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ORIGINAL ARTICLE

# Sputum transcriptomics implicates increased p38 signalling activity in severe asthma

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## ABSTRACT

*Background and objective:* **Severe asthma is responsible for a disproportionate burden of illness and healthcare costs spent on asthma. This study analyses sputum transcriptomics to investigate the mechanisms and novel treatment targets of severe asthma.**

*Methods:* **Induced sputum samples were collected in a cross-sectional study from participants with severe asthma (*n* = 12, defined as per GINA criteria), non-severe uncontrolled (*n* = 21) and controlled asthma (*n* = 21) and healthy controls (*n* = 15). Sputum RNA was extracted and transcriptomic profiles were generated (Illumina HumanRef-8 V2) and analysed (GeneSpring). Sputum protein lysates were analysed for p38 activation in a validation study (*n* = 24 asthma, *n* = 8 healthy) by western blotting.**

*Results:* **There were 2166 genes differentially expressed between the four groups. In severe asthma, the expression of 1875, 1308 and 563 genes was altered compared to healthy controls, controlled and uncontrolled asthma, respectively. Of the 1875 genes significantly different to healthy controls, 123 were >2-fold change from which four networks were identified. Thirty genes (>2-fold change) were significantly different in severe asthma compared to both controlled asthma and healthy controls. There was enrichment of genes in the p38 signalling pathway that were associated with severe asthma. Phosphorylation of p38 was increased in a subset of severe asthma samples, correlating with neutrophilic airway inflammation.**

*Conclusion:* **Severe asthma is associated with substantial differences in sputum gene expression that underlie unique cellular mechanisms. The p38 signalling pathway may be important in the pathogenesis of severe asthma, and future investigations into p38 inhibition are warranted as a 'non-Th2' therapeutic option.**

## SUMMARY AT A GLANCE

Sputum transcriptomic analysis revealed vast changes in gene expression in severe asthma. There was enrichment of genes in the p38 signalling pathway, four gene networks altered and 30 genes were identified that were highly differentially expressed in severe asthma compared with controlled asthma and healthy controls.

**Key words:** asthma, gene expression, immune system, inflammation, sputum.

## INTRODUCTION

Although most patients with asthma have mild to moderate disease that can be controlled with inhaled corticosteroid (ICS) therapy, 3–10% of patients have severe disease that remains uncontrolled despite maximal treatment.[1,2] Severe asthma is a major healthcare problem and is responsible for a disproportionate share of the healthcare costs associated with asthma. These patients suffer severe impairment of quality of life[3] and increased mortality.[4] Accordingly, current research is directed towards clarifying the definition, mechanisms and phenotypes of severe asthma in the hope of developing a deeper understanding, new diagnostic markers and targeted treatments to improve health outcomes.[5]

There are major challenges in the diagnosis and management of severe asthma.[6] A thorough understanding of the complex and heterogenous mechanisms underlying the pathogenesis of severe asthma is essential to meeting these challenges.[7] Severe asthma has been associated with persistent Th2-driven eosinophilic inflammation[8] that is unresponsive to high-dose ICS treatment, as well as the presence of neutrophilic inflammation.[9] However, the mechanisms of airway inflammation and the relationship to treatment responsiveness are unclear. The use of non-invasive methods paired with 'omics' technologies to identify underlying

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mechanisms and molecular pathways are important in the future treatment of severe asthma, including the identification of novel treatment targets for non-Th2/non-eosinophilic asthma.

We[10–12] and others[13–16] have reported sputum transcriptomics to investigate molecular phenotypes and mechanisms of asthma. This study builds upon our previous investigations to analyse differences in airway gene expression of patients with severe asthma, with a focus on identifying cellular pathways underlying inflammation and/or corticosteroid resistance. We hypothesized that there would be important differences in airway gene expression in severe asthma that would represent activation of inflammatory pathways, and that highly differentially regulated genes may represent severe asthma biomarkers signifying active mechanisms that warrant further investigation as novel treatment targets.

## METHODS

### Participants

This analysis is performed on two cross-sectional study populations. The first is a sputum transcriptomic discovery study ($n$ = 54 asthma, $n$ = 15 healthy controls).[10] The second is a validation study, consisting of severe asthma participants ($n$ = 24) and healthy controls ($n$ = 8). For both studies, adults with stable asthma (no respiratory tract infection, exacerbation or change in maintenance therapy in the past month) were recruited from the John Hunter Hospital Asthma Clinic, NSW, Australia. In the non-severe asthma groups, diagnosis was according to the American Thoracic Society guidelines based upon current (past 12 months) episodic respiratory symptoms, doctor's diagnosis (ever) and demonstrated evidence of airway hyperresponsiveness to hypertonic saline. Healthy control participants were recruited by advertisement, had no previous asthma diagnosis and normal lung function (forced expiratory volume in 1 s ($FEV_1$) %predicted >80, $FEV_1$/forced vital capacity (FVC) > 70). All participants gave written informed consent prior to their inclusion in the study and the Hunter New England Area Health Service (09/12/16/5.03 and 08/08/20/3.10) and the University of Newcastle Research Ethics Committees (H-2010-0044 and H-2009-0013) approved this study.

### Severe asthma characterization

Severe asthma was defined using the Global Initiative for Asthma (GINA) criteria,[17] by the presence of poor asthma control (Juniper Asthma Control Questionnaire (ACQ) score ≥ 1) or airflow limitation ($FEV_1$ % predicted < 80, $FEV_1$/FVC% < 70) despite treatment with high-dose ICS (≥1000 inhaled beclomethasone equivalent per day) along with long-acting β-agonist (LABA). Non-severe asthma patients used <1000 μg of inhaled beclomethasone equivalent per day and either had ACQ ≥ 1 (non-severe uncontrolled asthma group) or an ACQ < 1 (non-severe controlled asthma group).

### Sputum induction and analysis

Detailed methods for sputum collection and analysis were performed as previously described[18,19] and are provided in the Methods section of Appendix S1 (Supplementary Information).

### Sputum transcriptomics

Transcriptomic data were generated as previously described.[10] Briefly, sputum RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed into cRNA and biotin-UTP labelled using the Illumina TotalPrep RNA Amplification Kit (Ambion, Thermo Fisher Scientific, Scoresby, VIC, Australia) and hybridized to the Illumina Sentrix HumanRef-8 Version 2 Expression BeadChips (Illumina, San Diego, CA, USA).

### Western blotting and densitometry

For detailed methods, see Appendix S1 (Supplementary Information). Briefly, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using sputum protein (20 μg/sample, sputum protein extraction details provided in Appendix S1 in Supplementary Information) along with positive and negative controls for p38 phosphorylation (Cell Signalling Technologies, Danvers, MA, USA). The proteins were transferred onto a nitrocellulose membrane and western blots were run to detect phosphorylated p38 with β-actin used as a control. Data were expressed as densitometry-measured ratio of phospho-p38 to β-actin and the internal calibrator.

### Statistical analysis

Clinical and cell count data were analysed using Stata 15 (Stata Corporation, College Station, TX, USA) and reported as mean (SD) for normally distributed data and median (Q1, Q3) for non-parametric data. Statistical comparisons were performed using the multiple-comparison analysis of variance (ANOVA) for parametric data and the Kruskal–Wallis test for non-parametric data, $P < 0.05$ was considered significant. Details of transcriptomic data analysis are provided in detail Appendix S1 in Supplementary Information, and the data are available through GEO (Gene Expression Omnibus, accession number GSE137268). The differentially expressed genes and pathways that we found associated with severe asthma were further validated by investigating their differences in the U-BIOPRED data set GSE76262,[15] consisting of participants with severe asthma (SA, $n$ = 93), moderate asthma (MA, $n$ = 25) and healthy controls (HC, $n$ = 21).

## RESULTS

### Clinical features and inflammatory cells

Clinical details and inflammatory cell counts for the study participants are shown in Table 1. Out of 54 participants with asthma recruited in Study 1, 12 (22%) had severe asthma, 21 (39%) had non-severe uncontrolled asthma and 21 (39%) had non-severe controlled asthma. Participants with severe asthma were all

**Table 1** Clinical characteristics and sputum cell counts of inflammatory phenotypes of asthma

| | | Non-severe asthma | | | |
|---|---|---|---|---|---|
| | Severe asthma | Uncontrolled | Controlled | Healthy controls | *P*-value |
| *n* | 12 | 21 | 21 | 15 | |
| Age years, mean (SD) | 64 (13) | 56 (16) | 57 (13) | 45 (22) | 0.027 |
| Gender M\|F | 5\|7 | 12\|9 | 8\|13 | 7\|8 | 0.676 |
| Atopy, *n* (%) | 12 (100)[†] | 16 (76) | 10 (48) | 7 (47) | 0.003 |
| $FEV_1$ % predicted, mean (SD) | 63 (14)[‡§] | 64 (15)[‡§] | 92 (13) | 101 (13) | <0.001 |
| $FEV_1$/FVC %, mean (SD) | 64 (9)[‡] | 62 (9)[‡§] | 74 (8) | 79 (8) | <0.001 |
| BMI, mean (SD) | 31.7 (4.7) | 28.8 (6.1) | 32.6 (8.9) | 25.7 (4.2) | 0.018 |
| Exhaled nitric oxide (ppb), median (Q1, Q3) | 33.5 (16.7, 38.5) | 23.2 (13.6, 52.4) | 20.5 (13.4, 29.2) | 15.2 (12.5, 19.5) | 0.196 |
| Smoking, ex\|never | 6\|6 | 12\|9 | 8\|13 | 4\|11 | 0.286 |
| Pack years, median (Q1, Q3) | 16.5 (4.4, 25) | 8.8 (2.9, 26) | 5.4 (2.0, 14.5) | 23.9 (5.9, 41.9) | 0.689 |
| ACQ7 score, median (Q1, Q3) | 2.0 (1.1, 2.5)[§] | 1.4 (0.9, 1.7)[§] | 0.4 (0.3, 0.9) | N/A | <0.001 |
| ICS use, yes \| no | 12\|0[‡] | 11\|10 | 13\|8 | N/A | 0.011 |
| ICS dose (μg daily BDP equivalent) median (Q1, Q3) | 2000[¶††] (1000, 2000) | 400 (200, 1000) | 500 (400, 1600) | N/A | 0.003 |
| Total cell count × $10^6$/mL, median (Q1, Q3) | 5.4 (2.7, 10.8) | 3.2 (2.0, 4.1) | 2.9 (2.3, 5.5) | 3.7 (1.9, 4.8) | 0.267 |
| Neutrophils, %, median (Q1, Q3) | 65.1 (28.9, 72.3) | 41.0 (16.0, 64.0) | 46.0 (36.5, 66.5) | 28.4 (10.5, 44.7) | 0.081 |
| Eosinophils, % median (Q1, Q3) | 6.5 (0.8, 30.5)[‡‡] | 4.5 (0.8, 13.5)[‡‡] | 0.5 (0.0,3.3) | 0.4 (0.0, 0.8) | 0.007 |
| Inflammatory phenotype, *n* (%) | | | | | 0.228 |
| EA | 4 (33) | 9 (43) | 4 (19) | — | |
| NA | 3 (25) | 4 (19) | 4 (19) | — | |
| PGA | 1 (8) | 5 (24) | 10 (48) | — | |
| MGA | 4 (33) | 3 (14) | 3 (14) | — | |

[†]Fishers exact test $P < 0.013$ versus healthy and non-severe controlled asthma.
[‡]Bonferroni post hoc $P < 0.013$ versus healthy.
[§]$P < 0.013$ versus non-severe controlled asthma.
[¶]$P < 0.008$ versus non-severe controlled asthma.
[††]$P < 0.008$ versus non-severe uncontrolled asthma.
[‡‡]Kwallis2 $P < 0.008$ versus healthy.

ACQ, Asthma Control Questionnaire; BDP, Beclometasone dipropionate; BMI, body mass index; EA, eosinophilic asthma; $FEV_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroid; MGA, mixed granulocytic asthma; NA, neutrophilic asthma; PGA, paucigranulocytic asthma.

atopic, compared to 48% of controlled asthma and 47% of the healthy controls. Severe asthma participants had lower lung function, higher ACQ score and used more ICS. Participants with uncontrolled asthma also had lower lung function and higher ACQ score compared with controlled asthma. Increased sputum eosinophils were observed in severe and uncontrolled asthma.

## Differential gene expression in severe asthma

In sputum transcriptomic analysis, 2166 entities were differentially expressed between the four groups. In severe asthma, the expression of 1875 entities was altered compared to healthy controls. Pathway analysis showed that genes in the p38 signalling ($P$ = 0.005, 8/35 (22.9%) genes altered, Table S1 in Supplementary Information), EGFR1 (epidermal growth factor receptor 1; $P$ = 0.001, 27/176 (15.3%) genes altered) and TNF-α/NF-κB (tumour necrosis factor-α/nuclear factor-κB, $P$ = 0.007, 26/188 (13.8%) genes altered) pathways were overrepresented in severe asthma. There were 123 genes that were highly differentially expressed by >2-fold change (96 upregulated and 27 downregulated in severe asthma). Four gene networks among these 123 genes were identified from known protein–protein interactions (Fig. 1).

In severe asthma, the expression of 1308 entities were altered compared to controlled asthma. Pathways analysis showed that genes in the TGFBR (transforming growth factor β receptor, $P$ = 0.007, 16/137 (11.7%) genes altered) pathway were significantly overrepresented. Although not quite reaching a more stringent $P$-value cut off of 0.01, genes in the p38 signalling pathway were also overrepresented ($P$ = 0.043, 5/35 (14.2%), Table S1 in Supplementary Information). Of the 1308 entities, 35 were highly differentially expressed by >2-fold change, and 32 (91%, corresponding to 30 genes) of these were also highly differentially expressed compared to healthy controls (Table 2, Fig. 2).

When severe asthma was compared with uncontrolled asthma, there were 563 altered entities; however, there were no significant pathways and no genes were >2-fold different. Although not reaching significance, several genes in the p38 pathway were also altered ($P$ = 0.051; 3/35 (8.6%), Table S1 in Supplementary Information).

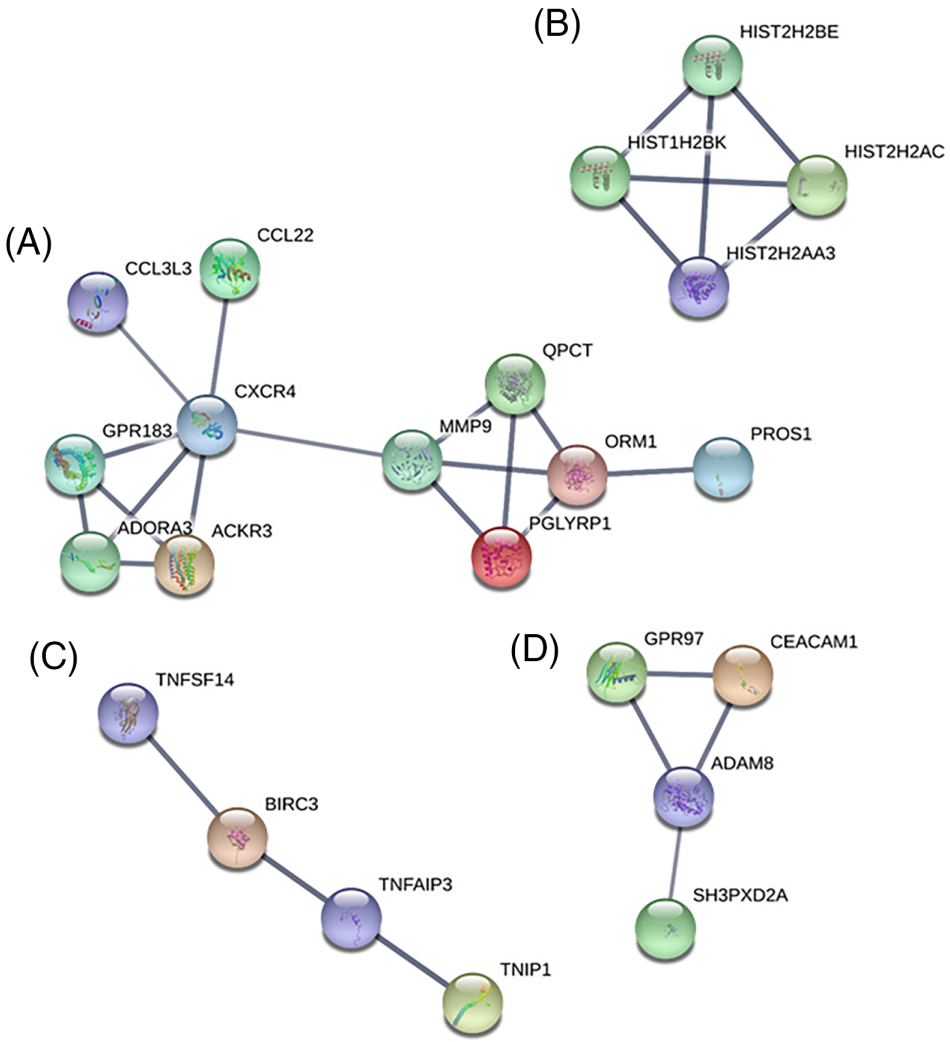


**Figure 1** We identified four networks (A–D), mapping protein–protein interactions among differentially expressed genes (adjusted $P < 0.05$ and >2-fold change) in severe asthma versus healthy controls. Network A is the largest containing 11 genes, with CXCR4 as the key node with six connections. Network B contains four members of histone cluster 2A/2B genes. Network C contains four tumour necrosis factor/nuclear factor-κB signalling molecules and network D contains four immune-related molecules involved in cell–cell interactions.

## Differential gene expression in non-severe asthma

In uncontrolled asthma, 59 genes were differentially expressed compared to controlled asthma, with 0 genes by >2-fold change. In uncontrolled asthma compared with healthy controls, there were 458 genes significantly differentially expressed, with nine genes that were >2-fold upregulated (Table S2 in Supplementary Information). Pathway analysis showed genes in the EGFR1 pathway were overrepresented ($P < 0.001$; 13/176 (7.4%)). In controlled asthma compared with healthy controls, there were 494 genes that were significantly differentially expressed, with five genes that were >2-fold change (four upregulated (*VNN2*, *HIST2HAA3*, *CXCR4* and *CLC*, Table S2 in Supplementary Information) and one downregulated (nuclear receptor subfamily 4, group A, member 3, *NR4A3*)).

## Validation of differentially expressed genes and pathways in the U-BIOPRED cohort

We further investigated these changes in the data set GSE76262, where we confirmed alterations in the p38 signalling ($P = 0.002$, 17/35 (49%) genes altered), EGFR1 ($P = 3.8 \times 10^{-6}$, 71/176 (40%) genes altered), TNF-α/NF-κB ($P = 6.0 \times 10^{-17}$, 100/188 (53%) genes altered) and TGFBR ($P = 2.7 \times 10^{-10}$, 68/137 (50%) genes altered) pathways in severe asthma compared to healthy controls. In severe asthma compared with moderate asthma, the p38 signalling ($P = 0.007$, 14/35 (49%) genes altered), EGFR1 ($P = 6.6 \times 10^{-7}$, 64/176 (36%) genes altered), TNF-α/NF-κB ($P = 1.9 \times 10^{-9}$, 74/188 (39%) genes altered) and TGFBR ($P = 3.3 \times 10^{-8}$, 56/137 (41%) genes altered) pathways were also altered.

For the 30 highly differentially expressed genes we identified (Table 2, Fig. 2), all but 2 of these genes were also found to be significantly differentially expressed in the U-BIOPRED data set, being regulated in the same direction (Table 2).

## Sputum p38 activation

Owing to the differences in expression of genes in the p38 pathway in severe asthma compared with our other asthma and healthy control groups, we sort to further investigate and validate p38 activity (protein phosphorylation) in severe asthma. We performed western blotting on sputum protein lysates from participants with severe asthma ($n = 24$; Table 3) and healthy

**Table 2** Highly differentially expressed genes in the airways of subjects with severe asthma

| | | | Newcastle data (GSE137268) | | U-BIOPRED data (GSE76262) | |
|---|---|---|---|---|---|---|
| | | | Fold change | | | |
| Gene symbol | Gene name | Accession | SA vs HC | SA vs CA | SA vs HC | SA vs MA |
| Upregulation | | | | | | |
| CLC | Charcot–Leydon crystal protein | NM_001828.4 | +8.5 | +2.9 | +6.1 | +3.6 |
| TNFSF14 | Tumour necrosis factor (ligand) superfamily member 14 | NM_003807.2 | +3.9 | +2.6 | +1.6 | +1.7 |
| SPINK1 | Serine peptidase inhibitor, Kazal type 1 | NM_003122.2 | +3.6 | +2.4 | +2.3 | +2.0 |
| PADI4 | Peptidyl arginine deiminase type IV | NM_012387.1 | +3.4 | +2.4 | +1.7 | +1.6 |
| OLIG2 | Oligodendrocyte transcription factor 1 | NM_138983.1 | +3.4 | +2.2 | +1.8 | +1.8 |
| FFAR3 | Free fatty acid receptor 3 | NM_005304.2 | +3.3 | +2.4 | +1.9 | +1.7 |
| GPR97 | G protein coupled receptor 97 | NM_170776.3 | +3.3 | +2.2 | +2.5 | +2.5 |
| MARCKSL | MARCKS-like 1 | NM_023009.4 | +3.2 | +2.1 | +1.7 | +1.7 |
| OLIG1 | Oligodendrocyte transcription factor 1 | NM_138983.1 | +3.1 | +2.3 | +1.8 | +1.6 |
| MMP9 | Matrix metalloproteinase 9 | NM_004994.2 | +3.0 | +2.9 | +1.8 | +1.5 |
| HES4 | Hairy and enhancer of split 4 | NM_021170.2 | +3.0 | +2.5 | +1.3 | +1.2 |
| CEBPE | CCAAT/enhancer binding protein (C/EBP) epsilon | NM_001805.2 | +2.9 | +2.1 | +1.4 | +1.4 |
| ADAM8 | ADAM metalloproteinase domain 8 | NM_001109.3 | +2.8 | +2.6 | +2.1 | +1.8 |
| CAMK1 | Calcium/calmodulin-dependent protein kinase I | NM_003656.3 | +2.7 | +2.1 | +1.7 | +1.3 |
| RASSF4 | Ras association (RalGDS/AF-6) domain family member 2 | NM_170774.1 | +2.7 | +2.2 | +2.0 | +1.8 |
| PGLYRP1 | Peptidoglycan recognition protein | NM_005091.1 | +2.6 | +2.1 | +1.2 | +1.2 |
| MBOAT7 | Membrane bound O-acyltransferase domain containing 7 | NM_024298.2 | +2.4 | +2.1 | +1.5 | +1.5 |
| MUCL1 | Mucin-like 1 | NM_058173.2 | +2.4 | +2.0 | NS | NS |
| PRR7 | Proline rich 7 | NM_030567.3 | +2.4 | +2.1 | +1.2 | +1.3 |
| DDIT4 | DNA damage inducible transcript 4 | NM_019058.2 | +2.3 | +2.2 | +2.1 | +1.9 |
| ATG2A | ATG2 autophagy related homologue A | NM_015104.1 | +2.3 | +2.0 | +2.1 | +1.5 |
| PIM2 | Pim-2 oncogene | NM_006875.2 | +2.2 | +2.0 | +1.5 | +1.4 |
| PTPN7 | Protein tyrosine phophatase, non-receptor type 7 | NM_080588.1 | +2.2 | +2.0 | +1.6 | +1.4 |
| RNF19B | Ring finger protein 19B | NM_153341.1 | +2.1 | +2.0 | +1.9 | +1.5 |
| Downregulation | | | | | | |
| IGFBP2 | Insulin-like growth factor binding protein 2 | NM_000597.2 | −2.7 | −2.0 | NS | NS |
| SLC47A1 | Solute carrier family 47, member 1 | NM_018242.2 | −2.5 | −2.0 | −3.0 | −2.8 |
| MSR1 | Macrophage scavenger receptor 1 | NM_002445.3 | −2.2 | −2.0 | −2.6 | −2.4 |
| COLEC12 | Collectin sub-family member 12 | NM_130386.1 | −2.2 | −2.1 | −2.5 | −2.4 |
| ALDH2 | Aldehyde dehydrogenase 2 | NM_000690.2 | −2.1 | −2.0 | −2.2 | −1.9 |
| PCOLCE2 | Procollagen C-endopeptidase enhancer 2 | NM_013363.2 | −2.0 | −2.2 | −2.7 | −2.2 |

CA, controlled asthma; HC, healthy control; MA, moderate asthma; SA, severe asthma.

controls ($n$ = 8; Table 3). Phosphorylation of p38 was not significantly increased in severe asthma compared to healthy controls (Fig. 3A). However, p38 activation was significantly increased in neutrophilic severe asthma compared to all other groups (Fig. 3B,C), and correlated with proportion of sputum neutrophils (Fig. 3D).

## DISCUSSION

This study demonstrates the vast changes in sputum gene expression present in severe asthma, with expression of genes belonging to the p38 signalling, TNF-α/NF-κB and EGFR1 pathways enriched in severe asthma compared with both controlled asthma and healthy controls. Moreover, we have identified four networks active in severe asthma and 30 highly differentially regulated genes in severe asthma compared to non-severe controlled asthma and healthy controls. Further investigation of p38 activity in sputum protein lysates found that increased phosphorylation of p38 was associated with neutrophilic airway inflammation in severe asthma. This study implicates p38 activity in severe asthma compared with neutrophilic inflammation, and thus p38 inhibition is a potential treatment target for this severe asthma phenotype.

 

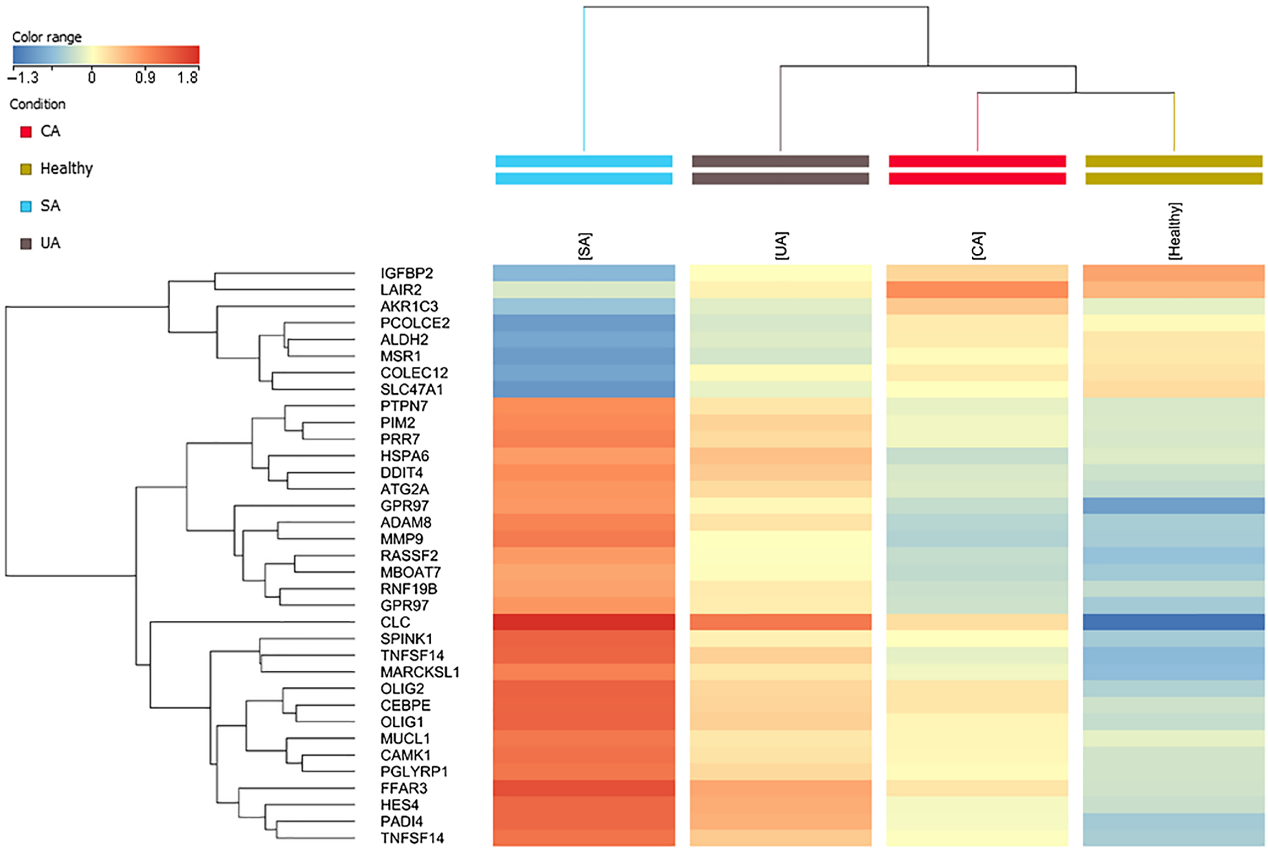


**Figure 2** Hierarchical clustering of average transcriptomic profiles across the four severity groups (CA, controlled asthma; HC, healthy control; SA, severe asthma; UA, uncontrolled asthma) and the 32 differentially expressed entities between severe asthma versus controlled asthma and healthy controls. Red represents a higher level of gene expression and blue represents a lower level of gene expression.

The use of high-throughput 'omics' technologies has gained much interest of late, with systems biology and clustering approaches being employed for biomarker discovery in programmes such as U-BIOPRED.[20] We[10] and others[15,16] have used sputum transcriptomics to identify phenotypes of asthma that included both Th2-driven eosinophilic and non-Th2 phenotypes, either featuring neutrophilic (featuring IL-1/TNF-α/inflammasome-driven inflammation) or non-inflammatory paucigranulocytic patterns (including metabolic pathways). Our sputum transcriptomic study lead to the development of a six-gene expression signature as a biomarker for identifying asthma inflammatory phenotypes, corticosteroid treatment responsiveness[11,21] and future exacerbations.[22]

Sputum transcriptomic analysis of severe asthma has been a focus of the U-BIOPRED programme, which has reported three types of analysis strategies so far. The first performed clustering on clinical characteristics to define phenotypes, and subsequently analysed differential gene expression,[23] and although this proved a robust approach in terms of clinical definitive phenotypes, relatively few differentially expressed genes were identified, which limits further broadening of mechanistic pathways. A second strategy performed hierarchical clustering on genes associated with eosinophilic inflammation whereby three transcriptional associated clusters (TAC) were identified,[15] and gene expression differences were associated with inflammatory phenotypes; however, this approach did not identify genes that are specific to asthma severity. A third strategy employed Gene Set Variation Analysis (GSVA) to identify enriched mechanisms and pathways.[13,14] These studies represent a strong advancement in the transcriptomic field of asthma research; however, to date, they have not directly reported genes dysregulated in severe asthma, nor what molecular pathways underlie those distinct changes.

By reporting vast changes in the gene expression profiles and upregulation of related pathways, we can potentially identify novel treatment targets and potential biomarkers for severe asthma. We have identified 30 genes that were highly differentially expressed in severe asthma compared with both controlled asthma and healthy controls. The most upregulated gene was Charcot–Leydon crystal (CLC), previously shown to be a strong marker of eosinophilic inflammation,[11] which likely represents the elevated sputum eosinophils in the severe asthma group. Genes involved in granulocyte function and development were also altered, including *PADI4*, *ADAM8*, *MMP9* and *CEBPE*. Multiple pathways were overrepresented in the differentially expressed genes, and of particular interest we followed up our observation of transcriptomic p38 signalling changes with direct measurement of p38 activation in a

 

**Table 3** Clinical characteristics and sputum cell counts of severe asthma participants for p38 investigations

| | Severe asthma | Healthy controls | *P*-value |
|---|---|---|---|
| *n* | 24 | 8 | |
| Age years, mean (SD) | 58 (15) | 40 (20) | 0.014 |
| Gender M\|F | 11\|13 | 3\|5 | 0.504 |
| Post β2 $FEV_1$ % predicted, mean (SD) | 72 (22) | 103 (9) | <0.001 |
| Post β2 $FEV_1$/FVC %, mean (SD) | 67 (12) | 83 (8) | 0.003 |
| BMI, mean (SD) | 32.3 (7.5) | 27.3 (5.6) | 0.098 |
| Smoking, current\|ex\|never | 2\|13\|9 | 0\|1\|7 | 0.055 |
| Pack years, median (Q1, Q3) | 11.0 (0.8, 18.0) | 0.0 (0.0, 0.0) | 0.074 |
| ACQ7 score, median (Q1, Q3) | 2.5 (1.3, 3.1) | — | |
| ICS use, yes: *n* (%) | 24 (100%) | — | |
| ICS dose (μg daily BDP equivalent) median (Q1, Q3) | 2000 (2000, 2000) | — | |
| Total cell count × $10^6$/mL, median (Q1, Q3) | 8.6 (3.8, 13.0) | 3.8 (2.5, 4.4) | 0.092 |
| Neutrophils, %, median (Q1, Q3) | 48.0 (18.6, 60.3) | 21.3 (8.0, 49.3) | 0.076 |
| Eosinophils, % median (Q1, Q3) | 1.3 (0.3, 17.6) | 0.8 (0.5, 1.0) | 0.265 |
| Inflammatory phenotype | | | |
| EA, *n* (%) | 10 (41.7%) | — | |
| NA, *n* (%) | 6(25%) | — | |
| PGA, *n* (%) | 8(33.3%) | — | |

BDP, Beclometasone dipropionate; BMI, body mass index; EA, eosinophilic asthma; $FEV_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroid; NA, neutrophilic asthma; PGA, paucigranulocytic asthma.

secondary substudy, strengthening our findings. Of note, there were relatively fewer transcriptomic differences between severe asthma and non-severe uncontrolled asthma, with those changes also being of lesser magnitude; however, similar trends exist in terms of pathways that were altered, including the p38 signalling pathway.

Consistent with our findings of the association of p38 signalling in severe asthma, p38 activity has been shown to be increased in peripheral blood mononuclear cells (PBMC) and alveolar macrophages (AM) from subjects with severe asthma.[24–26] Relative corticosteroid resistance of pro-inflammatory signalling in peripheral blood monocytes and AM correlates with clinical corticosteroid resistance in asthma.[24,26,27] Airway dysbiosis, via endotoxin-activated TLR4 signalling, may drive p38 activation, production of neutrophil chemokine IL-8 and treatment-refractory pro-inflammatory signalling in AM in corticosteroid-resistant asthma.[25] Altered cellular response to bacterial ligands likely underlie enhanced p38 activation in severe asthma, as AM from severe asthmatic patients show significantly reduced induction of the p38 inhibitory phosphatase MKP1 compared to AM from healthy and non-severe asthma patients.[24] Importantly, inhibitors of p38 activity restore corticosteroid sensitivity in severe asthma AM and PBMC.[28–30] Our analysis indicated that p38 activation was elevated in severe asthma associated with neutrophilic airway inflammation. Thus, we hypothesize that enhanced p38 activation may contribute to steroid-resistant inflammation and continuing recruitment of neutrophils to the airways in severe asthma.

As p38 activation specifically correlated with neutrophil proportion in severe asthma sputum samples, it is possible that neutrophils may be a major cellular source of increased phospho-p38 signal, or alternatively, neutrophil recruitment is a result of p38 activation. p38 Promotes a number of important facets of neutrophil function, including migration towards chemotactic signals and oxidative burst.[31,32] p38 Inhibitors are being investigated in clinical trials for neutrophilic diseases and inhaled p38 inhibitors are of particular interest in neutrophilic airway diseases including COPD and severe asthma.[33] Increased p38 phosphorylation has also been reported in bronchial epithelium of asthma patients, particularly in severe asthma, compared to healthy controls.[34,35] In the epithelium, p38 can be activated in response to stresses such as mechanical stretch and inflammatory cytokines including TNF-α, and p38 activity is linked to production of IL-8 and neutrophil recruitment to the airways.[34,36]

Although differences for sputum gene expression in non-severe asthma versus healthy controls were smaller than that of severe asthma, there remained some key changes. Nine genes were significantly differentially expressed in non-severe uncontrolled asthma versus healthy controls, and four of these (*CLC*, *HIST2H2AA3*, *CXCR4* and *VNN2*) were also upregulated in non-severe controlled asthma compared to healthy controls. This suggests that these genes may be potential biomarkers for distinguishing severities of asthma. Interestingly, *CXCR4* was a key node in the main gene network found in severe asthma, suggesting it may play a central role in pathogenesis.

However, this study does have its limitations, we have a small sample size and transcriptomic data are generated from RNA extracted whole sputum plugs, thereby containing a mix of airway inflammatory cells. In many ways, this can be considered a strength, as this is a truer representation of the multicellular interactions and pathway activations that occur in vivo, and thus increases the feasibility of this sample type for future diagnostics. The transcriptomic data are also

 

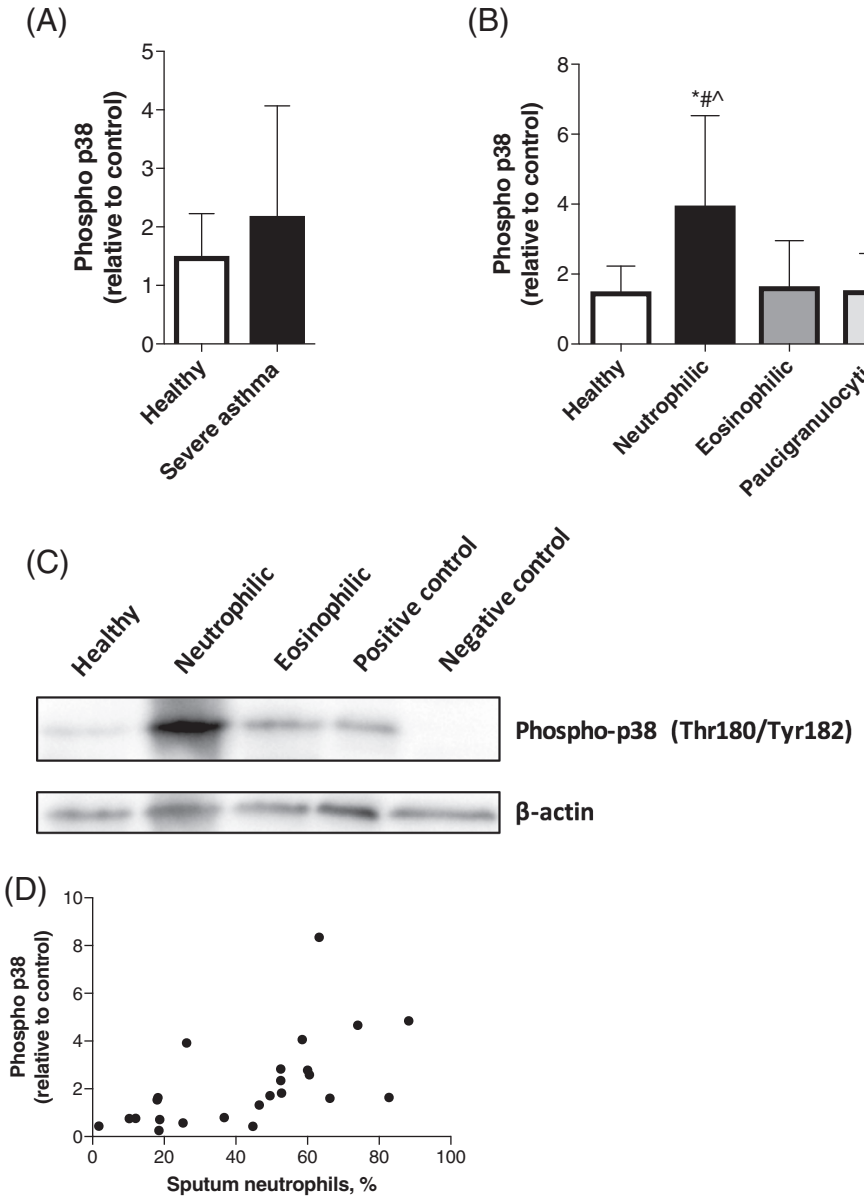


**Figure 3** p38 Activation in severe asthma is associated with neutrophilic inflammation. (A) Densitometry-quantified phospho-p38 normalized to β-actin loading control in sputum protein lysates from participants with severe asthma ($n = 24$) and healthy controls ($n = 8$). (B) Comparison of normalized sputum phospho-p38 levels in healthy controls with airway inflammatory phenotypes of severe asthma (neutrophilic $n = 6$, eosinophilic $n = 10$, paucigranulocytic $n = 8$). (C) Representative western blotting image for phospho-p38 and β-actin in sputum lysates, with positive and negative cell lysate controls for p38 activation. (D) Spearman correlation of normalized phospho-p38 western blot signal versus sputum neutrophil proportion ($r = 0.721$, $P < 0.001$). $*P < 0.05$ neutrophilic asthma versus healthy control, $\#P < 0.05$ neutrophilic asthma versus eosinophilic asthma and $^{\wedge}P < 0.05$ neutrophilic asthma versus paucigranulocytic asthma.

generated from the Illumina HumanRef-8 platform, with this technique now being superseded by RNA-seq methodologies that allow more in-depth data collection. With the smaller sample size, we were not able to investigate phenotypes of severe asthma in detail, but rather generalized differences associated with the severity of disease, and alterations of networks and pathways regardless of underlying phenotype. Our validation study did further confirm elevated p38 activity in the neutrophilic severe asthma, and therefore future studies should take underlying phenotypes into account.

In conclusion, severe asthma is associated with substantial differences in sputum gene expression that relate to unique cellular mechanisms. The p38 signalling pathway may be important in the pathogenesis of neutrophilic severe asthma, and future investigation into p38 inhibition for severe asthma as a 'non-Th2' therapeutic option is warranted, whereby a subgroup of patients with neutrophilic inflammation may have the potential for the greatest benefit.

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**Abbreviations:** ACQ, Asthma Control Questionnaire; AM, alveolar macrophage; BDP, Beclometasone dipropionate; CA, controlled asthma; CLC, Charcot–Leydon crystal; COPD, chronic obstructive pulmonary disease; EA, eosinophilic asthma; EGFR1, epidermal growth factor receptor 1; $FEV_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; GINA, Global Initiative for Asthma; HC, healthy control; ICS, inhaled corticosteroid; IL, interleukin; MA, moderate asthma; NA, neutrophilic asthma; NF-κB, nuclear factor-κB; PBMC, peripheral blood mononuclear cell; PGA, paucigranulocytic asthma; SA, severe asthma; TGFBR, transforming growth factor β receptor.

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## Supplementary Information

Additional supplementary information can be accessed via the *html* version of this article at the publisher's website.

**Appendix S1** Methods.
**Table S1** Genes significantly altered in severe asthma in the p38 pathway.
**Table S2** Highly differentially expressed genes in the airways of subjects with uncontrolled asthma.