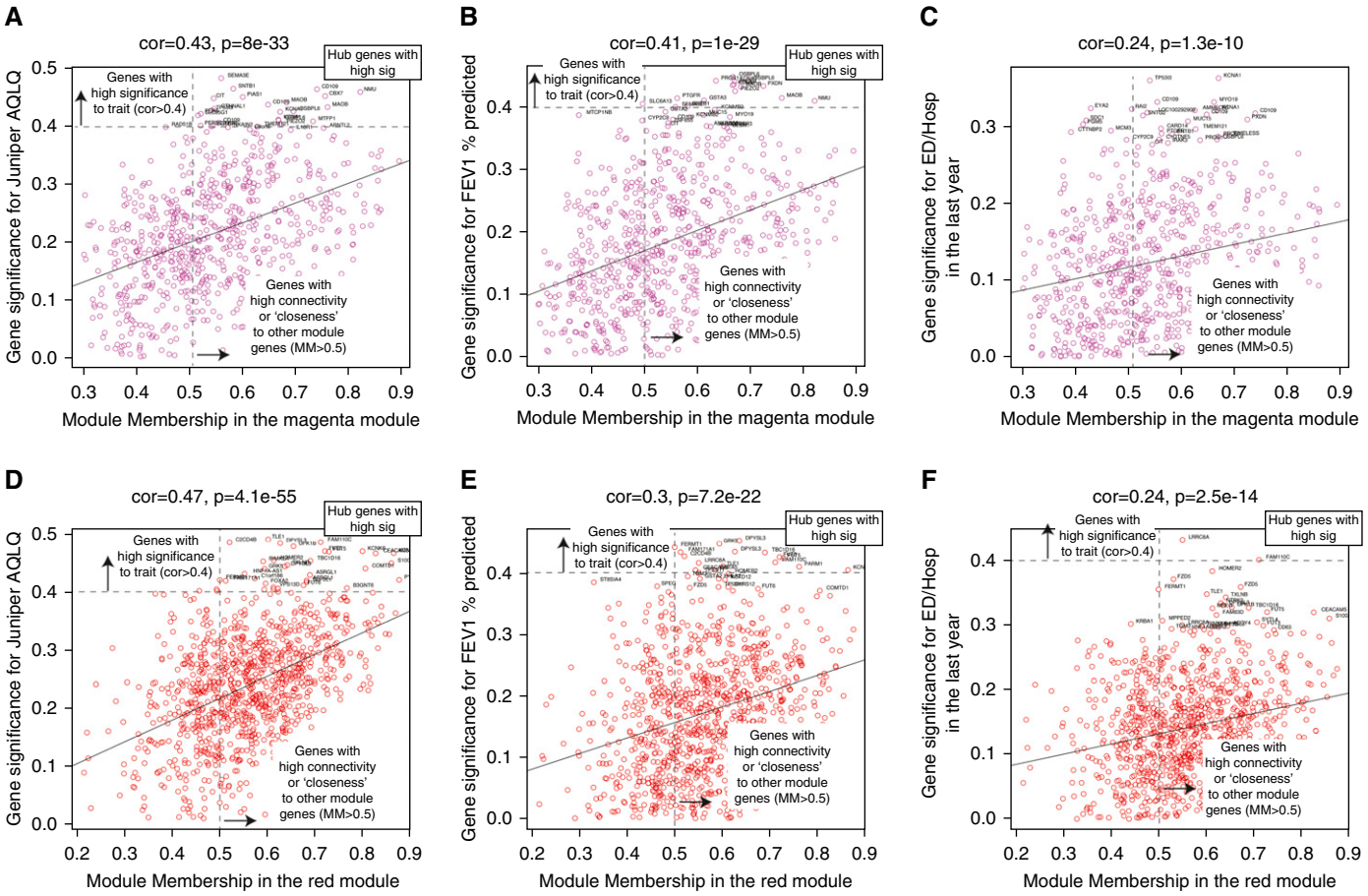

Figure 3. Magenta/mitosis and red/T2 absolute gene significance (GS) versus module membership (MM). Weighted gene coexpression network analysis calculation of GS to sample traits versus MM. In oversimplified terms, MM is a measure of how "tight" genes cluster within the module, or mathematically, how close gene expression is to the module eigenvalue. A gene with high MM and GS identifies hub genes that are both key components to the underlying biological process and highly correlated with the trait of interest. MM was plotted against GS for (*A* and *D*) Juniper Asthma Quality of Life Questionnaire (AQLQ), (*B* and *E*) FEV_1% predicted, and (*C* and *F*) emergency department visit or hospitalization (ED/Hosp) in the last year. These significant hub genes (detailed in Table 2) are found in the *top right corner* of each graph (except for *C*, which had no genes with correlation greater than 0.4 for ED/Hosp in the last year). cor = correlation or associations of modules with individual traits as calculated by weighted gene coexpression network analysis software utilizing standard Pearson's correlation; sig = significance.

correlations with SA traits were approximately two times as significant.

Identification of a T1-High Severe Asthma Cohort

There is increasing evidence that a subset of individuals with asthma, particularly those with severe disease, have up-regulation of airway IFN-γ (T1) immune responses (22, 23). The darkorange module, designated as "T1," contained 246 tightly correlated genes that encode T1-related chemokines (*CXCL9*, *CXCL10*, and *CXCL11*), transcription factors (*STAT1*), and cytokines (*IL15*). Gene enrichment analysis (IPA) reflected "INF signaling" and "antigen presentation pathway" as the top two signaling pathways ($P = 1.7 \times 10^{-21}$; $P = 2.5 \times 10^{-19}$).

Overall, T1/darkorange expression did not significantly correlate with asthma severity or any particular demographic trait. Nonetheless, 33 of 128 (26%) subjects with asthma had elevated T1 gene expression (>25th percentile of all subjects with asthma and HCs). T1-high asthma demonstrated equal severity compared with all non–T1-high asthma (45% vs. 39% were severe, respectively), with the latter group consisting primarily of subjects severe asthma with high T2 inflammation. Compared with all non–T1-high patients, T1-high patients were younger ($P = 0.027$).

Of T1-high asthma, seven subjects (21%) had concomitant up-regulation of T2 genes (>25th percentile of all subjects with asthma and HCs), and were designated as a T1-high/T2-high group (Figure 5). Another seven (21%) subjects had low expression of T2 genes (<25th percentile) and were designated as T1 high/T2 low. Finally, 19 (58%) subjects had moderate levels (25–75th percentile), and were designated as T1 high/T2 mod. T1 high/T2 low were the youngest (median, 24 yr; interquartile range [IQR], 19–35), and statistically younger than T2 mod. They had the lowest FE_{NO} (median, 16 ppb; IQR, 11–20), were statistically lower than T1 high/T2 high, had the lowest incidence of CRS, highest AQLQ (median, 5; IQR, 4–5.5), highest FEV_1% predicted (median, 78%; IQR, 71–100), and the lowest rate of ED/hosp. T1 high/T2 high were more than 50% African American, had very high FE_{NO}

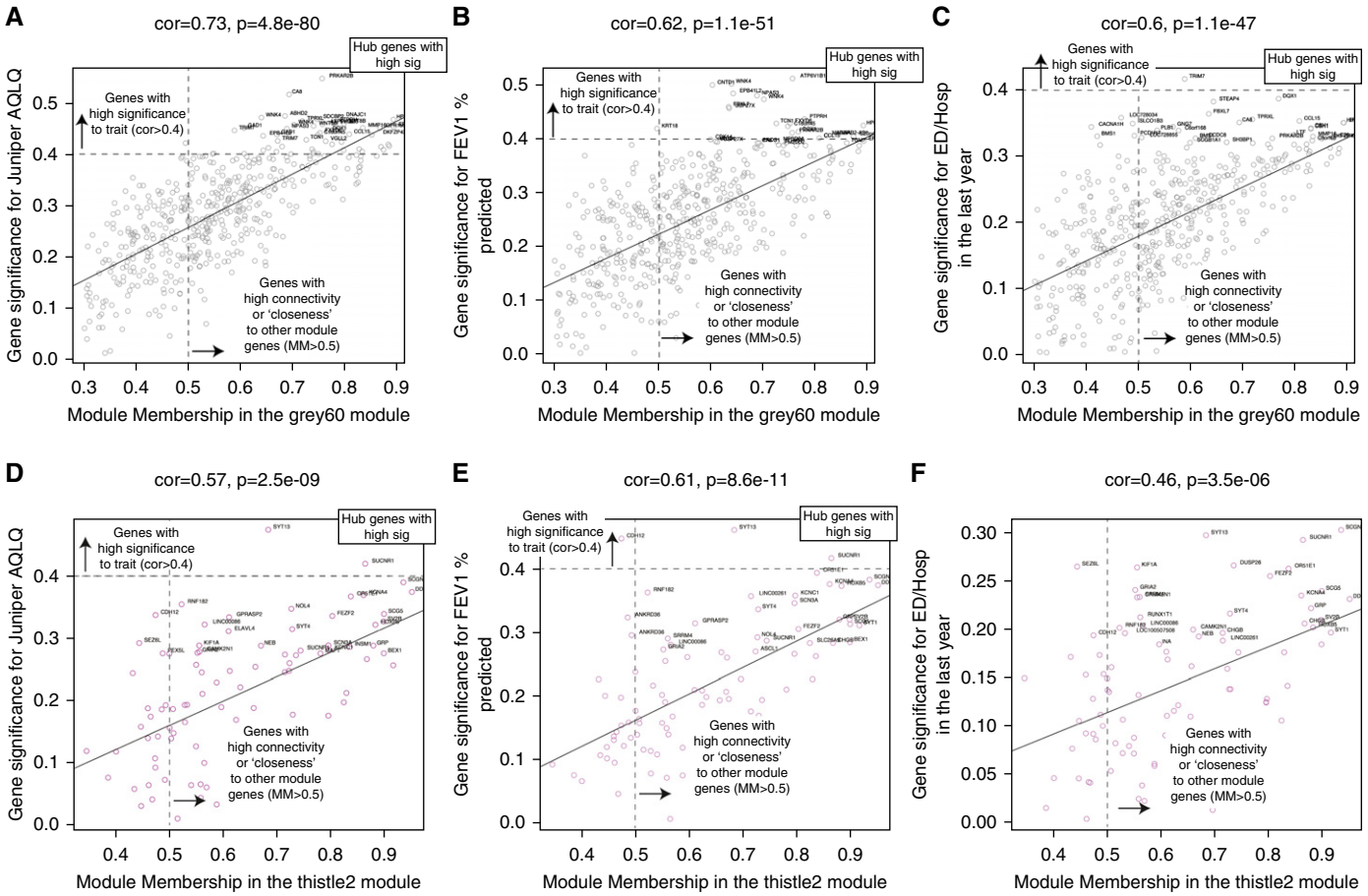

Figure 4. Grey60/epithelial growth and repair and thistle2/neuro absolute gene significance (GS) versus module membership (MM). Weighted gene coexpression network analysis calculation of GS to sample traits versus MM. In oversimplified terms, MM is a measure of how tight genes cluster within the module, or mathematically, how close gene expression is to the module eigenvalue. A gene with high MM and GS identifies hub genes that are both key components to the underlying biological process and highly correlated with the trait of interest. MM was plotted against GS for (*A* and *D*) Juniper Asthma Quality of Life Questionnaire (AQLQ), (*B* and *E*) FEV_1% predicted, and (*C* and *F*) emergency department visit or hospitalization (ED/Hosp) in the last year. These significant hub genes (detailed in Table 2) are found in the *top right corner* of each graph (except for *F*, which had no genes with correlation greater than 0.4 for ED/Hosp in the last year). In comparison to magenta/mitosis and red/T2 (Figure 3), grey60 and thistle2 demonstrate a significantly stronger correlation in GS versus MM. cor = correlation or associations of modules with individual traits as calculated by weighted gene coexpression network analysis software utilizing standard Pearson's correlation; sig = significance.

(median, 76 ppb; IQR, 41–218), with relatively normal FEV_1% predicted (median, 77%; IQR, 66–88), despite nearly 50% incidence of ED/hosp. Finally, the T1 high/T2 mod had slight elevation in F_{ENO} (median, 22 ppb; IQR, 17–43), lower AQLQ (median, 4; IQR, 3–5), the lowest FEV_1% predicted (median, 67%; IQR, 56–81), and more than 50% had been in the ED or hospitalized in the previous year. Interestingly, three HC subjects were in the T1-high group. Each of these HC subjects had markedly elevated levels of F_{ENO} (52, 87, and 257 ppb) and blood IgE (208, 368, 1,822 IU/ml), despite normal lung function and no asthma symptoms. T2 gene expression did not differentiate these three HC subjects.

Gene Expression Profiles Associated with SA Traits While Adjusting for Potential Confounders

Because the relationship between gene expression and clinical traits may potentially be confounded by batch effects and numerous, nonrelevant sample traits, LIMMA was used to correlate gene expression with FEV_1% predicted, AQLQ, and ED/hosp while adjusting for all potential confounders (including age, sex, race, BMI, and use of ICS or OCS). Conversely, adjustments for covariables directly tied to asthma severity (e.g., use of corticosteroids), but not responsible for gene expression differences, could incorrectly mask true biological findings. Therefore, the results are plotted as $-\log_{10}(P$ values) across all modules both before and after adjustment for confounders (Figure 6). Red/T2 and thistle2/neuro demonstrated a strong relative increase in gene expression after adjusting for confounders, largely due to adjusting for corticosteroid use. No hidden batch effects were found by a surrogate variable analysis. Both before and after adjustment for confounders, grey60/EGR and thistle2/neuro expression were most correlated with FEV_1% predicted, followed by red/T2 and magenta/mitosis (Figures 6A and 6D). Grey60/EGR and red/T2 were correlated highest with AQLQ (near equally after adjustments), followed by thistle2/neuro

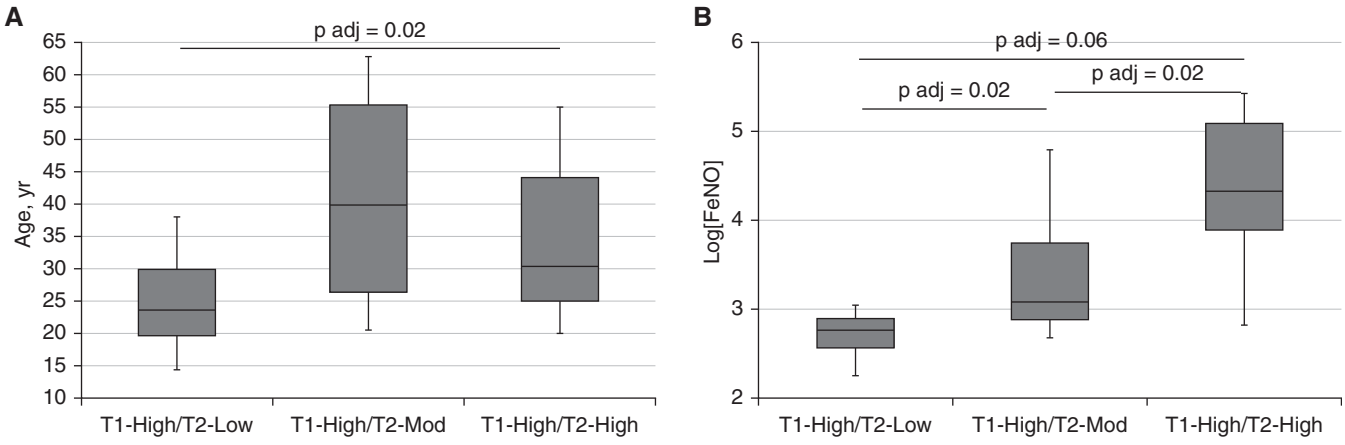

Figure 5. Demographics of T1-high asthma. The darkorange module, designated as T1, contained 246 tightly correlated genes that encoded genes of T1 inflammation. A total of 33 of 128 (26%) subjects with asthma had significantly elevated T1 gene expression ($P < 0.001$), designated as T1-high asthma. Of T1-high asthma, 7 participants (21%) had concomitant up-regulation of T2 genes (T1 high/T2 high), 7 (21%) had low expression of T2 genes (T1 high/T2 low), and 19 (58%) had modest levels, designated as T1 high/T2 mod. This figure compares and contrasts differences in (*A*) age and (*B*) fractional exhaled nitric oxide (Fe_{NO}) among these three T1 subphenotypes. *Box plots* and *error bars* represent medians, ranges, and interquartile ranges. adj = adjusted.

(Figures 6B and 6E). Finally, grey60/EGR was correlated strongest with ED/hosp, followed by red/T2, thistle2/neuro, and magenta/mitosis.

Overall, 2,512 genes correlated with FEV_1% predicted, 2,555 with AQLQ, and 963 with ED/hosp using a threshold Benjamini-Hochberg–adjusted *P* value <0.05 to adjust for multiple comparisons. Figure 7 plots the top 25 genes correlated with each of these three asthma characteristics (ranked by Benjamini-Hochberg–adjusted *P* value), and identifying different gene profiles that correlated with each of these SA characteristics, suggesting that they are pathogenically distinct (*see* the online supplement for full gene lists). Interestingly, although the top genes correlated with FEV_1% predicted and ED/hosp were scattered across grey60, red, magenta, and other modules, the top genes correlated with AQLQ were mostly red/T2 genes.

Of the top 200 genes that were correlated with FEV_1% predicted, AQLQ, and ED/hosp, 53 were common to all three ranked gene lists. Twenty of these 53 genes were also found to have high gene significance (cor > 0.40) and high module membership (MM > 0.50) in at least two of three of these same characteristics in the WGCNA (genes found in upper right corners of plots in Figures 3 and 4). A summary of the function and biological significance of these 20 genes is provided in Table 2.

Validation of T2 and T1 Modules in an External Cohort with Nonsevere Asthma

WGCNA was next performed on an external data set of mild-to-moderate asthma not on inhaled steroids and HCs (12). WGCNA constructed a T2 module that was similar to our red/T2 module and strongly correlated with the authors' label of "T2-high" asthma (cor = 0.60; $P = 2 \times 10^{-12}$) (*see* the online supplement for detailed comparisons). To better illustrate the similarity in our red/T2 module and the T2 module of the external data, both T2 gene coexpression networks were graphed using a relatively high correlation cutoff (Spearman's $\rho > 0.75$) (*see* the online supplement for analysis details) (Figure 8). Central hub genes between the T2 internal and T2 external gene networks were found to be roughly 50% common, suggesting that these highly coexpressed genes (e.g., CST1, FUT3, and so on) may provide a more robust biomarker for T2 inflammation than any singular gene. Using geometric means of the T2 module gene expression, we found T2 expression is up-regulated in mild, untreated asthma at nearly equal levels as SA (Figure 8C), with levels significantly elevated compared with HCs in both our data and the external data set (Figure 8D) ($P < 0.001$).

WGNA also replicated a T1 module (in the external data) similar to our darkorange/T1 module, and a cilia-related module that demonstrated the strongest inverse correlation with asthma (cor = -0.5; $P = 7 \times 10^{-8}$) (*see* the online supplement for comparisons). Twenty-nine (7%) of the cilia-related genes were common to the grey60/EGR module, including the #4 hub gene dead box helicase 5 (*DDX5*), which encodes an RNA helicase that regulates key hub gene *ERBB2* from the grey60/EGR. Otherwise, the cilia-related module was a distinct module not found in the SARP cohort.

Discussion

Bronchial epithelial cell gene expression in relation to asthma severity and its specific characteristics (i.e., FEV_1% predicted, AQLQ, ED/hosp, steroid use) suggests that a diverse array of immune and nonimmune mechanisms may be involved with the development SA. Individuals with mild-to-moderate asthma with elevated T2 gene expression have previously demonstrated improvement in FEV_1 when treated with ICS (5). The same is true for moderate-to-SA with clinical evidence of T2 inflammation (i.e., elevated blood and sputum eosinophils, Fe_{NO}, Eotaxin-3 [*CCL26*]) when treated with an antibody that blocks T2 signaling (24). In agreement with these previous studies, T2 expression was up-regulated in patients with mild, CS-naive asthma, but was less elevated in mild to moderate disease on ICS. However, the T2 signal was progressively higher in those on ICS as severity worsened. These findings suggested either worsening adherence or

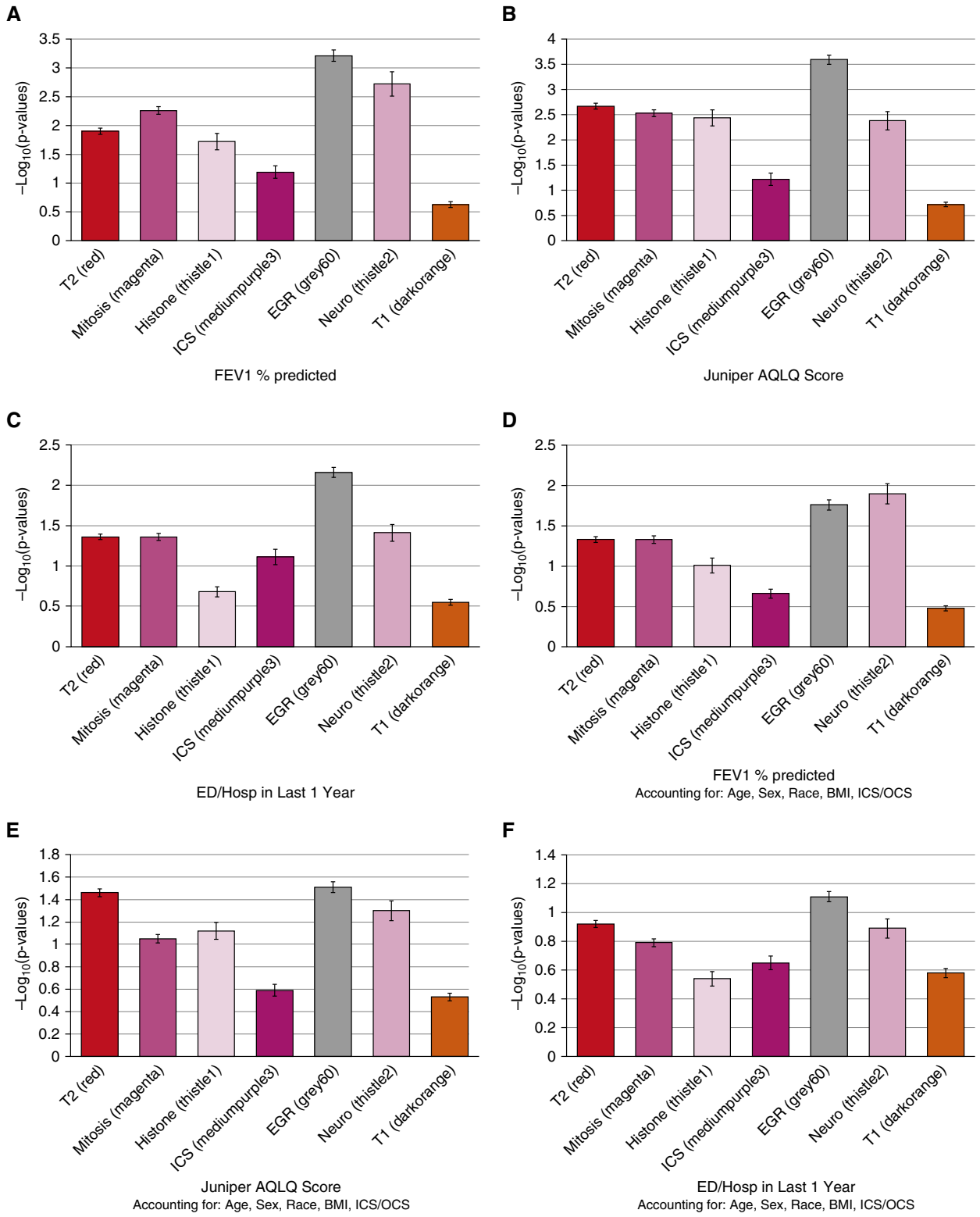

Figure 6. Correlation of modules to severe asthma traits while adjusting for potential confounders. Because the relationship between gene expression and clinical traits may potentially be confounded by batch effects and numerous, nonrelevant sample traits, software package LIMMA was used to correlate gene expression with FEV_1% predicted, Asthma Quality of Life Questionnaire (AQLQ), and emergency department visit or hospitalization (ED/Hosp) while adjusting for all potential confounders. (*A–C*) Overall module significance to FEV_1% predicted, Juniper AQLQ, and ED/Hosp are plotted as $-\log_{10}(P$ values) across all modules without adjusting for potential confounders. (*D–F*) The same plots are made while adjusting for age, sex, race, body mass index (BMI), and use of inhaled (ICS) or oral (OCS) corticosteroids. *Error bars* show SE. EGR = epithelial growth and repair.

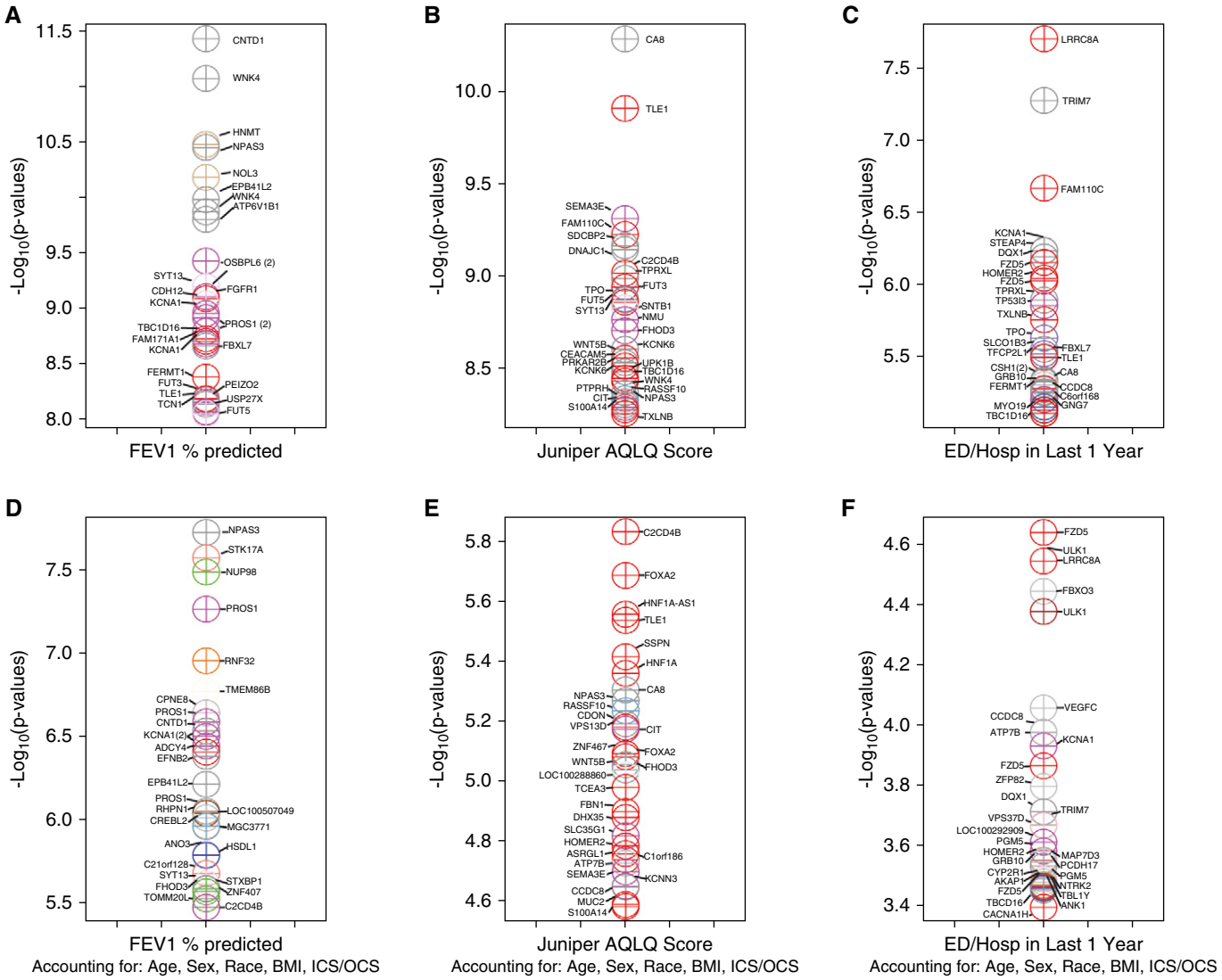

Figure 7. Top 25 genes correlated with severe asthma traits while adjusting for potential confounders. The top 25 genes correlated with each of these three asthma characteristics (ranked by Benjamini-Hochberg–adjusted *P* value), identifying gene profiles that correlate with each of these severe asthma characteristics (*see* the online supplement for full gene lists). Interestingly, although the top genes correlated with FEV_1% predicted and emergency department visit or hospitalization (ED/Hosp) were scattered across grey60, red, magenta and other modules, the top genes correlated with Asthma Quality of Life Questionnaire (AQLQ) were mostly red/T2 genes. BMI = body mass index; ICS = inhaled corticosteroids; OCS = oral corticosteroids.

the emergence of poor CS responsiveness as severity increased. ICS-induced gene expression (mediumpurple3 module) also increased with disease severity, suggesting that noncompliance was not the main reason for high T2 inflammation. FE_{NO} levels, a marker of T2 inflammation, matched this pattern of T2 inflammation in asthma severity classes. This observation might explain the often discordant studies featuring FE_{NO} (and similarly T2 inflammation), which identifies an at-risk phenotype when markedly elevated despite maximum therapy (25), but can also be elevated in mild asthma, particularly when untreated (26).

T2 expression correlated strongly with FEV_1% predicted, AQLQ, and ED/hosp, although most significantly with AQLQ (cor = 0.42), suggesting uncontrolled T2 inflammation decreases quality of life, which was likely related to T2 cytokine-induced airway smooth muscles contraction, mucus hypersecretion, edema, and eventually airway remodeling (27). Red/T2 gene *TLE1,* which demonstrated a strong positive relationship with asthma severity and inverse relationship to AQLQ, was the #4 gene to correlate with low AQLQ scores (after adjustment). Interestingly, a known susceptibility locus for childhood asthma in the Mexican population is located at or near the *TLE1* gene, and its effect is more pronounced when considering only children with asthma with atopy (28). *TLE1* encodes a transcriptional corepressor that inhibits transcriptional factor *FOXA2,* which was the #2 gene correlated with AQLQ after adjustments, demonstrating an inverse relationship with asthma severity and a positive relationship to AQLQ (29). In

Table 2. Summary of Hub Genes (Module Membership > 0.5) That Were Also among the Top 200 Genes Correlated with All Three Asthma Severity Characteristics by LIMMA Analysis

Gene	Protein Name	Module	Relationship to Severe Asthma	Chromosome Location	Protein Function and Potential Significance to Asthma (if Known)
CA8	Carbonic anhydrase 8	Grey60/EGR	↓	8 (q12.1)	Similar to salivary protein, although gene product likely lacks carbonic anhydrase activity
CCL15	Chemokine ligand 15	Grey60/EGR	↓	17 (q12)*	Airway smooth muscle are potent source, which may directly participate in the recruitment of inflammatory cells to asthmatic airways through *CCL15/CCR1* axis (40). *CCL15* was reduced in patients with asthma by omalizumab (41)
CSH1	Chorionic somatomammotropin hormone 1	Grey60/EGR	↓	17 (q23.3)*	Member of the somatotropin/prolactin family of hormones, plays an important role in cell growth
DPYSL3	Dihydropyrimidinase-like 3	Red/T2	↑	5 (q32)	Necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton. Plays a role in axon guidance, neuronal grown cone collapse, and cell migration
ERBB2	Erb-B2 receptor tyrosine kinase 2	Grey60/EGR	↓	17q12*	Required for airway epithelial repair following neutrophil elastase exposure (36). In a region of chromosome 17q21 (between 35.0 and 35.5 Mb) associated asthma with genes such as *ORMDL3*, *GSDM1/L*, *PSMD3*, *CSF3*, *ZPBP2*, *IKZF3*, *PERLD1*, and *PNMT* (42)
FERMT1	Fermitin family homolog 1	Red/T2	↑	20 (p12.3)	A member of the fermitin family proteins that are important to epithelial integrity; is specifically involved in integrin signaling and linkage of the actin cytoskeleton to the ECM
FOXA2	Forkhead box A2	Red/T2	↓	20 (p11.21)	Highly correlated with asthma in humans and animal models. *See* the DISCUSSION for further details
FUT3 and *FUT5*	Fucosyltransferase 3 and 5	Red/T2	↑	19 (p13.3)	Catalyzes the addition of fucose to precursor polysaccharides in the last step of Lewis antigen biosynthesis. Both *FUT3* and *FUT5* demonstrate strong transcription up-regulation in chronic viral infections, including VZV and CMV (43)
HOMER2	Homer scaffolding protein 2	Red/T2	↑	15 (q25.2)	Postsynaptic density scaffolding protein that is a member of homer family of dendritic proteins. As a cytosolic adaptor, it regulates G-protein–coupled receptors (44) and aids in coupling of surface receptors
HPN	Hepsin	Grey60/EGR	↓	19 (q13.11)*	Encodes a type II transmembrane serine protease that may be involved in diverse cellular functions, plays an essential role in cell growth and maintenance of cell morphology
KCNK6	Potassium channel, two-pore domain subfamily K, member 6	Red/T2	↑	19 (q13.2)*	Cell membrane potassium channels (pH-dependent, voltage insensitive, outward rectifying) that are widely expressed and stimulated by arachidonic acid. Two-pore potassium channels found in the apical membrane of airway epithelial cells that support mucociliary clearance (45)
MMP16	Matrix metallopeptidase 16	Grey60/EGR	↓	8 (q21.3)	Important to normal lung development and polymorphisms in this gene are associated with risk of bronchopulmonary dysplasia (46). Implicated in the development of pulmonary fibrosis (47)
NMU	Neuromedin U	Magenta/Mitosis	↑	2 (q31.2)	An active neuropeptide, part of the neuromedin family, that has a role in the pain, stress, feeding regulation, and immune-mediated inflammation
OSBPL6	Oxysterol-binding protein–like 6pr	Magenta/Mitosis	↓	4 (q12)	An intracellular lipid receptor and member of the OSBP family
PROS1	Protein S	Magenta/Mitosis	↓	3 (q11.1)	Plasma protein that acts as cofactor for the anticoagulant protease APC to inhibit anticoagulation
PTPRH	Protein tyrosine phosphatase, receptor type H	Grey60/EGR	↑	19 (q13.42)*	Contains extracellular region and involved in a variety of cellular processes, including contact inhibition of cell growth and motility, induced apoptotic cell death, inhibits T-cell receptor signaling by interactions with *LCK*, *LAT*, and *ZAP70*
SYT13	Synaptotagmin XIII	Thistle2/Neuro	↓	11 (p11.2)	Member of a large protein family that form membrane complexes and act as membrane traffickers

(Continued)

Table 2. (*Continued*)

Gene	Protein Name	Module	Relationship to Severe Asthma	Chromosome Location	Protein Function and Potential Significance to Asthma (if Known)
SUCNR1	Succinate receptor 1	thistle2/Neuro	↓	3 (q25.1)	G-protein–coupled receptor for succinate, a molecule in the citric acid cycle, this receptor is likely involved in the promotion of hematopoietic progenitor cell development
TLE1	Transducin-like enhancer of split 1	Red/T2	↑	9 (q21.32)	Found in a susceptibility locus for childhood asthma in the Mexican population, something that is more pronounced when considering only children with asthma with atopy (28). *TLE1* interacts with *RUNX3* to inhibit dendritic cell maturation (48), and loss of *RUNX3* function results in allergic asthma phenotype in mice (49). Inhibits NF-κ-B–regulated gene expression and transcriptional activation mediated by *FOXA2*

Definition of abbreviations: APC = activated protein C; CMV = cytomegalovirus; ECM = extracellular matrix; EGR = epithelial growth and repair; OSBP = oxysterol-binding protein; VZV = varicella zoster virus.
FEV_1% predicted, Asthma Quality of Life Questionnaire, and emergency department visit or hospitalization in the past year. In summary, 53 genes were found to be common to the top 200 genes (ranked by adjusted *P* value) correlated with FEV_1% predicted, Asthma Quality of Life Questionnaire, and emergency department visit or hospitalization in the past year (LIMMA analysis). Of these 53 genes, 20 were also found to have high gene significance (cor > 0.40) and module membership (MM > 0.50) in two or more of the three asthma severity characteristics (weighted gene coexpression network analysis) (Figures 3 and 4). Protein name and function were obtained from GeneCards: The Human Gene Database (www.genecards.org). Chromosome location was obtained from UCSC Genome Browser (www.genome.ucsc.edu). Up arrows indicate that gene expression is positively correlated/associated with asthma severity measures; that is, expression increases with severity. Down arrows indicate that gene expression is negatively correlated/associated with asthma severity measures; that is, expression decreases with severity, suggesting a protective effect.
*Gene positions that are at or near known asthma susceptibility loci (50).

mouse models, deletion of *FOXA2* in early lung development induced expression of T2 cytokines and chemokines, which resulted in spontaneous pulmonary eosinophilic inflammation and goblet cell metaplasia (30, 31). Thus, it could be hypothesized that the genetic variations that lead to overexpression of *TLE1* might in turn inhibit a key inhibitor of T2-mediated airway inflammation, *FOXA2* (32). Alternatively, *FOXA2* expression is also reduced by IL-13 signaling in airway epithelial cells, which suggests additive or synergistic effects (33). Finally, *FOXA2* was among the top hub genes (Table 2), identified by its strong MM and strong down-regulation in relation to all 3 asthma severity characteristics.

Conversely, EGR and neurological function and/or development (neuro) both demonstrated a strong inverse correlation with asthma severity, and were the 2 modules most correlated with FEV_1% predicted. Both EGR and neuro gene expression decreased linearly with increasing ATS-defined asthma severity scores. Taken together, these findings supported the concept that epithelial wound repair and neural features, as an integral parts of a healthy epithelial layer, might be at the heart of preventing loss of lung function and severe disease. The *ERBB2* gene demonstrated the highest combination of membership within the EGR module (i.e., correlation in expression with all other genes in the EGR module) and inverse correlation with asthma severity. *ERBB2* encodes a member of the epidermal growth factor (EGF) receptor family that is a well-known proto-oncogene found on the cell surface of epithelial cells and is required for normal epithelial repair (34–36). Interestingly, *ERBB2* is located at chromosome 17q12, approximately 200 kilo base pairs from genome-wide association studied asthma susceptibility loci at 17q21, and in linkage disequilibrium to *PGAP3* (37). Single nucleotide polymorphisms located at the *STARD3*/*PGAP3* loci are associated with highly atopic asthma compared with those with less atopic disease (38). The *DDX5* gene, which was a hub gene of the cilia module in mild-to-moderate asthma and a known regulator of *ERBB2*, is downstream of these genes at chromosome 17q23.3. These findings underscore the growing evidence that single nucleotide polymorphisms in these loci systematically contribute to the pathogenesis of asthma (*see* the online supplement), and suggest the importance of integrated analyses (39).

In our data, subjects with asthma with high levels of T1 gene expression were found to be significantly younger. Within the T1-high group, we found three subphenotypes with marked differences in clinical characteristics. T1 high/T2 low were young, white, relatively healthy subjects with asthma with low F_{ENO}, a low incidence of CRS, good quality of life, few exacerbations, and normal lung function. In contrast, when T1-high genes were associated with high expression of T2 genes, the clinical characteristics were markedly different. These subjects were mostly African American, with high F_{ENO} and near-normal lung function but with moderate incidence of CRS, reduced AQLQ, and a strong history of severe exacerbations. Finally, those with T1-high inflammation, but more moderate persistent elevations in T2 genes had very low lung function, highly urgent healthcare use, often in association with CRS.

In a previous study that involved this same patient cohort, hypothesis-free clustering of gene expression data identified a group of patients with asthma with high F_{ENO} levels and severe disease, moderate levels of T2 inflammation, and some indication for enhanced T1 immunity (increased *DUOX2* and *IL18RA*) (designated "subject cluster 3") (4). Internally validating and expanding on those earlier results, we found that nearly 50% of participants with T1 high/T2 mod, and nearly 30% of those with T1 high/T2 low were common to subject cluster 3 (*see* the online supplement).

Despite the large numbers of samples, there were important limitations to this

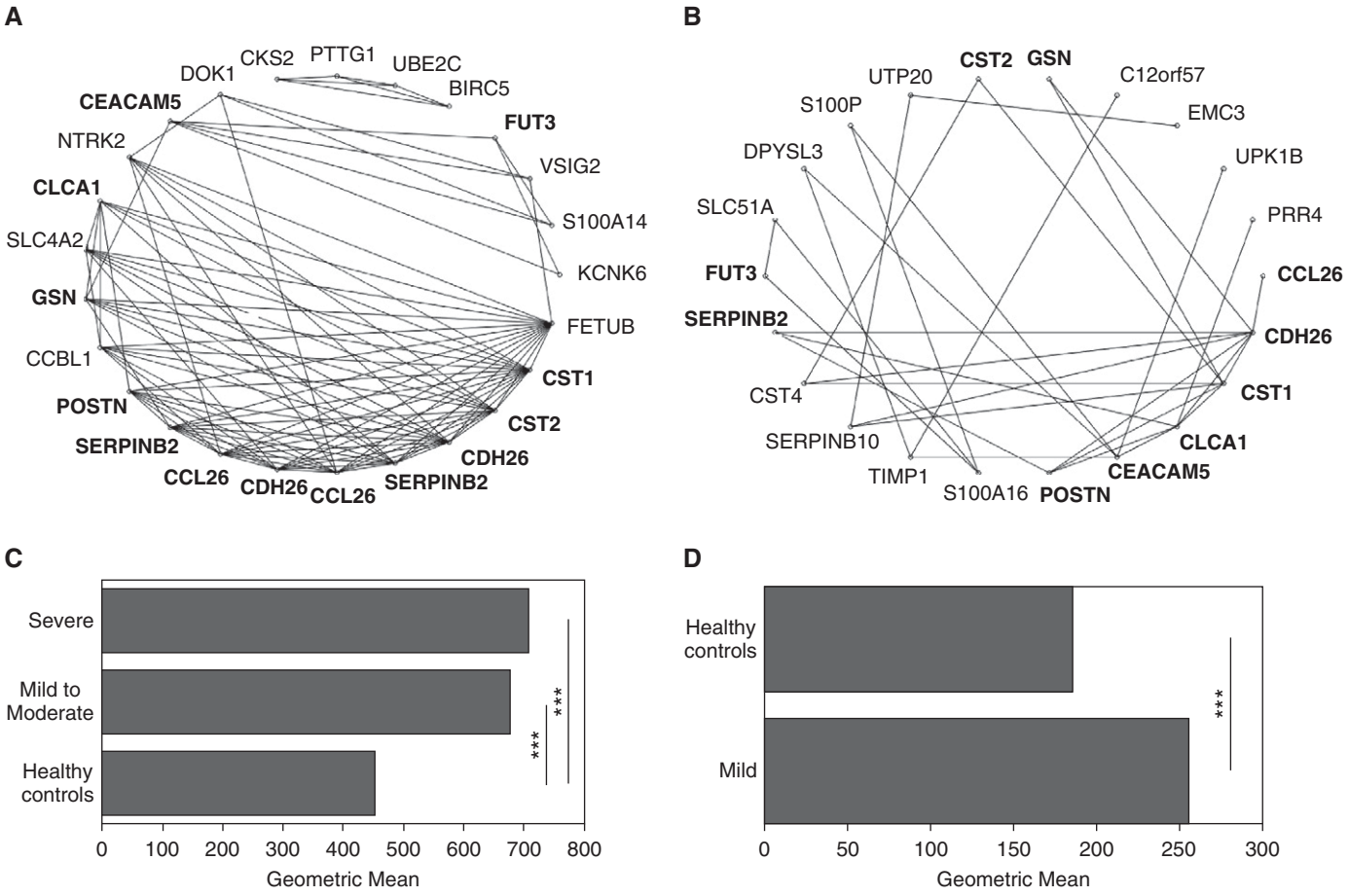

Figure 8. Similarity of T2 gene coexpression network (GCN) in internal and external cohorts. T2 GCN hub genes were strikingly similar between our data and the external data of subjects with mostly mild asthma not on an inhaled corticosteroid. (*A* and *B*) *Nodes* represent hub genes in the internal (*A*) and external (*B*) T2 GCN. *Edges* (i.e., connecting lines) demonstrate tight coexpression ($\rho > 0.75$). The enlarged, boldface genes are central hub genes that were found to be common between the T2 internal and T2 external gene networks, suggesting these genes (e.g., *CST1*, *FUT3*, and so on) may be used together as a more robust biomarker for T2 inflammation than a single gene alone. (*C*) The geometric mean of the T2 GCN was used to show that T2 gene expression was significantly higher in mild to moderate and severe asthma versus healthy controls in the internal cohort. (*D*) Similarly, T2 gene expression was significantly higher in subjects with mild asthma versus healthy controls in the external cohort. ***$P < 0.001$.

study. First, adherence to prescribed medications was not formally addressed in these patients. Although low adherence might have influenced levels of control, the effect on gene expression should be different between those who were adherent and those who were nonadherent. To support a minimal impact of nonadherence, the mediumpurple3/ICS module was up-regulated in relationship to both increasing severity and use of ICS. Second, although we discovered strong associations between genes with biological function that might explain severe disease, we could not confirm causality. Finally, data from the initial published study confirmed that the microarray data were strongly correlated with quantitative polymerase chain reaction measurements in more than 90% of the genes tested. However, the only hub gene in this study specifically confirmed was *ERBB2*. Additional protein confirmation will be required for many of these pathways.

In this hypothesis-generating analysis, we found that a diverse array of immune and nonimmune mechanisms might be involved with SA. Overall, T2 inflammation increased along with increasing severity and was closely tied to AQLQ, but only after initiation of ICS therapy, as those subjects with mild asthma who were not on ICS had similar expression levels as seen in SA. T1 inflammation overall did not increase with disease severity, although when elevated concomitantly with moderate-to-high T2 inflammation, it identified more severe disease in younger patients. Intriguingly, expression of low EGR and neuronal function genes were most strongly associated with SA and its characteristics than T2 inflammation, which suggested that epithelial integrity and related processes were of primary importance to the development of asthma and SA, and in line with current genetic findings. Future mechanistic research is needed to determine the influence that factors such as environmental exposures, medication usage, and genetic polymorphisms have on all of these inflammatory and repair mechanisms, potentially further unraveling the molecular roots of asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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