

Distinct Gene Expression Patterns between Nasal Mucosal Cells and Blood Collected from Allergic Rhinitis Sufferers

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Keywords

Allergic rhinitis · Gene expression · NanoString nCounter · Nasal mucosa

Abstract

Background: Investigations of gene expression in allergic rhinitis (AR) typically rely on invasive nasal biopsies (site of inflammation) or blood samples (systemic immunity) to obtain sufficient genetic material for analysis. New methodologies to circumvent the need for invasive sample collection offer promise to further the understanding of local immune mechanisms relevant in AR. **Methods:** A within-subject design was employed to compare immune gene expression profiles obtained from nasal washing/brushing and whole blood samples collected during peak pollen season. Twelve adults (age: 46.3 ± 12.3 years) with more than a 2-year history of AR and a confirmed grass pollen allergy participated in the study. Gene expression analysis was performed using a panel of 760 immune genes with the NanoString nCounter platform on nasal lavage/brushing cell lysates and compared to RNA extracted from blood. **Results:** A total of 355 genes were significantly differentially expressed between sample types (9.87 to $-9.71 \log_2$ fold change). The top 3

genes significantly upregulated in nasal lysate samples were Mucin 1 (*MUC1*), Tight Junction Protein 1 (*TJP1*), and Lipocalin-2 (*LCN2*). The top 3 genes significantly upregulated in blood samples were cluster of differentiation 3e (*CD3E*), FYN Proto-Oncogene Src Family Tyrosine Kinase (*FYN*) and cluster of differentiation 3d (*CD3D*). **Conclusions:** Overall, the blood and nasal lavage samples showed vastly distinct gene expression profiles and functional gene pathways which reflect their anatomical and functional origins. Evaluating immune gene expression of the nasal mucosa in addition to blood samples may be beneficial in understanding AR pathophysiology and response to allergen challenge.

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Introduction

Allergic rhinitis (AR) is a common upper respiratory tract disease estimated to affect between 10 and 40% of the global population. The nasal mucosa is the initial site for allergen exposure, which leads to a cascade of inflam-

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Table 1. Demographic, physical, and disease characteristics of the study cohort ($n = 12$)

Age, years	46.3±12.3
Sex (F/M), n	6/6
Body mass index	28.27±4.55
Self-assessed symptom severity	
mRQLQ (0–6)	2.68±1.69
Overall symptom severity (0–10)	3.0±3.07
Sum of individual symptom scores (0–70)	15.08±15.35
Family history of allergic disease	92
Personal history of	
Eczema	17
Hand dermatitis	17
Urticaria (hives)	25
Food allergy	25
Sensitisation profile of cohort (skin prick test)	
Bermuda grass (<i>Cynodon dactylon</i>)	100
House dust mite mix (<i>Dermatophagoides farinae</i> and <i>D. pteronyssinus</i>)	58
Dust mite (<i>Blomia tropicalis</i>)	25
Dust mix	42
Orchard grass (<i>Dactylis glomerata</i>)	75
Rye grass (<i>Lolium perenne</i>)	83
Johnson grass (<i>Sorghum halepense</i>)	92
Plantain grass (<i>Plantago lanceolata</i>)	50
Bahia grass (<i>Paspalum notatum</i>)	75
Ragweed grass mix (<i>Ambrosia trifida</i> and <i>A. artemisiifolia</i>)	8
Acacia (<i>Acacia longifolia</i>)	8
Co-allergy to pollen and dust mites	58

Values are mean ± SD or %, unless otherwise indicated.

Table 2. Immune variables of the study cohort ($n = 12$)

White cell count, $\times 10^9/L$	6.63±1.56
Eosinophils, $\times 10^9/L$	0.28±0.20
IgE for Bermuda grass, kU/L	6.33±8.08
ESR, mm/h	7.33±5.65
Total IgE, kU/L	404.90±567.05
Total IgG, mg/dL	1,234.85±495.16

Values are mean ± SD.

matory reactions that cause AR symptoms. Common treatments for symptoms include intra-nasal antihistamines and intra-nasal steroid sprays, which target H_1 and glucocorticoid receptors in nasal mucosal cells, respectively. Despite the importance of studying the nasal mucosa in AR pathophysiology and drug action, most AR investigations have typically analysed immune function via blood due to its abundance of immune cells and accessibility of sampling.

Microarray analyses that investigate the effect of glucocorticoid treatment [1], the pathogenesis of AR [2, 3], and nasal polyposis [4], have typically relied on nasal biopsies to obtain sufficient sample material for analysis. Given that nasal biopsies are highly invasive procedures that require local anaesthesia and specialised medical professionals, multiple consecutive sample collections over short time frames are often unfeasible. In contrast, non-invasive methods such as nasal brushing and nasal lavage allow for frequent and cost-effective collection of mucosal cells. To date, the primary limitation of nasal brushing and nasal lavage is that the techniques typically yield insufficient RNA quantity for multiplex gene expression experiments. However, the recently developed NanoString nCounter permits multiplex RNA analysis based on direct digital counting of molecular barcodes with reported sensitivity to a single molecule. The current study investigated the utility of the NanoString nCounter with a panel of 800 genes by comparing gene expression profiles from cellular material collected via nasal brushing and modified nasal wash technique with gene expression profiles obtained from whole blood.

Materials and Methods

A within-subject design was employed in this study. Twelve adults volunteered to participate in the study, which involved a single appointment at the Queensland Allergy Services Clinic in Southport (QLD, Australia) during the peak pollen season. To be included in the study, participants were required to have a history of AR exceeding 2 years, and a positive allergic response to Bermuda grass (Couch). Participants were excluded on a number of criteria including existing medical disease and medication usage. At the single appointment, skin prick testing, collection of nasal wash fluid, nasal brushings, and venous blood samples were obtained for gene expression analysis and assessment of immune variables. Participants were also asked to complete the mini Rhinoconjunctivitis Quality of Life Questionnaire (mRQLQ) [5] and symptom questionnaires to assess severity of symptoms. Detailed study design and experimental procedures are provided as online supplementary material (for all online suppl. material, see www.karger.com/doi/10.1159/000489609). Demographic, physical, and disease characteristics of the study cohort are presented in Table 1. Immune variables of the study cohort are presented in Table 2.

Nasal wash samples were collected by instilling 100 mL of PBS in each nostril (see online suppl. material). Expelled fluid was collected in a sterile container and supplemented with 20 mL of RPMI. Nasal brushings were collected with a brush placed between the nasal septum and inferior turbinate of each nostril. Harvested nasal mucosal cells were shaken from nasal brushes into 3.5 mL of RPMI to release cells. Nasal wash and nasal brushing samples were combined, and cellular material concentrated via centrifugation with subsequent lysis using a commercially available lysis buffer (RLT; Qiagen, Hilden, Germany). For whole blood samples, RNA

was extracted from PAXgene tubes using a Maxwell® RSC automated RNA extraction instrument using the commercially available Maxwell® RSC miRNA Tissue Kit (Promega Corporation, Fitchburg, WI, USA).

Immune gene expression analysis of nasal cell lysate and isolated blood RNA was performed using a commercially available NanoString nCounter PanCancer Immune Profiling panel (NanoString Technologies, Seattle, WA, USA). This panel contained 40 reference (housekeeping) genes and 730 immune genes, and was used in combination with the nCounter panel plus probe set (see online suppl. material) which contained an additional 30 immune genes (see online suppl. Table 1) (760 immune genes in total). Raw gene expression data was normalised against a set of 6 positive and 8 negative controls to account for background noise and platform-associated variation. Reference gene normalisation was performed using the GeNorm Algorithm where 36 of 40 housekeeping genes were selected based on the lowest variance across samples. Differentially expressed genes from blood and nasal lysate samples were assessed with a Benjamini-Yekutieli adjusted *t* test with significance accepted at $p < 0.05$. Functional gene annotation and pathway analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) using genes expressing $>1 \log_2$ fold change (FC) with significant pathway enrichment accepted at $p < 0.05$ and a false discovery rate of $p < 0.05$.

Results

Of the 760 genes tested on the NanoString arrays, 490 genes were expressed above background noise in both nasal lysate and blood samples, and therefore included in subsequent analyses. As shown in Figure 1a, a total of 95 genes were upregulated ($>1 \log_2$ FC) and 250 genes were downregulated ($<-1 \log_2$ FC) in nasal lysate samples compared to blood samples. A total of 145 genes had similar expression levels between nasal lysate and blood samples (0.96 to $-0.95 \log_2$ FC). While both sample types underwent the same NanoString protocols, the blood samples exhibited lower variability of average normalised $\log_2$ counts for all genes compared to the nasal lysate (Fig. 1b). Unsupervised hierarchical clustering of the 490 genes confirmed that the nasal lysate and blood samples were separated into two main clusters, thereby indicating minimal similarity in gene expression profiles between tissue types (Fig. 1c).

A total of 355 genes were significantly differentially expressed between sample types (9.87 to $-9.71 \log_2$ FC; refer to online suppl. Table 2). The top 5 genes significantly upregulated in nasal lysate samples were Mucin 1 (*MUC1*), Tight Junction Protein 1 (*TJP1*), Lipocalin-2 (*LCN2*), Integrin subunit alpha 2 (*ITGA2*), and Carcinoembryonic Antigen-Related Cell Adhesion Molecule 6 (*CEACAM6*). The top 5 genes significantly upregulated

in blood samples were cluster of differentiation 3e (*CD3E*), FYN Proto-Oncogene, Src Family Tyrosine Kinase (*FYN*), cluster of differentiation 3d (*CD3D*), Zeta-chain-associated protein kinase 70 (*ZAP-70*), and Fms-related tyrosine kinase 3 ligand (*FLT3LG*).

Upregulated genes in nasal lysate samples were significantly enriched for DAVID disease categories of ovarian cancer, respiratory syncytial virus bronchiolitis, and asthma (top three; for the full list of enriched pathways see online suppl. Tables 3–7). Upregulated genes in blood samples (systemic immunity) were also significantly enriched in respiratory tract disease categories and included asthma, bronchiolitis, and viral/respiratory syncytial virus infections.

Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis revealed that upregulated genes in nasal lysate samples were enriched in salmonella infection, NF- κ B signalling pathway, and cytokine-cytokine receptor interaction. In contrast, upregulated genes in blood samples were enriched in hematopoietic cell lineage, cytokine-cytokine receptor interaction, and tuberculosis KEGG pathways. The 145 genes that were similarly expressed in both nasal lysate and blood samples (Fig. 1a) were significantly enriched in herpes simplex infection, Toll-like receptor signalling, and hepatitis B KEGG pathways.

Discussion

Studies that examine AR pathophysiology and drug action have typically analysed immune function via blood. However, given that the nasal mucosa is the primary site of the allergic response and symptom manifestation, and is recognised as a source of local IgE production [6], additional insights are likely gained from examining immune gene expression from nasal mucosa directly. To date, gene expression analysis of nasal lavage and nasal brushing samples have traditionally been limited to amplification methods and analysis of a small number of genes [7, 8] or use of pooled samples from multiple participants to yield enough material for microarray experiments [9]. The current study provides important data supporting the use of the newly developed NanoString nCounter system to generate multiplex gene expression profiles in nasal lysate and nasal brushing samples as a new tool to further examine local mucosal immune gene expression profiles important in AR.

The modified nasal wash and nasal brushing procedures to obtain genetic material were non-invasive, time-efficient, and well tolerated by the participants. Further-

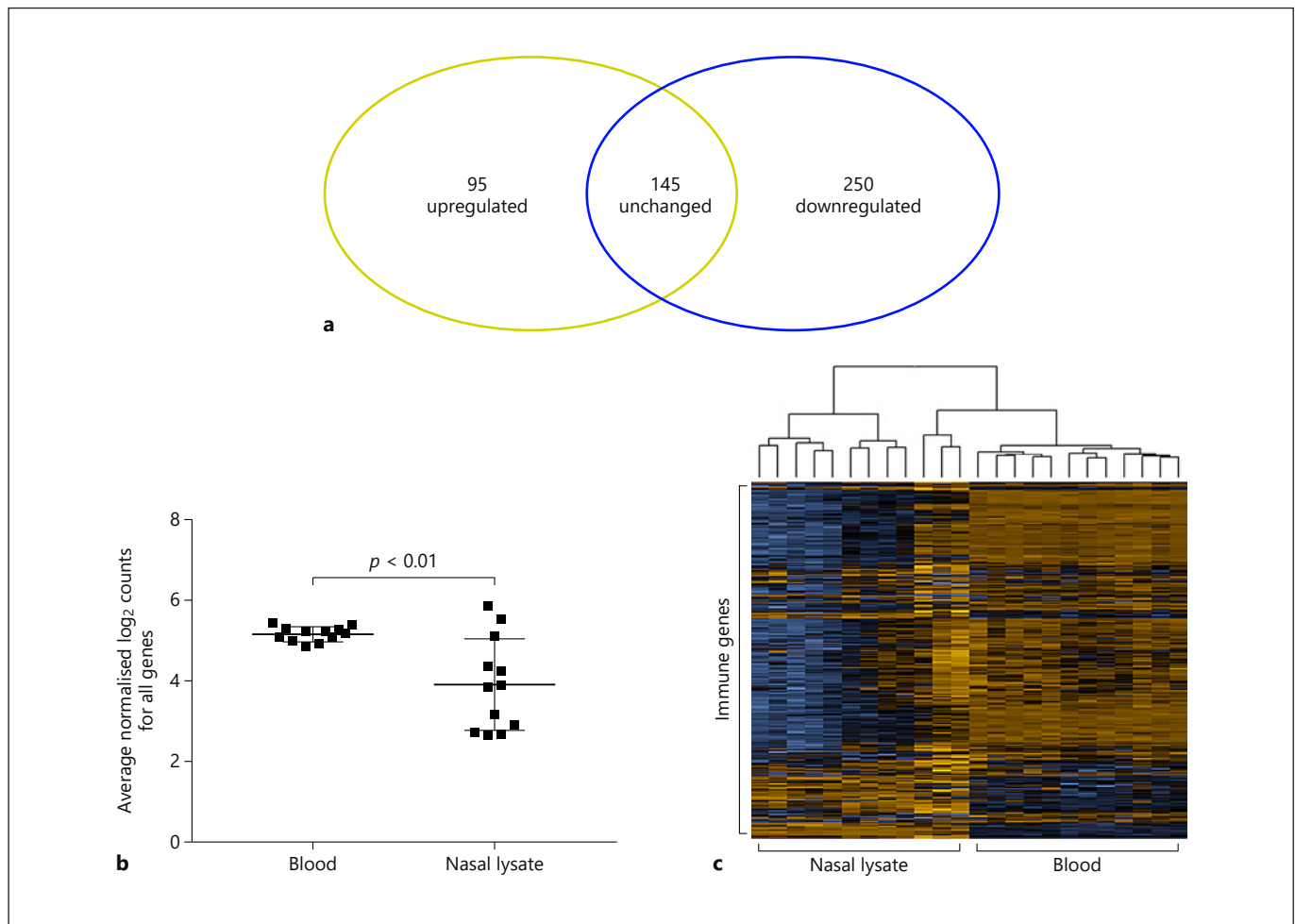


Fig. 1. **a** The Venn diagram shows the number of upregulated genes ($>1 \log_2$ fold change, FC) and downregulated genes ($<-1 \log_2$ FC) in nasal lysate samples compared to blood samples and the number of genes unchanged between each sample type. **b** Average normalised $\log_2$ counts for all nasal lysate and blood sample genes. Central bars indicate the mean and error bars indicate the standard deviation of the mean. Average $\log_2$ counts for each blood and nasal lysate sample are represented as squares. Blood samples have

similar average gene counts across all samples compared to nasal lysate samples where average counts vary more across samples. **c** Heat map of all 490 included genes by sample. Each row represents a gene and each column represents a sample. Unsupervised hierarchical clustering separated nose and blood samples into distinct clusters. Gene expression is represented on a Z scale whereby orange indicates high expression and blue indicates low expression.

more, the nasal lysate yielded sufficient quantity of immune cells from epithelial and luminal cells for multiplex gene expression experiments. As expected, larger variation occurred in normalised gene counts from nasal lysate compared to blood. This heterogeneity across nasal lysate samples may be caused by between-subject differences in nasal cell sampling technique or individual variation in the quantity and/or activity of infiltrating immune cells in the nasal mucosa. Regardless, the experimental protocol resulted in a substantial number of genes being ex-

pressed above background noise, confirming the sensitivity of the NanoString nCounter protocol for direct assessment of mucosal gene expression in individuals with AR.

The blood and nasal lysate samples revealed strikingly distinct overall gene expression profiles as depicted in the differential gene expression and heat map analysis of all genes. These results may reflect the compartmentalised nature of the immune system, where circulating blood and the nasal mucosa have a different cellular composition and biological roles. Jochems et al. [10] have previ-

ously compared immune cell populations and cytokine profiles in blood samples to nasal mucosal samples from healthy adults and reported that immune cells collected from the nose differed in composition and activation state than those in blood. In AR sufferers, nasal washing and brushing samples typically yield epithelial cells along with infiltrating immune cells such as eosinophils, neutrophils, and basophils [11–13]. In contrast to nasal mucosal samples, blood samples do not contain epithelial cells and are more abundant in natural killer cells and monocytes [10, 14].

Compared to the heat map analysis, additional insight into the pathogenesis of AR was gained when highly expressed genes were grouped into functional pathways. For example, the most significantly enriched disease category in nasal lysate was ovarian cancer. While this finding was unexpected, it should be noted that many of the genes that were enriched in the ovarian cancer disease category have dual functions in activating immune cells involved in allergic inflammation and stimulating tumour growth. In contrast to the heat map analysis of all genes, KEGG pathway analysis revealed similarities between blood and nasal lysate samples. In particular, enrichment of the cytokine-cytokine receptor pathway and enrichment of pathways involved in pathogen defence (i.e., salmonella, tuberculosis, and herpes simplex) was observed in both sample types.

Important information on the systemic immunological condition in AR can be gained from analysis of blood samples. However, examining the nasal mucosal system is of importance when studying AR pathophysiology and direct response to allergen and drug treatment. This proof-of-concept study provides initial evidence that the NanoString nCounter system can directly assess local mucosal immune gene expression patterns important to AR. Given that this study was conducted with a modest sample size, the findings presented here should be con-

firmed in a larger cohort of AR subjects. Inclusion of an allergy-free group would further assist in the identification of biomarkers specific to AR. In conclusion, the non-invasive, simple and data-rich technique described here will facilitate further studies in AR pathophysiology and the local effects of drug treatments.

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Statement of Ethics

This study was approved by the Griffith University Human Research Ethics Committee (Ref. #2015/564). All subjects provided written and informed consent prior to participation.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.M.W., N.P.W., and A.J.C. designed the study. A.M.W. performed the experiments and carried out the data analysis. A.M.W. and A.J.C. drafted the manuscript. A.W.C., N.P.W., and P.K.S. revised the manuscript. All authors approved the final version of the paper.

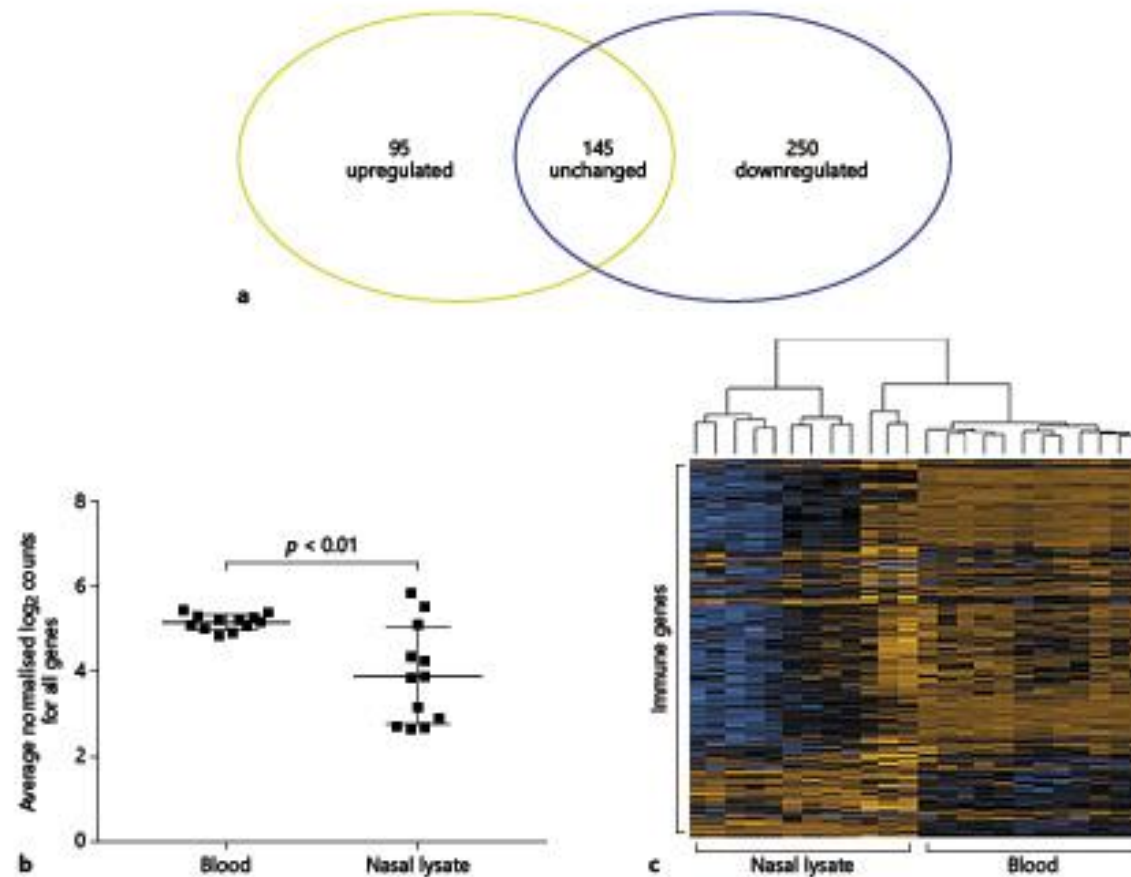
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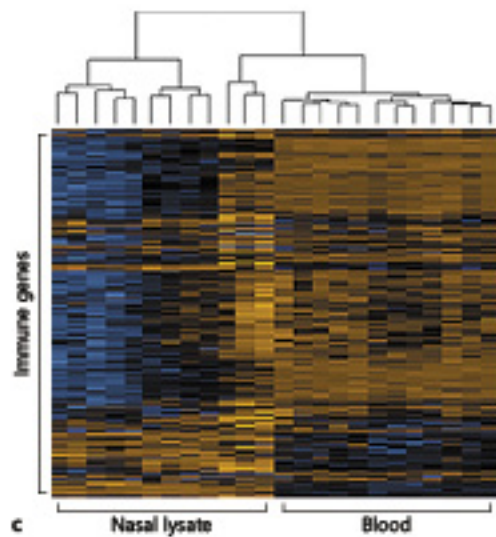
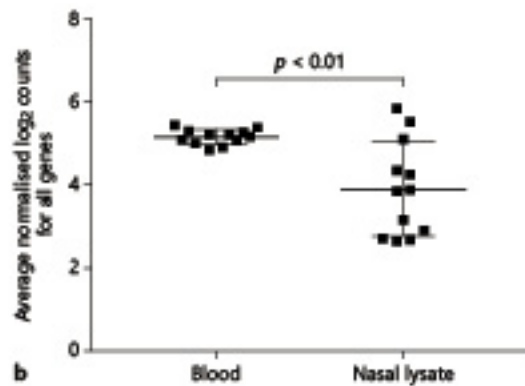
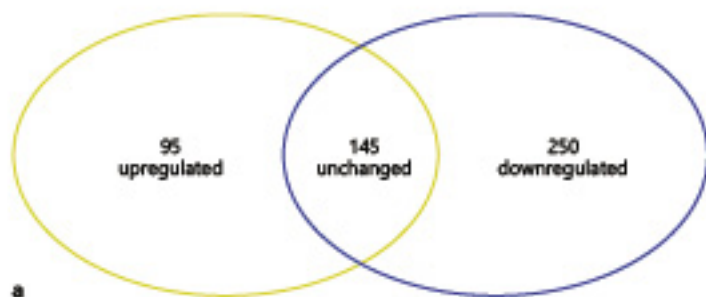
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