Systems Biology Based Approaches to Identify Biomarkers in Seasonal Allergic Rhinitis

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A Promise Is A Promise.

To my parents with deep love and gratitude
To my beloved girlfriend
To my friends

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Abstract:

Glucocorticoids (GC) are the most effective anti-inflammatory treatment for seasonal allergic rhinitis (SAR). However, a few patients with SAR show poor response to GC treatment. Hence, there is a clinical need to find biomarkers to predict and monitor treatment response. Given that GC may affect the expression of a large amount of genes and proteins in different cells and tissues from SAR, it is a formidable challenge to understand these complex changes and to identify candidate biomarkers by studying individual genes. The aim of the study was to develop systems biology based approaches to identify biomarkers for GC treatment response in SAR.

To achieve this goal, clinical investigations, experimental studies and bioinformatics analyses were combined. We profiled gene- and/or protein expression in nasal mucosa, nasal fluids and *in vitro* allergen-challenged CD4+ T cells from patients with SAR by gene expression microarray- and quantitative proteomics analysis. Ingenuity pathway analysis (IPA) and/or multivariate analysis were employed to prioritize candidate biomarkers and genes of importance to allergy. We further validated candidate biomarkers by ELISA.

We showed that several pathways, such as the *acute phase response pathway*, were enriched with genes-coding proteins that may be candidate biomarkers. We identified several novel biomarkers for GC treatment response in SAR including orosomucoid (ORM), apoliprotein H (ApoH) and fibrinogen alpha chain (FGA). With integrated multivariate and pathway analyses we also demonstrated that the expression of allergen-induced genes in CD4+ T cells from patient*s* with SAR was reversed by GC treatment. We indentified that increased IFN-γ activity in allergen-challenged CD4+ T cells was decreased by GC treatment.

In conclusion, we developed systems biology based approaches for the identification of novel biomarkers in SAR. These approaches may be generally applicable to identify biomarkers in clinical studies of complex diseases.

Keywords: seasonal allergic rhinitis; glucocorticoids; gene expression microarray analysis; proteomics; multivariate analysis; pathway analysis; biomarkers

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ORIGINAL PUBLICATIONS

The thesis is based on the following articles, which are referred to in the text by their Roman numerals (I-IV).

I. **Wang H.**, Barrenäs F., Bruhn S., Mobini R. & Benson M.
Increased IFN-γ activity in seasonal allergic rhinitis is decreased by corticosteroid treatment.
J Allergy Clin Immunol (2009); 124(6):1360-2. (Joint first co-author)

II. **Wang H.**, Chavali S., Mobini R., Muraro A., Barbon F., Boldrin D., Åberg N. & Benson M.
A pathway-based approach to find novel markers of local glucocorticoid treatment in intermittent allergic rhinitis.
Allergy (2011); 66(1):132-40.

III. **Wang H.**, Gottfries J., Barrenäs F. & Benson M.
Identification of Novel Biomarkers in Seasonal Allergic Rhinitis by Combining Proteomic, Multivariate and Pathway Analysis.
PLoS One (2011); 6(8):e23563.

IV. Zhao Y., **Wang H.**, Gustafsson M., Muraro A., Bruhn S. & Benson M.
Combined Multivariate and Pathway Analyses Show That Allergen-Induced Gene Expression Changes in CD4+ T cells Are Reversed by Glucocorticoids.
PLoS One (2012); 7(6):e39016. (Joint first co-author)

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ABBREVIATIONS

ALB	Albumin
A2M	Alpha-2-macroglobulin
ApoH	Apoliprotein H
CC16	Secretoglobin, family 1A, member 1
CCL2	Chemokine (C-C motif) ligand 2
CTSD	Cathepsin D
CXCL6	Chemokine (C-X-C motif) ligand 6
ECP	Eosinophil cationic protein
FcεRI	Fc epsilon receptor I
FGA	Fibrinogen alpha chain
GC	Glucocorticoids
GM-CSF	Granulocyte macrophage colony stimulating factor
HR	High responders
HRG	Histidine-rich glycoprotein
IFNG	Interferon-γ
IL-4	Interleukin 4
IPA	Ingenuity pathway analysis
iTRAQ	isobaric tags for relative and absolute quantification
LR	Low responders
MBP	Major basic protein
M-CSF	Macrophage colony-stimulating factor 1
MIF	Macrophage migration inhibitory factor
OPLS-DA	Orthogonal partial least squares-discriminant analysis
ORM	Orosomucoid
PBMC	Peripheral blood mononuclear cells
PCA	Principal component analysis
PPI	Protein-protein interaction
SAR	Seasonal Allergic Rhinitis
SCGB1D2	Secretoglobin, family 1D, member 2
SERPINB3	Serpin peptidase inhibitor, clade B, member 3
Th2	T helper type 2
TMT	Tandem Mass Tag
TNF- α	Tumor necrosis factor α
TNFSF10	Tumor necrosis factor ligand superfamily member 10
Treg	Regulatory CD4+ T cells
VEGFB	Vascular endothelial growth factor B

INTRODUCTION

Seasonal allergic rhinitis

Seasonal allergic rhinitis (SAR) is a common airway disease, which is caused by inhalant allergens such as birch or grass pollen during the pollen season. Following nasal exposure to the inhaled allergens, antigen presenting cells such as dendritic cells in the nasal mucosa present allergen peptides to CD4+ T cells [1, 2]. In allergic patients, this results in activation and proliferation of allergen-specific T helper type 2 (Th2) cells, which release cytokines including interleukin-4 (IL-4) and IL-13. These cytokines are required for IgE synthesis by B cells, while IL-5 is required for the differentiation of eosinophils [2-6]. IgE is captured by Fc epsilon receptor I (FcεRI) on mast cells. Cross linking of the FcεRI on mast cells by the interaction between IgE and allergen leads to the release of cytokines and mediators including tumor necrosis factor α (TNF-α), histamine, leukotriene C4 and prostaglandin D2 that together elicit the early phase response of SAR characterized by sneezing, rhinorrhoea and nasal congestion [2, 7, 8] (**Figure 1**). Accumulated eosinophils in the nasal mucosa release proinflammatory mediators including eosinophils cationic protein (ECP) and major basic protein (MBP), which contribute to enhanced membrane permeability and tissue damage [2, 9].

Effects of glucocorticoids in the treatment of allergy

It is well known that glucocorticoids (GC) are one of the most effective drugs for controlling the inflammation in allergic rhinitis [2, 10-12]. The anti-inflammatory effect can be explained at the cellular level by that GC inhibit a number of pro-inflammatory cells and induce regulatory CD4+ T cells (Treg) that are anti-inflammatory cells in nasal mucosa in allergic patients (**Figure 2**) [13-16]. For instance, GC inhibit the maturation, IgE-dependent FcεRI expression and the production of granulocyte macrophage colony stimulating factor (GM-CSF) and TNF-α of mast cells [17]. GC treatment decreases the number of dendritic cells in perennial allergic rhinitis and may inhibit the differentiation, maturation, and function of dendritic cells [15, 18, 19]. GC induce apoptosis of eosinophils and basophils [14, 20-22], IL-4 expression and histamine release of basophils [23]. GC inhibit class-switching to IgE in nasal B cells [16]. GC treatment decreases not only the numbers of Th2 cells and the production of Th2 cytokines including IL-4, IL-5 and IL-13, but also Th1 cells and their key cytokines IFN-γ and IL-12 [24-26]. In asthmatic patients, GC treatment

increases the IL-10 expression and Treg and restores the inhibitory effect of Treg on the proliferation and production of Th2 cells and their cytokines [13, 27]. GC also induce apoptosis of nasal epithelial cells that, in turn, affect cytokine production from these cells [28].

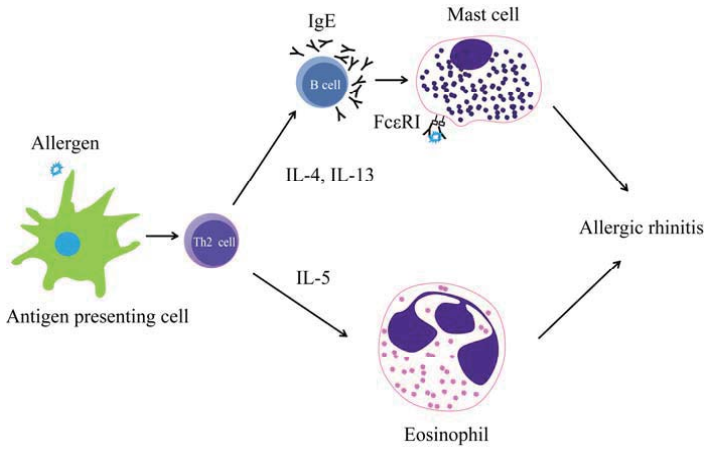

Figure 1. An illustration of allergic reaction in SAR. Allergen is processed by antigen presenting cells, which present antigen peptide to CD4+ T cells initiating Th2 response in patients with SAR. Th2 cells produce IL-4 and IL-13 required for IgE synthesis by B cells and IL-5 required for the differentiation of eosinophils, which contribute to enhanced membrane permeability and tissue damage. IgE is captured by FcεRI on mast cells. Cross linking of the FcεRI on mast cells by the interaction between IgE and allergen leads to the release of cytokines and mediators that together elicit SAR.

However, while GC generally decrease the expression of inflammatory genes, GC treatment may also increase the expression of anti-inflammatory genes [29]. The effects of GC on gene expression in different cell types show considerable variation [30-34]. For example, GC induce apoptosis in eosinophils, but have the opposite effect on neutrophils [35]. Moreover, GC affect not only inflammatory cells but also vascular permeability, and thereby plasma transudation, which has an important role in SAR [36, 37].
Although GC have wide inhibitory effects on inflammatory cells, 10-30% patients with inflammatory diseases including asthma and allergic rhinitis show poor or absent response to GC treatment [38, 39]. One of the mechanisms for GC resistance is that increased glucocorticoid receptor β in

GC resistant patients with inflammatory diseases including asthma and rheumatoid arthritis competes with glucocorticoid receptor α for the binding of glucocorticoid response elements and therefore act as a dominant-negative inhibitor [38, 39].

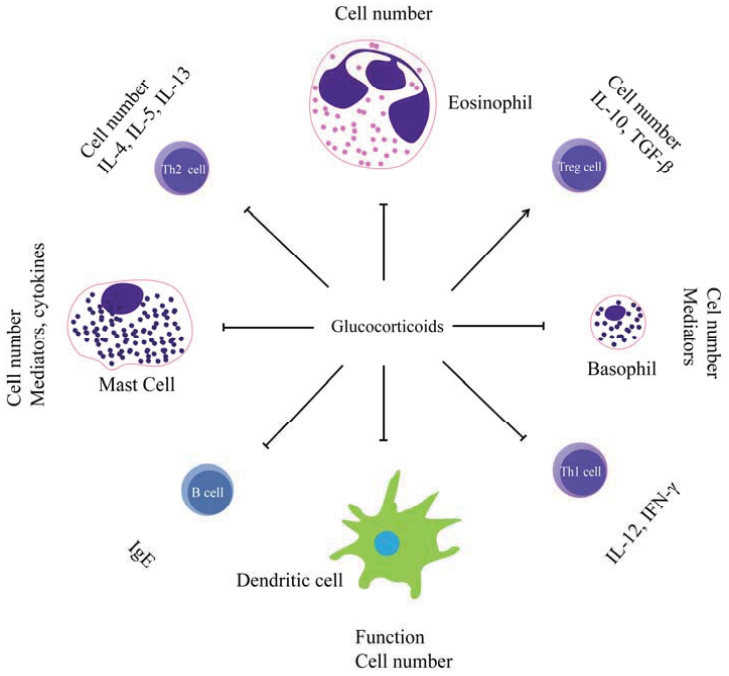

Figure 2. Effects of glucocorticoids on immune cells in inflammatory diseases. GC exert anti-inflammatory effects on inflammatory diseases by widely inhibiting different inflammatory cells including dendritic cells, Th1 cells, Th2 cells, B cells, mast cells, eosinophils and basophils and induction of Treg.

Biomarkers for GC treatment response in SAR

A biomarker is a biological sign that serves as an indicator of pathophysiological processes or response to treatment intervention. Biomarkers may help to establish the diagnosis of diseases and monitor the intervention effects of drugs on diseases. One of the key features of a good biomarker for diseases is that it has clinical relevance to the disease. Several biomarkers have been identified for GC treatment response in allergic

rhinitis. For instance, treatment with intranasal fluticasone result in more than 80% reduction of ECP, which is derived from eosinophils that increased in nasal fluids from symptomatic patients with SAR during the pollen season [35, 40, 41]. Albumin and alpha-2-macroglobulin (A2M) are leakage markers that indicate nasal permeability following allergen challenge. Topical GC treatment reduces the recovery of A2M and albumin in nasal fluids from patients with SAR following nasal allergen challenge [41, 42]. Tryptase is a mast cell-derived biomarker, which increases after allergen challenge but decreases following topical GC treatment [42, 43]. However, there are large variations observed in biomarkers. Also, many inflammatory proteins such as IL-13 and tryptase might be too low to be detected [42]. Furthermore, large variations of biomarkers between individuals may result in poor reproducibility [42].

The large variations may be caused by many factors such as variable dilution of nasal fluids and expression variations. This may be addressed by using combinations of proteins as diagnostic markers. In this way, it is possible to study altered relations between the markers, as opposed to absolute changes in the concentrations of the markers. In the former case, the dilution problem is less important because it is the same for all markers. However, there is a large number of genes that are involved in SAR and are affected by GC treatment [29, 35, 44]. Thus, it is a formidable challenge to identify novel biomarkers in SAR following GC treatment by studying individual genes using low-throughput techniques.

Systems biology based approaches for biological studies

Systems biology focuses on analyzing the complex interactions within biological systems using a more holistic perspective approach such as integrated transcriptional high-throughput- and network analysis [45, 46]. Therefore, systems biology has been mostly associated with high-throughput methods that allow for quantitative analysis of components interactions within biological systems. Increasing studies have shown that high-throughput omics analyses including gene expression microarray- and quantitative proteomics analysis are most efficient and widely used for exploring large amounts of genes and proteins simultaneously in many diseases [29, 47-49]. Another feature of systems biology is that computational approaches are developed for integration and interpretation of the large datasets that generated by high-throughput analyses [46, 50]. For example, network analysis has been firstly used to dissect individual genes of interest from a large amount of genes in SAR [44]. Multivariate analysis

has been shown to be beneficial for interpretation of omics data and prioritization of components of interest [51-53].

AIMS OF THE STUDY

The overall aim of the thesis was to develop systems biology based approaches to identify biomarkers for GC treatment response in SAR.

The specific aims of this thesis were:

Paper I:
To study the interferon-γ activity in CD4+ T cells following allergen challenge and GC treatment in SAR by integrated gene expression microarray- and bioinformatics analysis.

Paper II:
To identify pathways affected by GC treatment and test if those pathways could be used to find novel biomarkers of local GC treatment in nasal fluids from patients with SAR.

Paper III:
To identify novel biomarkers for GC treatment response in SAR by combined proteomics-, pathway- and multivariate analysis.

Paper IV:
To investigate whether allergen-induced gene expression changes in CD4+ T cells could be reversed by GC treatment in SAR by integrated genome-wide analysis and multivariate analysis.

MATERIALS AND METHODS

Subjects (Paper I-IV)

In this thesis, nasal mucosa, nasal lavage fluid, nasal fluid cells and blood from patients with SAR and/or healthy were analyzed. The written consent was obtained on a special form with information about the study and the conditions of the study. The study was approved by the Regional Ethics Committee of the University of Gothenburg and the Ethics Committee of the University of Padua.

SAR was defined by a positive seasonal history and a positive skin prick test or by a positive ImmunoCap Rapid (Phadia, Uppsala, Sweden) to birch and/or grass pollen. Patients with perennial symptoms or asthma were not included. All patients before and after treatment with fluticasone were asked to mark their symptoms (rhinorrhea, congestion, and itching) on a visual analogue scale of 10. These values were added to the total symptom score, as previously described [35].

Paper I:

Peripheral blood mononuclear cells (PBMC) were collected from 19 asymptomatic patients with SAR outside of the pollen season [54]. The median (range) age was 23 (19-28) and 8 were women. Nasal fluid cells were obtained from 15 symptomatic patients with SAR and 28 healthy controls during the pollen season.

Paper II:

Seven patients with SAR were used for nasal fluid cell gene expression microarray- and nasal fluid proteomic studies. The median (range) age of these patients was 28 (20-37) and 5 were women. The mean ± SEM symptom score of these patients before and after treatment was 16.1 ± 2.9 vs 9.9 ± 2.2. Nasal biopsies from three patients with SAR and three healthy controls during the pollen season were used for gene expression microarray analysis [55].

Nasal polyps from patients with SAR outside of the pollen season before and after GC treatment were also collected for gene expression microarray analysis [29]. Nasal fluids from 23 Italian patients with SAR during the pollen season before and after treatment with fluticasone were used for ELISA measurement. The median (range) age of these patients was 32 (15-

47) and 10 were women. The mean ± SEM symptom score of these patients was 20.9 ± 1.5 vs 7.2 ± 1.4.

Paper III:

Nasal fluids from 40 patients with SAR were collected in this study. Their median (range) age was 23 (17-49) and 24 were women. High-responders (HR) and low-responders (LR) to GC treatment were defined as follows. For each patient the ratio between the total symptom before and after GC treatment was computed. HR were defined as the ten patients with the highest ratios, while LR were defined as the ten patients with the lowest ratios (**Table 1**).

Paper IV:

CD4+ T cells from twelve patients with SAR outside the pollen season were analyzed and will be referred to as the training set. The two independent test data sets consisted of 21 patients and 28 patients with SAR outside the pollen season (these materials will be referred to as Test1 and Test2, respectively). The median (range) age of the training set was 23 (20-25) and 3 were women. For the Test1, the median (range) age was 27 (16-46) and 12 were women. For the Test2, the median (range) age was 31 (15-47) and 10 were women.

Collection of nasal lavage fluid and nasal mucosa (Paper II and III)

Nasal lavage samples from the patients were obtained after the start of symptoms during the pollen season, and after 2 weeks of treatment with two doses of 50 μg per dose fluticasone nasal spray in each nostril once daily. Sterile normal saline solution at room temperature was aerosolized into each nostril, while alternatingly clearing the other. The nasal fluids were allowed to return passively and collected in a graded test tube that was submerged in iced water, until 6 mL were recovered. The fluids were then filtered through a 30 μm Pre-Separation Filter (Miltenyi Biotec Inc., Bergisch-Gladbach, Germany) and centrifuged in 4 ºC. The supernatant was separated from the nasal fluid cell pellet and stored in aliquots in -70 ºC until use. In paper I and II, the cell pellet were lysed in 700 μL QIAzol (QIAGEN, Inc., Valencia, CA, USA) and stored in -70 ºC.

Table 1. Characteristics of HR and LR

ID	Gender	Age	Symptom score		Allergy tests	
			Pre	Post	Blood test	Skin prick test
HR1	F	25	28	4	+	+
HR2	F	18	22	6	+	+
HR3	M	22	17	5	+	+
HR4	M	29	16	4	-	+
HR5	F	46	22	6	-	+
HR6	M	18	15	4	+	+
HR7	F	26	25	9	-	+
HR8	F	44	16	3	-	+
HR9	M	35	20	4	+	-
HR10	F	47	27	9	+	+
LR1	M	19	14	21	-	+
LR2	F	18	20	22	-	+
LR3	M	47	8	13	+	+
LR4	M	18	15	15	-	+
LR5	F	22	6	15	-	+
LR6	F	18	13	11	-	+
LR7	F	38	21	24	+	-
LR8	F	40	20	19	-	+
LR9	F	18	7	15	-	+
LR10	F	35	21	29	+	-

HR, high responders; LR, low responders; F, female; M, male; +, positive; -, negative.

Nasal polyposis was identified on the basis of clinical symptoms and the visualization of polyps was done by anterior rhinoscopy [29]. Nasal biopsies were obtained as described from the nasal inferior turbinate, frozen immediately in liquid nitrogen, and then stored at -70 ºC [55].

Cell purification (Paper I and IV)

PBMC were enriched from blood from patients with SAR using Lymphoprep (Axis-Shield PoC, Oslo, Norway) according to the manufacturer's protocol. The purification of CD4+ T cells from *in vitro* cultured PBMC were performed using the CD4 Microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. The typical purity of sorted CD4+ T cells was > 90%.

In vitro stimulation of PBMC (Paper I and IV)

In paper I and IV, PBMC were challenged with diluent (D), allergen extracts from grass pollen (100 μg/mL, ALK Abello´, Hørsholm, Denmark) (A), or allergen + hydrocortisone (10^{-7} M, Sigma-Aldrich, St. Louis, Missouri, USA) (T) for seven days. In paper IV, PBMC in the test1 were challenged with diluent (Test1 D) or allergen extracts from grass pollen (Test1 A) for one week. In the Test2, PBMC were challenged by allergen extracts from birch (one patient) or grass pollen for one week with (Test2 T) or without (Test2 A) GC treatment. RPMI 1640 supplemented with 2 mM L-glutamine (PAA Laboratories, Linz, Austria), 5% human AB serum (Lonza, Switzerland), 5 μM β–mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri, USA) and 50 μg/mL gentamicin (Sigma-Aldrich, St. Louis, Missouri, USA) was used for cell culture.

Quantitative proteomic analysis (Paper II and III)

In paper II, nasal fluids from seven patients during the pollen season, before and after GC treatment, were analyzed with isobaric tags for relative and absolute quantification (iTRAQ)-based proteomic analysis. In paper III, nasal fluids from 10 HR and 10 LR during the pollen season before GC treatment were selected for tandem mass tag (TMT)-based quantitative proteomic analysis.

Prior to labelling, all nasal fluids samples were lyophilized and the protein was quantified using Pierce BCA Protein Assay (Thermo Scientific, Basel, Switzerland). Non-protein impurities were removed by quantitative precipitation clean-up using ProteoExtract® Protein Precipitation (Calbiochem, San Diego, CA, USA). The protein was dissolved, alkylated and digested with trypsin according to the manufacture's protocol (Applied Biosystems, Foster City, CA, USA). In paper II, each four-plex set consisting of one pooled standard sample and three nasal fluids samples was labelled with iTRAQ reagent 114, 115, 116 and 117 respectively following manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). In paper III, each five-plex set, using five reporters from a six-plex, consisting of one pooled standard sample and four nasal fluid samples were labelled with TMT reagents respectively following manufacturer's instructions (Pierce, Rockford, IL, USA). The concentrated peptides were fractionated by Strong Cation Exchange Chromatography **(**SCX) on an ÄKTA purifier system (GE Healthcare, Waukesha, WI, USA). The peptide containing 10 fractions were desalted on PepClean C18 spin columns according to manufacturer's instructions (Thermo Fisher Scientific, Inc., Waltham, MA,

USA). The desalted and dried fractions were reconstituted into 0.1% formic acid and analyzed on a LTQ-Orbitrap XL (Paper II) and LTQ-Orbitrap Velos instrument (Paper III), which were interfaced with an in-house constructed nano-LC column (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Mass spectrometry data analysis was performed using Proteome Discoverer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Database search for each set were performed by Mascot search engine. The detected protein threshold in the software was set to 99% confidence and identified proteins were grouped by sharing the same sequences to minimize redundancy. Only peptides unique for a given protein were considered for relative quantitation, excluding those common to other isoforms or proteins of the same family.

RNA preparation and gene expression microarray analysis (Paper I, II and IV)

Total RNA was isolated using the miRNeasy mini kit (QIAGEN, Inc., Valencia, CA, USA). RNA concentrations was analyzed with NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). The RNA quality was examined in Agilent 2100 Bioanalyzer using RNA 6000 Pico kit and the RNA Integrity Number were calculated in Agilent 2100 Bioanalyzer expert software (Agilent Technologies, Inc., Palo Alto, CA, USA).

In Paper I, II and IV, the microarray analyses of CD4+ T cells, nasal mucosa biopsies from patients with SAR and healthy controls, as well as of nasal polyps before and after GC treatment were performed with Illumina HumanRef-6 Expression BeadChip (Illumina, San Diego, CA). Nasal fluid cells were pooled into one patient- and one control pool for gene expression microarray analysis with Affymetrix HG U133A array. The experiment was performed according to the manufacturer's protocol. Prior to differential expression analysis, the gene expression microarray data was quantile normalized using R package.

ELISA (Paper I, II and III)

In paper I, IFNG, Granzyme A (GZMA) and GZMB in cell supernatants from diluent-, allergen- and allergen + GC-challenged PBMC were analyzed with ELISAs (R&D Systems Ltd, Abingdon, United Kingdom). In paper II and III, Albumin (ALB) was analyzed with an ELISA kit from Bethyl Laboratories (Montgomery, TX, USA). Apoliprotein H (ApoH) was

analyzed with an ELISA kit from United States Biological (Swampscott, MA, USA). Chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 6 (CXCL6), macrophage colony-stimulating factor 1 (M-CSF) and tumor necrosis factor ligand superfamily member 10 (TNFSF10) were analyzed with ELISA kits from R&D Systems Inc (Minneapolis, MN, USA). Secretoglobin, family 1A, member 1 (CC16) was analyzed with an ELISA kit from Bio Vender Laboratory Medicine (Brno, Czech Republic). ECP was analyzed with an ELISA kit from IG Instrumenten-Gesellschaft AG (Zürich, Switzerland). Macrophage migration inhibitory factor (MIF) was analyzed with an ELISA kit from RayBiotech (Norcross, GA, USA). Vascular endothelial growth factor B (VEGFB) was analyzed with an ELISA kit from Gentaur (Brussels, Belgium). Orosomucoid 1/ Orosomucoid 2 (ORM1/ORM2) was analyzed with an ELISA kit from R&D Systems Inc. (Minneapolis, MN, USA). Albumin (ALB) was analyzed with an ELISA kit from Bethyl Laboratories (Montgomery, TX, USA). Apoliprotein H (ApoH) was analyzed with an ELISA kit from United States Biological (Swampscott, MA, USA). Cathepsin D (CTSD), secretoglobin, family 1D, member 2 (SCGB1D2), fibrinogen alpha chain (FGA) and serpin peptidase inhibitor, clade B, member 3 (SERPINB3) were analyzed with ELISA kits from Uscnlife Life Sciences and Technology (Wuhan, China). Histidine-rich glycoprotein (HRG) was analyzed with an ELISA kit from Cusabio Biotech Co., Ltd (Wuhan, China). All experiments were performed according to the manufacturer's protocol.

Ingenuity pathway analysis (Paper I-IV)

Ingenuity Pathways Analysis program (IPA) (www.ingenuity.com) utilizes the Ingenuity Knowledge Base, a repository of biological interactions and functional annotations that is manually created from millions of individually modelled relationships between proteins, genes, complexes, cells, tissues, metabolites, drugs, and diseases from literatures. In paper I, IPA was used to define genes induced by IFNG. The criteria for growing out the genes that are downstream of interferon-γ included that: a) species including human, rat and mice, b) direct and indirect interactions, c) data source Ingenuity Expert Information, d) experimentally observed confidence level and e) relationship type including activation, inhibition, transcription and expression. In paper II, III and IV, IPA was used to map differentially expressed transcripts and/or proteins on to known pathways [56]. A Fisher's exact test was used to calculate a *P* value determining the probability that the association between the proteins and/or transcripts in the dataset and the canonical pathway is

explained by chance alone. Pathways with a *P* value less than 0.05 were considered to be statistically significant.

Protein-protein interaction analysis (Paper I)

Cytoscape is an open source software platform for visualizing complex networks and integrating these with any type of attribute data including protein-protein interaction (PPI). In paper I, IFNG-induced genes were mapped to known protein-protein interaction network using Cytoscape software [57, 58].

Principal component analysis (Paper IV)

Principal component analysis (PCA) is a mathematical approach that uses an orthogonal linear transformation to transform the data of sets of observations to a new coordinate system where the largest variance within sets of observations was projected to the first principal component, the second greatest variance on the second component, and so on. In paper IV, PCA was used to systematically interpret the variations between *in vitro* diluent-, allergen- and allergen + GC challenged CD4+ T cells. Prior to PCA modelling, gene expression microarray data were pre-processed with log-transformation. PCA was performed in SIMCA-P+ 12.0.1 software (UMETRICS, Umeå, Sweden).

Orthogonal partial least squares-discriminant analysis (Paper III and IV)

Orthogonal partial least squares-discriminant analysis (OPLS-DA) is a supervised multiple regression analysis for classification in which systematic variation in the X block such as gene expression microarray- and proteomics data is divided into two model parts, plus the residual noise: the first part which models the X variation correlated to Y variable and is referred to as the predictive component and the other part which comprise the X variation that is un-correlated to the discriminant Y variable and is referred to as the orthogonal component [53, 59], as judged by a leave out data cross validation (all data are left out once in a 7 leave out series). In this thesis the X variation correlated to Y was modelled in the first component, which is referred to as the predictive component. The cross validated, i.e. jack knifed loadings were used to select candidate proteins that best contributed to the discrimination between two groups [59]. In paper III and IV, OPLS-DA was used to interpret differences in nasal fluid protein profiles between symptomatic HR and LR, as well as differences between changes in gene

expression in *in vitro* challenged CD4+ T cells in two different groups. In paper III, the jack knifed loading plot was used to select candidate proteins. In paper IV, the SUS-plot that combines the Cor(T_p, X) profiles from two models where classes were compared to a common reference (group **A**) was used to identify the shared and unique structure between classes (**Figure 3**). The shared genes between two models with a | Cor(T_p, X) | $\geq$ 0.5 were extracted for pathway analysis [60]. Prior to OPLS-DA modelling, input data such as the proteomics- and gene expression microarray data were pre-processed with log-transformation. OPLS-DA was performed in SIMCA-P+ 12.0.1 software (UMETRICS, Umeå, Sweden).

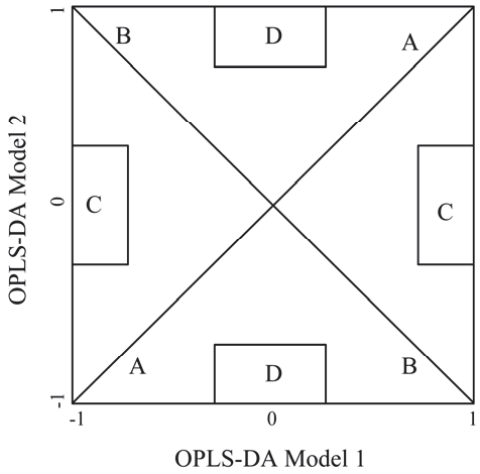

Figure 3. Illustration of the SUS-plot. A SUS-plot combining the Cor(**T_p**, X) from OPLS-DA model 1 and 2 was illustrated. X variables in the diagonal **A** were shared by both models while X variables in the diagonal **B** were inversely shared. X variables in the square **C** and **D** were unique in the model 1 and 2, respectively.

Hierarchical clustering analysis (Paper IV)

Hierarchical clustering analysis is a method of cluster analysis, which measures the dissimilarity between sets of observations. This is generally achieved by using an appropriate metric that measures the distance between pairs of observations, and a linkage criterion, which specifies the dissimilarity of sets as a function of the pairwise distances of observations in the sets. In paper IV, Pearson centered metric and centroid linkage rule were

used for hierarchical clustering analysis performed with GeneSpring GX software (Agilent Technologies, Palo Alto, CA, USA).

Statistical analysis (Paper I-IV)

In paper I, II and III, the Wilcoxon signed ranks test was performed to compare the difference in gene expression and ELISA data between two paired groups and the Mann-Whitney U test was used for unpaired comparisons. In paper I, a χ^2-test was used to examine if more IFNG-induced genes than expected by chance were differentially expressed. In paper IV, Pearson correlation analysis was used to analyze the correlation of the change in gene expression between two groups. Data were expressed as the mean ± SEM. A *P* value less than 0.05 was considered significant.

RESULTS AND COMMENTS

GC may affect the expression of thousands of genes and proteins in different cells and tissues from patients with SAR. It is a formidable challenge to understand the complex changes and to identify candidate biomarkers by studying individual genes. In this thesis, we developed systems biology based approaches to identify biomarkers for GC treatment response in SAR by high-throughput omics-, multivariate- and pathway analysis.

Pathway analysis showed that increased interferon-γ activity in SAR is decreased by GC treatment

High-throughput omics analysis has been widely applied in the study of novel genes and proteins that are important in many biological processes and diseases. However, there is a big challenge for mining candidates from a large amount of genes and proteins from high-throughput omics analysis. In paper I, we performed gene expression microarray analysis with diluent- (**D**), allergen- (**A**), and allergen + GC challenged (**T**) CD4+ T cells from patients with SAR. We observed increased *IFNG* expression in allergen-challenged CD4+ T cells, which decreased after GC treatment (**Figure 4A**). The expression of *IFNG* also increased in the supernatants of allergen-challenged CD4+ T cells, and decreased after GC treatment (**Figure 4B**). It is well known that the allergic response is orchestrated by Th2 cells that release Th2 cytokines including IL-4, IL-5 and IL-13 when exposed to allergen peptides [2, 4, 7]. According to the Th2 paradigm Th2 cells are counter-regulated by Th1 cells through the release of IFNG. Increasing evidence has shown that other T cell subsets also play important roles. Although the clinical relevance of the Th2 paradigm for allergic disease is supported by a large number of reports [3, 4, 7, 61], several *in vivo* studies have shown variable and even increased IFNG levels in allergic patients [35, 61-63]. Therefore, we aimed to understand if the IFNG activity also increased in allergen-challenged CD4+ T cells and decreased after GC treatment.

To address this, we analyzed the IFNG activity by studying the enrichment of sets of IFNG-induced genes in genes differentially expressed after allergen challenge and GC treatment. We defined 496 genes known to be induced by IFNG using the IPA program. Of the 496 IFNG-induced genes, 296 genes changed significantly. This was a significant proportion ($P < 0.0001$). Moreover, a significant proportion of IFNG-induced genes were also differentially expressed after GC treatment ($P < 0.0001$). This indicates that IFNG activity increased after allergen-challenge but decreased after GC

treatment. This was also supported by gene expression microarray analysis of nasal fluid cells from symptomatic patients with SAR during the pollen season, which showed increased activity of IFNG-induced genes compared to nasal fluid cells from controls.

We mapped the IFNG-induced genes differentially expressed with adjusted *P* value < 0.01 to PPI network. We observed that *GZMB* located as a "bridge" of the network, indicating that *GZMB* may be important in the complex network. *GZMB* showed the most significant increase after allergen challenge, as well as reversal after GC treatment (**Figure 5** and **6A**). A similar pattern was found for *GZMA* (**Figure 6A**). Analysis of GZMA and GZMB protein levels in the cell supernatants also conformed to this pattern (**Figure 6B**). This result suggests that GZMB may serve as a biomarker for response to GC treatment.

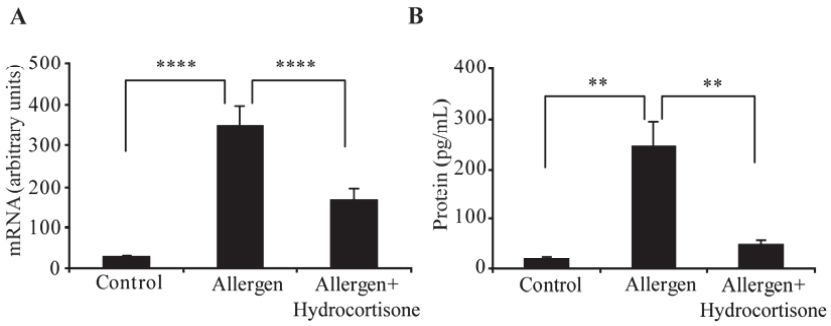

Figure 4. The mRNA (panel A) and protein levels (panel B) of IFNG in allergen-challenged CD4+ T cells. A, n =12 patients; B, n = 20 patients. ** *P* < 0.01, **** *P* < 0.0001.

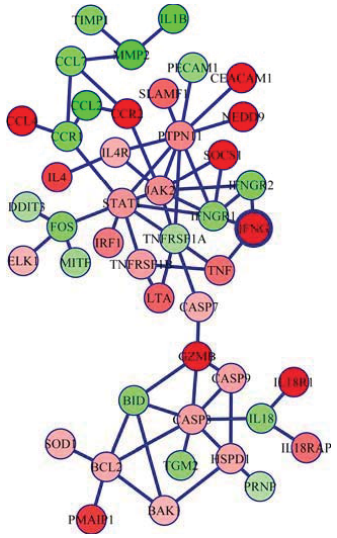

Figure 5. A module of IFNG-induced genes that were highly differentially expressed in allergen-challenged CD4+ T cells (adjusted $P < 0.01$). n = 12 patients. The green and red color presents decreased and increased gene expression, respectively.

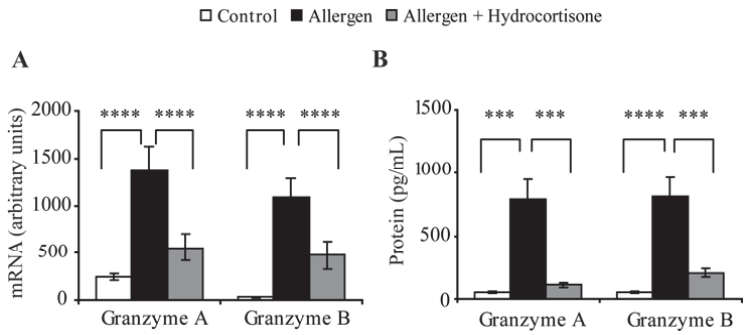

Figure 6. The mRNA (panel A) and protein levels (panel B) of GZMA and GZMB in allergen-challenged CD4+ T cells. A, n = 12 patients; B, n = 20 patients. *** $P < 0.001$, **** $P < 0.0001$.

Pathway analysis to identify biomarkers for GC treatment in SAR

In the above study, we showed that pathway analysis helped to enrich candidate genes of interest from high-throughput omics analysis, which permitted us to study tens of thousands of genes simultaneously. It is a huge challenge to identify novel biomarkers given that GC may affect the expression of thousands of genes in different cells and tissues from patients with SAR. This led us to change the strategy from studying individual genes in a low-throughput manner to integrated high-throughput omics- and pathway analysis. In paper II, we aimed to identify a) pathways affected by local GC treatment and b) examine if these pathways could be used to find novel markers of local GC treatment in nasal fluids from patients with SAR.

Changes in nasal fluid proteins may reflect the effects of GC on both nasal fluid cells and nasal mucosa. Since nasal fluid proteins may be derived from nasal fluid cells, nasal mucosa and plasma transudation, we analyzed a) nasal fluid proteins from patients with SAR before and after GC treatment by iTRAQ-based quantitative proteomics analysis, b) nasal fluid cells and nasal mucosa from patients with SAR before and after GC treatment [55], and c) nasal polyps from patients with SAR and healthy controls by gene expression microarray analysis [29] (**Figure 7**).

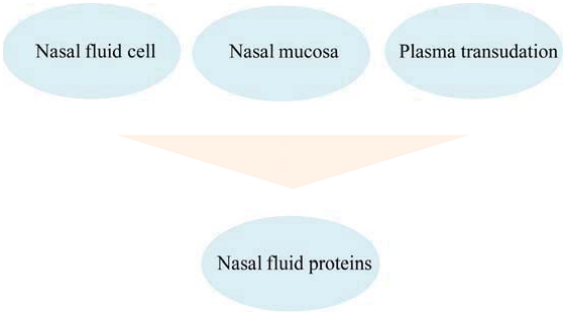

Figure 7. A simplified model of nasal fluid protein sources. Nasal fluid proteins may be derived from nasal fluid cells, nasal mucosa and plasma transudation.

Gene expression microarray analysis of nasal fluid cells showed 25 up-regulated genes and 68 down-regulated genes. Pathway analysis of the differentially expressed genes revealed that no known immune response

pathway was significantly enriched for those genes. We therefore selected the extracellular protein that encoded by the most differentially expressed gene CXCL6 for ELISA analysis. Gene expression microarray analysis of nasal polyps from patients with SAR outside of season before and after GC treatment identified 7151 differentially expressed genes. Pathway analysis showed many known immune pathways enriched for differentially expressed genes (**Table 2**). We selected four candidates from the *acute phase response signaling* (Albumin and ApoH) and *glucocorticoid receptor signaling pathways* (CC16 and CCL2).

We also examined if novel markers for response to GC treatment could be identified in pathways that differed in gene expression microarray data from nasal biopsies from patients with untreated SAR during the pollen season and healthy controls [55]. The top pathways enriched for differentially expressed genes were summarized in **Table 2**. We selected another four candidates from the *death receptor signaling* (TNFSF10), *role of macrophages, fibroblasts and endothelial cells in Rheumatoid Arthritis signaling* (MCSF, MIF) and *VEGF signaling* (VEGFB) (**Table 2**). iTRAQ-based protein profiling of nasal fluids from patients with SAR during the pollen season, before and after GC treatment identified 451 proteins, of which 62 proteins increased and 71 proteins decreased. Pathway analysis showed that two immunological pathways were significantly enriched for differentially expressed proteins, namely the *acute phase response signaling* and *complement system signaling* (**Table 2**).

We analyzed if the selected proteins representing the different pathways in nasal fluids and mucosa would also change in an independent material consisting of nasal fluids from 23 patients with active SAR before and after GC treatment. As a control we measured ECP, which decreased significantly after treatment (**Figure 8**). We observed that CCL2 and M-CSF increased and CXCL6 and ApoH decreased significantly after GC treatment (**Figure 8**). However, other proteins did not change significantly.

Table 2. Prioritization of candidate biomarkers with pathway analysis

Samples	Pathways	Candidates
Nasal fluid cells	No significant pathways	CXCL6
Nasal polyps	Acute phase response signaling	Albumin, ApoH
	Chemokine signaling	
	GC receptor signaling	CC16, CCL2
	T helper cell differentiation signaling	
	IL8 signaling	
Nasal biopsy	Acute phase response signaling	
	Complement system signaling	
	Chemokine signaling	
	Death receptor signaling	TNFSF10
	Glucocorticoid receptor signaling	
	IL4 signaling	
	Role of macrophages, fibroblasts and endothelial cells in Rheumatoid Arthritis signaling	MCSF, MIF
	VEGF signaling	VEGFB
Nasal fluids	Acute phase response signaling	
	Complement system	

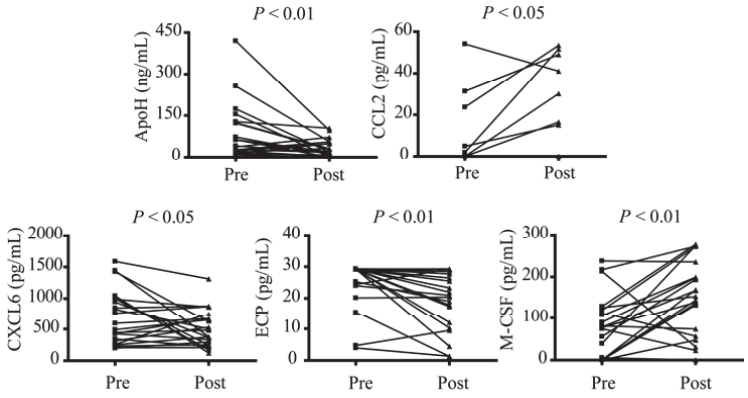

Figure 8. Analysis of proteins representing different pathways with ELISA. Pre, patients before GC treatment; Post, patients after GC treatment.

Identification of novel biomarkers in SAR by combining proteomic-, multivariate- and pathway analysis

GC have beneficial effects in the treatment of SAR [64]. However, 10-30% of patients with SAR and other inflammatory diseases show low or limited response to GC treatment [38]. We reasoned that HR and LR might be distinguished by differences in nasal fluid protein profiles, that might be targeted by GC treatment and therefore be potential biomarkers for GC treatment response.

To find such proteins, we first profiled nasal fluids from symptomatic HR and LR during the pollen season before GC treatment with a quantitative proteomic analysis on a LTQ-Orbitrap Velos instrument [65]. With 99% confidence and one peptide as threshold, this led to the identification of 953 unique proteins, compared to 451 nasal fluid proteins in the previous study [56]. A functional overview of these identified proteins in both HR and LR by IPA showed that the *acute phase response pathway* ($P = 1.39 \times 10^{-27}$, 54 proteins) and *complement signalling* pathway ($P = 1.52 \times 10^{-22}$, 23 proteins) were most significantly enriched for nasal fluid proteins (**Figure 9**). This is in agreement with the findings in nasal fluids from the previous study (**Table 2**).

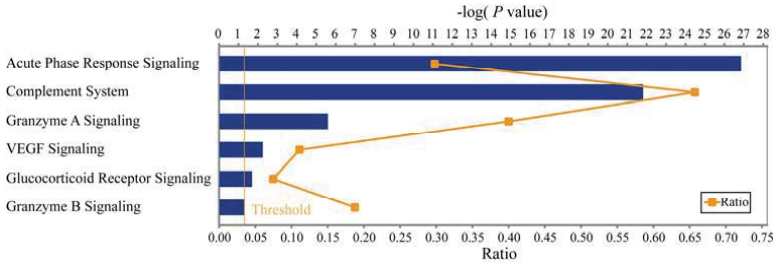

Figure 9. Pathways enriched with nasal fluid proteins identified in both symptomatic HR and LR to GC treatment. A total of 953 unique proteins were identified in nasal fluids from both symptomatic HR and LR and were mapped onto canonical pathways using the IPA software. The yellow threshold indicates 95% confidence.

Next, we searched for the combination of proteins that best separated HR and LR using OPLS-DA [53, 66]. To increase the feasibility and chance of the identification of potential biomarkers, we excluded proteins comprising more than 50% missing data in either HR or LR, which resulted in 161 proteins for modelling. OPLS-DA modelling with the 161 proteins showed that HR and LR were partially discriminated (**Figure 10A**). We extracted the top 40 proteins (25% of the input proteins in the OPLS-DA modelling) that correlated to the discrimination between HR and LR, using OPLS-DA predictive loadings plot with significant confidence intervals (**Figure 10B**). Pathway analysis with the top 40 proteins showed that the *acute phase response pathway* ($P = 2.45 \times 10^{-26}$, 19 proteins) was significantly enriched **(Figure 11)**. Of note, all the 19 proteins enriched in the *acute phase response pathway* increased in HR compared to LR (**Table 3**). This indicates increased inflammatory response in symptomatic HR compared to LR.

We prioritized candidate biomarkers for response to GC treatment based on OPLS-DA modelling as well as pathway analysis. We selected ORM1, ORM2, ApoH, HRG, ALB and FGA from the *acute phase response pathway* based on their estimated contribution to the discrimination between HR and LR in the OPLS-DA model (**Figure 11, Table 3**). Moreover, SERPINB3 and SCGB1D2, which did not belong to the *acute phase response pathway*, were also selected as candidates based on their estimated contribution to the discrimination between HR and LR in the OPLS-DA model.

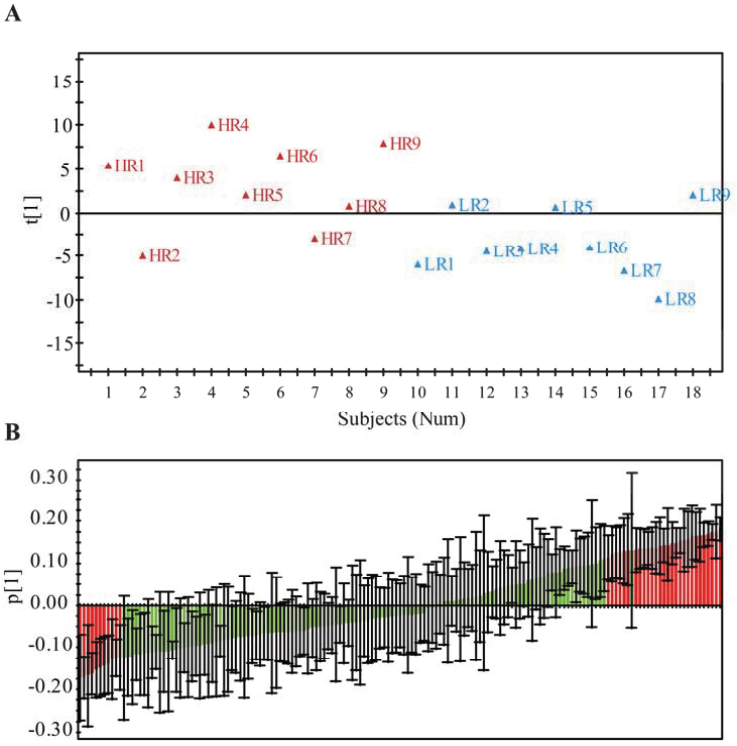

Figure 10. OPLS-DA modelling with nasal fluid proteins from symptomatic HR and LR. **A)** OPLS-DA score plot showed partial separation between HR and LR, where t[1] represents the predictive component. All samples were within a ± 2 standard deviation (SD) limit (according to Hotelling's T^2). **B)** OPLS-DA loading plot with confidence intervals (according to the cross validation procedure). The top 40 proteins that best correlated to the discrimination between HR and LR were highlighted in red. The black line represents error bar.

Table 3. Nasal fluid proteins enriched in the *acute phase response pathway*

Protein ID	Protein Symbol	Protein Name	FC
P01023	A2M	Alpha-2-macroglobulin	1.36
P02768	ALB	Albumin	1.36
P02647	APOA1	Apolipoprotein A-I	1.20
P02749	APOH	Apolipoprotein H (beta-2-glycoprotein I)	1.76
P01024	C3	Complement component 3	1.26
P0C0L5	C4	Complement component 4B	1.29
P01031	C5	Complement component 5	1.33
P00450	CP	Ceruloplasmin	1.04
P02671	FGA	Fibrinogen alpha chain	1.55
P02751	FN1	Fibronectin 1	1.32
P02790	HPX	Hemopexin	1.14
P04196	HRG	Histidine-rich glycoprotein	1.74
P19823	ITIH2	Inter-alpha (globulin) inhibitor H2	1.61
Q14624	ITIH4	Inter-alpha (globulin) inhibitor H4	1.33
P19652	ORM2	Orosomucoid 2	2.80
P02763	ORM1	Orosomucoid 1	1.57
P01009	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	1.23
P05155	SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	1.70
P02787	TF	Transferrin	1.31

FC, fold change of high responders/low responders.

We validated these candidate biomarkers with ELISA in nasal fluids from patients with SAR before and after GC treatment. Several proteins decreased significantly after GC treatment, namely ORM, APOH and FGA, which were selected from the *acute phase response pathway*, as well as SERPINB3 and CTSD (**Figure 12A**). Additionally, ORM, FGA and APOH that belonged to the *acute phase response pathway* were significantly decreased in the 10 HR but not in the 10 LR after GC treatment (**Figure 12B**).

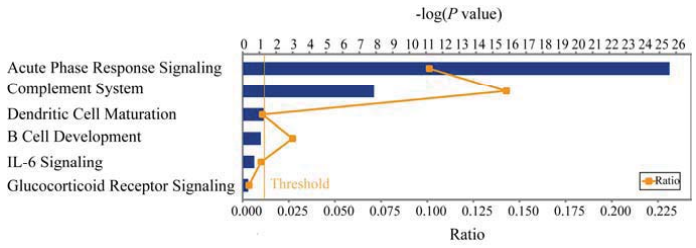

Figure 11. Pathway analysis with the top 40 proteins that highly correlated to the discrimination between HR and LR. The yellow threshold indicates 95% confidence.

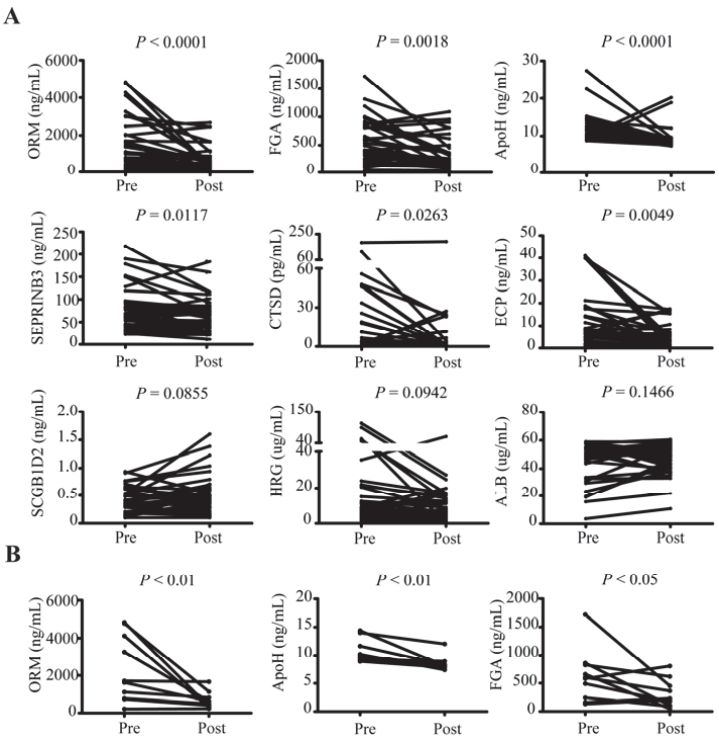

Figure 12. Identification of candidate biomarkers with ELISA**. A)** Nasal fluids from 40 patients with SAR before and after GC treatment were

analyzed. **B)** Nasal fluids from 10 HR. Pre, patients before GC treatment; Post, patients after GC treatment.

Integrated gene expression microarray- and multivariate analysis showed reversed gene expression pattern in allergen-challenged CD4+ T cells by GC treatment

We have shown that multivariate analysis helped to identify a combination of proteins that contributed to the discrimination between two subgroups of patients with SAR. In this study, we aimed to integrate gene expression microarray- and multivariate analysis to study the effect of GC treatment on the gene expression in allergen-challenged CD4+ T cells from patients with SAR.

Gene expression microarray analysis was performed to profile gene expression in diluent- (**D**), allergen- (**A**), and allergen + GC (**T**) challenged CD4+ T cells from twelve patients with SAR. PCA with the gene expression microarray data showed a good separation of the three groups (**Figure 13**). Allergen- and diluent challenged groups were most separated, while the GC treated group, **T**, was located between **D** and **A** (**Figure 13**). This indicates that GC tended to reverse allergen-induced gene expression changes in CD4+ T cells from patients with SAR.

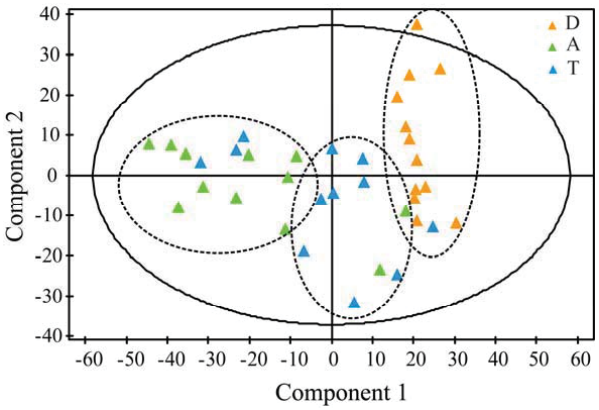

Figure 13. PCA modeling of the gene expression microarray data from diluent- (**D**), allergen- (**A**) and allergen + GC treated (**T**) CD4+ T cells from patients with SAR in the training set.

We identified the correlation between changes in gene expression in allergen-challenged CD4+ T cells before and after GC treatment by OPLS-DA modeling with diluent- and allergen-challenged groups (Model DvsA), as well as with allergen- and allergen + GC challenged groups (Model

AvsT). Pearson correlation analysis with the covariance and correlation of the two models revealed that allergen-induced genes were widely reversed by GC treatment (r = -0.77 and -0.97, respectively) (**Figure 14**). We extracted 547 genes reversed by GC treatment using a SUS-plot from OPLS-DA models based on their high contribution to the discrimination (**Figure 14B**). We found that those genes belonged to several different inflammatory pathways including *TNFR2 Signalling*, *Interferon Signalling*, *Glucocorticoid Receptor Signalling* and *T Helper Cell Differentiation*. These pathways included inflammatory genes of known importance for allergy, such as *CSF2*, *TNF*, *IFNG*, *GZMA*, *GZMB*, *IRF4*, *STAT1 and IL13*, or potential relevance for allergy such as *NR3C1* and *IL21R* (**Table 4**). Taken together, these findings indicate that GC treatment reversed gene expression changes in a wide variety of pathways and genes in allergen-challenged CD4+ T cells.

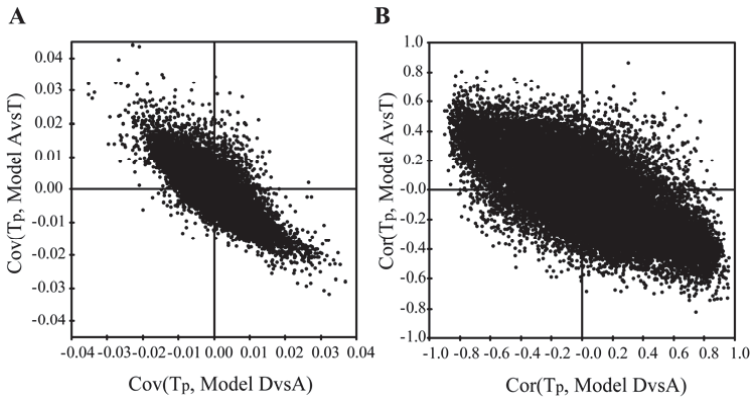

Figure 14. Comparison between Model DvsA and Model AvsT derived from the training set. Comparison of the Cov(Tp) (**A**) and of the Cor(Tp) (**B**) of all genes between Model DvsA and Model AvsT. Cov(Tp), the covariance of the predictive component; Cor(Tp), the correlation of the predictive component.

Table 4. Genes of known relevance for allergy, whose expression increased following allergen-challenge and were reversed by GC treatment

Gene symbols	Model DvsA		Model AvsT	
	Cov(Tp)	Cor(Tp)	Cov(Tp)	Cor(Tp)
CSF2	0.034	0.95	-0.027	-0.54
TNF	0.018	0.95	-0.017	-0.63
IFNG	0.022	0.88	-0.022	-0.55
GZMA	0.023	0.81	-0.028	-0.56
GZMB	0.030	0.89	-0.025	-0.50
IRF4	0.025	0.92	-0.022	-0.52
STAT1	0.022	0.85	-0.025	-0.71
IL13	0.013	0.87	-0.018	-0.69
NR3C1	0.009	0.66	-0.011	-0.53
IL21R	0.016	0.79	-0.021	-0.60

Cov(Tp), the covariance of the predictive component; Cor(Tp), the correlation of the predictive component.

The observed gene expression changes were supported by analyses of two independent materials. PCA and hierarchical clustering showed that the 547 genes clearly separated the two groups (**Figure 15**).

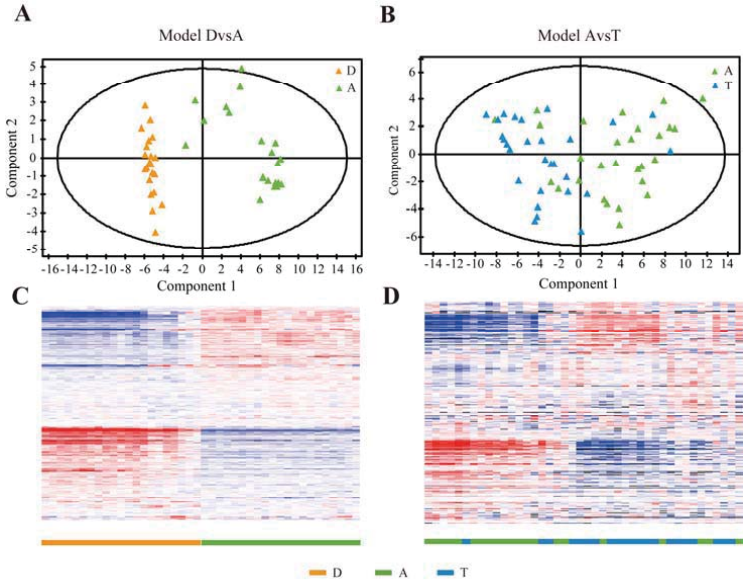

Figure 15. Validation studies of top 547 genes whose expression changed in CD4+ T cells from patients with SAR after allergen-challenge and were reversed by GC treatment. The CD4+ T cells were obtained from two independent materials and analyzed with gene expression microarray. PCA (**A** and **B**) and hierarchical clustering analysis (**C** and **D**) of Test1 (**A** and **C**) and Test2 (**B** and **D**) with the top 547 genes that were changed by allergen challenge and were reversed by GC treatment.

GENERAL DISCUSSION

GC are one of the most effective treatments for allergy and other infammatory diseases [67, 68]. The identification of nasal fluid protein markers for response to GC treatment in SAR is complicated by the involvement of multiple cells and mediators in a complex immunological network [61, 67, 68]. It is a huge challenge to understand the complex changes and to identify candidate biomarkers for response to GC treatment by studying individual genes given that a large number of genes and proteins may change in response to GC treatment. In this thesis, we have developed integrated high-throughput omics-, multivariate- and pathway analysis for the identification of biomarkers for response to GC treatment in SAR.

The advantage and disadvantage of the approaches used

The general principle of developing such systems biology based approaches is that high-throughput omics analysis can be used to detect and quantify the expression of a large number of genes and proteins in different tissues and fluids simutanerously while multivariate analysis may help to statistically identify combinations of proteins with potential diagnostic value and pathway analysis helps to biologically enrich potencial combinantions of biomarkers of relevance to allergy [56, 69].

Gene expresion microarray analysis may help to profile all known genes in biological tisuses such as nasal mucosa in SAR in a high-throughput manner. The gene expression microarray data from nasal mucosa may add information for the identification of potential biomarkers for response to GC treatment in the transcripts level. However, changes in gene expression of nasal mucosa and cells may not completely reflect the expression changes in proteins from nasal fluids that are contributed by different compartments. For instance, several genes including *CC16* and *TNFSF10* that differed in transcripts level showed no statistical change in nasal fluid. Quantative proteomics analysis with nasal fluids can help to directly identify and quantify a large number of nasal fluid proteins. However, this analysis is limited by the proteomics technique itself, which is not sensitive enough to detect low-abundance proteins, some of which may have diagnostic potential. For instance, the known biomarker ECP, which differed in nasal fluids from patients with SAR before and after GC treatment, was only detectable in a few samples by the quantitative proteomics analysis in the both two independent studies. In paper II, only 451 proteins were detected by the quantitative proteomics analysis on a LTQ-Orbitrap XL instrument. In paper III, we improved the productivity by performing the proteomics analysis on

an advanced mass spectrometer, namely LTQ Orbitrap Velos instrument. In agreement with our previous study [56], the nasal fluid proteins from patients with SAR were most significantly enriched in the *acute phase response* and *complement signalling* pathways.

The reason for using multivariate analysis was that univariate analysis does not take into account the relationships between variables and their correlations to the classification between groups. Searching combination of proteins that were correlated to the classification may increase the possibility of finding potential biomarkers. Compared to univariate analysis, multivariate analysis allows us to interpret and visualize multiple variables, such as gene expression microarray- and proteomics data, providing integrated information with less error and more validity. For instance, PCA modeling with the gene expression microarray data in paper IV enabled us to find that the gene expression pattern induced by allergen challenge was potentially reversed by GC treatment. Using the OPLS-DA, we further indentified the covariance and correlation of the whole genes to the classification caused by allergen challenge and GC treatment. The scatter plot of the covariance of the whole genes and also the SUS plot allowed us to identify that the expression pattern of whole genes in allergen-challenged CD4+ T cells was systematically reversed by GC treatment. These plots also permitted us to determine allergen-induced genes that were reversed by GC treatment.

Multivariate analysis may mathematically identify combinations of potential biomarkers. To increase the feasibility of the identification of potential protein markers, we utilized pathway analysis to obtain a functional overview of the combinations of proteins. Pathway-based analysis has the advantage of detecting combination of functionally related genes and proteins whose expression change together and enrich in the same pathways. This may imply greater biological significance that changes of individual genes. Therefore, we focused on differentially expressed extracellular proteins and extracellular proteins-coding genes, which had high expression levels and represented pathways of relevance to allergy. However, pathway-based analysis can be confounded by limited knowledge about pathways and how those differ between cells and tissues. This may cause a bias for manually selecting candidate biomarkers. For example, the proteomic analysis of nasal fluid in paper II showed that three cystatins, namely CST1, CST4 and CST5, were among the proteins whose levels increased most following GC treatment. These cystatins were not part of a pathway that

changed significantly and therefore not selected for analysis in the validation material [70]. However, these proteins may have an important role in SAR as well as in explaining the beneficial effects of GC treatment: they may decrease the IgE-inducing immunogenicity of cysteine protease allergens [70]. On the other hand, pathway-based analysis has the advantage of detecting groups of functionally related genes whose expression change together. It is likely that in the near future improved knowledge of pathways may increase the efficacy of pathway-based analysis to identify diagnostic markers.

The effect of GC treatment on SAR

GC have wide-ranging effects on different tissues and cell types [38, 71, 72]. Interestingly, while GC mainly decrease the expression of inflammatory genes, they may also increase the expression of anti-inflammatory genes. It is also of note that the expression of several inflammatory genes is not affected [29]. We found that, in general, allergen-induced genes expression changes were reversed by GC treatment. The genes that were most affected by allergen challenge and reversed by GC treatment belonged to a wide variety of inflammatory pathways and cellular functions. Several of those pathways have known roles in allergy, namely *TNFR2 Signalling*, *Interferon Signalling*, *Glucocorticoid Receptor Signalling* and *T Helper Cell Differentiation.*

Previous studies have shown important roles for several proteins in those pathways. For example, IFNG is a master cytokine of Th1 differentiation, while IRF4 has a key role in Th2 differentiation [73, 74]. The soluble IL-2RB is known to reflect T cell involvement and was found to be increased in patients with allergic disease but to be reduced by GC treatment [75, 76]. Our approaches may also help to identify novel candidate genes in allergy. The rationale for this assertion is that a gene whose expression is induced by allergen challenge and also reversed by GC treatment, has an increased likelihood to be relevant for the disease. Examples of such genes included *NR3C1*, which is also known as the glucocorticoid receptor, from the *Glucocorticoid Receptor Signalling*, which can be activated by GC [77]. It has recently been reported that NR3C1 increased in nasal mucosa from patients with allergic rhinitis [78]. IL-21R from the *T helper cell differentiation pathway* is important in the development of Th2 response and has been demonstrated to be essential for allergic skin inflammation in human and mice [79, 80].

Known functions of identified biomarkers related to allergy

ORM is an acute phase serum protein, which is synthesized by liver as well as epithelial cells and macrophages [81, 82]. It is found to be a secondary granule protein of neutrophils, which is released immediately in response to activation [83, 84]. This indicates that it exerts immunomodulatory activities not only systemically but also locally during the acute phase immune response. CCL2 and CXCL6 are chemoattractants for T cells and eosinophils that increase in asthma [85-88]. M-CSF induces proliferation of T cells [89], but has not been previously described in allergy. ApoH is a pleiotropic serum protein, which is novel in allergy, but has been implicated in Th2-like responses in Sjögren's syndrome [90]. SERPINB3 has been shown to be upregulated in bronchial epithelial cells from asthma patients by Th2 cytokines IL-4 and IL-13 [91]. Taken togerher, this indicates that these protein biomarkers may biologically reflect the changes in symptom of patients with SAR after GC treatment. Given the large variations observed in individuals, further studies of large materials are warranted to examine such individual variations and if combinations of proteins can be used as diagnostic markers in SAR and other allergic diseases.

Pre-treatment differences in patients with SAR

We speculated that variations in response to GC treatment in SAR might be due to individul pre-treatment differences in the inflamatory response. This was confirmed by pathway analysis with the proteomics data from symptomatic HR and LR, which showed that these proteins were most enriched in the *acute phase response pathway* and all the proteins enriched in this pathway were higher in HR compared to LR. This indicates that the *acute phase response pathway* was more active in HR. Pre-treatment differences in the inflammatory response between symptomatic HR and LR may explain the variability in biomarkers for GC treatment response. This was further confirmed by the ELISA analysis with nasal fluids from HR and LR before and after GC treatment. Three proteins ORM (ORM1/ORM2), APOH and FGA from the *acute phase response pathway* were differentially expressed in HR but not in LR. To our knowlegde, such differences have not been previously examined. Although our material was relatively small and HR and LR only partially separated by the proteins, elucidation of such differences could have an important diagnostic implication, namely to predict response to GC and possibly to other treatments. Ideally, diagnostic protein combinations could be identified in order to routinely determine the optimal medication for individual patients. This would be a step towards personalized treatment in SAR and other allergic diseases.

CONCLUSIONS

In this thesis, we showed increased IFNG activity in allergen-challenged CD4+ T cells but was decreased by GC treatment from patients with SAR by integrating gene expression microarray- and pathway analysis. Moreover, we identified pathways affected by GC treatment in SAR and these pathways were used to identify novel biomarkers for response to GC treatment by integrated high-throughput omics-, multivariate- and pathway analysis. With the developed approaches, we further showed that allergen-induced gene expression changes in CD4+ T cells from patients with SAR were reversed by GC treatment. The top allergen-induced genes that reversed by GC treatment belonged to several inflammatory pathways and genes of known or potential relevance for allergy.

In summary, we developed systems biology based approaches by integrating high-throughput omics-, multivariate- and pathway analysis for the identification of novel biomarkers for response to GC treatment in SAR. The analytical principles may be generally applicable to identify biomarkers in clinical studies of complex diseases.

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