# Identification of Biomarkers and Pathogenesis in Severe Asthma by Co-expression Network Analysis

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# Abstract

Background

Severe asthma is a heterogeneous inflammatory disease. The rise of precise immunotherapy for severe asthmatics underlines more understanding of molecular mechanisms and biomarkers. In this study, we aim to identify underlying mechanisms and hub genes that define asthma severity.

Methods

Differentially expressed genes were screened out based on bronchial epithelial brushings from mild and severe asthmatics. Then, the weighted gene co-expression network analysis was adopted to identify gene networks and the most significant module associated with asthma severity. Meanwhile, hub genes screening and functional enrichment analysis was performed. Receiver operating characteristic was conducted to validate the hub genes.

Results

Weighted gene co-expression network analysis identified 6 modules associated with asthma severity. Three modules were positively correlated ($P < 0.001$) with asthma severity, containing genes upregulated in severe asthmatics. Functional enrichment analysis found genes in the highlighted module mainly enriched in neutrophil degranulation and activation, leukocyte migration and chemotaxis. Hub genes identified in the module were CXCR1, CXCR2, CCR1, CCR7, TLR2, FPR1, FCGR3B, FCGR2A, ITGAM, and PLEK. Combining these hub genes possessed a moderate ability for discriminating between severe asthmatics and mild-moderate asthmatics with an area under the curve of 0.75.

Conclusion

Our results identified biomarkers and potential pathogenesis of severe asthma, which provides sight into treatment targets and prognostic markers.


# Introduction

Asthma is a chronic, heterogeneous inflammatory disease with complex pathological mechanisms and diverse clinical phenotypes. Severe asthma is one of the phenotypes, which is defined as uncontrolled asthma despite adherence to maximal optimized therapy and asthma worsens when high-dose treatment is decreased [1]. Patients with severe asthma attempt to achieve control and prevent life-threatening exacerbations with high doses of inhaled corticosteroids or even oral corticosteroids [2], while at a 3.1 fold higher risk of developing osteoporotic fracture and a 2.7 fold higher risk of pneumonia [3]. Furthermore, corticosteroids-resistance is common in severe asthma patients, making corticosteroid therapy less effective [4, 5]. In consideration of side effects and limitations of traditional therapies, novel treatments surrounding immunity were developed. Nevertheless, early attempts at immunosuppressive

therapies have been unsuccessful [2], underlining a comprehensive understanding of molecular mechanism and endotypes of severe asthma.

Weighted gene co-expression network analysis (WGCNA) is a bioinformatics method for exploring the complex relationships between gene expression profiles and phenotypes. It is widely used in studies of multigene diseases to identify potential biomarkers and provide molecular targets for treatment. Some researchers also applied this method to explore pathogenesis of asthma and demonstrate pathways and genes associated with asthma severity [6-8]. Nevertheless, differentially expressed genes (DEGs) between healthy controls and asthmatics or genes from all asthmatics were considered to construct a co-expression network in studies mentioned above, rather than DEGs between mild and severe asthmatics. Analysis of DEGs from mild-severe asthmatics could identify genes especially contributing to disease progression. In this study, such genes were considered for WGCNA and further biologically functional analysis to define hidden mechanisms and key genes in severe asthmatics. The results would shed light on treatment targets and prognosis assessment of severe asthma.

# Materials And Methods

Data processing and differential gene expression analysis

Dataset related to severe asthma was obtained from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) with accessing number GSE89809. It consisted of healthy controls and asthma patients varying from mild to severe with different tissue types. As bronchial epithelial cells are thought to be highly informative in describing changes in gene expression in asthma [9, 10], data of epithelial brushings from mild and severe asthmatics were extracted for WGCNA. In total, 14 mild asthmatics and 11 severe asthmatics were included. Raw microarray gene expression data were normalized using RMA in R and subjected to several quality control procedures. Then gene IDs were respectively mapped to the microarray probes using annotation information. Probes matching more than one gene were eliminated from the dataset, and the mean expression value for genes measured by multiple probes was calculated. Differentially expressed genes (DEGs) in severe asthmatics compared to mild asthmatics were screened using limma package in R software. A gene with log 2 Fold Change > 0.5 and P value adjusted by false discovery rate <0.05 was considered significantly differently expressed.

Construction of co-expression modules

A scale-free co-expression network using obtained DEGs was performed using the R package “WGCNA”. The soft-thresholding power β was calculated in the construction of each module using the pickSoftThreshold function of WGCNA, which provides a suitable power value for network construction by calculating the scale-free topology fit index for a set of candidate powers, ranging from 1 to 20. If the index value for the reference dataset exceeded 0.85, the appropriate power was determined. Then a one-step network construction method was used to identify co-expression modules. The minimum number of genes for each module was set at 50, and the strength of interaction between each module was analyzed and visualized using the heatmap tool package. The hclust function was used to cluster samples and

check for outliers. The relationships between modules and severity of asthma were assessed. Other clinic traits obtained from the dataset were also included to analyze the correlations with each module.

Identification of clinically significant module and hub genes

Module eigengene (ME) represents the first principal component of a given module and the gene expression profiles in this module. Gene significance (GS) and module membership (MM) was defined as the absolute value of the correlation between the gene and the clinic trait and the correlation of gene expression with the ME, respectively. The most important module for asthma severity was identified if:

1. the correlation between the module and the asthma severity $\geq |0.5|$ ;
2. the correlation between MM and GS in this module was statistically significant ( $P < 0.05$ ).

The selected module was visualized using STRING.

Hub genes are those inside co-expression modules tend to have high connectivity. Genes in the selected module with $|MM| > 0.6$ and $|GS| > 0.5$ were imported to Cytoscape, then, the top 10 degree genes were filtered as the hub genes.

Enrichment analysis

To further understand the functions of genes in the key module, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed on genes in the key module, using the R package “clusterProfiler”. GO terms included biological process (BP), cellular component (CC), and molecular function (MF). A p-value <0.05 was regarded as the cut-off criterion. The top 10 categories associated with GO analysis and KEGG analysis were showed respectively.

Validation of hub genes

Hub genes were validated using the GSE43696 dataset. Receiver operating characteristic (ROC) curve analysis was conducted for each hub gene and combined hub genes with the ROC package. The area under the curve (AUC) was used to evaluate sensitivity and specificity of the ten hub genes.

# Results

Dataset selection and DEGs identification

The microarray gene expression dataset GSE89809 was used in this study. After data normalization and quality control, 1035 genes were identified as the DEGs of which 634 were upregulated and 401 were downregulated between mild asthma and severe asthma. A volcano plot of all probesets is shown in Figure S1 and a heatmap of the top 25 changed genes is shown in Figure S2.

Co-expression network construction and disease-specific module identification

The expression profiles of the 1035 DEGs were used to conduct WGCNA. Hierarchical clustering analysis was then performed. When the threshold was set to 50, GSM2389953 was considered to be outliers (Figure S3) and removed prior to further analyses. The optimal power β for which the scale-free topology index exceeded 0.85 was computed as 8 (Figure 1). After this soft threshold of 8 was implemented, 6 significant gene modules were thus detected ranging in size from 27 genes to 585 genes (Figure 2). The gray module contained DEGs not clustered in any module.

Identification of clinically significant module and hub genes

Module-trait associations were identified based on the correlation between module eigengene and clinic traits. Results (Figure 3) indicated that all modules were significantly associated with asthma severity. Three modules (brown, blue, and green) were positively correlated to three clinic traits, namely severity of asthma, ACQ score, and GINA control, while negatively correlated with FEV1 and FVC. That means genes in these modules are predominantly up-expressed in severe asthma. In contrast, two modules (yellow and turquoise) were found to negatively correlate with asthma severity, ACQ score, and GINA control. Besides, modules positively associated with asthma severity were also found to be correlated with smoking status. No modules were found to be correlated with allergic rhinitis or nasal polyps. In addition, the eigengene dendrogram and heatmap were used to quantify modules similarity (Figure S4).

Among these modules, the module brown had the strongest correlation to asthma severity($r = 0.72$, $p < 0.001$). GS and MM of brown module were further calculated using WGCNA. Figure 4 showed that brown module had a strong GS-MM correlation ($p < 0.001$), which was identified as clinically significant module and visualized in String database (Figure S5). Totally 48 genes with $|GS| > 0.5$ and $|MM| > 0.6$ in brown module were imported into Cytoscape, then the top 10 degree genes were filtered as hub genes named CXCR1, CXCR2, CCR1, CCR7, TLR2, FPR1, FCGR3B, FCGR2A, ITGAM and PLEK ( Figure 5 ).

Functional enrichment analysis

GO and KEGG enrichment analysis of gene modules were explored. GO enrichment results showed that the brown module genes were significantly associated with immune responses such as neutrophil degranulation and activation, leukocyte migration and chemotaxis (Figure 6). The KEGG pathway enrichment results indicated that genes in the brown module were primarily enriched in cytokine-cytokine receptor interaction, phagosome, chemokine signaling pathway (Figure 7).

In the analysis of interactions between the 5 modules, results indicated that modules positively related to asthma severity (brown, blue, green module) had correlation with each other. The similar correlation was also observed in modules negatively related to asthma severity. Thus, enrichment analysis was also conducted for genes in positively related modules. Go and KEGG enrichment results of these modules were in consistent with the results of brown module ( Figure S6, S7 ).

Validation of hub genes in ROC curve analysis

To verify hub gene expression, dataset GSE43696 was retrieved from GEO. This dataset provided gene expression data of healthy control, mild-moderate and severe asthmatics from bronchial epithelial brushing. Subsequently, expression data of 10 hub genes from mild-moderate and severe asthmatics were extracted. ROC curve analysis was used to evaluate the diagnostic prediction value of the hub genes for asthma severity. This analysis revealed that the area under the ROC curve for CXCR2 genes was 0.66, followed by CXCR1, TLR2, FPR1, CCR1, FCGR3B and ITGAM (Figure S7-S16). Combining 10 hub genes possessed a moderate ability for discriminating between severe asthmatics and mild-moderate subjects with an area under the curve ( AUC ) of 0.75 (Figure 8).

# Discussion

Severe asthma patients contribute to 50-60% of asthma costs and are associated with poor quality of life and high mortality and morbidity [11]. The unclear molecular mechanism and refractory response to traditional asthmatic therapies seen in these patients have been challenging for clinicians to treat this subtype of asthma. In this study, we used the DEGs between severe and mild asthma samples for the first time to construct a co-expression network by WGCNA and carried out a comprehensive analysis of key genes and pathological processes associated with asthma severity, hoping that the findings will provide ideas for the understanding and future treatment of severe asthma.

In total 6 modules were identified in this paper, of which 3 modules were positively related to asthma severity while 3 modules were negatively related to asthma severity. The brown module with the strongest relation to asthma severity and the significant MM-GS correlation was identified as the critical module. Enrichment analysis showed that genes in brown module were enriched in neutrophil degranulation and activation, leukocyte migration and chemotaxis, cytokine-cytokine receptor interaction, phagosome, chemokine signaling pathways. Then 10 hub genes in brown module were filtered and verified in another dataset, showing a moderate ability for discriminating severe asthma from mild-moderate asthma with AUC of 0.75.

Results of module-trait relationships showed modules positively related to asthma severity(brown, blue and green module) also positively related to ACQ score and GINA control grade while negatively related to FEV1 and FVC, which means the higher genes expression level in these modules, the worse asthma control and lung function. Besides, positive relationships were found between smoking status and high expression of genes in severe asthma. Previous studies showed that asthma patients exposed to smoke are typically steroid-refractory and result in uncontrolled asthma [12]. One of the probable mechanisms has been linked with Th17 pathway [13]which mediates neutrophilic activation and recruitment in airway. This is consistent with our enrichment analysis results that genes positively to smoking status were enriched in neutrophil degranulation, activation and migration. In addition, no modules were found to be linked with phenotype allergic rhinitis and nasal polyps.

For brown module genes, the significant enriched terms in GO and KEGG were as followings: neutrophil degranulation and activation, leukocyte migration and chemotaxis, cytokine-cytokine receptor interaction,

phagosome, chemokine signaling pathway. Similar enrichment results were found when all modules positively related to asthma severity were considered. Asthma can be classified as “T-helper 2 (Th2)/type 2 asthma” and “non-Th2/type 2 asthma” based on the inflammatory pattern [14]. Neutrophil inflammation, characterized by the lack of Th2-mediated inflammatory response and increasing airway neutrophils [15], has been linked with asthma severity [16, 17]. In fact, neutrophils contribute to the pathology of asthma, regardless of whether asthma is eosinophilic or non-eosinophilic [18, 19]. This is in accordance with the present enrichment analysis results that from mild to severe asthma, more neutrophils activated and migrated, as well as more cytokines produced and functioned. Besides, neutrophil inflammation is more prominent in patients fail to respond to inhaled corticosteroids, also referred to as severe asthma than other levels severity of asthma [20]. Thus, novel treatments aiming at decreasing neutrophils may benefit patients with severe asthma.

Identified hub genes further provided biomarkers for severe asthma. CXCR1 and CXCR2 are chemokine receptors respond to IL-8, which is an essential chemokine enhances neutrophils migration into airways and contributes to asthma severity and lung damage [21]. In this way, CXCR1/2 inhibition might be a rational therapeutic strategy for severe asthma treatment. For example, a selective CXCR2 antagonist named SCH527123 was reported to reduce sputum neutrophils and mild exacerbations [22]. However, AZD5069, which is also an antagonist of CXCR2 was investigated and results showed it failed to reduce asthma exacerbations or improve lung function compared with placebo [23]. Recently, a study suggested that KLF2, as a regulator of CXCR1/2, may represent an indicator of asthma severity when combined with CXCR1/2 [24]. Thus provides another direction for the treatment of severe asthma target on CXCR1/2. Unlike CXCR1/2, CCR1 and CCR7 are chemokine receptors in Th2/type 2 pathway, which is supposed as the dominant inflammatory pathway underpinning severe asthma. CCR1 mainly expressed in eosinophils, macrophages, lymphocytes. It plays a role in the progression of asthma by promoting chemotaxis of leukocytes in the airway epithelium and probably by modulating the Th1 or Th2 cytokine balance [25]. Biopsy of the airways has demonstrated elevated expression of CCR1 mRNA in mild-to-severe asthma [26]. CCR7 involved in the migration and maturation of dendritic cells (DCs), which have been shown to facilitate the development of asthma [27-29]. CCR7 could also take part in the airway remodeling of severe asthma by enhancing fibrocytes transmigration [30]. In addition, findings of CCR7 on immune tolerance in allergy-induced asthmatic [31, 32] may provide ideas on the treatment of severe asthma.

Toll-like receptors (TLR) family is the first line of defense against invading microbes [33]. They cause Th17 responses, leading to increased production of IL-8, IL-17, which can modify airway structures to contribute to the lower FEV1, remodeling, and airway obstruction seen in patients with severe neutrophilic asthma [34]. This paper indicated TLR2 had increasing expression in severe asthma. In line with our results is the upregulation of TLR2 in sputum T cell of non-eosinophilic asthma and TLR2 pathway in severe asthma. However, recent study showed that TLR2 may reduce Th17 cytokines by suppressing a Th17 phenotype of Treg cells. That means TLR2 may induce remission of asthma [35]. Another study in mouse also suggested appropriate stimulation of the TLR2/4 pathway may help to prevent asthma later in life [36]. Thus, further studies are needed to reveal the effect of TLR2 on asthma and disease severity.

FPR1 is a powerful neutrophils chemotactic factor and have been linked to chronic inflammation diseases. Though FPR1 was reported to react to cigarette smoke [37, 38] and involve in anti-inflammatory activities of glucocorticoid [39], little was known about its effect on the process of asthma.

FCGR3B, FCGR2A and ITGAM are immune-related genes. FCGR2B and FCGR3A are associated with several immune-mediated diseases such as systemic lupus erythematosus and severe nephropathy. ITGAM is also known as the biomarker for systemic lupus erythematosus. PLEK is major protein kinase C substrate of platelets, monocytes, macrophages and lymphocytes. The exact function of these genes on asthma patients is not known.

For the first time, co-expression modules was built via WGCNA using DEGs of mild-severe asthmatics from bronchial epithelial brushings to discover mechanisms and hub genes in severe asthma. There are some limitations in our study. First, samples in this study are small, mainly due to the lack of related gene expression profiles with rich clinical phenotypes. Second, our research results are based on public online database information and future studies are needed to prove and it. Third, detailed mechanisms of identified hub genes required to be elucidated by more experiments.

In conclusion, we identified neutrophil degranulation and activation were key pathways in the development of asthma. Meanwhile, hub genes such as CXCR1, CXCR2, CCR1, CCR7, TLR2 and FPR1 were discovered act as core parts in asthma severity through either neutrophil inflammation pathway or T2 immune pathway. Our results can be useful to serve as potential immunotherapy targets and prognostic marker. Further mechanism studies are required to validate and elucidate our results.

# Declarations

*Ethics approval and consent to participate*

Not applicable.

## *Consent for publication*

Not applicable.

## *Availability of data and materials*

The datasets generated and analysed during the current study are available in Gene Expression Omnibus (GEO), [https://www.ncbi.nlm.nih.gov/gds/].

## *Competing interests*

The authors declare that they have no competing interests.

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### *Authors' contributions*

Zeyi Zhang and Jingjing Wang analyzed the data. Zeyi Zhang was a major contributor in writing the manuscript. All authors read and approved the final manuscript. Ou chen is responsible for review and modification of the manuscript.

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Not applicable.

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# Figures

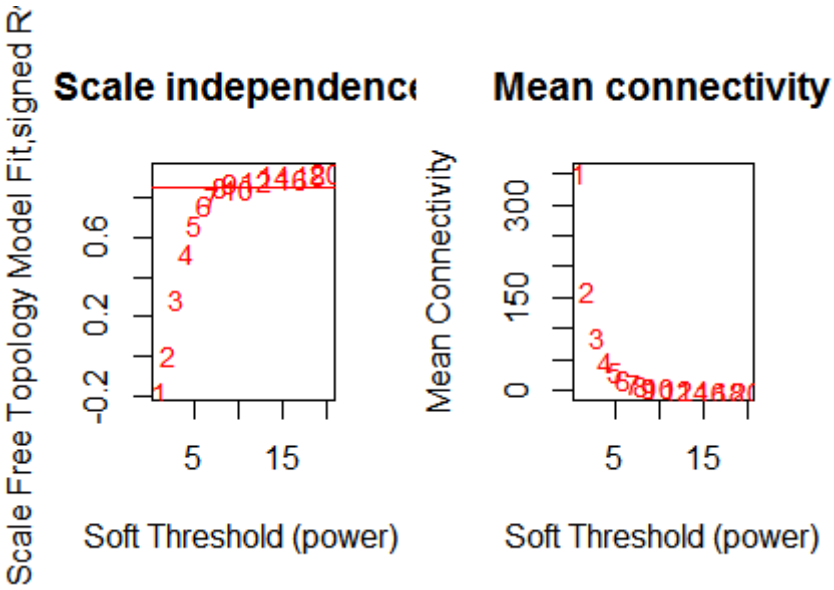



**Figure 1**

Analysis of network topology for a set of soft-thresholding powers.

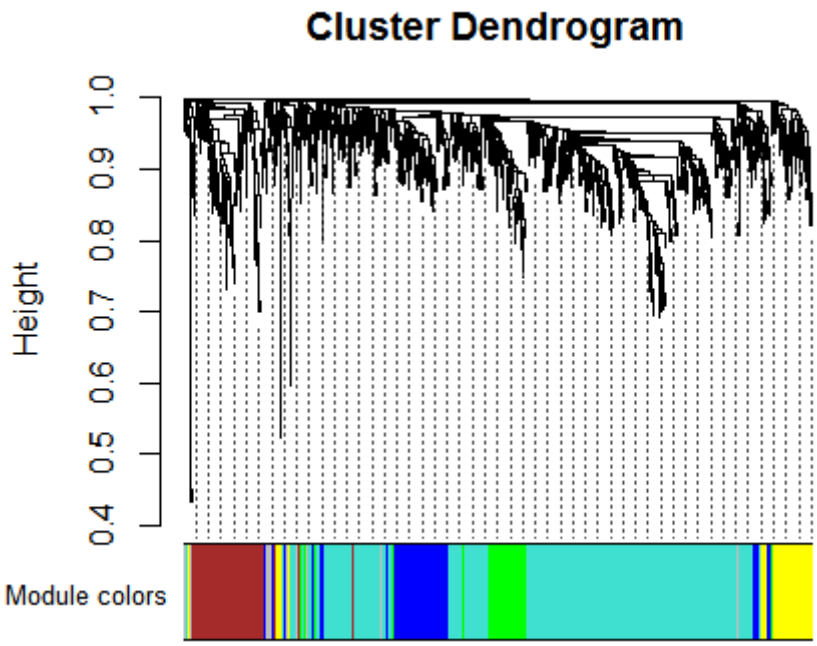



Figure 2

Clustering dendrograms of genes

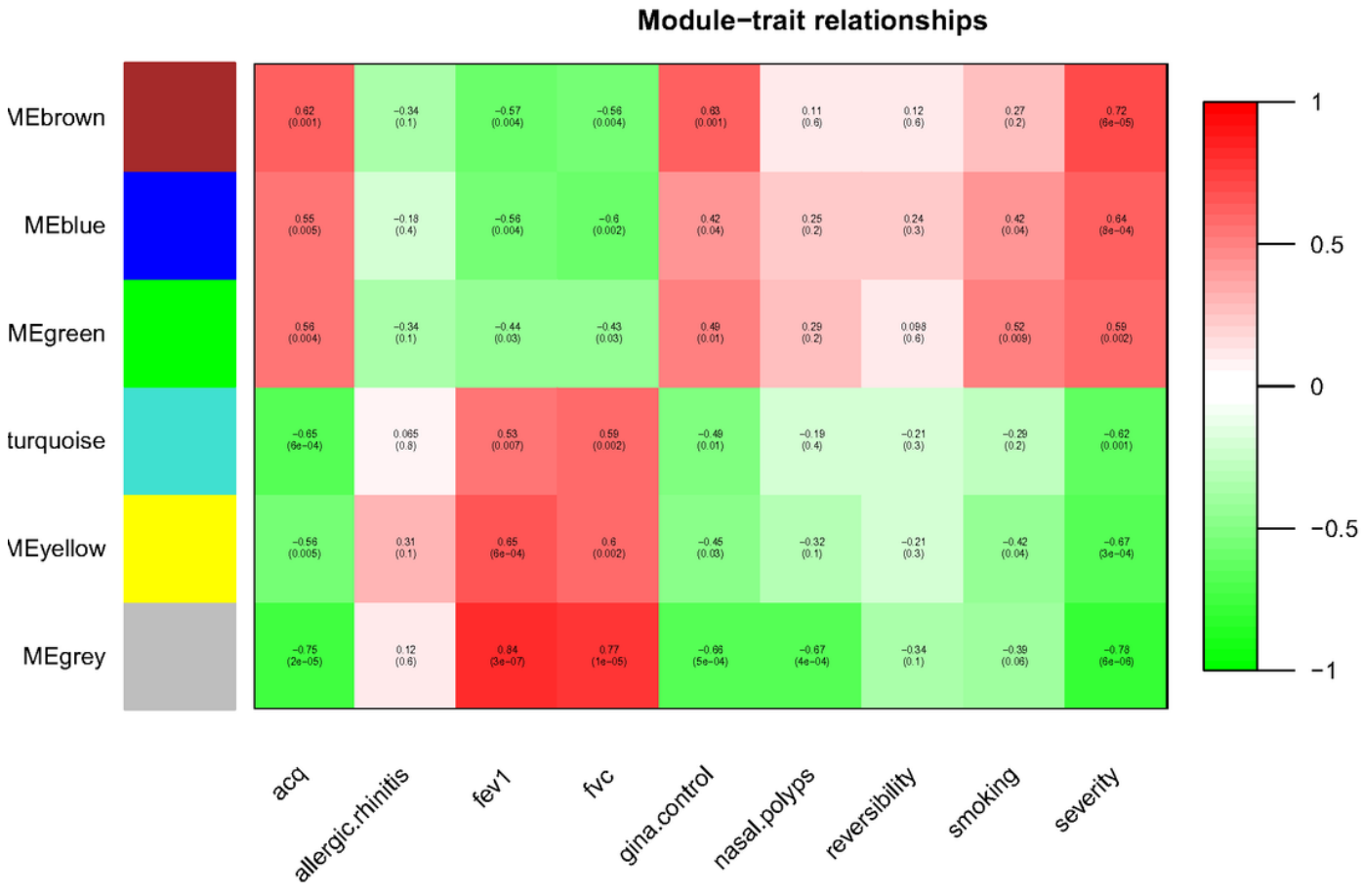



Figure 3

Module-trait relationships

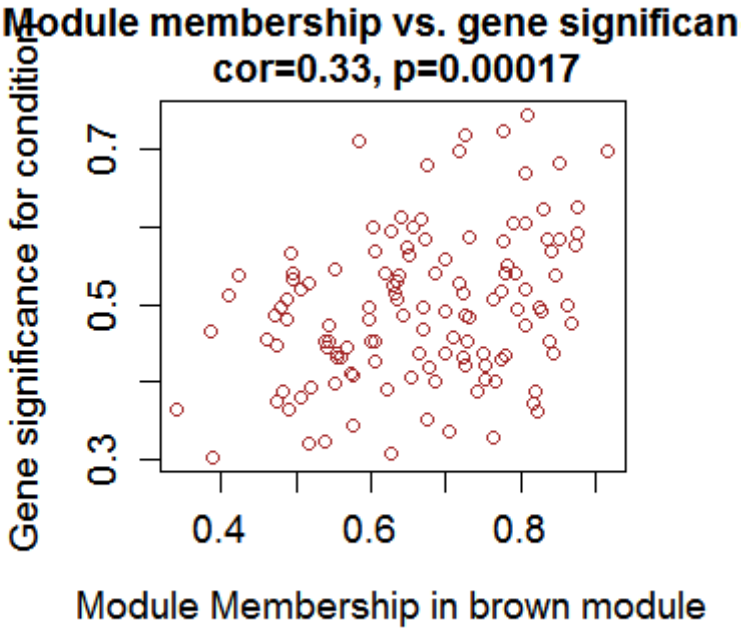



**Figure 4**

Gene significance for asthma severity vs module membership in brown module

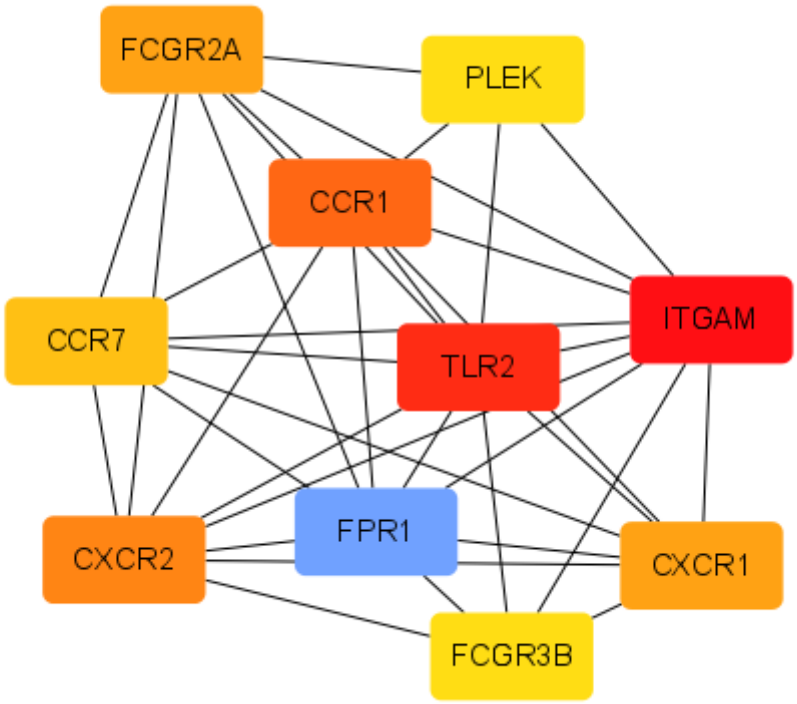
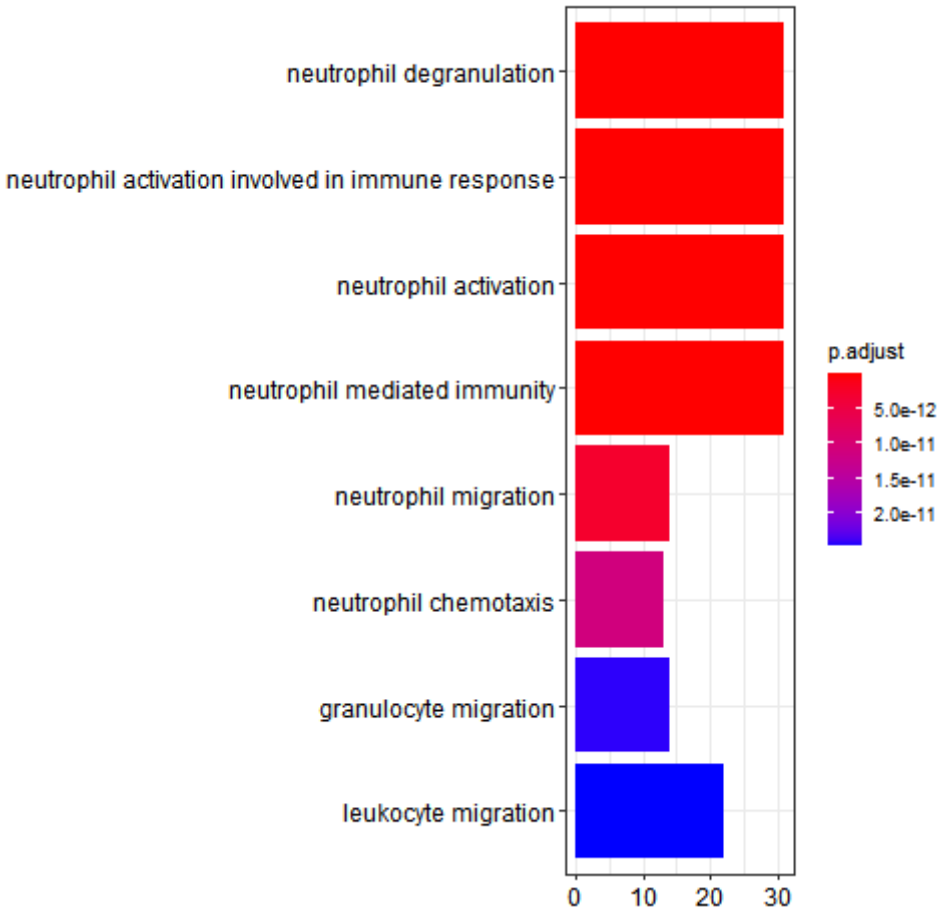


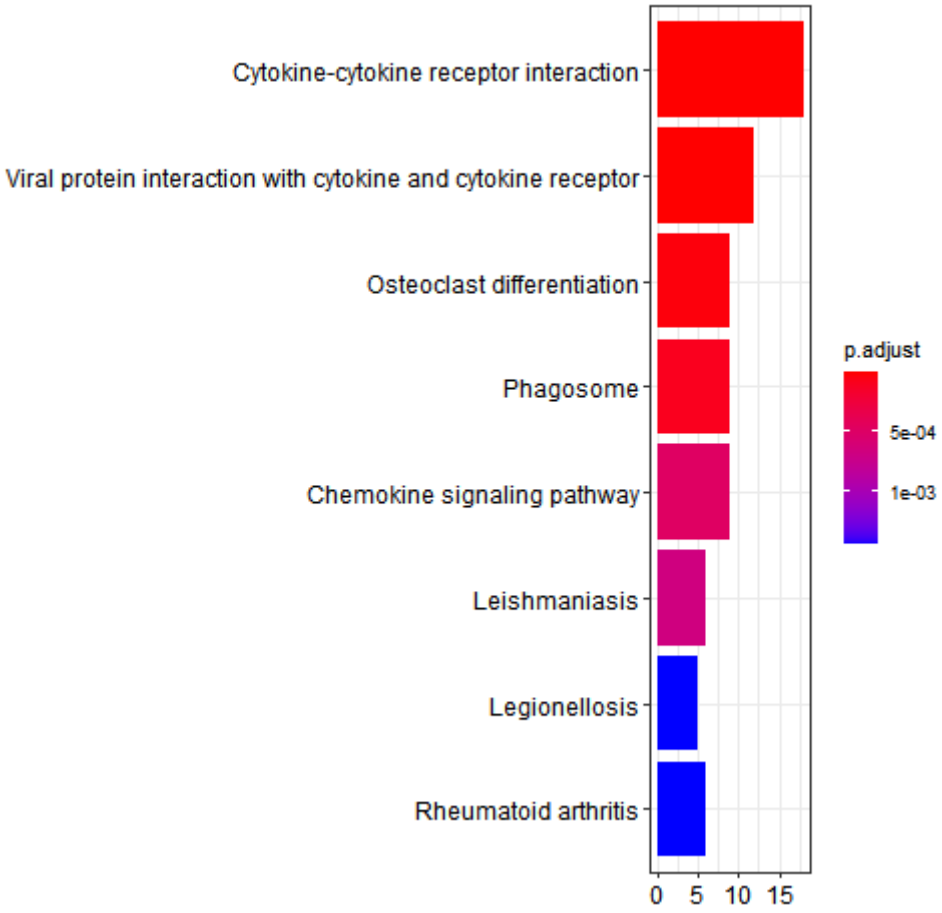

**Figure 5**

Hub genes visualized in Cytoscape




## Figure 6

GO enrichment analysis of genes in brown module




## Figure 7

KEGG enrichment analysis of genes in brown module

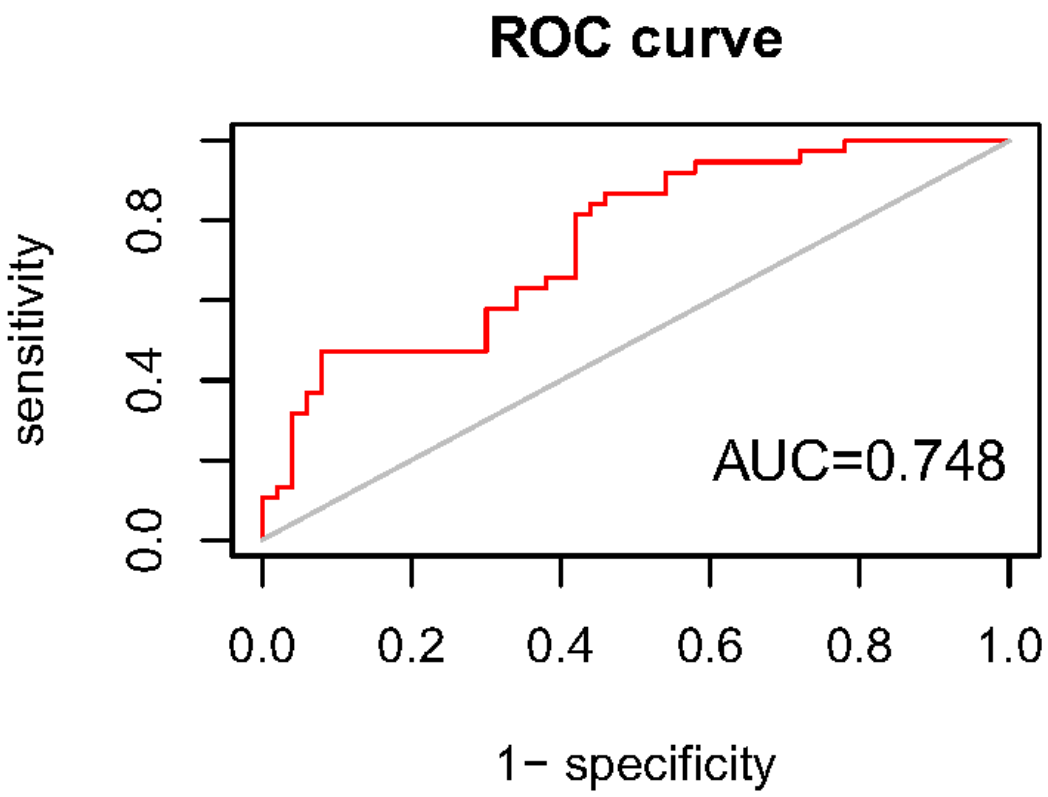



**Figure 8**

Receiver operating characteristics curve analyses of combined hub genes

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement.docx