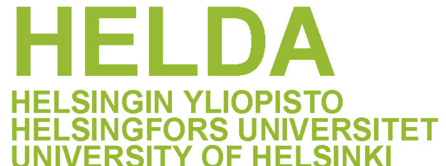

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ORIGINAL ARTICLE

Tonsillar transcriptional profiles in atopic and non-atopic subjects

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Abstract

Background: Emerging research suggests that local lymphatic tissue such as tonsils have important role in regulating the immune responses. However, allergen sensitization-induced alterations in transcriptome of tonsils are not known.

Objectives: To examine the key differences in tonsillar gene expression between atopic and non-atopic subjects and further by type of sensitization.

Methods: RNA-sequencing was performed on 52 tonsillar samples from atopic and non-atopic tonsillectomy patients. Sensitization to common food- and aero-allergen was defined by allergen specific IgE. Following groups were studied: (1) aero- and food-allergen sensitized (AS+FS) versus non-sensitized (NS), (2) aeroallergen-sensitized (AS) versus food-allergen sensitized (FS), (3) AS versus NS, (4) FS versus NS. Bioinformatics analysis was done using DESeq2(v3.10.2), WGCNA and GATK pipeline in R software (v3.3.1). Protein–protein interaction network was made from String database.

Results: We studied 13 aeroallergen-sensitized, 6 food-allergen sensitized, 4 both food-and aero-allergen-sensitized and 29 non-sensitized tonsillectomy patients.

Abbreviations: AS:, Aeroallergen-sensitized; AS+FS:, Aero- and food-allergen sensitized; BP:, Biological process; CC:, Cellular Component; CPM:, Counts per millions; DEGs:, Differentially expressed genes; FDR:, False discovery rate; FS:, Food-allergen sensitized; GO:, Gene Ontology; IgE:, Immunoglobulin E; MF:, Molecular Function; NS:, Non-sensitized; RNA-seq:, RNA-sequencing; TMM:, Trimmed mean of M values.

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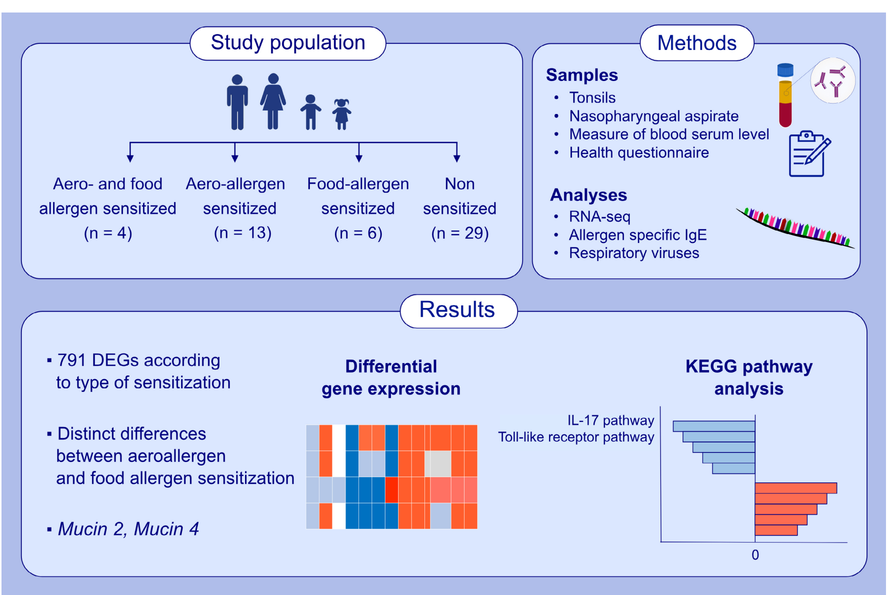
Overall, 697 unique differentially expressed genes (DEGs) were detected in all sensitized subgroups including chemokines (*CXCL2*, *CXCL8*, *CXCL10*, *CXCL11*), *IL-20RA*, *MUC1* and *MUC20*. When comparing different groups, the gene expression profiles overlapped except the AS versus FS group comparison, suggesting significantly different gene expression between the two sensitization subgroups. Furthermore, aeroallergen-sensitized subjects had more prominent immune responses compared with non-sensitized and food-allergen sensitized subjects including gene expression for IL-17 pathway and Toll-like receptor signalling pathway.

Conclusion: Allergic sensitization is associated with extensive tonsillar transcriptomic alterations and changes in immune related genes and pathways. Distinct differences were found between aero-allergen and food-allergen sensitization.

KEYWORDS
aeroallergen, atopy, IL-17, tonsil, transcriptome



GRAPHICAL ABSTRACT

Allergic sensitization, especially to aeroallergens, is extensively associated with tonsillar transcriptomics. Aeroallergen sensitized subjects were characterized by increased expression of cytokines, such as IL-17, and chemokines.

1 | INTRODUCTION

The prevalence of allergic sensitization is approximately 30% in developed countries.[1] Upper airways serve as the main route of allergens, irritants and pathogens to the body. Hence, interactions between environment, airway barriers and the immune system play an important role in health and pathobiology of allergy.[2-5] The palatine tonsils are the secondary nasopharyngeal lymphoid tissue and constitute the first contact point of the immune system to inhaled or ingested pathogens.[5] The impaired functioning of tonsils may lead to tonsillar hypertrophy and affect the quality of life especially in children. The most common indications for tonsillectomy in children are recurrent tonsillitis, obstructive sleep disorders and complications of tonsillitis.[6,7]

Atopy can affect the tonsillar immune response especially when combined with bacterial or viral infection.[7] After contact with antigens, tonsils induce several immune responses where production of pro-inflammatory cytokines and chemokines is integral.[8] It has been studied previously that different clinical characteristics and viral infections are associated with distinct immune responses. We have previously shown that tonsillar microbiome also exhibited difference in the atopic compared with non-atopic patients.[9] Thus, it is important to study the difference of tonsillar gene expression in atopic compared with non-atopic patients since this has been relatively little studied.

Tonsils provide a unique and important in vivo model for studying the immune responses to microbes and other antigens.[9] Although

there are many studies related to diseases of palatine tonsils, for example hypertrophy and recurrent tonsillitis; the gene expression in palatine tonsils in atopic and non-atopic subjects is not well studied in detail.[10-12] Studying transcriptomic changes of palatine tonsil samples from children with different sensitization patterns will help to understand the lymphoid tissue dynamics in relation to sensitization.

1.1 | Aims and hypothesis

The aim was to analyse palatine tonsillar transcriptome to identify key pathways in relation to sensitization pattern by using the RNA-sequencing (RNA-seq). The hypothesis was that there is an association between different sensitization patterns and gene expression profiles of key cytokines.

2 | METHODS

The methods for inclusion of study subjects and collection of clinical and laboratory data are shown in Appendix S1. Self-reported allergies and other clinical data were asked with standard questionnaire (Table S2).[13] Allergic sensitization was tested with allergen specific immunoglobulin IgE antibodies to any of the common food-allergen (codfish, cow's milk, egg, peanut, wheat, soybean) or aeroallergens (cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum* and *Dermatophagoides pteronyssinus*) with cut-off level 0.35 kU/L (Phadiatop Combi®, Phadia, Uppsala, Sweden) (Table 1 and Table 2). Animal sensitization was defined as positive IgE antibodies to cat, dog, horse or *Dermatophagoides pteronyssinus*. Birch, mugwort, timothy and *Cladosporium herbarum* were considered as pollen aeroallergens.[14] Respiratory viruses were detected from nasopharyngeal aspirates and intratonsillar samples by using PCRs (including reverse transcription step when applicable) on nucleic acid extracts.[13,15,16]

2.1 | Definitions

- Atopy (sensitization) was defined as positive immunoglobulin (Ig) E antibody (≥0.35 kU/L) to any of the following allergens: codfish, cow's milk, egg, peanut, soybean, wheat, cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum* and *Dermatophagoides pteronyssinus* (Phadiatop Combi®, Phadia, Uppsala, Sweden).
- Food-allergen sensitization was defined as positive IgE antibodies to any of the former 6 allergens.
- Aero-allergen sensitization was defined as positive IgE antibodies to any of the latter 8 allergens.
- Highly sensitized was defined as IgE > 10 kU/L to any of the allergens detected.
- Allergic symptoms were defined as self-reported allergic rhinitis, doctor-diagnosed atopic eczema (asked in the questionnaire, Table S2) and doctor-diagnosed asthma (asked in the questionnaire).
- Virus infection was defined by positive virus PCR test from nasopharyngeal aspirate (NPA) or tonsil, and virus coinfection if two or more virus PCR tests were positive.
- Tonsillar hypertrophy was defined as enlarged tonsils causing symptoms, for example obstructive sleep disorder, snoring and eating problems.
- Recurrent tonsillitis was defined as several pharyngitis in the medical records.

2.2 | Comparisons

Tonsillar whole-transcriptome gene expression was compared between the following predefined groups:

Atopic (allergic sensitized) versus non-atopic (non-sensitized).

2.3 | Subgroup's analysis

1. Aero- and food-allergen sensitized (AS+FS) versus non-sensitized (NS).
2. Aeroallergen-sensitized (AS) versus food-sensitized (FS).
3. Aeroallergen-sensitized (AS) versus non-sensitized (NS).
4. Food-sensitized (FS) versus non-sensitized (NS).

2.4 | Additional, exploratory comparisons

5. Highly sensitized (allergen specific IgE sensitization >10 kU/L) versus non-sensitized.
6. Allergic + sensitized versus non-allergic-non-sensitized.

2.5 | RNA-sequencing, data processing and read mapping

Total RNA from cell samples was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed with the Revert Aid M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) using random hexamer primers according to the manufacturers. RNA-sequencing on the samples was carried out with Illumina HiSeq 2500. Preprocessing and quality control of reads were performed with FastQC (v0.10.1)[17] and the filtered single-end reads were aligned to the human genome (GRch38) using HISAT (v2.1.0).[18] The number of uniquely mapped reads varied between 41 and 50 million per sample.

2.6 | Bioinformatics analysis of RNA-sequencing data

Raw read counts were transformed into normalized data using the counts function in Deseq2[19] R software (v3.3.1). Differentially

TABLE 1 Patient characteristics

Factor	Atopic ($n = 23$)	Non-atopic ($n = 29$)	p-value
Median age (range), years	12.4 (2.0–38.1)	7.5 (3.0–21.1)	0.14
Male	12 (52%)	16 (55%)	0.70
Self-reported allergy[a]	11 (47%)	8 (28%)	0.069
Indication for adeno−/tonsillectomy			
Recurrent tonsillitis	11 (48%)	13 (45%)	0.70
Tonsillar hypertrophy	11 (48%)	13 (45%)	0.94
Other indication[b]	1 (4%)	3 (10%)	0.62
Allergen specific sensitization			
Food allergen	6 (26%)	0 (0%)	
IgE kU/L[c]	1.1 (0.7, 1.7)		
Aeroallergen	13 (56%)	0 (0%)	
IgE kU/L[c]	4.3 (1.1, 39.1)		
Both food and aeroallergen	4 (17%)	0 (0%)	
IgE kU/L, food[c]	1.5 (0.6, 10.1)		
IgE kU/L, aero[c]	46.9 (0.6, 98.2)		
Physician diagnosed asthma	5/21 (27%)	3/26 (12%)	0.44
Self-reported allergic rhinitis	10/19 (53%)	3/27 (11%)	0.003
Physician-diagnosed atopic dermatitis	2/20 (10%)	6/29 (21%)	0.32
Upper respiratory symptoms on the operation day[d]	4/18 (22%)	4/25 (16%)	0.70
Cough on the operation day	2/18 (4%)	1/25 (11%)	0.56
Respiratory viruses in tonsillar tissue	11 (48%)	15 (52%)	0.78
Respiratory viruses in nasopharynx	18 (75%)	24 (83%)	0.49
Smoking or exposure to smoking	10/22 (45%)	15/28 (54%)	0.57
Use of antibiotics within 1 year	15/19 (79%)	18/28 (64%)	0.28
Season of sampling			
April–August (in-season)	9 (39%)	11 (38%)	0.93
September–March (off-season)	14 (61%)	18 (62%)	0.93

Note: Data are expressed as number of subjects (%) except age. Differences between atopic vs. nonatopic subjects were calculated with Mann–Whitney *U* test, Chi square test or Fischer exact test (when counts <5). Allergen specific sensitization was defined as positive immunoglobulin (Ig) E antibody (≥0.35 kU/L) to any of the following allergens: codfish, cow's milk, egg, peanut, soybean, wheat, cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum* and *Dermatophagoides pteronyssinus* (Phadiatop Combi®, Phadia, Uppsala, Sweden). The latter eight allergens were considered as aeroallergens.

[a]Animal or housedust mite, pollen, food or drug allergy.

[b]Chronic white patches in tonsils ($n = 1$), teeth braces ($n = 1$), periodic fever ($n = 2$).

[c]Data expressed as median (IQR).

[d]Two had rhinitis, two had throat pain, two had cough and rhinitis, one had cough and throat pain and one had rhinitis and throat pain.

expressed genes (DEGs) were identified with Deseq2 (v3.10.2) threshold for statistical significance was set at a false discovery rate (FDR) < 0.05 and log2 (foldchange) greater than 1 or less than −1. Hierarchical clustering of genes was done using Pearson correlation and ward linkage. A heatmap of gene expression matrix was produced with Pheatmap R package (v3.3.1). The weighted gene coexpression network analysis[20] algorithm was used to construct networks for gene expression from all study participants.

2.7 | Gene ontology analysis and functional protein network analysis

Gene ontology (GO) and pathway enrichment analysis was performed with cluster profiler.[21] Functional protein association networks were investigated by using STRING (http://string-db.org/). Cytokines clustering analysis was performed by using in-house collected clusters of cytokines.

TABLE 2 Patient characteristics between the groups

Factor	Aeroallergen sensitized (AS) $n = 13$	Food allergen sensitized (FS) $n = 6$	Aero- and food-allergen sensitized (A + FS) $n = 4$	Non-sensitized (NS) $n = 29$	p-value
Median age (range), years	14.2 (7.5–38.1)	4.6 (2.0–15.5)	6.8 (4.1–18.5)	7.5 (3.0–21.1)	0.006
Male	8 (62%)	2 (33%)	2 (50%)	16 (55%)	0.71
Self-reported allergy[a]	6/11 (55%)	2/5 (40%)	3 (75%)	8/26 (31%)	0.28
Indication for adeno−/tonsillectomy					
Recurrent tonsillitis	9 (69%)	1 (17%)	1 (25%)	13 (45%)	0.13
Tonsillar hypertrophy	4 (31%)	5 (83%)	2 (50%)	13 (45%)	0.20
Other indication[b]	0	0	1 (25%)	3 (10%)	0.31
Physician diagnosed asthma	2/12 (17%)	2/5 (40%)	1 (25%)	3/26 (12%)	0.46
Self-reported allergic rhinitis	6/10 (60%)	1/5 (20%)	3 (75%)	3/27 (11%)	0.004
Physician-diagnosed atopic dermatitis	0	0	2 (50%)	6 (21%)	0.088
Upper respiratory symptoms on the operation day[c]	2/9 (22%)	1/5 (20%)	1 (25%)	4/25 (16%)	0.96
Cough on the operation day	1/5 (20%)	1/9 (11%)	0	1/25 (4%)	0.54
Respiratory viruses in tonsillar tissue	7 (54%)	2 (33%)	2 (50%)	15 (52%)	0.86
Respiratory viruses in nasopharynx	9 (69%)	6 (100%)	3 (75%)	24 (83%)	0.44
Smoking or exposure to smoking	7/12 (58%)	3 (50%)	0	15/28 (54%)	0.21
Use of antibiotics within 1 year	8/10 (80%)	5/5 (100%)	2/4 (50%)	18/28 (64%)	0.28
Season of sampling					
April–August	4 (31%)	2 (33%)	3 (75%)	11 (38%)	0.45
September–March	9 (69%)	4 (67%)	1 (25%)	18 (62%)	0.45

Note: Data are expressed as number of subjects (%) except age. Differences between the groups were calculated with Kruskal–Wallis test, Chi square test or Fischer exact test (when counts <5). Allergen specific sensitization was defined as positive immunoglobulin (Ig) E antibody (≥0.35 kU/L) to any of the following allergens: codfish, cow's milk, egg, peanut, soybean, wheat, cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum* and *Dermatophagoides pteronyssinus* (Phadiatop Combi®, Phadia, Uppsala, Sweden). The latter eight allergens were considered as aeroallergens.

[a]Animal or housedust mite, pollen, food or drug allergy.

[b]Chronic white patches in tonsils ($n = 1$), teeth braces ($n = 1$), periodic fever ($n = 2$).

[c]Two had rhinitis, two had throat pain, two had cough and rhinitis, one had cough and throat pain and one had rhinitis and throat pain.

2.8 | Variants analysis

In order to find the regulatory variants responsible for the differential expression, we performed eQTL analysis which identifies cis and trans regulatory elements. For this purpose, variant calling from the HISat2 alignments was performed using Genome Analysis Toolkit (GATK)[22] following the best practices (https://gatk.broadinstitute.org/hc/en-us/articles/360035531192-RNAseq-short-variant-discovery-SNPs-Indels-; last accessed 03.08.2020). The SNPs were functionally annotated using ANNOVAR.[23] The correlation between the gene expression and the expressed SNPs was assessed using ReQTL implemented in R.[24] Finally, SNPs that passed the filtering criteria were subjected to eQTL analysis (p value cis < 1e-6; p value trans <1e-6) along-with the batch corrected gene expression matrix, sensitization pattern and other covariates (Age groups and sex).

3 | RESULTS

3.1 | Differential gene expression in palatine tonsil samples

3.1.1 | Atopic versus non-atopic

To obtain an overview of the tonsillar gene expression differences in atopic ($n = 23$) and non-atopic ($n = 29$) subjects, we compared the RNA-seq data in these groups. A linear regression model was fitted to the data to analyse the variation at the group level. However, the comparison of atopic and non-atopic subjects showed no statistically significant results (FDR <0.05). Most interesting findings were found from subgroup comparisons (see below).

3.1.2 | Aero- and food-allergen sensitization versus no sensitization

When comparing AS+FS and NS groups, a total of 97 (5 up- and 92 down-regulated) DEGs were identified (FDR <0.05 and log2 (fold-change) greater than 1 or less than −1) (Figure 1A, Table E1 in this article's Online Repository). Of these 97 DEGs, the top upregulated genes were *CXCL10*, *CXCL11* and *CXCL9* (Table 3). DEGs in this group were found to be enriched in KEGG pathways viral protein interaction with cytokine and cytokine receptor, cytokine–cytokine receptor interaction, chemokine signalling pathway and Toll-like receptor signalling pathway (Figure 2A). The downregulated genes were enriched in GO BP cornification, keratinization and cornified envelope (Table GO results in this article's Online Repository).

3.1.3 | Aeroallergen sensitization versus food-allergen sensitization

When comparing AS and FS groups, a total of 680 (495 up- and 185 down-regulated) DEGs were identified (FDR <0.05 and log2 (foldchange) greater than 1 or less than −1) (Figure 1A, Table E2 in this article's Online Repository). Of these 680 DEGs, some DEGs were linked to immune response, for example *CXCL2*, *CXCL3*, *CXCL5*, *CXCL8*, *CXCL9*, *CXCL10*, *CXCL11*, *CXCL2*, *MUC1*, *MUC4*, *MUC20*, *DEFB4A and DEFB1* (Table 3). Upregulated genes in AS subjects were enriched in GO BP including inflammatory response, cytokine mediated signalling pathway, neutrophil chemotaxis and neutrophil degranulation. Upregulated genes were also enriched for KEGG pathways such as Pertussis, IL-17 signalling pathway, viral protein interaction with cytokine and cytokine receptor and Toll-like receptor signalling pathway (Figure 2A). KEGG enrichment for downregulated genes was found for oxidative phosphorylation and Fanconi anaemia pathway (Table GO results in this article's Online Repository).

3.1.4 | Aeroallergen sensitization versus no sensitization

When comparing AS and NS groups, a total of 76 (56 up- and 20 down-regulated) DEGs were identified (FDR <0.05 and log2 (fold-change) greater than 1 or less than −1) (Figure 1A, Table E3 in this article's Online Repository). We found several interesting, upregulated genes in the comparison AS versus NS group including *VSIG2*, *IL-20RA*, *LGALS7*, *DEFB4A*, *FOS*, *CXCL2*, *CXCL3*, *CXCL5*, *CXCL8*, *DEFB1* and *CXCR2* (Table 3). KEGG pathway enrichment showed the up-regulation of genes for IL-17 pathway, viral protein interaction with cytokine and cytokine receptor, cytokine–cytokine receptor interaction, chemokine signalling pathway and Toll-like receptor signalling pathway, NF-Kappa B signalling pathway and NOD-like receptor signalling pathway (Figure 2A).

The upregulated genes in this comparison and from other comparisons were clustered into a fine string network (Figure 2B). These upregulated genes were found to be enriched in chemokine-mediated signalling pathway, cytokine-mediated signalling pathway, neutrophil chemotaxis, inflammatory response and response to bacterium. There were no enrichment results found for the downregulated genes in this group.

3.1.5 | Food-allergen sensitization versus no sensitization

When comparing FS and NS groups, a total of 174 (14 up- and 160 down-regulated) DEGs were identified (FDR <0.05 and log2 (fold-change) greater than 1 or less than −1) (Figure 1A, Table E4 in this article's Online Repository) These DEGs included *CXCL5*, *CXCL9*, *CXCL10* and *CXCL11* (Table 3). Upregulated genes were enriched in several GO BP including Electron transport coupled proton transport, oxidative phosphorylation and aerobic respiration (Figure 2A & B). Downregulated genes were enriched in GO BP Cornification, epithelial cell differentiation and programmed cell death.

3.2 | Overview of tonsillar RNA-sequencing transcriptome

Pairwise comparisons (Figure 1A) showed different levels of differentially expressed genes with significant differences in AS versus FS group (Table E2). Many DEGs were observed overlapping in all group comparisons except the AS versus FS group that had 442 different DEGs (Figure 1C), suggesting that the gene expression is significantly different in these two types of sensitizations. The overall unique DEGs in all group comparisons are shown in heatmap (Figure 1B), with 97, 680, 76 and 174 DEGs in AS+FS versus NS, AS versus FS, AS versus NS and FS versus NS respectively.

Interestingly, AS+FS versus NS and FS versus NS exhibited same overall expression profile while AS versus NS and AS versus FS showed different pattern of gene expression. Mainly, DEGs in AS subjects showed a distinct expression profile in the heatmap. In addition, immune-related gene cluster analysis showed distinct gene expression in all group comparisons (Figure 3A,B).

3.3 | Network analysis

We constructed weighted gene co-expression networks of the RNA-seq data to perform functional classification related to each subject group. WGCNA (Weighted Gene Co-expression Network Analysis) analysis can identify clusters or modules of genes that have similar gene expression pattern. Hereby, we hypothesize that modules of genes that are co-expressed are most likely to participate in same biological functions. In this analysis, we identified 22 modules (Figure 4A,C); 5 modules were found statistically significant

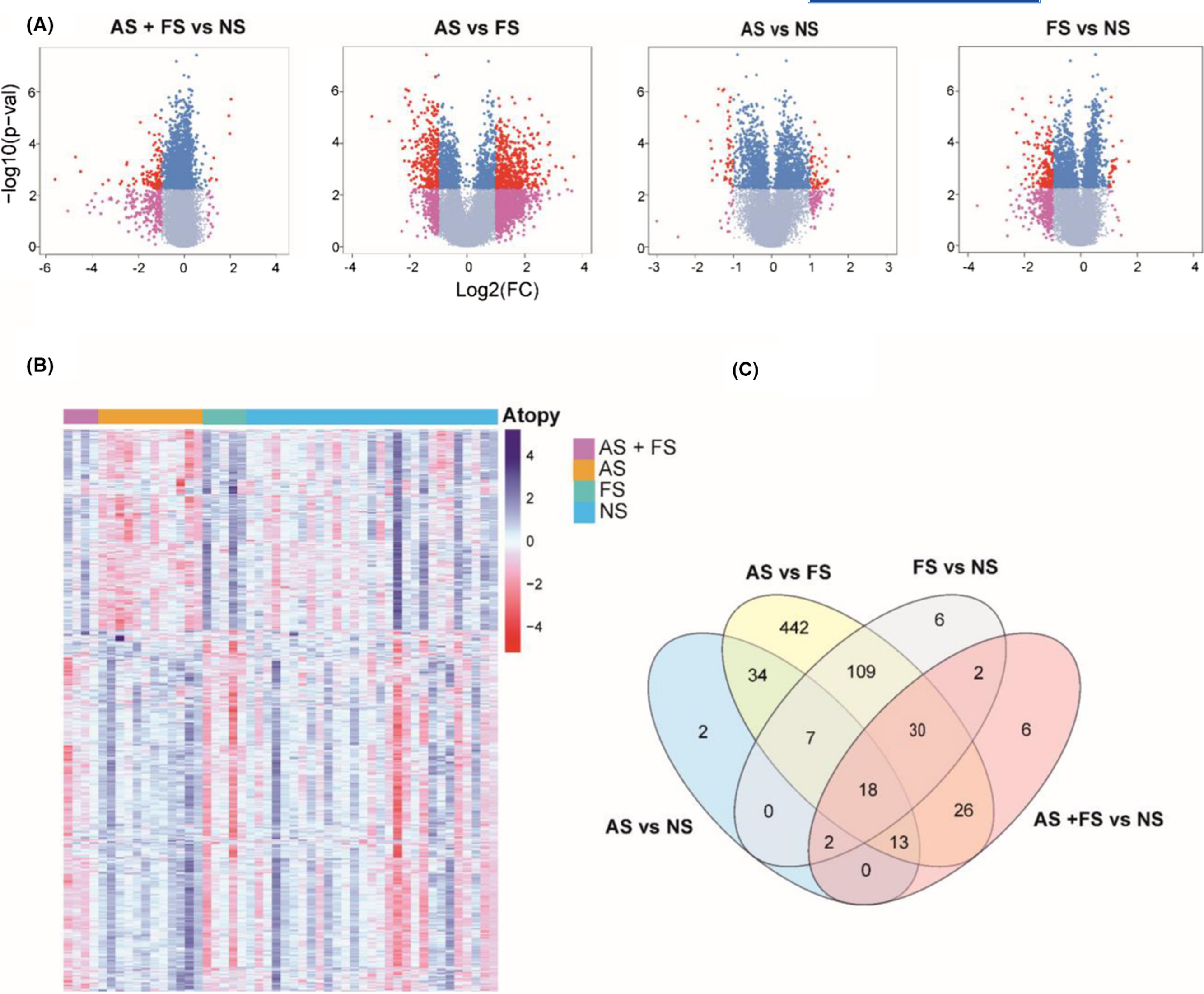


FIGURE 1 Differential gene expression in sensitized and non-sensitized subjects. (A) Volcano plots show the DEGs measured by the RNA-seq analysis in the four groups, that is aero- and food-allergen sensitized versus non-sensitized (AS+FS versus NS), aeroallergen sensitized (AS) versus food-allergen sensitized (FS), AS versus NS, FS versus NS and (please see methods for details). Red, pink and blue dots represent DEGs at FDR <0.05 & log2|FC| > 1, log2|FC| > 1, FDR <0.05 respectively. (B) Heatmap exhibits gene expression in unique DEGs (FDR <0.05 & log2|FC| > 1) in all the subject groups. Gene expression changes (log2 fold changes from their mean values) are represented by colors (blue to red) in the heat map. (C) Venn diagram shows DEGs (FDR <0.05 & log2|FC| > 1) between each pairwise comparison of the four groups; numbers show DEGs.

in self-reported allergy, food and aeroallergen sensitization. Blue module (Figure 4C) was positively associated with food-allergen sensitization and negatively associated with aeroallergen sensitization. Functional enrichment of genes in blue module showed enrichment in Fanconi anaemia pathway, B-cell receptor signalling pathway and viral carcinogenesis. This functional characterization suggests that genes co-expressed in blue module is related to atopy. GO BP (Biological Process) enrichment showed results in viral process. Pink module that was enriched in allergy, the genes in this module were enriched in antigen processing: Ubiquitination and Proteasome degradation and ISG1-antiviral mechanism. We identified significantly overrepresented biological pathways and gene ontology terms for each module in the network. GO analysis revealed the enrichment in response to organic substance and viral process. Protein–protein interaction network of genes clustered in firebrick4 module showed the interaction of the proteins (Figure 4B). GO enrichment of genes in red colour nodes exhibited response to cytokine stimulus, regulation of inflammatory response, regulation of cytokine production and viral process.

3.4 | Variants analysis

We performed variant analysis of RNA-seq data to identify the genomic variants that were associated with sensitization and types of sensitizations. Altogether, 56,872 filtered SNPs were employed

TABLE 3 Immune system-related DEGs

Rank	Genes	Description	Function	Fold Change	Log2FC	FDR	Comment (references)
Immune related DEGs in AS+FS versus NS subjects							
1.	CXCL9	C-X-C motif chemokine Ligand 9	This antimicrobial gene encodes secreted proteins involved in immunoregulatory and inflammatory processes.	2.25	1.17	0.01	
2.	IP-10 (CXCL10)	C-X-C motif chemokine Ligand 10	Stimulation of monocytes, natural killer and T-cell migration	4.08	2.03	0.001	Allergic inflammation,[25] Asthmatic airway[26]
3.	CXCL11	C-X-C motif chemokine Ligand 11	Development, homeostasis and function of the immune system	3.91	1.97	0.005	Allergic inflammation[25]
Immune related DEGs in AS versus FS subjects							
1.	CXCL2	C-X-C motif chemokine Ligand 2	Antimicrobial gene involved in immunoregulatory and inflammatory processes	2.5	1.3	0.04	
2.	CXCL3	C-X-C motif chemokine Ligand 3	Plays a role in inflammation and as a chemoattractant for neutrophils	3.6	1.85	0.04	
3.	CXCL5	C-X-C motif chemokine Ligand 5	Involved in recruitment of leukocytes and neutrophils		6.6	0.002	
4.	CXCL8	C-X-C motif chemokine Ligand 8 (also known as IL-8)	Major mediator of the inflammatory response	0.25	2.11	0.03	
5.	CXCL9	C-X-C motif chemokine Ligand 9		5.76	2.4	0.02	
6.	IP-10 (CXCL10)	C-X-C motif chemokine Ligand 10	Stimulation of monocytes, natural killer and T-cell migration	4.84	2.2	0.002	Allergic inflammation,[25] Asthmatic airway[26]
7.	CXCL11	C-X-C motif chemokine Ligand 11	Development, homeostasis, and function of the immune system	5.6	2.5	0.005	Allergic inflammation[25]
8.	IL20RA	Interleukin 20 receptor subunit alpha	May be involved in epidermal function	2.8	1.5	0.01	
9.	MUC1	Mucin 1, Cell Surface Associated	Forms protective mucous barriers on epithelial surfaces	5.6	2.5	0.02	
10.	MUC4	Mucin 4, Cell Surface Associated	Plays important roles in the protection of the epithelial cells and have been implicated in epithelial renewal and differentiation	5.2	2.4	0.04	
11.	MUC20	Mucin 20, Cell Surface Associated	Mucous barrier	3.5	1.8	0.01	
12.	DEFB4A	Defensin Beta 4A	Antibiotic peptide which is locally regulated by inflammation	10.5	3.4	0.03	
13.	DEFB1	Defensin Beta 1	Antimicrobial peptide implicated in the resistance of epithelial surfaces to microbial colonization	3.24	1.7	0.04	
Immune related DEGs in AS versus NS subjects							

TABLE 3 (Continued)

Rank	Genes	Description	Function	Fold Change	Log2FC	FDR	Comment (references)
1.	VSIG2	V-Set and Immunoglobulin Domain Containing 2	Predicted to be integral component of plasma membrane	2.2	1.13	0.04	
2.	LGALS7	Lectin, Galactoside-Binding, Soluble, 7	Carbohydrate binding	4.02	2.01	0.01	
3.	FOS	Fos Proto-Oncogene	Regulators of cell proliferation, differentiation, and transformation	2.6	1.41	0.01	
4.	DEFB4A	Defensin Beta 4A	Antibiotic peptide which is locally regulated by inflammation	2.75	1.46	0.03	
5.	CXCL2	C-X-C motif chemokine Ligand 2	Expressed at sites of inflammation, antimicrobial gene involved in immunoregulatory and inflammatory processes	2.26	1.18	0.03	Related to asthma[27]
6.	CXCL3	C-X-C motif chemokine Ligand 3	Plays a role in inflammation and as a chemoattractant for neutrophils	2.46	1.30	0.047	
7.	CXCL5	C-X-C motif chemokine Ligand 5	Involved in the recruitment and activation of leukocytes	18.37	4.20	0.002	
8.	CXCL8	C-X-C motif chemokine Ligand 8 (also known as IL-8)	Major mediator of the inflammatory response	2.49	1.32	0.03	
9.	IL1B	Interleukin-1 B	An important mediator of the inflammatory response	2.28	1.19	0.03	
7.	CXCR2	C-X-C Motif Chemokine Receptor 2 (also known as IL8RA)	Receptor for interleukin-8 which is a powerful neutrophil chemotactic factor	2.5	1.31	0.01	
9.	MUC1	Mucin 1, Cell Surface Associated	Forms protective mucous barriers on epithelial surfaces	2.08	1.06	0.02	Suppresses response to bacterial infection[27]
10.	MUC20	Mucin 20, Cell Surface Associated	Mucous barrier	2.07	1.05	0.01	Related to asthma[28]
11.	IL-20RA	Interleukin-20 Receptor Subunit Alpha	A cytokine that may be involved in epidermal function	2.04	1.03	0.01	
Immune-related DEGs in FS versus NS subjects							
1.	CXCL11	C-X-C motif chemokine Ligand 11	Development, homeostasis, and function of the immune system	0.20	−2.26	0.005	Allergic inflammation[25]
2.	IP-10 (CXCL10)	C-X-C motif chemokine Ligand 10	Stimulation of monocytes, natural killer and T-cell migration	0.35	−2.07	0.002	Allergic inflammation[25] Asthmatic airway[26]

Note: References correspond to the phenotype that has been studied before in relation to the susceptible gene. Foldchanges refer to the first group compared with the second group, for example foldchange of 1 would mean gene is upregulated in the first group.

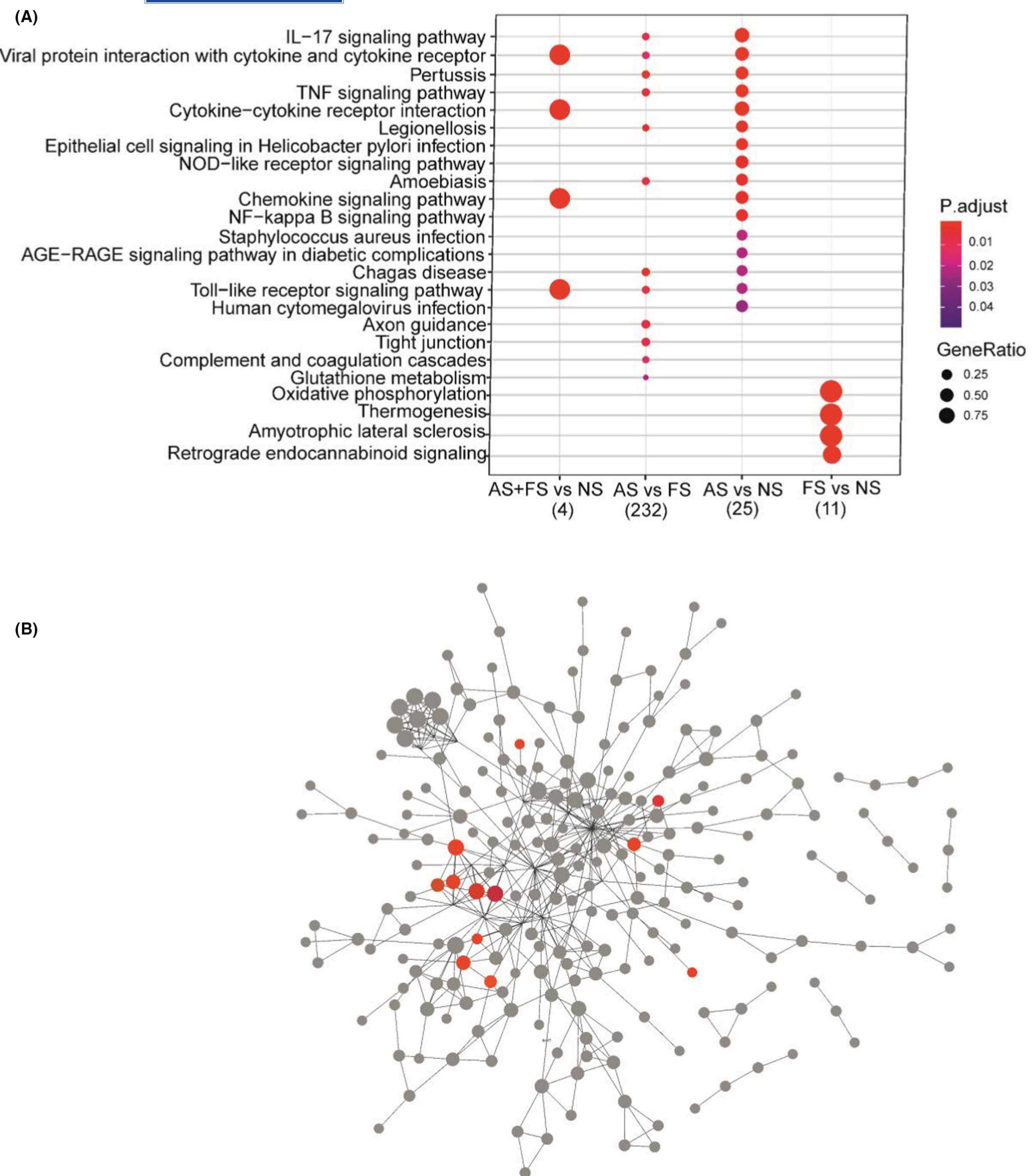


FIGURE 2 Functional enrichment and protein–protein interaction network of upregulated genes from all groups of comparisons. (A) The figure shows KEGG pathways enriched for DEGs (FDR <0.05 & log2(FC) > 1). *p*. adjust represents adjusted *p* value for the KEGG pathway enrichment. (GO Table). (B) String protein–protein interaction network analysis of the upregulated genes (FDR <0.05 & log2(FC) > 1) from all groups of comparisons. The nodes in red colour indicate KEGG pathways enrichment from Figure 2A.

for the eQTL analysis. Of these, a total of 640 susceptibility regulatory variants (2 cis- and 638 trans) were having an FDR value of <0.05, indicating the most robust changes that were associated with sensitization. Our results showed that these susceptibility variants can affect the expression of 109 DEGs in tonsillar RNA-seq data (see variants data table in this article's Online Repository). The results suggested significant SNPs including cis regulators rs1049092 (missense variant) and rs118027159 (intron variant), for *HLA-DQB1* and

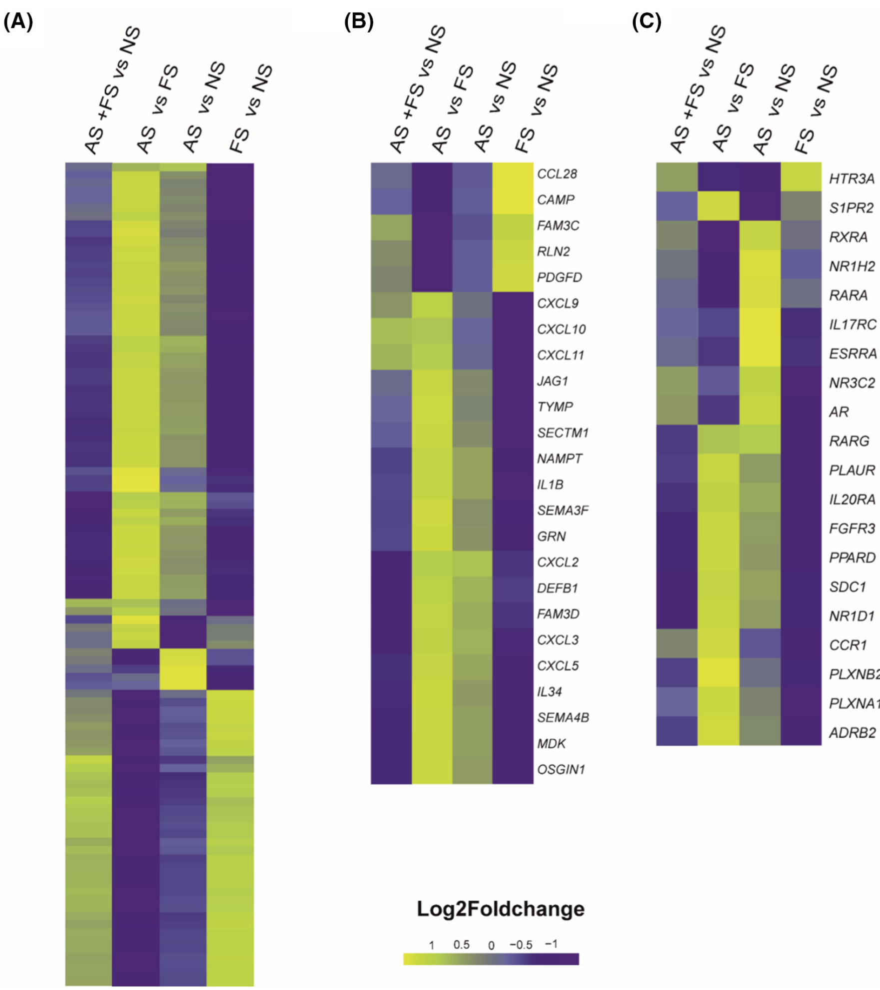


FIGURE 3 The figure shows the examples from manually collected database of differentially expressed genes (FDR <0.05) for transcription Factors (A), cytokines and chemokines (B & C) in our data. The figure shows the varying expression of cytokines, chemokines, and transcription factors in all groups of comparisons from tonsillar RNA-seq data. Log2FoldChange values are colour coded from blue (low) to yellow (high). Expression of genes in groups differ that indicates genes can express distinctively under the effect of different kind of sensitization (Please see this in article's Online repository table for abbreviations).

TUG1 respectively. Other clinically interesting variants included 116 trans SNPs that were significantly associated with the expression of *IL-1B* gene. (Table 4, see variants data table in this article's Online Repository). Furthermore, we identified novel trans variants for *MAPK3*, *MUC-21*, *IL-1B and HLA-DQB1*. The variants with the known association with the phenotype and genes with large number of variants are shown in Table 4.

4 | DISCUSSION

This is the first study to evaluate palatine tonsillar RNA-seq transcriptomic alterations in relation to sensitization patterns. Our data provide new insights into the gene expression of tonsils. No overall DEGs were found between atopic and non-atopic groups. However, large number of DEGs were found in comparing sensitization subgroups. As expected, many subgroup comparisons showed that specific sensitization was associated with overrepresented chemokines (*CXCL2*, *CXCL8*, *CXCL10* and *CXCL11*) and genes from MUC-family (*MUC1*, *MUC20*). Interestingly, aeroallergen versus food sensitized subjects showed 63% distinctively different genes. Furthermore, aeroallergen sensitized subjects had more prominent immune responses including gene expression for IL-17 pathway. We are also first to investigate the effect and association of gene variants with the allergic sensitization across the tonsillar transcriptome.

The exploration of the DEGs identified in AS+FS versus NS group found that in addition to other highly upregulated genes, especially the chemokines (*CXCL10* and *CXCL11*) showed elevated expression. These are proinflammatory chemokines that regulate and maintain inflammatory immune responses by mobilizing the immune cells to the infection site. *CXCL10* can worsen allergic airway inflammation.[29] In addition, *CXCL10* has recently been found to be associated with food allergy in very young children.[30] AS versus NS subjects on the other hand, showed the upregulation of chemokines (*CXCL2*, *CXCL8*/*IL-8*) which are involved in regulating immune responses in inflammatory diseases.[27,31,32,33] *IL-8* is a major inflammatory mediator[31] known to be stimulated by environmental changes, bacterial infection or *IL-1* gene expression.[34] *IL-8* has been found in airway epithelial cells upon the exposure of allergens and may act as chemoattractant in allergic individuals.[35] *IL-8* binds to the G-protein couple receptors *CXCR1* and *CXCR2* for inducing the inflammatory response.[32] Interestingly, we also found the upregulation of *IL-8* receptor, that is *CXCR2* in AS subjects. *IL-8* is mainly involved in inducing the inflammatory response by recruiting the neutrophils, mononuclear phagocytes, mast cells and T cells. *IL-8* contributes to the pathogenesis of

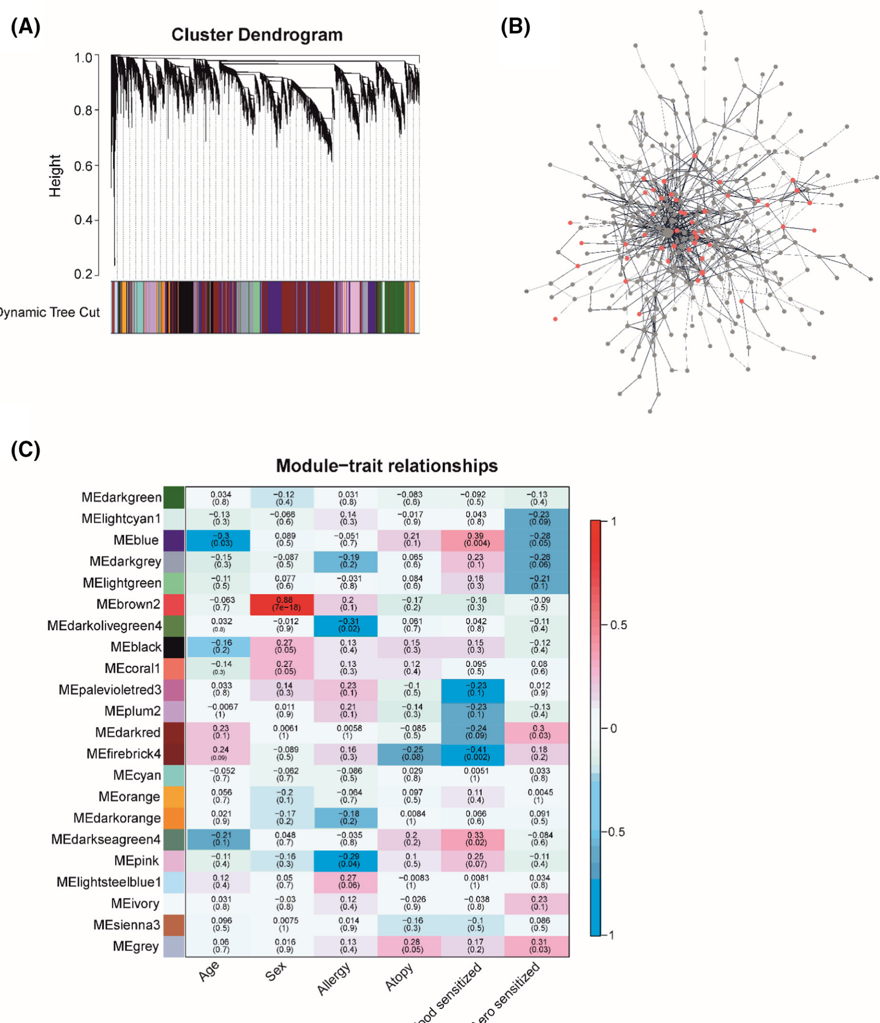


FIGURE 4 Identification of coexpressed genes in Network modules found in tonsil gene expression data. (A) Weighted gene coexpression network which was used to identify modules of coexpressed genes. Figure shows cluster dendrograms for the networks containing 22 modules where dendrogram represents the weighted correlation network analysis across all the tonsillar samples. Each network module is represented by a different colour. (B) Protein–protein interaction network of genes clustered in firebrick4 module. The figure shows the interaction of the proteins and GO enrichment of red colour nodes in response to cytokine stimulus, regulation of inflammatory response, regulation of cytokine production and viral process. (C) Trait-module relationship heatmap, y-axis shows modules and x-axis shows traits. Heatmap shows correlation and *p*-values for the correlation values in the same box.

several respiratory diseases including bronchial asthma, acute respiratory distress syndrome, respiratory syncytial virus infections and viral bronchiolitis.[32,33,36] Moreover, *IL-8* may play crucial role in the onset of acute asthma exacerbations or severity of allergic asthma.[1,2] Therefore, it is implicated to control the atopic asthma by mediating the IgE production.[37] Since *IL-8* and *CXCL2* are important part of inflammatory processes, their aberrant expression can also mediate the inflammatory processes in tonsils of aero-sensitized subjects.

In addition, DEGs from MUC family (*MUC1*, *MUC20*) were upregulated in AS versus NS and AS versus FS subjects. *MUC1* is an anti-inflammatory gene of mucin family expressed in nasal, pharyngeal, bronchial and tracheal cells in the human airway and in lung epithelial cells.[38,39] *MUC1* is known for its function in many inflammatory diseases. For example, Li-Bo et al[40] reported significantly decreased expression of *MUC1* in nasal epithelium of AR patients and rats with AR. This may suggest that deficiency of *MUC1* can be involved in the inflammation of the tonsils of allergic sensitized subjects. Further exploration of the genes more highly expressed in the AS subjects compared with NS subjects indicated that in addition to the changes in chemokines and MUC genes, *IL-20RA* gene is also upregulated. *IL-20RA* is an *IL-10* related cytokine that is potentially associated to eczema. Higher expression of *IL-20RA* is associated with excessive cell proliferation and psoriasis such as skin disease.[41] Since tonsils are made of several small crypts which are surrounded by epithelium, higher expression of *IL-20RA* indicates that it may play a role in sensitized tonsils.

The potentially most important and clinically relevant finding was the identification of upregulated genes in IL-17 pathway in AS versus NS and AS versus FS subjects. IL-17 is a proinflammatory cytokine well known for its immune protective function in several inflammatory diseases.[42] This is also known to play crucial role in type-III inflammatory response against extracellular pathogens and fungi.[43] A major function of IL-17 is the recruitment of neutrophils during an infection.[44] Moreover, IL-17 induces the gene expression of some other genes in inflammatory response by activating immune pathways including NF-κB.[44] However, the role of IL-17 pathway in human tonsils in relation to allergic sensitization has been unexplored previously. We identified that genes enriched in IL-17 pathways from our data are statistically significant; however, the gene ratio in this pathway is small but smaller alterations in the genes can alter the pathways.[45] In addition, one gene can change and affect

the gene expression, a group of genes expressed statistically significant, should not be ignored. NF-κB and MAPK signalling pathway are activated in NOD-like receptor (NLRs) signalling pathway. NLRs induce innate immunity by activating NF-κB and MAPK signalling pathways.[46] Activation of these pathways induces the inflammatory cytokines or chemokines. NLRs are also considered intracellular sensors of microbial infection. In addition, NOD1 has been associated with asthma and atopic eczema.[47] In our data, we identified the elevated expression of NOD-like receptor pathway that suggests the potential role of NLRs in tonsils of sensitized individuals.

The present study is the first one that has investigated the effect and association of variants with the allergic sensitization across the tonsillar transcriptome. The variant calling and subsequent expression quantitative trait locus (eQTL) analysis was performed to assess the presence of regulatory variants (*cis* or *trans*) that directly influence the expression of differentially expressed genes between atopic and non-atopic individuals.[48] We observed 2 *cis* and 638 *trans*-eQTLs which have shown association with the expression of the DEGs in our data. Novel genes that were targets of these regulatory variants include the following: *IL-1B*, *HLA-DQB1*,[49-51] *MAPK3*, *MUC-21* and *TUG1*. Many of these genes are known candidates for allergic or tonsillar diseases revealed through genome-wide association studies (GWAS). For example, Bønnelykke et al[52] has already described an association of *HLA-DQB1* variant with allergic sensitization. Furthermore, present study also illustrates the complex regulation of *IL-1B* expression with largest number of *trans*-eQTLs (116, FDR < 0.05). *IL-1B* is a member of cytokine family *IL-1* which plays pivotal role in the activation of proinflammatory innate and adaptive immune response to a wide range of stimuli. *IL-1B* is very strong in exerting proinflammatory reactions to the extra cellular pathogens.[53] Overall, these finding may suggest that these variants can have influence on the expression level of associated DEGs and with allergic sensitization.

The strengths of the study include carefully characterized patients and high laboratory standards. Our study has some limitations. All our study samples were taken from tonsillectomy patients suffering mainly from tonsillar hypertrophy or recurrent tonsillar infections. We did not have any samples from control/healthy subjects. Second, the ratio of the subject groups was slightly uneven, but the tools including DESeq2 enabled us to handle this problem quite well. DESeq2 distribute the read count to solve the batch problem before doing the differential expression analysis. Additionally, only subsets of patients had serum available for allergen specific IgE testing. Also, this study is entirely based on transcriptional profile and cannot identify potential cells involved. However, overall sample size of this study is supposed to decrease the bias. In addition, the findings of this study should be confirmed in an independent sample set.

5 | CONCLUSIONS

The type of allergic sensitization is associated with extensive tonsillar transcriptomic alterations and changes in immune-related genes

TABLE 4 Effect of top *cis* and *trans* risk variants associated with DEGs in tonsils

Ensembl gene id	Regulation	Number of SNPs	Mapped gene	Location	Related Trait (s) GWAS catalogue	Beta	t-stat	*p*-value	FDR
ENSG00000179344	cis	1	HLA-DQB1	6:32662025_A>G	Asthma, allergic sensitization	−1.383	−8.081	1.24E-10	9.47E-06
ENSG00000099985	cis	1	OSM	22:30974057_A>G		6.268	5.748	5.37E-07	0.00583
ENSG00000125538	trans	116	IL-1B	(see SNPs table)					
ENSG00000204544	trans	1	MUC21	11:1956322_G>A	Asthma (childhood onset)	−6.222	−5.660	7.33E-07	0.03
ENSG00000102882	trans	1	MAPK3	12:49269847_T>C	Tonsillectomy	−1.910	−5.764	5.08E-07	0.03
ENSG00000173391	trans	112	OLR1	(see SNPs table)					
ENSG00000105963	trans	100	ADAP1						
ENSG00000081041	trans	53	CXCL2						
ENSG00000099985	trans	50	OSM						

Note: References correspond to the phenotype that has been studied before in relation to the susceptible gene.

and pathways. Distinct differences were found between aeroallergen and food allergen sensitization groups, which could reflect different pathobiology behind them.

AUTHOR CONTRIBUTIONS

TJ and CA involved in conception and study design. TP, LI and EM collected the data. TH, FA, GT and LI involved in data management and analysis. TJ was a lead study coordinator. TH and TJ involved in drafting and writing of the manuscript. TJ, CA, STS, GT, FA, TH, LI, EM and TP involved in critical revision of the manuscript. All authors read, edited and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in relation to this work.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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