

## ORIGINAL ARTICLE

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Asthma and rhinitis

# Transcriptomics of biopsies identifies novel genes and pathways linked to neutrophilic inflammation in severe asthma

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## Abstract

**Background:** Severe asthma is a complex disease. Transcriptomic profiling has contributed to understanding the pathogenesis of asthma, especially type-2 inflammation. However, there is still poor understanding of non-type-2 asthma, and consequently, there are limited treatment options.

**Objective:** The aim of this study was to identify differentially expressed genes (DEGs) and pathways in endobronchial biopsies associated with inflammatory phenotypes of severe asthma.

**Methods:** This cross-sectional study examined endobronchial biopsies from 47 adults with severe asthma (neutrophilic asthma (NA)  $n = 9$ , eosinophilic asthma (EA)  $n = 22$  and paucigranulocytic asthma (PGA)  $n = 16$ ) and 13 healthy controls (HC). RNA was extracted and transcriptomic profiles generated (Illumina Humanref-12 V4) and analysed using GeneSpring GX14.9.1. Pathway identification using Ingenuity Pathway Analysis.

**Results:** NA had the most distinct profile, with signature of 60 top-ranked DEGs ( $FC > \pm 2$ ) including genes associated with innate immunity response, neutrophil degranulation and IL-10 signalling. NA presented enrichment to pathways previously linked to neutrophilic inflammation; dendritic cell maturation, Th1, TREM1, inflammasome, Th17 and p38 MAPK, as well as novel links to neuroinflammation, NFAT and PKC $\theta$  signalling. EA presented similar transcriptomic profiles to PGA and HC. Despite the higher proportion of bacterial colonization in NA, no changes were observed in the transcriptomic profiles of severe asthma culture positive compared with severe asthma culture negative.

**Conclusions & Clinical Relevance:** NA features a distinct transcriptomic profile with seven pathways enriched in NA compared to EA, PGA and HC. All those with severe asthma had significant enrichment for SUMOylation, basal cell carcinoma signalling and Wnt/ $\beta$ -catenin pathways compared to HC, despite high-dose inhaled corticosteroids. These findings contribute to the understanding of mechanistic pathways in endobronchial biopsies associated with NA and identify potential novel treatment targets for severe asthma.

## KEYWORDS

endobronchial biopsies, inflammatory phenotypes, Severe asthma

## 1 | INTRODUCTION

Severe asthma is a complex disease with diverse clinical and inflammatory phenotypes.<sup>1</sup> While severe asthma affects 5–10% of those with asthma, it is refractory to treatment with inhaled corticosteroids (ICS), leading to poor symptom control and increased exacerbations. Recent advances have seen treatments targeting type-2 immunity, resulting in improved outcomes for those with severe allergic and eosinophilic asthma.<sup>2</sup> However, more than 50% of patients with asthma present with non-type-2 mechanisms of inflammation either alone or in combination with type-2 mechanisms.<sup>3–7</sup> These mechanisms are relatively unresponsive to corticosteroids and not targeted by current monoclonal antibodies.<sup>7–9</sup> A greater understanding of the mechanisms leading to different phenotypes of severe asthma is a key step towards personalized management, as well as being crucial for the discovery of new therapies targeting non-type-2 pathways.<sup>10,11</sup>

Previous transcriptomic analyses have improved our understanding of asthma.<sup>5,12–20</sup> Analysis of luminal airway inflammation using induced sputum from subjects with asthma revealed distinct molecular pathways associated with inflammatory phenotypes.<sup>5,12</sup> Assessment of epithelial brushings identified a specific signature associated with Th2-high inflammation and was able to relate this to responsiveness to ICS.<sup>17,18</sup> Furthermore, in severe asthma, the study of gene expression has demonstrated specific mechanisms associated with clinical features,<sup>5,13,19</sup> adult onset of disease<sup>14</sup> and Th2-high inflammation<sup>16,20</sup> and has even identified subtypes of patients with eosinophilic inflammation and corticosteroid insensitivity from epithelial brushings and endobronchial biopsies.<sup>15</sup> These findings demonstrate the importance of transcriptomic profiling in the context of asthma and severe asthma, as well as providing insights on pathogenesis. However, the underlying mechanistic pathways in different inflammatory phenotypes, specifically in those with non-type-2 inflammation, remain largely unknown. Endobronchial biopsies sample the airway wall and include immune cells, the epithelium, fibroblasts, smooth muscle and other structural components, all of which play a vital role in asthma. In this study, we hypothesized inflammatory phenotypes of severe asthma are associated with distinct transcriptomic profiles in endobronchial biopsies. This study aimed to identify differentially expressed genes and examine potential mechanistic pathways associated with different inflammatory phenotypes.

## 2 | METHODS

### 2.1 | Study design and participants

This is a cross-sectional study of adults with severe asthma ( $n = 47$ ) and healthy controls (HC;  $n = 13$ ) recruited from the respiratory

#### Key Message

- A 'neutrophilic signature' identified in neutrophilic asthma associated with innate response, degranulation and IL-10.
- Novel links between neutrophilic asthma and neuroinflammation, NFAT and PKC $\theta$  signalling were identified.
- Findings contribute to the understanding of neutrophilic asthma and in the identification of novel treatment targets.

ambulatory care clinics at John Hunter Hospital (Newcastle, NSW, Australia). Written informed consent was obtained from all HC and asthma participants. Bronchoscopy was conducted in HC participants and had no underlying lung or cardiac disease. All HC had FEV<sub>1</sub> percentage predicted  $\geq 80\%$  assessed by spirometry, and current smokers were excluded. Severe asthma was defined as treatment GINA step 4 or 5<sup>21</sup> requiring high doses of ICS ( $>500 \mu\text{g}$  fluticasone or equivalent per day) plus a second controller that remained 'uncontrolled', either with persistent symptoms (ACQ  $\geq 1.5$ ) and/or frequent severe exacerbations ( $\geq 3$  exacerbations that required courses of prednisone for at least 3 days in the last 12 months).<sup>21</sup>

### 2.2 | Bronchial lavage fluid and endobronchial biopsies

Endobronchial biopsies and bronchial lavage fluid (BLF) were obtained from the second to third generation airways in the right or left lower lobe as previously described.<sup>22</sup> BLF total cell count (TCC) and differential cell count were performed as previously described.<sup>12</sup>

### 2.3 | Inflammatory phenotyping

We used BLF to evaluate inflammatory profile present in the airway lumen due to its accessibility during bronchoscopy. Inflammatory phenotypes were defined using differential count and TCC using the 95th percentile from a larger HC cohort ( $n = 86$ ) as previously described in induced sputum<sup>23</sup> (Table S1). Percentage cut-offs were defined as eosinophils (3.5%), neutrophils (71.75%) and total cell count ( $0.99 \times 10^6/\text{mL}$ ). Inflammatory phenotypes were defined as neutrophilic asthma (NA) when differential count was  $\geq 71.75\%$  neutrophils  $+ \geq 0.99 \times 10^6/\text{mL}$  TCC and  $< 3.5\%$  eosinophils; eosinophilic asthma

(EA)  $\geq 3.5\%$  eosinophils,  $< 71.75\%$  neutrophils, paucigranulocytic asthma (PGA) when  $< 3.5\%$  eosinophils,  $< 71.75\%$  neutrophils, mixed granulocytic asthma (MGA) when  $\geq 3.5\%$  eosinophils,  $\geq 71.75\%$  neutrophils and healthy control (HC) when  $< 3.5\%$  eosinophils,  $< 71.75\%$  neutrophils.

## 2.4 | Microarray data and technical validation

Endobronchial biopsy tissue was lysed (RNeasy Lysis Buffer; Qiagen) and homogenized (TissueLyser II; Qiagen) prior to RNA extraction (RNeasy Mini Kit and RNase-free DNase; Qiagen). RNA was quantified (Quant-iT RiboGreen RNA Quantitation Assay Kit; Life Technologies Corporation), and a quality assessment was done (Agilent 2100 Bioanalyzer). Samples had adequate quality for microarray analysis with RNA integrity number (RIN) mean 7.5 (SD 1.4).<sup>24–26</sup> cRNA was prepared and biotin-UTP labelled using Illumina TotalPrep RNA amplification kit (Life Technologies Corporation), and 750 ng of cRNA was hybridized to Illumina's HT-12 V4 Beadchips and scanned in Illumina Bead Station, captured using BeadScan 3.5.11 (Illumina). Array raw data were processed (GenomeStudio software, Illumina) and analysed (GeneSpring GX14.9.1; Agilent Technologies). Data were log2-transformed and quantile-normalized. Genes were included in further analysis when probe set expression quality was flagged as present or marginally present in all samples as previously defined.<sup>12</sup> Differentially expressed genes (DEGs) were determined using ANOVA with Tukey's *post hoc* testing ( $P \leq 0.05$  adjusted for multiple comparisons using Benjamini-Hochberg false discovery rate FDR) and top-ranked DEGs when fold change (FC)  $> +2$  or  $< -2$ . Probe sets with highest average expression were used when more than one probe with the same gene annotation was found. Hierarchical clustering was performed using Euclidean Ward method. Technical validation was performed using qPCR to confirm selected differentially expressed genes in inflammatory phenotypes and HC as previously described.<sup>22</sup> We calculated  $\Delta CT$  as cycle of target gene threshold, subtracting the cycle threshold of housekeeping gene 18s rRNA, with a Ct  $< 20$  considered acceptable. Normalized microarray data and  $2^{-\Delta CT}$  value for matching data from *IL1B*, *CLC* and *FKBP5* were correlated using Spearman's rank correlation.

## 2.5 | Statistical analysis

Results were analysed using Stata 15 (StataCorp, College Station, USA) and figures created using GraphPad Prism 8.3.1 (GraphPad Software, Inc.). Data are reported as mean (SD) for normally distributed data or median (quartile (Q1–Q3)). Continuous measures were analysed using a Wilcoxon rank-sum or Student's *t* test, depending on outcome distribution. Fishers' exact test was used for categorical data. Spearman's rank correlation measured the associations between gene expression and lung function and cell count in BLF.

## 2.6 | Pathways identification

We performed pathway analysis to gain mechanistic insight into the lists of genes.<sup>27</sup> We inputted DEGs (FDR  $\leq 0.05$ ) and FC values into Ingenuity Pathway Analysis Software (IPA) (Qiagen, Hilden, Germany). Significance was calculated with right-tailed Fisher's exact test ( $P$ -value  $\leq 0.05$ ). The z-score indicated predicted activation state of the canonical pathway. A positive z-score indicated predicted activation, and a negative z-score predicted inhibition of the pathway. Top pathways were identified for having a  $P$ -value  $\leq 0.05$  and z-score of  $\geq \pm 2$ .

## 2.7 | Protein interaction network

STRING database (version 11.0. <https://string-db.org/>) was used to study the interaction of protein products and functional enrichment of the overlapping DEGs in NA. A minimum required interaction score of high confidence ( $> 0.7$ ) was applied, and only query proteins were included in the analysis for the maximum number of interactions. Gene Ontology (GO) terms and Reactome pathways with FDR  $\leq 0.05$  were considered significant.

# 3 | RESULTS

## 3.1 | Clinical features and inflammatory cells

The characteristics of study participants are summarized in Table 1. Participants with severe asthma were significantly older than healthy controls and had reduced lung function. Of the 47 participants with severe asthma, there were 9 (19.2%) NA, 22 (46.8%) EA and 16 (34.0%) PGA (Table 2). This cohort contained no participants with MGA phenotype. There was no difference between the inflammatory phenotypes for age, lung function, symptoms or use of medications. Participants with NA had a significantly higher viability, TCC and neutrophil proportion compared with HC, EA and PGA, and lower proportion of macrophages compared with HC and PGA. Those with EA had a significantly higher proportion of eosinophils and epithelial cells compared with HC and PGA. There was a significant difference in the proportions of bacterial positive culture between NA and HC (Table 2).

## 3.2 | Severe asthma compared with healthy controls

We identified 2,331 DEGs (FDR  $\leq 0.05$ ) between severe asthma and HC. There were 15 genes top-ranked expressed with FC of  $> \pm 2$ . Four of these were up-regulated (*CD163*, *LOC649143*, *CA12* and *ADORA3*) and 11 were down-regulated (*MGC72104*, *CCNY*, *CYP2F1*, *LOC650737*, *H19*, *CCL5*, *PLEKHA1*, *LOC731992*, *TPSB2*, *HLA-DQB2* and *TPSAB1*, Table 3) in severe asthma. Pathway analysis identified

**TABLE 1** Clinical characteristics of severe asthma and healthy controls

| Characteristic                                         | Healthy Control   | Severe asthma     | P-value |
|--------------------------------------------------------|-------------------|-------------------|---------|
| N                                                      | 13                | 47                | —       |
| Age(years), mean (SD)                                  | 45 (13)           | 59 (13)           | 0.002   |
| Sex, Female n (%)                                      | 6 (46)            | 30 (64)           | 0.250   |
| Atopy, n (%)                                           | 1 (8)             | 24 (51)           | 0.004   |
| FEV <sub>1</sub> %predicted, mean (SD)                 | 101.4 (10.7)      | 75.2 (21.5)       | <0.001  |
| FVC %predicted, mean (SD)                              | 104.2 (9.9)       | 91.8 (18.2)       | 0.022   |
| FEV <sub>1</sub> /FVC, mean (SD)                       | 79.8 (3.9)        | 66.0 (12.7)       | <0.001  |
| Former smoker, n (%)                                   | 2 (15)            | 9 (19)            | 0.756   |
| Pack years, median (Q1, Q3)                            | 5 (5, 5)          | 10 (5, 15)        | 0.275   |
| Total cell count x10 <sup>6</sup> /mL, median (Q1, Q3) | 0.1 (0.1, 0.2)    | 0.2 (0.1, 0.6)    | 0.170   |
| Viability, median (Q1, Q3)                             | 75 (68, 76)       | 80 (57, 91)       | 0.440   |
| Neutrophils %, median (Q1, Q3)                         | 40.3 (15.3, 62.8) | 56.0 (24.5, 81.5) | 0.193   |
| Eosinophils %, median (Q1, Q3)                         | 0.5 (0.3, 1)      | 2 (0.8, 9.8)      | <0.001  |
| Macrophages %, median (Q1, Q3)                         | 33 (20.8, 43.8)   | 23.8 (11.5, 40.8) | 0.149   |
| Lymphocytes %, median (Q1, Q3)                         | 1.5 (0.5, 2)      | 0.5 (0, 2.3)      | 0.183   |
| Epithelial cells %, median (Q1, Q3)                    | 14.5 (7.5, 23.3)  | 3.5 (0.8, 15.8)   | 0.010   |
| Squamous cells %, median (Q1, Q3)                      | 2 (1, 2.4)        | 1.5 (0, 3.2)      | 0.342   |

Data was analysed using Fishers' exact test for categorical data, Wilcoxon rank-sum or a Student's t test depending on the outcome distribution (significant P-value ≤0.05).

eight canonical pathways enriched in all severe asthma compared with HC: SUMOylation pathway, VDR/RXR activation, thrombin signalling, NER pathway, NRF2-mediated oxidative stress response, role of Wnt/GSK-3 $\beta$  signalling in pathogenesis of influenza, basal cell carcinoma and Wnt/ $\beta$ -catenin signalling (Figure 1). Details of the pathways identified are provided in Table S3.

### 3.3 | Inflammatory phenotypes of severe asthma

We identified 2,195 DEGs between inflammatory phenotypes and HC. Hierarchical clustering of the identified genes revealed four clusters: PGA/EA group, HC group, a predominantly EA group and a cluster that was predominantly NA, clearly distinct from the other three groups (Figure 2). *Post hoc* analysis identified 1793 (197 FC  $\pm$ 2) DEGs in severe NA compared with HC, 921 (128 FC  $\pm$ 2) when compared to EA and 896 (124 FC  $\pm$ 2) compared with PGA (Table 3).

There were 60 top-ranked DEGs (FC  $\geq$ 2 or  $\leq$  -2) that overlapped when NA was compared with all other groups, suggesting they are specific to severe NA. These overlapping genes were identified as a 'neutrophilic signature' in endobronchial biopsies (Figure 3a). The protein interaction network created on this signature shows the close involvement of several of these genes in processes relevant to the pathogenesis of NA (Figure 3b). The neutrophilic signature corresponded to GO functional enrichment of defence response, immune response, immune system process, response to stress and inflammatory response (Figure 3c). Reactome pathways enriched in the signature indicate immune, innate, cytokine signalling, neutrophilic degranulation and interleukin-10 (Figure 3d).

Of the comparisons with EA, there were 16 (1 FC  $\pm$ 2) DEGs in comparison with PGA and 1219 (17 FC  $\pm$ 2) in comparison with HC, with CLC up-regulated in all comparisons (Figure 3e). While when those with severe PGA were examined, there were 1117 (8 FC  $\pm$ 2) DEGs in comparison with HC (Table 3). *PHACTR3* overlapped in PGA when compared with HC and NA (Figure 3f). Complete lists of differentially expressed genes are provided in Tables S9–S11.

### 3.4 | Pathways in inflammatory phenotypes

Enriched pathways were identified using IPA using DEGs for each phenotype comparison. In NA compared with HC, there were ten pathways enriched (Figure 4a), whilst in NA compared with EA there were 17 pathways enriched (Figure 4b), and when NA was compared with PGA there were 16 pathways enriched (Figure 4c). Of these, there were seven pathways consistently enriched and predicted to be activated (positive z-score) in NA when compared with all other groups, including neuroinflammation signalling, dendritic cell maturation, Th1 pathway, TREM1 signalling, role of nuclear factor of activated T cells (NFAT) in regulation of the immune response, PKC $\theta$  signalling in T lymphocytes and inflammasome pathway (Figure 4d). There were nine pathways enriched and overlapping when NA was compared with EA and PGA: role of PRRs in recognition of bacteria and viruses, iCOS-iCOSL signalling in T helper cells, Th17 activation pathway, production of nitric oxide and reactive oxygen species in macrophages, calcium-induced T lymphocyte apoptosis, Fc $\gamma$  receptor-mediated phagocytosis in macrophages and monocytes, iNOS signalling, Cdc42 signalling and IL-8 signalling (Figure 4d).

TABLE 2 Clinical characteristics of different severe asthma inflammatory phenotypes and healthy controls

| Characteristic                                         | Healthy Control           | Severe asthma                                                                                                                                                                         |                                                                                                                                                                          |                                                                                                                                                                                                                 | P-value |
|--------------------------------------------------------|---------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------|
|                                                        | HC                        | NA                                                                                                                                                                                    | EA                                                                                                                                                                       | PGA                                                                                                                                                                                                             |         |
| N                                                      | 13                        | 9                                                                                                                                                                                     | 22                                                                                                                                                                       | 16                                                                                                                                                                                                              | —       |
| Age(years), mean (SD)                                  | 45 (13)                   | 62 (12) <sup>a</sup>                                                                                                                                                                  | 58 (13) <sup>a</sup>                                                                                                                                                     | 55 (14)                                                                                                                                                                                                         | 0.008   |
| Sex, female n (%)                                      | 6 (46)                    | 7 (78)                                                                                                                                                                                | 12 (55)                                                                                                                                                                  | 11 (69)                                                                                                                                                                                                         | 0.391   |
| Atopy, n (%)                                           | 1 (8)                     | 6 (67)                                                                                                                                                                                | 13 (54)                                                                                                                                                                  | 6 (40) (n = 15)                                                                                                                                                                                                 | 0.014   |
| BMI (kg/m <sup>2</sup> ), mean (SD)                    | 31 (n = 1)                | 24.4 (2.6) (n = 5)                                                                                                                                                                    | 30.1 (6.4) (n = 18)                                                                                                                                                      | 30.8 (6.4) (n = 12)                                                                                                                                                                                             | 0.254   |
| Age of onset (%)                                       |                           |                                                                                                                                                                                       |                                                                                                                                                                          |                                                                                                                                                                                                                 | 0.101   |
| Childhood                                              | N/A                       | 5/7 (71)                                                                                                                                                                              | 6/20 (30)                                                                                                                                                                | 8/14 (57)                                                                                                                                                                                                       |         |
| Adult                                                  | N/A                       | 2/7 (29)                                                                                                                                                                              | 14/20 (70)                                                                                                                                                               | 6/14 (43)                                                                                                                                                                                                       |         |
| FEV <sub>1</sub> %predicted, mean (SD)                 | 101.4 (10.7)              | 75.3 (14.1) <sup>a</sup>                                                                                                                                                              | 74.3 (18.1) <sup>a</sup>                                                                                                                                                 | 73.5 (26.9) <sup>a</sup>                                                                                                                                                                                        | 0.002   |
| FVC %predicted, mean (SD)                              | 104.2 (9.9)               | 97.4 (19.3)                                                                                                                                                                           | 88.5 (16.7)                                                                                                                                                              | 91.9 (19.4)                                                                                                                                                                                                     | 0.088   |
| FEV <sub>1</sub> /FVC, mean (SD)                       | 79.8 (3.9)                | 64.5 (5.8) <sup>a</sup>                                                                                                                                                               | 66.8 (10.9) <sup>a</sup>                                                                                                                                                 | 64.5 (17) <sup>a</sup>                                                                                                                                                                                          | 0.003   |
| Former smoker, n (%)                                   | 2 (15)                    | 1 (11)                                                                                                                                                                                | 5 (23)                                                                                                                                                                   | 3 (19)                                                                                                                                                                                                          | 0.879   |
| Pack years, median (Q1, Q3)                            | 5 (5, 5)                  | 10 (10, 10)                                                                                                                                                                           | 10 (5, 20)                                                                                                                                                               | 12.5 (5, 20)                                                                                                                                                                                                    | 0.738   |
| ACQ score, mean (SD)                                   | N/A                       | 1.4 (1.3) (n = 7)                                                                                                                                                                     | 2.0 (1.2) (n = 20)                                                                                                                                                       | 2.3 (0.9) (n = 15)                                                                                                                                                                                              | 0.268   |
| ICS dose µg/day, median (Q1, Q3)                       | N/A                       | 2000 (1600, 2000)                                                                                                                                                                     | 1600 (800, 2000)                                                                                                                                                         | 2000 (1300, 2000)                                                                                                                                                                                               | 0.276   |
| OCS use (%)                                            | N/A                       | 1 (11)                                                                                                                                                                                | 5 (23)                                                                                                                                                                   | 1 (6)                                                                                                                                                                                                           | 0.760   |
| OCS dose µg/day, median (Q1, Q3)                       | N/A                       | 15 (15, 15)                                                                                                                                                                           | 10 (7.5, 10)                                                                                                                                                             | 15 (15, 15)                                                                                                                                                                                                     | 0.180   |
| OCS courses, past 12 months, median (Q1, Q3)           | N/A                       | 2 (1, 2)                                                                                                                                                                              | 2 (1, 4)                                                                                                                                                                 | 2.5 (1, 4.5)                                                                                                                                                                                                    | 0.524   |
| Total cell count x10 <sup>6</sup> /mL, median (Q1, Q3) | 0.1 (0.1, 0.2)            | 2.6 (1.9, 6.4) <sup>a,b,d</sup>                                                                                                                                                       | 0.1 (0.1, 0.3)                                                                                                                                                           | 0.1 (0.1, 0.3)                                                                                                                                                                                                  | <0.001  |
| Viability, median (Q1, Q3)                             | 75 (68, 76)               | 95.1 (94, 96) <sup>a,b,d</sup>                                                                                                                                                        | 66.9 (50, 85)                                                                                                                                                            | 73.9 (50, 85)                                                                                                                                                                                                   | 0.001   |
| Neutrophils %, median (Q1, Q3)                         | 40.3 (15.3, 62.8)         | 89 (84.5, 91.3) <sup>a,b,d</sup>                                                                                                                                                      | 31.1 (22.3, 65.8)                                                                                                                                                        | 44.1 (13.3, 60.6)                                                                                                                                                                                               | <0.001  |
| Eosinophils %, median (Q1, Q3)                         | 0.5 (0.3, 1)              | 1.3 (0.5, 2)                                                                                                                                                                          | 11.3 (4.8, 25.8) <sup>a,c,d</sup>                                                                                                                                        | 0.9 (0.5, 1.1)                                                                                                                                                                                                  | <0.001  |
| Macrophages %, median (Q1, Q3)                         | 33 (20.8, 43.8)           | 9 (8, 11.5) <sup>a,d</sup>                                                                                                                                                            | 22.9 (15.5, 40)                                                                                                                                                          | 39.4 (28.5, 54)                                                                                                                                                                                                 | <0.001  |
| Lymphocytes %, median (Q1, Q3)                         | 1.5 (0.5, 2) <sup>c</sup> | 0.5 (0, 1)                                                                                                                                                                            | 0.5 (0, 3.3) <sup>c</sup>                                                                                                                                                | 1 (0.3, 2.5) <sup>c</sup>                                                                                                                                                                                       | 0.361   |
| Epithelial cells %, median (Q1, Q3)                    | 14.5 (7.5, 23.3)          | 0 (0, 0.8)                                                                                                                                                                            | 5.5 (1.3, 23.8) <sup>a,d</sup>                                                                                                                                           | 8 (3, 17.8)                                                                                                                                                                                                     | <0.001  |
| Squamous cells %, median (Q1, Q3)                      | 2 (1, 2.4)                | 0 (0, 0.3)                                                                                                                                                                            | 2 (0.4, 4)                                                                                                                                                               | 1.5 (0.1, 4.4)                                                                                                                                                                                                  | 0.045   |
| Bacteriological culture positive, n (%)                | 0 (0)                     | 7 (77.8) <sup>a</sup><br><i>Pseudomonas aeruginosa</i> (n = 3)<br><i>Haemophilus influenzae</i> (n = 2)<br><i>Escherichia coli</i> (n = 1)<br><i>Streptococcus pneumoniae</i> (n = 1) | 8 (36.4)<br><i>Pseudomonas aeruginosa</i> (n = 4)<br><i>Haemophilus influenzae</i> (n = 2)<br><i>Escherichia coli</i> (n = 1)<br><i>Streptococcus pneumoniae</i> (n = 1) | 5 (31.3)<br><i>Pseudomonas aeruginosa</i> (n = 1)<br><i>Haemophilus influenzae</i> (n = 1)<br><i>Acinetobacter baumannii</i> (n = 1)<br><i>Staphylococcus aureus</i> (n = 1)<br><i>Escherichia coli</i> (n = 1) | 0.002   |

Data were analysed using Fishers' exact test for categorical data, Wilcoxon rank-sum or a Student's *t* test depending on the outcome distribution with Bonferroni correction (Significant *P*-value ≤0.008).

Abbreviations: ACQ, Asthma Control Questionnaire; and PGA, paucigranulocytic asthma; EA, eosinophilic asthma; HC, healthy control; ICS, inhaled corticosteroid; N/A, not applicable; NA, neutrophilic asthma; OCS, oral corticosteroid.

<sup>a</sup>*P* < 0.008 versus HC.

<sup>b</sup>*P* < 0.008 versus EA.

<sup>c</sup>*P* < 0.008 versus NA.

<sup>d</sup>*P* < 0.008 versus PGA.

TABLE 3 Top-ranked differentially expressed genes (FDR  $\leq 0.05$ , FC  $\geq +2$  or  $\leq -2$ )

| SA vs HC  |      | NA vs HC <sup>d</sup> |      | NA vs EA <sup>d</sup> |     | NA vs PGA <sup>d</sup> |      | EA vs HC          |      | EA vs PGA        |     | PGA vs HC            |      |
|-----------|------|-----------------------|------|-----------------------|-----|------------------------|------|-------------------|------|------------------|-----|----------------------|------|
| Gene      | FC   | Gene                  | FC   | Gene                  | FC  | Gene                   | FC   | Gene              | FC   | Gene             | FC  | Gene                 | FC   |
| CD163     | 2.3  | SAA1                  | 11.3 | SAA1                  | 6.7 | CCL20                  | 6.3  | CLC <sup>a</sup>  | 2.3  | CLC <sup>a</sup> | 2.1 | FKBP5                | 2.4  |
| LOC649143 | 2.3  | IDO1                  | 6.6  | CXCL10                | 5.9 | TNFRSF6B               | 5.1  | CCL13             | 2.1  |                  |     | PHACTR3 <sup>c</sup> | 2.4  |
| CA12      | 2.2  | CCL20                 | 6.3  | CCL20                 | 5.9 | MGC29506               | 5    | EIF2C2            | -2   |                  |     | PRICKLE1             | -2   |
| ADORA3    | 2    | CD163                 | 5.5  | IDO1                  | 5.8 | S100A9                 | 4.9  | RSPRY1            | -2   |                  |     | CCL5                 | -2.2 |
| MGC72104  | -2   | TNFRSF6B              | 5.4  | CXCL9                 | 5.7 | IDO1                   | 4.8  | LOC646808         | -2   |                  |     | PLEKHA1              | -2.4 |
| CCNY      | -2.1 | SAA4                  | 5.3  | SAA4                  | 5.2 | UBD                    | 4.5  | PLEKHA1           | -2   |                  |     | HLA-DQB2             | -3   |
| CYP2F1    | -2.1 | S100A9                | 5.1  | HLA-DRB1              | 5.2 | CXCL10                 | 4.4  | CCNY              | -2   |                  |     | TPSB2                | -3.4 |
| LOC650737 | -2.1 | UBD                   | 4.9  | UBD                   | 4.4 | CD79A                  | 4.4  | NFIC              | -2   |                  |     | TPSAB1               | -4.8 |
| H19       | -2.2 | CXCL10                | 4.8  | TNFRSF6B              | 4.1 | HLA-DRB1               | 4.3  | MGC72104          | -2   |                  |     |                      |      |
| CCL5      | -2.2 | IL8                   | 4.6  | CD79A                 | 3.9 | SAA1                   | 4.1  | FOXA2             | -2.1 |                  |     |                      |      |
| PLEKHA1   | -2.3 | RARRES1               | 4.5  | MGC29506              | 3.9 | FCN1                   | 3.7  | LOC650737         | -2.1 |                  |     |                      |      |
| LOC731992 | -2.4 | CXCL9                 | 4.3  | CHI3L2                | 3.8 | CHI3L2                 | 3.6  | CYP2F1            | -2.1 |                  |     |                      |      |
| TPSB2     | -2.7 | CHI3L2                | 4.2  | RARRES1               | 3.6 | CXCL9                  | 3.4  | TPSB2             | -2.2 |                  |     |                      |      |
| HLA-DQB2  | -2.9 | CFB                   | 4    | S100A9                | 3.6 | C1QB                   | 3    | LOC731992         | -2.4 |                  |     |                      |      |
| TPSAB1    | -3.1 | ADORA3                | 3.8  | C1QB                  | 3.3 | CSF3R                  | 2.9  | CCL5 <sup>b</sup> | -2.6 |                  |     |                      |      |
|           |      | CCL13                 | 3.7  | FCN1                  | 3.3 | SOD2                   | 2.9  | TPSAB1            | -2.7 |                  |     |                      |      |
|           |      | SOD2                  | 3.6  | HLA-DQA1              | 3.2 | FPR1                   | 2.9  | HLA-DQB2          | -3.1 |                  |     |                      |      |
|           |      | FCN1                  | 3.6  | C1QC                  | 3.1 | CCL13                  | 2.9  |                   |      |                  |     |                      |      |
|           |      | SIGLEC10              | 3.6  | CSF3R                 | 3   | C1QC                   | 2.8  |                   |      |                  |     |                      |      |
|           |      | INDO                  | 3.5  | CD163                 | 3   | IFI30                  | 2.8  |                   |      |                  |     |                      |      |
|           |      | HLA-DRB1              | 3.2  | CFB                   | 3   | IL8                    | 2.8  |                   |      |                  |     |                      |      |
|           |      | CSF3R                 | 3.2  | SIGLEC10              | 2.9 | PLEK                   | 2.8  |                   |      |                  |     |                      |      |
|           |      | FPR1                  | 3    | STAT1                 | 2.9 | CD163                  | 2.7  |                   |      |                  |     |                      |      |
|           |      | HLA-DRB6              | 3    | HLA-DMB               | 2.8 | CD14                   | 2.7  |                   |      |                  |     |                      |      |
|           |      | NNMT                  | 2.8  | SOD2                  | 2.8 | IL1B                   | 2.7  |                   |      |                  |     |                      |      |
|           |      | STAT1                 | 2.2  | INDO                  | 2.8 | GZMB                   | 2.6  |                   |      |                  |     |                      |      |
|           |      | CKB                   | -2.5 | HLA-DRA               | 2.7 | CFB                    | 2.6  |                   |      |                  |     |                      |      |
|           |      | KRT15                 | -2.6 | FPR1                  | 2.7 | HLA-DQA1               | 2.6  |                   |      |                  |     |                      |      |
|           |      | HLA-DQB2              | -2.6 | FGR                   | 2.6 | STAT1                  | 2.6  |                   |      |                  |     |                      |      |
|           |      | ATP6V1B1              | -2.6 | IFI30                 | 2.6 | ALOX15                 | -2   |                   |      |                  |     |                      |      |
|           |      | CLDN8                 | -2.7 | FCER1G                | 2.5 | BNIPL                  | -2.1 |                   |      |                  |     |                      |      |

(Continues)

TABLE 3 (Continued)

| SA vs HC |    | NA vs HC <sup>d</sup> |      | NA vs EA <sup>d</sup> |      | NA vs PGA <sup>d</sup> |      | EA vs HC |    | EA vs PGA |    | PGA vs HC |    |
|----------|----|-----------------------|------|-----------------------|------|------------------------|------|----------|----|-----------|----|-----------|----|
| Gene     | FC | Gene                  | FC   | Gene                  | FC   | Gene                   | FC   | Gene     | FC | Gene      | FC | Gene      | FC |
|          |    | HOOK1                 | -2.8 | LCP1                  | 2.5  | RBM20                  | -2.2 |          |    |           |    |           |    |
|          |    | CYP2S1                | -2.8 | HLA-DMA               | 2.5  | KRT5                   | -2.2 |          |    |           |    |           |    |
|          |    | EPHX1                 | -2.8 | HLA-DRB6              | 2.5  | UGT2B7                 | -2.2 |          |    |           |    |           |    |
|          |    | TPSB2                 | -2.8 | CD14                  | 2.5  | CKB                    | -2.2 |          |    |           |    |           |    |
|          |    | KRT5                  | -2.9 | IL8                   | 2.4  | GATS                   | -2.3 |          |    |           |    |           |    |
|          |    | AHNAK2                | -3.1 | CCL5 <sup>b</sup>     | 2    | ZDHHC11                | -2.3 |          |    |           |    |           |    |
|          |    | PLEKHA1               | -3.1 | LMTK3                 | -2   | ATP6V1B1               | -2.3 |          |    |           |    |           |    |
|          |    | CCDC81                | -3.1 | CLC <sup>a</sup>      | -2   | PHACTR3 <sup>c</sup>   | -2.4 |          |    |           |    |           |    |
|          |    | SULT2B1               | -3.5 | CDH26                 | -2   | SULT2B1                | -2.5 |          |    |           |    |           |    |
|          |    | CYP26A1               | -3.8 | CKB                   | -2.1 | CYP2F1                 | -2.7 |          |    |           |    |           |    |
|          |    | TPSAB1                | -3.8 | AADAC                 | -2.2 | CLDN8                  | -2.8 |          |    |           |    |           |    |
|          |    | CYP2F1                | -3.8 | SULT2B1               | -2.3 | DAPL1                  | -3   |          |    |           |    |           |    |
|          |    | DAPL1                 | -4.5 | OLFML2A               | -2.3 | CCDC81                 | -3   |          |    |           |    |           |    |
|          |    | GSTA2                 | -4.8 | CYP2S1                | -2.3 | AHNAK2                 | -3.1 |          |    |           |    |           |    |
|          |    | KRT4                  | -8.9 | KRT5                  | -2.6 | CYP26A1                | -3.1 |          |    |           |    |           |    |

Gene names in boldface correspond to NA top-ranked overlapping genes, Figure 3a.

<sup>a</sup>CLC up-regulation in EA compared with HC, NA and PGA.

<sup>b</sup>CCL5 down-regulated in EA compared with HC and NA.

<sup>c</sup>PHACTR3 up-regulated in PGA compared with HC and NA.

<sup>d</sup>For complete list of top-ranked expressed genes, see Tables S9–S11.

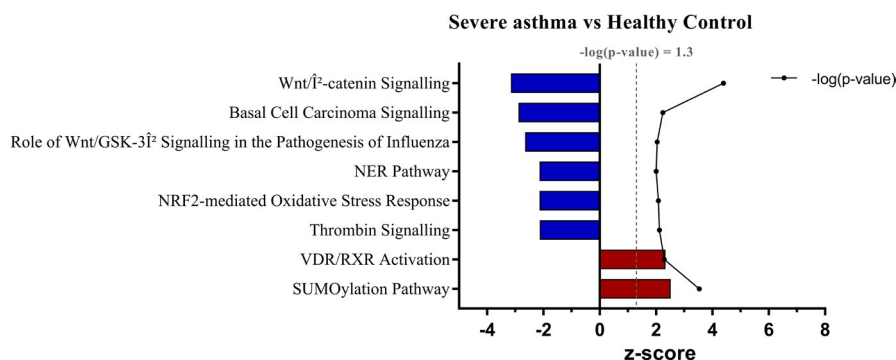


**FIGURE 1** Identified canonical pathways associated with severe asthma compared with healthy controls. Pathways depicted are false discovery rate  $\leq 0.05$ . SA: severe asthma, HC: healthy control. Significantly enriched canonical pathways were identified with a right-tailed Fisher's Exact Test corrected for multiple testing using the Benjamini-Hochberg method (false discovery rate  $\leq 0.05$ ), dotted grey line indicates significance threshold of  $-\log(P\text{-value}) = 1.3$ . The z-score indicates predicted activation state of the canonical pathway. Red colour indicates a positive z-score and predicted activation of the pathway, and blue indicates a negative z-score and predicted inhibition of the pathway

In EA compared with HC, we identified 13 enriched pathways (Figure 5a), and when PGA was compared with HC, there were ten enriched pathways (Figure 5b). Of these, the role of Wnt/GSK-3 $\beta$  signalling in the pathogenesis of influenza, CXCR4 signalling, Fc $\gamma$  receptor-mediated phagocytosis in macrophages and monocytes were overlapping in EA and PGA when compared with HC (Figure 5c). No pathways were enriched when EA was compared with PGA. All severe asthma inflammatory phenotypes (NA, EA and PGA) presented enrichment for the SUMOylation pathway (predicted activation) and basal cell carcinoma (predicted inhibition) (Figures 4d and 5c). Details of the pathways identified are provided in Tables S4–S8.

### 3.5 | Colonization with potentially pathogenic bacteria

As there was an increased proportion of BLF samples culture positive for potentially pathogenic bacteria in participants with NA (bacteria cultured, described in Table 2), the effect of these was evaluated. An analysis of severe asthma participants who had a positive BLF bacteriological compared with BLF culture negative was performed (Clinical characteristics of groups can be found in Table S2). No significant differences in gene expression were detected between BLF culture-positive compared with BLF culture-negative severe asthma participants.

### 3.6 | Clinical correlations with gene expression

Associations between top-ranked DEGs in inflammatory phenotypes, including the NA gene signature with clinical metadata, were then examined to identify potential clinical impacts of these genes. Higher expression of *CSF3R*, *FKBP5*, *ADORA3* and *CD14* correlated negatively with mean ACQ score. In contrast, there was a positive correlation between mean ACQ score and the expression of *ALOX15* and *CDH26* in severe asthma. High expression of *ADORA3* was

associated with high ICS dose, whereas low expression of *PRICKLE1*, *CCNY*, *TPSAB1* and *PLEKHA1* was associated with high ICS dose. High BMI correlated with high expression to *FKBP5*. Expression of *GMZB* and *CDH26* negatively correlated with FEV<sub>1</sub>% predicted. No additional correlations were identified for other clinical parameters (Figure S1).

### 3.7 | Quantitative real time PCR validation of DEGs

To validate the DEGs, three genes with a fold change of greater than 2 in one of the inflammatory phenotypes were selected for qPCR analysis. Significant correlations were observed between microarray data and qPCR for selected differentially expressed genes, *IL1B* ( $r = 0.64$ ,  $P < 0.001$ ), *CLC* ( $r = 0.72$ ,  $P < 0.001$ ), *FKBP5* ( $r = 0.35$ ,  $P = 0.033$ ) (See (Figure S2)).

## 4 | DISCUSSION

Transcriptomic profiling of severe asthma in tissue from endobronchial biopsies identified genes and pathways associated with specific inflammatory phenotypes. Participants with NA had the most distinct transcriptomic profile, represented by large numbers of DEGs, a 'neutrophilic signature', a clear clustering of gene expression patterns and higher proportion of BLF bacterial culture positive. We identified pathway enrichment associated with neutrophilic inflammation; Th1, TREM1 signalling, dendritic cell maturation, and inflammasome activation; as well as we established novel links between severe NA and enrichment of neuroinflammation signalling, role of NFAT in regulation of the immune response and PKC $\theta$  signalling in T lymphocytes pathways. Those with severe EA and PGA had similar transcriptomic profiles within the biopsy tissue, suggesting similar mechanisms of disease pathogenesis within this compartment in these groups. We showed significant enrichment for SUMOylation, basal cell carcinoma signalling and Wnt/ $\beta$ -catenin pathways across

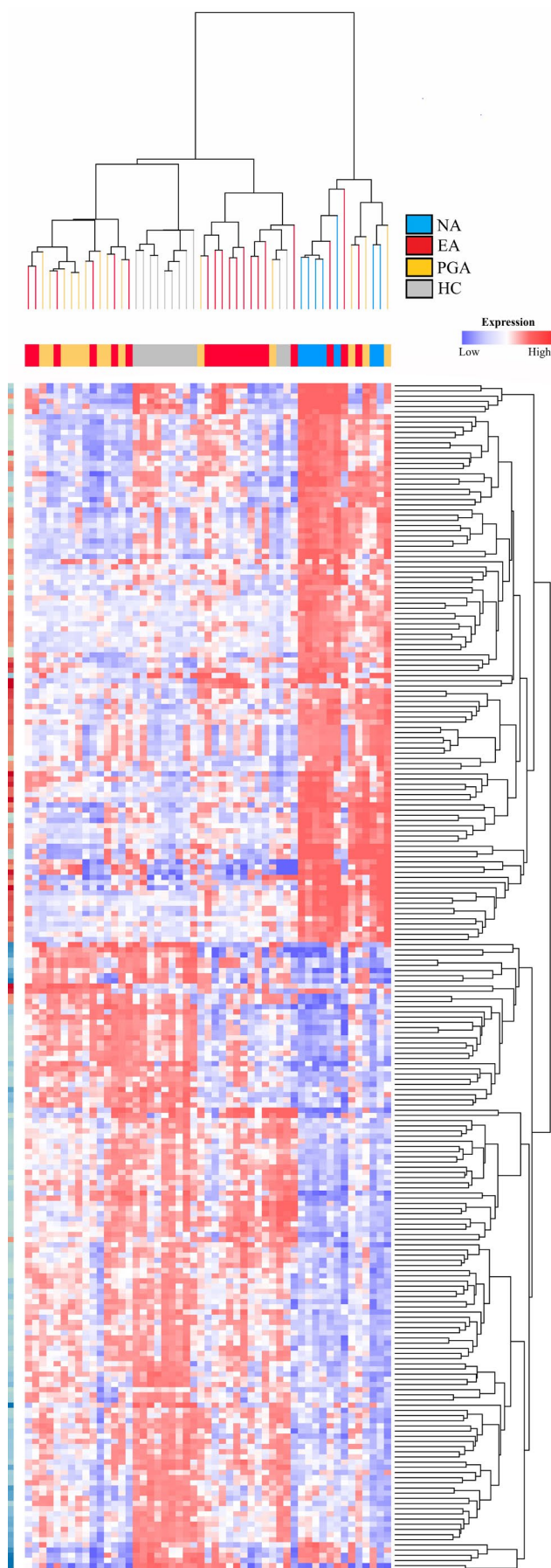


**FIGURE 2** Heat map depicting unsupervised hierarchical clustering of differentially expressed genes in severe asthma inflammatory phenotypes. Distances calculated with Euclidean correlation metric and Ward's method. Red represents a higher level of gene expression and blue a lower level of expression. Each column represents a participant with severe asthma or healthy control. EA (red): eosinophilic asthma, HC (grey): healthy control, NA (light blue): neutrophilic asthma, PGA (yellow): paucigranulocytic asthma

all inflammatory phenotypes of severe asthma. This study contributes to an important gap in knowledge in severe asthma research where mechanisms/pathways associated with non-type-2 inflammatory phenotypes in the tissue remain poorly understood.

In neutrophilic severe asthma, a 'neutrophilic signature' comprising 60 top-ranked DEGs was identified. This signature presented enrichment to immune response, innate immunity, neutrophil degranulation and IL-10 signalling and included genes which have previously been associated with non-type-2 mechanisms and neutrophilic inflammation in asthma, such as *SAA1*, *S100A9*, *CD163*, *IL1B*, *STAT1*, *CCL20* and *IL8*<sup>28,29</sup> and other novel links to neutrophilia such as *GZMB* and *IDO*. A strong link between NA and inflammasome has been described. *SAA1* is an acute phase protein that activates the inflammasome by binding to Toll-like receptors, it increases IL1 $\beta$  and IL10 production and promotes neutrophilia.<sup>30-33</sup> In this cohort, *SAA1* was the highest up-regulated gene in NA, confirming the link between neutrophilic airway inflammation, innate immunity and inflammasome activation in this phenotype.<sup>28,33-35</sup>

The *S100A9* protein belongs to the damage-associated molecular patterns and is responsible for initiating and amplifying the innate immune response. It has been proposed as a key feature in neutrophil-dominant obstruction airway disease as it promotes mucus hypersecretion from epithelial cells.<sup>36</sup> Protein *CD163* is expressed in monocytes and macrophages and provides tissue protection against oxidative damage. Neutrophil elastase is responsible for *CD163* shedding, and soluble-*CD163* has been reported as a marker for neutrophil and macrophage activity<sup>37</sup> and has been shown to increase in the serum of people with severe asthma.<sup>37</sup> We demonstrated up-regulation of *IL1B* in NA. This is consistent with previous studies that found *IL1B* expression linked to airway inflammation,<sup>38</sup> neutrophilic asthma<sup>12</sup> and disease severity.<sup>20</sup> *STAT1* has been shown to be induced by IFN- $\gamma$  and reduced by secretory leukocyte protease inhibitor. Increased action of IFN- $\gamma$  has also previously been associated with non-type-2 mechanisms in severe asthma.<sup>39</sup>

Other genes appearing in the neutrophilic signature included *GZMB* and *IDO1*. The expression of these has not been previously described in association with neutrophilic inflammation, but has been associated with airway remodelling<sup>40</sup> and exacerbations in COPD.<sup>41</sup> *GZMB* levels are increased in BAL,<sup>42</sup> blood<sup>43</sup> and sputum in asthma following allergen challenge<sup>44</sup> and even in fatal asthma cases.<sup>40</sup> *GZMB* is a potent cytotoxin that mediates targeted apoptosis and serves as an innate immune barrier against bacteria.<sup>45</sup> A variety of cell types can secrete *GZMB*, neutrophils among them.<sup>46</sup> *GZMB* can be released to the extracellular environment causing

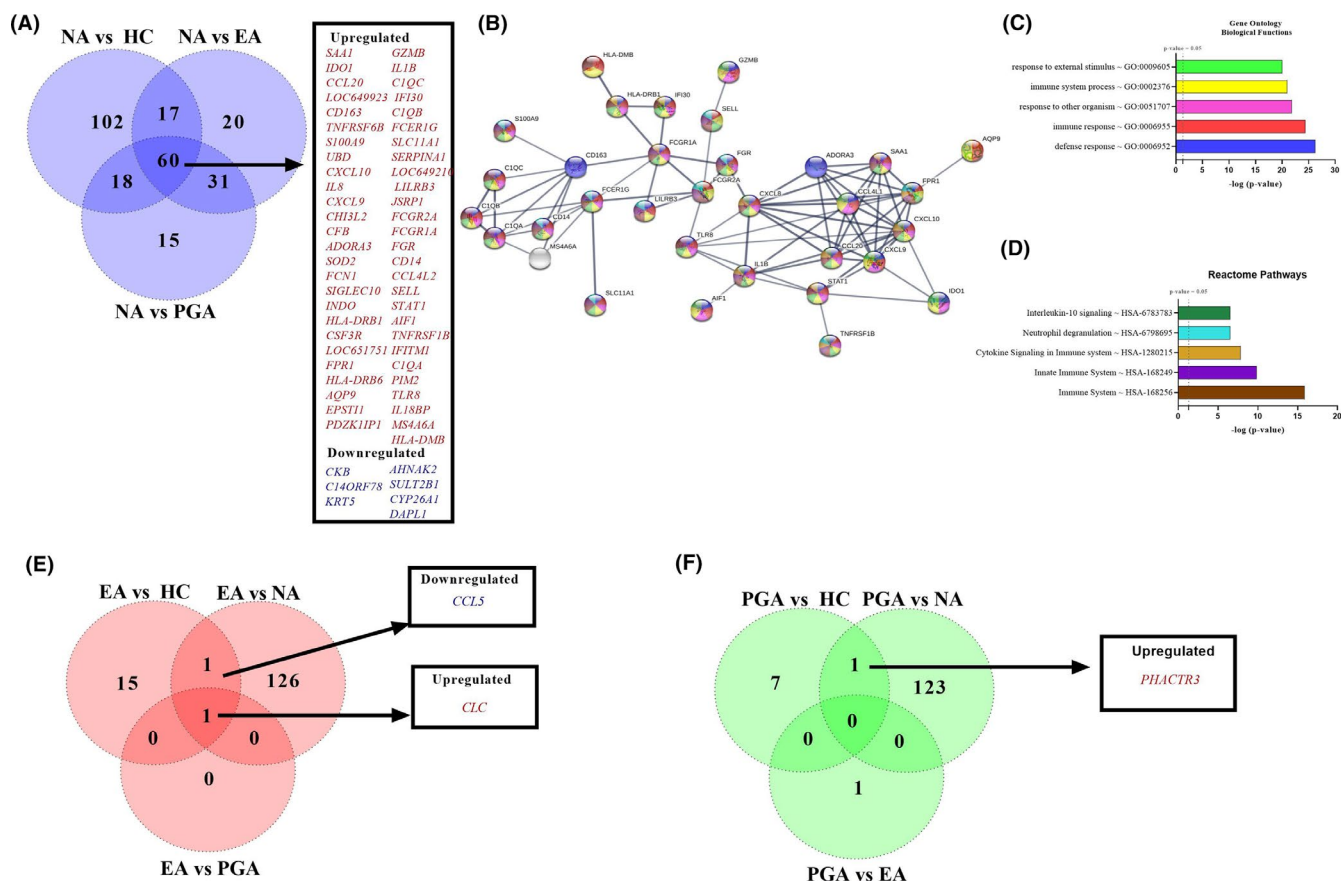


**FIGURE 3** Functional enrichment and network interaction in overlapped NA genes. (a) Venn diagram depicting top-ranked differentially expressed genes (false discovery rate  $\leq 0.05$  and fold change (FC)  $> +2$  or  $< -2$ ) between severe asthma neutrophilic asthma (NA) compared with healthy control (HC), eosinophilic asthma (EA) and paucigranulocytic asthma (PGA). (b) Protein interaction network representing protein interaction of gene products from overlapping NA genes. (c) Gene Ontology (GO) analysis represent the top enriched GO terms (false discovery rate (FDR)  $\leq 0.05$ ) with arbitrary colours assigned to biological GO terms. A grey dotted line indicating  $P$ -value = 0.05 is shown here. Colour of each node depicts respective GO terms. Blue = defence response, red = immune response, magenta = response to other organism, yellow = immune system process, green = response to external stimulus. (d) Top enriched Reactome pathways FDR  $\leq 0.05$  with arbitrary colours assigned to pathways. A grey dotted line indicating  $P$ -value = 0.05 is shown here. Colour of each node depicts respective pathway. Brown = Immune system, purple = Innate Immune system, mustard = cytokine signalling in immune system, aqua blue = neutrophil degranulation, dark green = interleukin-10 signalling. (e) Venn diagram depicting differentially expressed genes (false discovery rate  $\leq 0.05$  and fold change (FC)  $\geq 2$  or  $\leq -2$ ) between severe asthma EA and (f) PGA compared with NA, HC and other phenotypes

degranulation and remodelling of the extracellular matrix proteins.<sup>47</sup> GZMB is up-regulated by neutrophils and in this study is likely to reflect infiltration and persistent presence of neutrophils in the airway while potentially contributing to airway remodelling.

IDO1 encodes an enzyme with antimicrobial properties and is vital to the metabolism of tryptophan and maintenance of immune response homeostasis in experimental asthma.<sup>48</sup> Tryptophan catalysis via IDO has been demonstrated in acute exacerbations in COPD<sup>41</sup>; its potential as a biomarker of exacerbations in asthma remains unknown.

The neutrophilic signature together with the higher prevalence of bacteria in the NA phenotype compared with other phenotypes confirms previous findings where NA was associated with microbial dysbiosis.<sup>49-51</sup> The strong link between neutrophilic signature, and mechanisms of innate immunity could potentially be accounting for the relatively steroid insensitivity that has been linked previously

with NA.<sup>35,52,53</sup> This study only detected bacterial presence by conventional bacteriological methods, and a thorough study of the microbiome and gene expression in a larger sample size of people with neutrophilic severe asthma might reveal the underlying mechanisms further.

Pathway analysis revealed seven pathways enriched in NA when compared with HC, EA and PGA. Of those, Th1,<sup>54</sup> TREM1 signalling,<sup>55</sup> dendritic cell maturation<sup>56</sup> and inflammasome<sup>35,53</sup> are known mechanisms of neutrophilic airway inflammation, whereas neuroinflammation signalling, role of NFAT in regulation of the immune response and PKC $\theta$  signalling in T lymphocytes pathways represent novel links to severe NA. The role of neuroinflammation in the pathogenesis of airway disease is a recent area of interest.<sup>57</sup> Evidence supports the close relationship between the immune and nervous systems, with direct interaction of sensory neurons and immune cells such as eosinophils, T cells and dendritic cells, although

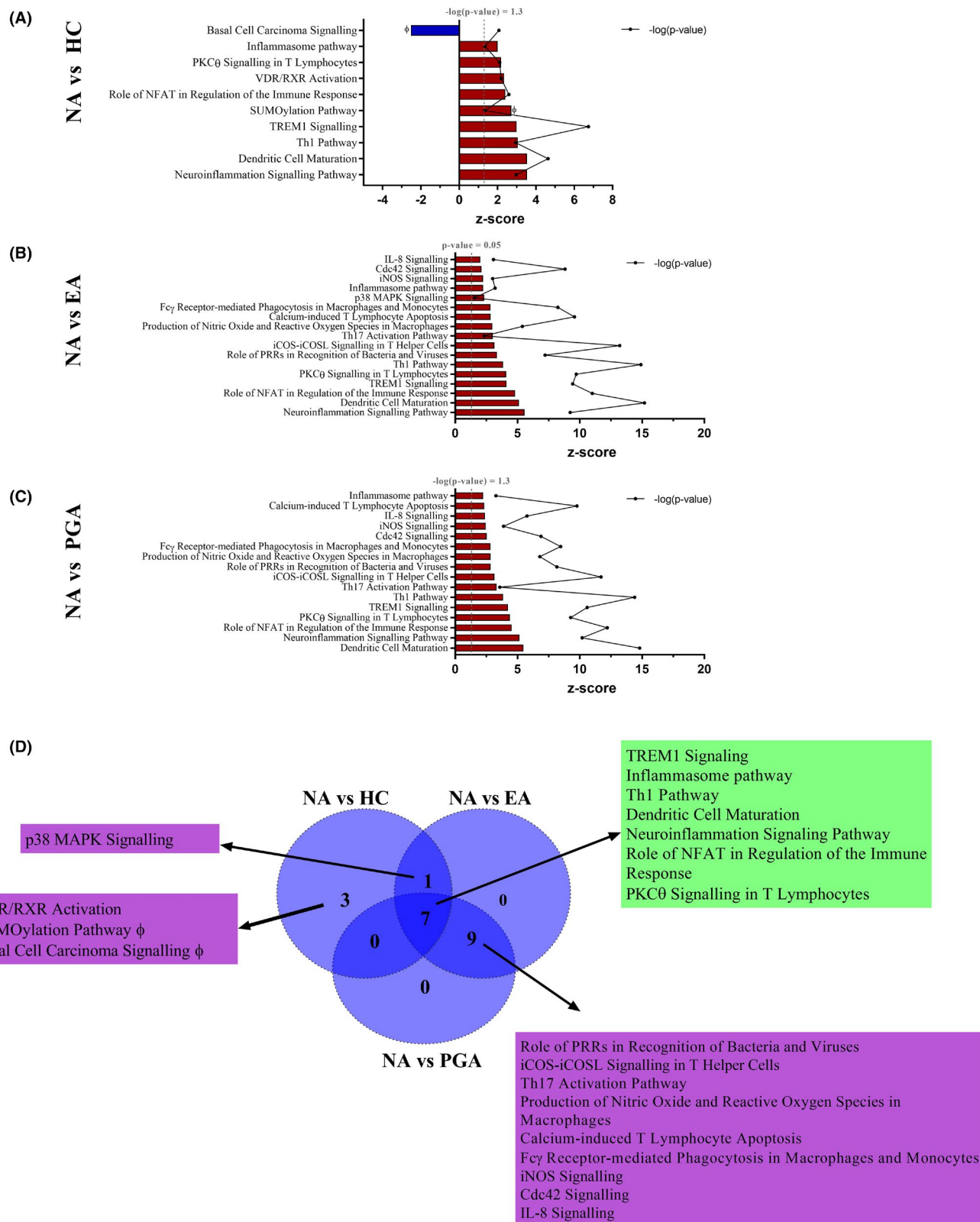


**FIGURE 4** Canonical pathways identified associated with severe neutrophilic asthma compared with other inflammatory phenotypes of severe asthma and healthy control. Significantly enriched canonical pathways were identified with a right-tailed Fisher's Exact Test corrected for multiple testing using the Benjamini-Hochberg method (false discovery rate  $\leq 0.05$ , dotted grey line indicates significance threshold of  $-\log(P\text{-value}) = 1.3$ ). Dot-plot Indicate  $-\log(P\text{-value})$ . The x-axis depicts z-score indicating predicted activation state of the canonical pathway. Red colour bars indicate a positive z-score and predicted activation of the pathway, and blue colour bars indicate a negative z-score and predicted inhibition of the pathway. (a) NA compared with HC; (b) NA compared with EA; (c) NA compared with PGA; (d) Venn diagram depicting shared canonical pathways identified in severe neutrophilic asthma compared with other inflammatory phenotypes of severe asthma and healthy controls.  $\phi$  = shared by EA, NA and PGA phenotypes compared with HC. HC, healthy controls; EA, eosinophilic asthma; NA, neutrophilic asthma, PGA, paucigranulocytic asthma

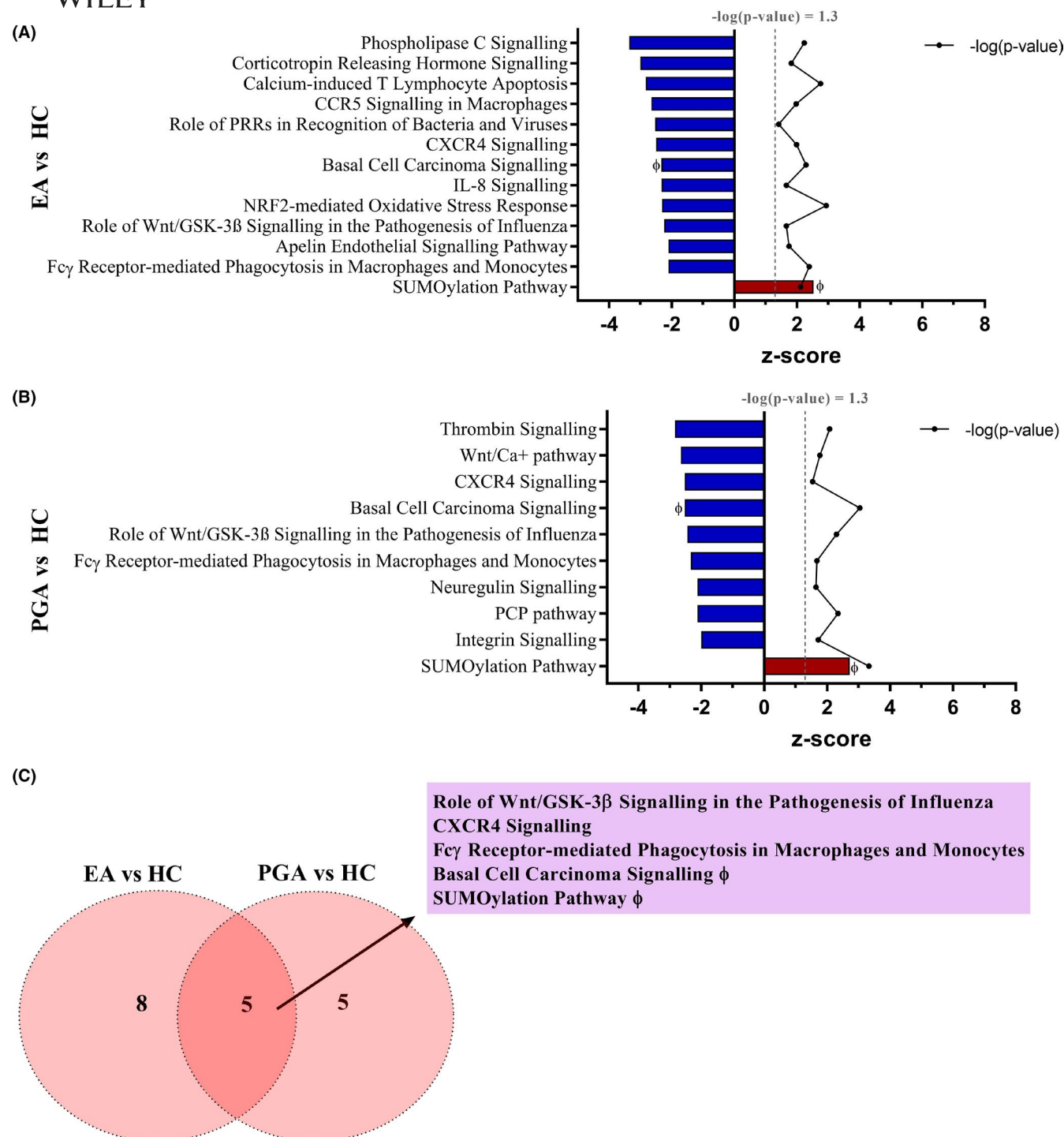


**FIGURE 5** Canonical pathways identified associated with severe eosinophilic and paucigranulocytic asthma compared with healthy control. Significantly enriched canonical pathways were identified with a right-tailed Fisher's Exact Test corrected for multiple testing using the Benjamini-Hochberg method (false discovery rate  $\leq 0.05$ ). The z-score indicates predicted activation state of the canonical pathway. Red colour indicates a positive z-score and predicted activation of the pathway, and blue colour indicates a negative z-score and predicted inhibition of the pathway. (a) EA compared with HC; (b) PGA compared with HC. (c) Venn diagram depicting overlapping canonical pathways between contrasts. No pathways were overrepresented when EA was compared with PGA.  $\phi$  = shared by EA, NA and PGA phenotypes compared with HC. EA, eosinophilic asthma; PGA, paucigranulocytic asthma; HC, healthy controls

interactions with neutrophils have not been established.<sup>57</sup> Both NFAT and PKC $\theta$  signalling pathways play important roles in the immune response and are expressed in a variety of cells.<sup>58,59</sup> NFAT

comprises a family of calcium-dependent transcription factors.<sup>60</sup> The specific mechanisms by which NFAT acts in asthma remain largely unknown; however, NFATc1 is increased in children

with allergic asthma.<sup>61</sup> NFAT1-mediated IL8 production promotes migration and neutrophil infiltration in breast tumours,<sup>62</sup> suggesting a key mechanism of neutrophil recruitment and infiltration into the airway that can be investigated as a potential target for NA. PKC $\theta$  is a phospholipid-dependent protein kinase that promotes T cell activation by mediating interaction between T cell receptor and CD28.<sup>63</sup> Interestingly, NFAT and PKC $\theta$  pathways can induce activation signals essential for NF- $\kappa$ B activation. NF- $\kappa$ B has been described and proposed as a mechanism leading to the chronic cycle of inflammation and injury in asthma.<sup>64</sup> Further studies are needed to characterize the roles of these transcription factors in NA.

Additionally, p38 MAPK signalling was enriched in NA when compared with EA, and the Th17 pathway was enriched when compared with EA and PGA. The p38 MAPK signalling pathway has been identified as one of the mechanisms leading to glucocorticoid resistance<sup>65</sup> in severe asthma,<sup>66</sup> and its inhibition can reduce neutrophilic inflammation.<sup>67</sup>

Th17 activation has been associated with severe asthma,<sup>54,68</sup> with Th17-mediated neutrophilic airway inflammation<sup>69-71</sup> and a decreased response to corticosteroids.<sup>70,72</sup> CCL20 and IL8 are key players in the Th17 pathway and part of the neutrophilic signature identified in this study. CCL20 attracts Th17 cells by binding to their receptor CCR6,<sup>69</sup> whereas IL8 is produced and released by Th17 cells attracting neutrophils to the site of airway inflammation.<sup>69</sup> These findings suggest predominant roles for p38 MAPK signalling and Th17 pathway activation in severe NA that are not observed in EA or PGA. In the context of targeting inflammation in those with severe NA, these pathways provide areas for further study as potential therapeutic targets, although they are less likely to be ineffective in those with EA and PGA.

EA and PGA presented less robust differences in gene expression when compared with HC, NA and between each other. This might be due to the presence of similar mechanisms of disease pathogenesis within this compartment but also potentially due to the presence of a PGA subgroup in which type-2 mechanisms have been successfully suppressed by treatment with high doses of ICS that were being used in our population with severe asthma. Other mechanisms related to metabolic pathways, ubiquitination and mitochondrial function,<sup>16</sup> such as those described previously in sputum, were not detected by this transcriptomic study in biopsies, reflective of the differences in composition of these two samples. Some findings from EA and PGA can be highlighted. We identified up-regulation of CLC in severe persistent EA compared to other phenotypes, consistent with previous findings in induced sputum.<sup>12</sup> FKBP5, up-regulated in PGA compared with HC, encodes a chaperone protein previously described as a steroid-response gene and associated with type-2-low.<sup>17,73</sup>

In addition, the lack of activation of pathways of type-2 inflammation, even in those with EA is likely to reflect the cells sampled by endobronchial biopsy, as well as the effect of high-dose corticosteroids presumably reducing the number and activation of eosinophils in the tissue. The identified processes were associated with airway fibrosis and remodelling, processes that are likely to be active in

airway walls and also likely to be steroid-insensitive,<sup>74,75</sup> but clearly demonstrate highly active and ubiquitous pathways in the setting of severe refractory asthma.

SUMOylation is the process by which the Small Ubiquitin-like Modifier (SUMO) proteins undergo post-transcriptional modifications to regulate gene expression, subcellular localization and protein-protein interaction.<sup>76</sup> In asthma, it has been implicated in tissue remodelling,<sup>77,78</sup> fibroblast transformation and innate immunity, especially suppressing type-1 interferon responses.<sup>79-81</sup> Enrichment of the SUMOylation pathway represents a potential key regulator of airway remodelling, and further investigation is warranted to determine the therapeutic potential of this area.

Genes altered from basal cell carcinoma signalling and Wnt/ $\beta$ -catenin pathways included WNT10A, DVL2, SMO, AXIN1, WNT7B and WNT5B and have also been associated with mechanisms of airway remodelling.<sup>82</sup> Treatment with ICS can reduce inflammation, but airway wall remodelling is known to be insensitive to suppression by corticosteroids and largely thought to be an irreversible feature of asthma.<sup>83</sup> The SUMOylation, basal cell signalling and genes from the Wnt/ $\beta$ -catenin pathway are likely mediators of structural changes in the airway. In addition, although endobronchial biopsies only sample the proximal airway, this study has proven to be a useful tool in the identification of mechanisms associated with airway remodelling that might not be detectable when sampling the airway lumen. The ubiquitous dysregulation of these pathways, in all severe asthma, suggests not only their importance of these pathways in the pathogenesis, but also its failure to be controlled even with high doses of ICS.

The exploratory nature of our study has some limitations. It is a single-site cross-sectional study, and it is unclear how these findings would reflect those within the wider population. The stability of the inflammatory phenotypes was not evaluated over time. We did not include participants with mild or moderate asthma, and it remains unclear if the mechanisms observed here reflect disease state specific to the inflammatory profile presented.

We used BLF cell count cut-offs to identify phenotypes. The granulocytic composition of BLF is less studied than induced sputum. There is evidence that supports the BLF eosinophil count to closely resemble that found in induced sputum; however, neutrophil cell counts are less well studied, variable in both sputum and BLF, and it is unclear what should constitute a cut-off for neutrophilic asthma.<sup>84,85</sup> Sample size was limited although comparable to previous transcriptomic studies in severe asthma.<sup>13-15,19</sup> We consider it important to note that this is the first study to perform transcriptomic profiling of inflammatory phenotypes in biopsies to investigate underlying mechanisms associated with these phenotypes. A validation on an independent cohort was not possible due to limited availability of matching BLF or induced sputum cell counts and transcriptomic data from endobronchial biopsies of participants with severe asthma in current publicly available data. It would be interesting to investigate these findings in future larger cohorts, particularly including a greater representation of the neutrophilic and mixed granulocytic asthma group. Our study utilized whole endobronchial

biopsies to sample diverse components of the airway wall, and therefore, the genes and pathways found here reflect this diversity. Future investigation of individual contributions of different cellular components using sequencing technologies is encouraged. Lastly, studying gene expression alone provides a valuable yet limited view of the mechanisms that might be involved. Studies involving other profiling using other 'omics' would enhance our understanding of this disease state.

In summary, improving our understanding of the mechanisms leading to different phenotypes of severe asthma is a key step towards personalized management as well as being crucial for the discovery of new therapies targeting non-type-2 pathways in severe asthma. This study is one of the largest to investigate gene expression and pathways in endobronchial biopsies from participants with different inflammatory phenotypes of severe asthma. Adults with severe asthma had enrichment for SUMOylation, basal cell carcinoma signalling and Wnt/ $\beta$ -catenin pathways associated with both fibrosis and airway remodelling were identified. EA and PGA presented similar transcriptomic profiles, whereas NA had a distinct profile confirming DEGs and pathways previously reported in neutrophilic asthma. We also established novel links between severe NA and genes and pathways; neuroinflammation signalling, the role of NFAT in regulation of the immune response, and PKC $\theta$  signalling in T lymphocytes.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

## AUTHOR CONTRIBUTIONS

All authors designed the study. JLS supervised and coordinated the study. SSO performed the experiments and wrote the manuscript which was further refined and edited by JLS, PABW, KJB and DB. PABW performed the bronchoscopy, KJB supervised the laboratory analysis and DB supervised the statistical analysis. All authors approved the final manuscript for submission.

## ETHICAL STATEMENT

The study was approved by the University of Newcastle (H-163-1205) and Hunter New England Human Research Ethics Committee (05/08/10/3.09).

## DATA AVAILABILITY STATEMENT

The data set supporting the conclusions of this article is available in the Gene Expression Omnibus repository, accession number GSE143303 in [www.ncbi.nlm.nih.gov/projects/geo](http://www.ncbi.nlm.nih.gov/projects/geo).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplementary File 1

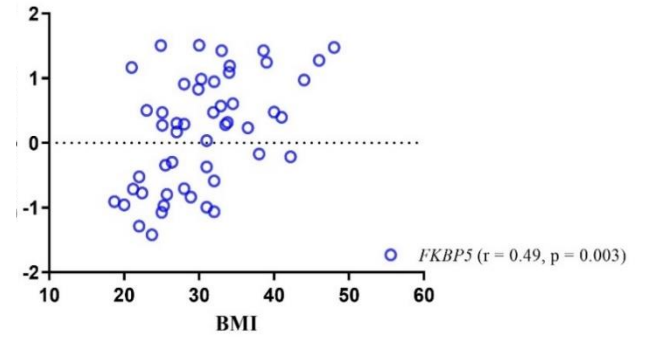
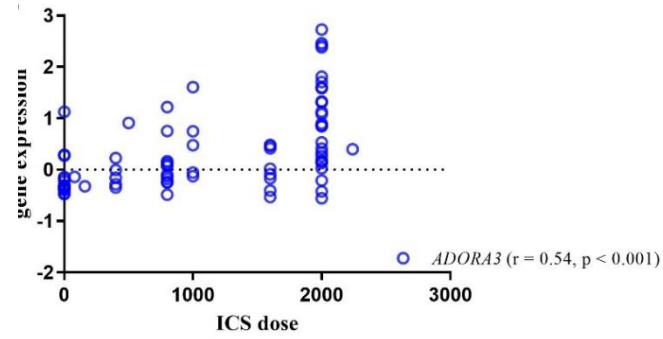
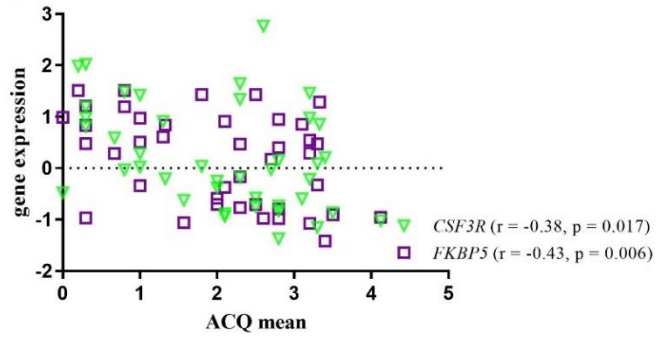
Table S1: Bronchial Lavage Fluid 95<sup>th</sup> percentile of healthy controls

| Variable                                                           | Value                      |
|--------------------------------------------------------------------|----------------------------|
| N                                                                  | 86                         |
| Total cell count x10 <sup>6</sup> /mL, 95 <sup>th</sup> percentile | 0.99 × 10 <sup>6</sup> /mL |
| Viability, 95 <sup>th</sup> percentile                             | 100                        |
| Neutrophils %, 95 <sup>th</sup> percentile                         | 71.75                      |
| Eosinophils %, 95 <sup>th</sup> percentile                         | 3.5                        |

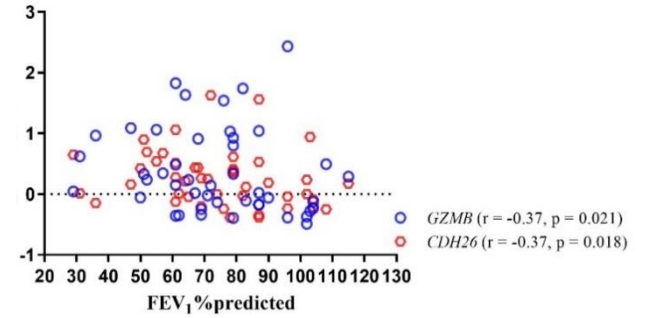
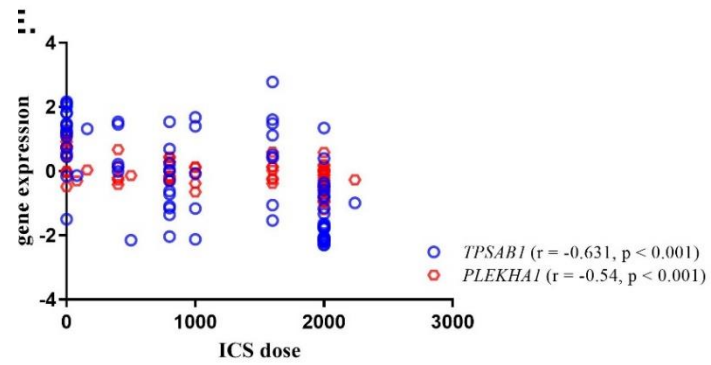
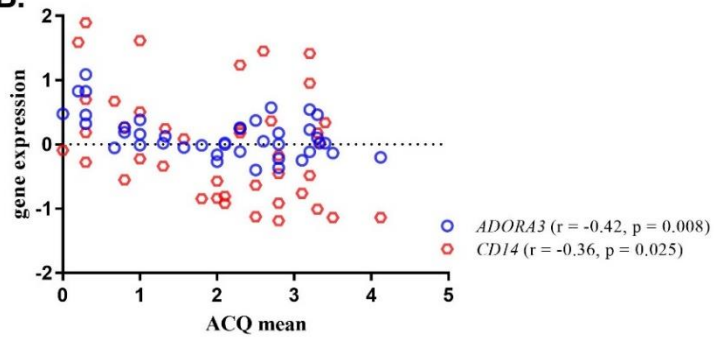
Table S2: Clinical characteristics of severe asthma culture negative compared with severe asthma culture positive

| Characteristic                                         | Severe asthma     |                     | p-value        |
|--------------------------------------------------------|-------------------|---------------------|----------------|
|                                                        | Culture negative  | Culture positive    |                |
| N                                                      | 27                | 20                  | -              |
| Age(years), mean (SD)                                  | 62 (12)           | 54 (14)             | 0.057          |
| Sex, F n (%)                                           | 17 (63)           | 13 (65)             | 0.886          |
| Atopy, n (%)                                           | 12 (44.4)         | 12 (60)             | 0.351          |
| BMI (kg/m <sup>2</sup> ), mean (SD)                    | 31 (n = 22)       | 27.3 (5.6) (n = 13) | 0.098          |
| Age of Onset, n (%)                                    |                   |                     | 0.758          |
| Childhood                                              | 10 (37)           | 9 (45)              |                |
| Adult                                                  | 13 (48)           | 9 (45)              |                |
| Unknown                                                | 4 (15)            | 2 (10)              |                |
| FEV <sub>1</sub> %predicted, mean (SD)                 | 72.7 (20.3)       | 78.3 (23.1)         | 0.390          |
| FEV <sub>1</sub> /FVC, mean (SD)                       | 65.2 (10.7)       | 66.9 (15.2)         | 0.667          |
| Former smoker, n (%)                                   | 4 (15)            | 5 (25)              | 0.380          |
| Pack years, median (Q1, Q3)                            | 7.5 (5, 10)       | 15 (7, 30)          | 0.372          |
| ACQ score, mean (SD)                                   | 2.1 (1)           | 1.8 (1.3)           | 0.423          |
| ICS use, yes n (%)                                     | 27 (100)          | 20 (100)            | 0.410          |
| ICS dose µg/day, median (Q1, Q3)                       | 1600 (800, 2000)  | 2000 (1300, 2000)   | 0.062          |
| OCS use (%)                                            | 4 (15)            | 3 (15)              | 0.986          |
| OCS dose µg/day, median (Q1, Q3)                       | 10 (10, 15)       | 10 (5, 15)          | 0.637          |
| OCS courses, past 12 months, median (Q1, Q3)           | 2 (1, 4)          | 2 (1, 4)            | 0.962          |
| Total cell count x10 <sup>6</sup> /mL, median (Q1, Q3) | 0.1 (0.1, 0.2)    | 0.5 (0.2, 2)        | < <b>0.001</b> |
| Viability, median (Q1, Q3)                             | 62.5 (50, 86.7)   | 86.5 (79, 95)       | <b>0.002</b>   |
| BLF Neutrophils %, median (Q1, Q3)                     | 40.3 (15.3, 62.8) | 56.0 (24.5, 81.5)   | <b>0.010</b>   |
| Eosinophils %, median (Q1, Q3)                         | 2 (0.75, 13.3)    | 2 (1, 4)            | 0.643          |
| Macrophages %, median (Q1, Q3)                         | 29.8 (15, 43.5)   | 20.3 (10.1, 26.8)   | 0.109          |
| Lymphocytes %, median (Q1, Q3)                         | 0.8 (0, 2.3)      | 0.5 (0, 1.9)        | 0.703          |
| Epithelial cells %, median (Q1, Q3)                    | 8.5 (1.8, 24.8)   | 1.1 (0, 4.4)        | <b>0.003</b>   |
| Squamous cells %, median (Q1, Q3)                      | 1.7 (0.3, 3.6)    | 0.3 (0, 2.1)        | 0.120          |

A.



B.



C.

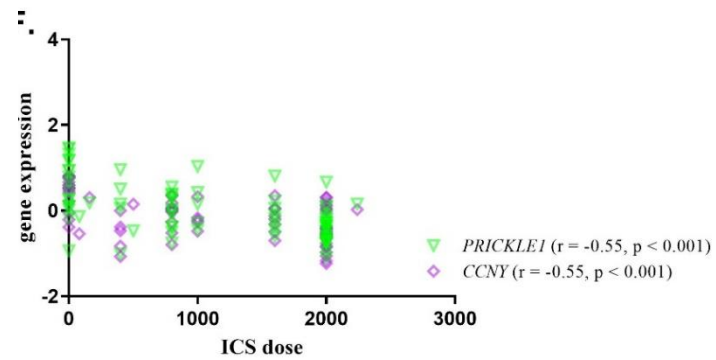
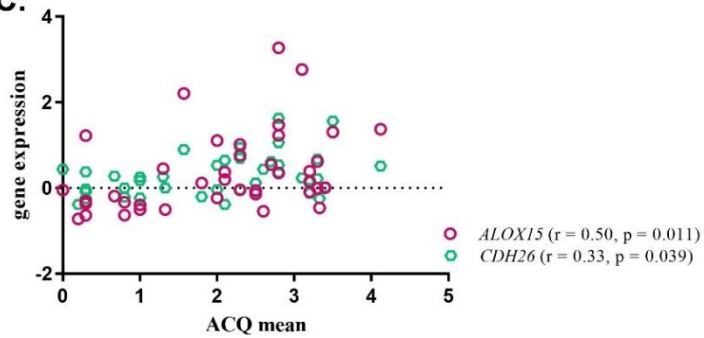


**Figure S1. Scatterplot of correlation between gene expression of top differentially expressed genes and clinical features.** Y-axis presents normalised gene expression while x-axis shows clinical feature. A). and B). Expression of CSF3R, FKBP5, ADORA3, and CD14 negatively correlate with ACQ mean; C). Gene expression of ALOX15, and CDH26 positively correlate with ACQ mean; D). ADORA3 correlates with ICS dose; E). and F). TPSAB1, PLEKHA1, PRICKLE1 and CCNY negatively correlate with ICS dose G). FKBP5 correlates with BMI; H). Expression of GMZB, and CDH26 negatively correlate with FEV1 % predicted. ACQ: asthma control questionnaire, BMI: body mass index.

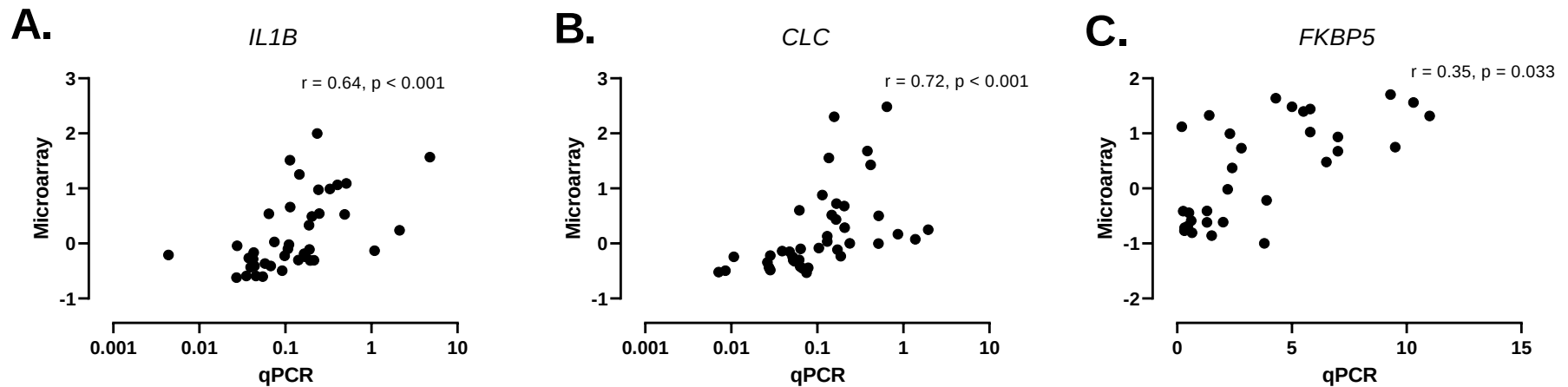


**Figure S2. Validation of transcriptomic data by quantitative polymerase chain reaction for differentially expressed genes *IL1B*, *CLC* and *FKBP5*.** A.-C. Normalized microarray expression correlates with qPCR for highly expressed differentially genes. Matching data was used for genes *IL1B* (n=40), *CLC* (n=42) and *FKBP5* (n=37).