INVESTIGATING MOLECULAR AND GENETIC DIFFERENCES IN ALLERGIC RHINITIS PHENOTYPES

by

SIMRANJIT SAMRA

B.Sc., The University of British Columbia, 2018

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2021

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

Investigating Molecular And Genetic Differences In Allergic Rhinitis Phenotypes

submitted by Simranjit Samra in partial fulfillment of the requirements for

the degree of Master of Science

in Experimental Medicine

Examining Committee:

Dr. Scott J Tebbutt, Medicine, UBC

Supervisor

Dr. Andrew Sandford, Medicine, UBC

Supervisory Committee Member

Dr. Kelly Brown, Pediatrics, UBC

Additional Examiner

Additional Supervisory Committee Members:

Dr. Robert Schellenberg, Medicine, UBC

Supervisory Committee Member

Abstract

Allergic rhinitis (AR) is a heterogeneous disorder that is associated with inflammation of the upper airways. The prevalence of AR has increased rapidly in recent years, and currently affects 10 - 40% of the global population. Common examples of symptoms experienced after allergen exposure include nasal congestion, rhinorrhea, sneezing, and nasal itching. Additional symptoms include conjunctivitis and exacerbation of comorbid asthma. AR is characterized by an early phase response (EPR) and, in some individuals, a subsequent late-phase response (LPR). The induction of allergic responses can be studied using controlled allergen challenge facilities (CACF). Multiple CACFs have identified three response phenotypes in AR: early responders, protracted early responders, and dual responders. Molecular and genetic differences between AR phenotypes have not been well investigated.

In order to identify molecular differences between phenotypes, we used baseline peripheral blood collected from individuals with AR. Blood samples from discovery and validation cohorts were profiled for biomarker candidates using a custom gene expression assay. Using univariate and multivariate analyses, we were unable to identify and validate a clear discriminatory signal between AR phenotypes.

Next, we investigated the relationship between single nucleotide polymorphisms (SNPs) in cholinergic synapse pathway genes and the development of the LPR. We specifically looked at the cholinergic synapse pathway because polymorphisms in these genes have previously been associated with late asthmatic responses. Participants were split into two categories based on late-onset congestion, which is the predominant nasal symptom experienced during the LPR: low congestion (LC) and high congestion (HC). Allele frequencies of 25 SNPs located in cholinergic synapse pathway genes (*ADCY3*, *AKT3*, *CACNA1S, CHRM3, CHRNB2, GNG4,* and *KCNQ4)*,

were found to be significantly different between HC and LC subgroups. Additionally, we identified that the minor allele content of the HC subgroup was significantly higher than that of the LC subgroup. The cholinergic system may be a potential therapeutic target for the LPR.

Lay Summary

Allergic rhinitis (AR), also known as hay fever, is the most prevalent allergic disorder worldwide. Common symptoms of AR include runny/stuffy nose, sneezing, and itching. People with AR experience nasal inflammation and symptoms after breathing in an allergy-causing substance, such as pollen or dust. These responses occur because a body's immune system mistakes a harmless allergen as a harmful foreign substance. Individuals develop an immediate response after allergen exposure. A subgroup of individuals will also develop a late-phase response (LPR), which occurs hours after allergen exposure. There is limited understanding of the mechanisms responsible for the development of the LPR. We identified a relationship between the LPR and differences in genes involved in certain synapses (synapses are found between two nerve cells and are used to transfer information from one cell to another). These differences may be leading to the development of the LPR.

Preface

I participated in the experimental design and performed all the experiments and analyses described in this thesis. This study was approved by Institutional Review Boards and written informed consents were obtained in compliance with the Research Ethics Board of the University British Columbia (UBC) (UBC Ethics number: H09-02114).

Chapter 3 contains parts of the following manuscript in preparation:

Samra SK, Rajasekaran A, Sandford AJ, Ellis AK, Tebbutt SJ. Cholinergic synapse pathway gene polymorphisms associated with late-phase responses in allergic rhinitis. Manuscript in preparation.

I performed all statistical analyses, developed the phenotyping protocol, and wrote the first draft of the manuscript. DNA extraction, DNA quality assessment, and sample preparation were performed by myself under the supervision of SJT. AKE and SJT participated in provision of blood samples. AJS and SJT provided statistical support. The Environmental Exposure Unit studies were performed by AKE. SKS, AR, AKE, and SJT participated in the research design. All authors contributed to the final version of the manuscript.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

APC	antigen presenting cells
AR	allergic rhinitis
AUC	area under the curve
BH-FDR	Benjamini–Hochberg false discovery rate
bp	base pair
CBC	complete blood count
CBC/diffs	complete blood cell counts and differentials
CR	chronic rhinitis
DNA	deoxyribonucleic acid
DR	dual responders
EEU	environmental exposure unit
EPR	early-phase response
ER	early responders
FOV	field of view
GO	gene ontology
GTEx	genotype-tissue expression
GWAS	genome-wide association study
HC	high congestion
HDM	house dust mite
IgE	immunoglobulin E
IL	interleukin
ILC2	type 2 innate lymphoid cells
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	low congestion
limma	linear models for microarray and RNA-Seq data
LOOCV	leave-one-out cross-validation
LPR	late-phase response
NAC	nasal allergen challenge
NAR	nonallergic rhinitis
PCA	principal component analysis
PNIF	peak nasal inspiratory flow
QOL	quality of life

RIN	RNA integrity number
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
Th2	type 2 T helper cells
TNSS	total nasal symptom score
TSLP	thymic stromal lymphopoietin

Acknowledgements

I would like to thank my supervisor Dr. Scott J Tebbutt who supported me and guided me through my degree. Through his mentorship I was able to become a confident researcher and develop understanding in biomarker workflow and computational analysis. I want to thank my supervisor committee members, Dr. Robert Schellenberg and Dr. Andrew Sandford for their guidance and feedback regarding my project. I also want to thank the researchers at the Prevention of Organ Failure (PROOF) Centre of Excellence for their valuable input and suggestions, and for answering my many questions regarding statistics. I would also like to thank our research collaborator, Dr. Anne Ellis and her team at Kingston General Hospital because without them this project would not be possible as they recruited research participants, performed the environmental exposure unit studies, collected blood samples and provided insight regarding allergic rhinitis phenotypes. Additionally, I want to acknowledge the financial support from Mitacs, AllerGen NCE, BC Lung Association, and PROOF Centre.

I would like to express my sincere thanks to Mr. Daniel He and Dr. Amrit Singh for their help on statistical and computational data analysis. I also want to thank Ms. Basak Sahin for teaching me laboratory techniques. Additionally, thank you to previous and current Tebbutt laboratory members, Dr. Ashwini Rajasekaran, Ms. Jinelle Panton, Mr. Abhinav Checkervarty, and Dr. Young Woong Kim for your help.

Lastly, I want to thank my friends and family, Mr. Balvir Samra, Ms. Daljit Samra, Mr. Premvir Samra, Ms. Samantha Lang, Ms. Emilie Theberge, Mr. Samuel Shin, Mr. Miguel Prieto, Ms. Vivian Fung, Ms. Ravneet Hansi, Ms. Adriana Cabrera, Ms. Katarzyna Kabacinska, Ms. Marcia Jude, and Mr. Casey Dheensaw for their love and support.

To My Parents

Chapter 1: Introduction

1.1 Thesis overview

Allergic rhinitis (AR), also known as hay fever, is an inflammatory disorder that results when an inhaled substance (such as pollen, dust, or pet hair) causes an allergic reaction. It affects 20-25% of Canadians and is the most common allergic disorder worldwide[1-2]. Several phenotypes of AR have been identified using controlled allergen challenge facilities (CACF): early responders (ER), protracted ERs (PER), and dual responders (DR)[3-4]. Progression of symptoms in a CACF can be monitored using total nasal symptom scores (TNSS; composite of rhinorrhea, nasal congestion, nasal itching, and sneezing).

AR is characterized by an early-phase response (EPR) and, in some individuals, a subsequent late-phase response (LPR). Individuals phenotyped as ERs and PERs only develop an EPR, however PERs experience nasal symptoms for an extended period of time. Individuals that develop both EPR and LPR are phenotyped as DRs. This thesis includes four goals: 1) to develop blood-based biomarker panels that can discriminate between AR phenotypes, 2) to investigate the relationship between single nucleotide polymorphisms (SNPs) in the cholinergic synapse pathway and the LPR, 3) to investigate re-phenotyping AR responses using nasal congestion scores, and 4) to identify genetic variants that may be influencing gene expression in AR phenotypes.

The first part of this thesis (Chapter 2) focused on development of biomarker panels for AR. Using a custom gene expression assay[5-6], I was unable to identify and validate blood-based biomarker panels that could discriminate between AR phenotypes at baseline. Chapter 3 investigated if enrichment of minor alleles in cholinergic synapse pathway genes may be

influencing the development of the LPR in AR, similar to allergic asthma[7]. Chapter 3 also investigated re-phenotyping AR using nasal congestion, one of the four parameters of TNSS. After the study participants were re-phenotyped, I identified that participants in the high congestion subgroup had an enrichment of minor alleles in cholinergic synapse pathway genes compared to participants in the low congestion subgroup. Chapter 4 focused on identifying expression quantitative trait loci (eQTL) in AR phenotypes. The final chapter of this thesis summarizes all the results of this project and describes its limitations as well as future directions for investigation.

1.2 Chronic rhinitis (CR)

Chronic rhinitis (CR) represents a common symptomatic disorder of the nose affecting up to 40% of the global population[1]. Patients with CR have persistent inflammation of the inner lining of the nose. Based on underlying etiology and type of nasal inflammation, CR can be classified into nonallergic rhinitis (NAR), allergic rhinitis (AR) and mixed rhinitis[8-9]. Mixed rhinitis has all the aspects of AR however symptoms can also be initiated by nasal hyperreactivity, similar to NAR.

1.2.1 Nonallergic rhinitis (NAR)

Patients with NAR or AR exhibit similar nasal symptoms however, NAR is independent of immunoglobulin E (IgE)-mediated mechanisms. Usually adult onset, the age of presentation for NAR is between 30 and 60 years[10]. NAR can be divided into multiple subtypes, however vasomotor rhinitis (VMR) is the most prevalent subtype [11]. Common triggers for VMR include cigarette smoke, perfumes, barometric pressure, drugs, and ingestion of alcohol. Approximately

60% of patients with AR will also have VMR and hence will develop symptoms in response to non-allergic environmental irritants[12-15].

1.2.2 Allergic rhinitis (AR)

AR is a heterogeneous disorder characterized by IgE-mediated inflammation of the upper respiratory tract. Nasal congestion, rhinorrhea, sneezing, and nasal itching are common examples of symptoms experienced after allergen exposure. However, some individuals may also experience conjunctivitis, and throat and palatal itch[16]. Common triggers of AR include pollen, domestic animals, and house dust mites. Significant quality of life (QOL) and socioeconomic impacts are associated with AR[17]. The impact of AR on QOL extends to sleep, cognitive function, and mood[18-19]. AR is also associated with decreased school and work performance during peak pollen season[20].

1.3 Diagnosis of AR

A diagnosis of AR requires a thorough history and physical examination. A further allergy test, such as a skin prick test, is needed to confirm that underlying allergies are the cause of rhinitis[21]. During the history examination, patients detail the extent of nasal symptoms they have experienced recently and describe any family history of atopic disorders. The physical examination consists of an assessment of outward signs of AR. These include persistent breathing through the mouth which is indicative of nasal congestion and frequent sniffling. A general practitioner may also perform an internal endoscopic examination of the nose to assess for nasal ulcerations and nasal polyps[21]. Other assessments may include examination of the

posterior oropharynx for evidence of postnasal drip, and examination of the chest and skin for evidence of concurrent allergic disorders[21].

After a diagnosis of AR is made, a skin prick test is recommended to identify allergens that initiate allergic responses. To perform a skin prick test, a small drop of a specific allergen is placed on an individual's skin. Then the skin is pricked, and the allergen extract is pushed into the epidermis. Within half an hour, a wheal-and-flare response will be observed if the individual is allergic to the extract. A panel of common environmental allergens are assessed during a skin prick test.

1.4 Intermittent AR and persistent AR

Previously, AR was classified as seasonal or perennial depending on the type of allergen and the time of year nasal symptoms were experienced. However, this classification was not sufficient because polysensitized patients will experience symptoms throughout the year and certain allergens may be seasonal or perennial depending on the climate[22]. Therefore, the classification of AR was updated by the Allergic Rhinitis and its Impact on Asthma (ARIA) world health initiative. Now AR is classified based on symptom duration. Patients with intermittent AR experience symptoms less than 4 days a week or for less than 4 consecutive weeks[23]. Patients with persistent AR experience symptoms for more than 4 days a week and for more than 4 consecutive weeks[23]. Presently, both the original and new classification systems are in use.

1.5 The pathophysiology of AR

AR is caused by environmental allergen exposure in genetically predisposed individuals. Allergic responses are initiated after allergen particles are inspired through the nasal cavity and deposited on the nasal epithelium.

1.5.1 Anatomy of the nasal cavity

The nasal cavity is the uppermost part of the respiratory system, it extends from the nostrils to the pharynx. The nasal septum divides the nasal cavity, and a set of superior, middle, and inferior turbinates are present on either side. The nasal cavity is lined by mucus and an epidermal layer of nasal mucosa. Nasal mucosa facilitates humidification, temperature regulation, and filtration of inspired air. Healthy nasal airway epithelium contains basal cells, ciliated cells, and goblet cells. Basal cells are progenitors of other cell types, and they line the basement membrane zone and do not have contact with the lumen of the airway[24]. Ciliated cells facilitate mucus motility and push foreign bodies trapped in mucus towards the posterior nasopharynx. A major constituent of mucus is mucins[25], which are secreted by goblet cells. Nasal mucosa has inherent antioxidant, antiprotease, and antimicrobial properties to defend against foreign bodies. However, nasal mucosa in AR patients has decreased antiprotease activity, making it more susceptible to protease attack by allergens[26].

1.5.2 Sensitization process

Sensitization occurs when previously unexposed allergen particles pass through the epidermal layer. These particles release allergenic proteins that diffuse into the nasal mucosa. Allergenic proteins cleave tight junctions in the airway epithelium, resulting in the activation of epithelial

cells[27]. Activated nasal epithelial cells secrete type 2 polarizing cytokines which include interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP). The secreted cytokines induce activation of type 2 innate lymphoid cells (ILC2) and antigen presenting cells (APCs)[28-30]. Activated ILC2s secrete large amounts of type 2 cytokines including IL-5, IL-9 and IL-13[31]. Both IL-5 and IL-9 recruit and activate eosinophils and mast cells while IL-13 is imperative for goblet cell hyperplasia[32].

Present within and below the nasal epithelium are immature dendritic cells. After capturing allergen particles, immature dendritic cells rapidly mature and migrate to the lymph nodes and then present processed allergen peptides to naïve T cells that preferentially differentiate towards the type 2 T helper cell (Th2) lineage[33-35]. Activated T cells proliferate into effector T cells that release Th2 cytokines including IL-4, IL-5, IL-9, and IL-13[36-37]. Continued expression of IL-4 is needed to maintain the Th2 cell lineage and recruit more naïve T cells into this lineage[38]. Additionally, IL-4 and IL-13 act together with the CD40 ligand expressed on Th2 cells to influence heavy-chain class switching in B cells to IgE production. Next, IgE antibodies bind to mast cells, basophils and dendritic cells using high-affinity receptors (FcεRI)[39]. This binding results in mast cells and basophils releasing large amounts of histamine[39-40]. Activated mast cells also secrete other mediators of allergic responses including leukotrienes, prostaglandins, and cytokines[39]. The binding of allergen specific IgE antibodies to high-affinity receptors (FcεRI), results in sensitization of an atopic individual's mast cells and other effector cells to release certain immune mediators in response to subsequent encounters with the specific allergen.

1.5.3 Early-phase response

Allergen exposure in sensitized individuals results in the early-phase response (EPR), which occurs within 30 minutes of exposure and is caused by the rapid cross-linking of IgE antibodies with the inspired allergen (Figure 1-1). This results in degranulation of mast cells and basophils and the release of preformed mediators including histamine, cysteinyl leukotrienes (leukotrienes C_4, D_4, and E_4), and prostaglandin D_2[39-42]. These mediators are responsible for the nasal symptoms experienced during an allergic response. Histamine promotes the activation of H1 receptors on sensory nerves[43-44], which then transmit signals to the central nervous system and result in nasal itching and sneezing[45-46]. Histamine, leukotrienes, and prostaglandin D_2 stimulate mucus secretion, vasodilation, increase in vascular permeability, plasma extravasation and pooling of blood in the capacious venous sinusoids, and together these processes lead to rhinorrhea and nasal congestion[47-50].

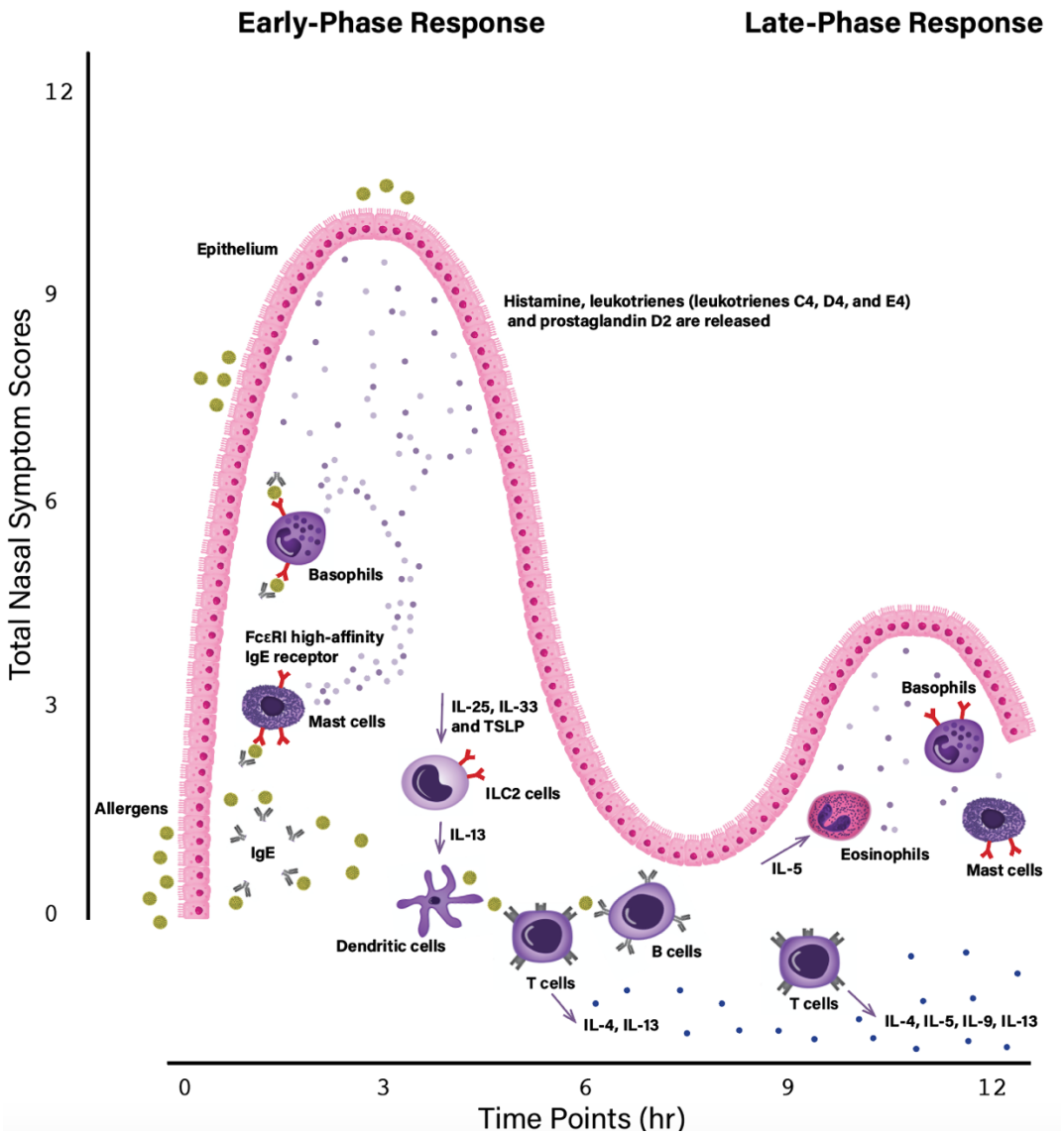


Figure 1-1 Summary of mechanisms associated with early- and late-phase responses (EPR and LPR).

During the EPR, histamine, leukotrienes, and prostaglandin D2 are released which result in the development of nasal symptoms and the recruitment and activation of inflammatory cells. Cytokines and chemoattractants released during the EPR promote the infiltration of nasal mucosa by eosinophils, basophils, and T cells. T cells release IL-4, IL-5, IL-9 and IL-13, which play an important role in the development of the LPR.

1.5.4 Late-phase response

A subgroup of AR patients will develop an additional allergic response 6 to 9 hours after allergen exposure, which is referred to as a late-phase response (LPR)[51-52]. Mediators released during EPR, promote the release of cytokines and chemokines that sustain nasal inflammation by inducing an influx of immune cells towards the nasal mucosa[53-54]. Circulating eosinophils, basophils, and neutrophils can adhere to nasal endothelial cells using adhesion molecules, such as vascular cell adhesion molecule 1, E-selectin, and intercellular adhesion molecule 1[55-57]. Additionally, chemoattractants such as IL-5 promote the infiltration of nasal mucosa by eosinophils, basophils, and T cells[58-60]. Together these immune cells sustain inflammation and nasal symptoms up to 24 hours after allergen onset.

1.6 Systemic aspect of AR

Immune responses in allergic rhinitis can be studied using nasal lavage samples (local immunity) or blood samples (systemic immunity). Vastly distinct gene expression profiles are found between nasal lavage samples and blood samples collected from individuals with AR during peak pollen season[61]. Many studies have focused on investigating local immune responses in AR. There is limited research investigating systemic immune responses. The Tebbutt laboratory has identified signatures of immune gene expression in blood samples collected from individuals with AR after allergen challenge[62]. Using this systemic immune gene signature analysis[62], genes have been identified that have significant differential expression patterns in blood samples collected before and after treatment with peptide immunotherapy[63].

1.7 Comorbidities of AR

AR coincides with numerous associated disorders which can be divided into four categories: allergic disorders, disorders related to the nose, disorders related to sleep, and turbinate hypertrophy[64]. Allergic disorders associated with AR include atopic dermatitis (AD), food allergies, and asthma. The strongest association is between asthma and rhinitis and has been recognized for several decades[65]. A majority of individuals with inflammatory asthma have inflammation in both the upper and lower respiratory tracts[66], suggesting the presence of an associated upper airway disorder such as AR or NAR[67]. Comorbidities that are anatomically related to the nose include sinusitis, conjunctivitis, and middle ear complications. AR is also associated with sleep impairment and disorders such as obstructive sleep apnea (OSA)[68]. Individuals with AR may have difficulty going to sleep and suffer from nocturnal awakenings[68], ultimately leading to fatigue and learning impairment[69]. There is also an association with AR and turbinate hypertrophy, which may result in persistent nasal congestion and headaches[70].

1.8 Treatment options for AR

The first approach to managing AR is avoiding allergens that trigger allergic reactions. However, this is not a solution for airborne allergens. In these cases, pharmacotherapy or allergen immunotherapy may be prescribed.

1.8.1 Efficacy of pharmacologic treatment

Pharmacologic treatment options include oral antihistamines and intranasal glucocorticoids. Oral antihistamines have a fast onset of action and are utilized when needed. Second- and third-generation antihistamines are preferred because they are as effective as first-generation

antihistamines but without the sedative effect[71]. Antihistamines have a modest impact on AR symptomology, especially nasal congestion[72]. Intranasal glucocorticoids are the best pharmacologic treatment option for intermittent AR; however, they are only moderately effective at treating persistent AR[72-74].

1.8.2 Efficacy of allergen immunotherapy

Even with pharmacologic therapy, 61% of AR patients still report their symptoms as not well-controlled[23]. The next step to treat such patients would be allergen immunotherapy, which can confer long-term benefit after treatment completion[75-78]. Allergen immunotherapy can be administered either subcutaneously (SCIT) or sublingually (SLIT)[79-87], although the clinical effectiveness of SCIT and SLIT is similar[87]. Allergen immunotherapies are the only disease-modifying treatment option that can induce immune tolerance and prevent the progression of AR to allergic asthma[85,88-92]. Remission of allergic responses is observed in patients after continuous treatment for 3 years[75-78]. However, these treatments are not successful in some AR patients and it is difficult to identify these patients before starting treatment[79].

1.9 Human model of AR

AR responses can be studied using established human models: the nasal allergen challenge (NAC) and controlled allergen challenge facilities (CACF). These models use different methods to administer allergen extract to research participants[93-95]. The NAC model administers a standardized allergen dose directly to a participant's nasal mucosa through the nostrils. The allergen dose can be customized for each participant to ensure everyone achieves similar levels of nasal symptoms[96]. CACFs are custom-designed rooms that house study participants in a

controlled environment for allergen exposure. The environmental exposure unit (EEU) is one example of a CACF, and it consists of a large room with a feeder system that continuously delivers standardized levels of allergen into a seating area, allowing for simultaneous exposure of airborne allergens to large groups of people[97].

1.9.1 Environmental exposure unit (EEU)

The EEU model is located at Kingston General Hospital and was developed in 1981 by Dr. James H. Day[93,97]. The experiments described in this thesis use clinical data and samples collected from three different EEU studies. The studies were performed in similar conditions but used different allergens during the exposure session: birch pollen (*Betula pendula*), rye grass pollen (*Lolium perenne*), or house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*). The setup of the EEU allows for the allergen concentration to be maintained within a narrow range (Figure 1-2). The air in the EEU is continuously circulated using fans and allergen levels are measured every 30 minutes using impact type particle samplers (Rotorod® counters)[97]. The pollen emission rate is then modified based on these counts.

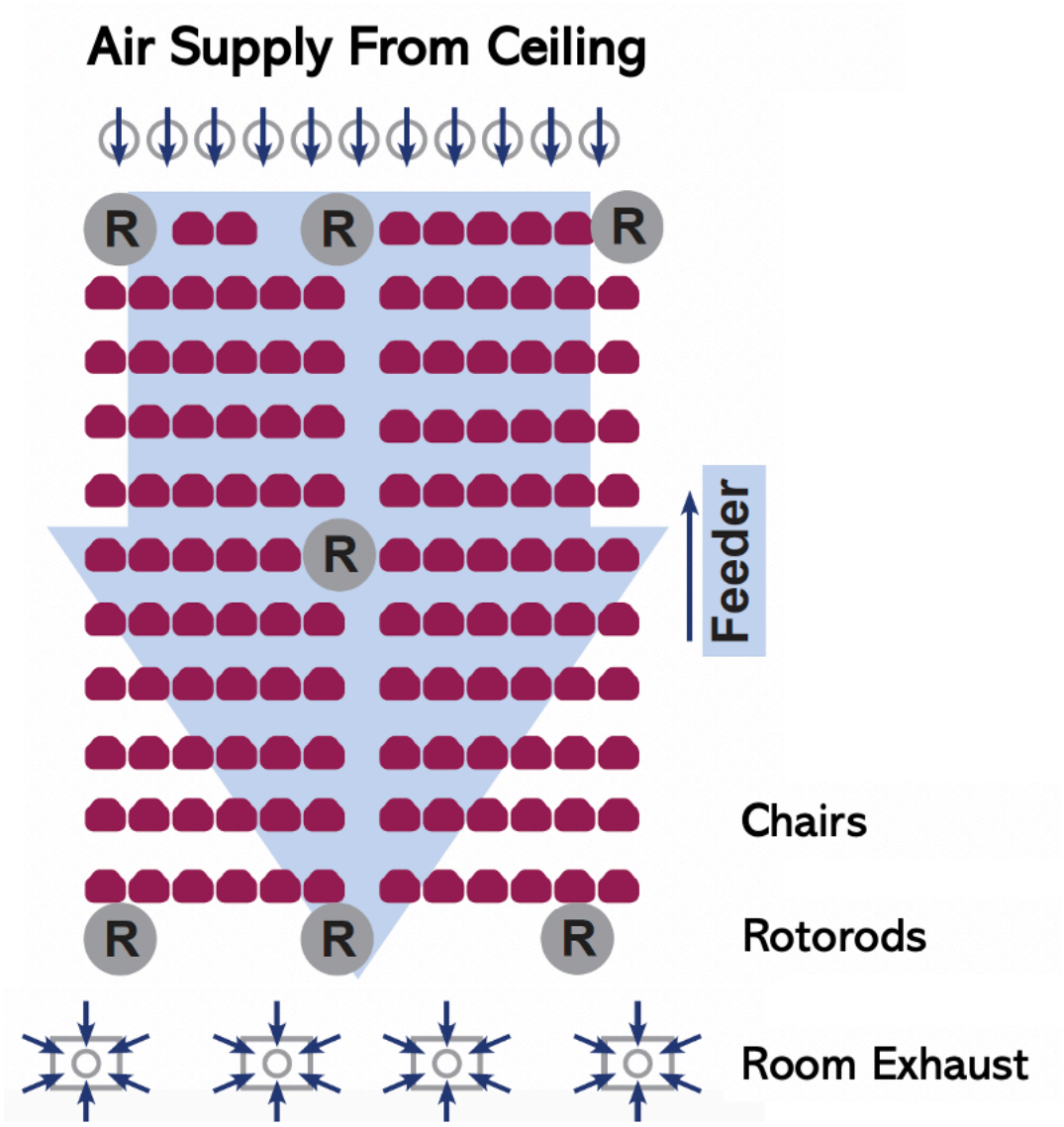


Figure 1-2 Layout of the environmental exposure unit (EEU). The EEU has a feeder system that continuously delivers allergens into a large seating area. The allergens are propelled, by selectively placed groups of fans, over the seating area. Allergen levels are measured using seven impact type particle samplers (Rotorod® counters).

1.9.2 Clinical symptoms scores

Following allergen exposure, clinical symptoms of AR are assessed using two measurements: total nasal symptom score (TNSS) and peak nasal inspiratory flow (PNIF).

1.9.2.1 Total nasal symptom score (TNSS)

TNSS is a subjective assessment of clinical symptoms of AR. At each timepoint, participants record their symptoms on diary cards using a four-point scale (0-3) for rhinorrhea, nasal congestion, sneezing, and nasal itching[98-100]. The scores were defined as the following: 0 indicates absence, 1 indicates mild symptoms, 2 indicates bothersome yet tolerable symptoms, and 3 indicates that symptoms were severe and difficult to tolerate. The scores for each symptom were added together at each timepoint to calculate the TNSS (maximum score of 12).

1.9.2.2 Peak nasal inspiratory flow (PNIF)

PNIF is an objective assessment of nasal airway patency[97]. At each timepoint, the PNIF was measured in liters per minute (L/min) using a noninvasive In-Check PNIF meter (Clement Clarke International Ltd., Essex, UK). To measure nasal patency, participants inhale through their nose to draw air through the device which results in a cursor moving along a scale to indicate the speed of inhalation.

1.9.3 Phenotyping AR

Research participants with AR can be grouped into one of three categories based on late-onset symptoms: early responders (ERs), protracted ERs (PERs), or dual responders (DRs)[3-4]. Participants phenotyped as ERs and PERs develop only an EPR. Dual responders instead develop both EPRs and LPRs. Phenotypes are assigned based on the pattern of hourly reported TNSS data. Most participants reach a peak in TNSS after 1.5 to 3 hours of allergen exposure. ERs experience at least a 50% reduction in TNSS by Hour 6 compared to Hour 3 and return to baseline by Hour 12. In comparison, PERs do not experience a 50% reduction in TNSS by Hour

6 and do not return to baseline by Hour 12. DRs experience at least a 50% reduction in TNSS at Hour 6 compared to Hour 3 and maintain decreased symptom severity for at least 2 hours followed by a clear and sustained increase in symptoms, indicative of an LPR.

1.10 Thesis Summary

Chapter 2 describes my first project which investigated gene expression differences between AR phenotypes in peripheral blood at baseline. Using a multivariate biomarker approach, I was able to identify key combinations of gene transcripts that were capable of discriminating between ERs from DRs, healthy controls from DRs, and Grouped ERs and PERs from DRs. However, I was unable to validate these results in an independent test set. Next, I studied genetic differences between AR phenotypes. Chapter 3 describes my second project which investigated the enrichment of minor alleles in the cholinergic synapse pathway genes and the development of the LPR. I was unable to identify significant genetic analysis results when using traditional AR phenotypes, which are assigned using TNSS. In chapter 3, I re-phenotyped AR using nasal congestion. I identified that individuals in the high congestion subgroup had an enrichment of minor alleles in cholinergic synapse pathway genes compared to the low congestion subgroup. In chapter 4, I used the molecular and genetic datasets created in the previous two chapters and performed a cis-expression quantitative trait loci (cis-eQTL) analysis. I identified SNPs that were associated with *AKT3* expression in AR phenotypes. Finally, chapter 5 summarizes all the results and discusses future directions.

Chapter 2: Transcriptomic biomarkers of allergic rhinitis

2.1 Introduction

The prevalence of AR has increased rapidly in industrialized societies, currently affecting 10 – 40% of the global population[1]. Similar to the field of allergic asthma[101], AR phenotypes may be a useful selection criterion during clinical trial enrollment as novel therapeutics may be more appropriate for a certain phenotype. However, CACFs, such as the EEU, require considerable resources, staff, and time. Thus, there remains a need for rapid and cost-effective biomarker-based phenotyping.

Blood-based transcripts can discriminate between subgroups of allergic asthma and predict the development of late asthmatic responses[5]. Similarly, blood-based transcripts may be able to discriminate between AR phenotypes. The aim of this chapter was to identify biomarker panels (combinations of single transcripts) that could predict a participant's response to allergen exposure in the EEU[95]: ER, DR (EPR and LPR) or PER (intermediate response)[3-4]. Molecular characterization of the phenotypes may also provide further evidence that PERs are a distinct phenotype of AR as an intermediate allergic response is not observed in allergic asthma[102]. In this chapter, biomarker panels were developed using the NanoString nCounter platform. Panels were identified in a discovery cohort, and the performance of the models was assessed using cross-validation. The performance of the models was then assessed in an independent cohort.

2.2 Hypothesis

We hypothesized that molecular differences detectable in baseline peripheral blood samples can be used to discriminate between AR phenotypes.

2.3 Materials and Methods

2.3.1 Research participants

Research participants were screened and selected by our collaborators at Kingston General Hospital. Written informed consents were obtained from participants before undergoing allergen exposure. All participants underwent skin prick testing to common environmental allergens (rye grass, birch, timothy grass, ragweed, tree mix, dog, cat, and house dust mite). Allergic participants selected for EEU studies had a positive skin prick test to rye grass, birch or house dust mite (defined as a wheal diameter of 3 mm or greater than that produced by the negative control)[103] and a 2-year documented history of allergic rhinoconjunctivitis symptoms. Healthy participants had a negative skin prick test to common environmental allergens and did not have a documented history of allergic rhinoconjunctivitis symptoms. Exclusion criteria included participants with asthma requiring the use of a short-acting beta agonist greater than twice a week and a known history of positive test results for Hepatitis B, Hepatitis C, HIV or tuberculosis. Participants were also excluded if they had a history of alcohol or drug abuse, and if they were currently receiving allergen specific immunotherapy injections (additional details about the exclusion criteria and medication washouts can be found in the Bibliography)[97].

Study participants were pooled from three independent EEU studies. The discovery cohort consisted of 67 allergic participants: 33 were ERs, 26 were PERs and 8 were DRs (Figure 2-1A). The discovery cohort also included 17 healthy participants. Phenotypes were assigned using TNSS data[3-4] as previously described in Chapter 1.9.3. Demographics of the discovery cohort are shown in Table 2-1. An additional 44 participants were used for validation: 16 were ERs, 11 were PERs, 7 were DRs and 10 were healthy participants (Figure 2-1B). Demographics of the validation cohort are shown in Table 2-2.

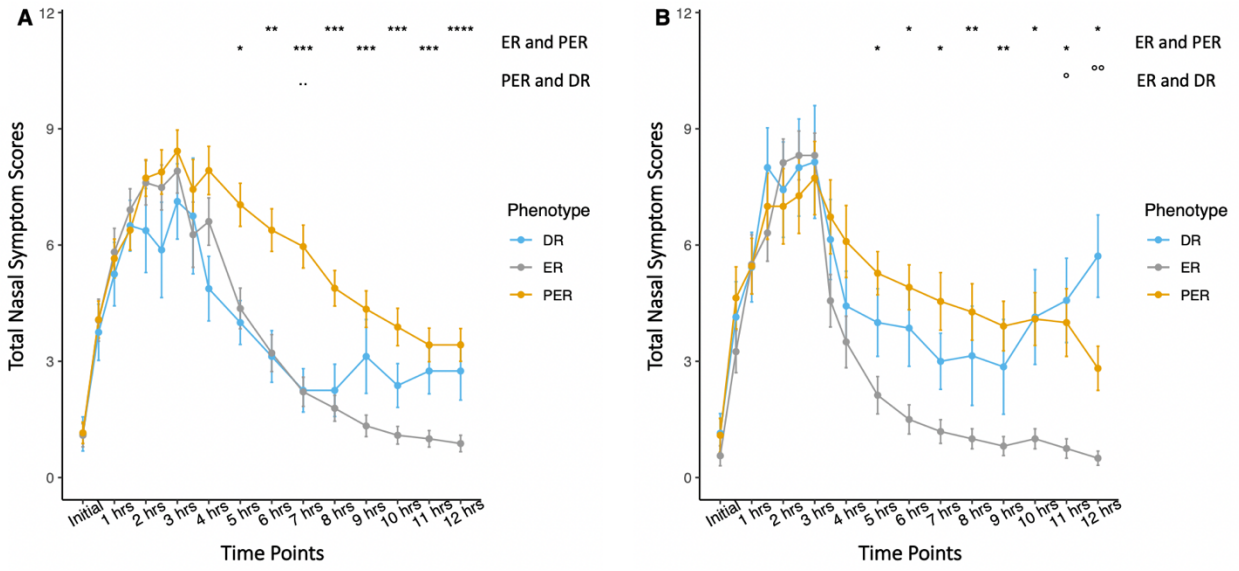


Figure 2-1 Phenotyping the discovery and validation cohorts using total nasal symptom scores (TNSS). Research participants were classified as early responders (ERs), protracted ERs (PERs) or dual responders (DRs) based on changes in TNSS data. **A** In the discovery cohort significant differences were found between ERs and PERs (*) at hours 5 to 12 and between PERs and DRs (•) at hour 7. **B** In the validation cohort significant differences were found between ERs and PERs (*) at hours 5 to 12 and between ERs and DRs (○) at hours 11 to 12. Analysis was performed using Wilcoxon Signed Rank test with Bonferroni corrections. The number of symbols indicates the *P*-value: 1 symbol indicates that the *P*-value was less than 0.1, 2 symbols indicates that the *P*-value was less than 0.01, 3 symbols indicates that the *P*-value was less than 0.001, and 4 symbols indicates that the *P*-value was less than 0.0001.

Table 2-1 Demographics of the discovery cohort

	Allergic Participants			Healthy Participants	*P*-Values								
	ER (n=33)	**PER (n=26)**	**DR (n =8)**	**CP (n=17)**	**ER vs DR**	**PER vs DR**	**ER vs PER**	**CP vs ER**	**CP vs PER**	**CP vs DR**	**ER and PER vs DR**	**ER vs PER and DR**	**CP vs AP**
Female, %	48%	69%	50%	65%									
Height, cm*	169.31 ± 8.76	169.27 ± 8.95	171.22 ± 9.24	167.85 ± 6.29	0.60	0.61	0.98	0.50	0.55	0.37	0.59	0.84	0.78
Weight, kg†	85.40 (68.80-90.50)	85.90 (64.05-95.40)	73.05 (72.03-80.70)	85.50 (76.60-102.80)	0.24	0.51	0.83	0.44	0.33	0.14	0.31	0.79	0.25
Age, yr†	39.00 (30.00-47.00)	36.50 (31.00-43.50)	37.00 (28.00-44.25)	37.00 (25.00-47.00)	0.46	0.64	0.80	0.48	0.71	0.72	0.62	0.51	0.63
Allergen													
Birch	15	16	4	9									
Grass	18	10	4	8									
Leukocytes, X 10^9 cells/L*	6.10 ± 1.84	6.32 ± 1.66	6.33 ± 1.61	6.00 ± 0.83	0.74	0.99	0.64	0.78	0.41	0.60	0.84	0.61	0.46
Neutrophils, %*	55 ± 8	58 ± 7	56 ± 7	60 ± 7	0.80	0.59	0.24	0.07	0.43	0.31	0.92	0.29	0.12
Lymphocytes, %*	32 ± 7	31 ± 6	31 ± 6	29 ± 6	0.81	0.78	0.44	0.16	0.45	0.43	0.99	0.47	0.20
Monocytes, %*	8 ± 2	7 ± 2	8 ± 1	7 ± 2	0.49	0.14	0.43	0.23	0.59	0.08	0.26	0.66	0.22
Eosinophils, %†	4 (2-5)	2 (2-4)	3 (2-3)	2 (2-5)	0.26	0.56	**0.02**	0.06	0.95	0.48	0.69	**0.02**	0.23

*Variable is assumed to be normally distributed. Descriptive statistics are presented as mean SD.

†Variable is assumed to not be normally distributed. Descriptive statistics are presented as median (25–75th percentiles).

Definition of abbreviations: ER = early responders, PER = protracted early responder, DR = dual responders, CP = control participants, AP = all allergic participants

Table 2-2 Demographics of the validation cohort

	Allergic Participants			Healthy Participants	*P*-Values								
	ER (n=16)	**PER (n=11)**	**DR (n =7)**	**CP (n=10)**	**ER vs DR**	**PER vs DR**	**ER vs PER**	**CP vs ER**	**CP vs PER**	**CP vs DR**	**ER and PER vs DR**	**ER vs PER and DR**	**CP vs AP**
Female, %	81%	64%	71%	70%									
Height, cm*	164.13 ± 9.48	167.35 ± 11.21	169.14 ± 13.28	166.82 ± 10.63	0.39	0.77	0.44	0.52	0.91	0.71	0.51	0.29	0.87
Weight, kg†	106.50 (72.15-126.43)	107.50 (76.20-112.10)	89.00 (81.75-93.75)	87.25 (78.68-98.63)	0.49	0.60	0.64	0.64	0.76	0.99	0.48	0.48	0.70
Age, yr†	47.50 (44.75-50.75)	40.00 (35.50-51.00)	39.00 (28.50-51.50)	47.50 (30.00-52.00)	0.38	0.86	0.28	0.83	0.91	0.73	0.51	0.22	0.98
Leukocytes, X 10^9 cells/L*	7.53 ± 2.35	7.44 ± 2.07	6.57 ± 1.58	7.11 ± 1.53	0.27	0.33	0.91	0.59	0.68	0.49	0.27	0.56	0.75
Neutrophils, %*	57 ± 9	59 ± 9	56 ± 8	60 ± 6	0.70	0.49	0.72	0.40	0.66	0.28	0.56	0.94	0.32
Lymphocytes, %*	29 ± 6	30 ± 8	33 ± 8	31 ± 7	0.39	0.65	0.63	0.83	0.81	0.51	0.46	0.40	0.84
Monocytes, %*	8 ± 2	7 ± 1	8 ± 1	6 ± 1	0.63	0.07	0.29	0.09	0.47	**0.01**	0.24	0.61	**0.04**
Eosinophils, %†	3 (2-4)	2 (2-3)	3 (2-3)	2 (2-4)	0.49	0.59	0.16	0.29	0.89	0.67	0.87	0.17	0.52

*Variable is assumed to be normally distributed. Descriptive statistics are presented as mean SD.

†Variable is assumed to not be normally distributed. Descriptive statistics are presented as median (25–75th percentiles).

Definition of abbreviations: ER = early responders, PER = protracted early responder, DR = dual responders, CP = control participants, AP = all allergic participants

2.3.2 Environmental exposure unit (EEU)

Research participants were continuously exposed to 3500 grain/m^3 of allergen using the EEU model (3 hours of allergen exposure for rye grass study and 4 hours of allergen exposure for birch and house dust mite studies). Research participants recorded their TNSS and PNIF at the beginning of an EEU session, every half-hour throughout the session, and every hour from the end of the session until 12 hours had passed since allergen exposure was initiated.

2.3.3 Blood collection and processing

Whole peripheral blood samples were collected from research participants at baseline (before allergen exposure) using PAXgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and K2 EDTA Vacutainer tubes (BD, Franklin Lakes, NJ, USA). PAXgene tubes were processed (incubation at room temperature for 2 hours) and then stored at -80°C before and after shipment to the Tebbutt laboratory in Vancouver, Canada. Complete blood counts and differentials (CBC/diffs) were obtained from the EDTA tubes using an automated hematology analyzer (Sysmex XE-2100TM, Sysmex, Kobe, Japan).

2.3.4 Experimental techniques

2.3.4.1 RNA extractions

Intracellular RNA was extracted from 5 mL of each PAXgene tube using the PAXgene Blood miRNA kit **(**PreAnalytiX-Qiagen, Germany). The concentration and quality of extracted RNA was assessed using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively.

2.3.4.2 NanoString nCounter gene expression assay

The NanoString nCounter system allows for multiplexed measurement of gene expression and has reproducibility comparable to that of qPCR and exceeding that of RNA sequencing[104-107]. Additionally, nCounter technology is robust, non-enzymatic, and relies on direct hybridization (target molecules to probes) and imaging instead of amplification of target molecules[108]. This technology can be easily implemented in a clinical setting and can generate data in less than 24 hours, making it ideal for clinical research biomarker studies[109]. Positive and negative control probes are included in NanoString assays, and they can be utilized to assess assay performance and determine quality control metrics[110].

Purified RNA samples (100 ng) were analyzed with a custom NanoString nCounter Elements assay (NanoString Technologies, USA). This assay was developed by the Tebbutt laboratory to predict the late-asthmatic response in allergic asthma[5] and diagnose western red-cedar asthma[6]. The assay quantifies the expression of 166 transcripts using nCounter Elements TagSets (capture and reporter probes) and target-specific oligonucleotide probe pairs (Figure 2-2). The reporter probe is fluorescently labeled with a unique barcode (sequence of six colours) for each target transcript sequence, allowing for multiplexed digital counting of RNA[108]. NanoString assay workflow consisted of three steps. The first step in the protocol was to mix the RNA samples with the various reporter and capture probes. After hybridization at 67°C for 18 hours using a thermocycler, the reporter and capture probes bound to probes A and B which then further bound to the target transcript. Hybridization reactions were performed in sets of 12 using a strip of 12 tubes. Next, the samples were placed in the fully automated nCounter Prep Station for purification and immobilization of the tag-target complexes to the cartridge. The cartridges were processed at high sensitivity for 3 hours and scanned with the nCounter Digital Analyzer

under the maximum field of view (FOV) setting (555 FOVs) to obtain transcript counts.

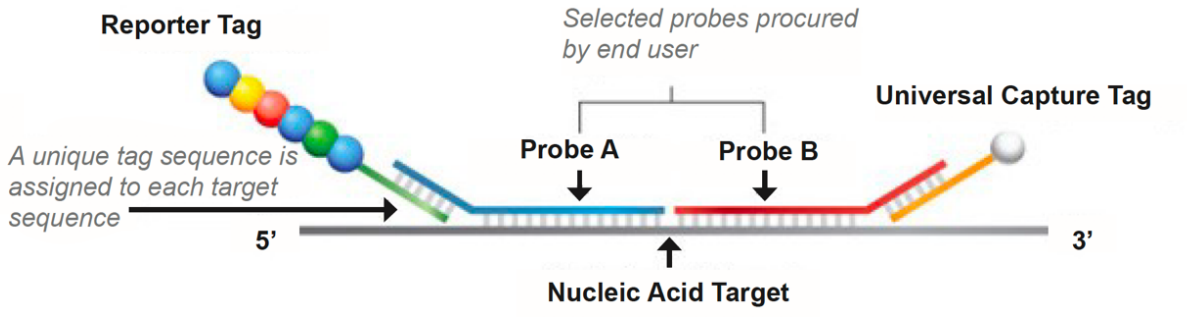


Figure 2-2 NanoString nCounter Elements tag-target complex.

2.3.5 Statistical methodologies

2.3.5.1 Data normalization

Data obtained from the nCounter Digital Analyzer was normalized using the statistical computing software program, R (version 3.6.3). First, the data was assessed for quality control metrics including FOV, binding density, linearity of positive control spike-ins and background signals (see appendix A.1 for details). To control for assay-to-assay variability, NanoString uses six positive control spike-ins at the following concentrations: 128fM, 32fM, 8fM, 2fM, 0.5fM and 0.125fM. The geometric mean of the six positive controls was calculated for each sample. Then, the mean of all sample geometric means was divided by each sample's geometric mean in order to obtain a normalization factor for each sample. The gene expression data for each sample was scaled by the corresponding normalization factor. To control for biological variation across samples, top housekeeping genes with the lowest coefficient of variations were used to perform housekeeping normalization. Technical variability was assessed using replicates of the same sample across multiple cartridges.

2.3.5.2 Differential gene expression

Differential gene expression analysis was performed using the Linear Models for Microarray and RNA-Seq data[111] (limma) R-library. Limma utilizes a moderation factor that shrinks the variance of each gene towards a common value[112]. This approach is advantageous for studies with small sizes as it minimizes the number of false positives. The Benjamini-Hochberg method was used to control the false discovery rate (BH-FDR) and to adjust for multiple hypotheses testing[113].

2.3.5.3 Biomarker analysis

A biomarker development pipeline was used to identify biomarker panels that could discriminate between AR phenotypes. The pipeline used different classification algorithms, such as elastic net[114] and random forest[115], to build predictive biomarker panels. The pipeline also incorporated a pathway-directed approach in which a set of genes was pre-selected if they belonged to the same pathway. Next, these genes were further evaluated using elastic net and random forest.

2.3.5.3.1 Elastic Net

Elastic net is a predictive model which assumes a linear relationship between independent variables (gene expression profiles) and dependent variables (phenotypes). Elastic net produces a biomarker panel by incorporating the penalties of lasso and ridge regression:

$$\frac{\sum_{i=1}^{n}(y_i - x_i^J \hat{\beta})^2}{2n} + \lambda \left(\frac{1-\alpha}{2} \sum_{j=1}^{m} \hat{\beta}_j^2 + \alpha \sum_{j=1}^{m} |\hat{\beta}_j| \right)$$

Incorporation of these penalties allows for variable selection (selects a limited number of genes from a large dataset) and shrinkage of regression coefficients (increases predictive performance). The size of a biomarker panel is controlled by the α parameter, which can range from 0 (all variables have non-zero regression coefficients) to 1 (many variables have zero regression coefficients and hence are excluded). The shrinkage of regression coefficients is controlled by the λ parameter, which is estimated using cross-validation (see Section 2.3.5.3.3).

2.3.5.3.2 Random forest

Random forest is an ensemble method which combines the predictions of tree classifiers. Tree classifiers are predictive models where the branches are made by splitting variables (genes expression profiles) at particular values that best separate the observations into the correct phenotypic groups. Each tree classifier was built by splitting randomly selected m variables:

$$m = \sqrt{p}\text{ , p = number of variables}$$

at each split. To identify an effective biomarker panel, random forest uses Gini importance score to rank variables based on how often a certain variable is selected at each split and its ability to divide observations into their phenotypic groups.

2.3.5.3.3 Cross-validation

The test error of a biomarker panel can be estimated using cross-validation, which splits the observations into a training and test set. After training a biomarker model on the training set, the test error is computed using the test set. In this chapter, leave-one-out cross-validation (LOOCV)

was performed, which trains a model on all the observations except for one and a prediction is made using that one observation. This process is repeated until all observations have been used as a test set and then the average test error is calculated. The training and test sets used for cross validation were not biased by a previous univariate statistical filter. Cross validation produced a list of probabilities (likelihood of being a certain phenotype) for all participants that were used to calculate the area under the receiver operating curve (AUC), a measure of classification performance. The AUC can range from 0 (all predictions made by the model are incorrect) to 1 (all predictions made by the model are correct). An AUC of 0.50 indicates that the classification model is randomly splitting the observations into two phenotypic groups. For this analysis, an AUC threshold of 0.70 was applied because it is recommended for further clinical biomarker implementation[116]. Once optimal final tuning parameters (result in an AUC of 0.70 or greater) were selected using cross validation, the final model was refit using all the observations. The performance of the final model was then assessed using an additional cohort.

2.4 Results

2.4.1 Demographics of discovery and validation cohorts

There were no significant differences in age, height, weight or total leukocyte counts for all phenotypic comparisons within the discovery and validation cohorts (P-value < 0.05). Significant differences were found in leukocyte subtypes. In the discovery cohort, eosinophils were significantly different between 1) ERs versus PERs and 2) ERs versus grouped PERs and DRs. In the validation cohort, monocytes were significantly different between 1) healthy controls versus DRs and 2) healthy controls versus all allergic participants.

2.4.2 Reproducibility assessment and batch correction

Aliquots of a single RNA sample were run on different cartridges as part of the discovery cohort (January 16th, April 11th and 14th 2019) and the validation cohort (September 2nd and 3rd 2020) to assess inter-assay variability. Figure 2-3 depicts sample correlations (Pearson) among the five technical replicates using log2 transformed data (before housekeeping normalization) of all 166 transcripts. Excellent correlations ranging between 0.978-0.991 were obtained between all replicates except one (April 14th, 2019). Correlations between this one replicate and the other four replicates ranged between 0.902-0.934. Intra-assay variability was assessed by running duplicate aliquots of RNA samples on the same cartridges. Excellent correlations, ranging between 0.970-0.996, were obtained for both intra-assay replicates (Figure 2-4).

The discovery and validation cohorts were analyzed using two different NanoString TagSets and thus were normalized together (discovery cohort: TagSet 192 and extension TagSet 24; validation cohort: TagSet 192 and extension TagSet 36). A batch effect between the discovery and validation cohorts was identified using principal component analysis (PCA) (Figure 2-5A). Batch correction was performed using the Combat R-library, which uses parametric and non-parametric empirical Bayes (EB) frameworks to remove batch effects[117] (Figure 2-B).

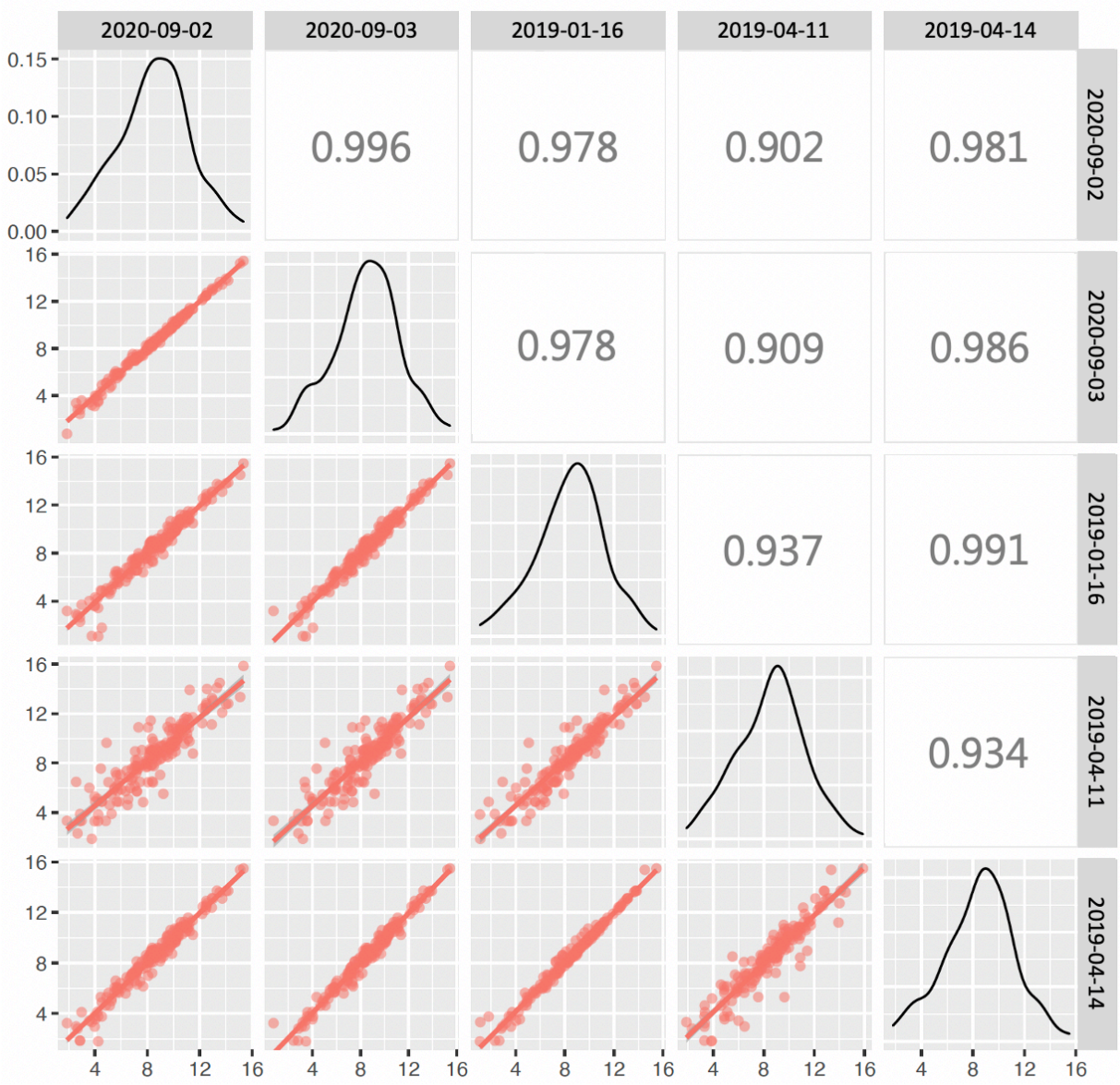


Figure 2-3 Scatter plots and Pearson correlations of 166 genes measured on aliquots of a single RNA sample across multiple batches. The x-axis and y-axis of the plot are log2 transformed counts of gene expression for the sample shown along the top and the right-hand side, respectively.

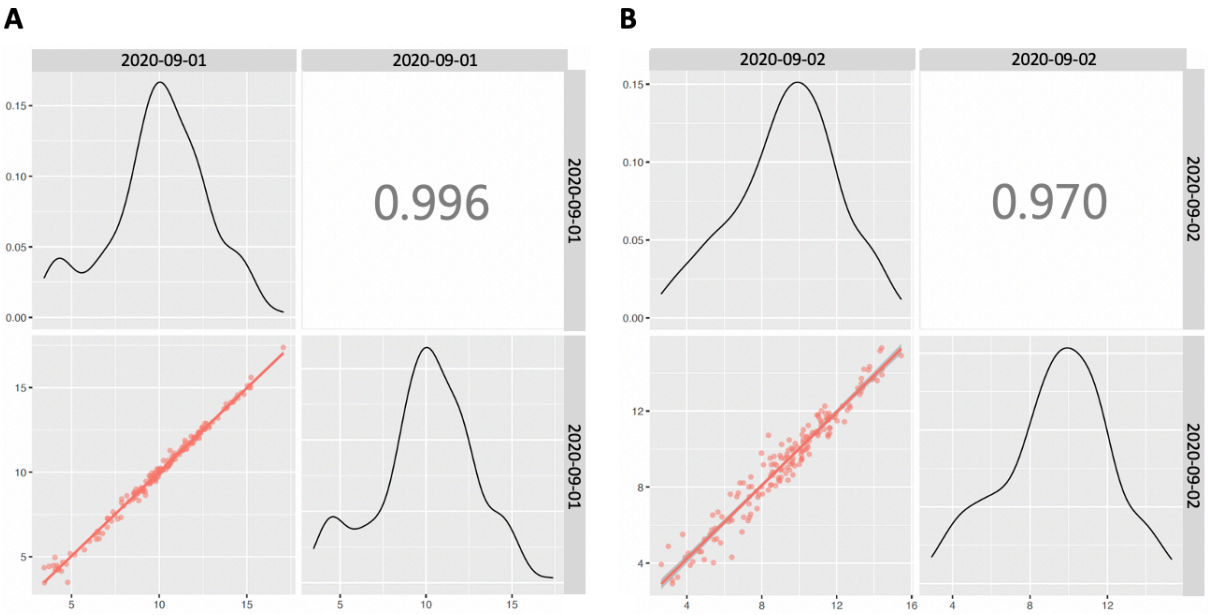


Figure 2-4 Scatter plots and Pearson correlations of 166 genes measured on duplicate aliquots of RNA samples on the same cartridge. The x-axis and y-axis of the plot are log2 transformed counts of gene expression for the sample shown along the top and the right-hand side, respectively. A Duplicate aliquots from September 1st, 2020 B Duplicate aliquots from September 2nd, 2020.

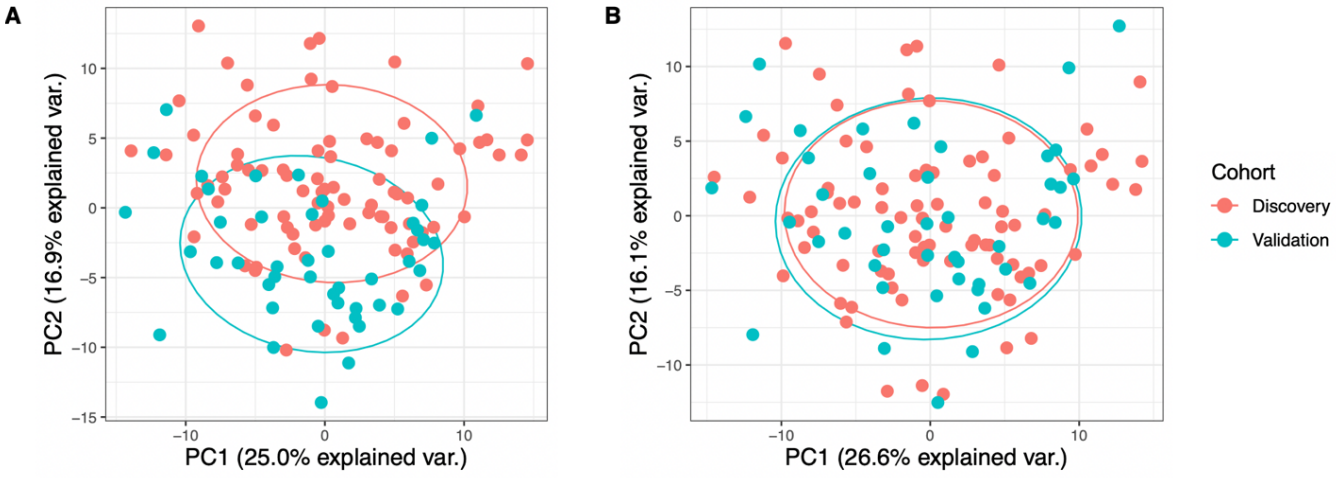
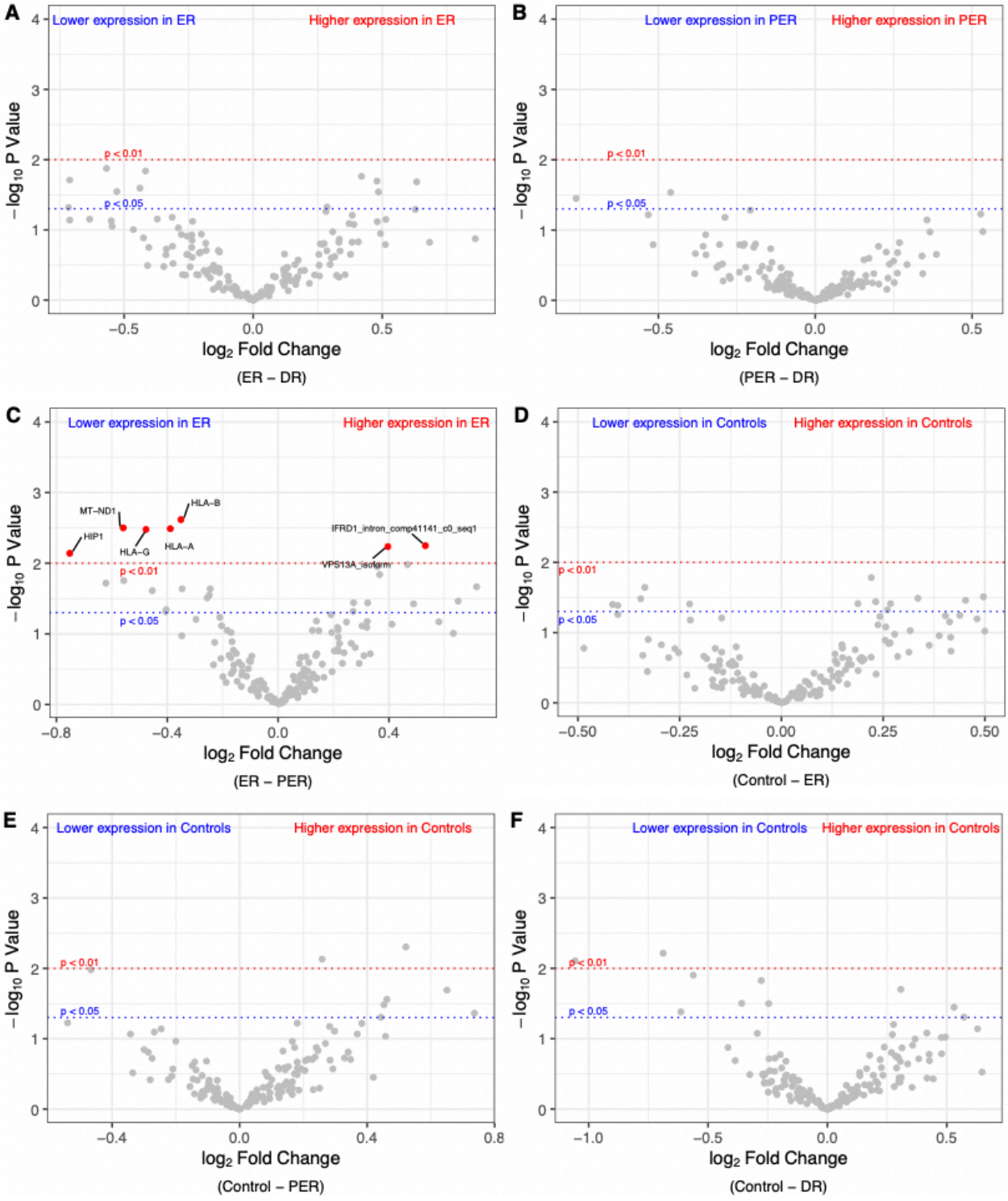


Figure 2-5 Principal component analysis before and after batch effect correction. Batch effect correction was performed using the ComBat R-library. A Before batch-correction B After batch-correction.

2.4.3 Differentially expressed RNA in discovery and validation cohorts

At an FDR cut-off of 0.20, transcripts were differentially expressed in only two comparisons in the discovery cohort: ERs versus PERs (Figure 2-6C) and ERs versus grouped PERs & DRs (Figure 2-6H). In Figure 2-6C, ERs had significantly higher expression of *IFRD1_intron_comp41141_c0_seq1* and *VPS13A_isform* while PERS had significantly higher expression of *HLA-A*, *HLA-B*, *HLA-G, MT-ND1*, and *HIP1*. These genes were also differentially expressed between ERs versus grouped PERs & DRs (Figure 2-6H). No transcripts were differentially expressed in the other seven comparisons in the discovery cohort: ERs versus DRs, PERs versus DRs, DRs versus healthy participants, ERs versus healthy participants, PERs versus healthy participants, grouped ERs & PERs versus DRs, and all allergic participants versus healthy controls. In the validation cohort, no transcripts were differentially expressed in any comparison at an FDR cut-off of 0.20. Differential gene expression analysis was also performed after combining all the samples from the discovery and validation cohorts together and after stratifying by sex, no transcripts were differentially expressed in any phenotypic comparison.


A
Lower expression in ER
Higher expression in ER
p < 0.01
p < 0.05
−log10 P Value
log2 Fold Change
(ER – DR)
B
Lower expression in PER
Higher expression in PER
p < 0.01
p < 0.05
−log10 P Value
log2 Fold Change
(PER – DR)
C
Lower expression in ER
Higher expression in ER
MT−ND1
HLA−B
HIP1
HLA−G
HLA−A
IFRD1_intron_comp41141_c0_seq1
VPS13A_isoform
p < 0.01
p < 0.05
−log10 P Value
log2 Fold Change
(ER – PER)
D
Lower expression in Controls
Higher expression in Controls
p < 0.01
p < 0.05
−log10 P Value
log2 Fold Change
(Control – ER)
E
Lower expression in Controls
Higher expression in Controls
p < 0.01
p < 0.05
−log10 P Value
log2 Fold Change
(Control – PER)
F
Lower expression in Controls
Higher expression in Controls
p < 0.01
p < 0.05
−log10 P Value
log2 Fold Change
(Control – DR)

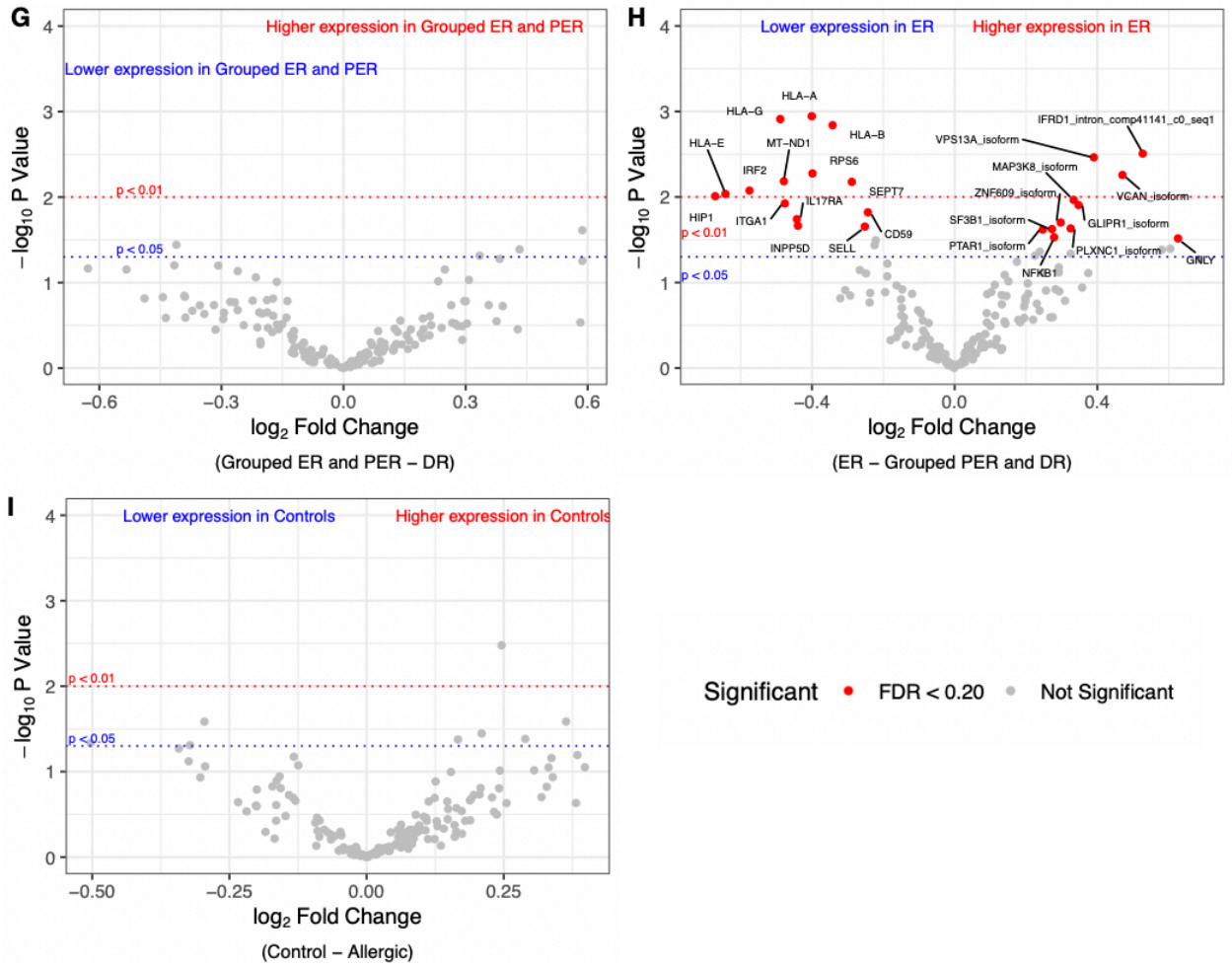


Figure 2-6 Volcano plots of gene expression data from the discovery cohort. A Early responders (ERs) versus Dual responders (DRs), **B** Protracted early responders (PERs) versus DRs, **C** ERs versus PERs, **D** Controls versus ER, **E** Controls versus PER, **F** Controls versus DR, **G** Grouped ERs & PERs versus DRs, **H** Grouped PERs & DRs versus ERs, **I** Controls versus Allergic.

2.4.4 Principal component analysis (PCA)

Using all genes measured on the custom NanoString assay, a PCA was performed for samples in the discovery and validation cohorts. In Figure 2-7, separation is not observed in the PCA plots between ERs versus DRs, PERs versus DRs, ERs versus PERs, Grouped ERs & PERs versus DRs, ERs versus Grouped PERs & DRs, and all allergic participants versus healthy controls.

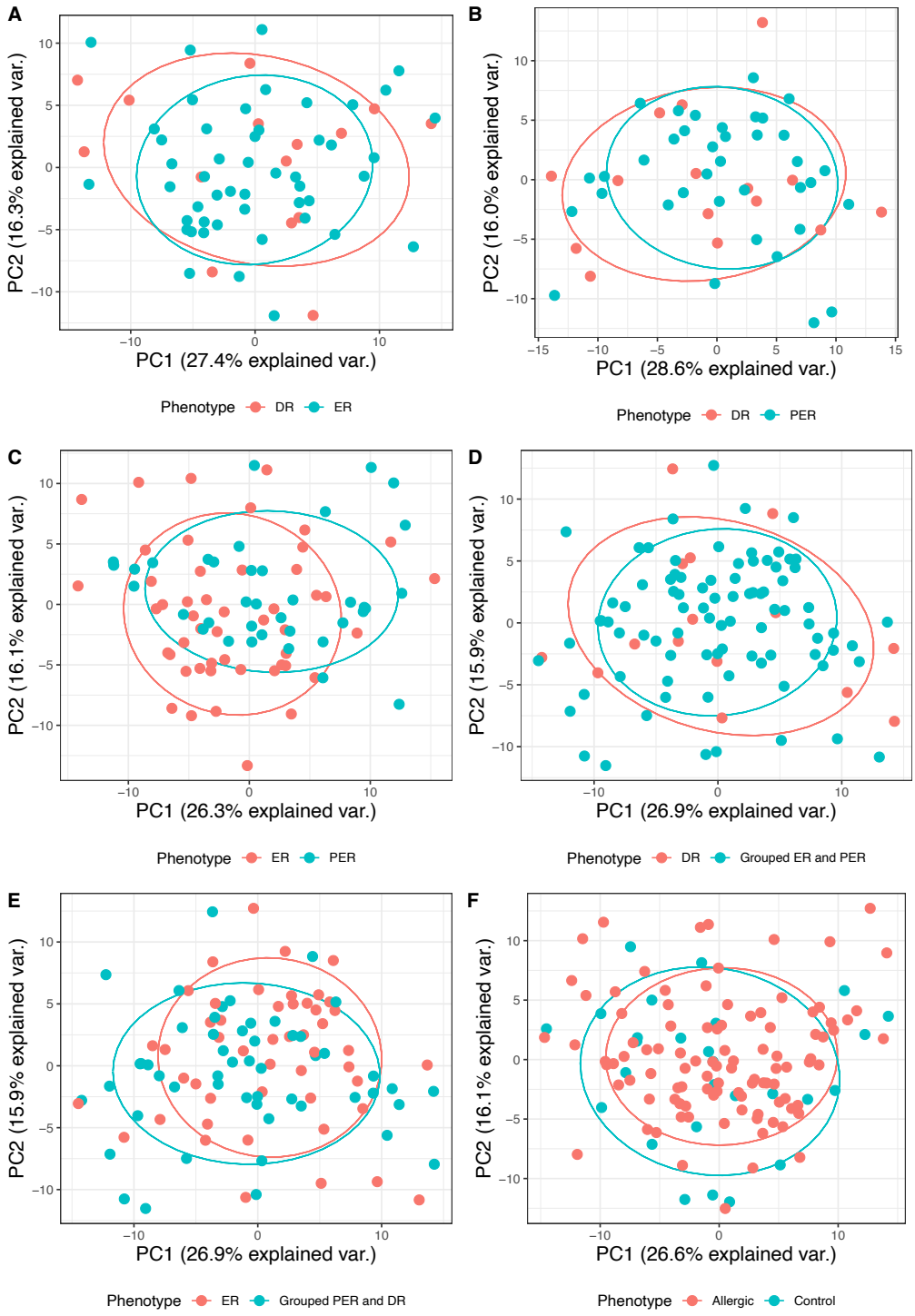


Figure 2-7 Principal component analysis plot of all samples (discovery + validation). **A** Early responders (ERs) versus Dual responders (DRs), **B** Protracted early responders (PERs) versus DRs, **C** ERs versus PERs, **D** Grouped ERs & PERs versus DRs, **E** Grouped PERs & DRs versus ERs, **F** Allergic participants versus healthy controls.

2.4.5 Biomarker analysis

A biomarker development pipeline was used to identify biomarker panels that could discriminate between AR phenotypes at baseline. Multiple panels with AUC performance greater than 0.70 were identified in the discovery cohort using a pathway-directed approach. However, these panels were unable to be validated in the validation cohort for all phenotypic comparisons.

Participants in the discovery cohort were exposed to seasonal allergens (rye grass and birch) in the EEU, and these studies were performed out of season. In contrast participants in the validation cohort were exposed to a perennial allergen (house dust mite) in the EEU. Because allergen exposure frequency of seasonal allergy is limited, seasonal allergens result in less chronic inflammation compared to perennial allergens. Slightly different molecular mechanisms may be underlying allergic responses caused by seasonal and perennial allergens. Therefore, the biomarker analysis was performed again using training and test sets which included samples from all three allergens and had a similar composition of sex. Training and test sets were created by combining the discovery and validation cohorts together and then randomly splitting the samples using the Caret R-library.

Using the training sets, biomarker panels were identified that could discriminate between phenotypes. Figure 2-8 shows the AUC performance (LOOCV) of the identified panels (orange line) and after the phenotypic labels were reshuffled (blue line). A six-gene panel (*BCL6*, *FNIP1*, *PDCD1*, *FADD*, *HCLS1*, and *RORC*, pathway = GO regulation of leukocyte apoptotic process) could discriminate between ERs and DRs (AUC=0.73) in the training set (Figure 2-8A). Combinations of genes were also identified that could discriminate between DRs and healthy controls (*CCR2*, *CARM1*, *SETX*, *BCL6*, *LTF*, *SEMA4D*, *CDK5RAP3*, *MAP2K2*, *GATA3*, *LTK*, and *SMAD2*, pathway = GO regulation of cell development). This panel had an AUC of 0.76 in

the training set (Figure 2-8B). Using a fifteen-gene panel (*NFKBIA*, *CTSS*, *CLEC4E*, *CASP8*, *IL17RA*, *MSN*, *CCR2*, *HIP1*, *GATA3*, *IL17A*, *HCLS1*, *PLA2G6*, *ATP8A1*, *PPP3R1*, *PLAGL2*, and *CD4*, pathway = GO positive regulation of transport), grouped ERs and PERs could be discriminated from DRs (AUC=0.72) in the training set (Figure 2-8C). *GATA3* plays a pivotal role in allergic immune responses and was included in two of the three biomarker panels used to discriminate between AR phenotypes. *GATA3* had a nominal p value of 0.37 between DRs and healthy controls and a nominal p value of 0.57 between DRs and grouped ERs and PERs. In both comparisons, DRs had decreased expression of *GATA3*.

The predictive performance of the three panels was then assessed in the test sets. In the test sets, the panels only had AUC performance greater than 0.70 after recalibration. Recalibration refers to re-training the model in the test set, which results in different regression coefficients for each transcript part of the panel. The AUC performance of biomarker panels that could discriminate ERs from DRs, healthy controls from DRs, and grouped ERs and PERs from DRs were 0.81, 0.75, and 0.78, respectively.

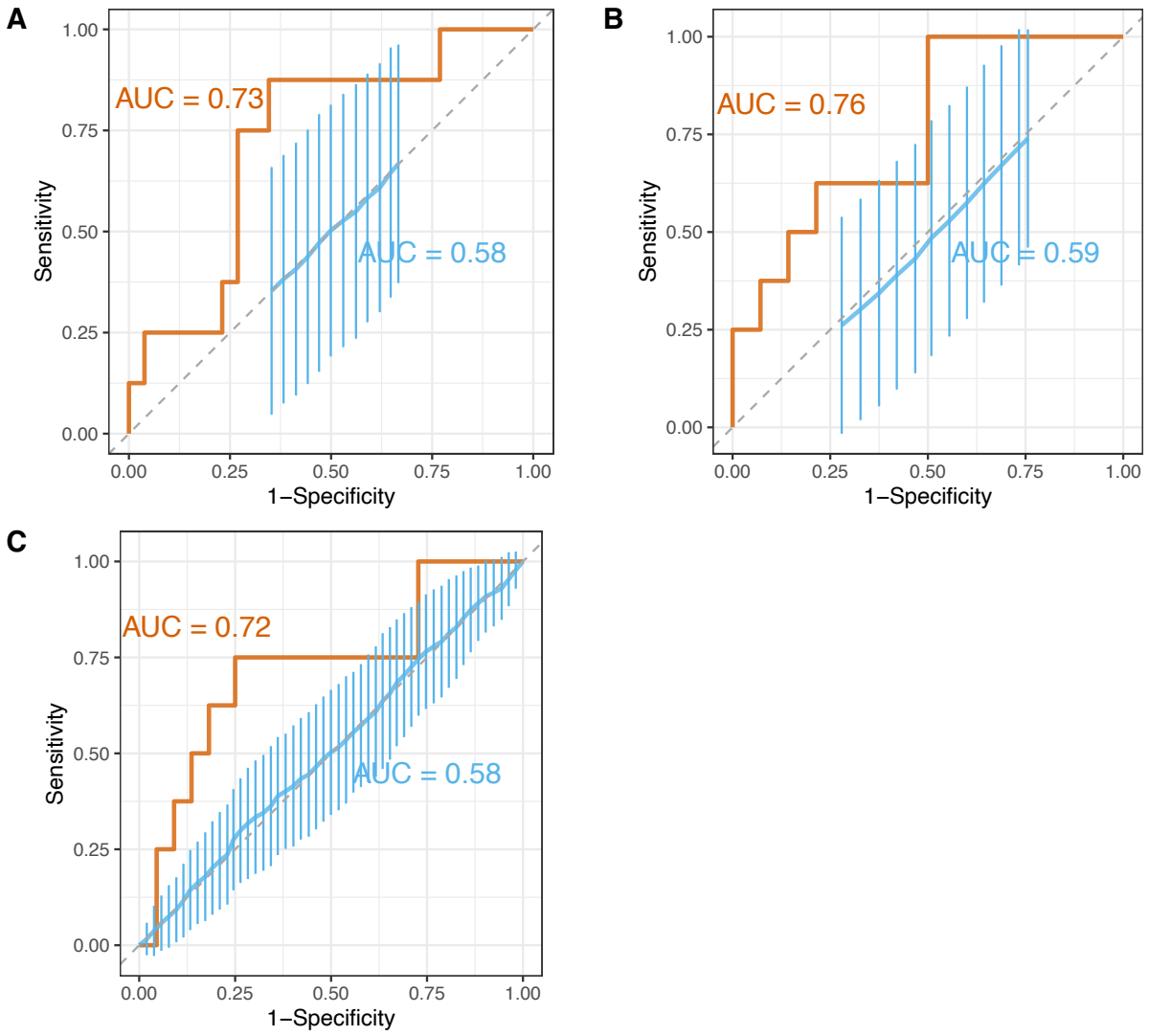


Figure 2-8 AUC performance of biomarker panels (orange line) and after reshuffling phenotypic labels (blue line) in training set. A: Early responders (ERs) and dual responders (DRs), **B**: DRs and healthy controls, **C**: Grouped ERs & protracted ERs and DRs.

2.4.6 Additional analyses

The biomarker analysis detailed in section 2.4.5 was performed again after re-phenotyping allergic participants using nasal congestion scores (see section 3.3.2.2 for re-phenotyping details). Participants were divided into two categories based on their nasal congestion scores: high congestion (HC) and low congestion (LC). We were unable to identify and validate

biomarker panels that could discriminate between the HC and LC phenotypes. A differential expression analysis was also performed using the new phenotypes. However, no genes were identified to be significantly different between the HC and LC phenotypes (FDR < 0.20). Using both the traditional (TNSS) and new (nasal congestion) phenotypes, unsupervised clustering analyses were performed. The clustering analyses were unable to identify distinct clusters in the expression dataset.

2.5 Discussion

In this study, we were interested in identifying blood-based molecular differences between different phenotypes of AR. We determined that standard differential expression analysis was able to identify statistically significant genes in only two comparisons in the discovery cohort: ERs and PERs (Figure 2-6C) and ERs and grouped PERs & DRs (Figure 2-6H). Statistically significant genes were not identified in the validation cohort. Additionally, the fold change directions of the statistically significant genes in the discovery cohort were different from those identified in the validation cohort (Appendix A2). An exploratory differential expression analysis after combining the cohorts together and after stratifying by sex also did not identify statistically significant genes different between phenotypes, suggesting that little variation exists between the genes measured on the NanoString assay and AR phenotypes.

One of the aims of this chapter was to identify blood-based biomarker panels that could discriminate between AR phenotypes at baseline. Using a multivariate biomarker approach, we were unable to validate panels (AUC > 0.70) identified in the discovery cohort. However, the discovery cohort used seasonal allergens and the validation cohort used a perennial allergen. Seasonal and perennial AR result in slightly different nasal symptoms[118], suggesting different

molecular mechanisms may be underlying the two AR subtypes. Therefore, all samples (discovery + validation) were combined and then a training and a test set were randomly created to identify and test biomarker panels. Using a pathway-directed biomarker approach, we found multiple panels in the training set that could discriminate between phenotypes. These signals were unable to be replicated in the test set. Only after recalibration were panels able to discriminate between phenotypes in three comparisons: ERs and DRs, DRs and healthy controls, and grouped ERs & PERs and DRs. ERs and DRs could be discriminated with a biomarker panel that was developed using the GO regulation of leukocyte apoptotic process pathway. This panel included the gene *FNIP1*, which is also part of a biomarker panel that can discriminate between ERs and DRs in allergic asthma[5]. The GO regulation of cell development and GO positive regulation of transport pathways were used to develop biomarker panels that could discriminate DRs from healthy controls and grouped ERs and PERs from DRs, respectively. *GATA3* was included in both of these panels. The dual response is the classic biphasic response observed in allergic asthma. Thus, it is not surprising that *GATA3* was implicated in these comparisons, as it plays a critical role in the differentiation of Th2 cells[119] and its inactivation results in a significantly attenuated late asthmatic response[120].

These three biomarker panels had discriminatory ability only after recalibration. However, there should not be a need to recalibrate the panels because all samples were normalized together, and batch-correction was performed. Next, the directions of regression coefficients of the models used in the training and test sets were compared (Appendix A3). For many genes, the directions of the regression coefficients flip before and after recalibration. This suggests that the relationship between the predictor variable and the response variable is different

between the two models, and that the signal identified in the training set is not truly being validated.

The second aim of this chapter was to provide further evidence that the protracted early response is a distinct phenotype of AR as an intermediate allergic response is not observed in allergic asthma, unlike the early response and dual response. Using all genes measured on the custom NanoString assay, a PCA was performed for all samples (discovery + validation). In Figure 2-7, separation between AR phenotypes is not observed (significant overlap between 95% confidence ellipses).

Together, these findings suggest that based on the 166 genes measured in blood at baseline, limited molecular variation exists between AR phenotypes. In the future, RNA samples should be profiled with a NanoString assay that measures a larger number of genes, such as the PanCancer Immune Profiling panel, which measures the expression of 730 immune genes in peripheral blood.

2.6 Conclusion

Limitations of this study include small sample size of DRs, small expression dataset (only measured 166 genes), and different types of allergens were used in the discovery and validation cohorts. In this chapter, a thorough biomarker analysis was performed for AR phenotypes (TNSS phenotypes and nasal congestion phenotypes). Leveraging previous biological knowledge, blood-based biomarker panels were identified that could discriminate between phenotypes at baseline. The performance of the panels was then assessed using an independent validation cohort. Despite not being able to validate the identified panels, this work is still of importance

because it is one of the few studies that has investigated molecular differences between AR phenotypes.

Chapter 3: Association between polymorphisms in cholinergic synapse pathway genes and late-onset congestion in allergic rhinitis

3.1 Introduction

AR is characterized by an immediate EPR and, in some individuals, an LPR which occurs 6 to 9 hours after allergen exposure[51-52]. Although the LPR includes symptoms of rhinorrhea, sneezing and nasal congestion and itching similar to the EPR, it is primarily characterized by nasal congestion[71]. It is not well understood why only a subgroup of AR patients develop an LPR.

This chapter of the thesis focuses on studying the association between SNPs, genetic variants occurring at specific sites in the genome, in cholinergic synapse pathway genes and the development of the LPR. In asthmatics, enrichment of minor alleles in cholinergic synapse pathway genes is associated with the development of late asthmatic responses[7]. Because allergic asthma and AR are inflammatory disorders with similar pathophysiology, enrichment of minor alleles in these pathway genes may be influencing the development of the LPR. This chapter also focuses on phenotyping AR using nasal congestion scores instead of total nasal symptom scores (TNSS; composite of rhinorrhea, nasal congestion, nasal itching, and sneezing). Nasal congestion is the predominate symptom experienced in the LPR and hence may be a useful measurement to use to phenotype allergic responses.

3.1.1 Cholinergic synapse pathway

Present in both parasympathetic and sympathetic systems, cholinergic synapses convert a presynaptic electrical signal into a chemical signal using acetylcholine (Figure 3-1). Acetylcholine is synthesized from acetyl coenzyme A (acetyl-CoA) and choline in a reaction catalyzed by choline acetyltransferase (CAT). During increased neuronal activity, release of

acetyl-CoA from mitochondria is upregulated and an influx of choline uptake is observed at nerve endings in the synaptic cleft. After diffusing across the synaptic cleft, acetylcholine can bind to two types of receptors: ionotropic nicotinic acetylcholine receptors (nAChRs) and metabotropic muscarinic acetylcholine receptors (mAChRs).

3.1.2 Acetylcholine receptors

Activation of nAChRs result in a rapid influx of sodium and calcium ions (Na^+ and Ca^{2+}), leading to cellular depolarization and generation of an excitatory action potential. Activation of mAChRs is slower because they are G-protein coupled receptors (GPCRs). After activation of GPCRs by ligand binding, a second messenger system is generated using intracellular signaling molecules to produce an excitatory or inhibitory response. Activation of mAChRs alters cellular homeostasis of proteins such as phospholipase C, adenylate cyclase, and cyclic adenosine monophosphate (cAMP), depending on the type of muscarinic receptors (M1–M5) present at the postsynaptic side[121].

Odd-numbered muscarinic receptors (M1, M3, M5) are coupled to $G_{q/11}$ proteins and activate phospholipase C (PLC)[122-123]. This leads to an increase in neuronal excitability through activation of cation channels which release Ca^{2+} from intracellular stores or inhibit calcium-regulated potassium (SK) channels[124]. Even-numbered muscarinic receptors (M2, M4) are coupled to G_i/G_o proteins and inhibit adenylate cyclase and cAMP[125]. Inhibition of these proteins decreases neuronal activity by inhibition of Ca^{2+} channels[126] and reduction of Ca^{2+} priming of SK channels[127]. Furthermore, muscarinic receptors can couple with signaling pathways that involve subsequent activation of serine/threonine protein kinases that modulate gene expression.

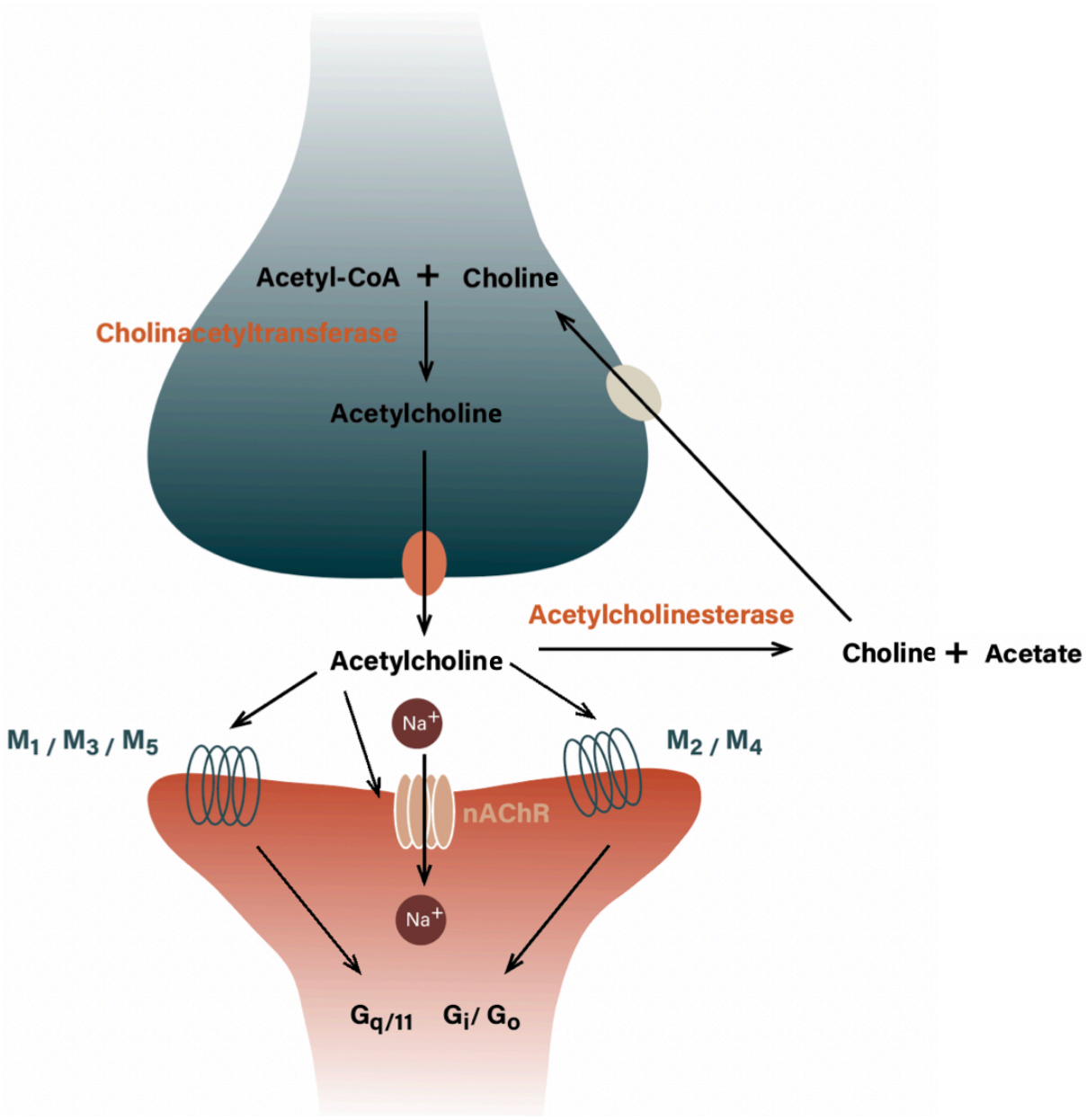


Figure 3-1 Summary of cholinergic synapse events.

Action potentials trigger presynaptic neurons to release acetylcholine molecules, which diffuse across the synaptic cleft and interact with acetylcholine receptors: ionotropic nicotinic acetylcholine receptors (nAChRs) and metabotropic muscarinic acetylcholine receptors (mAChRs). *Definition of abbreviations:* M1-M5 = type of muscarinic receptors; $G_{q/11}$ and $G_{i/o}$ = types of G proteins.

3.1.3 Importance of cholinergic synapses in atopic disorders

Clinical manifestations of allergy are controlled by the parasympathetic nervous system (PNS). Presynaptic and postsynaptic parasympathetic nerves are almost entirely cholinergic and when stimulated, increase digestive secretions and decrease respiration and heart rate. The nose is supplied by parasympathetic nerves which traverse the vidian nerve and innervate serous and mucous cells of submucosal glands, arteries and veins[128-130]. Surgical removal of nerves supplying the nose (vidian neurectomy) prevents allergy-associated overactive secretion and surgical removal of the nerves supplying the airway (vagotomy) prevents asthmatic symptoms and inflammation[131]. A similar effect can be achieved using pharmacologic agents, such as atropine, which block parasympathetic nerve signaling.

3.2 Hypothesis

We hypothesized that enrichment of SNPs in cholinergic synapse pathway genes may be contributing to the development of the LPR in AR.

3.3 Materials and Methods

3.3.1 Research participants

Upon written informed consent, participants were recruited into EEU studies at Kingston General Hospital (74 allergic participants and 20 healthy controls). For this project, blood samples were pooled from three EEU studies (rye grass, birch and house dust mite). The same participants from the rye grass and birch EEU studies were used in chapters 2 and 3. Allergic participants had a positive skin prick test to rye grass, birch or house dust mite (defined as a wheal diameter of 3 mm or greater than that produced by the negative control)[103] and a 2-year

documented history of allergic rhinoconjunctivitis symptoms. Healthy participants had a negative skin prick test to common environmental allergens and did not have a documented history of allergic rhinoconjunctivitis symptoms. Details regarding subject exclusion criteria can be found in Chapter 2.3.1. Participants underwent allergen exposure in the EEU, details regarding the EEU and collection of clinical system scores can be found in Chapter 2.3.2. Blood samples were collected before an EEU session and processed as previously described in Chapter 2.3.3.

3.3.2 Phenotyping research participants

3.3.2.1 Phenotyping using total nasal symptom scores (TNSS)

Participants were grouped into one of three categories based on the pattern of hourly reported TNSS: 32 participants were early responders (ERs), 27 participants were protracted ERs (PERs), and 15 participants were dual responders (DRs) (Figure 3-2). Phenotypes were assigned using guidelines[3-4] previously described in Chapter 1.9.3 (Table 3-1).

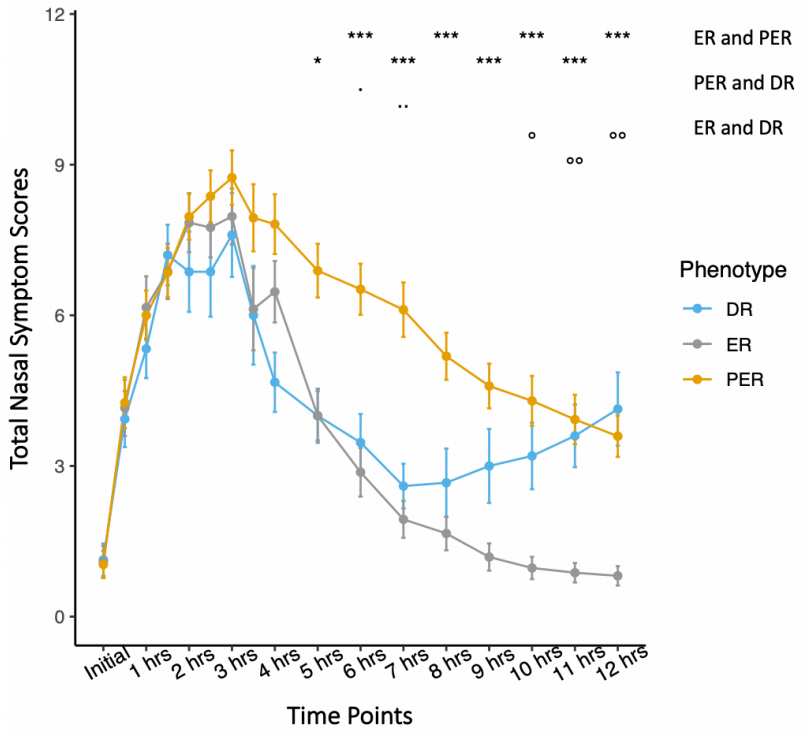


Figure 3-2 Phenotyping using total nasal symptom scores (TNSS). Research participants were classified as early responders (ERs), protracted ERs (PERs) or dual responders (DRs) based on changes in TNSS data. Significant differences were found between ERs and PERs (*) at hours 5 to 12, between ERs and DRs (○) at hours 10 to 12, and between PERs and DRs (•) at hours 6 to 7. Analysis was performed using Wilcoxon Signed Rank test with Bonferroni corrections. The number of symbols indicates the *P*-value: one symbol indicates that the *P*-value was less than 0.1, two symbols indicate that the *P*-value was less than 0.01, three symbols indicate that the *P*-value was less than 0.001, and four symbols indicate that the *P*-value was less than 0.0001.

Table 3-1 Demographics of participants phenotyped using total nasal symptom scores

	Allergic Participants			Healthy Participants	*P*-Values								
	ER (n=32)	**PER (n=27)**	**DR (n =15)**	**CP (n=20)**	**ER vs DR**	**PER vs DR**	**ER vs PER**	**CP vs ER**	**CP vs PER**	**CP vs DR**	**ER and PER vs DR**	**ER vs PER and DR**	**CP vs AP**
Female, %	47%	69%	60%	75%									
Height, cm*	169.08 ± 8.92	169.80 ± 10.93	170.25 ± 9.28	166.83 ± 6.48	0.72	0.89	0.77	0.30	0.20	0.29	0.79	0.69	0.14
Weight, kg†	85.45 (71.95-91.50)	88.20 (66.10-97.35)	79.70 (72.30-89.65)	85.30 (75.70-101.68)	0.60	0.45	0.68	0.63	0.83	0.33	0.48	0.96	0.55
Age, yr†	39.00 (30.00-48.50)	39.00 (32.50-43.50)	39.00 (28.50-45.00)	38.50 (25.75-48.00)	0.53	0.64	0.88	0.62	0.82	0.75	0.54	0.68	0.80
Allergen													
Birch	13	12	4	9									
Grass	16	10	4	8									
House Dust Mite	3	5	7	3									
Leukocytes, X 10^9 cells/L*	6.19 ± 1.76	6.45 ± 1.55	6.44 ± 1.55	6.09 ± 0.84	0.63	0.97	0.54	0.79	0.31	0.44	0.78	0.51	0.36
Neutrophils, %*	57 ± 8	58 ± 7	56 ± 8	59 ± 7	0.85	0.43	0.49	0.25	0.61	0.24	0.61	0.69	0.26
Lymphocytes, %*	31 ± 7	31 ± 7	32 ± 7	29 ± 7	0.86	0.72	0.82	0.40	0.53	0.39	0.78	0.94	0.36
Monocytes, %*	8 ± 3	7 ± 1	8 ± 1	7 ± 2	0.33	**0.01**	0.29	0.22	0.72	**0.01**	0.06	0.30	0.16
Eosinophils, %†	3 (2-4)	2 (1-3)	3 (2-3)	2 (2-4)	0.34	0.28	**0.02**	0.08	0.83	0.61	0.99	**0.03**	0.36

*Variable is assumed to be normally distributed. Descriptive statistics are presented as mean SD.

†Variable is assumed to not be normally distributed. Descriptive statistics are presented as median (25–75th percentiles).

Definition of abbreviations: ER = early responders, PER = protracted early responder, DR = dual responders, CP = control participants, AP = all allergic participants

3.3.2.2 Phenotyping using nasal congestion scores

Allergic participants were also phenotyped using nasal congestion scores. Participants were divided into two categories based on hourly reported nasal congestions scores: 38 participants belonged to the high congestion (HC) subgroup and 36 participants belonged to the low congestion (LC) subgroup (Figure 3-3). The HC subgroup did not experience a 50% reduction in nasal congestion scores by Hour 6 compared to Hour 3 and did not return to baseline by Hour 12. In contrast, the LC subgroup did experience a 50% reduction in nasal congestion scores by Hour 6 compared to Hour 3 and did return to baseline by Hour 12. Demographics of the two subgroups are shown in Table 3-2.

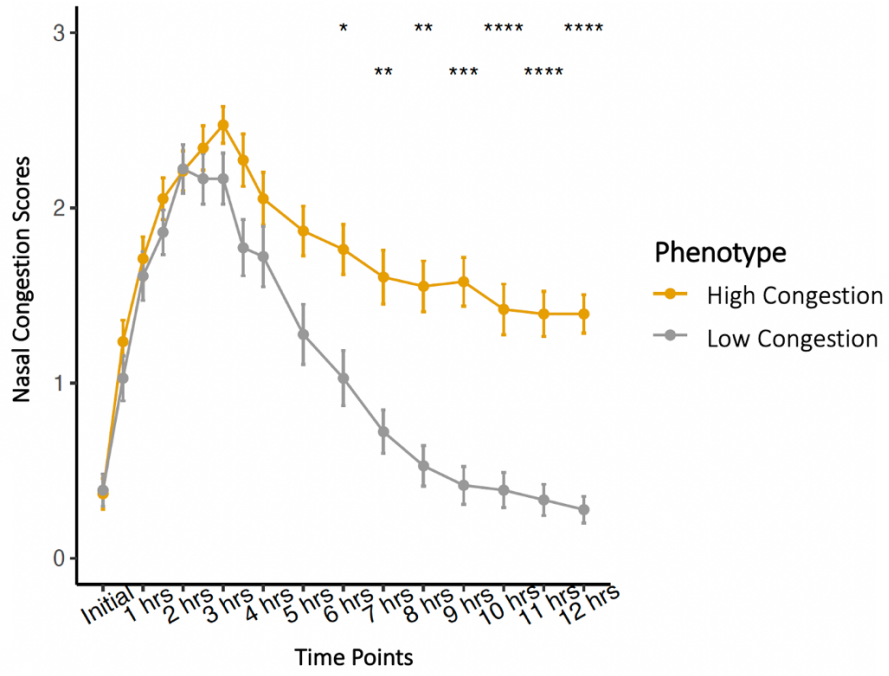


Figure 3-3 Phenotyping using nasal congestion scores. Research participants were divided into two subgroups, high congestion (HC) and low congestion (LC), based on changes in nasal congestion score data. Significant differences were found between HC and LC subgroups at hours 6 to 12. Analysis was performed using Wilcoxon Signed Rank test with Bonferroni corrections. The number of symbols indicates the *P*-value: one symbol indicates that the *P*-value was less than 0.1, two symbols indicate that the *P*-value was less than 0.01, three symbols indicate that the *P*-value was less than 0.001, and four symbols indicate that the *P*-value was less than 0.0001

Table 3-2 Demographics of participants phenotyped using nasal congestion scores

	HC (n = 38)	LC (n = 36)	*P*-Values
Female, %	53%	61%	
Height, cm*	170.99 ± 9.44	168.09 ± 9.17	0.19
Weight, kg†	85.45 (72.55-96.45)	85.75 (68.48-91.45)	0.83
Age, *yr*†	39.00 (31.00-44.75)	38.57 (30.00-45.75)	0.67
Allergen			
Birch	14	15	
Grass	16	14	
HDM	8	7	
Blood cell counts and frequencies before challenge			
Leukocytes, × 10^9 cells/L*	6.49 ± 1.61	6.17 ± 1.65	0.39
Neutrophils, %*	57 ± 7	56 ± 8	0.35
Lymphocytes, %*	31 ± 6	32 ± 7	0.50
Monocytes, %*	7 ± 2	8 ± 2	0.27
Eosinophils, %†	3 (2-3)	3 (2-4)	0.91

*Variable is assumed to be normally distributed. Descriptive statistics are presented as mean SD. A t test was used to compare between the two groups. †Variable is assumed to not be normally distributed. Descriptive statistics are presented as median (25–75th percentiles). A Wilcoxon rank-sum test was used to compare between the two groups.

Definition of abbreviations: HC = high congestion, LC = low congestion

3.3.3 Experimental techniques

3.3.3.1 DNA extractions

After thawing PAXgene tubes for 2 hours, DNA was extracted from 5 mL of PAXgene sample. Using the PAXgene Blood miRNA kit (PreAnalytiX-Qiagen, Germany), nucleic acid pellets were resuspended and then incubated in buffers together with proteinase K for protein digestion.

Next, the cell lysate was homogenized and then DNA was extracted using buffers from the PAXgene Blood DNA kit (PreAnalytiX-Qiagen, Germany). The concentration and quality of extracted DNA was measured using PicoGreen dsDNA quantitation assay, an ultrasensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (Thermo Scientific, Wilmington, DE, USA).

3.3.3.2 Affymetrix axiom arrays

Genotyping was performed using Affymetrix axiom SNP arrays (Affymetrix, Santa Clara, CA, USA). Axiom arrays are genome-wide, measure approximately 850,000 genotypes, and unlike TaqMan assays, do not require dual-labeled probes and PCR amplification which increase the cost of genotyping. DNA samples were added to a 96-well plate and then shipped to the Affymetrix research services laboratory in Santa Clara, USA. The axiom workflow consisted of the following steps: target preparation, hybridization, ligation, and signal amplification (Figure 3-4). Target preparation entailed fragmentation of genomic DNA into oligonucleotides. The fragments were then purified and hybridized to the surface of the array plate using colour-coded probes. After hybridization, the plate was washed to remove non-specific ligation events and minimize background noise. Following ligation, the plate was stained and imaged to obtain genotypes.

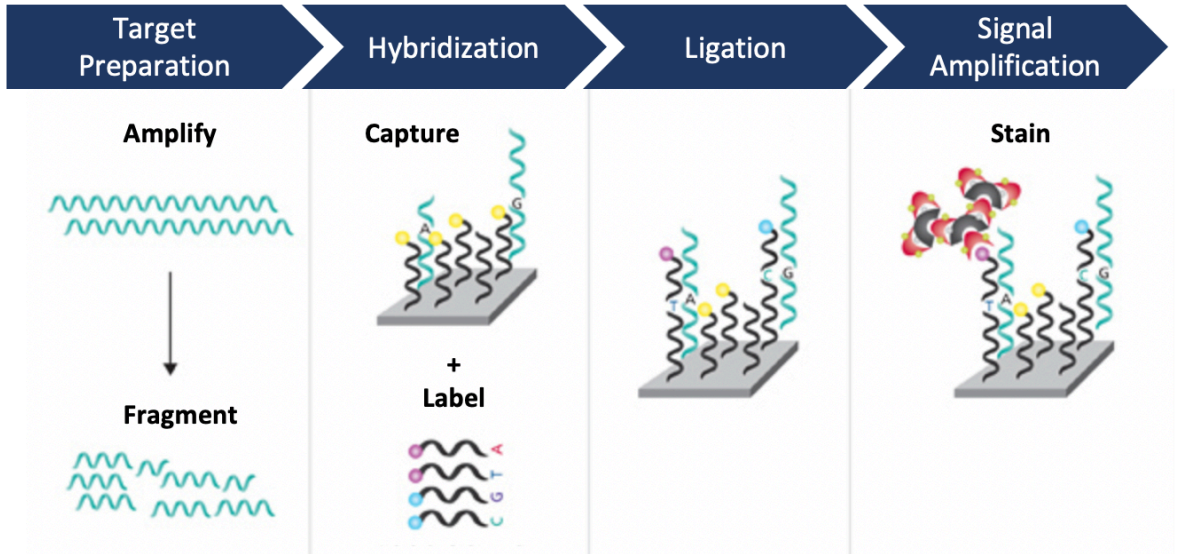


Figure 3-4 Workflow of Affymetrix axiom SNP arrays.

3.3.4 Data analysis

3.3.4.1 Differential allele frequency analysis

Downstream genetic analyses were performed using whole genome association analysis software, Plink (version 1.9). First, the genotype dataset was filtered to only include SNPs with minor allele frequency (MAF) of 10% or greater and that were in Hardy-Weinberg equilibrium ($p > 0.05$); deviations of genotype frequencies from Hardy-Weinberg equilibrium were assessed using Chi-square (χ^2) test. Also, the SNPs analyzed were within 50,000 bp upstream from the transcription start site and downstream of the 3' untranslated region of eight genes selected from the cholinergic synapse KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway: *ADCY3*, *AKT3*, *CACNA1S, CHRM3, CHRNB2, GNB1, GNG4* and *KCNQ4*. Logistic regression was used to analyze the differential allele frequency (218 SNPs). In order to include weaker associations, nominal statistical significance was set to *P*-value ≤ 0.1. Additionally, the *P*-values and odds ratios were adjusted for sex.

3.3.4.2 Distribution of minor alleles

The distribution of minor alleles was analyzed for statistically significant SNPs (*P*-value ≤ 0.1). First, genotypes were recoded as 0, 1 or 2 corresponding to the number of minor alleles present at each position (minor alleles were scored as 1 and major alleles were scored as 0). The cumulative effect and accumulation of minor alleles was then analyzed using an unweighted approach, which consisted of a linear sum of minor alleles divided by total SNPs analyzed. The minor allele content (MAC) was compared using Mann–Whitney U tests.

3.4 Results

3.4.1 Demographics of research participants

There were no significant differences in age, height, weight or total leukocyte counts for all phenotypic comparisons using TNSS or nasal congestion scores (*P*-value < 0.05). Significant differences were found in leukocyte subtypes. Monocytes were significantly different between 1) PERs versus DRs and 2) healthy controls versus DRs. Additionally, eosinophils were significantly different between 1) ERs versus PERs and 2) ERs versus grouped PERs and DRs.

3.4.2 Correlation of clinical symptom scores

Allergic participants had a weak negative correlation between TNSS and PNIF (Pearson correlations, $r = -0.37$) (Figure 3-5A). A reduction in PNIF indicates difficulty breathing through the nose and an increase in TNSS indicates the presence of nasal symptoms. A weak negative correlation was also identified between PNIF and nasal congestion scores ($r = -0.42$), which are objective and subjective measurements of nasal patency, respectively (Figure 3-4B).

Allergic participants had strong correlations between TNSS and nasal congestion (r =0.87), rhinorrhea (r =0.88), nasal itching (r =0.82), and sneezing (r =0.80) (Figure 3-4C – Figure 3-4F).

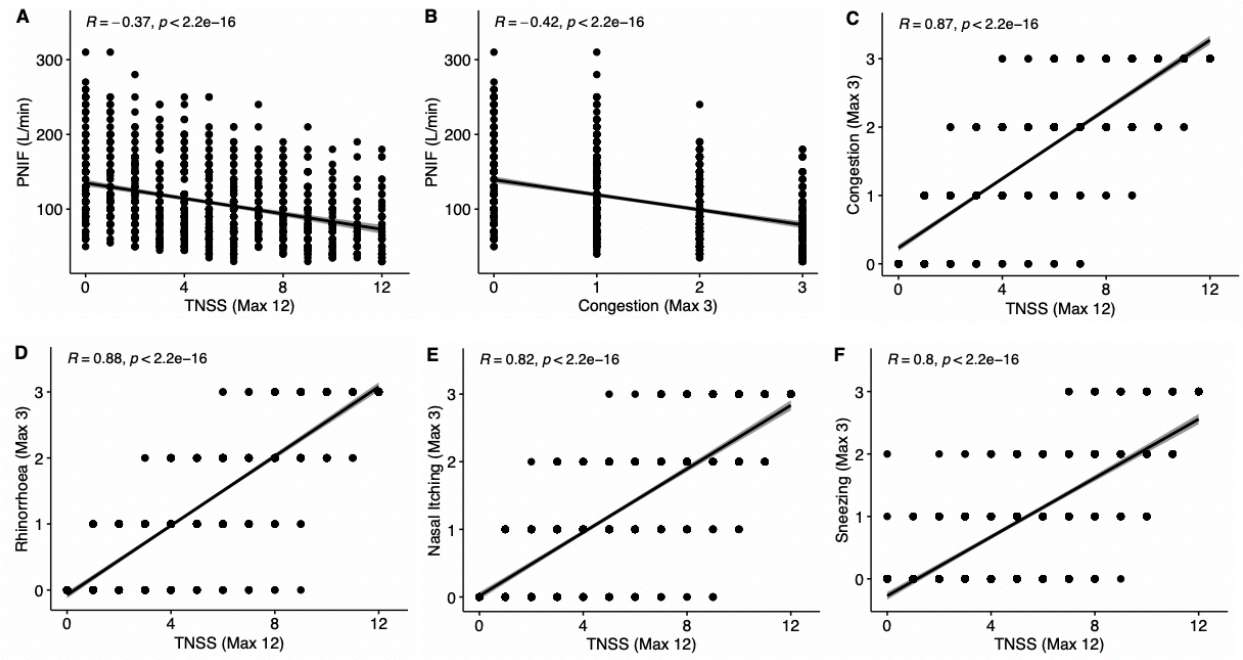


Figure 3-5 Correlation between clinical symptom scores. Pearson correlation (r) between **A**: Peak nasal inspiratory flow (PNIF) and total nasal symptom score (TNSS), **B**: PNIF and nasal congestion, **C**: Nasal congestion and TNSS, **D**: Rhinorrhea and TNSS, **E**: Nasal itching and TNSS, **F**: Sneezing and TNSS.

3.4.3 Comparison between phenotyping methods

Allergic participants were phenotyped using two different measurements: TNSS and nasal congestion scores. Phenotypes were assigned based on the pattern of late-onset symptoms. Figure 3-6 compares the phenotypes assigned using the two measurements. Using TNSS, 15 participants were classified as DRs, and 10 of these participants belonged to the HC subgroup and 5 belonged to the LC subgroup. A majority of the participants classified as ERs belonged to the LC subgroup (25 out of the 32 ERs), which is expected because ERs experience a reduction

in nasal symptoms by Hour 6 after allergen exposure and return to baseline by Hour 12. Using TNSS, 27 participants were classified as PERs. A majority of PERs belonged to the HC subgroup (22 out of the 27 PERs), which is expected because PERs continue to experience nasal symptoms even 12 hours after allergen exposure.

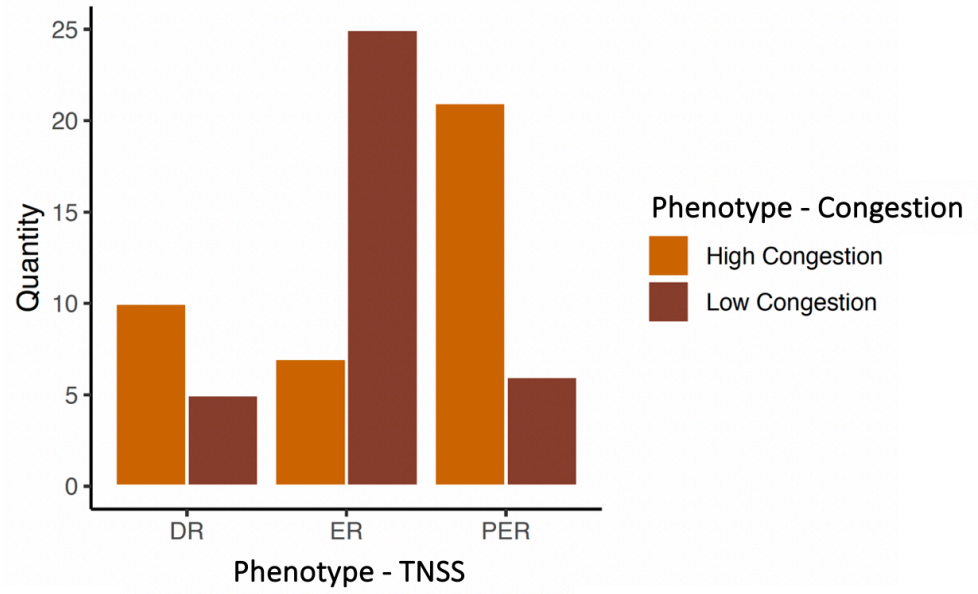


Figure 3-6 Comparing phenotypes assigned to research participants. A bar plot comparing between phenotypes assigned using total nasal symptom scores (TNSS) and nasal congestion scores. *Definition of abbreviations:* ER = early responders, PER = protracted early responders, and DR = dual responders.

3.4.4 Genetic analysis using total nasal symptom score (TNSS) phenotypes

Allele frequencies of nine phenotypic comparisons were analyzed (ERs and DRs, PERs and DRs, ERs and PERs, DRs and healthy participants, ERs and healthy participants, PERs and healthy participants, grouped ERs & PERs and DRs, ERs and grouped PERs & DRs, and all allergic participants and healthy controls). At a nominal *P*-value cut-off of 0.1, significantly different SNPs were identified in all comparisons (Appendix B1) when the variants were

considered individually. However, the minor allele content (MAC) of the phenotypes in each comparison was not significantly different.

3.4.5 Genetic analysis using nasal congestion phenotypes

Allele frequencies were analyzed between the HC and LC subgroups. At a *P*-value cut-off of 0.1, 25 SNPs were found to be significantly different between the two subgroups (Table 3-3). Some of the identified SNPs were in linkage disequilibrium (Figure 3-7). The MAC of the HC subgroup (MAC = 0.71) was significantly higher than that of the LC subgroup (MAC=0.61, p=0.009) (Figure 3-8). At a nominal *P*-value cut-off of 0.1, 26 SNPs were found to be significantly different between healthy participants and the LC subgroup (Appendix B2). Healthy participants had significantly higher MAC (MAC=0.64) than the LC subgroup (MAC=0.54, p=0.08). Between healthy participants and the HC subgroup, 29 SNPs were found to be significantly different (Appendix B2). The HC subgroup had significantly higher MAC (MAC =0.72) than healthy participants (MAC=0.64, p=0.02).

Table 3-3 Differential allele frequencies between the high congestion and low congestion subgroup

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
ADCY3	rs17046742	2	24942956	A	2.766	0.08432
	rs36029941	2	24904932	T	1.824	0.08664
AKT3	rs12691548	1	243656826	A	2.551	0.02473
	rs3856231	1	243605604	T	2.369	0.03715
	rs4430311	1	243852691	C	1.961	0.09573
	rs2953328	1	243860378	C	1.961	0.09573
CACNA1S	rs10920134	1	201148684	C	2.434	0.05842
CHRM3	rs1984165	1	239954965	T	0.4314	0.0262
	rs4659933	1	239955647	A	2.268	0.0271
	rs10926008	1	239898823	G	2.359	0.03645
	rs643040	1	239784120	C	0.5105	0.05141
	rs685550	1	239761108	G	0.427	0.05733
	rs2790336	1	239799386	G	0.5727	0.0891
CHRNB2	rs3766922	1	154604579	G	0.3891	0.008479
	rs11335288	1	154591260	G	2.112	0.03226
	rs2229857	1	154601491	T	2.112	0.03226
	rs7533471	1	154628860	G	1.886	0.05297
	rs3841062	1	154578468	-	1.877	0.07905
GNG4	rs61834659	1	235673662	T	4.529	0.04533
KCNQ4	rs71577632	1	40773241	TGGAG	0.2785	0.01629
	rs4660456	1	40773839	G	0.2785	0.01629
	rs751823	1	40875748	T	2.536	0.01666
	rs4660463	1	40799954	T	0.4024	0.02658
	rs823690	1	40796214	G	1.958	0.05236
	rs72949146	1	40860761	C	3.118	0.05742

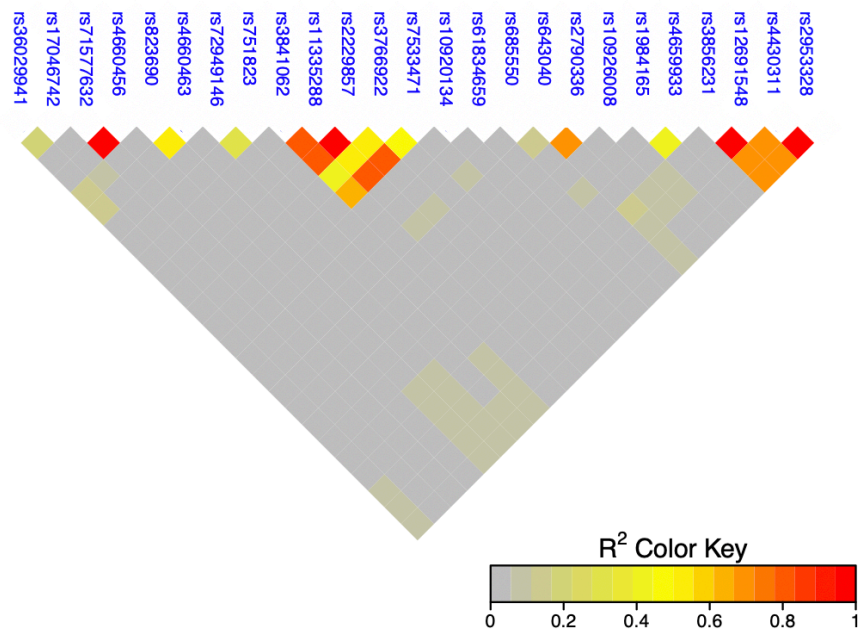


Figure 3-7 Pairwise linkage disequilibrium heatmap.

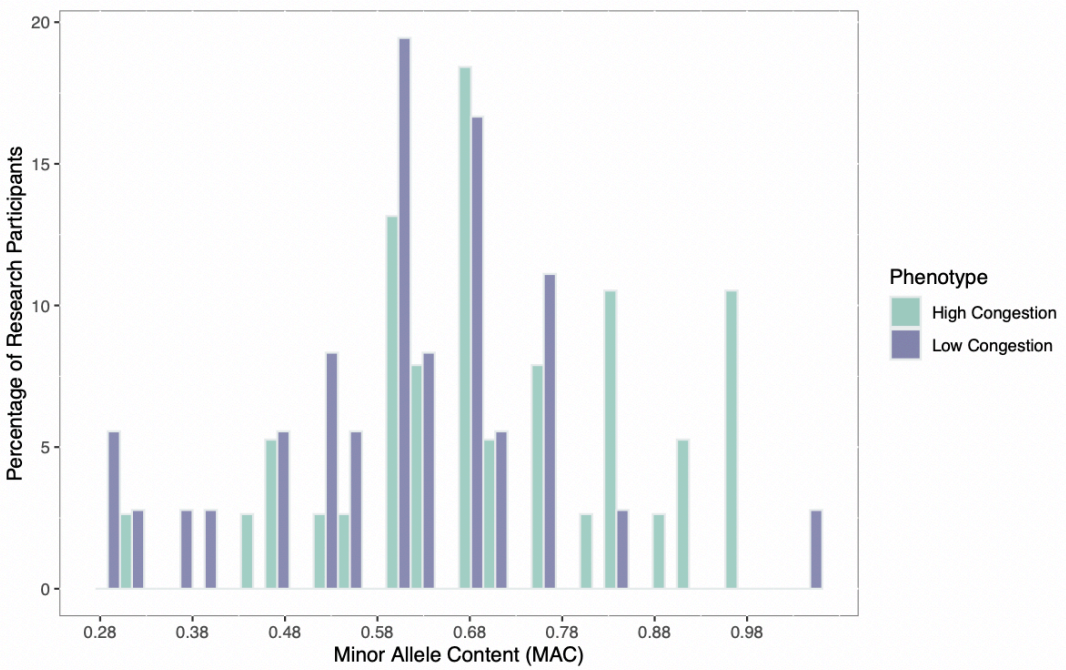


Figure 3-8 Minor allele distribution between nasal congestion phenotypes.A histogram showing the minor allele content (MAC) of the high congestion and low congestion subgroups.

3.5 Discussion

This chapter studied the association between SNPs in cholinergic synapse pathway genes and the development of the LPR. We specifically looked at the cholinergic synapse pathway because polymorphisms in these genes have previously been associated with late asthmatic responses[7]. Differential allele frequency analysis was performed between different comparisons; the resulting *P*-values and odds ratios were adjusted for sex but not for population stratification, a limitation of this study. Furthermore, due to the small sizes, multiple statistical testing corrections was not performed. Different allele frequencies were identified between TNSS phenotypes (Appendix B1). However, no significant difference was identified between MAC in all phenotypic comparisons.

Next, research participants were re-phenotyped using nasal congestion scores, one parameter of TNSS, and then divided into two subgroups: HC and LC. After re-phenotyping, 25 significantly different SNPs were identified between participants in the HC and LC subgroups (Table 3-3). The SNPs with significantly different allele frequencies were located in 7 cholinergic synapse pathway genes: *ADCY3*, *AKT3*, *CACNA1S, CHRM3, CHRNB2, GNG4,* and *KCNQ4*. *AKT3* mediates multiple cell signalling mechanisms and *CACNA1S* and *KCNQ4* encode subunits of calcium and potassium channels, respectively. *GNG4* encodes a G protein and *ADCY3* encodes an adenylate cyclase, which catalyzes the formation of the signaling molecule cAMP in response to G-protein signaling. Additionally, *CHRM3* encodes a muscarinic receptor (M3) and *CHRNB2* encodes a subunit of nicotinic receptors. In nasal mucosal glands, M3 is the dominant muscarinic receptor and mediates secretion and vasodilation[132]. Significantly higher MAC in these seven genes was found in the HC subgroup compared to the LC subgroup (Figure

3-8). Significantly different allele and genotype frequencies were also identified between healthy participants and participants part of the HC and LC subgroups (Appendix B2).

Our findings suggest that enrichment of minor alleles in cholinergic synapse pathway genes is associated with late-onset congestion in AR and could be a significant mechanism contributing to the development of the LPR. Genetic variants in cholinergic nicotinic receptor genes are also associated with nicotine dependence[133]. Some of the participants may have been smokers because participants were not excluded based on their smoking habits from the three EEU studies. However, this should not affect my results because the genetic variants that influence risk for nicotine dependence are located in genes (*CHRNB3*, *KCNJ6*, and *GABRA4*) that were not identified in my analysis[133].

Parasympathetic nerves, which are almost entirely cholinergic, regulate mucus secretion and vasodilation, both which can induce nasal congestion[134]. In the nose, parasympathetic nerves connect to nasal cavity arteries, venous sinusoids, mucus-producing acinar glands, and goblet cells in the nasal respiratory epithelium[134]. Cholinergic synapses use acetylcholine to convert electrical signals into chemical signals. Increased activity of the enzyme responsible for acetylcholine synthesis, choline acetyltransferase, is associated with AR[135]. This suggests that increased levels of acetylcholine are present in AR, resulting in increased mucus secretion and subsequently nasal congestion. Nasal congestion is also induced by increased vasodilation. Administration of histamine to atopic individuals to the nasal mucosa on one side of their nose can result in closure of the opposite side of the nose[136]. Histamine-induced nasal closure can be blocked using oxitropium[137], a muscarinic receptor antagonist. Another muscarinic receptor antagonist, atropine, can partially inhibit nasal congestion induced by stimulation of parasympathetic nerves[138-140]. These pharmacologic agents demonstrate the association between muscarinic receptors and vasodilation, and ultimately nasal congestion.

3.6 Conclusion

In conclusion, we have shown that nasal congestion scores may be a useful measurement to phenotype AR. In this chapter we have also identified a relationship between enrichment of minor alleles in cholinergic synapse pathways genes and late-onset congestion in AR. Genetic variants of cholinergic synapse pathway genes may have a significant influence on molecular mechanisms that lead to the development of the LPR. The cholinergic system may be a potential therapeutic target for the management and treatment of AR. In the future, we would like to study polymorphisms in the cholinergic synapse pathway genes in a larger sample size.

Chapter 4: Expression quantitative trait loci in allergic rhinitis phenotypes

4.1 Introduction

Many genetic loci have been associated with AR in genome-wide association studies (GWAS) on allergic rhinitis, and other loci have been suggested from GWAS on related disorders, including asthma plus hay fever and allergic sensitization[141-146]. Many of these genetic loci have regulatory effects on genes that affect a wide range of immune-cell types[146]. In this chapter, an expression quantitative trait loci (eQTL) analysis was performed was to further understand the pathobiology of AR. An eQTL analysis aims to identify genetic variants that affect the expression of close (cis) or distant (trans) genes. An eQTL refers to a gene-SNP pair for which the expression of the gene is associated with the allelic configuration of the SNP.

Using the molecular and genetic datasets created in the previous chapters, a cis-eQTL analysis was performed to identify genetic variants that influence expression levels of *AKT3* in AR phenotypes. This analysis focused on *AKT3* because it is the only gene out of the seven cholinergic synapse pathway genes identified in chapter 3 that was measured using the custom NanoString assay. Due to the small size, a trans-eQTL analysis was not performed as it tests tens of millions of gene-SNP pairs.

4.2 Hypothesis

We hypothesized that single nucleotide polymorphisms regulate *AKT3* expression in allergic rhinitis phenotypes.

4.3 Materials and methods

4.3.1 Research participants and collection of clinical samples

For this study we had access to blood samples collected from 74 allergic participants and 20 healthy controls. Research participants were phenotyped using two different measurements: total nasal symptom scores (TNSS) and nasal congestion scores. Participants were grouped into one of three categories based on the pattern of hourly reported TNSS: 32 participants were early responders (ERs), 27 participants were protracted ERs (PERs), and 15 participants were dual responders (DRs). Participants were then re-phenotyped using hourly reported nasal congestions scores: 38 participants belonged to the high congestion (HC) subgroup and 36 participants belonged to the low congestion (LC) subgroup. Phenotypes were assigned using guidelines previously described in Chapter 3.3.3.

4.3.2 Gene expression dataset

Extracted RNA samples (100 ng) were profiled using a NanoString nCounter Elements assay that was developed by the Tebbutt laboratory to predict the late-asthmatic response in allergic asthma[5] and diagnose western red cedar asthma[6] (NanoString Technologies, USA). The NanoString assay measured the expression of 166 genes including *AKT3*. Details regarding NanoString workflow and data normalization are described in sections 2.3.4.2 and 2.3.5.1.

4.3.3 Genotyping dataset

Extracted DNA samples (500 ng) were profiled using Affymetrix axiom SNP arrays (Affymetrix, Santa Clara, CA, USA). The resulting dataset contained measurements for over 850,000 SNP genotypes. Next, the dataset was filtered to only include SNPs with minor allele

frequency (MAF) of 10% or greater and that were in Hardy-Weinberg equilibrium ($p > 0.05$). Details regarding axiom workflow are described in section 3.3.4.2.

4.3.4 Expression quantitative trait loci analysis (eQTL)

Using the MatrixEQTL R-library[147], cis-eQTL analysis was performed. Sex, age, and leukocyte subtypes (eosinophils, monocytes, lymphocytes, and neutrophils) were included as covariates. Two different genetic models were used to compute cis-eQTLs: 1) additive and 2) additive and dominant. Using these models, three different analyses were performed. In the additive linear analysis, genotype is assumed to have only an additive effect on gene expression:

$$expression = \alpha + \sum_k \beta_k \cdot covariate_k + \gamma \cdot genotype\_additive$$

In the ANOVA analysis, genotype is allowed to have both additive and dominant effects:

$$expression = \alpha + \sum_k \beta_k \cdot covariate_k + \gamma_1 \cdot genotype\_additive + \gamma_2 \cdot genotype\_dominant$$

The third analysis assessed the interaction between genotype and gene expression in two groups of samples (different allergic rhinitis phenotypes):

$$expression = \alpha + \sum_k \beta_k \cdot covariate_k + \gamma \cdot genotype\_additive + \delta \cdot genotype\_additive \cdot covariate_K$$

4.4 Results

4.4.1 Cis-eQTLs computed using additive linear and ANOVA analyses

A cis-eQTL analysis was performed to link variations in *AKT3* expression levels to genotypes using two genetic models. The analysis was performed independently in all AR phenotypes, which were assigned using TNSS (ER, PER, and DR) or nasal congestions scores (HC and LC subgroups). At an FDR cut-off of 0.1, cis-eQTLs were identified in DRs using an additive linear analysis (Figure 4-1A). DRs that had a minor allele in rs10927033 had increased *AKT3*

expression. At an FDR cut-off of 0.1, cis-eQTLs were identified in the HC subgroup using an ANOVA analysis (Figure 4-1B-D). Participants in the HC subgroup who were heterozygous for rs1092700 and rs320320 or were homozygous for the minor allele for rs2256126 had increased *AKT3* expression. Significant cis-eQTLs were not identified in other phenotypes or in all allergic participants.

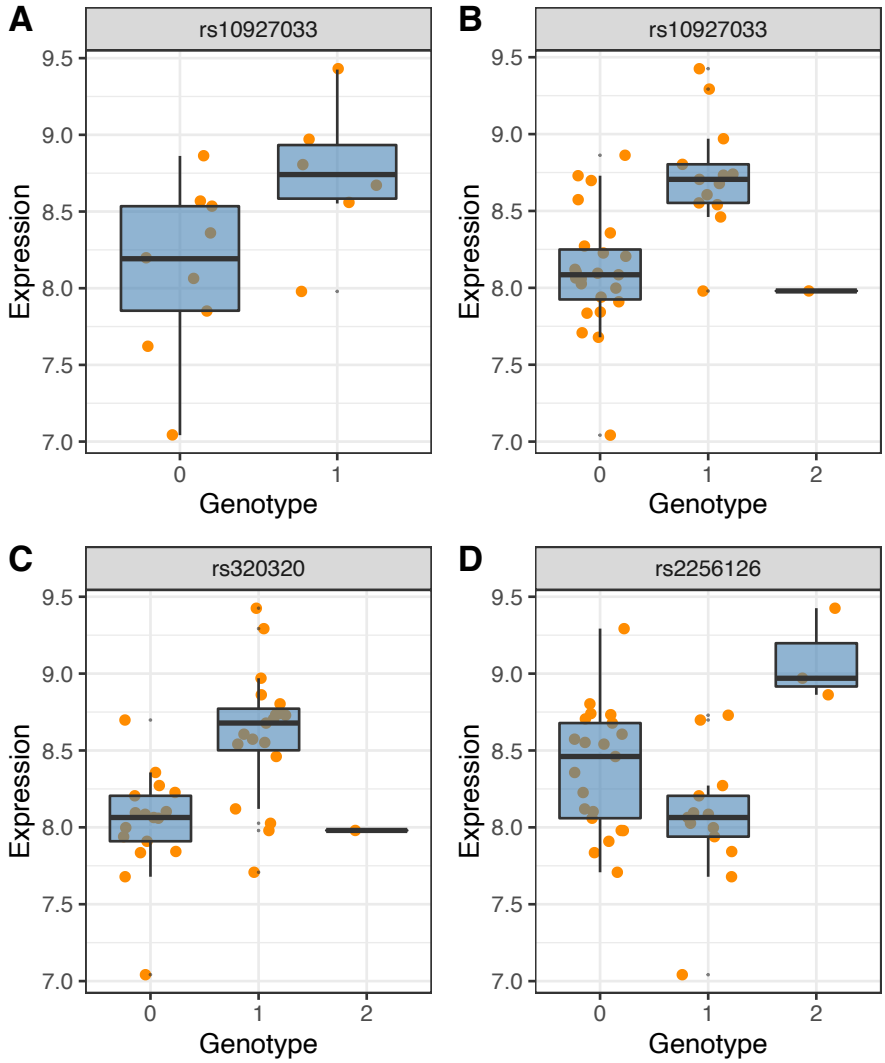


Figure 4-1 Expression quantitative trait loci (eQTL) in allergic rhinitis phenotypes. Boxplots depicting the effect of **A** rs10927033 on *AKT3* expression in dual responders **B** rs10927033 on *AKT3* expression in high congestion (HC) subgroup **C** rs320320 on *AKT3* expression in HC subgroup **D** rs2256126 on *AKT3* expression in HC subgroup.

4.4.2 Cis-eQTLs identified in phenotypic comparisons

The significance of the interaction between genotype and phenotype on gene expression was also analyzed.. The cis-eQTL analysis was performed independently in the following TNSS comparisons: ERs and DRs, PERs and DRs, ERs and PERs, grouped ERs & PERs and DRs, ERs and grouped PERs & DRs, ERs and healthy controls, PERs and healthy controls, DRs and healthy controls, and healthy controls and all allergic participants. At an FDR cut-off of 0.1, significant cis-eQTLs were identified in two comparisons, 1) ERs and DRs and 2) healthy controls and DRs (Figure 4-2). A cis-eQTL analysis was also performed between the HC and LC subgroups, however, significant cis-eQTLs were not identified (*P*-value <0.1).

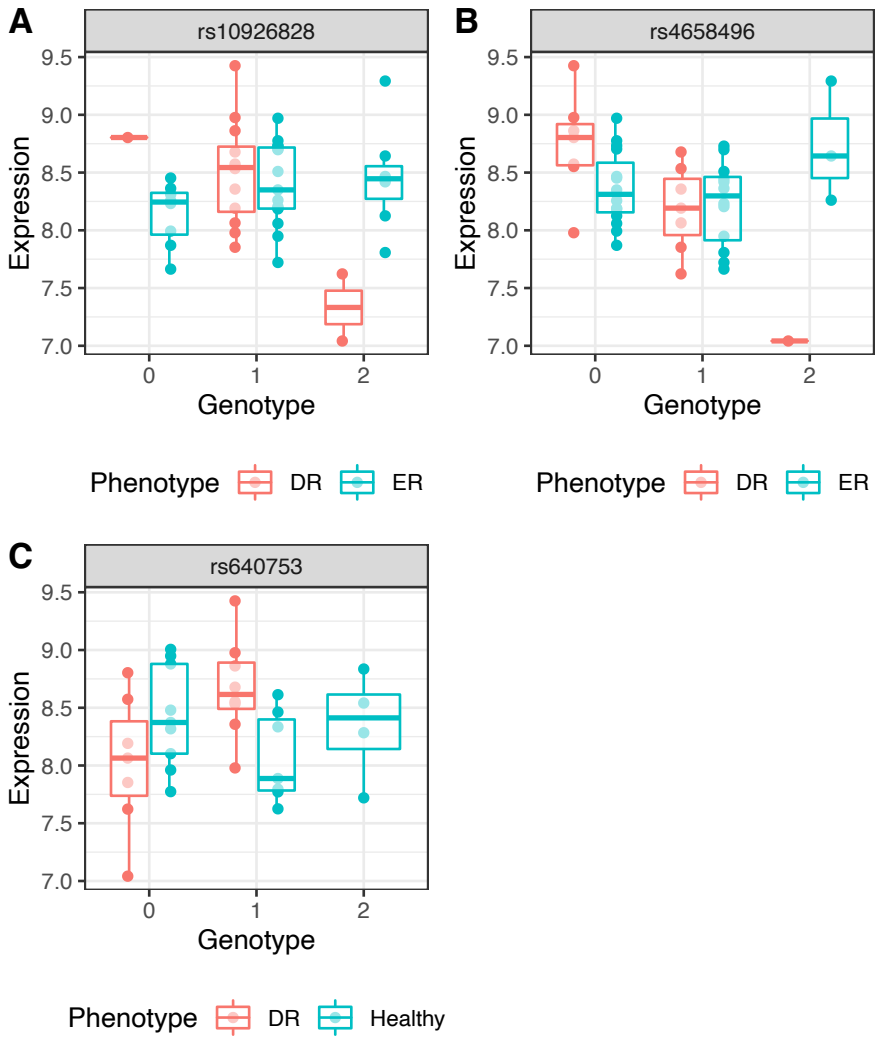


Figure 4-2 Expression quantitative trait loci (eQTL) in comparisons between allergic rhinitis phenotypes

Boxplots depicting the effect of: A rs10926828 on AKT3 expression in dual responders (DR) and early responders (ER) B rs4658496 on AKT3 expression in DRs and ERs C rs640753 on AKT3 expression in DRs and healthy controls.

4.5 Discussion

The aim of this chapter was to identify genetic variants that influenced expression levels of *AKT3* in AR phenotypes. We were specifically interested in the *AKT3* gene because it was the only gene in the cholinergic synapse pathway that was quantified at the transcript level using the custom NanoString assay. This chapter did not focus on identifying trans-eQTLs due to the small

sample size, as a trans-eQTL analysis tests a much larger number of SNP-gene pairs. Multiple GWAS on AR have identified SNPs associated with AR, and other SNPs have been suggested from GWAS on related disorders[141-145]. However, our genotyping dataset did not contain measurements for many of these SNPs. Normally, genome imputation would be performed to infer unobserved genotypes using statistics. However, this approach could not be utilized in this study due to the small number of participants.

Significant cis-eQTLs were only identified in DRs using an additive linear analysis and in the HC subgroup using an ANOVA analysis (Figure 4-1). In both phenotypes, rs10927033 was shown to influence *AKT3* expression. The interaction between genotype and *AKT3* expression was also analyzed in phenotypic comparisons. Significant cis-eQTLs were only identified in two comparisons, 1) ERs and DRs and 2) healthy controls and DRs (Figure 4-2). I investigated if the eQTLs identified in this chapter have previously been associated with AR or other disorders using the HaploReg website and the Genome-Tissue Expression (GTEx) portal. I found that these eQTLs had not previously been identified.

4.6 Conclusion

This chapter focused on identifying genetic variants that influence *AKT3* expression in different AR phenotypes. A key limitation of this project was the small sample size and hence all our analyses had minimal power. We were able to identify novel cis-eQTLs in AR phenotypes. However, it is necessary to perform this analysis in a larger sample size.

Chapter 5: Conclusions and future directions

5.1 Overall summary and conclusions

The immune system consists of a collection of cells, chemicals and processes that function to protect the human body from pathogens. Immune responses are categorized into type 1 and type 2 immune responses[148-149]. Immune defense against microorganisms is referred to as type 1 immunity and relies primarily on direct removal of pathogens or infected host cells. In contrast, type 2 immunity protects against macroparasites and relies on Th2 cells, IgE and a range of innate immune cells including basophils, eosinophils, mast cells and innate lymphoid cells (ILCs). However, nonharmful environmental stimuli, such as allergens, can also trigger type 2 immune responses. Allergic disorders are caused by inflammation induced by abnormal hypersensitive reactions to allergens.

AR is the most common clinical manifestation of allergy, affecting 400 million people worldwide, with prevalence increasing rapidly in industrialized societies[150-151]. Allergic responses are initiated following environmental allergen exposure in genetically predisposed individuals and results in allergic inflammation of the nasal mucosa. AR is characterized by an EPR and, in some individuals, a subsequent LPR. The underlying causes of the heterogeneity observed in AR are still not understood. This dissertation investigated molecular and genetic differences between AR phenotypes. This thesis had four goals: to develop biomarker panels that could discriminate between AR phenotypes, to investigate the relationship between SNPs in cholinergic synapse pathway genes and the LPR, to investigate re-phenotyping AR responses using nasal congestion, and to identify genetic variants that may be influencing the expression of *AKT3* in AR phenotypes.

Individuals with AR can be phenotyped as ERs, PERs or DRs, based on hourly reported TNSS measurements[3-4]. In chapter 2, we tested the hypothesis that molecular differences detectable in baseline peripheral blood samples can be used to discriminate between phenotypes. Using standard differential expression analysis, we were only able to identify statistically significant expression in two phenotypic comparisons in the discovery cohort. These signals were not validated (p value and directionality) in the validation cohort. Using a multivariate biomarker approach, biomarker panels that could discriminate (AUC > 0.70) between AR phenotypes were identified, however, were unable to be validated.

In chapter 3, we tested the hypothesis that enrichment of SNPs in cholinergic synapse pathway genes may be contributing to the development of the LPR. Different allele frequencies were identified between TNSS phenotypes. However, no significant difference was identified between MAC in all comparisons. Next, allergic participants were re-phenotyped using nasal congestion scores, and then divided into two subgroups: HC and LC. After re-phenotyping, 25 significantly different SNPs were identified between participants in the HC and LC subgroups. Additionally, we found that that HC subgroup had significantly higher MAC in cholinergic synapse pathway genes compared to the LC subgroup.

In chapter 4, we tested the hypothesis that SNPs regulate *AKT3* expression in AR phenotypes (TNSS phenotypes and nasal congestion phenotypes). We identified significant cis-eQTLs in DRs and participants part of the HC subgroup. We also analyzed the interaction - between genotype and *AKT3* expression in different phenotypic comparisons. We found significant cis-eQTLs in two comparisons: ERs and DRs and healthy controls and DRs.

In conclusion, we found that nasal congestions scores are a useful measurement to phenotype AR responses, and that there is association between minor alleles in cholinergic synapse pathways genes and the development of the LPR.

5.2 Limitations

We were unable to identify and validate a discriminatory molecular signal between AR phenotypes in chapter 2. Limitations of this analysis included small expression dataset (only measured the expression of 166 transcripts), and that the dataset was created using baseline blood samples. We used baseline blood samples for the biomarker analysis because the Tebbutt laboratory had previously identified blood-based biomarkers that could discriminate between allergic asthma phenotypes at baseline[5]. Additionally, our expression dataset had small sample sizes, especially for DRs, and there was a sex imbalance. Another limitation of this study is that the gene expression dataset may be affected by cellular heterogeneity.

The genotyping dataset used in chapters 3 and 4 had small sample sizes, did not adjust for population stratification, and comprised primarily of Caucasians, and therefore may be more sensitive to outliers than a larger study with randomized sampling. Additionally, the *P*-values reported in chapter 3 were not corrected for multiple statistical testing due to the very small sample size and insufficient power for a genetic association study. Furthermore, a weakness of our re-phenotyping protocol is that we used subjective nasal congestion measurements instead of PNIF measurements, which are an objective measurement of nasal congestion.

5.3 Future directions

This dissertation has contributed to further understanding molecular and genetic differences between different AR responses, and has shown that nasal congestion scores can be used to phenotype AR responses. In the future, molecular differences in nasal lavage samples should be investigated. There may be a strong discriminatory molecular signal directly in the nose at baseline (local immunity) compared to blood (systemic immunity). This dissertation provides evidence for the association between the cholinergic system and the development of the LPR in AR. However, in the future, polymorphisms in the cholinergic synapse pathway genes should be investigated in a large-scale study. The cholinergic system may be a potential therapeutic target for the LPR, and may be useful for other allergic disorders, such as anaphylaxis and skin reactions, which also have LPRs.

Bibliography

1. Brożek JL, Bousquet J, Agache I, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines—2016 revision. Journal of allergy and clinical immunology. 2017;140:950-958.

2. Keith PK, Desrosiers M, Laister T, Schellenberg RR, Waserman S. The burden of allergic rhinitis (AR) in Canada: perspectives of physicians and patients. Allergy, asthma, and clinical immunology. 2012;8:7-7.

3. Soliman M, Ellis, Anne K. Phenotyping allergic rhinitis as early- or dual-phase responses using the environmental exposure unit. Annals of allergy, asthma, & immunology. 2015;114:344-345.

4. Jacobs RL, Ramirez DA, Rather CG, et al. Redness response phenotypes of allergic conjunctivitis in an allergen challenge chamber. Annals of allergy, asthma, & immunology. 2016;2017;118:86-93.e2.

5. Singh A, Shannon CP, Kim YW, et al. Novel Blood-based Transcriptional Biomarker Panels Predict the Late-Phase Asthmatic Response. American journal of respiratory and critical care medicine. 2018;197:450-462.

6. Yang CX, Singh A, Kim YW, Conway EM, Carlsten C, Tebbutt SJ. Diagnosis of Western Red Cedar Asthma Using a Blood-based Gene Expression Biomarker Panel. American journal of respiratory and critical care medicine. 2017;196:1615-1617.

7. Rajasekaran A, He D, Yue A, et al. Cholinergic synapse pathway gene polymorphisms associated with allergen-induced late asthmatic responses. ERJ open research. 2019;5:00107-2019.

8. Chapter 3: Nonallergic vasomotor rhinitis. In: Bernstein J.A., editor. Rhinitis and Related Upper Respiratory Conditions: A Clinical Guide; Bernstein, J.A., Ed.; Springer: Cham, Switzerland, 2018.

9. Settipane RA, Lieberman P. Update on nonallergic rhinitis. Annals of allergy, asthma, & immunology. 2001;86:494-508.

10. Settipane RA, Charnock DR. Epidemiology of rhinitis: allergic and nonallergic. Clinical allergy and immunology. 2007;19:23.

11. Scarupa MD, Kaliner MA. Nonallergic Rhinitis, With a Focus on Vasomotor Rhinitis. The World Allergy Organization journal. 2009;2:20-25.

12. Scadding GK, Kariyawasam HH, Scadding G, et al. BSACI guideline for the diagnosis and management of allergic and non-allergic rhinitis (Revised Edition 2017; First edition 2007). Clinical and experimental allergy. 2017;47:856-889.

13. Halderman A, Sindwani R. Surgical Management of Vasomotor Rhinitis: A Systematic Review. American journal of rhinology & allergy. 2015;29:128-134.

14. Wheatley LM, Togias A. Clinical practice. Allergic rhinitis. The New England journal of medicine. 2015;372(5):456-463.

15. Settipane RA. Epidemiology of vasomotor rhinitis. World Allergy Organ J. 2009;2(6):115-118.

16. Broide DH. Allergic rhinitis: Pathophysiology. Allergy Asthma Proc. 2010;31(5):370-374.

17. Meltzer EO, Gross GN, Katial R, Storms WW. Allergic rhinitis substantially impacts patient quality of life: findings from the Nasal Allergy Survey Assessing Limitations. J Fam Pract. 2012;61(2 Suppl):S5-S10.

18. Thompson AK, Juniper E, Meltzer EO. Quality of life in patients with allergic rhinitis. Ann Allergy Asthma Immunol. 2000;1:338–348.

19. Baiardini I, Braido F, Cauglia S, Canonica GW. Sleep disturbances in allergic rhinitis. Allergy. 2006;1:1259–1267.

20. Bachert C, Bousquet J, Canonica GW, et al. Levocetirizine improves quality of life and reduces costs in long-term management of persistent allergic rhinitis. Journal of allergy and clinical immunology. 2004;114:838-844.

21. Small P, Frenkiel S, Becker A, Boisvert P, Bouchard J, Carr S, Cockcroft D, Denburg J, Desrosiers M, Gall R, Hamid Q, Hébert J, Javer A, Keith P, Kim H, Lavigne F, Lemièr C, Massoud E, Payton K, Schellenberg B, Sussman G, Tannenbaum D, Watson W, Witterick I, Wright E, The Canadian Rhinitis Working Group. Rhinitis: a practical and comprehensive approach to assessment and therapy. J Otolaryngol. 2007;36(Suppl 1):S5–27.

22. Lee P, Mace S. An approach to allergic rhinitis. Allergy Rounds. 2009;1:1.

23. Bousquet J, Khaltaev N, Cruz AA, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). Allergy (Copenhagen). 2008;63 Suppl 86:8-160.

24. Evans MJ and Plopper CG. The role of basal cells in adhesion of columnar epithelium to airway basement membrane. Am Rev Respir Dis. 1998;138:481-3.

25. Voynow JA, Rubin BK. Mucins, Mucus, and Sputum. Chest. 2009;135:505-512.

26. Tomazic PV, Birner-Gruenberger R, Leitner A, Obrist B, Spoerk S, Lang-Loidolt D. Nasal mucus proteomic changes reflect altered immune responses and epithelial permeability in patients with allergic rhinitis. Journal of allergy and clinical immunology. 2014;2013;133:741-750.

27. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJM, Heijink IH. E-cadherin: gatekeeper of airway mucosa and allergic sensitization. Trends in immunology. 2011;32:248-255.

28. GR, Farkas L, Scheel C, et al. A thymic stromal lymphopoietin responsive dendritic cell subset mediates allergic responses in the upper airway mucosa. Journal of allergy and clinical immunology. 2014;134:613–621.

29. Haenuki Y, Matsushita K, Futatsugi-Yumikura S, et al. A critical role of IL-33 in experimental allergic rhinitis. Journal of allergy and clinical immunology. 2012;130:184-194.

30. Licona-Limón P, Kim LK, Palm NW, Flavell RA. TH2, allergy and group 2 innate lymphoid cells. Nature immunology. 2013;14:536-542.

31. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature. 2010;463:540-544.

32. Lambrecht BN, Hammad H. The immunology of asthma. Nature immunology. 2015;16:45-56.

33. Takano K, Kojima T, Go M, et al. HLA-DR- and CD11c-positive dendritic cells penetrate beyond well-developed epithelial tight junctions in human nasal mucosa of allergic rhinitis. J Histochem Cytochem. 2005;53(5):611-619.

34. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll like receptor 4 triggering of airway structural cells. Nature Medicine. 2009;15:410–416.

35. Jahnsen FL, Gran E, Haye R, Brandtzaeg P. Human Nasal Mucosa Contains Antigen-Presenting Cells of Strikingly Different Functional Phenotypes. American journal of respiratory cell and molecular biology. 2004;30:31-37.

36. Mirchandani AS, Besnard A, Yip E, et al. Type 2 Innate Lymphoid Cells Drive CD4 + Th2 Cell Responses. The Journal of immunology (1950). 2014;192:2442-2448.

37. Halim TF, Steer C, Mathä L, et al. Group 2 Innate Lymphoid Cells Are Critical for the Initiation of Adaptive T Helper 2 Cell-Mediated Allergic Lung Inflammation. Immunity (Cambridge, Mass.). 2014;40:425-435.

38. Soumelis V, Reche PA, Kanzler H, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nature immunology. 2002;3:673–680.

39. Gould HJ, Sutton BJ. IgE in allergy and asthma today. Nature Reviews Immunology. 2008; 8:205–17.

40. Borriello F, Iannone R, Marone G. Histamine Release from Mast Cells and Basophils. Handb Exp Pharmacol. 2017;241:121-139.

41. Naclerio RM, Meier HL, Kagey-Sobotka A, et al. Mediator release after nasal airway challenge with allergen. The American review of respiratory disease. 1983;128:597.

42. Creticos PS, Peters SP, Adkinson J, N F, et al. Peptide leukotriene release after antigen challenge in patients sensitive to ragweed. The New England journal of medicine. 1984;310:1626.

43. Doyle WJ, Boehm S, Skoner DP. Physiologic responses to intranasal dose-response challenges with histamine, methacholine, bradykinin, and prostaglandin in adult volunteers with and without nasal allergy. Journal of allergy and clinical immunology. 1990;86:924-935.

44. Bachert C. The role of histamine in allergic disease: re-appraisal of its inflammatory potential. Allergy. 2002;57:287–296.

45. Schmelz M, Schmidt R, Bickel A, Handwerker HO, Torebjork HE. Specific C-receptors for itch in human skin. The Journal of neuroscience. 1997;17:8003–8008.

46. Andrew D, Craig AD. Spinothalamic lamina I neurons selectively sensitive to histamine: a central neural pathway for itch. Nature neuroscience. 2001;4:72–77.

47. Al Suleimani YM, Walker MJA. Allergic rhinitis and its pharmacology. Pharmacology & therapeutics (Oxford). 2007;114:233-260.

48. Secher C, Kirkegaard J, Borum P, Maansson A, Osterhammel P, Mygind N. Significance of H1 and H2 receptors in the human nose: rationale for topical use of combined antihistamine preparations. Journal of allergy and clinical immunology. 1982;70:211–218.

49. Wood-Baker R, Lau L, Howarth PH. Histamine and the nasal vasculature: the influence of H1 and H2-histamine receptor antagonism. Clinical otolaryngology and allied sciences. 1996;21:348–352.

50. Togias A. H1-receptors: localization and role in airway physiology and in immune functions. Journal of allergy and clinical immunology. 2003;112(4, Suppl.):S60–S68.

51. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature. 2008;454:445–454.

52. De Bruin-Weller, Weller, De Monchy. Repeated allergen challenge as a new research model for studying allergic reactions. Clinical & experimental allergy. 1999;29:159–165.

53. Howarth PH, Persson CGA, Meltzer EO, Jacobson MR, Durham SR, Silkoff PE. Objective monitoring of nasal airway inflammation in rhinitis. Journal of allergy and clinical immunology. 2005;115:S414-S441.

54. Westergren VS, Wilson SJ, Penrose JF, Howarth PH, Sampson AP. Nasal mucosal expression of the leukotriene and prostanoid pathways in seasonal and perennial allergic rhinitis. Clinical & experimental allergy. 2009;39:820–828.

55. Baroody FM, Lee BJ, Lim MC, Bochner BS. Implicating adhesion molecules in nasal allergic inflammation. European archives of oto-rhino-laryngology. 1995;252 Suppl 1:S50-S58.

56. Springer TA. Adhesion receptors of the immune system. Nature. 1990;346:425-434.

57. Bevilacqua MP, Nelson RM. Selectins. Journal of clinical investigation.1993;91:379-387.

58. Jutel M, Akdis M, Akdis CA. Histamine, histamine receptors and their role in immune pathology. Clinical & experimental allergy. 2009;39:1786–1800.

59. Dobrina A, Menegazzi R, Carlos T et al. Mechanisms of eosinophil adherence to cultured vascular endothelial cells: eosinophils bind to the cytokine induced endothelial ligand vascular cell adhesion molecule-1 via the very late activation antigen-4 integrin receptor. Journal of Clinical Investigation.1991;88:20–26.

60. Plewako H, Holmberg K, Oancea I, Gotlib T, Samolinski B, Rak S. A follow- up study of immunotherapy-treated birch-allergic patients: effect on the expression of chemokines in the nasal mucosa. Clinical & experimental allergy. 2008;1124–1131.

61. Watts A, West N, Cripps A, Smith P, Cox A. Distinct Gene Expression Patterns between Nasal Mucosal Cells and Blood Collected from Allergic Rhinitis Sufferers. International archives of allergy and immunology. 2018;177:29-34.

62. Kim YW, Singh A, Shannon CP, et al. Investigating Immune Gene Signatures in Peripheral Blood from Subjects with Allergic Rhinitis Undergoing Nasal Allergen Challenge. The Journal of immunology. 2017;199:3395-3405.

63. Kim YW, Tonti E, Hickey P, et al. Immunological changes in peripheral blood following nasal allergen challenge in subjects with allergic rhinitis pre- and post-peptide immunotherapy: An open-label clinical study. Allergy (Copenhagen). 2020.

64. Cingi C, Gevaert P, Mösges R, Rondon C, Hox V, Rudenko M, Muluk NB, Scadding G, Manole F, Hupin C, Fokkens WJ, Akdis C, Bachert C, Demoly P, Mullol J, Muraro A, Papadopoulos N, Pawankar R, Rombaux P, Toskala E, Kalogjera L, Prokopakis E, Hellings PW, Bousquet J. Multi-morbidities of allergic rhinitis in adults: European Academy of Allergy and Clinical Immunology Task Force Report. Clinical And Translational Allergy. 2017;7:17.

65. Brydon MJ. Skin prick testing in general practice. Journal of advanced nursing. 1998;27:442–444.

66. Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. The Journal of allergy and clinical immunology. 2001;108(5 Suppl):S147-S334.

67. Fokkens WJ, Lund VJ, Mullol J, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. Rhinology. Supplement. 2012;23:3 p preceding table of contents, 1-3 p preceding table of contents.

68. Léger D, Annesi-Maesano I, Carat F, et al. Allergic Rhinitis and Its Consequences on Quality of Sleep: An Unexplored Area. Archives of internal medicine (1960). 2006;166:1744-1748.

69. Simons FE. Learning impairment and allergic rhinitis. Allergy and asthma proceedings. 1996;17:185-189

70. Jose J, Coatesworth AP, Jose J. Inferior turbinate surgery for nasal obstruction in allergic rhinitis after failed medical treatment. Cochrane Database of Systematic Reviews. 2010;2010:CD005235-CD005235.

71. Wallace DV, Dykewicz MS, Bernstein DI, et al. The diagnosis and management of rhinitis: an updated practice parameter Journal of allergy and clinical immunology. 2008; 122(Suppl):S1–S84.

72. Benninger M, Farrar JR, Blaiss M, et al. Evaluating approved medications to treat allergic rhinitis in the United States: an evidence-based review of efficacy for nasal symptoms by class. Annals of allergy, asthma & immunology. 2010;104:13–29.

73. Weiner JM, Abramson MJ, Puy RM. Intranasal corticosteroids versus oral H1 receptor antagonists in allergic rhinitis: systematic review of randomised controlled trials. BMJ. 1998;317:1624–1629.

74. Bende M, Carrillo T, Vona I, da Castel-Branco MG, Arheden L. A randomized comparison of the effects of budesonide and mometasone furoate aqueous nasal sprays on nasal peak flow rate and symptoms in perennial allergic rhinitis. Annals of allergy, asthma & immunology. 2002;88:617–623.

75. Muraro A, Roberts G, Halken S, et al. EAACI guidelines on allergen immunotherapy: executive statement. Allergy. 2018;73:739-743.

76. Pfaar O, Bachert C, Bufe A, et al. Guideline on allergen-specific immunotherapy in IgE mediated allergic diseases – S2k Guideline of the German Society for Allergology and Clinical Immunology (DGAKI), the Society for Pediatric Allergy and Environmental Medicine (GPA), the Medical Association of German Allergologists (AeDA), the Austrian Society for Allergy and Immunology (ÖGAI), the Swiss Society for Allergy and

Immunology (SGAI), the German Society of Dermatology (DDG), the German Society of Oto-Rhino-Laryngology, Head and Neck Surgery (DGHNO-KHC), the German Society of Pediatrics and Adolescent Medicine (DGKJ), the Society for Pediatric Pneumology (GPP), the German Respiratory Society (DGP), the German Association of ENT Surgeons (BV-HNO), the Professional Federation of Paediatricians and Youth Doctors (BVKJ), the Federal Association of Pulmonologists (BDP) and the German Dermatologists Association (BVDD). Allergo Journal International. 2014;23:282-319.

77. Roberts G, Pfaar O, Akdis CA, et al. EAACI guidelines on allergen immunotherapy: allergic rhinoconjunctivitis. Allergy. 2018;73:765-798.
78. Di Bona D, Plaia A, Leto-Barone MS, La Piana S, Macchia L, Di Lorenzo G. Efficacy of allergen immunotherapy in reducing the likelihood of developing new allergen sensitizations: a systematic review. Allergy. 2017;72:691-704.

79. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. Journal of allergy and clinical immunology. 1998;102:558-562.

80. Shamji MH, Ljorring C, Francis JN, Calderon MA, Larche M, Kimber I et al. Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. Allergy. 2012;67:217-226.

81. Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W et al. Long-term clinical efficacy of grass-pollen immunotherapy. The New England journal of medicine. 1999;341:468-475.

82. Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. Cochrane Database of Systematic Reviews. 2007;CD001936.

83. Radulovic S, Calderon MA, Wilson D, Durham S. Sublingual immunotherapy for allergic rhinitis. Cochrane Database of Systematic Reviews. 2010;CD002893.

84. Radulovic S, Wilson D, Calderon M, Durham S. Systematic reviews of sublingual immunotherapy (SLIT). Allergy. 2011;66:740-752.

85. Calderon MA, Penagos M, Sheikh A, Canonica GW, Durham SR. Sublingual immunotherapy for allergic conjunctivitis: Cochrane systematic review and meta-analysis. Clin Exp Allergy 2011;41:1263-1272.

86. Compalati E, Braido F, Canonica GW. An update on allergen immunotherapy and asthma. Current Opinion in Pulmonary Medicine. 2014;20:109-117.

87. Nelson H, Cartier S, Allen-Ramey F, Lawton S, Calderon MA. Network Meta-analysis Shows Commercialized Subcutaneous and Sublingual Grass Products Have Comparable

Efficacy. The journal of allergy and clinical immunology in practice (Cambridge, MA). 2015;3:256-266.

88. Jutel M, Agache I, Bonini S, et al. International consensus on allergy immunotherapy. Journal of allergy and clinical immunology. 2015;136:556-568.

89. Shamji MH, Durham SR. Mechanisms of immunotherapy to aeroallergens. Clinical & experimental allergy. 2011;41:1235-1246.

90. Matsuoka T, Shamji MH, Durham SR. Allergen immunotherapy and tolerance. Allergology International. 2013;62:403-413.
91. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens. World Allergy Organization Journal. 2015;8:17-17.

92. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. Journal of allergy and clinical immunology. 2014;133:621-631.

93. Ellis AK, North ML, Walker T, Steacy LM. Environmental exposure unit: a sensitive, specific, and reproducible methodology for allergen challenge. Annals of allergy, asthma, & immunology. 2013;111:323–328.

94. North ML, Soliman M, Walker T, Steacy LM, Ellis AK. Controlled Allergen Challenge Facilities and Their Unique Contributions to Allergic Rhinitis Research. Current allergy and asthma reports. 2015;15:1-10.

95. Hohlfeld JM, Holland-Letz T, Larbig M, Lavae-Mokhtari M, Wierenga E, Kapsenberg M, et al. Diagnostic value of outcome measures following allergen exposure in an environmental challenge chamber compared with natural conditions. Clinical & experimental allergy. 2010;40:998–1006.

96. Ellis AK, Soliman M, Steacy L, et al. The Allergic Rhinitis - Clinical Investigator Collaborative (AR-CIC): nasal allergen challenge protocol optimization for studying AR pathophysiology and evaluating novel therapies. Allergy, asthma, and clinical immunology. 2015;11:16-16.

97. Ellis AK, Steacy LM, Hobsbawn B, Conway CE, Walker TJ. Clinical validation of controlled grass pollen challenge in the Environmental Exposure Unit (EEU). Allergy, asthma, and clinical immunology. 2015;11:5-5.

98. Akerlund A, Andersson M, Leflein J, Lildholdt T, Mygind N. Clinical trial design, nasal allergen challenge models, and considerations of relevance to pediatrics, nasal polyposis, and different classes of medication. Journal of allergy and clinical immunology. 2005;115:S460–S482.

99. Juniper EF, Stahl E, Doty RL, Simons FE, Allen DB, Howarth PH. Clinical outcomes and adverse effect monitoring in allergic rhinitis. Journal of allergy and clinical immunology. 2005;115:S390–S413.

100. Food and Drug Administration. Guidance for industry allergic rhinitis: clinical development programs for drug products. 2000.

101. Gauvreau GM, Watson RM, Rerecich TJ, Baswick E, Inman MD, O'Byrne PM. Repeatability of allergen-induced airway inflammation. Journal of allergy and clinical immunology. 1999;104:66-71.

102. Herxheimer H. Bronchial obstruction induced by allergens, histamine and acetyl-beta-methylcholinechloride. International archives of allergy and applied immunology. 1951;2:27.

103. Bousquet J, Heinzerling L, Bachert C, et al. Practical guide to skin prick tests in allergy to aeroallergens. Allergy. 2012;67:18-24.

104. Veldman-Jones MH, Brant R, Rooney C, Geh C, Emery H, Harbron CG, et al. Evaluating Robustness and Sensitivity of the NanoString Technologies nCounter Platform to Enable Multiplexed Gene Expression Analysis of Clinical Samples. Cancer research. 2015;75:2587-2593.

105. Radke L, Giese C, Lubitz A, Hinderlich S, Sandig G, Hummel M, et al. Reference gene stability in peripheral blood mononuclear cells determined by qPCR and NanoString. Microchimica Acta. 2014;181:1733-1742.

106. Malkov VA, Serikawa KA, Balantac N, Watters J, Geiss G, Mashadi-Hossein A, et al. Multiplexed measurements of gene signatures in different analytes using the Nanostring nCounter Assay System. BMC research notes. 2009;2:80-80.

107. Prokopec SD, Watson JD, Waggott DM, Smith AB, Wu AH, Okey AB, et al. Systematic evaluation of medium-throughput mRNA abundance platforms. RNA (New York, NY). 2013;19:51-62.

108. Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nature biotechnology. 2008;26:317-325.

109. Northcott PA, Shih DJH, Remke M, et al. Rapid, reliable, and reproducible molecular sub-grouping of clinical medulloblastoma samples. Acta neuropathologica. 2011;2012;123:615-626.

110. Kulkarni MM. Digital multiplexed gene expression analysis using the NanoString nCounter system. Current protocols in molecular biology. 2011;Chapter 25:Unit25B.10.

111. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic acids research. 2015;43:e47.

112. Smyth GK. Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments. Statistical applications in genetics and molecular biology. 2004;3:1-25.

113. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B, Methodological. 1995;57:289-300.

114. Zou H, Hastie T. Regularization and variable selection via the elastic net. Journal of the Royal Statistical Society. Series B, Statistical methodology. 2005;67:301-320.

115. Breiman L. Random Forests. Machine learning. 2001;45:5-32.

116. Sin DD, Hollander Z, DeMarco ML, McManus BM, Ng RT. Biomarker Development for Chronic Obstructive Pulmonary Disease. From Discovery to Clinical Implementation. American journal of respiratory and critical care medicine. 2015;192:1162-1170.

117. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostat Oxf Engl. 2007;8:118–127.

118. Skoner DP. Allergic rhinitis: Definition, epidemiology, pathophysiology, detection, and diagnosis. Journal of allergy and clinical immunology. 2001;108:S2-S8.

119. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4+ T cells. Cell 1997;89:587–596.

120. Krug N, Hohlfeld JM, Kirsten A, et al. Allergen-Induced Asthmatic Responses Modified by a GATA3-Specific DNAzyme. The New England Journal of Medicine. 2015;372:1987-1995.

121. Eglen RM, Hegde SS, Watson N. Muscarinic receptor subtypes and smooth muscle function. Pharmacological reviews. 1996;48:531-565.

122. Grandordy BM, Cuss FM, Sampson AS, Palmer JB, Barnes PJ. Phosphatidylinositol response to cholinergic agonists in airway smooth muscle: relationship to contraction and muscarinic receptor occupancy. The Journal of pharmacology and experimental therapeutics. 1986;238:273.

123. Meurs H, Timmermans A, Van Amsterdam RGM, Brouwer F, Kauffman HF, Zaagsma J. Muscarinic receptors in human airway smooth muscle are coupled to phosphoinositide metabolism. European journal of pharmacology. 1989;164:369-371.

124. Brown DA, Abogadie FC, Allen TGJ, et al. Muscarinic mechanisms in nerve cells. Life sciences (1973). 1997;60:1137-1144.

125. Muñoz W, Rudy B. Spatiotemporal specificity in cholinergic control of neocortical function. Current opinion in neurobiology. 2014;26:149-160.

126. Wess J, Liu J, Blin N, Yun J, Lerche C, Kostenis E. Structural basis of receptor/G protein coupling selectivity studied with muscarinic receptors as model systems. Life sciences (1973). 1997;60:1007-1014.

127. Brown DA, Selyanko AA. Membrane currents underlying the cholinergic slow excitatory post-synaptic potential in the rat sympathetic ganglion. The Journal of Physiology. 1985;365:365-387.

128. Lundberg JM, Anggard A, Emson P, Fahrenkrug J, Hokfelt T. Vasoactive Intestinal Polypeptide and Cholinergic Mechanisms in Cat Nasal Mucosa: Studies on Choline Acetyltransferase and Release of Vasoactive Intestinal Polypeptide. Proceedings of the National Academy of Sciences - PNAS. 1981;78:5255-5259.

129. Cauna N. Blood and nerve supply of the nasal lining. In: Proctor DF, Andersen IB, eds. The nose: upper airway physiology and the atmospheric environment. Amsterdam: Elsevier.1982:45-69.

130. Klassen ABm, van Megen JB, Kuihpers W, vasn den Brock P. Autonomic innervation of the nasal mucosa. Journal for oto-rhino-laryngology and its related specialties. 1988;50:32-34.

131. Balogh G, Dimitrov-Szokodi D, Husveti A. Lung denervation in the therapy of intractable bronchial asthma. The Journal of thoracic surgery. 1957;33:166184.

132. Okayama M, Mullol J, Baraniuk Jn, et al. Muscarinic receptor subtypes in human nasal mucosa: characterization, autoradiographic localization, and function in vitro. American journal of respiratory cell and molecular biology. 1993;8:176-187.

133. Saccone SF, Hinrichs AL, Saccone NL, et al. Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. Human Molecular Genetics. 2007;16 :36–49.

134. Kaliner MA. The physiology and pathophysiology of the parasympathetic nervous system in nasal disease: An overview. Journal of allergy and clinical immunology. 1992;90:1044-1045.

135. Kubo N, Minami T, Hori Y, Yamashita T, Kumazawa T. Enhanced Parasympathetic Nerve Activities in Experimentally-induced Nasal Hypersensitivity. Acta oto-laryngologica. 1989;108:14-20.

136. Sheahan P, Walsh RM, Walsh MA, Costello RW. Induction of nasal hyper-responsiveness by allergen challenge in allergic rhinitis: the role of afferent and efferent nerves. Clinical and experimental allergy. 2005;35:45-51.

137. Birchall MA, Henderson JC, Pride NB, Fuller RW. A comparison of the effects of an alphaagonist, an anti-muscarinic agent and placebo on intranasal histamine challenge in allergic rhinitis. Clin Otolaryngol Allied Sci. 1996; 21:212–217. [PubMed: 8818489]

138. Änggård A. The Effects of Parasympathetic Nerve Stimulation on the Microcirculation and Secretion in the Nasal Mucosa of the Cat. Acta oto-laryngologica. 1974;78:98-105.

139. Eccles R, Wilson H. The autonomic innervation of the nasal blood vessels of the cat. The Journal of Physiology. 1974;238:549-560.

140. Gadlage R, Behnke EE, Jackson RT. Is the Vidian Nerve Cholinergic? Archives of otolaryngology (1960). 1975;101:422-425.

141. Bunyavanich S, Schadt EE, Himes BE, et al. Integrated genome-wide association, coexpression network, and expression single nucleotide polymorphism analysis identifies novel pathway in allergic rhinitis. BMC medical genomics. 2014;7:48-48.

142. Ramasamy A, DPhil, Curjuric I, MD, Coin LJ, DPhil, et al. A genome-wide meta-analysis of genetic variants associated with allergic rhinitis and grass sensitization and their interaction with birth order. Journal of allergy and clinical immunology. 2011;128:996-1005.

143. Hinds DA, McMahon G, Kiefer AK, et al. A genome-wide association meta-analysis of self-reported allergy identifies shared and allergy-specific susceptibility loci. Nature Genetics. 2013;45(8):907-911.

144. Bønnelykke K, Matheson MC, Pers TH, et al. Meta-analysis of genome-wide association studies identifies ten loci influencing allergic sensitization. Nature Genetics. 2013;45:902-906.

145. Ferreira MA, Matheson MC, Tang CS, et al. Genome-wide association analysis identifies 11 risk variants associated with the asthma with hay fever phenotype. Journal of allergy and clinical immunology. 2014;133:1564-1571.

146. Waage J, Standl M, Curtin JA, et al. Genome-wide association and HLA fine-mapping studies identify risk loci and genetic pathways underlying allergic rhinitis. Nature Genetics. 2018;50:1072-1080.

147. Shabalin AA. Matrix eQTL: ultra fast eQTL analysis via large matrix operations. Bioinformatics. 2012;28:1353-1358.

148. Licona-Limón P, Kim LK, Palm NW, Flavell RA. TH2, allergy and group 2 innate lymphoid cells. Nature Immunology. 2013;14(6):536–542.

149. Iwasaki A, Medzhitov R. Regulation of Adaptive Immunity by the Innate Immune System. Science. 2010;327(5963):291–295.

150. Greiner AN, Hellings PW, Rotiroti G, Scadding GK. Allergic rhinitis. Lancet. 2011;378(9809):2112-2122.

151. Björkstén B, Clayton T, Ellwood P, et al. Worldwide time trends for symptoms of rhinitis and conjunctivitis: Phase III of the International Study of Asthma and Allergies in Childhood. Pediatric allergy and immunology. 2008;19:110-124.

Appendix

Appendix A Supplementary material for Chapter 2

A.1 NanoString quality control criteria

1. Imaging QC: % FOV (field of view) must be greater than 75 FOV.
2. Binding Density QC: Binding density must be between 0.05 and 2.25.
3. Positive Control Linearity QC: The R^2 must be greater than 0.9 between the counts and concentrations of the 6 positive controls.
4. Positive Control Limit of Detection QC: The second lowest positive control spike in (0.5fM) must have counts greater than the Mean ± 2SD of the negative controls for each sample.

A.2 Differential expression analysis

A. Differential expression analysis in early responders and protracted early responders

	Discovery Cohort			Validation Cohort		
Genes	**logFC**	***P*-Value**	**BH-FDR**	**logFC**	***P*-Value**	**BH-FDR**
HLA-B	-0.35	0.002	0.13	0.06	0.72	0.89
MT-ND1	-0.56	0.003	0.13	0.34	0.17	0.89
HLA-A	-0.39	0.003	0.13	0.12	0.53	0.89
HLA-G	-0.47	0.003	0.13	0.16	0.49	0.89
IFRD1_intron_comp41141_c0_seq1	0.53	0.005	0.16	-0.15	0.52	0.89
VPS13A_isform	0.39	0.005	0.16	-0.25	0.19	0.89
HIP1	-0.75	0.007	0.17	0.59	0.17	0.89

B. Differential expression analysis in early responders and grouped protracted early responders & dual responders

	Discovery Cohort			Validation Cohort		
Genes	**logFC**	***P*-Value**	**BH-FDR**	**logFC**	***P*-Value**	**BH-FDR**
HLA-A	-0.41	0.001	0.08	0.11	0.55	0.84
HLA-B	-0.34	0.001	0.08	0.07	0.64	0.84
HLA-G	-0.49	0.001	0.08	0.14	0.51	0.84
IFRD1_intron_comp41141_c0_seq1	0.53	0.003	0.11	-0.11	0.60	0.84
VPS13A	0.39	0.003	0.11	-0.27	0.12	0.76
SEPT7	-0.29	0.007	0.12	0.06	0.71	0.87
MT-ND1	-0.48	0.007	0.12	0.29	0.19	0.77
RPS6	-0.40	0.005	0.12	0.01	0.97	0.98
VCAN_isoform	0.47	0.006	0.12	-0.23	0.31	0.79
HIP1	-0.67	0.010	0.13	0.69	0.07	0.76
HLA-E	-0.64	0.009	0.13	0.35	0.29	0.79
IRF2	-0.58	0.008	0.13	0.29	0.33	0.79
GLIPR1_isoform	0.35	0.012	0.14	-0.08	0.65	0.84
ITGA1	-0.48	0.012	0.14	-0.06	0.84	0.90
MAP3K8_isoform	0.33	0.011	0.14	-0.17	0.30	0.79
CD59	-0.24	0.015	0.15	0.27	0.09	0.76
IL17RA	-0.44	0.018	0.17	0.37	0.16	0.77
INPP5D	-0.44	0.022	0.17	0.15	0.57	0.84
PLXNC1_isoform	0.33	0.023	0.17	0.11	0.55	0.84
PTAR1_isoform	0.25	0.024	0.17	-0.08	0.58	0.84
SELL	-0.25	0.022	0.17	0.31	0.05	0.76
SF3B1_isoform	0.27	0.024	0.17	-0.04	0.81	0.90
ZNF609_isoform	0.30	0.020	0.17	-0.27	0.11	0.76
GNLY	0.63	0.030	0.20	-0.46	0.28	0.79
NFKB1	0.28	0.030	0.20	-0.06	0.77	0.90

A.3 Regression coefficients of elastic net models

A. Regression coefficients of elastic model used to discriminate between early responders and dual responders

	Discovery cohort	Validation cohort
BCL6	-1.7240125	18.700072
FADD	0.1420327	-5.435858
FNIP1	5.5773950	-17.544964
HCLS1	-1.7976698	-27.748739
PDCD1	1.0982856	6.977360
RORC	-1.1336487	1.045253

B. Regression coefficients of elastic model used to discriminate between dual responders and healthy controls

	Discovery cohort	Validation cohort
BCL6	-2.8686763	-8.862091
CARM1	1.1877088	-3.648510
CCR2	0.6192682	4.442497
CDK5RAP3	1.5309976	-4.486903
GATA3	-0.9506529	3.088319
LTF	0.4281719	-1.706503
LTK	-0.1761912	5.236743
MAP2K2	1.0512223	7.608112
SEMA4D	1.4832828	-4.748312
SETX	-5.0998500	9.593554
SMAD2	2.2983478	4.706440

C. Regression coefficients of elastic model used to discriminate between grouped early responders & protracted early responders and dual responders

	Discovery cohort	**Validation cohort**
ATP8A1	1.73923700	7.0173673
CASP8	-1.56512329	-12.8509396
CCR2	0.59266042	-8.5786596
CD4	0.75560623	-10.4524233
CLEC4E	-1.39427366	12.6922697
CTSS	0.86455051	-2.8788272
GATA3	-1.11783118	-2.4174927
HCLS1	-4.42930616	-1.2476609
HIP1	0.02037303	-4.3854978
IL17A	-0.55265588	4.3330260
IL17RA	-0.61858145	2.2008268
MSN	1.54395104	6.3460700
NFKBIA	3.30772607	-0.8976699
PLA2G6	-1.12492780	11.3508790
PLAGL2	2.25620826	1.5174822
PPP3R1	0.20481852	-10.6974181

Appendix B Supplementary material for Chapter 3

B.1 Differential allele frequencies between total nasal symptom score (TNSS) phenotypes

A. Differential allele frequencies between early responders and dual responders

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
AKT3	rs950399	1	243898804	C	3.592	0.03935
	rs4658589	1	243699678	G	4.446	0.05091
	rs3856231	1	243605604	T	3.305	0.054
CACNA1S	rs10920134	1	201148684	C	3.977	0.02357
CHRM3	rs4617391	1	239438742	A	0.1723	0.01398
	rs10802815	1	239892999	G	0.2479	0.01825
	rs10495447	1	239888040	A	0.1898	0.02142
	rs72758710	1	239610588	G	5.974	0.02257
	rs12749330	1	239806444	C	0.2957	0.03226
	rs16839051	1	239900066	C	8.73	0.05943
	rs12133100	1	239414036	T	0.2031	0.06128
	rs3063601	1	239676361	TGT	2.967	0.06457
	rs10925941	1	239649238	A	2.551	0.08224
	rs72760759	1	239929933	C	0.2359	0.09145
CHRNB2	rs57783436	1	154539233	G	3.376	0.01907
	rs3841062	1	154578468	-	2.804	0.03363
	rs7533471	1	154628860	G	2.324	0.05149
	rs11335288	1	154591260	G	2.22	0.0873
	rs2229857	1	154601491	T	2.22	0.0873
KCNQ4	rs72305291	1	40735162	-	3.99	0.02524
	rs1057925	1	40738897	C	0.2506	0.02524
	rs4660456	1	40773839	G	0.1645	0.03289
	rs71577632	1	40773241	TGGAG	0.164	0.03289
	rs10789206	1	40750908	G	2.56	0.08227

B. Differential allele frequencies between protracted early responders and dual responders

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
ADCY3	rs73920612	2	24924420	G	0.3202	0.09797
CACNA1S	rs3767498	1	201051599	A	6.127	0.01338
	rs2297909	1	200991179	A	3.701	0.03113
	rs3753967	1	201155282	T	0.3698	0.07981
CHRM3	rs1111249	1	239802010	G	3.916	0.01618
	rs4617391	1	239438742	A	0.1956	0.01811
	rs663927	1	239772051	A	0.3124	0.0553
	rs12133100	1	239414036	T	0.1923	0.05694
	rs865213	1	239768258	T	0.3529	0.08103
	rs6688669	1	239612517	G	2.631	0.0832
CHRNB2	rs3841062	1	154578468	-	3.293	0.02412
	rs57783436	1	154539233	G	3.309	0.03062
	rs7533471	1	154628860	G	2.198	0.06888
GNG4	rs2481094	1	235569196	C	0.2954	0.0675
	rs2131922	1	235541701	G	0.4051	0.08654
	rs58308716	1	235558951	T	3.044	0.09261

C. Differential allele frequencies between early responders and protracted early responders

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
CACNA1S	rs10920134	1	201148684	C	2.727	0.08721
CHRM3	rs10926008	1	239898823	G	4.463	0.004588
	rs16839051	1	239900066	C	12.63	0.01939
	rs10926012	1	239902344	G	2.699	0.02936
	rs34737866	1	239939249	A	0.3572	0.03038
	rs12125436	1	239477902	G	0.2784	0.03087
	rs1111249	1	239802010	G	0.4062	0.03882
	rs3063601	1	239676361	TGT	2.428	0.04229
	rs10495443	1	239496101	A	0.3103	0.04779
	rs10925941	1	239649238	A	2.162	0.05265
	rs663927	1	239772051	A	2.567	0.05944
	rs631873	1	239777318	T	2.815	0.06008
	rs72758710	1	239610588	G	3.473	0.07123
	rs5782090	1	239652817	A	0.4553	0.07491
	rs6697471	1	239619915	C	0.4553	0.07491
	rs621060	1	239828986	G	0.2999	0.07816
	rs2184857	1	239918447	C	2.19	0.0785
	rs10495447	1	239888040	A	0.3842	0.07908
	rs72760794	1	239950110	T	0.4047	0.08459
	rs614992	1	239782471	T	2.49	0.0892
	rs61834667	1	239379880	C	0.4919	0.09031
	rs865213	1	239768258	T	2.281	0.09259
	rs16839045	1	239898428	G	4.085	0.09325
GNB1	rs142445070	1	1881007	-	0.3644	0.03858
KCNQ4	rs4660456	1	40773839	G	0.3111	0.04317
	rs71577632	1	40773241	TGGAG	0.311	0.04317
	rs751823	1	40875748	T	2.396	0.04638
	rs35486269	1	40839214	T	5.69	0.04651

D. Differential allele frequencies between healthy controls and early responders

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
AKT3	rs2953328	1	243860378	C	0.2202	0.008142
	rs4430311	1	243852691	C	0.2202	0.008142
	rs12691548	1	243656826	A	0.2147	0.01121
	rs3856231	1	243605604	T	0.2246	0.01894
	rs4658589	1	243699678	G	0.2846	0.06765
	rs6682456	1	243779161	C	0.2846	0.06765
	rs10927033	1	243532237	A	0.3132	0.073
CACNA1S	rs3767498	1	201051599	A	2.729	0.06137
CHRM3	rs56202307	1	239394427	G	4.542	0.014
	rs10925907	1	239535562	G	3.226	0.02722
	rs12749330	1	239806444	C	3.789	0.03464
	rs16839051	1	239900066	C	0.1171	0.04877
	rs16839045	1	239898428	G	0.1746	0.05332
	rs1111249	1	239802010	G	2.399	0.05538
	rs34737866	1	239939249	A	2.672	0.05969
	rs66588531	1	239364959	G	2.911	0.0821
	rs72754630	1	239367551	G	2.748	0.08395
	rs1218666	1	239528460	T	0.4932	0.08847
CHRNB2	rs57783436	1	154539233	G	0.3238	0.04529
GNB1	rs2748983	1	1928648	C	4.743	0.006134
	rs142445070	1	1881007	-	2.91	0.03939
	rs4648624	1	1757074	T	2.587	0.04347
	rs59555069	1	1775447	A	0.3491	0.06489
GNG4	rs7554426	1	235639087	G	4.13	0.007569
	rs61834659	1	235673662	T	0.2068	0.01019
	rs6677212	1	235647036	G	0.1979	0.01047
	rs429328	1	235517074	G	0.2783	0.0198
	rs6692985	1	235626574	G	3.502	0.02085
	rs2131922	1	235541701	G	0.4168	0.0707
	rs10713673	1	235647448	-	0.4076	0.08612
KCNQ4	rs35486269	1	40839214	T	0.05865	0.006981

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
KCNQ4	rs3820523	1	40827517	T	0.1731	0.01463
	rs751823	1	40875748	T	0.3269	0.0201
	rs55679729	1	40820951	A	2.885	0.05515

E. Differential allele frequencies between healthy controls and protracted early responders

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
AKT3	rs2953328	1	243860378	C	0.3281	0.03214
	rs4430311	1	243852691	C	0.3281	0.03214
	rs12691548	1	243656826	A	0.3745	0.04939
	rs4132509	1	243779782	A	0.3907	0.07038
	rs320320	1	243671884	G	0.4262	0.09679
CACNA1S	rs10920134	1	201148684	C	4.49	0.0419
	rs2281845	1	201112815	T	2.734	0.06003
	rs12743065	1	201083434	T	2.311	0.081
CHRM3	rs6688669	1	239612517	G	0.2839	0.02648
	rs10925907	1	239535562	G	3.083	0.03325
	rs72760794	1	239950110	T	0.3228	0.04443
	rs10926008	1	239898823	G	3.27	0.04551
	rs10925941	1	239649238	A	2.424	0.05244
	rs3063601	1	239676361	TGT	2.192	0.08364
	rs1984165	1	239954965	C	0.417	0.08961
	rs12125436	1	239477902	G	0.3654	0.09172
	rs10495443	1	239496101	A	0.3654	0.09172
	rs2355227	1	239574489	T	0.468	0.09929
GNG4	rs6692985	1	235626574	G	3.147	0.03659
	rs7554426	1	235639087	G	2.513	0.05334
KCNQ4	rs4660456	1	40773839	G	0.2817	0.03331
	rs71577632	1	40773241	TGGAG	0.2817	0.03331
	rs55679729	1	40820951	A	3.097	0.03388
	rs10789206	1	40750908	G	2.58	0.03836

F. Differential allele frequencies between healthy controls and dual responders

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
AKT3	rs150276657	1	243438442	-	5.667	0.0529
	rs2953328	1	243860378	C	0.3475	0.09396
	rs4430311	1	243852691	C	0.3475	0.09396
CACNA1S	rs3767498	1	201051599	A	20.43	0.007026
	rs10920134	1	201148684	C	5.713	0.01999
	rs3767510	1	201075216	T	0.4013	0.05642
	rs3753967	1	201155282	T	0.296	0.06589
	rs12135240	1	201104427	C	4.852	0.09415
CHRM3	rs1111249	1	239802010	G	3.216	0.03068
	rs56202307	1	239394427	G	2.843	0.06536
	rs12026416	1	239338104	G	0.2252	0.09724
CHRNB2	rs7533471	1	154628860	G	5.301	0.008854
	rs11335288	1	154591260	G	3.127	0.04017
	rs2229857	1	154601491	T	3.127	0.04017
	rs3841062	1	154578468	-	3.127	0.04017
GNG4	rs7554426	1	235639087	G	4.652	0.01172
	rs6429197	1	235611717	C	17.27	0.0119
	rs429328	1	235517074	G	0.2201	0.01588
	rs2481094	1	235569196	C	0.1423	0.02447
	rs6677212	1	235647036	G	0.146	0.02599
	rs10713673	1	235647448	-	0.3319	0.06362
	rs10926274	1	235618234	A	2.619	0.06986
	rs17548391	1	235611611	A	3.86	0.08547
	rs74148718	1	235601777	C	3.037	0.09981
KCNQ4	rs55679729	1	40820951	A	4.834	0.02356
	rs4660456	1	40773839	G	0.1209	0.03032
	rs71577632	1	40773241	TGGAG	0.129	0.03032
	rs1057925	1	40738897	C	0.2569	0.03109
	rs10789206	1	40750908	G	4.233	0.03441
	rs3820523	1	40827517	T	0.1393	0.04578
	rs751823	1	40875748	T	0.3956	0.0757

G. Differential allele frequencies between grouped early responders & protracted early responders and dual responders

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
AKT3	rs950399	1	243898804	C	2.238	0.04274
	rs4658589	1	243699678	G	2.929	0.07365
CACNA1S	rs10920134	1	201148684	C	3.019	0.03077
CHRM3	rs10926008	1	239898823	G	3.231	0.007767
	rs72758710	1	239610588	G	5.085	0.01652
	rs10495447	1	239888040	A	0.3107	0.01862
	rs3063601	1	239676361	TGT	2.603	0.02112
	rs16839051	1	239900066	C	11.13	0.02245
	rs10925941	1	239649238	A	2.314	0.02858
	rs10926012	1	239902344	G	2.27	0.02948
	rs12125436	1	239477902	G	0.3761	0.04989
	rs10802815	1	239892999	G	0.5053	0.05037
	rs34737866	1	239939249	A	0.4638	0.05938
	rs10495443	1	239496101	A	0.4199	0.08154
	rs2184857	1	239918447	C	2.061	0.08441
	rs6697471	1	239619915	C	0.5205	0.0847
	rs66588531	1	239364959	G	0.4261	0.08857
	rs16839045	1	239898428	G	3.884	0.09206
GNB1	rs142445070	1	1881007	-	0.4434	0.06309
	rs2748983	1	1928648	C	0.5207	0.08486
KCNQ4	rs4660456	1	40773839	G	0.2592	0.01015
	rs71577632	1	40773241	TGGAG	0.2592	0.01015
	rs72305291	1	40735162	-	2.6	0.02427
	rs35486269	1	40839214	T	4.643	0.05843
	rs10789206	1	40750908	G	2.012	0.06572
	rs17361735	1	40856698	A	0.4263	0.08669
	rs1057925	1	40738897	C	0.5193	0.09263
	rs72949146	1	40860761	C	3.046	0.09372

H. Differential allele frequencies between early responders and grouped protracted early responders & dual responders

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
AKT3	rs3856231	1	243605604	T	2.363	0.08233
CACNA1S	rs10920134	1	201148684	C	3.016	0.03735
	rs3767498	1	201051599	A	2.162	0.09534
CHRM3	rs4617391	1	239438742	A	0.1784	0.008869
	rs10802815	1	239892999	G	0.3577	0.03232
	rs12133100	1	239414036	T	0.2001	0.04629
	rs12749330	1	239806444	C	0.4203	0.04772
	rs10495447	1	239888040	A	0.3169	0.05981
	rs1111249	1	239802010	G	2.097	0.07275
CHRNB2	rs57783436	1	154539233	G	3.214	0.009632
	rs3841062	1	154578468	-	3.007	0.01196
	rs7533471	1	154628860	G	2.279	0.03094
	rs11335288	1	154591260	G	2.176	0.05716
	rs2229857	1	154601491	T	2.176	0.05716
KCNQ4	rs1057925	1	40738897	C	0.3639	0.04878
	rs72305291	1	40735162	-	2.542	0.0745
	rs4660456	1	40773839	G	0.2595	0.09374
	rs71577632	1	40773241	TGGAG	0.2595	0.09374

I. Differential allele frequencies between healthy controls and all allergic participants

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
AKT3	rs2953328	1	243860378	C	0.2978	0.007053
	rs4430311	1	243852691	C	0.2978	0.007053
	rs12691548	1	243656826	A	0.3375	0.01413
	rs320320	1	243671884	G	0.405	0.05164
	rs3856231	1	243605604	T	0.4137	0.05451
	rs4132509	1	243779782	A	0.4162	0.05542
	rs10927033	1	243532237	A	0.4174	0.08245
CACNA1S	rs3767498	1	201051599	A	2.736	0.04572
	rs2281845	1	201112815	T	2.289	0.05581
	rs10920134	1	201148684	C	3.368	0.06339
	rs3767510	1	201075216	T	0.5432	0.07565
CHRM3	rs10925907	1	239535562	G	3.023	0.01865
	rs56202307	1	239394427	G	2.449	0.04332
	rs1218666	1	239528460	T	0.5457	0.07304
	rs6688669	1	239612517	G	0.4879	0.08636
	rs1111249	1	239802010	G	1.973	0.08851
CHRNB2	rs7533471	1	154628860	G	2.054	0.08886
GNB1	rs2748983	1	1928648	C	2.294	0.04843
GNG4	rs7554426	1	235639087	G	3.316	0.007818
	rs61834659	1	235673662	T	0.3533	0.01624
	rs6677212	1	235647036	G	0.3682	0.01737
	rs429328	1	235517074	G	0.3813	0.02086
	rs2131922	1	235541701	G	0.4831	0.06252
	rs10713673	1	235647448	-	0.4615	0.08032
	rs2481094	1	235569196	C	0.4703	0.09086
	rs6429197	1	235611717	C	2.369	0.09186
KCNQ4	rs55679729	1	40820951	A	3.06	0.0208
	rs3820523	1	40827517	T	0.3956	0.03168
	rs10789206	1	40750908	G	2.4	0.03862
	rs4660456	1	40773839	G	0.3924	0.03931
	rs71577632	1	40773241	TGGAG	0.3924	0.03931

B.2 Differential allele frequencies between healthy controls and nasal congestion phenotypes

A. Differential allele frequencies between healthy controls and high congestion subgroup

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
AKT3	rs4430311	1	243852691	C	0.4042	0.05871
	rs2953328	1	243860378	C	0.4042	0.05871
CACNA1S	rs10920134	1	201148684	C	4.259	0.0329
	rs2281845	1	201112815	T	2.708	0.05554
	rs3767498	1	201051599	A	2.852	0.0586
CHRM3	rs10925907	1	239535562	G	3.201	0.02871
	rs1984165	1	239954965	C	0.3433	0.02928
	rs12026416	1	239338104	G	0.2446	0.03644
	Affx-7849210	1	239394427	G	2.583	0.04282
	rs6688669	1	239612517	G	0.3861	0.06698
	rs10925941	1	239649238	A	2.124	0.08771
	rs72760794	1	239950110	T	0.4332	0.08882
	rs1218666	1	239528460	T	0.528	0.09148
CHRNB2	rs7533471	1	154628860	G	2.835	0.02718
	rs11335288	1	154591260	G	2.209	0.06916
	rs2229857	1	154601491	T	2.209	0.06916
GNB1	rs2748983	1	1928648	C	2.515	0.05572
GNG4	rs6692985	1	235626574	G	5.094	0.003882
	rs7554426	1	235639087	G	3.614	0.008761
	rs6677212	1	235647036	G	0.4085	0.06266
KCNQ4	rs71577632	1	40773241	TGGAG	0.2314	0.0146
	rs4660456	1	40773839	G	0.2314	0.0146
	rs55679729	1	40820951	A	3.474	0.01532
	rs10789206	1	40750908	G	2.497	0.05381
	rs3820523	1	40827517	T	0.3661	0.05612
	rs1576122	1	40842470	C	0.4381	0.07005
	rs72305291	1	40735162	-	2.283	0.08315

B. Differential allele frequencies between healthy controls and low congestion subgroup

Gene	RS Number	Chromosomal Location	Biological Position	Minor Allele	Odds Ratios	*P*-Value
ADCY3	rs17046742	2	24942956	A	0.2352	0.05128
AKT3	rs12691548	1	243656826	A	0.1385	0.002975
	rs4430311	1	243852691	C	0.1771	0.003301
	rs2953328	1	243860378	C	0.1771	0.003301
	rs3856231	1	243605604	T	0.159	0.007481
	rs10927033	1	243532237	A	0.258	0.02926
	rs320320	1	243671884	G	0.3141	0.03155
	rs4132509	1	243779782	A	0.3178	0.03397
	rs150276657	1	243438442	-	4.224	0.04931
	rs12046990	1	243454545	C	5.011	0.05644
CACNA1S	rs3767498	1	201051599	A	2.618	0.06814
CHRM3	rs10925907	1	239535562	G	2.98	0.03533
	rs2790336	1	239799386	G	2.008	0.07848
	rs1111249	1	239802010	G	2.095	0.08596
	rs56202307	1	239394427	G	2.409	0.08609
GNB1	rs2748983	1	1928648	C	2.133	0.09576
GNG4	rs61834659	1	235673662	T	0.0936	0.004637
	rs429328	1	235517074	G	0.2225	0.00649
	rs6677212	1	235647036	G	0.2726	0.01616
	rs7554426	1	235639087	G	3.07	0.02033
	rs6692985	1	235626574	G	2.957	0.03488
	rs2131922	1	235541701	G	0.3886	0.05017
	rs6429197	1	235611717	C	2.617	0.0797
	rs10713673	1	235647448	-	0.4339	0.09238
KCNQ4	rs751823	1	40875748	T	0.4015	0.02089
	rs55679729	1	40820951	A	2.786	0.06145
	rs3820523	1	40827517	T	0.3861	0.06315
	rs10789206	1	40750908	G	2.111	0.09199