

## **Transcriptome analysis reveals upregulation of bitter taste receptors in severe asthmatics**

Christina Orsmark Pietras, PhD,<sup>1</sup> Anna James, PhD,<sup>2</sup> Jon R Konradsen, MD, PhD,<sup>3,4</sup> Björn Nordlund, RN,<sup>3,4</sup> Cilla Söderhäll, PhD,<sup>1</sup> Ville Pulkkinen, PhD,<sup>2,5</sup> Christophe Pedroletti, MD, PhD,<sup>4</sup> Kameran Daham, MD,<sup>6,7</sup> Maciek Kupczyk, MD, PhD,<sup>2</sup> Barbro Dahlén MD, PhD,<sup>6,7</sup> Juha Kere, MD, PhD,<sup>1,8</sup> Sven-Erik Dahlén, MD, PhD,<sup>2</sup> Gunilla Hedlin, MD, PhD,<sup>3,4\*</sup> and Erik Melén, MD, PhD,<sup>2,3\*</sup>

<sup>1</sup> Department of Biosciences and Nutrition at Novum, Karolinska Institutet (KI)

<sup>2</sup> Institute of Environmental Medicine, KI

<sup>3</sup> Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm

<sup>4</sup> Department of Women's and Children's Health, KI

<sup>5</sup> Department of Medical Genetics, Biomedicum Helsinki, University of Helsinki, and Folkhälsan Institute of Genetics, Helsinki, Finland,

<sup>6</sup> Lung/allergy clinic at Karolinska University Hospital Huddinge,

<sup>7</sup> Department of internal medicine at Huddinge University Hospital, KI

<sup>8</sup> Science for Life Laboratory, KI

All belonging to The Centre for Allergy Research at Karolinska Institutet, Stockholm, Sweden and being members of GA<sup>2</sup>LEN, The Global Allergy and Asthma European Network.

\*Shared last authorship.

Correspondence and reprint requests: Sven-Erik Dahlén, MD, PhD, The National Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden.

E-mail: sven-erik.dahlen@ki.se

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## ABSTRACT

The causes of severe childhood asthma are poorly understood. Our aim was in this study to define global patterns of gene expression in children with severe therapy-resistant and controlled asthma.

White blood cells were isolated and the global transcriptome profile was characterised using the Affymetrix Human Gene ST 1.0 chip in children with severe, therapy-resistant asthma (SA, n=20), controlled asthma (CA, n=20) and healthy controls (Ctrl, n=19). Receptor expression was studied in separated fractions of leukocytes in adults asthmatics (n=12).

1378 genes were differentially expressed in one or several of the SA vs. Ctrl, SA vs. CA or CA vs. Ctrl contrasts. Three significantly enriched KEGG pathways were represented; natural killer cell mediated cytotoxicity (upregulated in CA), N-Glycan biosynthesis (downregulated in SA) and bitter taste transduction, *TAS2Rs* (upregulated mostly in SA). qPCR experiments confirmed upregulation of *TAS2Rs* in the SA group compared to the Ctrl group. Further analysis of sorted leukocyte fractions from adult asthmatics indicated that *TAS2R* expression was highest in blood lymphocytes. Significant correlations between expression of the *TAS2Rs* and clinical markers of asthma severity were found in both adults and children.

In conclusion, specific gene expression patterns were observed in children with severe, therapy-resistant asthma. The increased expression of bronchodilatory bitter taste receptors suggests a new target for treatment of asthma.

## INTRODUCTION

Asthma is a heterogeneous disease that affects millions of adults and children worldwide. Around 5% of children with asthma suffer from chronic symptoms and/or severe exacerbations despite treatment with a variety of drugs, including high doses of inhaled corticosteroids [1]. The term “Problematic severe asthma” has been proposed to classify these children, of whom a subgroup suffers from severe, therapy-resistant asthma that probably requires novel therapeutic approaches [2]. Therapy-resistant children should be distinguished from children with other forms of “problematic asthma” that is difficult to treat due to unidentified exacerbating factors, social issues or poor compliance. The causes of severe therapy-resistant childhood asthma are still poorly understood.

Asthma is known to run in families, and studies also suggest that there is a considerable hereditary component for disease severity [3]. To date, there are few genetic studies on therapy-resistant asthma in children, and few studies have explored the gene expression profile that characterises this phenotype [4]. The clinical features of severe asthma: chronic inflammation and subsequent structural changes of the airways, lung function deterioration and comorbidities [5-7] suggest that genes involved in these processes may be of particular interest. Using a genomics-based approach involving microarray profiling in blood cells from children collected during acute asthma exacerbation and convalescence, gene expression patterns have been characterised and highlight pathways related to e.g. prostaglandin metabolism and innate immunity [8].

Our primary aim was to perform genome-wide expression array analyses on leukocytes obtained from extensively characterised children taking part in a national study on problematic severe asthma [5]. Fresh white blood cells from 17 children with asthma resistant to therapy, 19 patients with controlled persistent asthma and 18 healthy controls were

assessed and our data indicate clearly different gene expression patterns between severe, therapy-resistant asthma and controlled persistent asthma.

One particular novel pathway found to characterize the severe therapy resistant asthma phenotype was that of bitter taste receptor signalling. This finding was somewhat unexpected as until recently bitter taste receptors (TAS2Rs) were thought to be exclusively located on the tongue, where their stimulation by specific ligands enables our perception of bitter taste. However, Deshpande et al demonstrated the presence of these receptors in the human lung and furthermore, that their stimulation induces bronchodilation [9]. A secondary aim of the current study was therefore to perform a more detailed examination of *TAS2R* expression and function in isolated blood leukocytes. For this purpose, blood was collected from an additional 12 asthmatic subjects and our findings revealed particularly abundant *TAS2R* expression on lymphocytes, as well as inhibitory effects of TAS2R agonists on pro-inflammatory mediator release. We also found correlations between *TAS2R* expression and clinical characteristics, suggesting that this pathway may be one important endogenous protective mechanism in severe asthma.

## **METHODS**

### **Study design**

Two different cohorts of patients were used in the current study. Genome-wide expression array analyses were performed in mixed blood leukocytes from children taking part in a national study on problematic severe asthma as described [5]. Subsequently, a more detailed examination of bitter taste receptor expression and function was performed on isolated blood leukocytes from an additional 12 adult asthmatic subjects.

### **STUDY SUBJECTS, CHILDREN**

The nation-wide study on problematic severe asthma in Sweden is an observational, multi-center investigation in which school aged children with problematic severe asthma were compared to age-matched peers with controlled persistent asthma (CA). All patients were required to have been without airway infections or exacerbations of their asthma during a two-week period prior to examination. The inclusion criteria are presented in Table E1 (see Supplementary material), whereas details concerning enrolment are presented elsewhere [5]. Among children who fulfilled the definition of problematic severe asthma, we identified children who were severely resistant to therapy (called severe asthma, SA) based on detailed clinical characterisation (insufficient asthma control despite level 4 treatment, according to GINA)[10]. The sample set used in this study comprised 20 children with SA (with an Asthma Control Test, ACT score less than 19), 20 children with CA (selected from the controlled, persistent asthma group, with an ACT score more than 20 but less than 24) and 19 healthy controls (Ctrl) recruited at Astrid Lindgren Children's Hospital, Stockholm. The controls had been admitted for elective surgical procedures such as scrotal hernia, and blood samples were collected prior to the planned surgical procedure. Informed consent was

obtained from all participating children and their parents and the study was approved by the local ethics committee.

### **Asthma control test**

Self-reported asthma control was assessed using the Asthma Control test® (ACT) [11, 12]. A total score of: 25-27 points were considered to reflect very well controlled asthma; 20-24 points controlled asthma and 19 points or less uncontrolled asthma.

### **Nitric oxide, pulmonary function and assessment of bronchial hyperresponsiveness**

Employing a NIOX<sup>TM</sup> apparatus (Aerocrine AB, Solna, Sweden), the fraction of exhaled nitric oxide (FE<sub>NO</sub>) was measured prior to spirometry, in accordance with international guidelines [13]. Treatment with short- (SABA) and/or long-acting (LABA)  $\beta$ -2 agonists and/or leukotriene antagonists (LTRA) was withheld for 8, 24 and 72 hours, respectively, prior to measurement of baseline pulmonary function and provocation with methacholine. Spirometry was performed with a Vitalograph<sup>®</sup> 2120 (Vitalograph<sup>®</sup>, Ennis, Ireland), in accordance with published recommendations [14] and using the reference values reported by Polgar[15].Bronchial hyperresponsiveness (BHR) to a challenge with methacholine was assessed utilizing a Spira nebuliser (Spira Respiratory Care Centre, Hämeenlinna, Finland) [16]and results presented as the slope of the dose-response curves (DRS<sub>methacholine</sub>) [17].

### **Blood analyses**

Following application of local anesthesia (EMLA cream, AstraZeneca, Sweden), samples of venous blood were collected. White blood cells (WBC) and Immunoglobulin E (IgE) were measured. Atopy was defined as specific IgE >0.35kU/L to inhalant allergens (Phadiatop®, a mixture of cat, dog, horse, birch, timothy, mugwort, *Dermatophagoides pteronyssinus*, and *Cladosporium* allergens. Phadia AB, Uppsala, Sweden). Atopy was, however, not measured in healthy controls as a history of atopy was an exclusion criterion for that group. WBC were freshly isolated from the blood, immediately put into RNeasy® (Applied Biosystems /Ambion, Austin, TX, USA) and stored at -20° and -80° C until RNA extraction.

### **RNA Extraction**

Total RNA was extracted from WBC (buffy coat) using RiboPure™-Blood extraction kit (Applied Biosystems /Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA quality and quantity were assessed using NanoDrop 8000 (Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

### **Microarray Profiling**

For transcriptome profiling the Affymetrix Human Gene ST 1.0 chip was used (Affymetrix, Santa Clara, CA). The Gene ST 1.0 chip contains 28,875 genes/transcription sets with a resolution of 26 (median) probes per transcription set. cDNA synthesis and hybridisation were performed according to the manufacturer's recommendation. A total of 59 hybridizations from 59 subjects were carried out using standard Affymetrix protocols for gene expression ([www.affymetrix.com](http://www.affymetrix.com)). Experimental data was pre-processed using the statistical software R (<http://www.R-project.org>) and Bioconductor (<http://www.bioconductor.org>) by implementing the *Affy* package and expression values were extracted from the .CEL files and



normalized using Robust Multi-array Average (*justRMA*). For quality assessment of the data post normalisation the ArrayQualityMetrics package was applied [18]. Detected outliers were removed and the data set normalised again. Before further analysis normalisation control genes were filtered away. Data analysis and annotation were performed implementing the *limma* package and the hugene10sttranscriptcluster library.

In order to validate the results of the microarray profiling, additional qPCR experiments were performed to examine expression of all TAS2Rs found to be differentially expressed between the subject groups. cDNA synthesis and qPCR for 11 commercially available human *TAS2Rs* were performed exactly as described below in the “qPCR for Bitter Taste Receptors” section. These experiments were performed in a subset of children, healthy controls (n=18) and children with severe therapy resistant asthma (n=19), for whom sufficient RNA was available. Normally distributed data were compared by Students’ unpaired t-test and skewed data using the Mann Whitney test.

### **Statistics of clinical characteristics in children and microarray data**

A two-tailed Student’s t-test or Analysis of variance (ANOVA) were used to test for differences in clinical variables between SA, MA and Ctrl groups and Wilcoxon ranksum test for variables with a skewed distribution (SA vs CA only). A chi-square test was used for differences in categorical variables (sex). A p-value  $\leq 0.05$  was regarded as significant. F-statistics was used on the microarray data as a global test to generate a list of genes differentially expressed in any of the three contrasts; controlled asthma vs. healthy controls (CA-Ctrl), severe asthma vs. controlled asthma (SA-CA) and severe asthma vs. healthy controls (SA-Ctrl). Multiple testing was corrected for using the default Benjamini and Hochberg’s method and all genes with an adjusted p-value  $\leq 0.05$  were regarded as

significant, generating a top table of 1378 genes significantly differentially expressed in any of the contrasts. The moderated t-statistics revealed each contrast's contribution to the global test of differential expression.

### **Clustering and Functional Enrichment Analysis**

Unsupervised hierarchic clustering and heatmap were generated implementing the *gplots* package using all 1378 significant genes implicated by the F-statistics. Functional annotation and enrichment analysis were performed to determine if more genes than expected by chance were from a given biological pathway or process. Biological process (BP\_FAT) and pathways (KEGG) were assessed using DAVID 6.7 (<http://david.abcc.ncifcrf.gov>) [19, 20]. Enrichment was considered significant if the Benjamini and Hochberg's adjusted p-value was  $\leq 0.05$  relative to the Affymetrix Human Gene ST 1.0 background. All microarray data are available in the Gene Expression Omnibus repository ([www.ncbi.nlm.nih.gov/projects/geo/](http://www.ncbi.nlm.nih.gov/projects/geo/)) (accession no. GSE27011).

### **STUDY SUBJECTS, ADULTS**

In order to investigate the expression and function of bitter taste receptors on isolated blood leukocyte fractions, blood was collected from an additional 12 adult patients with intermittent allergic asthma, documented airway hyperresponsiveness to methacholine and a positive skin prick test to pollen or animal dander, subject characteristics are shown in Table E2. These subjects were recruited from the outpatient clinic at the Dept. of Respiratory medicine and Allergy, Karolinska University Hospital, Huddinge and all provided written consent to participate in the study which was approved by the local ethics committee.

### **Isolation of blood leukocyte fractions**

Peripheral venous blood was collected into Vacutainer® tubes containing heparin (Beckton Dickinson, Franklin Lakes, NJ, USA). Following dextran sedimentation of erythrocytes, Lymphoprep® (Axis-Shield, Dundee, Scotland) density gradient separation was performed to separate a granulocyte-rich pellet and PBMC-rich interphase. Neutrophils and monocytes were isolated by positive selection using CD16 and CD14 MACS® microbeads respectively, and lymphocytes by negative selection following the removal of monocytes according to the manufacturers' instructions (Miltenyi Biotech, BergischGladbach, Germany). These preparations yielded a purity of >95%.

### **qPCR for Bitter Taste Receptors**

RNA was extracted using the RNeasy® Mini kit with on-column DNase digestion according to the manufacturers' instructions (Qiagen, Hilden, Germany). Briefly, fresh cells were homogenised in the RLT lysis buffer supplied, and stored at -80°C before proceeding with the RNA extraction protocol. RNA concentration and quality was assessed using a NanoDrop 8000 (Wilmington, DE, USA). cDNA synthesis was carried out using the QuantiTect Reverse Transcription kit (Qiagen, Hamburg, Germany) according to manufacturers' instructions. Quantitative real-time PCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with TaqMan® reagents (Life Technologies, Invitrogen, Carlsbad, CA, USA). 11 commercially available TaqMan® primers for human TAS2Rs (hTAS2R4, 5, 10, 13, 14, 19, 20, 31, 45, 46 and 50) and the housekeeping gene glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) were used. As negative controls, PCR reactions were performed using water or products of the cDNA reaction

performed in the absence of the reverse transcriptase enzyme (-RT control). Gene expression relative to that of GAPDH was calculated according to the  $2^{-\Delta CT}$  formula.

### **Effect of TAS2R agonists on leukocyte function**

In order to examine the effect of TAS2R activation on leukocyte function, the TAS2R agonists chloroquine and denatonium were applied to a standardised assay known to increase pro-inflammatory mediator release by blood leukocytes. Briefly, whole blood was incubated with 100µg/ml LPS for 24 hours, with or without 10, 100 or 1000µM of the bitter taste receptor agonist or relevant vehicle control. Plasma was then removed and stored at -80°C prior to measurement of pro-inflammatory cytokines and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). A panel of 17 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, G-CSF, GM-CSF, IFNγ, MCP-1, MIP-1β, and TNFα) were assayed in serum (diluted 1:4), using a Bio-Plex Pro™ 17-plex kit with a Luminex® analyser (BIO-RAD Laboratories, Hercules, CA, USA). PGE<sub>2</sub> levels were measured by enzyme immunoassay (Cayman Chemical, Ann Arbor, Michigan, USA).

### **Statistics, bitter taste receptor expression in adults**

The majority of datasets showing bitter taste receptor expression in isolated blood leukocytes were normally distributed according to the Kolmogorov-Smirnov test and were therefore compared using Student's paired t-test. Correlations between receptor expression and clinical characteristics were examined using Pearson's test. A p-value of <0.05 was accepted as statistically significant.

## RESULTS

### Identification of genes subdividing severe asthma, controlled asthma and controls

The transcriptomes of 59 subjects were assayed on Affymetrix Human Gene ST 1.0 expression arrays. After pre-processing and quality control of the post-hybridised arrays, five samples were regarded as outliers and removed. The remaining sample set of 17 severe asthmatics (SA), 19 controlled asthmatics (CA) and 18 healthy controls (Ctrl) were used for downstream analyses. Application of linear modelling approach and F-test statistics identified a top table of 1378 transcription clusters/genes that were significantly differentially expressed in one, or several, of the CA vs. Ctrl, SA vs. CA or SA vs. Ctrl contrasts (see Table E3 in the Supplementary material). T-test statistics was applied to identify which of the contrasts contributed to the overall significance, and the Venn diagram (Figure 1) illustrates this distribution. Based on these 1378 genes, a large number seemed to uniquely differentiate the SA group from the CA (n=351 genes) and ctrl (n=315) groups, whereas fewer genes differentiated the CA from the Ctrl group (n=149). Three hundred and fifty five genes were also shared between the SA-Ctrl and SA-CA contrasts, which supported the view that SA was characterized by a unique set of genes compared with the other two groups.

The clinical data of the three groups (SA, CA, Ctrls) are presented in Table 1. A significant increase was seen for SA compared to CA for bronchial hyperresponsiveness ( $DRS_{\text{methacholine}}$ ,  $p=0.01$ ) and Ctrls were slightly younger than children with SA and MA. There were however no significant differences seen for  $FEV_1$ , total WBC count, Eosinophils, Neutrophils,  $FE_{NO}$ , atopy or total IgE. No statistics were performed on ACT or ICS, of which a derivation in score or dose, respectively, was an inclusion criterion for the expression analysis.

An unsupervised hierarchical clustering and heat map of the normalised raw intensity of the 1378 differentially expressed genes illustrated a more refined grouping of the pre-defined

SA, CA and Ctrl groups (Figure 2). The column dendrogram separated the majority of SA from CA and Ctrl individuals (cluster I and II, respectively), and also separated the majority of Ctrl individuals from SA and CA (all but two Ctrl individuals found in II b). CA patients were found both in II a and II b.

### **Novel pathways and processes in asthma**

Pathways enrichment analyses using the DAVID database were done utilizing two separate approaches. First, all 1378 differentially expressed genes in the top table were used, revealing three significantly enriched KEGG pathways; taste transduction (mostly upregulated in SA), natural killer cell mediated cytotoxicity (upregulated in CA) and N-Glycan biosynthesis (downregulated in SA) (Table 2). Second, we made use of the dendrogram row clusters comprising the heatmap (Figure 2). We crudely dissected out four blocks from the row dendrogram (A, B, C and D in Figure 2) and performed functional annotation on these clusters (Table 2). Cluster A contained the natural killer cell mediated cytotoxicity pathway but also revealed two additional pathways, T cell receptor signalling and citrate cycle, all of these being up regulated in controlled asthma. Cluster D contained the bitter taste receptor pathway, being up-regulated in all severe asthmatics and some controlled asthmatics with a remarkably improved p-value (from 0.03 using the top table genes to  $4.2 \times 10^{-10}$ ).

To look for additional functional enriched groupings we performed gene ontology (GO) enrichment analysis. In severe asthma (SA) vs. healthy controls (Ctrl), sensory perception of taste was significantly enriched. Other significant biological processes related to metabolic processes and immune processes were also identified. For a complete list see Table E3 in the Supplementary material. Out of the total list of 1378 differentially expressed genes, fifteen percent of the transcript clusters were annotated as known or, in most cases, novel non-coding RNA (ncRNA), mostly represented by snRNA, snoRNA, rRNA, miscRNA and microRNA

(see Table E4 in the Supplementary material). The majority of ncRNA were found to be upregulated in severe asthma compared to controlled asthma or controls.

### **Confirmation of *TAS2R* expression in children by qPCR**

Expression of mRNA for 11 different *TAS2Rs* (4, 5, 10, 13, 14, 19, 20, 31, 45, 46 and 50) was confirmed by qPCR in mixed blood leukocyte samples from healthy children and children with severe therapy resistant asthma (figure E1 in the Supplementary material). In general, the expression of most bitter taste receptors was higher in children with severe asthma compared to the healthy controls and these differences reached statistical significance for *TAS2R13* (p=0.028), *TAS2R14* (p=0.009) and *TAS2R19* (p=0.037).

### **Co-expression of genetically associated asthma genes**

Against our list of 1378 differentially expressed genes we compared a list of 97 previously recognised asthma candidate genes, identified either through well replicated association studies or through genome wide association studies [21]. Twelve genes were present in both lists (Table 3). Most genes were upregulated in controlled asthma alone with three exceptions; *RORA*, that was upregulated in both severe and controlled asthma and *PEX19* and *MYB*, significantly downregulated in severe asthma alone. Functional annotation revealed no shared pathways, but several of the genes (*RORA*, *TBX21*, *ADRB2*, *IL2RB*, *PDE4D*, *MYB*, *PAFAH2*) were involved in the same biological processes such as positive regulation of various metabolic processes, regulation of cell death, and leukocyte activation.

### **Relative expression of *TAS2Rs* in isolated blood leukocytes**

Expression of mRNA for the 11 commercially available human bitter taste receptors relative to GAPDH in mixed leukocytes, lymphocytes, neutrophils and monocytes is shown in Figure 3. Expression of all bitter taste receptors was significantly greatest in the lymphocyte population compared to monocytes. Expression of the *TAS2Rs* was also greater in lymphocytes than neutrophils, but for *TAS2R50*, *TAS2R45*, *TAS2R31* and *TAS2R5* these differences did not reach statistical significance. In lymphocytes, the relative expression of *TAS2R10* was higher than that of the other *TAS2Rs* examined. The expression of *TAS2R5* on mixed blood leukocytes showed a significant inverse correlation with lung function in these subjects, both FEV1 % predicted ( $p=0.011$ , Pearson's correlation coefficient=-0.83) and FVC % predicted ( $p=0.029$ ). Interestingly, in the asthmatic children, *TAS2R5* mRNA expression in mixed blood leukocytes ( $\text{Log}^2$  *TAS2R5* expression derived from the Affymetrix Human Gene ST 1.0 chip) also showed a statistically significant correlation with bronchial hyperresponsiveness to methacholine, measured as the slope of the dose-response curve ( $p=0.008$ , Pearson's correlation coefficient=0.47).

### **Effect of TAS2R agonists on LPS-induced inflammatory mediator release**

In order to examine the possible role of TAS2R activation on blood leukocyte function, the effect of the prototype TAS2R agonists chloroquine and denatonium (10-1000  $\mu\text{M}$ ) on LPS-induced inflammatory mediator release was examined. Results showed that both chloroquine and denatonium significantly inhibited the release of  $\text{TNF}\alpha$ , IL-13 and MCP-1. Chloroquine also inhibited the release of IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-10, IL-17, G-CSF, GM-CSF,  $\text{IFN}\gamma$  and  $\text{PGE}_2$  in a dose dependent manner (Figure 4).



## DISCUSSION

Using genome-wide expression arrays to analyse the transcriptomes of fifty-four asthmatic patients and healthy controls, we identified a separation in gene expression between severe, therapy-resistant asthma and well-controlled persistent asthma. Whereas traditional immune related functions, such as T-cell receptor signalling and natural killer cell mediated cytotoxicity, were found to be up-regulated in controlled asthmatics, we could not observe such a pattern in the severe asthmatics. Instead, we saw a profound enrichment and up-regulation of bitter taste receptors (*TAS2Rs*), which highlighted a pathway previously not associated with severe therapy-resistant asthma. We thus observed a profound enrichment and up-regulation of the bitter taste receptor pathway in leukocytes from the severe asthmatics, which became even more significant from the cluster analyses in the heatmap dendrogram. These findings were further strengthened by our qPCR analyses, also showing increased *TAS2R* expression in these subjects.

In this study we looked at the genome-wide expression pattern in whole white blood cells (WBC) with the aim to find unique expression profiles that could separate the predefined patient groups. By using a global approach that identified genes differentially expressed in any of the contrasts, we were able to identify 1378 significantly ( $p \leq 0.05$ ) altered genes. Unsupervised hierarchical clustering and heatmap of the gene expression in all samples (Figure 2) separated most of the severe asthmatics (cluster I) from controlled asthmatics and the healthy control group (cluster II). Although the expression patterns could classify the patient groups rather well, it is evident that other biological and physiological parameters also contributed to these complex asthma phenotypes.

We thus observed a profound enrichment and up-regulation of the bitter taste receptor pathway in leukocytes from the severe asthmatics, which became even more significant from

the cluster analyses in the heatmap dendrogram. Until recently, the bitter taste receptors (TAS2Rs) were not known to have any role in asthma. However, TAS2Rs have now been connected to the respiratory system by four independent studies. Shah *et al* showed that *TAS2Rs* were expressed on the motile cilia of human airway epithelial cells and that bitter compounds increased the intracellular  $\text{Ca}^{2+}$  concentration and stimulated ciliary beat frequency [22]. The motile cells are responsible for propelling mucus and harmful substances out of the lung and it has been shown that viral infections and cigarette smoke causes a loss of these cilia and might disrupt the defensive mechanism [23]. Very recent data suggest that a specific *TAS2R* (38) is involved in the innate immune defence of upper airways and that variation in this gene contributes to individual differences in susceptibility to respiratory infection [24]. Deshpande and co-workers found that the *TAS2Rs* were expressed in human airway smooth muscle (ASM) and that bitter tastants caused relaxation of isolated ASM and dilation of airways. Inhaled bitter tastants also caused decreased airway obstruction in a mouse model of asthma [9]. We have recently confirmed and extended these observations by documenting that bitter taste receptor agonists also induce relaxation of guinea pig trachea, and that they may relax also maximally contracted bronchi that are unresponsive to betaagonist [25]. In addition, our present findings of inhibitory effects of two *TAS2R* agonists on leukocyte function, suggests that activation of bitter taste receptors may have anti-inflammatory actions as well. Taken together, these studies suggest a protective role for the *TAS2Rs* in asthma. Our study is the first to report upregulation of *TAS2Rs* in human white blood cells in patients suffering from severe, therapy resistant asthma.

In order to examine in more detail which white blood cells express bitter taste receptors we performed qPCR for 11 *TAS2Rs* in isolated lymphocyte, monocyte and neutrophil fractions from a subset of 12 well-characterised adult asthmatic subjects. Results showed that expression of all BTR receptors was greatest in the lymphocyte fraction, and furthermore that

*hTAS2R10* appeared to be the most abundantly expressed. Although *hTAS2R10* was not differentially expressed between the different groups of children it was included in the panel of receptors examined by qPCR as *TAS2R10* was found to be one of the most highly expressed bitter taste receptors on airway smooth muscle in the study by Deshpande et al [9]. While these findings relate to a different group of patients, specific differences in *TAS2R* expression on lymphocytes may underlie the differences observed between the controlled and severe asthmatics, as *TAS2R* expression in lymphocytes was often similar to that observed in mixed leukocyte samples. As similar white blood cell counts were observed between the children with controlled and severe asthma (Table 1), the observed *TAS2R* upregulation is unlikely to be due to differences in leukocyte numbers. Exactly which lymphocyte subsets, and the biological relevance of *TAS2R* expression on these cells, should be the focus of future investigations. Our initial experiments have shown that the *TAS2R* agonists chloroquine and denatonium are able to inhibit the release of several pro-inflammatory cytokines and eicosanoid release from blood leukocytes (Figure 4) suggesting that these agents may have anti-inflammatory as well as bronchodilatory properties, however, more detailed functional studies are still required. The broader profile of activity of chloroquine compared with denatonium, however confirms recent findings in airway smooth muscle [25].

The pathway analyses also demonstrated that T-cell related pathways such as NK T-cell mediated cytotoxicity, were upregulated in controlled asthmatics whereas N-glycan biosynthesis was downregulated in the severe asthma group. The role of natural killer (NK) cells in asthma has been debated and studies have both shown a vast upregulation of NK T-cells in bronchoalveolar-lavage fluid from asthmatic patients [26], as well as no increase at all.[27] Murine models have supported the role of NK T-cells in asthma by showing that NK T-cell deficient mice do not develop, or showed impaired, allergen-induced airway hyperreactivity.[28, 29] Our results point towards a role for NK-T cells in asthma. The N-

glycan biosynthesis pathway is responsible for the production of glycans that couple to proteins and lipids on the cell membrane. Alterations in the distribution of glycoproteins at the cell surface has been suggested to contribute to many chronic human diseases, including autoimmunity [30].

A great number of the genes identified, primarily in severe asthma, were annotated as non-coding RNA (ncRNA), or novel ncRNA. Recent genome-wide expression studies have shown that the human genome produces many thousands of these regulatory, non-protein-coding RNAs and that they play roles in virtually all developmental processes, but primarily in transcriptional and post-transcriptional regulation [31]. Subsequently, ncRNAs have been linked to various human diseases such as cancer, central nervous system disorders and cardiovascular disease [31]. The role of ncRNA in asthma, and in particular therapy-resistant asthma is, however, unclear and remains a target for further investigation.

Twelve out of 97 well-replicated asthma genes were found among the 1378 differentially expressed genes (Table 3). These genes have thus been associated with asthma at the DNA as well as the RNA level, which supports their role in the pathogenesis of disease. Most of the genes were up-regulated only in controlled asthma (*PEX19*, *RORA* and *MYB* being exceptions). This might be due to the fact that most association studies, as well as GWAS, that were consulted to generate the asthma gene list were conducted on combined patient materials consisting mostly of mild/moderate asthmatics.

Our study has limitations. First, we measured gene expression patterns in peripheral blood cells (mainly leukocytes), which are only one of several possible target cells in controlled /severe asthma. However the immune system is a large component in the etiology of asthma. Some other studies have measured gene expression levels from cells obtained from bronchial alveolar lavage or bronchial biopsies [32-34]. A direct comparison with these studies is therefore difficult to make, however, combining data performed in various relevant

tissues might help us paint a more complete picture of asthma. Our pathway analyses were stringent and only explored known pathways, which is always a limiting factor. A liberal inclusion of differentially expressed genes ( $p \leq 0.05$ , regardless of fold change) was compensated with a stricter approach for the functional annotation. This was done in an attempt to increase power but reduce false positives. A larger study size would have been preferred but almost 60 study subjects were nevertheless a rather large number for genome-wide expression analyses. Although history of atopy was an exclusion criterion for the healthy control group, we cannot completely rule out that some control children had specific IgE  $>0.35$  kU/L (since this was not measured).

In conclusion, the study provided new insight into the characteristics of severe therapy-resistant asthma in children. It is of note that this sufficiently powered genome-wide transcriptome analysis was possible only because of the accessibility to a group of asthmatic patients who were exceptionally well phenotyped. Our data in the controlled asthmatics supported differential expression of known pathways, such as T cell related networks. The novel finding was however that the study revealed specific up-regulation of pathways previously not associated with asthma in the severe asthma group, for example N-glycan biosynthesis and in particular bitter taste transduction. The increased expression of bronchodilatory bitter taste receptors and the initial indications of anti-inflammatory responses mediated by these receptors certainly warrant further explorations of their potential as a new target for asthma therapy. It may finally be more than co-incidental that beneficial therapeutic effects have been reported for treatment with chloroquine in asthma [35].

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**Table 1.** Clinical data for the 54 children included in the transcriptome analysis

	SA (n=17)	CA (n=19)	Ctrl (n=18)	p-value for difference†
Age	14 (3)	14 (3)	10 (5)	0.002
Sex (F/M)	7/10	7/12	3/15	Ns
ACT	16(3)	23(2)	NA	NA
ICS*	800 (800-800)	360 (200-400)	NA	NA
FEV <sub>1</sub> , % of predicted	91 (20)	89 (11)	NA	Ns
DRS <sub>methacholine</sub> *	31 (1.6-59)	0.8 (0.2-7.7)	NA	0.01
Total WBC (10 <sup>9</sup> x L <sup>-1</sup> )	6.4 (2.1)	6.0 (1.5)	5.6 (0.9)	Ns
Eosinophils (10 <sup>9</sup> x L <sup>-1</sup> )	0.3 (0.2)	0.3 (0.2)	0.2 (0.2)	Ns
Neutrophils (10 <sup>9</sup> x L <sup>-1</sup> )	3.3 (1.5)	2.5 (0.7)	2.7 (0.8)	Ns
Lymfocytes (10 <sup>9</sup> x L <sup>-1</sup> )	2.3 (0.7)	2.7 (0.7)	2.2 (0.8)	Ns
FE <sub>NO</sub> , ppb*	18 (11-38)	16 (9-21)	NA	Ns
Total IgE, kUA/l*	270 (120-740)	200 (66-545)	NA	Ns
Atopic (%):‡	14 (82)	15 (79)	NA	Ns

Abbreviations in order of appearance: SA, severe asthma; CA, controlled asthma; Ctrl, healthy controls; F, female; M, male; ACT, asthma control test; NA, not applicable; ICS, inhaled corticosteroids; FEV<sub>1</sub>, forced expiratory volume during 1 second; DRS<sub>methacholine</sub>, slope of the dose-response curve for provocation with methacholine; WBC, white blood cells; FE<sub>NO</sub>, fraction of nitric oxide in exhaled air (ppb, parts per billion); IgE, immunoglobulin E (kUA/L, kilounits of antibody per liter). The values are presented as means (standard deviation), unless otherwise indicated. \*Median (interquartile range). † Student's t-test between SA and CA or ANOVA for overall difference between the groups (when possible), and chi2-test for sex and atopy difference. Wilcoxon rank-sum test was used for DRS<sub>methacholine</sub>, FE<sub>NO</sub> and Total IgE difference between SA and CA. ns = not significant (p >0.05).

‡ Atopy was defined as specific IgE >0.35kU/L to inhalant allergens (Phadiatop®)

**Table 2.** Enriched pathways in children with severe or controlled asthma.

KEGG Pathway	Genes			Adj. P*
	CA vs. Ctrl	SA vs. CA	SA vs. Ctrl	
Taste transduction (bitter)		-	<i>ITPR3</i>	0.03
		+ +	<i>TAS2R45</i>	
	+		<i>TAS2R13, TAS2R14, TAS2R19,</i>	
	+		<i>TAS2R20, TAS2R31, TAS2R50</i>	
		+	<i>TAS2R4, TAS2R46, TAS2R5</i>	
Natural killer cell mediated cytotoxicity	+		<i>CD244, CD247, FYN, SH2D1B, GZMB,</i>	0.02
	+		<i>ICAM2, KIR2DL1/ KIR2DL2, KLRD1,</i>	
	+		<i>NCRI, NFATC2</i>	
	+	-	<i>HCST, NCR3, PRF1, ZAP70</i>	
		-	<i>ITGAL, LAT</i>	
	-		<i>TNFRSF10D</i>	
N-Glycan Biosynthesis		-	<i>VAV2</i>	0.02
		-	<i>B4GALT3, ALG3, DPAGT1</i>	
		-	<i>ALG1, ALG12, ALG9, DPM2, GANAB,</i>	
		-	<i>MOGS, RPN2</i>	
Dendrogram row cluster†				
Natural killer cell mediated cytotoxicity	A		<i>CD244, CD247, FYN, SH2D1B, GZMB,</i> <i>HCST, ITGAL, ICAM2, KIR2DL1, LAT,</i> <i>NFATC2, PRF1, ZAP70</i>	0.02
T cell receptor signaling pathway	A		<i>CD247, CD4, CBLB, FYN, CARD11,</i> <i>LAT, NFATC2, NFKB1, PRKCQ,</i> <i>AKT2, ZAP70</i>	0.03
Citrate cycle (TCA cycle)	A		<i>ACLY, ACO2, IDH2, IDH3A, IDH3B,</i> <i>MDH2</i>	0.04
NA	B		NA	
NA	C		NA	
Taste transduction (bitter)	D		<i>TAS2R13, TAS2R14, TAS2R19,</i> <i>TAS2R20, TAS2R31, TAS2R4,</i> <i>TAS2R45, TAS2R46, TAS2R5, TAS2R50</i>	4.2x10 <sup>-10</sup>

Abbreviations: SA, severe asthma; CA, controlled asthma; Ctrl, healthy controls;

Comments: + or - indicates if the genes are significantly up- or down-regulated in each respective contrast. \*Benjamini and Hochberg's correction for multiple testing. NA implies no significant pathways. †See Figure 2 for dendrogram row cluster classification.

**Table 3.** Twelve previously identified asthma candidate genes enriched in our list of 1378 differentially expressed genes.

ID	Gene	CA-Ctrl		SA-CA		SA-Ctrl		Adj. F, P*†	GO function‡
		FC	Adj. P*	FC	Adj. P*	FC	Adj. P*		
8043791	<i>INPP4A</i>	1.16	0.006	-1.06	0.26	1.09	0.10	0.004	Phosphatidylinositol-3,4 / -4-5-bisphosphate 4-phosphatase activity. hydrolase activity
7921571	<i>PEX19</i>	-1.00	0.99	-1.17	0.01	-1.17	0.01	0.005	Protein binding
7989365	<i>RORA</i>	1.26	0.02	-1.03	0.78	1.22	0.02	0.006	DNA, protein and zinc ion binding. transcription factor activity. steroid hormone receptor
8112220	<i>PDE4D</i>	1.28	0.01	-1.09	0.40	1.18	0.08	0.007	Catalytic and hydrolase activity. metal ion binding 3',5'-cyclic-nucleotide phosphodiesterase activity
8075886	<i>IL2RB</i>	1.58	0.02	-1.29	0.15	1.22	0.35	0.01	Interleukin-2 receptor activity
8008029	<i>TBX21</i>	1.36	0.03	-1.23	0.11	1.11	0.56	0.02	DNA binding. transcription factor activity
8122202	<i>MYB</i>	-1.08	0.62	-1.11	0.17	-1.20	0.01	0.03	DNA replication and transcriptional repression
8014916	<i>ORMDL3</i>	1.16	0.10	-1.16	0.054	1.01	0.97	0.04	Protein binding
7913883	<i>PAFAH2</i>	1.25	0.047	-1.13	0.24	1.11	0.44	0.04	1-alkyl-2-acetyl-glycerophosphocholine esterase activity. hydrolase activity
8109086	<i>ADRB2</i>	1.22	0.052	-1.09	0.38	1.12	0.29	0.04	beta2-adrenergic receptor activity. protein binding
7974363	<i>PTGDR</i>	1.64	0.057	-1.23	0.41	1.34	0.28	0.04	Prostaglandin receptor activity. protein binding. signal transducer
8115455	<i>HAVCR1</i>	1.16	0.18	-1.18	0.050	-1.01	0.93	0.048	Receptor activity

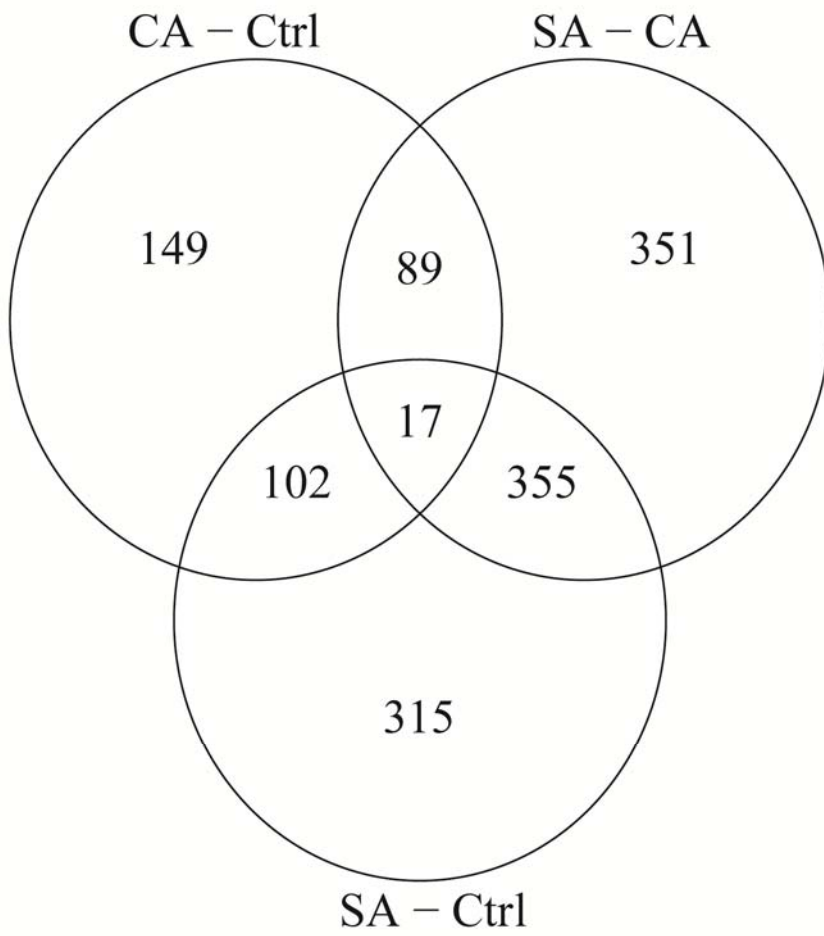
Abbreviations: CA, controlled asthma; SA, severe asthma; Ctrl, healthy controls; FC, Fold change. \*Benjamini and Hochberg's correction for multiple testing. †A global F-test to test for significance in any of the contrasts. ‡A summary of GO function from the EBI database quickGO.

**Figure 1. A Venn Diagram illustrating the distribution of differentially expressed genes between SA, CA and Ctrl children.** The numbers in the circles depict the number of genes uniquely differentially expressed (DE) or shared between the contrasts; controlled asthma vs. healthy controls (CA-Ctrl) severe asthma vs. controlled asthma (SA-CA) and severe asthma vs. healthy controls (SA-Ctrl). The number of down- or up-regulated genes in each of the contrasts is indicated below the diagram.

**Figure 2. Global gene expression patterns in SA, CA and Ctrl children.** Unsupervised hierarchical clustering and heat map illustrates each individual's expression pattern in all 1378 significant ( $p \leq 0.05$ ) differentially expressed genes. The column dendrogram separates the subjects into three main clusters (I, II a and II b). Row dendrogram represents gene clusters crudely divided into cluster A, B, C and D. Normalized gene expression is indicated by the row Z-score where yellow-red represents up- and blue down-regulated genes. SA, severe asthma; CA, controlled asthma; Ctrl, healthy controls.

**Figure 3. Expression of human bitter taste receptors in isolated leukocytes from adult asthmatics.** The relative expression of hTAS2Rs (4, 5, 10, 13, 14, 19, 20, 31, 45, 46 and 50) compared to GAPDH, as measured by qPCR, was examined in mixed leukocytes and isolated lymphocytes, monocytes and neutrophils (n=12). Results are shown as mean+SEM and expression levels of the different TAS2Rs in the different cell populations were compared by Students t-test, \*  $p < 0.05$ .

**Figure 4. The effect of TAS2R agonists on LPS-induced pro-inflammatory cytokine and PGE<sub>2</sub> release.** Blood samples from 12 asthmatic adults was incubated for 24 hours, either unstimulated (C) or stimulated with LPS (100µg/ml), in the absence (LPS) or presence of chloroquine (●) or denatonium (■) (10-1000µM). Serum levels of 17 cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, TNFα, G-CSF, GM-CSF, IFNγ, MCP-1 and MIP-1α, as well as PGE<sub>2</sub>, were analysed by luminex and enzyme immunoassay respectively. Results for IL-6, IL-8 and MIP-1α are not shown as these were present at levels above the detection limit of the assay used. The scale use for all graphs is pg/ml apart from for TNFα, IFNγ, IL-1β and PGE<sub>2</sub> where it is ng/ml. \*p<0.05 compared to LPS alone.



	CA - Ctrl	SA - CA	SA - Ctrl
down	29	510	261
up	328	302	528

Figure 1.

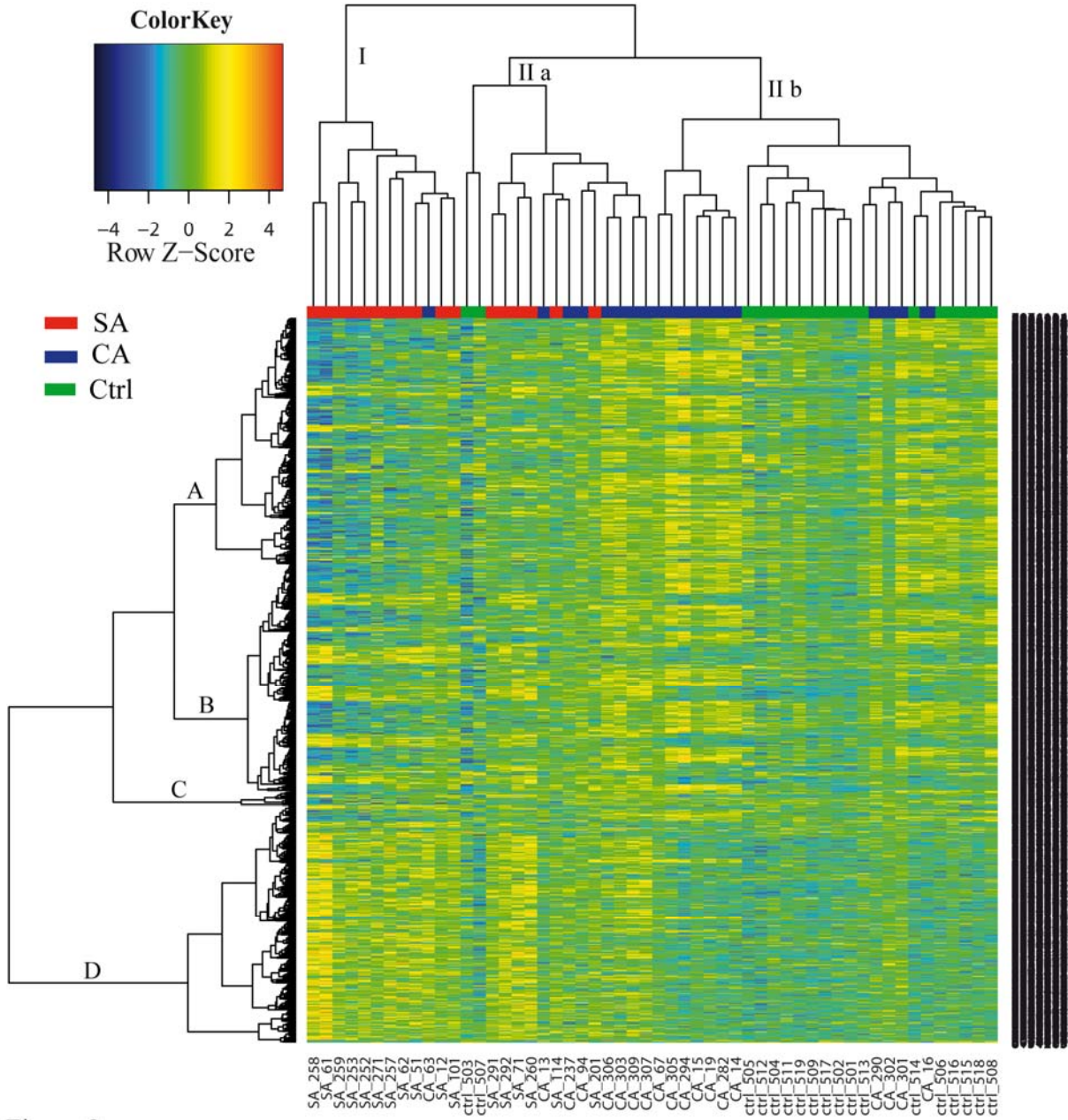


Figure 2.



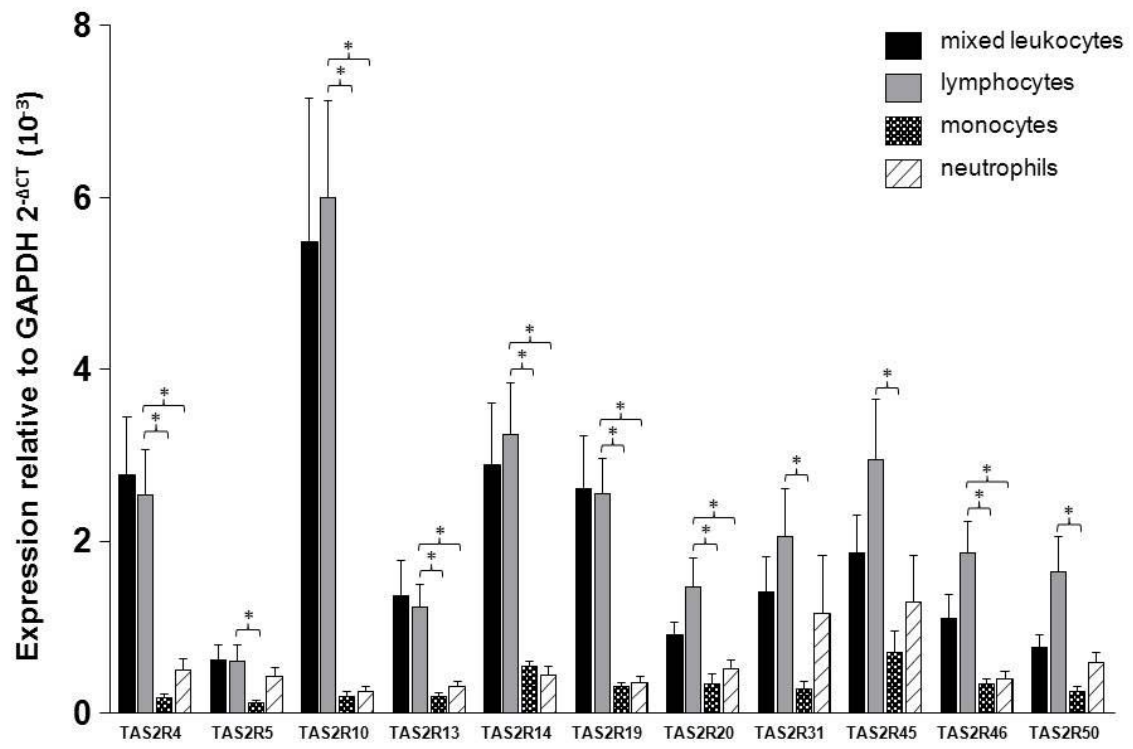


Figure 3.

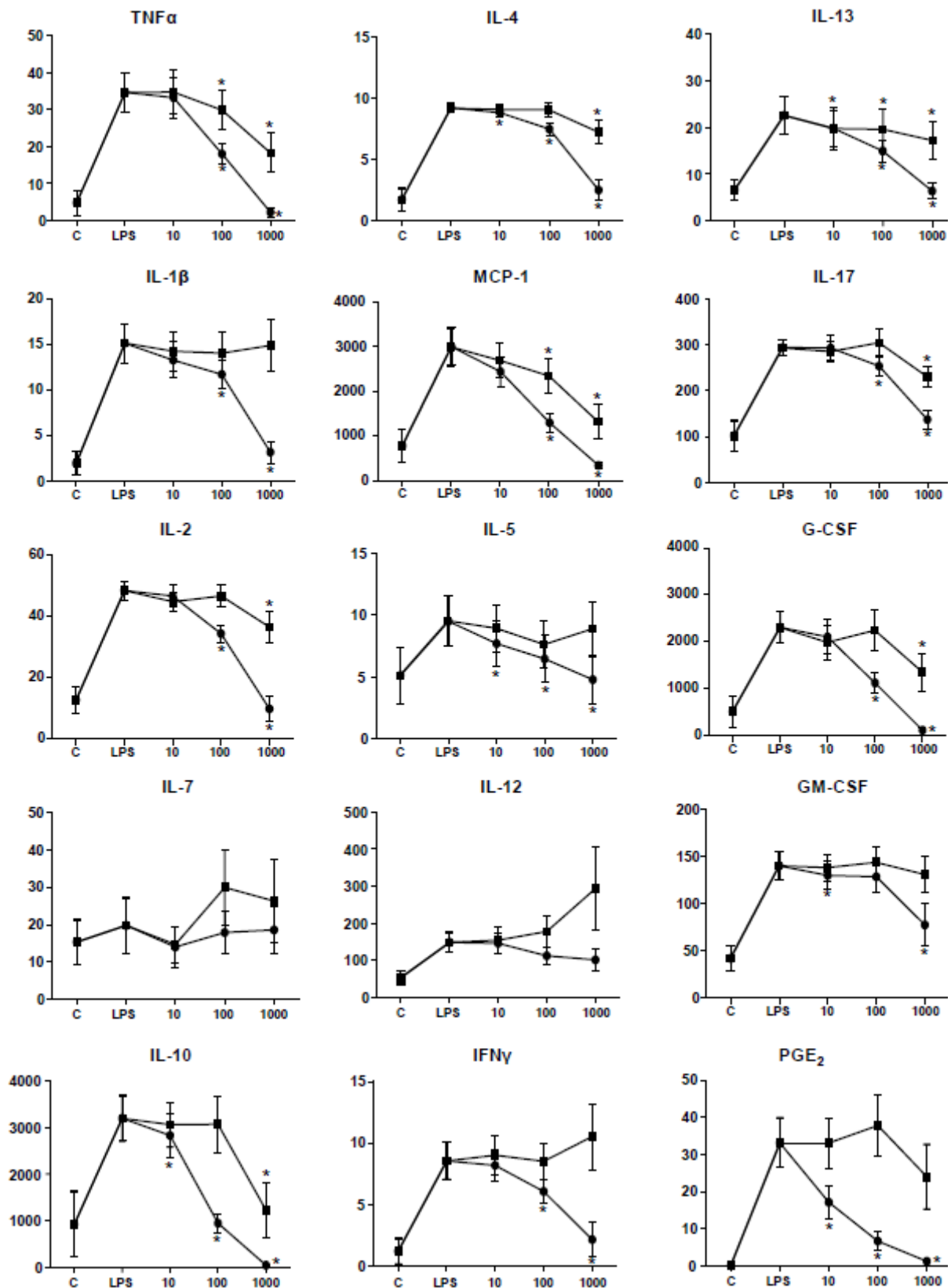


Figure 4.