Immune and microbiome regulation in allergic rhinitis

Annabelle Monica Watts

BAppSc, MAppSc (research)

Menzies Health Institute Queensland

School of Medical Science

Griffith Health

Griffith University

Submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

May 2019

ABSTRACT

Allergic rhinitis (AR) is a common chronic upper respiratory disease where exposure to allergens causes an IgE mediated inflammatory response. AR is estimated to affect between 10-40% of the population worldwide and is responsible for significant economic and medical burden. The primary symptoms of AR include rhinorrhoea, nasal congestion, itchy nose and eyes and sneezing. There is currently no cure for AR and the current treatment options are typically focused on achieving symptom relief. Whilst the symptoms of AR manifest predominately in the upper respiratory tract, the pathophysiology of the disease is complex, and involves interactions between the mucosal and systemic immune systems. As such, there is an increasing need to better understand the complex immunological mechanisms which underpin the disease. Doing so may lay the foundation for strategies to reduce AR symptoms through modifying the disease process itself or via the development of novel therapies.

In a series of six studies, this thesis investigated the pathophysiology and treatment of AR via immune and molecular phenotyping of the gut microbiome, peripheral blood, and nasal mucosa. Chapter 1 presents a comprehensive review of the pathophysiology and treatment strategies for AR and provides direction for the investigative route undertaken in the PhD thesis. Chapter 2 sought to evaluate the gut microbial composition of adults with AR (n=57) compared with controls without AR (n=23). Prior to this study it was not known if aberrant bacterial colonisation patterns, as reported for atopic infants, also occurred in adults with AR. Sequencing of the prokaryotic 16s rRNA gene isolated from stool samples, revealed that adult AR sufferers have a distinct microbiome profile compared to non-AR controls. The adult AR microbiome was marked by a reduced microbial diversity and altered abundance of certain microbes. Namely, the phyla Bacteroidetes and Actinobacteria were more abundant in AR samples compared with control samples. Whereas, the Firmicutes were less abundant in the AR group compared with controls. The Firmicutes phyla are major contributors of butyrate production in the gut which helps maintain the integrity of the intestinal barrier. Reduced butyrate production may lead to increased gut permeability and antigen transfer. Altered abundance of *Parabacteroides*, bifidobacteria, *Oxalobacter* and Clostridiales, was also observed between groups. Notably, altered abundance of bifidobacteria and Clostridiales has also been observed in other studies of atopy. Overall, this study provides evidence that adult AR sufferers have a distinct gut microbiome profile compared with controls. Mechanistic studies are needed to evaluate the effect of the AR microbiome on disease pathophysiology. However, this study provides a basis for possible modification of disease processes via modulating the microbiome with complementary therapies including probiotics, prebiotics and faecal transplant.

Based on the findings from the gut microbiome study, Chapters 3 and 4 investigated whether modifying the microbiome with probiotics had beneficial clinical effects on the symptoms of AR. Probiotics

transiently colonise the intestine and their therapeutic potential for AR is thought to be related to the known role of the microbiome in health and disease. The probiotic supplement investigated in this study contained six bacterial strains from the *Lactobacillus*, *Bifidobacterium* and *Lactococcus* genera. A Simon's-Two Stage design including 40 intermittent/seasonal AR sufferers was developed to determine if the probiotic supplement had sufficient biological activity to warrant further study. Using this design, 63% of those with AR had a clinically meaningful response to probiotic treatment based on assessment of disease specific quality of life scores. The proportion of participants exhibiting improvement in quality of life metrics was encouraging and the data generated in this study provides important information to support the development of larger phase III trials.

Gene expression analysis is a powerful tool which characterises the activity of many immune genes and enables studies of disease pathophysiology and response to pharmaceutical treatment. Gene expression studies of the nasal mucosa in individuals with AR typically rely on invasive nasal biopsies to obtain enough genetic material for analysis. Chapter 5 sought to develop a novel gene expression protocol to circumvent the need for invasive sample collection. Nasal washing and brushing samples collected via non-invasive means yielded enough molecular material for multiplex gene expression analysis of 760 immune genes using the NanoString nCounter. A within-subject design including 12 individuals with intermittent AR was utilised to compare immune gene expression profiles obtained from nasal washing/brushing samples and whole blood samples collected into Paxgene tubes. Overall, the blood and nasal samples showed vastly distinct gene expression profiles which reflects their unique anatomical and functional origins. The differences observed between sites proves that collection of blood samples is a poor surrogate for direct sampling of nasal mucosa when investigating local immune mechanisms pertinent to AR physiology.

The diagnosis of AR is confounded by the common occurrence of positive allergen-specific IgE or skin prick test to allergens in patients with other chronic respiratory diseases/rhinitis endotypes that share similar symptoms and clinical presentation, but have different underlying pathophysiology. Greater understanding of the immune networks underpinning AR pathophysiology may assist with further defining rhinitis endotypes and enhance knowledge of treatment responses. The gene expression protocol designed in Chapter 5 was utilised in Chapter 6 to compare immune gene expression profiles in nasal lysate and peripheral blood samples of adults with persistent AR (n=45) to otherwise healthy controls without AR (n=24). Overall, distinct gene expression profiles in the nasal mucosa and peripheral blood were observed in the AR cohort compared with controls. A total of 113 immune genes were significantly differentially expressed in peripheral blood samples between groups. In contrast, 14 genes were significantly differentially expressed in nasal lysate samples between groups. Allergy-related genes such as CCL17, CCL26 and TPSAB1 were upregulated in the nasal lysate samples of AR sufferers. Chemokines CCL17 and CCL26 are involved the chemotaxis of key effector cells and

TPSAB1 encodes tryptase which is an inflammatory mediator released from activated mast cells and basophils. In blood samples, the Prostaglandin D2 receptor was significantly upregulated in the AR group compared with the non-AR group. Interaction of prostaglandin with the prostaglandin receptor stimulates activation and chemotaxis of key inflammatory cells pertinent to the allergic response. The results of this study also provided further insights into the interaction between the mucosal and systemic immune system. Many of the clinical markers measured in blood such as eosinophil counts and IgE levels were correlated with counts of certain differentially expressed genes in nasal mucosa samples. The AR specific genes and gene pathways identified in this study may contribute to the refinement of rhinitis endotypes or as biomarkers to evaluate the effectiveness of treatment regimens.

Following on from the investigation of AR pathophysiology, Chapter 7 used the same gene expression protocol to compare changes to immune gene expression in nasal mucosal and blood samples from adults with AR in response to treatment with topical nasal sprays. Previous studies have found that the antihistamine azelastine hydrochloride (AZE) and corticosteroid fluticasone propionate (FP) combination spray is more effective at reducing symptoms than monotherapy with AZE or FP, however the biological basis for the enhanced effects of the combination spray remain unclear. A parallel group design was used to compare the immune gene expression profiles for individuals with persistent AR following seven days administration of either AZE (n=14), FP (n=16) or the combination spray AZE/FP (n=16). Severity of symptoms during the study period were also assessed with self-report symptom questionnaires. Overall, distinct gene expression profiles in the nasal mucosa were observed across all intervention groups following treatment. The gene expression profiles for FP and AZE were the most different from each other which is consistent with the separate mechanism of action between corticosteroids and antihistamines. An intriguing finding of this study was that FP and AZE/FP had similar effects on symptom reduction, but had unique effects on immune gene expression. In particular, FP altered the expression of 206 immune genes in the nasal mucosa with the majority of these genes being downregulated following treatment. In comparison, AZE/FP significantly altered 16 immune genes in the nasal mucosa with a mix of downregulated (n=10) and upregulated (n=6) genes following treatment. The moderate number (n=16) of genes modulated by AZE/FP is sufficient to markedly reduce AR symptoms, whilst also preventing total suppression of the local immune system.

Overall, this thesis provides novel and important insights into the pathophysiology of AR through examination of the gastrointestinal microbes, local tissues (nasal mucosa) and systemic immune system. Specific bacterial species and specific groups of bacteria were reported as altered in individuals with a history of AR. In addition, the novel gene-expression approach developed in this thesis identified biomarkers in the nasal mucosa and blood samples that were unique to AR. The biomarkers described in this thesis pave the way for the development of diagnostic tests to better define rhinitis endotypes

and monitor response to pharmaceutical treatment. The second theme of this thesis was to evaluate the efficacy and mechanisms of treatments that act on mucosal tissues (nasal and gut). This thesis provides support for the ongoing clinical development of a specific probiotic supplement for AR symptoms. In addition, a major finding of this thesis was that AZE/FP combination spray provides superior symptom relief, and unlike the commonly used corticosteroid monotherapy FP, has minimal suppressive effects on the mucosal immune system.

STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

7th May 2019

Annabelle Monica Watts

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to Professor Allan Cripps. I have thoroughly enjoyed working under your direction. Thank you for your help shaping this thesis and for your guidance in all PhD and personal matters. I would especially like to thank you for making the effort to get to know me as a student, for making me feel like a priority among your hectic schedule, and for your calming and respectful approach to supervision. I would also like to sincerely thank my co-supervisor's Dr Amanda Cox and Dr Nic West. To Amanda, thank you for your management of a well-organised and effective laboratory, and for teaching me how to write manuscripts and develop the 'story'. Your exceptional training during my time as Research Assistant provided me with the skills needed to complete this PhD. To Nic, thank you for handling the commercial aspects of my studies, for your understanding nature, your positive outlook, and for encouraging me to see the 'silver lining'. Your commitment to the success of this research group is truly inspiring.

This PhD would not have been possible without the individuals who participated in my studies. Squirting salty water up your nose and donating numerous blood samples could not have been pleasant, but your contribution was invaluable to this research. I am grateful for the data analysis and statistical guidance provided by Dr Ping Zhang. I would also like to acknowledge Associate Professor Evelin Tiralongo and Dr Claire Williams for allowing me to work in your well-managed and welcoming Clinical Trial Unit. Furthermore, the Queensland Allergy Services clinic kindly allowed me to use their clinic space for study appointments and performed the skin prick testing. I would also like to recognise the assistance of Winclove Probiotics, Nutriimmun and Mylan N.V for funding the intervention studies.

I have been fortunate to receive guidance from many people in my life. Although Professor Flavia Huygens and Professor Iain Lamont were not directly involved in my PhD, their training during my early scientific years has helped me immensely. In particular, Flavia was very supportive of the 'researcher' in me and encouraged me to have a career in science, and Iain taught me how to think about data and the importance of experimental rigor. I would also like to express my gratitude towards my fellow PhD students Candice Colbey, Fiona Chen and Michelle Chamoun who have been a positive influence and support network during my PhD. Rebecca Ramsey, Sofia Jimenez and Elisabeth Kolarich, thank you all for being such a wonderful office buddies and friends. You both brought so much joy and light to this PhD experience.

I am eternally grateful for the nurturing support system I have in my personal life. To the Watts and the Kavanagh families, thank you for your words of encouragement, offers of help, and interest in my research projects. To my parents, thank you for instilling resilience and a strong work ethic in me, and for inspiring me to achieve my goals. To my new family, thank you for your genuine enthusiasm towards my field of research and for welcoming me into your lives with open arms. Finally, I would like to thank my beautiful fiancé Justin for his undying support and love. You were always there when I needed you. For every time it got too much, and I wanted to quit, you were there encouraging me to keep going. For every success, you were there cheering me on.

ABBREVIATIONS

µg	Microgram
%	Percentage
°C	Degrees centigrade
µL	Microlitre
AD	Atopic dermatitis
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
AP-1	Activation protein 1
APC	Antigen presenting cell
AR	Allergic rhinitis
ARC	Activator-recruited cofactor
ARIA	Allergic Rhinitis and Its Impact of Asthma guidelines
AZE	Azelastine hydrochloride
BMI	Body mass index
CBP/p300	CREB-binding protein
cc	Cubic centimetre
CCL	Chemokine ligand
CFU	Colony forming units
CG	Control group
CI	Confidence interval
COX	Cyclooxygenase
DAG	1,2-diacyl-glycerol
DAVID	Database for annotation, visualization and integrated discovery
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
ECP	Eosinophil cationic protein
EFSA	European food safety authority
ELISA	Enzyme-linked immunosorbent assay
EPO	Eosinophil peroxidase
ESR	Erythrocyte sedimentation rate
FC	Fold change
FDR	False discovery rate
FP	Fluticasone propionate
FPR	Formal peptide receptor
g	Gram
GAD	Genetic association database
GALT	Gut associated lymphoid tissue
GDP	Guanosine diphosphate
GILZ	Glucocorticoid-induced leucine zipper
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPR35	G protein coupled receptor 35
GR	Glucocorticoid receptor
GRE	Glucocorticoid response elements
GRIP	Glucocorticoid receptor interacting protein
GTP	Guanosine triphosphate
Gα	Guanine nucleotide-binding protein alpha
Gβ	Guanine nucleotide-binding protein beta
Gγ	Guanine nucleotide-binding protein gamma
H1	Histamine 1 receptor

H2	Histamine 2 receptor
H3	Histamine receptor 3
H4	Histamine receptor 4
HDAC	Histone deacetylase
hr	Hour
HSP	Heat shock protein
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC2	Type 2 innate lymphoid cells
INAH	Intranasal antihistamine
iNKT	Invariant natural killer T cell
iNOS	Inducible nitric oxide synthase
IP3	Inositol 1,4,5-triphosphate
IR	Inositol 1,4,5-triphosphate receptor type 1
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
K	Kilo
KEGG	Kyoto Encyclopaedia of Genes and Genomes
kg	Kilogram
L	Litre
LPS	Lipopolysaccharide
m	Metre
MAMP	Microbial associated molecular pattern
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
min	Minute
MKP-1	Mitogen-activated protein kinase phosphatase 1
ml	Millilitre
mm	Millimetre
MPO	Myeloperoxidase
mRQLQ	Mini rhinoconjunctivitis quality of life questionnaire
n	Number
NADPH	Nicotinamide adenine dinucleotide phosphate
N-CoR	Nuclear receptor corepressor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
NO	Nitric oxide
NOD	Oligomerization domain protein
OARSS	Other Allergic Rhinitis Symptom Score
OR	Odds ratio
p/CAF	CBP/p300 associated factor
p/Cip	CBP/p300 co-integrator associated protein
Pa	Pascals
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIP2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein kinase C
PLCβ	Phospholipase C Beta
PP2A	Protein phosphate 2A

PPRS	Pattern recognition receptors
QLD	Queensland
RANTES	Regulated on activation, normal T cell expressed and secreted
RAST	Radio-allergosorbent test
RBL	Rat basophilic leukemia
RCT	Randomised controlled trial
RLR	Retinoic acid-inducible gene-1-like receptor
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell park memorial institute medium
RTSS	Rhinitis Total Symptom Score
s	Second
SCORAD	Scoring of Atopic Dermatitis
SD	Standard deviation
SLPI	Secretory leukocyte protease inhibitor
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
SPSS	Statistical package for the social sciences
SPT	Skin prick test
SRC-1	Steroid receptor coactivator 1
STRING	Search Tool for the Retrieval of Interacting Genes
TARC	Thymus and activation regulated chemokine
Th2	T helper 2
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
TNSS	Total nasal symptom score
TOSS	Total ocular symptom score
TRAP	Thyroid hormone receptor associated protein
Treg	T regulatory cell
TRP	Transient receptor potential
TSLP	Thymic stromal lymphopoietin
U	Units
VAS	Visual analogue scale
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
WCC	White cell count

ACKNOWLEDGEMENT OF PAPERS INCLUDED IN THIS THESIS

Section 9.1 of the Griffith University Code for the Responsible Conduct of Research ("Criteria for Authorship"), in accordance with Section 5 of the Australian Code for the Responsible Conduct of Research, states:

> To be named as an author, a researcher must have made a substantial scholarly contribution to the creative or scholarly work that constitutes the research output, and be able to take public responsibility for at least that part of the work they contributed. Attribution of authorship depends to some extent on the discipline and publisher policies, but in all cases, authorship must be based on substantial contributions in a combination of one or more of:
>
> - conception and design of the research project
> - analysis and interpretation of research data
> - drafting or making significant parts of the creative or scholarly work or critically revising it so as to contribute significantly to the final output.

Section 9.3 of the Griffith University Code ("Responsibilities of Researchers"), in accordance with Section 5 of the Australian Code, states:

> Researchers are expected to:
>
> - Offer authorship to all people, including research trainees, who meet the criteria for authorship listed above, but only those people.
> - accept or decline offers of authorship promptly in writing.
> - Include in the list of authors only those who have accepted authorship
> - Appoint one author to be the executive author to record authorship and manage correspondence about the work with the publisher and other interested parties.
> - Acknowledge all those who have contributed to the research, facilities or materials but who do not qualify as authors, such as research assistants, technical staff, and advisors on cultural or community knowledge. Obtain written consent to name individuals.

Included in this thesis are manuscripts in Chapters 1, 3, 4 and 5 which are co-authored by other researchers. My contribution to each co-authored paper is outlined at the beginning of each relevant Chapter. The bibliographic details and status of these manuscripts are:

Chapter 1:

Watts AM, Cripps AW, West NP, Cox AJ. Modulation of allergic inflammation in the nasal mucosa of allergic rhinitis sufferers with topical pharmaceutical agents. Front Pharmacol. 2019;10:294.

Chapter 3:

Watts AM, West NP, Smith PK, Cripps AW, Cox AJ. Probiotics and Allergic Rhintis: A Simon Two-Stage Design to Determine Effectiveness. J Altern Complement Med. 2016;**22**(12):1007-1012.

Chapter 4:

Watts AM, Cox AJ, Smith PK, Besseling-van der Vaart I, Cripps AW, West NP. A Specicially Designed Multispecies Probiotic Supplement Relieves Seasonal Allergic Rhinitis Symptoms. J Altern Complement Med. 2018;24(8):833-840.

Chapter 5:

Watts AM, West NP, Cripps AW, Smith PK, Cox AJ. Distinct Gene Expression Patterns between Nasal Mucosal Cells and Blood Collected from Allergic Rhinitis Sufferers. *Int Arch Allergy Immunol* 2018;**177**(1):29-34.

Supplementary material in Appendix 2

Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in each paper.

_____7th May 2019

Annabelle Monica Watts

_____7th May 2019

Supervisor: Professor Allan Cripps

CHAPTER ONE

Review of the literature, gap analysis and thesis aims

MODULATION OF ALLERGIC INFLAMMATION IN THE NASAL MUCOSA OF ALLERGIC RHINITIS SUFFERERS WITH TOPICAL PHARMACEUTICAL AGENTS

This Chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

> **Watts AM**, Cripps AW, West NP, Cox AJ. Modulation of allergic inflammation in the nasal mucosa of allergic rhinitis sufferers with topical pharmaceutical agents. Front Pharmacol. 2019;10:294.

My contribution to the paper involved:

The critical review of the literature, preparation of figures and tables, and primary writer of the manuscript text.

_____7th May 2019

Annabelle Monica Watts

_____7th May 2019

Supervisor: Professor Allan Cripps

THE GUT MICROBIOTA IN ALLERGIC RHINITIS SUFFERERS AND MODULATION OF THE GUT MICROBIOTA WITH PROBIOTICS

1.0 Introduction

The gastrointestinal microbiota is unofficially described as an immune compartment which plays an important role in the development and regulation of local and systemic immunity. Indeed, several immune-mediated conditions (1-7) including allergic disease (8-11), have been linked with abnormal gut microbiome composition. Over the last few decades, probiotics have been increasingly recognised as an alternative therapeutic strategy to treat AR symptoms. The rationale for probiotic supplementation stems from the known immunoregulatory capacity of commensal gut microbes. This review will: (i) describe the diverse function of the gut microbiota in humans (ii) outline factors that influence gut microbiome composition, including mode of delivery, infant diet and household living environment (iii) describe the theories proposed to explain the rise of allergic disease, including the hygiene hypothesis, microbiota hypothesis and old friends' hypothesis (iv) discuss the composition of the microbiota in allergic disease (v) describe the immunoregulatory mechanisms of commensal gut bacteria (vi) define probiotics and outline their general uses (vii) discuss the efficacy of probiotics in allergic disease and (viii) identify the mechanism of action of probiotics in allergic diseases.

2.0 The microbiota and its function

The microbiota and its associated genetic content (microbiome) is an extremely complex and dynamic community of microbes (12). The collection of genes within the microbiota is estimated to outnumber the genes in the human genome by a factor of 100-150 (13, 14) and total microbial cells outnumber human cells by a factor of 10 (15, 16). The majority of microbes reside in the gut, particularly in the large intestine. It has been estimated that an approximate 100 trillion microbes (17) or 10^8 to 10^{12} CFU/g of faecal material exist in the gastrointestinal tract (GIT). The gut of a healthy human harbours 500-1000 distinct bacterial phylotypes (18) of which the most predominant phyla are Firmicutes and Bacteroidetes (17). Species belonging to the Actinobacteria, Proteobacteria and Verrucomicrobia phyla, are also common within the gut microbial community (19).

Commensal gut bacteria are involved in many biological functions. Gut bacteria aid in: digestion of food; absorption of nutrients from otherwise indigestible foods; biosynthesis of hormones and vitamins; production of short-chain fatty acids which is an energy source for enterocytes and occurs via fermentation of complex carbohydrates; protection against pathogens through production of antimicrobial compounds and competitive exclusion, and contributing to the structure of the gut mucosa

by maintenance of mucous glycoproteins (18, 20-22). Gut bacteria also play an important role in the development and regulation of the immune system and the induction of immune tolerance (20). The role gut bacteria play in immunity is discussed in subsequent sections of this review.

3.0 Factors influencing infant microbiota composition

Until recently, the first colonization with microbes was thought to begin at birth. However, the 'sterile womb paradigm' has been challenged by the advent of molecular biology techniques that have demonstrated the presence of microbes in the amniotic fluid, placenta and meconium from healthy pregnancies (23). However, the degree of colonization that occurs in utero is still debated. At birth, newborns are further colonised with an enormous variety of microorganisms at exposed skin and mucosal sites such as the mouth, urogenital tracts and the gut (20, 22). The gut microbiota undergo active transformation in infancy, increasing in bacterial diversity, and later stabilising at around three years of age, to yield a microbiome similar to that of adults (24). The colonising bacteria mostly originate from the mothers vaginal and gastrointestinal tract (25). Early colonisation of the gut microbiota is heavily influenced by environmental and host factors such as mode of delivery, feeding strategies, levels of hygiene, exposure to disinfectants, pets or livestock and antibiotic exposure (18, 25). The main environmental and host factors associated with changes to the gut microbiota are described in further detail below.

3.1 Mode of delivery

Infants born via caesarean section are not directly exposed to maternal microbes and are instead colonised by bacteria originating from the hospital environment and skin bacteria, resulting in a delayed colonisation of the gut with beneficial bacteria. As such, caesarean born infants display a dissimilar gut microbial composition pattern compared to vaginally delivered infants. In a large birth cohort study (n=700) conducted in Copenhagen Demark, delivery by means of caesarean section was associated with a unique composition pattern of the neonatal gut (26). Colonisation of the intestinal tract by *Citrobacter freundii, Clostridium* species, *Enterobacter cloacae, Enterococcus faecalis, Klebsiella oxytoca, Klebsiella pneumoniae,* and *Staphyloccus aureus* at one week of age was associated with caesarean born infants, whereas colonisation with *Escherichia coli* was associated with vaginal delivery (26). Similarly, Backhed et al. also identified a differential gut microbiome in their cohort of Swedish infants (n=98) delivered via caesarean section compared to vaginal delivery. The caesarean section microbiome was enriched with *Enterobacter hormaechei, E. cancerogenus, Haemophilus parainfluenzae, H. aegyptius. H. influenzae, H. haemolyticus, Staphylococcus australis* and *Veillonella dispar, V. parvula* (27). Interestingly, these microbes have been previously associated with oral, skin and hospital sources, indicating that caesarean-delivered infants acquire their early intestinal bacteria from these sources. In

contrast, the gut microbiome of vaginally delivered infants were enriched with beneficial bacteria from the genera *Bacteroides, Bifidobacterium, Parabacteroides* and *Escherichia*. Vertical mother to neonate transfer of gut microbiota was confirmed in this study, and was more pronounced in the vaginally delivered infants (27). Specifically, a total of 72% of the early colonising microbes in stool samples from vaginally-delivered newborns was equivalent to stool samples from their associated mother, while only 41% of these microbes were detected in newborns delivered via caesarean section.

Based on the aberrant microbiome profiles observed in caesarean delivered infants, associations between the mode of delivery and the risk for atopic disease have been explored. A recent meta-analysis of 26 cohort studies, indicated that infants born via elective caesarean section have a 16% higher risk of developing asthma compared to vaginally delivered infants (28). In a large retrospective cohort study of children aged 3-10 years (n=8953), an increased risk of subsequent AR diagnosis was associated with caesarean section delivery compared to vaginal delivery (adjusted odds ratio 1.37%, 95% Confidence Interval (CI) 1.14 - 1.63) (29). However, no association with other atopic disorders such as atopic dermatitis, food allergy and asthma in boys was reported in this study.

3.2 Infant diet

A major source for bacterial colonisation in the infant gut is through the microbiota present in the mother's breast milk (30, 31). Breast milk also contains complex oligosaccharides with prebiotic activity that support the growth of colonic bacteria. It is therefore unsurprising, that breastfed infants display a different gut microbiome from that of formula-fed infants. In a cohort of 4-month-old Swedish infants, exclusively breast-fed infants have higher levels of taxa commonly used in probiotic formulations such as *Lactobacillus johnsonii, L. gasseri, L. paracasei, L. casei* and *Bifidobacterium longum*, whereas, exclusively formula-fed infants had increased levels of *Clostridiodes difficile, Granulicatella adiacens, Citrobacter spp., Enterobacter cloacae* and *Bilophila wadsworthia* (27).

The association between mode-of-feeding and later development of allergic disease, has been extensively studied. In a large birth cohort study (n=2705) conducted in the Netherlands longer duration of breast-feeding was associated with a decreased risk for recurrent wheeze in the first two years of life, and a decreased risk of self-reported atopic dermatitis, in infants of mothers without a history of allergy or asthma. These findings were supported by a meta-analysis of 75 studies examining history of asthma and mode-of feeding, whereby infants that were breastfed 'longer' had a lower risk of developing asthma (pooled odds ratio 0.78, 95% CI 0.74 – 0.84) compared to 'lesser' breastfeeding. From these collective findings, it appears that breastfeeding supplies beneficial microbes and prebiotic nutrition that helps shape the structure of the gut microbiome, which in turn aids in the immune development of the host.

3.3 Household living environment

Exposure to a farming environment, namely the contact with livestock and animal feed, or exposure to furry animals provide an additional source of contact with microbial agents. Indeed, Ege et al identified a greater diversity of microbes in mattress dust samples from farming households compared to non-farming households (32). It is plausible that increased exposure to a diverse source of microbes in the household, could translate to a more diverse gut microbiome. Increased richness and diversity of the microbiota in stool samples from 4-month old infants (n=24) living with pets was observed compared to infants living in households without any household pets. Additional support for the transfer of microbes from pets to the infant gut microbiota was provided by Nermes et al. 2005. *Bifidobacterium pseudolongum*, an animal derived *Bifidobacterium*, was significantly detected more often in the stool samples of 1-month old infants living in a household with at least one furry indoor pet compared to non-pet exposed infants (33.3% vs 14.1%, p=0.01).

Associations between the exposure to farms or household pets and the development of atopic conditions have been previously explored (32-35). Ege and colleagues reported that children living on farms had a lower prevalence of asthma and atopy compared to the non-farming households (32). Similarly, a meta-analysis of 21 birth cohort studies, reported a favourable effect of exposure to dogs and pets overall on the risk of atopic dermatitis development in infants or children (35). However, no association emerged with exposure to cats (35). The favourable effect of pet and farm exposure on the later development of allergic disease is likely explained by the increased contact with microbial agents in early life and thereby affecting the development of the immune system.

4.0 Theories to explain the rise of allergic disease

In 1989 Strachan proposed the 'hygiene hypothesis' to explain the observed rise in the prevalence of allergic diseases in industrialised countries. Strachan reported an inverse relationship between the prevalence of allergic disorders (allergic rhinitis and atopic dermatitis) and the number of children in the household. It was postulated that improvements in hygiene and reduced family size limited the opportunity for cross infection and maturation of the immune system, thus resulting in the increased presentation of allergic diseases (36). Since then, numerous epidemiological studies have described associations between the composition of gut bacteria and allergic diseases, prompting an update of the hygiene hypothesis to the 'microbiota hypothesis'. This theory suggests that changes in microbial colonisation patterns early in life, influenced by the lifestyle factors of the western world, cause a polarisation towards a Th2 dominant immune response and therefore a higher incidence of allergies (37, 38). In 2003 Rook proposed a modification to the hygiene and microbiota hypotheses to explain the rise in allergic disease (39). Rook's 'Old Friends' hypothesis postulates that the western lifestyle

and urbanisation has depleted 'old' infections such as helminths, *Helicobacter pylori*, saprophytic mycobacteria, and hepatitis A virus that persisted in the hunter-gather groups and needed to be tolerated (39-41). Rook has hypothesized that it is these 'old friend' microbes rather than crowd infections that prime immunoregulatory mechanisms involved in preventing allergic disease (40).

5.0 The gut microbiome in allergic disease

Table 1 describes studies that have examined the composition of the gut bacteria in allergic versus non-allergic individuals. The majority of these studies were prospective birth cohort studies examining the early composition of the gut microbiota and the later development of allergy. Notably, many gut microbiota studies have been conducted in paediatric populations, however few studies have been conducted in adults with existing disease. Atopic dermatitis was the most common clinical manifestation of allergy investigated in these collective studies. Bacteriological culture was the primary method used to characterise the microbial composition of the faecal samples. The limitation of bacteriological culture to extensively characterise the gut microbes is well recognised (42). As such, more recent investigations have employed quantitative PCR to identify a specific set of microbes or 16S gene sequencing to characterise the microbial community. While differences in microbial composition between allergic and non-allergic subjects were identified in a number of these studies (Table 1), a consensus of an 'allergic microbiome profile' cannot be reached and it still not known which microorganisms have protective effects against allergies. Heterogeneity in study design features, including allergic disease classification, age of subjects, and microbiome identification tools used, complicate our understanding of the microbiome profile of allergic individuals. Despite this, some general patterns in the microbial composition of allergic individuals were observed in the studies described in Table 1. A consistent finding across studies is that lower microbial diversity occurs in the intestine of allergic individuals compared to non-allergic individuals. In addition, many studies also identified a reduced abundance of bifidobacteria in the allergic individuals in comparison to the non-allergic individuals, although this was not reported in all studies.

The gut microbiome is known to undergo rapid increases in bacterial diversity up to approximately three years of age (24). As such, it remains unclear if findings from paediatric studies extend to adult populations. Only a limited number of studies have examined the microbiota of adults with existing allergic disease. Of these few studies conducted, reduced total count of bacteria and anaerobic bacteria were reported in adult atopic dermatitis patients compared to the healthy controls (43). Hevia et al. employed 16s rRNA gene-based sequencing to examine the gut microbiome profile of adult allergic asthma sufferers compared to healthy non-allergic individuals and identified a greater abundance of genera *Faecalibacterium* and *Bifidobacterium* in stool samples from the asthma sufferers. Similar microbial identification tools were used in the large-scale 'American Gut Project' which comprised of 1879 participants. The results of this trial revealed a decreased microbial diversity in participants with

self-reported drug, food and seasonal allergies, a decreased abundance of Clostridiales, and a higher abundance of Bacteroidales in participants with self-reported seasonal allergies (10). To date, no faecal microbiota studies employing next generation sequencing technology have been conducted in adult AR sufferers and represents a major gap in the literature.

Table 1: Studies examining gastrointestinal microbial composition in individuals with allergies in comparison to healthy controls

	Participant age	**Number of participants**	**Allergic condition**	**Sampling point(s)**	**Detection Method**	**Country of Origin**	**Outcome compared to Healthy controls**
Bjorksten et al. 1999 (44)	2 years old	Total (n=29 Estonian and 33 Swedish): 27 allergic and 36 non-allergic	Atopic dermatitis (Hanifin and Rajka) and at least one positive SPT for egg, cow's milk, cat, dog, timothy and birch pollen at 2 years of age	Single time point	Bacteriological culture	Estonia and Sweden	↓ incidence of *Lactobacilli*; ↑ coliforms counts; ↑ *Staphyloccus aureus* counts; ↓ proportion of *Bacteroides*
Bjorksten et al. 2001(25)	Infants followed up to 2 years	24 Estonian and 20 Swedish. 9 from each group developed allergies.	Atopic dermatitis (Hanifin and Rajka); recurrent wheezing (≥ 3 times) and/or positive SPT at 3, 6, 12 months and 2 years of life	Stool samples collected at 5/6 days of life and at 1,3,6 and 12 months of age	Bacteriological culture	Estonia and Sweden	↓ colonisation with *Enterococci* and *Bifidobacteria*; ↑ counts Clostridia ↑ prevalence of *Staphylococcus aureus* ↓ counts of *Bacteroides*
He et al 2001 (45)	Infants (2-7 months of age)	4 allergic 6 non-allergic	Food allergy and atopic dermatitis (Hanifin)	Single time point	Bacteriological culture-based methods – search for *Bifidobacteria*	Finland	↑ counts *Bifidobacterium adolescentis;* ↓ counts *Bifidobacterium bifidum*
Kalliomaki et al.2001 (46)	Infants followed up to 1 year	22 allergic and 54 non-allergic	High risk for developing allergies. Allergy status determined via SPT at 12 months	Stool collection prior to allergy outcome assessment. 3 weeks and 3 months of age	Bacteriological culture and Quantitative fluorescence in situ hybridisation	Finland	↑ Clostridia; ↓ bifidobacteria

	Participant age	Number of participants	Allergic condition	Sampling point(s)	Detection Method	Country of Origin	Outcome compared to Healthy controls
Watanbe et al. 2003 (9)	Allergic: 7.6 ± 5.0 years, 16 boys and 14 girls vs non-allergic: 6.5 ± 4.4 years, 31 boys and 37 girls	30 allergic and 68 non-allergic	Atopic dermatitis (Japanese Dermatological Association)	Single time point	Bacteriological culture	Japan	↓ counts bifidobacteria*;* ↓ counts bifidobacteria in severe vs mild AD; ↑ occurrence of *Staphylococcus*
Matusomoto 2004 (43)	Allergic: aged average 28.6 years and non-allergic 27.1 years	11 allergic and 14 non-allergic	Physician diagnosed severe atopic dermatitis	Single time point	Bacteriological culture	Japan	↓ total bacteria counts ↑ proportion *Enterobacteriaceae* ↑incidence of moulds
Sepp et al. 2005 (47)	5 years of age	19 allergic and 19 non-allergic	Atopic dermatitis, asthma and allergic rhinitis and positive SPT or serum IgE against at least one allergen	Single time point	Bacteriological culture	Estonia	↓ Incidence of bifidobacteria; ↑ Clostridia
Penders et al. 2006 (48)	Infants followed up to 1 year	26 allergic and 52 non-allergic	Atopic dermatitis symptoms and IgE sensitisation to cow's milk, hens egg, or peanut at one year of age.	1 month of age	16s rRNA qPCR and DGGE	The Netherlands	↔ entire bacterial profile or bifidobacteria*;* ↑ colonisation *E.coli*

	Participant age	Number of participants	Allergic condition	Sampling point(s)	Detection Method	Country of Origin	Outcome compared to Healthy controls
Alderbeth et al. 2007 (49)	Infants followed up to 18 months	Göteborg (n=116) London (n=108), Rome (n=100). 74 participants (23%) of the entire cohort developed atopic dermatitis	Atopic dermatitis (Williams test) and food specific IgE at 18 months	Stool collection prior to allergy outcome assessment: Stool collection at 7, 14, 28 days and 2, 6 and 12 months	Bacteriological culture	England, Italy, Sweden	↔ time of acquisition of any bacterial species
Songjinda et al. 2007 (50)	Infants 2 months old (followed until 2 years of age)	8 allergic and 7 non-allergic	History of atopic dermatitis, asthma and food allergy (ISAAC questionnaire) at 2 years of age	Stool collection prior to allergy outcome assessment. Stool sample collected within 5 days of life, one month, two months of age	qPCR	Japan	↑ proportion *Bacteroidaceae;* ↔ *Enterobacteriaceae*, bifidobacteria, enterococci, lactobacilli and *Clostridium perfringens*
Suzuki et al. 2007 (51)	Followed up to 6 months of age	10 allergic and 16 non-allergic	Atopic dermatitis (Hanifin & Rajka), recurrent wheezing (≥ 3 episodes) and positive SPT to at least one allergen	Stool collection prior to allergy outcome assessment. Stool collection at one, three and six months of age	qPCR	rural Japan	↑ prevalence *Bifidobacterium catenulatum* at one month of age ↑ *B. bifidum* at six months of age
Vael et al.2008	Newborns with a three-year follow-up	26 allergic and 91 non-allergic	Asthma, wheeze and atopic dermatitis symptoms (ISAAC questionnaire and the Asthma Predictive Index)	Stool sample collected before allergy assessment outcome. Sample collected at 3 weeks of age.	Bacteriological culture	Belgium	↑ *Bacteroides fragilis*, ↑ total anaerobe counts

	Participant age	Number of participants	Allergic condition	Sampling point(s)	Detection Method	Country of Origin	Outcome compared to Healthy controls
Sjogren et al. 2009 (52)	Newborns followed to five years of age	16 allergic and 31 non allergic	Clinical evaluation of asthma, atopic dermatitis, allergic rhinitis/conjunctivitis symptoms at three, six, and 12 months of age and 2 and 5 years of age	Stool collection prior to allergy outcome assessment. Samples collected at five to six days and at one month and 2 months of age	qPCR	Sweden	↓ colonisation *lactobacilli* group 1 (*L. rhamnosus, L. casei, L. paracasei*) ↓*Bifidobacterium adolescentis* ↓ *Clostridiodes difficile*
Storro et al. 2011 (53)	Newborns with a 2-year follow-up	42 allergic and 52 non-allergic	ISAAC questionnaire for allergic symptoms (Allergic rhinoconjunctivitis, asthma, atopic dermatitis). Atopic dermatitis (UKWP criteria). Diagnosis of allergic disease was confirmed by a paediatrician and dermatologist. Serum specific IgE >0.35 kU/mL	Stool sample collected before allergy assessment outcome. Samples collected at 10 days, 4 months and one and two years of age.	qPCR	Norway	↓ counts of *E. coli*, ↑ *Bifidobacterium longum*, ↓ *Bacteroides fragilis*
Waligora-Dupriet et al. 2011 (54)	Infants aged between 3 and 24 months of age	10 allergic and 20 non-allergic	Allergic symptoms and at least one positive SPT or serum specific IgE	Sample collected during clinical assessment. Single time point	Bacteriological culture, PCR and Box-PCR fingerprinting	France	↔ colonisation of aerobic and anaerobic genre, ↔ diversity of *Bifidobacterium* colonisation

	Participant age	Number of participants	Allergic condition	Sampling point(s)	Detection Method	Country of Origin	Outcome compared to Healthy controls
van Nimwegen et al. 2011 (55)	Infants followed until seven years of age	1176 newborns at ages 6-7 years. 6.9% of the cohort had parent-reported asthma, 8% had wheeze, 12.4% had atopic dermatitis, 21.6% had food allergy and 28.9% had inhalant allergies.	Asthma, wheeze and atopic dermatitis (ISAAC questionnaire), at least one positive serum IgE for hen's egg, cow's milk, peanut, birch, grass pollen, cat, dog and house dust mite	Stool collection prior to allergy outcome assessment. Stool sample collected at 1 month of age	qPCR	The Netherlands	Colonisation with *Clostridiodes difficile* was associated with wheeze and atopic dermatitis
Abrahamsson et al. 2012(56)	Followed up to 2 years of age	20 allergic and 20 non-allergic	Atopic dermatitis symptoms and at least one positive skin prick test or allergen-specific IgE	5-7 days, 1 month, 12 months of age	16S rDNA 454-pyrosequencing	Sweden	↓ microbiota diversity at one month of age ↓ Proteobacteria at 12 months of age
Candela et al. 2012 (57)	Participants aged between four and fourteen years of age	19 allergic and 12 non-allergic	Clinical diagnosis of allergy (rhinitis, asthma, grass pollen sensitisation, atopic dermatitis, oral allergy syndrome, cow's milk allergy)	Single time point	qPCR	Italy	↓ *Clostridium* cluster IV ↓ *Faecalibacterium prausnitzii*, ↓ *Akkermansia muciniphila* ↑ *Enterobacteriaceae*
Hevia et al. 2015 (8)	Allergic aged 39.43 ± 10.98 years non-allergic 39.29 ± 10.98 years	21 allergic and 22 non-allergic	Allergic asthma (Global Initiative for Asthma) and positive SPT or serum-specific IgE to at least one allergen	Single time point	Ion Torrent sequencing of 16s rRNA	Spain	↔ microbial diversity ↑ *Bifidobacterium* ↑ *Faecalibacterium*

	Participant age	Number of participants	Allergic condition	Sampling points	Detection Method	Country of Origin	Outcome compared to Healthy controls
Hua et al. 2016 (10)	1879 adults; aged 45.5 ± 15.7 years	81.5% of participants with at least one self-reported allergy	Self-reported allergies in a questionnaire (food, drug, seasonal, asthma, atopic dermatitis, pet dander)	Stool sample collected at the time of allergy questionnaire	16s rRNA V4 sequencing	United States of America	↓ reduced species richness associated with each allergy except bee sting, asthma and eczema; ↑ higher abundance of Bacteroidales in nut and seasonal pollen allergy ↓ reduced abundance of Clostridiales in nut and seasonal pollen allergy

↑ increased; ↓ decreased; ↔ no difference; mean ± standard deviation; SPT, Skin Prick Test

6.0 Gut bacteria and immune function

The human immune system functions in a complex balance between tolerance to the commensal microbiota inhabiting the gut and defending against infectious agents and opportunistic pathogens. The gut microbiota is known to play an integral role in both mucosal and systemic immunity. Indeed, extensive cross-talk exists between the gut microbiota and the hosts' innate and adaptive immune systems. Immune surveillance of the gut commensal community involves the recognition of microbial associated molecular patterns (MAMPs) such as lipopolysaccharide, flagellin, peptidoglycan via pattern-recognition receptors (PPRS) located on immune cells of the gut, including nucleotide binding oligomerization domain proteins (NODs), Toll-like receptors (TLRs), and retinoic acid-inducible gene-1-like receptors (RLRs) (19).

The postnatal development of the mucosal immune system is dependent on immune stimulation with colonising microbes. A major component of the gut mucosal immunity is the gut associated lymphoid tissue (GALT) which is organised in specialised immune compartments including Peyer's patches, mesenteric lymph nodes and isolated lymphoid follicles. Lymphocytes are also distributed throughout the epithelium and lamina propria. In germ-free or antibiotic treated mice, Peyer's patch structures and mesenteric lymph nodes are reduced in number or underdeveloped (20, 58-60). In addition to regulating the development of lymphoid structures, the composition of the gut microbiota is involved in inducing oral tolerance. Oral tolerance to ingested antigenic proteins occurs via induction of antigen-specific regulatory T cells that supress immune activation upon repeated exposure with the same antigenic protein (61). Evidence suggests that a full gut microbiota is necessary for induction of oral tolerance. Indeed, germ-free mice as well as mice monocolonised with either *E. coli* or *L. plantarum*, were significantly less capable of producing tolerogenic serum factor after an antigen feed. In contrast, the conventionally colonised mice could induce oral tolerance (61).

The activity of the gut microbiota has been shown to affect immune responses at distant sites, including the respiratory tract. Increased numbers of eosinophils and lymphocytes were reported in the airway cellular infiltrate of germ-free ovalbumin sensitised/challenged mice compared to colonised mice. Remarkably, the airway inflammation was reversed by recolonisation of the germ-free mice with the same bacteria from the colonised mice (62). Accumulation of invariant natural killer (iNKT) cells has been observed in the lamina propria and lungs of germ-free mice. iNKT cells secrete abundant amounts of proinflammatory cytokines such as IL4 and IL13 upon activation, all of which leads to increased morbidity in asthma sufferers (63). Interestingly, recolonisation of the germ-free mice with a conventional microbiota, protected the mice from mucosal iNKT accumulation and related pathology (63). Similar findings of weakened immune function in germ-free mice have also been observed in antibiotic-treated mice. Russell et al. identified increased airway responsiveness, increased total

inflammatory infiltrates and eosinophils in bronchiolar lavage fluid and higher serum antigen specific IgE in the antibiotic (vancomycin) treated mice, compared to the control animals (64). Effects of the vancomycin treatment were also observed in the gut, with a lower overall diversity of gut microbes and reduced numbers of Treg associated markers CD4+ CD25+ and FoxP3+ (64). These studies provide evidence that alterations to the gut flora can affect immune responses at distant sites. The study of Chua et al. identified that colonisation of the gut with specific gut microbes is also an important factor in the pathogenesis of allergic respiratory disease (65). A higher incidence of *Ruminococcus gnavus* was identified in stool samples from infants who later developed respiratory allergies. In a follow-up experimental asthmatic mouse study, ovalbumin sensitised/challenged mice were infected with *R. gnavus* via oral gavage intragastric administration. The *R. gnavus* infected mice showed a greater secretion of IL-25, IL33 and Thymic Stromal Lymphopoietin by colonic tissues, thereby promoting Th2 differentiation and further cytokine release and an enhanced infiltration of eosinophils and mast cells to the colon and lung parenchyma. In addition, the *R. gnavus* infected mice displayed increased airway-hyperresponsiveness and histologic airway inflammation (65). The gut microbiota may modify allergic disease systemically and at other mucosal sites through the common mucosal immune system. In the context of allergic disease, the common mucosal theory suggests that the gut and respiratory mucosa function as a single immune organ, sharing functions of immune surveillance and regulation of host responses. Antigen presentation at one mucosal site can stimulate migration of lymphoid cells to other mucosal sites thereby affecting immune responses at distant sites (22, 66). Indeed, T cells, involved in respiratory inflammatory responses, are capable of migrating from one mucosal site to another (67). Interestingly, biopsies of the small intestine taken from patients with asthma and AR were shown to have an accumulation of T cells, eosinophils, mast cells, macrophages and an increased expression of Th2 cytokines IL4 and IL5, an immune phenotype similar to that of sensitised respiratory airways (68).

7.0 Probiotics: description and general uses

Beneficial microbes have long been utilised by mankind. In 1907, Russian immunologist Élie Metschnikow reported findings on probiotic studies in his book ‘The Prolongation of Life’. Since then, the World Health Organisation (WHO) officially defined probiotics as ‘live microorganisms that, when administered in adequate amounts, confer a health benefit to the host’ (69, 70). On this basis, microorganisms must satisfy several conditions to be considered a probiotic (21, 71, 72) including:

- Microbial organisms identified at a genus, species and strain level
- Yield functional or clinical benefit when consumed by the host (clinically documented by at least 1 phase 2 study)

- Survive transit through the gastric system (acid and bile tolerant) or able to be developed into enteric coated capsules
- Be compatible with product medium and remain viable and stable throughout the processing and storage procedures
- Safe for nutrition and clinical use (low risk for side effects, be non-pathogenic, non-toxic, does not carry antibiotic resistance genes and susceptible to antibiotics)
- Able to temporarily colonise the gut and adhere to mucosal surfaces

Over the last few decades, probiotics have been increasingly recognised as an alternative therapeutic strategy to treat a range of infectious and immune conditions owing to their wide availability, reasonable price and good safety profile. Supplementation with probiotics has yielded positive clinical outcomes in disorders such as bacterial vaginosis (73), intestinal related diseases/infections (74-76), urinary tract infections (77), migraine (78), ulcerative colitis (79) Type 2 diabetes (80) and respiratory infections (81, 82). Strains from the genera *Lactobacillus* and *Bifidobacterium* are the most commonly used species in probiotic supplements and dietary foods (71). These genera are typically found in the human gut and their intrinsic features allow them to thrive in the gut and prevail over pathogenic microorganisms (83).

8.0 Probiotics in treatment of allergic diseases

The rationale for the use of probiotics to treat allergic disease stems from the 'microbiota hypothesis' and the reduced abundance of beneficial bacteria from the genera *Lactobacillus* and *Bifidobacterium* in stool samples of allergy sufferers compared to controls, as shown in some studies (11, 84). Since the late 1990's, at least 70 randomised placebo-controlled trials have been conducted to examine the effect on probiotic supplementation on the prevention and treatment of allergic disease, including atopic dermatitis, asthma and AR.

A meta-analysis of 16 randomised-controlled trials (RCTs) examining probiotic supplementation in gestation and early infancy (up to six months of life) reported that probiotic supplementation reduced the risk of developing atopic dermatitis (AD) in infants by 26% (18%-33%, 95% CI). These findings were confirmed by another meta-analysis of 26 RCTs whereby probiotic supplementation significantly reduced the risk of AD occurrence (0.64, 0.56 – 0.74, OR, 95% CI) (85). Meta-analyses conducted on probiotic supplementation and the reduction of AD symptoms in established disease has also been conducted. Kim et al. analysed 26 RCTs on probiotic supplementation and improvement in AD measured via the validated Scoring of Atopic Dermatitis (SCORAD) which incorporates intensity and

coverage of skin lesions and subjective symptoms on a visual analogue scale. The authors reported a significant reduction in SCORAD scores following probiotic supplementation in adults (mean and 95% CI, -5.74, -7.27, -4.20) and children aged 1-18 years (-8.26, -13.28, -3.25) with AD. No effect of probiotic supplementation on SCORAD scores was observed in infants (<1 year) (86).

The effects of probiotic supplementation on the prevention of asthma diagnosis or reduction in asthmatic symptoms are limited. A recent meta-analysis of 11 studies identified that probiotic supplementation was associated with fewer asthma episodes (1.3, 1.06 – 1.59; RR, 95% CI). However, no effect of probiotic supplementation on asthma symptoms, number of symptom free days and forced expiratory volume and peak expiratory flow was found (87) Azad et al. reported similar findings, in a subgroup analysis of nine RCTs, the pooled data showed no association between probiotic supplementation and doctor diagnosed asthma or childhood wheeze (88).

Meta-analyses identifying associations between AR symptoms and probiotic supplementation are limited by heterogeneity of outcome measures. Zajec et al, identified 23 studies on probiotic supplementation in AR sufferers, however, only four studies could be included in each of the meta-analyses. Pooled data from four studies (n=335 probiotic group and n=287 placebo group) with the Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ) as the outcome measure, showed a significant improvement in RQLQ scores in the probiotic group compared to the placebo group (standard mean difference -2.23, 95% CI, -4.07 to -0.40) (89). In contrast, analysis of pooled data from four studies (n=270 probiotic group and n=263 placebo group) showed no effect of probiotic supplementation on Rhinitis Total Symptom Score (RTSS). There is currently insufficient evidence to support any effect of probiotic supplementation on AR symptoms, with studies reporting mixed results across the board. Guidelines regarding the efficacious use of probiotic supplementation in allergic diseases, particularly AR, are difficult to establish due to the large degree of variation across studies in key factors such as geographic location, trial design, dosage and route of administration. In addition, the immunomodulatory effects of probiotics are known to be species and strain specific (90-96) further limiting assessment of meaningful clinical outcomes.

9.0 Mechanisms of action of probiotics in allergic disease

The mechanisms of action of probiotics in alleviating symptoms of allergic disease including AR is poorly defined. Reports from *in vitro*, animal and human studies have indicated that probiotics may exert action through several postulated mechanisms at both a local and systemic level. A description of these mechanisms and supporting evidence are detailed in Table 2 on the next page.

Table 2: A non-exhaustive summary of the proposed mechanisms of probiotics in allergic disease, with examples

Proposed mechanism	**Supporting evidence**			**Example reference**
	Experimental Model	**Probiotic strain(s)**	**Outcomes**	
Local effects and mucosal barrier *Improved integrity of the gut barrier (reduced permeability) reduces systemic antigen load and associated inflammation.*	Clinical trial	*Lactobacillus rhamnosus* 19070-2 and *L. reuteri* DSM 12246	Probiotic supplementation reduced intestinal permeability measured via the lactulose-mannitol test in children with AD	(97)
	Murine	*VSL#3: Bifidobacterium longum, B. infantis, B. breve, L. acidophilus, L. casei, L. delbrueckii subsp bulgaricus, L. planetarium* and *Streptococcus thermophilus*	Excised tissue from mice following administration of probiotics showed improved epithelial barrier function	(98)
	Murine	*Lactobacillus rhamnosus* GG	In mice with defective barrier function, probiotic administration increased expression of tight junction protein claudin-3 and improved intestinal paracellular permeability.	(99)

Proposed mechanism	Supporting evidence			Reference
	Experimental Model	**Probiotic strain(s)**	**Outcomes**	
Th1/Th2 balance, induction of cytokines *A polarization towards a Th2 phenotype is recognised in allergic disorders. Probiotics stimulate the production of Th1 cells to restore immune homeostasis*	Murine	*Lactobacillus reuteri* *L. brevis*	Increased expression of pro-inflammatory /Th1 cytokines TNF-α, IL-2 and IL-β were observed in sections of the small intestine following oral-administration of probiotics.	(95)
	Murine	*Bifidobacterium longum*	Administration of immunostimulatory DNA sequence from *Bifidobacterium longum* prevented antigen-induced Th2 immune responses	(100)
	Murine model – food allergy	*Lactobacillus acidophilus* AD031, *Bifidobacterium lactis* AD011, *L. acidophilus* AD031 *B. lactis* AD011	In ovalbumin sensitised mice, administration of probiotics reduced IL-4 levels and increased INF-γ and IL-10 compared to non-treated mice	(101)
	Clinical trial	*L. gasseri* TMC0356	Oral-administration of a probiotic supplement daily for four weeks in a cohort of perennial AR sufferers (n=15) increased the proportion of Th1 cells in PBMC's	(102)

Proposed mechanism	Supporting evidence			Reference
	Experimental Model	**Probiotic strain(s)**	**Outcomes**	
Toll-like receptor stimulation *Recognition of bacteria is mediated by pattern recognition systems such as TLRs. TLR's are located on the surface of DC appendices, and upon ligation with bacterial components of the probiotic, signal the maturation of DC's.*	Cell culture	*Lactobacillus plantarum CCFM634, L. plantarum CCFM734, L. fermentum CCFM381, L. acidophilus CCFM137* and *Streptococcus thermophilus CCFM218*	Probiotic bacteria stimulated TL2/TLR6. Stimulation of TL2/TLR6 was confirmed with blocking antibodies.	(103)
	Murine	*Lactobacillus reuteri*	Administration of probiotics to ovalbumin-sensitised mice lessened the asthmatic response (eosinophil infiltration, local cytokine production, airway hyper-responsiveness) following allergen-challenge. These effects were not replicated in TLR9 deficient mice	(104)
	Cell culture	*Bifidobacterium breve*	Probiotics induced maturation and prolonged survival of dendritic cells. These effects occurred via probiotic induced TLR2 activation.	(105)

Proposed mechanism	Supporting evidence			Reference
	Experimental Model	**Probiotic strain(s)**	**Outcomes**	
Dendritic cell activity *Probiotics enhance activity of DC's. DC's are vital for bacterial recognition, shaping T-cell responses (conversion of naïve T cells to T-helper cells) and inducing tolerance*	Cell culture	*VSL#3: Bifidobacterium longum, B. infantis, B. breve, L. acidophilus, L. casei, L. delbrueckii subsp bulgaricus, L. planetarum* and *Streptococcus thermophilus*	IL-10 production was upregulated by dendritic cells in a co-culture of human blood and lamina propria immune cells with probiotic cell wall components	(106)
	Cell culture	*Streptococcus pyogenes*	DC's stimulated with a probiotic strain displayed an increased expression of costimulatory molecules CD80, CD83 and CD86 and increased production of Th1 cytokines and chemokines	(107)
	Cell culture	*Lactobacillus reuteri* and *L. casei*	Probiotic strains prime monocyte derived DC's to initiate development of Treg cells via ligation of C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). Blocking antibodies to DC-SIGN prevented the induction of Tregs by probiotics.	(92)
	Cell culture	*Lactobacillus gasseri, L. johnsonii and L. reuteri*	Lactobacillus exposed myloid DC's upregulated costimulatory molecules HLA-DR, CD40, CD80 and CD86 and stimulated T helper cells towards a Th1 phenotype	(108)

Proposed mechanism	Supporting evidence			Reference
	Experimental Model	**Probiotic strain(s)**	**Outcomes**	
T-regulatory cell activity *Probiotics induce production and activation of T regulatory cells. Tregs are a specialised subpopulation of T cells that suppress the immune response thereby maintaining immune homestasis and immune tolerance against non-self antigens*	Murine	heat-killed *Lactobacillus acidophilus* L-92	Oral administration of probiotics induced TGF- β production in Peyer's Patches. TGF-β is associated with activation of regulatory T cells (Tregs)	(109)
	Murine	*Lactobacillus rhamnosus* GG	Oral-administration of probiotics in sensitised mice following allergen challenge suppressed characteristic asthmatic responses (airway reactivity, pulmonary eosinophil influx, IgE production) and was associated with an increase in TGF-β producing CD4+/CD3+ T cells in the mesenteric lymph nodes and an upregulation of Foxp3-expressing cells (Treg marker) in the peribronchial lymph nodes. Overall probiotic supplementation reduced makers of allergic airway disease via the induction of Tregs.	(110)
	Murine	*Escherichia coli Nissle* 1917	Oral administration of probiotics improved allergen induced dermatitis and was associated with increased number of FoxP3 (+) cells in skin	(111)

	Supporting evidence			Reference
	Experimental Model	**Probiotic strain(s)**	**Outcomes**	
	Murine	*Lactobacillus reuteri*	Sensitised mice administered probiotics by gavage had higher total numbers of spleen CD4(+)CD25(+)Foxp3(+)T cells compared to the vehicle control. The CD4(+)CD25(+) cells isolated from probiotic administered mice had greater capacity to suppress T-effector cell proliferation.	(112)

10.0 Conclusion

The gut microbiome plays an integral role in the regulation of immune homeostasis pertinent to the development of allergic disease. Unique gut microbial composition patterns have been associated with the onset of allergic disease in infants, however there is insufficient evidence to determine if these composition patterns persist in atopic adults. Further, heterogeneity in study design features, including allergic disease classification, age of participants, and microbiome identification tools used, complicate our understanding of the microbiome profile of allergic individuals. Microbiota studies employing next generation sequencing technology in well-defined cohorts of adult AR sufferers are needed to better define the microbiome profile of adult AR sufferers. Probiotic supplements have been proposed as a potential treatment option for AR sufferers. The rationale for probiotic use in allergic disease, stems from the aberrant composition patterns observed in atopic individuals and the known immunomodulatory capacity of commensal bacteria. To date, many studies have sought to examine the effect of probiotic supplementation on AR symptoms. However, the results of these studies are mixed and a conclusion on their efficacy in allergic disease cannot be reached. Further, the beneficial effects of probiotics are known to be strain-specific, and differences in trial design, dosage, route of administration and outcome measures make determining their efficacy difficult. As such, additional well-controlled studies with clinically relevant outcomes are needed to better elucidate the effectiveness of probiotics in the management of AR.

SUMMARY OF THE LITERATURE

AR is a chronic upper respiratory disease and is driven by an IgE mediated reaction in the nasal mucosa in response to allergen exposure. The nasal mucosa of AR sufferers is typically characterised by an influx of innate immune cells such as eosinophils, neutrophils and mast cells, and increased production of chemokines, cytokines and other mediators, which are responsible for the perpetuation of inflammation and manifestation of symptoms. The four prime symptoms of AR are rhinorrhoea, nasal congestions, sneezing and watery/itchy eyes. Other symptoms experienced by some sufferers include post-nasal drip, itchy throat and sinus pain. House dust mites, animal dander and mould spores are responsible for persistent/perennial presentation of symptoms where as intermittent/seasonal symptoms are typically initiated by exposure to plant pollen. Complete avoidance of allergens is not possible and without a cure the current therapeutic options are typically focused on achieving symptomatic relief.

The gut microbiome, unofficially described as an 'immune compartment', plays an integral role in the development and regulation of the immune system and induction of immune tolerance. In the context of allergy, insufficient microbial stimulus during infancy is hypothesised to drive polarisation towards a Th2 phenotype, resulting in persistent atopy. Indeed, multiple studies have observed altered gut microbial composition in paediatric atopic cohorts compared with non-atopic controls. Modification of the microbiome with probiotic supplements has been evaluated as a strategy to modify the disease process and reduce allergic symptoms. To date, many studies have examined the effect of probiotic supplementation on AR symptoms. However, the results of these studies have yielded mixed findings.

Antihistamines and corticosteroids are the most commonly used medications to manage AR symptoms and have been developed in intranasal formulations. The primary mechanism of action of antihistamines is to alter the activity of the histamine receptor towards an inactive state. Topical antihistamines are therefore effective at reducing histamine-related symptoms such as sneezing, itch and rhinorrhoea. However, they have limited activity on nasal congestion. Intranasal corticosteroids subdue many components of the allergic response. Corticosteroids exert their anti-inflammatory action by modifying multiple signal transduction pathways via transactivation and transrepression. These actions result in the downregulation of many inflammatory cytokines, chemokines and mediators pertinent to the allergic response. A combination of an antihistamine and corticosteroid in a single spray has been shown to be more effective at reducing symptoms of AR than either monotherapy alone. Each active ingredient in the combination spray has a different mechanism of action and therefore there is potential for synergistic effects that may contribute to additional symptomatic relief.

GAPS IN THE CURRENT KNOWLEDGE IDENTIFIED BY REVIEW OF THE LITERATURE

1) Precision medicine is a novel therapeutic approach which seeks to address disease heterogeneity and variability in response to standard treatment. Biomarkers are an integral component of endotype-driven precision medicine. In AR, the development of biomarkers to evaluate treatment responses are hindered by the complexity of the disease and absence of broad analytical methods that can accurately assess the nasal mucosa in AR sufferers via non-invasive means.

2) There is conflicting evidence on the mechanism of action of specific antihistamines. AZE treatment *in vitro* reduced TNF-α in human monocyte cultures, whereas no effect on TNF-α production was observed in nasal lavage samples from AR participants following treatment with AZE. In addition, AZE treatment had variable effects on histamine and tryptase levels measured in nasal lavage fluid across clinical studies. Additional studies are needed to clarify the effects of AZE treatment on these biomarkers of allergic inflammation.

3) The combination of AZE and FP in a single spray has been shown to be significantly more effective at reducing nasal symptom scores compared to treatment either monotherapy. However, the biological mechanisms underpinning the superior clinical effects observed are unknown. Studies employing a broad analytical approach are needed to identify possible synergistic targets of the combination therapy.

4) Unique gut microbial composition patterns have reported in paediatric cohorts, however there is insufficient evidence to determine if aberrant composition patterns also persist in atopic adults. Microbiota studies utilising next generation sequencing technology are needed to better delineate the microbiome profile of adult AR sufferers.

5) While many studies have examined the effect of probiotic supplements on AR symptoms, the results are mixed and a conclusion on their efficacy in AR cannot be reached. The beneficial effects of probiotics are known to be strain-specific and design heterogeneity between studies makes determining their efficacy difficult. Additional studies are needed to provide further information on the utility of probiotic supplements in the treatment of AR symptoms. In addition, the results of one probiotic formulation cannot be extrapolated to another, and as such, each formula should be independently examined for clinical efficacy.

THE SPECIFIC AIMS OF THIS THESIS ARE TO:

1) Examine the gut microbial composition and diversity in adults with AR and controls without AR.

2) Determine if supplementation with a multi-species probiotic supplement for eight weeks provides clinical benefit in intermittent/seasonal AR sufferers.

3) Develop a novel method to examine gene expression profiles in the nasal mucosa via non-invasive sampling.

4) To compare gene expression profiles in the nasal mucosa to peripheral blood in AR sufferers using the newly developed gene expression protocol.

5) Examine gene expression profiles in nasal mucosal samples and peripheral blood in AR sufferers compared with controls without AR.

6) Determine the effect of a topical antihistamine, topical steroid, and combination treatment on gene expression profiles in the nasal mucosa and peripheral blood.

CHAPTER TWO

Study one: The gut microbiome of adults with allergic rhinitis is characterised by reduced diversity and an altered abundance of key microbial taxa compared to controls

THE GUT MICROBIOME OF ADULTS WITH ALLERGIC RHINITIS IS CHARACTERISED BY REDUCED DIVERSITY AND AN ALTERED ABUNDANCE OF KEY MICROBIAL TAXA COMPARED TO CONTROLS

This Chapter is formatted in a style suitable for publication in Allergology International. *Supplementary material for the Chapter is provided in Appendix 1*.

My contribution to the paper involved:

Recruitment of study participants and sample collection, DNA extraction experiments, statistical analysis of the 16s rRNA abundance data and participant clinical and demographic measures, preparation of figures and tables, and primary writer of manuscript.

_____7th May 2019

Annabelle Monica Watts

_____7th May 2019

Supervisor: Professor Allan Cripps

The gut microbiome of adults with allergic rhinitis is characterised by reduced diversity and an altered abundance of key microbial taxa compared to controls

Annabelle M Watts[1,2], Nicholas P West[1,2], Ping Zhang[1], Pete K Smith [3,4], Allan W Cripps[1,3], and Amanda J Cox[1,2]

[1] Menzies Health Institute of Queensland, Griffith University, Southport, QLD, Australia

[2] School of Medical Science, Griffith University, Southport, QLD, Australia

[3] School of Medicine, Griffith University, Southport, QLD, Australia

[4] Queensland Allergy Services Clinic Southport, Australia

Email addresses:

a.watts@griffith.edu.au

n.west@griffith.edu.au

p.zhang@griffith.edu.au

pksn@mac.com

allan.cripps@griffith.edu.au

a.cox@griffith.edu.au

Corresponding Author:

Dr Amanda Cox

Email: a.cox@griffith.edu.au

Phone: +61 (07) 56780898
Menzies Health Institute Queensland
Griffith University
Parklands Drive
Southport, QLD, 4215
Australia

Author contributions:

Design: AMW, NPW, AJC; Experiments: AMW, AJC; Data analysis: AMW, PZ; Drafting of Manuscript: AMW; Manuscript revision: AMW, AJC, AWC, NPW, PZ, PKS. All authors approved the final version of the manuscript.

Conflict of Interest

The authors declare no commercial or financial conflict of interest

Abstract

Background: Unique gut microbial colonisation patterns are associated with the onset of allergic disease in infants, however there is insufficient evidence to determine if aberrant microbial composition patterns also occur in adult allergic rhinitis (AR) sufferers.

Methods: This study compared the gut microbial composition in stool samples between 57 adult AR sufferers (39.06 ± 13.29 years) and 23 controls (CG; 36.55 ± 10.51 years) via next-generation sequencing of the V3-V4 hypervariable regions of the 16S rRNA gene.

Results: Species richness determined via the Shannon index was significantly reduced in the AR cohort compared to the CG (4.35 ± 0.59 in AR vs 4.65 ± 0.55 in CG, p=0.037). Trends for reduced species richness in the AR group were also observed with the observed OTU counts, inverse Simpson, and CHAO1 diversity indices. The phyla Bacteroidetes (p=0.021) and Actinobacteria (p=0.032) were significantly more abundant in the AR group compared to the CG. The Firmicutes phylum was significantly less abundant in the AR group compared to the CG (p=0.005). An increased abundance of *Parabacteroides* (p=0.003) and *Bifidobacterium* (p=0.017) and a reduced abundance of *Oxalobacter* (p=0.023) and Clostridiales (p=0.008) was also observed in the AR cohort compared to the CG.

Conclusion: Adult AR sufferers have a distinct gut microbiome profile, marked by a reduced microbial diversity and altered abundance of certain microbes compared to controls. The results of this study provide evidence that unique gut microbial patterns occur in AR sufferers in adulthood and warrants further examination in the form of mechanistic studies.

Keywords

microbiome, allergic rhinitis, 16s rRNA gene, species richness, gastrointestinal

Abbreviations

Allergic rhinitis, AR; controls without allergic rhinitis, CG.

Introduction

The gastrointestinal microbiota plays an important role in the development and regulation of local and systemic immunity. Indeed, several immune-mediated conditions (1-7) including allergic disease (8-11), have been associated with abnormal gut microbiome composition. AR is the most prevalent allergic disease and is characterised by a T-helper 2 polarised response which promotes IgE mediated inflammation in the nasal mucosa following allergen exposure. Polarisation of the Th2 phenotype develops in utero and persists in neonates at birth (12, 13). Immune homeostasis and maturation towards a Th1 phenotype is dependent on colonisation of the gut with commensal microbes (14). Data generated from germ-free mouse models has shown that commensal gut microbes regulate Th2 responses (15), with recent evidence suggesting this occurs via induction of enteric Th17 and T regulatory cells (16).

The relationship between the gut microbial composition and the onset of allergic disease has been extensively studied in paediatric populations and has been reviewed elsewhere (11). A consistent finding in these paediatric studies is that lower microbial diversity occurs in the intestine of allergic infants compared to non-allergic infants. While differences in microbial composition between allergic and non-allergic infants have been reported, a consensus of an 'allergic microbiome profile' cannot be reached. Heterogeneity in study design features, including allergic disease classification, age of subjects, and microbiome identification tools used, complicate our understanding of the microbiome profile of allergic infants. Furthermore, the microbiome profile of allergic infants has been shown to differ by time of sample collection, where samples collected

before the onset of allergic disease differs from samples collected during active disease. This data suggests that there is a time or age-related effect on the development of gut microbiome, relative to atopy.

The gut microbiome is known to undergo rapid increases in bacterial diversity up to approximately three years of age (17). As such, it remains unclear if findings from paediatric studies extend to adult populations. Only a limited number of studies have examined the microbiota of adults with existing allergic disease. Of these few studies conducted, reduced total count of bacteria and anaerobic bacteria were reported in adult atopic dermatitis patients (n=11) compared to the healthy controls (n=14) (18). This study employed bacterial-culture techniques to count and identify specific bacterial taxa and its drawbacks including exclusion of 'nonculturable' bacteria, are well-recognised. 16s rRNA gene-based sequencing has been recently used to circumvent these restrictions, whereby a greater abundance of genera *Faecalibacterium* and *Bifidobacterium* are reported to exist in the faecal microbiota of adult allergic asthma sufferers (n=21) compared to heathy individuals (n=22) (8). Similar microbial identification tools were used in the large-scale 'American Gut Project' which comprised of 1879 participants. The results of this trial revealed a decreased microbial diversity in participants with self-reported drug, food and seasonal allergies, a decreased abundance of Clostridiales, and a higher abundance of Bacteroidales in participants with self-reported seasonal allergies (10). Given that allergy subtypes are mediated by different inflammatory pathways (19), and perhaps relate to different gut microbial profiles, there may be merit in classifying subjects by allergy phenotypes rather than self-reported allergen sensitivity.

To the author's knowledge, no faecal microbiota studies employing next generation sequencing technology have been conducted in adult AR sufferers. This study investigated this gap in knowledge by comparing the gut microbial composition between adults with well-defined AR and in controls without AR.

Materials and methods

Study design

This study was designed as a cross-sectional study to characterise differences in the gut microbiota between adults with established AR (n=57) and adults with no history of AR (n=23; control group). All participants attended appointments at the Queensland Allergy Services Clinic in Southport (Gold Coast, Australia) and the Clinical Trial Unit at Griffith University (Gold Coast, Australia) for allergy testing and collection of blood and stool samples. This study was approved by the Griffith University Human Research Ethics Committee (approval #s: 2015/564/HREC; 2016/279).

Participant selection

Both men and women, aged 18 to 65 years were recruited to the study. Adults with established AR included both seasonal and perennial AR sufferers with a greater than two-year history of AR symptoms and a positive allergic response to dust mites or grass pollens. Allergy status was confirmed with a skin prick test against a panel of dust mite and plant pollen allergens as described previously (20, 21). Symptom severity was determined using the validated mini Rhinoconjuctivitis Quality of Life Questionnaire (mRQLQ) consisting of 14 questions separated into five domains: activities, practical problems, nose symptoms, eye symptoms and other symptoms (22). All items on the questionnaire were rated on a 7-point likert scale (0-6) with each item averaged to give a maximum overall score of six.

AR subjects were excluded from participating if they suffered from non-allergic rhinitis (vasomotor rhinitis), consumed probiotics in the previous 8-12 weeks, were treated with oral corticosteroids within the previous six months or antibiotics within the previous 30 days, used anti-inflammatory or immune-modulating medications, had existing respiratory disease including asthma, nasal polyposis or chronic-obstructive pulmonary disorder, had existing immune dysfunction (other than allergies) or gastrointestinal tract diseases or disorders, were ill or had infectious disease at the time of enrolment or were pregnant at the time of enrolment.

Individuals were recruited to the study as controls (CG) if they reported no history of allergic rhinitis, tested negative to the panel of dust mite and plant pollens and were free from chronic disease. Participants were excluded from the study if they consumed probiotics in the previous 8-12 weeks, had taken antibiotics within the previous 30 days, used anti-inflammatory or immune-modulating medications, had existing respiratory disease, immune dysfunction or gastrointestinal disease or disorder, were ill or had an infectious disease at the time of enrolment or were pregnant at the time of enrolment.

Blood sample collection

Venous blood samples were collected for analysis of full blood count including white cell differential (QML Pathology, Murarrie, Queensland, Australia). In addition, erythrocyte sedimentation rate (ESR) over one hour was measured using fresh blood samples collected in sodium citrate tubes and using commercially available Vacuette ESR pipettes (Greiner Bio-One, Kremsmünster, Austria) as per the Westergren method (23).

Stool sample collection

Subjects were provided with a sample collection kit and instructed to collect a stool sample within 24 hours prior to their scheduled study visit. Collection instructions included not contaminate the sample with urine or water and to store the sample at room temperature until their study visit. Stool samples were frozen at -80 °C upon receipt until processing.

Faecal microbial composition

DNA was extracted from defrosted stool samples using the method described by Yu *et al.* (2004) which included homogenisation, a combination of chemical and mechanical lysis (using silica/zirconia beads; Daintree Scientific, Tasmania, Australia), salt/alcohol precipitation and purification using a Qiagen DNAeasy kit (Qiagen, Hilden, Germany). The quality and quantity of DNA was assessed with the NanoDrop 1000 UV-Vis spectrophotometer (ThermoScientific, Massachusettes, United States).

Isolated DNA was amplified using universal primers for the V3-V4 region of the microbial 16s rRNA marker gene (F:5'-CCTACGGGNGGCWGCAG-3'; R:5'-GACTACHVGGGTATCTAATCC-3'), as described previously (24) and PCR products sequenced on an Illumina MiSeq system (Illumina, California, USA) by a commercial provider (Macrogen, Seoul, Korea). Sequence data were processed with CD-HIT-OUT (25) to filter out erroneous and chimeric reads. Taxonomic classification and identity assignment was performed using a reference-based approach with the NCBI database of 16S rRNA gene sequences.

Statistics

Differences in demographic and clinical measures between groups was assessed with an independent t-test for continuous variables and a Chi-squared test for categorical variables. A one-way analysis of variance (ANOVA) or an independent t-test was used to perform the differential α-diversity and, abundance analyses from the phylum to species levels. Welch's t-test /unequal variances t-test was used for heteroscedastic data. The analysis was confined to taxa with a relative abundance (detected) >0% and prevalence of >50% in either group (AR or CG). Differences in detection rate of taxa (i.e. detected, >0%; or not detected, 0%; in a given sample) between groups was identified with a Chi-square test. Statistical significance was accepted at $p < 0.05$. Partial least squares-discriminant analysis (PLS-DA) implemented in mixOmics R package (26) was employed for the multivariate analysis. Taxa with a relative abundance of >0% and prevalence <50% in both groups (AR and CG) where excluded from the PLS-DA.

Results

The demographic and clinical characteristics of the cohorts are included in Table 1. The groups were matched in key physical attributes. However consistent with diagnosis of atopic conditions, the AR group had significantly higher white blood cell, lymphocyte and eosinophil counts compared to the control group (CG). The AR group had mild-moderate symptoms based on mRQLQ scores (2.84 ± 1.23 [out of a maximum possible score of 6]) with the majority sensitised to both plant pollens and dust mites. Several participants also reported allergies other than AR; a total of 44% of the cohort reported a history of skin allergies (eczema, hand dermatitis, urticaria and itchy rash), 28% also reported a history of food allergy, and 12% also reported a history of drug allergy (including Codeine (opioid), acetylsalicylic acid (nonsteroidal anti-inflammatory drug), antibiotics and metoclopramide (dopamine D_2 receptor antagonist/5-HT_3 receptor antagonist/5-HT_4 receptor agonist).

Microbial Diversity

A trend for greater microbial diversity (~7%; p=0.129) was noted among the CG. Higher OTU count, CHAO1 and Inverse Simpson α-diversity measures were observed in the CG compared to the AR group (Figure 1). The Shannon Diversity index, which considers both species richness and evenness, was significantly higher (p=0.037) in the controls (CG) compared to the AR group (Figure 1).

Microbial Composition / Taxonomic Classification

Phyla

Taxonomic classification at the phylum level revealed that Firmicutes, Bacteroidetes, Verrucomicrobia and Proteobacteria were the dominant bacterial phyla in both groups (Figure 2). The Bacteroidetes (36.33 ± 12.14% in AR vs 29.79 ± 8.29% in CG, p=0.021) and Actinobacteria phyla (1.03 ± 1.91% vs 0.44% ± 0.45%, p=0.032) were significantly more abundant in the AR group compared to the CG. In contrast, the Firmicutes phylum was significantly less abundant in the AR group compared to the CG (56.37% ± 13.13% vs 64.89% ± 8.34%, p=0.005). Further, there was a significant difference in the Firmicutes to Bacteroidetes ratio between the AR and CG (1.85 ± 1.16 vs 2.40 ± 0.90, p=0.047).

Order

Within the order taxonomic rank, the OTUs obtained were assigned to 26 unique orders; of these 12 orders were considered prevalent (detected in >50% of samples from either group) and 14 were less prevalent detected in <50% of samples), supplementary data Table 1. The most abundant order present in the both AR and CG cohorts were Clostridiales (53.14%), Bacteroidales (34.13%), Negativicutes unclassified (4.32%) and Verrucomicrobiales (3.76%). The orders Bifidobacteriales and Bacteroidales were significantly more abundant in the AR cohort compared to the CG cohort. In contrast, the Clostridiales were significantly less abundant in the AR cohort compared to the CG cohort (Table 2).

Genus

At the genus level, 112 unique genera were identified; 57 of these genera were considered prevalent and 55 were less-prevalent (supplementary data Table 2). The most abundant genera in both the AR and CG were *Bacteroides* (21.55%), *Faecalibacterium* (20.03%), *Lachnospiraceae* unclassified genera (6.25%), *Alistipes* (4.15%), *Akkermansia* (3.77%), *Prevotella* (3.71%) and *Oscillibacter* (3.51%). The genera *Bifidobacterium*, *Parabacteroides* and *Bacteroides* were significantly more abundant in the AR group compared to the CG (Table 2). In contrast, *Oxalobacter* and *Coriobacteriaceae*, unclassified were significantly less abundant in the AR group (Table 2).

Among the less-prevalent genera, some differences in rates of detection between AR and CG were noted (Table 3). For example, the *Acidiminococcus* genera was detected significantly more frequently in the AR group compared to the CG (~25% of AR samples vs ~4% of non-AR samples). In contrast, the genre *Rothia* and *Coriobacteriaceae* unclassified were detected significantly more often in the CG compared to the AR group.

Species

At the species level, 290 unique species were identified – 122 of these species were considered prevalent and 168 were less-prevalent. A number of species were significantly more abundant in AR group compared to the CG including *Parabacteroides distasonis*, *Bacteroides vulgatus* and *Anaerotruncus colihominis* (Table 2). In contrast, *Eubacterium xylanophilum*, *Murimonas intestini*, *Oscillibacter valericigenes*, *Agathobaculum*

butyriciproducens and *Oxalobacter formigenes* were less abundant in the AR group compared to the CG (Table 2).

Differences in the detection rate of less-prevalent species (<50% of either cohort) were observed between the AR and CG cohort (Table 3). *Clostridium hylemonase*, *Ruminococcus gnavus* and *Acidaminococcus intentini* species were present significantly more in the AR cohort compared to the CG. In contrast, *Rothia mucilaginosa*, *Muricomes intestini*, *Clostridium papyrosolvens*, *Clostridium straminisolvens* and *Dialister succinatiphilus* were detected significantly less frequently in the AR cohort compared to the CG.

PLS-DA Multivariate Analysis

Multivariate analysis using partial least squares-discriminant analysis (PLS-DA) was performed on phylum to species level with microbiota abundance data. As shown in Figure 3A and 3B, PLS-DA discriminated the AR and CG cohorts based on the genus and species abundance data. Clustering of samples was observed at the genus and species levels for the first two principal coordinates, suggesting a distinct microbial structure between cohorts. Although the observed clustering of samples accounts for a small amount of total variance.

Discussion

The current study analysed the faecal microbial community of adult AR sufferers and controls. The AR cohort had a distinct gut microbiome profile, marked by a reduced microbial diversity and altered abundance of certain gut microbes compared to the controls. The results presented here provide evidence that unique gut microbial patterns occur in adult AR sufferers and warrants further investigation in the form of mechanistic studies.

A key finding from the current study was related to overall microbial diversity. Species richness (α-diversity) measured via the Shannon index was significantly reduced in the AR cohort compared to the CG. A similar trend of reduced species richness in the AR cohort was also observed with the inverse Simpson, observed OTU counts, and CHAO1 diversity indices. Lack of sufficient richness or evenness in the gastrointestinal microbial community appears to impair its ability to withstand exogenous disturbances (27). Indeed, it has been suggested that reliable microbial richness indices may be useful indicators to determine the relative stability or "fitness" of the gut microbiome (27). Other studies have also identified reduced richness in atopic individuals compared to controls. Hua et al, examined publicly available 16S rRNA data collected from the 'American Gut Project' and reported that species richness was significantly negatively associated with self-reported seasonal allergy in adult sufferers (10). Similarly, Bisgaard et al. reported that reduced faecal bacterial diversity at 1 and 12 months after birth significantly increased the risk of developing allergic sensitisation and allergic rhinitis by the age of 6 years (n=346) (28).

Other key findings from the current study relate to the differential abundance/detection of particular microbial taxa or specific microbes in the AR group compared to the controls. At the phylum level, a significantly increased abundance of Actinobacteria in the AR group and a significantly different Firmicutes to Bacteroidetes ratio between groups, with a higher abundance of Bacteroidetes and lower abundance of Firmicutes in the AR cohort was detected. Increased abundance of Bacteroidetes in the AR cohort translated through to the Class (Bacteroidia), Order (Bacteroidales), Family (*Bacteroidaceae*) and Genus level (*Bacteroids*) taxa. Other studies of atopic cohorts have also identified a higher abundance of Bacteroidetes classifications in stool samples from atopic subjects. Analysis of data from the American Gut Project identified a higher abundance Bacteroidales in adults with self-reported nut and seasonal pollen allergies (10). In a small study of Japanese infants (n=15), the abundance of *Bacteroidaceae* was significantly higher in infants who later went on to develop atopic disease by the age of two, as determined by the International Study of Asthma and Allergies in Childhood (ISACC) questionnaire (29). It is worth noting that the Bacteroidetes and the Firmicutes are the most abundant phyla present in the western faecal microbiome (30, 31) and this observation was also reflected in this study. The metabolites generated by these phyla play a significant role in colonic health and immune regulation. The Bacteroidetes phyla are generally associated with a greater production of acetate and proprionate whereas the Firmcutes phyla are associated with a greater production of butyrate (32). Butyrate, is a key energy source for colonic epithelial cells and contributes to maintaining the intestinal barrier via modulation of tight junction expression (33). A dysfunctional gut barrier allows for increased pro-inflammatory molecules and antigen transfer into submucosa and systemic circulation, resulting in local and systemic inflammatory responses. A

dominance of Bacteroidetes over Firmicutes may reduce overall butyrate production and thereby affect the integrity of the gut barrier. Interestingly, clinical and experimental studies have shown that gut permeability is increased in subjects with allergic disease compared to healthy controls (34-39). Given these results were generated from independent studies, future work is needed to explore the potential link between abundance of butyrate producing bacteria and gut permeability in atopy.

At the class and order level, this study reports a reduced abundance of Clostridia/Clostridiales (Firmicutes) in the AR cohort compared to the controls. A reduced abundance of Clostridiales was also observed by Hua et al. in adults with self-reported nut and seasonal pollen allergies. Reduced Clostridia has also been detected in faecal samples from atopic infants and children. Candela et al. reported a lower count of *Clostridium IV* in their cohort of Italian children (n=19 allergic, 12 non-allergic; aged 4-14 yrs) (40). Verhulst et al. observed an association between reduced prevalence of *Clostridium* in stool samples of infants (n=154) at three weeks of age and the occurrence of wheezing symptoms at 12 months of age (41). Indigenous intestinal *Clostridium* clusters IV, XIVa and XVIII have been recognised as effective inducers of Tregs in the colon of mice (42, 43). Tregs are known for their ability to maintain immune homeostasis and promote immune tolerance to allergens, which is particularly relevant in the pathogenesis of allergic diseases (44). Notably, reduced Clostridia have also been identified in other immune mediated diseases such as Crohn's (45).

At the genus level, we observed a higher abundance of *Bifidobacterium* (Actinobacteria) and Parabacteroides (Bacteroidetes) in the AR group compared to the CG. This finding is consistent with the study of Hevia et al. who reported a higher abundance of *Bifidobacterium* in their cohort of adult asthma sufferers. Hevia et al. also reported an increased abundance of the genera *Faecalibacterium* (Firmicutes), however this finding was not replicated in our study. Notably, most of the participants in the allergic asthma group (81%) also reported a history of AR in this study which a comorbidity of asthma. Despite their relatively low numerical abundance in the gastrointestinal tract, *Bifidobacteria* are being increasingly recognised for their beneficial effect on colon and immune function (46). Indeed, in experimental animal models of allergy, oral administration of *Bifidobacteria* species has been found to supress Th2 responses, enhance Treg activity and reduce infiltration of neutrophils and eosinophils to the airway (47-49) all of which would markedly reduce AR symptoms if these effects where translated to humans. In addition, *Bifidobacteria* strains are commonly included in probiotic supplements targeted for alleviation of allergy symptoms (50) including our own prior investigation of a multi-strain probiotic (which included *Bifidobacteria* strains) in which significant improvements in AR symptoms were noted over eight weeks of supplementation (21). Given the apparent anti-allergic effects associated with certain *Bifidobacteria* strains, the observed greater abundance of *Bifidobacteria* in adults with atopy is a surprising result. A further interesting and novel finding in the present study was the reduced abundance of the genera *Oxalobacter* (Proteobacteria) in the AR cohort. Members of the *Oxalobacteraceae* are known to colonise the rhizosphere and roots of many plant species (51). In relation to human health, *Oxalobacter* species metabolise oxalate in the intestinal tract and is protective against the formation of calcium oxalate

kidney stones and other oxalate-associated pathologies (52). Furthermore, a link between the presence of *Oxalobacteraceae* in house dust and the prevalence of atopy has been observed. Indeed, members of the *Oxalobacteraceae* were found to be more abundant in dust samples from the Finnish Karelia homes compared to geographically adjacent Russian Karelia whereby the abundance of atopic disease in this region is fourfold lower (53). While microbial gut composition was not performed in allergy sufferers living in these regions, these findings provide a potential link between exposure to plant related microbes such as *Oxalobacteraceae* and the prevalence of atopy.

In this study numerous species were differentially abundant/detected (9 abundant/ 14 detected) in the AR group compared to the controls. Among these species, several were identified as previously associated with atopy, however, for a large proportion of the differentially abundant/detected species, the relationship with atopy is unknown and warrants further investigation. Species differentially abundant/detected and of particular interest include *Ruminicoccus gnavus* (Firmicutes), *Bacteroidetes vulgatus* (Bacteroidetes) and *Bifidobacteria adolescentis* (Actinobacteria). In the current study a significantly increased detection of *R. gnavus* in the AR group when compared to the CG was observed. *R. gnavus* has been previously associated with the development and pathogenesis of atopy, especially respiratory allergies (54). Chua et al. reported a higher incidence of *R. gnavus* in stool specimens from infants who later developed respiratory allergies (54). In a follow-up experimental asthmatic mouse model, ovalbumin sensitised/challenge mice were infected with *R. gnavus* via oral gavage intragastric administration. The *R. gnavus* infected mice showed greater secretion of interleukin (IL)-25, IL33 and Thymic Stromal Lymphopoietin by colonic tissues, thereby promoting Th2

differentiation and further cytokine release, and an enhanced infiltration of eosinophils and mast cells to the colon and lung parenchyma (54). In addition, the *R. gnavus* infected mice displayed increased airway-hyperresponsiveness and histologic airway inflammation (54) providing evidence of a clear link between gut bacterial species and mechanisms underpinning allergic disease.

The finding of an increased abundance of *B. vulgatus* in the AR group is consistent with previously reported links with atopy. In a small study of children aged 3-10 years (n=25 allergic, 22 non-allergic), serum IgG titres for *B. vulgatus* were significantly higher in the allergic group compared to the non-allergic group (55). These results were confirmed in a larger study of children aged 12-13 years (n=83 multiple allergies, 433 non-allergic), where IgG titres for *B. vulgatus* were significantly higher in children reporting multiple allergies (i.e. asthma, rhinitis, eczema, and food allergy) compared to non-allergic rhinitis children (56). Hevia et al. in their study of adults with allergic asthma, reported a significantly increased abundance of *B. adolescentis* in the allergic group. In the current study, an increased abundance of *B. adolescentis* in the allergy group was also observed, although this finding was not statistically significant (p=0.052). Studies involving newborns and children fail to resolve the potential role for *B. adolescentis* in AR; one study reported a lower prevalence of *B. adolescentis* in stool samples of newborns (n=47) that developed allergic symptoms by age five (57) where as a second study reported an increased prevalence in stool samples from 5-year-old Estonian children diagnosed with eczema (n=20 allergic; 20 non-allergic) (58).

Key differentially abundant species identified in previous reports of atopic children and infants including, *Akkermansia muciniphilia*, *Faecalibacterium prausnitzii*, *Bifidobacterium catenulatum*, *B. longum*, *Staphyloccoccus aureus*, *Bacteroides fragilis*, *Clostridium difficile* and *Escherichia coli*, were not significantly differentially abundant in this cohort of adults suffering AR. This finding may be due to differences in study features, cohort ethnicity, sample processing methodology, and microbial identification tools used. Nonetheless, the differentially abundant microbes present in infancy/childhood cannot be entirely extrapolated to adults, and therefore demonstrates the importance of sampling the microbiome of allergy sufferers in adulthood. Longitudinal studies that capture the early microbiome and the microbiome throughout childhood to adulthood, while a challenging task, is worth further investigation to elucidate shifts in the microbiome of allergic subjects over time. In addition, mechanistic studies in gnotobiotic mice should be conducted to elucidate how the taxa identified in the current study contribute to the pathophysiology of AR.

The strengths of the current study lie in the novelty of assessing gut microbiome composition in an adult population with clinically well-characterised allergic disease. In addition, notwithstanding population studies, the sample size in our investigation is larger than typical single-centre investigations. Despite the strength of the design the authors acknowledge that this study is not without its limitations. In particular, there are several considerations in the use and interpretation of 16s rRNA amplicon analysis. 16s rRNA sequencing is often employed in human microbiome studies due to its ability to resolve the microbial population structure and biodiversity and its relative affordability. However, the amplicons generated by this sequencing method are relatively short in size

and therefore may not provide a high level of confidence of the population structure at the species level. An alternative approach to 16s rRNA sequencing, is whole genome shot gun sequencing (WGS) which allows more accurate detection of taxa at the species level. The differentially abundant/detected species identified in this cohort of AR sufferers, may be worth confirming with an additional sequencing method such as WGS. While this study describes the microbial composition of stool samples from adults with AR and adults without AR, it should also be noted that stool samples may only capture luminal microbiota and not the mucosal-associated microbiota which may play a critical role in regulation of the mucosal immune system and local mucosal immune regulation relevant in AR.

Overall, a unique microbial community in the AR cohort, marked by a reduced microbial diversity, increased abundance of Bacteroidetes, *Parabacteroides*, Actinobacteria and *Bifidobacterium* and a reduced abundance of *Oxalobacter* and Clostridiales was observed in the current study. Several taxa identified in our study were consistent with previous reports in atopic adults. However, this study also identified taxa that were unique to our study and have not been previously associated with atopy. Interestingly, the differentially abundant/detected taxa reported here, were not always consistent with the findings presented in atopic paediatric cohorts. In light of the unique microbiome patterns in adult AR subjects presented here, identifying the metabolites and mechanisms underpinning the microbiota-host relationship will improve the understanding of how the composition of the microbiome regulates immune homeostasis and may advise potential therapeutic options for treating allergies (e.g. dietary intervention, probiotics, faecal transplant).

Acknowledgements

The authors appreciate the support of the Queensland Allergy Services Clinic for providing clinic access and for performing the skin prick tests.

References:

1. Alkanani AK, Hara N, Gottlieb PA, Ir D, Robertson CE, Wagner BD, et al. Alterations in Intestinal Microbiota Correlate With Susceptibility to Type 1 Diabetes. Diabetes. 2015;64(10):3510-20.

2. Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, et al. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. BMC Med. 2013;11(1):46.

3. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Elife. 2013;2:e01202.

4. Vaahtovuo J, Munukka E, Korkeamaki M, Luukkainen R, Toivanen P. Fecal microbiota in early rheumatoid arthritis. J Rheumatol. 2008;35(8):1500-5.

5. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut. 2011;60(5):631-7.

6. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, et al. Low counts of Faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis. 2009;15(8):1183-9.

7. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Paz Soldan MM, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. Sci Rep. 2016;6:28484.

8. Hevia A, Milani C, Lopez P, Donado CD, Cuervo A, Gonzalez S, et al. Allergic Patients with Long-Term Asthma Display Low Levels of Bifidobacterium adolescentis. PLoS One. 2016;11(2):e0147809.

9. Watanabe S, Narisawa Y, Arase S, Okamatsu H, Ikenaga T, Tajiri Y, et al. Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. J Allergy Clin Immunol. 2003;111(3):587-91.

10. Hua X, Goedert JJ, Pu A, Yu G, Shi J. Allergy associations with the adult fecal microbiota: Analysis of the American Gut Project. EBioMedicine. 2016;3:172-9.

11. Melli LC, do Carmo-Rodrigues MS, Araujo-Filho HB, Sole D, de Morais MB. Intestinal microbiota and allergic diseases: A systematic review. Allergol Immunopathol (Madr). 2016;44(2):177-88.

12. Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD, et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. J Immunol. 1998;160(10):4730-7.

13. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today. 1993;14(7):353-6.

14. McLoughlin RM, Mills KH. Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma. J Allergy Clin Immunol. 2011;127(5):1097-107; quiz 108-9.

15. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 2005;122(1):107-18.

16. Ohnmacht C, Park JH, Cording S, Wing JB, Atarashi K, Obata Y, et al. MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORgammat(+) T cells. Science. 2015;349(6251):989-93.

17. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 2012;486(7402):222-7.

18. Matsumoto M, Ohishi H, Kakizoe K, Benno Y. Faecal microbiota and secretory immunogloblin a levels in adult patients with atopic dermatitis. Microbial ecology in health and disease. 2004;16(1):13-7.

19. Agache I, Rogozea L. Endotypes in allergic diseases. Current opinion in allergy and clinical immunology. 2018;18(3):177-83.

20. Watts AM, West NP, Smith PK, Cripps AW, Cox AJ. Probiotics and Allergic Rhinitis: A Simon Two-Stage Design to Determine Effectiveness. J Altern Complement Med. 2016;22(12):1007-12.

21. Watts AM, Cox AJ, Smith PK, Besseling-van der Vaart I, Cripps AW, West NP. A Specifically Designed Multispecies Probiotic Supplement Relieves Seasonal Allergic Rhinitis Symptoms. J Altern Complement Med. 2018;24(8):833-40.

22. Juniper, Thompson, Ferrie, Roberts. Development and validation of the Mini Rhinoconjunctivitis Quality of Life Questionnaire. Clin Exp Allergy. 2000;30(1):132-40.

23. K. G. Von Boroviczeny LEB, B. S. Bull, A. Chattas, J. B. Dawson, K. Fukutake, F. W. Gunz, J. A. Koepke, S. M. Lewis, E. Rewald, M. Roubicek, G. Ruthenstroth-Bauer. Recommendation of measurement of erythrocyte sedimentation rate of human blood. Am J Clin Pathol. 1977;68(4):505-7.

24. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013;41(1):e1.

25. Li W, Fu L, Niu B, Wu S, Wooley J. Ultrafast clustering algorithms for metagenomic sequence analysis. Brief Bioinform. 2012;13(6):656-68.

26. Le Cao K, Rohart F, Gonzalez I, Dejean S, Gautier B, Bartolo F, et al. mixOmics: Omics Data Integration Project. R package version 6.1.1. https://CRANR-projectorg/package=mixOmics. 2016.

27. Bäckhed F, Fraser Claire M, Ringel Y, Sanders Mary E, Sartor RB, Sherman Philip M, et al. Defining a Healthy Human Gut Microbiome: Current Concepts, Future Directions, and Clinical Applications. Cell Host & Microbe. 2012;12(5):611-22.

28. Bisgaard H, Li N, Bonnelykke K, Chawes BLK, Skov T, Paludan-Müller G, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. J Allergy Clin Immunol. 2011;128(3):646-52.e5.

29. Songjinda P, Nakayama J, Tateyama A, Tanaka S, Tsubouchi M, Kiyohara C, et al. Differences in developing intestinal microbiota between allergic and non-allergic infants: a pilot study in Japan. Biosci Biotechnol Biochem. 2007;71(9):2338-42.

30. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-14.

31. Johnson EL, Heaver SL, Walters WA, Ley RE. Microbiome and metabolic disease: revisiting the bacterial phylum Bacteroidetes. J Mol Med (Berl). 2017;95(1):1-8.

32. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota and host energy metabolism. J Lipid Res. 2013:jlr. R036012.

33. Brahe LK, Astrup A, Larsen LH. Is butyrate the link between diet, intestinal microbiota and obesity-related metabolic diseases? Obesity reviews : an official journal of the International Association for the Study of Obesity. 2013;14(12):950-9.

34. Ukabam SO, Mann RJ, Cooper BT. Small intestinal permeability to sugars in patients with atopic eczema. Br J Dermatol. 1984;110(6):649-52.

35. Pike MG, Heddle RJ, Boulton P, Turner MW, Atherton DJ. Increased intestinal permeability in atopic eczema. J Invest Dermatol. 1986;86(2):101-4.

36. Rosenfeldt V, Benfeldt E, Valerius NH, Paerregaard A, Michaelsen KF. Effect of probiotics on gastrointestinal symptoms and small intestinal permeability in children with atopic dermatitis. J Pediatr. 2004;145(5):612-6.

37. Hijazi Z, Molla AM, Al-Habashi H, Muawad WM, Molla AM, Sharma PN. Intestinal permeability is increased in bronchial asthma. Arch Dis Child. 2004;89(3):227-9.

38. Benard A, Desreumeaux P, Huglo D, Hoorelbeke A, Tonnel AB, Wallaert B. Increased intestinal permeability in bronchial asthma. J Allergy Clin Immunol. 1996;97(6):1173-8.

39. Majamaa H, Isolauri E. Evaluation of the gut mucosal barrier: evidence for increased antigen transfer in children with atopic eczema. J Allergy Clin Immunol. 1996;97(4):985-90.

40. Candela M, Rampelli S, Turroni S, Severgnini M, Consolandi C, De Bellis G, et al. Unbalance of intestinal microbiota in atopic children. BMC Microbiol. 2012;12(1):95.

41. Verhulst SL, Vael C, Beunckens C, Nelen V, Goossens H, Desager K. A longitudinal analysis on the association between antibiotic use, intestinal microflora, and wheezing during the first year of life. J Asthma. 2008;45(9):828-32.

42. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science. 2011;331(6015):337-41.

43. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013;500(7461):232-6.

44. Noval Rivas M, Chatila TA. Regulatory T cells in allergic diseases. J Allergy Clin Immunol. 2016;138(3):639-52.

45. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. 2006;55(2):205-11.

46. Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L. Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut. Front Microbiol. 2016;7:979.

47. Ren J, Zhao Y, Huang S, Lv D, Yang F, Lou L, et al. Immunomodulatory effect of Bifidobacterium breve on experimental allergic rhinitis in BALB/c mice. Exp Ther Med. 2018;16(5):3996-4004.

48. Raftis EJ, Delday MI, Cowie P, McCluskey SM, Singh MD, Ettorre A, et al. Bifidobacterium breve MRx0004 protects against airway inflammation in a severe asthma model by suppressing both neutrophil and eosinophil lung infiltration. Sci Rep. 2018;8(1):12024.

49. Casaro MC, Crisma AR, Vieira AT, Silva GHM, Mendes E, Ribeiro WR, et al. Prophylactic Bifidobacterium adolescentis ATTCC 15703 supplementation reduces

partially allergic airway disease in Balb/c but not in C57BL/6 mice. Benef Microbes. 2018;9(3):465-76.

50. Zajac AE, Adams AS, Turner JH. A systematic review and meta-analysis of probiotics for the treatment of allergic rhinitis. Int Forum Allergy Rhinol. 2015;5(6):524-32.

51. Ofek M, Hadar Y, Minz D. Ecology of Root Colonizing Massilia (Oxalobacteraceae). PLoS One. 2012;7(7):e40117.

52. Liu M, Koh H, Kurtz ZD, Battaglia T, PeBenito A, Li H, et al. Oxalobacter formigenes-associated host features and microbial community structures examined using the American Gut Project. Microbiome. 2017;5(1):108.

53. Pakarinen J, Hyvärinen A, Salkinoja-Salonen M, Laitinen S, Nevalainen A, Mäkelä MJ, et al. Predominance of Gram-positive bacteria in house dust in the low-allergy risk Russian Karelia. Environ Microbiol. 2008;10(12):3317-25.

54. Chua HH, Chou HC, Tung YL, Chiang BL, Liao CC, Liu HH, et al. Intestinal Dysbiosis Featuring Abundance of Ruminococcus gnavus Associates With Allergic Diseases in Infants. Gastroenterology. 2018;154(1):154-67.

55. Kamiya T, Aiba Y, Noda S, Tanaka K, Iikura Y, Koga Y. Elevation of Serum IgG Antibody to Enteric Bacteria in Allergic Children. The Showa University Journal of Medical Sciences. 2002;14(1):49-55.

56. Fukuda S, Ishikawa H, Koga Y, Aiba Y, Nakashima K, Cheng L, et al. Allergic symptoms and microflora in schoolchildren. J Adolesc Health. 2004;35(2):156-8.

57. Sjogren YM, Jenmalm MC, Bottcher MF, Bjorksten B, Sverremark-Ekstrom E. Altered early infant gut microbiota in children developing allergy up to 5 years of age. Clin Exp Allergy. 2009;39(4):518-26.

58. Stsepetova J, Sepp E, Julge K, Vaughan E, Mikelsaar M, de Vos WM. Molecularly assessed shifts of Bifidobacterium ssp. and less diverse microbial communities are characteristic of 5-year-old allergic children. FEMS Immunol Med Microbiol. 2007;51(2):260-9.

Figure legends

Figure 1. Alpha diversity metrics for allergic rhinitis and control group (A-D). 16s rRNA sequencing was performed using stool samples collected from AR and CG participants and alpha diversity metrics were determined. (A) The total number of observed operational taxonomic units (OTUs) was lower in the AR group (B) The Chao1 index was lower in the AR group (C) The Shannon index was significantly lower in the AR group ($p=<0.05$*) (D) The Inverse Simpson index was lower in the AR group. Outer limits of each box represent the 25^{th} and 75^{th} percentiles, with the median shown as the line within the box. Whiskers (error bars) show the 5^{th} and 95^{th} percentiles, which filled circles showing the outliers.

Figure 2. The relative abundance of bacteria phyla for allergic rhinitis (AR) and control groups (CG) measured via 16s rRNA sequencing using collected stool samples *$p<0.05$ **$p<0.01$.

Figure 3. PLS-DA multivariate analysis at the genus and species level collated from 16s rRNA sequencing data performed using collected stool samples. A) at the genus level, clustering of samples within the AR and CG cohorts was observed for the first two principal coordinates. The first coordinate represents 7% variance explained and second coordinate represents 6% variance explained. B) at the species level, clear clustering of AR and CG samples was also observed for the first two principal coordinates. The first coordinate represents 7% of variance explained and the second coordinate represents 4% of variance explained. The blue full circles represent the AR samples and the orange full

triangles represent the CG samples. The coloured ellipses, blue for AR and orange for CG, are plotted to represent the 95% confidence level of the population.

Tables

Table 1. Demographic and clinical features of AR participants and controls (CG).

	All mean	AR mean ± SD	CG mean ± SD	P value
n	80	57	23	-
Age (years)	38.34 ± 12.54	39.06 ± 13.29	36.55 ± 10.51	0.421
Sex (M/F)	32/48 (60% F)	22/35 (61% F)	10/13 (57% F)	0.687
Height (cm)	172.16 ± 9.83	171.07 ± 9.80	174.85 ± 9.60	0.120
Weight (kg)	75.90 ± 16.20	76.27 ± 14.95	75.00 ± 19.31	0.779
BMI (kg/m^2)	25.47 ± 4.28	25.96 ± 4.08	24.25 ± 4.61	0.105
Ethnicity (% Caucasian)	87.50	84.21	95.65	0.161
Immune measures				
White cell count ($x10^9/L$)	6.48 ± 1.69	6.82 ± 1.72	5.63 ± 1.30	0.004
Lymphocytes ($x10^9/L$)	2.09 ± 0.66	2.21 ± 0.66	1.81 ± 0.59	0.014
Eosinophils ($x10^9/L$)	0.30 ± 0.26	0.37 ± 0.27	0.11 ± 0.08	<0.00001
Neutrophils ($x10^9/L$)	3.52 ± 1.16	3.64 ± 1.21	3.22 ± 0.98	0.136
Basophils ($x10^9/L$)	0.05 ± 0.04	0.05 ± 0.04	0.04 ± 0.03	0.110
ESR (mm/hr)	9.10 ± 9.83	10.12 ± 10.70	6.57 ± 6.81	0.144
Disease characteristics				
Co-allergy to dust mites and pollen (%)	42.5%	59.65%	0%	-
Dust-mite only (%)	23.75%	33.33%	0%	-
Pollen only (%)	5%	7.02%	0%	-

M- male; F- female; cm – centimetre; kg – kilogram; m^2 – meters squared; L – litre; mm- millimetre; % - percentage

Table 2. Relative abundance of differentially abundant taxa between the AR and controls (CG) at the Order, Genus and Species level.

	AR (%)	**CG (%)**	**P value**
Order			
Bifidobacteriales	0.87 ± 1.81	0.24 ± 0.34	0.013
Bacteroidales	36.30 ± 12.10	28.70 ± 7.97	0.007
Clostridiales	50.50 ± 14.70	59.80 ± 10.80	0.008
Genus			
Bifidobacterium	0.87 ± 1.81	0.26 ± 0.35	0.017
Parabacteroides	2.40 ± 2.55	1.15 ± 1.03	0.003
Bacteroides	23.40 ± 16.00	16.90 ± 9.40	0.028
Oxalobacter	<0.01 ± <0.01	<0.01 ± 0.01	0.023
Faecalibacterium	19.30 ± 12.10	21.80 ± 11.50	0.41
Species			
Parabacteroides distasonis	0.64 ± 0.87	0.30 ± 0.34	0.014
Bacteroides vulgatus	13.50 ± 14.10	7.83 ± 6.23	0.015
Eubacterium xylanophilum	0.11 ± 0.14	0.27 ± 0.30	0.021
Murimonas intestini	0.01 ± 0.01	0.02 ± 0.03	0.01
Oscillibacter valericigenes	0.23 ± 0.33	0.45 ± 0.47	0.021
Agathobaculum butyriciproducens	0.12 ± 0.09	0.24 ± 0.24	0.027
Anaerotruncus colihominis	0.03 ± 0.03	0.02 ± 0.01	0.001
Oxalobacter formigenes	<0.01 ± 0.01	0.01 ± 0.01	0.035
Bifidobacterium adolescentis	0.57 ± 1.49	0.16 ± 0.29	0.052

AR, allergic rhinitis, CG, control group. Data are presented as mean ± SD

Table 3. Differentially detected taxa between the AR and CG group at the family, genus and species level

	AR number (proportion)	CG number (proportion)	Fishers exact P value	Chi-Square p value
Family				
Micrococcaceae	4 (7.02%)	7 (30.43%)	0.003	0.0001
Oxalobacteraceae	10 (17.54%)	13 (56.52%)	0.0009	0.0005
Genus				
Rothia	4 (7.02%)	7 (30.43%)	0.003	0.0001
Coriobacteriaceae unclassified	7 (12.28%)	10 (43.48%)	0.0049	0.0020
Acidaminococcus	14 (24.56%)	1 (4.35%)	0.0548	0.0360
Oxalobacter	10 (17.54%)	13 (56.52%)	0.0009	0.0005
Sutterella	25 (43.86%)	19 (82.61%)	0.0025	0.0016
Species				
Rothia mucilaginosa	4 (7.02%)	7 (30.43%)	0.0147	0.0092
Bacteroides massiliensis	23 (40.35%)	16 (69.57%)	0.0258	0.0180
Christensenella minuta	45 (78.95%)	23 (100%)	0.0154	0.0170
Muricomes intestini	0 (0%)	2 (8.70%)	0.0801	0.0242
Murimonas intestini	31 (54.39%)	19 (82.61%)	0.223	0.0183
Clostridium asparagiforme	23 (40.35%)	15 (65.22%)	0.0517	0.0438
Clostridium hylemonae	27 (47.37%)	4 (17.39%)	0.0212	0.0127
Ruminococcus gnavus	26 (45.61%)	5 (21.74%)	0.0748	0.0473
Clostridium papyrosolvens	5 (8.77%)	6 (26.09%)	0.0687	0.0418
Clostridium straminisolvens	4 (7.02%)	8 (34.78%)	0.0036	0.0016
Acidaminococcus intestini	13 (22.81%)	1 (4.35%)	0.0568	0.0492
Dialister succinatiphilus	9 (15.79%)	9 (39.13%)	0.0371	0.0237
Oxalobacter formigenes	10 (17.54%)	13 (56.52%)	0.0009	0.0005
Sutterella wadsworthensis	23 (40.35%)	16 (69.57%)	0.0258	0.0180

In a given sample, taxa were considered ‘detected’ if the relative abundance as >0% and ‘not detected’ if the relative abundance was 0%. AR, Allergic rhinitis; CG, control group.

Figure 1

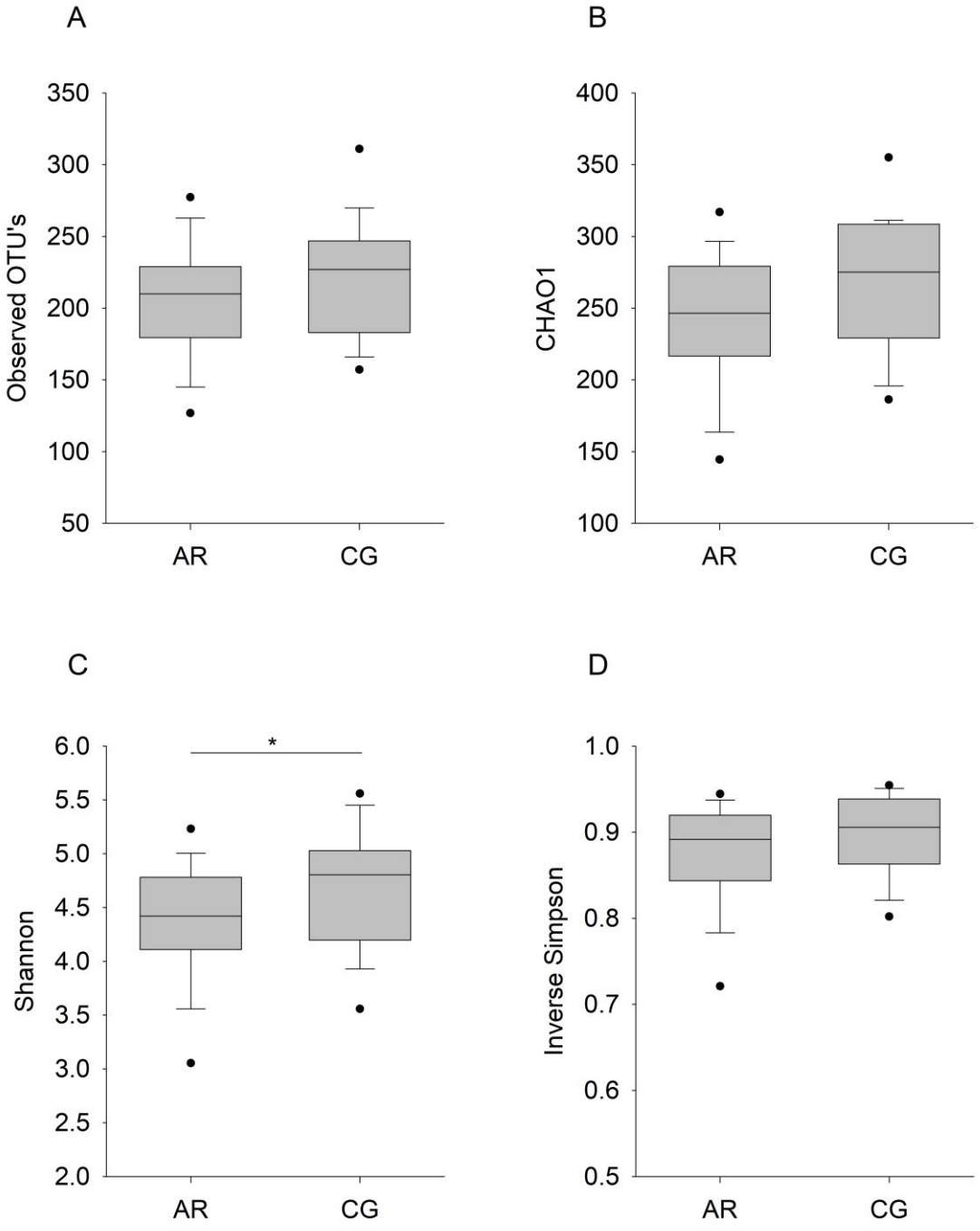

A
Observed OTU's
350
300
250
200
150
100
50
AR
CG
B
CHAO1
400
350
300
250
200
150
100
AR
CG
C
Shannon
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
*
AR
CG
D
Inverse Simpson
1.0
0.9
0.8
0.7
0.6
0.5
AR
CG

Figure 2

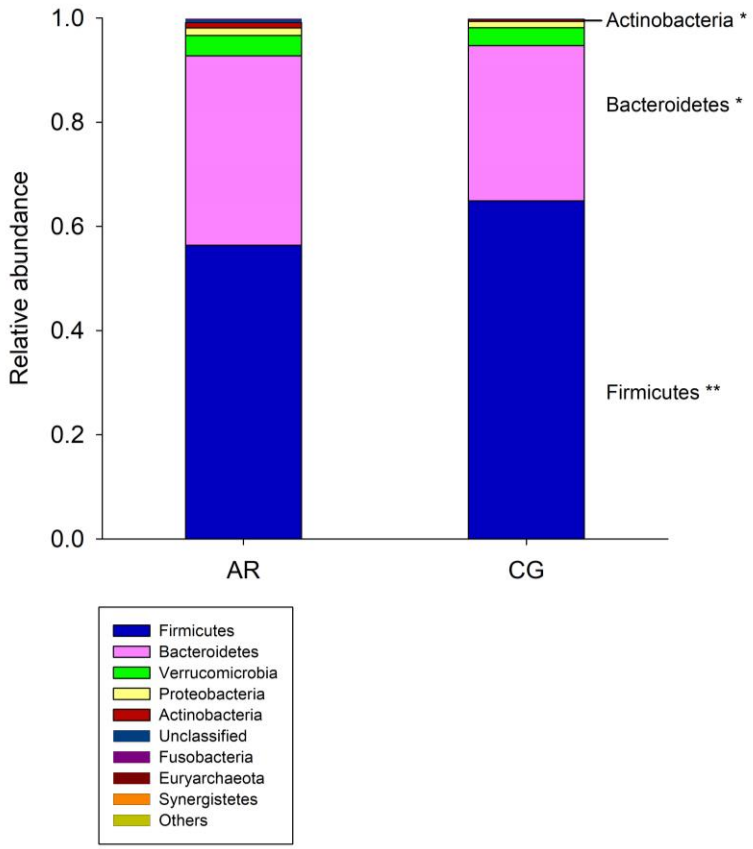
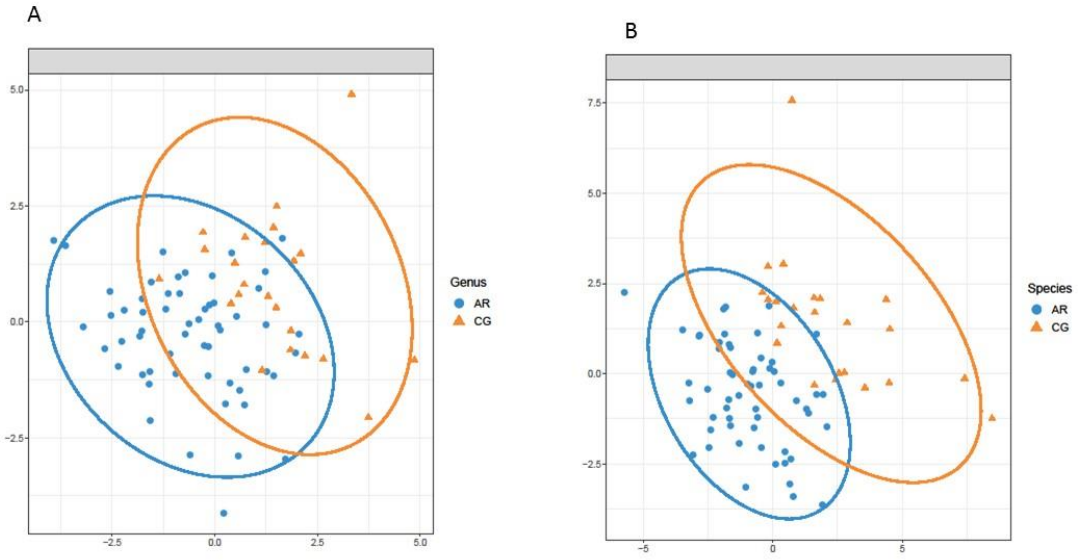

1.0
0.8
0.6
0.4
0.2
0.0
Relative abundance
AR
CG
Actinobacteria *
Bacteroidetes *
Firmicutes **
Firmicutes
Bacteroidetes
Verrucomicrobia
Proteobacteria
Actinobacteria
Unclassified
Fusobacteria
Euryarchaeota
Synergistetes
Others

Figure 3



CHAPTER THREE

Study two: Probiotics and Allergic Rhinitis: A Simon Two-Stage design to determine effectiveness

PROBIOTICS AND ALLERGIC RHINITIS: A SIMON TWO-STAGE DESIGN TO DETERMINE EFFECTIVENESS

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

> **Watts AM,** West NP, Smith PK, Cripps AW, Cox AJ. Probiotics and Allergic Rhintis: A Simon Two-Stage Design to Determine Effectiveness. J Altern Complement Med. 2016;**22**(12):1007-1012.

My contribution to the paper involved:

Critical review of the literature, elements of the study design and preparation of the manuscript text.

_____7th May 2019

Annabelle Monica Watts

_____7th May 2019

Supervisor: Professor Allan Cripps

CHAPTER FOUR

Study three: A specifically designed multi-species probiotic supplement relieves seasonal allergic rhinitis symptoms

A SPECIFICALLY DESIGNED MULTI-SPECIES PROBIOTIC SUPPLEMENT RELIEVES SEASONAL ALLERGIC RHINITIS SYMPTOMS

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

> **Watts AM**, Cox AJ, Smith PK, Besseling-van der Vaart I, Cripps AW, West NP. A Specicially Designed Multispecies Probiotic Supplement Relieves Seasonal Allergic Rhinitis Symptoms. J Altern Complement Med. 2018;24(8):833-840.

My contribution to the paper involved:

Recruitment of study participants and management of clinical trial, collection of samples, IgE and IgG ELISA experiments, statistical analysis of symptom data and demographic and clinical data, preparation of figures and tables, and primary writer of manuscript.

_____7th May 2019

Annabelle Monica Watts

_____7th May 2019

Supervisor: Professor Allan Cripps

CHAPTER FIVE

Study four: Distinct gene expression patterns between nasal mucosal cells and blood collected from allergic rhinitis sufferers

DISTINCT GENE EXPRESSION PATTERNS BETWEEN NASAL MUCOSAL CELLS AND BLOOD COLLECTED FROM ALLERGIC RHINITIS SUFFERERS

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

> **Watts AM,** West NP, Cripps AW, Smith PK, Cox AJ. Distinct Gene Expression Patterns between Nasal Mucosal Cells and Blood Collected from Allergic Rhinitis Sufferers. *Int Arch Allergy Immunol* 2018;**177**(1):29-34.
>
> *Supplementary material in Appendix 2*

My contribution to the paper involved:

Recruitment of study participants and collection of samples, optimisation of nasal mucosa sampling and gene expression protocol, design of panel plus gene expression panel, RNA extraction, quantification and gene expression experiments, analysis of gene expression data, demographic and clinical data, preparation of figures and tables, and primary writer of manuscript.

_____7th May 2019

Annabelle Monica Watts

_____7th May 2019

Supervisor: Professor Allan Cripps

CHAPTER SIX

Study five: Adult allergic rhinitis sufferers have unique nasal mucosal and peripheral blood immune gene expression profiles compared with controls

ADULT ALLERGIC RHINITIS SUFFERERS HAVE UNIQUE NASAL MUCOSAL AND PERIPHERAL BLOOD IMMUNE GENE EXPRESSION PROFILES COMPARED WITH CONTROLS

This Chapter is formatted in a style suitable for publication in Clinical and Experimental Allergy. *Supplementary material for the Chapter is provided in Appendix 3.*

My contribution to this Chapter involved:

Recruitment of study participants and collection of samples, RNA extraction, quantification and gene expression experiments, analysis of gene expression data, demographic, clinical and symptom data, preparation of figures and tables, and primary writer of manuscript.

_____7th May 2019

Annabelle Monica Watts

_____7th May 2019

Supervisor: Professor Allan Cripps

Abstract

Background: Allergic rhinitis is a complex disease involving both mucosal and systemic immune compartments. Greater understanding of the immune networks underpinning AR pathophysiology may assist with further refining the diagnosis of AR and associated endotypes.

Objective: To compare immune gene expression profiles in nasal mucosa and peripheral blood samples between adults with AR and controls without allergic rhinitis (CG).

Methods: This cross-sectional study included 45 adults with moderate-severe and persistent AR (37.6 ± 12.8 years; mean ± SD) and 24 adults without AR (36.6 ± 10.2). Gene expression analysis was performed using the NanoString nCounter PanCancerImmune profiling panel (n=730 immune genes) in combination with the panel plus probe set (n=30 allergy-related genes) with purified RNA from peripheral blood and cell lysates prepared from combined nasal lavage and nasal brushing.

Results: A total of 113 genes were significantly differentially expressed in peripheral blood samples between groups ($p<0.05$). In contrast, 14 genes were differentially expressed in nasal lysate samples between groups ($p<0.05$). Up-regulation of allergy-related genes in nasal mucosa samples in the AR group was observed. Namely, chemokines CCL17 and CCL26 are involved in the chemotaxis of key effector cells and TPSAB1 which encodes tryptase, an inflammatory mediator released from activated mast cells and basophils. A total of six differentially expressed genes (DEGs) were in common between the nasal mucosa and blood samples. In addition, counts of specific DEGs in nasal mucosa samples were positively correlated with eosinophil and dust mite-specific IgE counts in blood.

Conclusions and Clinical Relevance: Distinct gene expression profiles in blood and nasal mucosa samples were observed between AR sufferers and controls. The results of this study also provide evidence for a close interaction between the local site and systemic immunity. The genes identified in this study may contribute to the refinement of rhinitis endotypes or as biomarkers to evaluate effectiveness of treatment regimens.

Introduction

Allergic rhinitis (AR) is classified as a chronic upper respiratory disease estimated to affect between 10 – 30% of the global population (1-4) and is associated with significant medical and economic burden (5-7). AR symptoms occur primarily in the upper respiratory tract; however, the immunopathology of the disease is highly complex and involves interactions between the local site (nasal mucosa) and the systemic immune system (lymphoid tissues and peripheral blood). Indeed, changes to the activation status of peripheral blood leucocytes were observed following nasal allergen challenge in seasonal allergic rhinitis sufferers (8). Specifically, fluctuations in the frequency of peripheral blood CD107a, CD63 and CD203c basophil activation makers, CD80(+) and CD86(+) plasmacytoid dendritic cells and CD4(+) CD25lo memory T cells were observed following nasal allergen challenge (8). These results support the concept that allergen exposure at the local respiratory site can induce a systemic immunological response, particularly observed in cell types that are consistent with the AR phenotype.

Multiplex gene expression analyses, including microarray experiments, are an effective means of gaining a global representation of the cellular mechanisms behind complex diseases. Additionally, gene expression experiments are well suited to identifying genes involved in the pathophysiology of specific diseases and in identifying potential drug targets. Traditionally, microarray experiments using nasal mucosal samples have typically relied on nasal biopsy specimens (9, 10) or have pooled nasal lavage samples from multiple individuals to obtain sufficient sample material for analysis (11). We have successfully conducted gene expression experiments with cell lysate samples collected via nasal brushing and nasal washing using the NanoString nCounter system. This method provided a cost-effective and non-invasive means of sampling that yielded sufficient molecular material for expression analysis of a panel of 760 immune genes.

The diagnosis of AR is confounded by other chronic respiratory diseases/rhinitis endotypes that share symptoms but have different underlying immunopathology. The diagnosis of AR would be improved by the identification of AR-specific biomarkers that provide an objective measure of the disease endotype. As such, we have applied this gene expression technology to identify AR-specific genes and characterise immune-gene expression profiles of nasal mucosal and peripheral blood samples of participants with AR compared to a cohort of healthy non-AR controls. The combined approach of analysing both the local (nasal mucosa) and systemic immune system (peripheral blood) provides a powerful tool to further understand immunological networks underpinning AR pathophysiology.

Methods

Study design

This study was designed as a cross-sectional study to characterise differences in the immune gene expression profiles in peripheral blood and nasal mucosa samples between adults with existing AR (n=45) and adults with no history of AR (n=24 controls). Participants attended a screening appointment at the Queensland Allergy Services Clinic in Southport for evaluation of allergen sensitivities. Following screening, eligible participants were instructed to cease use of all intranasal and immune modulating medications for 14 days prior to their follow-up appointment. Participants then attended an appointment at the Clinical Trial Unit at Griffith University (Gold Coast, Australia) for provision of blood and nasal mucosal samples and completion of symptom surveys. This study was approved by the Griffith University Human Research Ethics Committee (approval #s: 2015/564; 2016/279).

Participant selection

Participants were both male and female aged between 18 to 65 years of age with a more than two-year history of AR symptoms. Participants had persistent AR and moderate-to-severe symptoms as defined by the Allergic Rhinitis and Its Impact on Asthma guidelines (ARIA) with symptoms occurring for more than four days per week and more than four weeks in a row, and one or more of the following conditions present: (1) sleep disturbance, (2) impairment of daily activities, (3) impairment of school or work, or (4) troublesome symptoms. Participants had a positive allergic response to dust mites determined via a skin prick test and/or serum specific IgE radioallergosorbent test (RAST) (QML Pathology, Murarrie, Queensland, Australia) to *Dermatophagoides pternyssinus* or *D. farinae*. Participants were also tested for against a panel of pollen allergens and an IgE RAST for grass pollen mix (Bermuda, Timothy, Meadow, Johnson, Rye and Paspalum) for characterisation of the cohort.

AR individuals were excluded from participating if they suffered from non-allergic rhinitis (vasomotor rhinitis), consumed probiotics in the previous 8-12 weeks, were treated with oral corticosteroids within the previous six months or antibiotics within the previous 30 days, used anti-inflammatory or immune-modulating medications, had existing respiratory disease including asthma, nasal polyposis, or chronic obstructive pulmonary disorder, had existing immune dysfunction (other than allergies), had recent nasal surgery or nasal trauma that could affect nasal mucosal sampling, were ill or had infectious disease at time of enrolment, reported hepatic impairment or excessive alcohol consumption as per the NHMRC alcohol guidelines Australia (12) and Bouchery et al. 2011 (13) or were pregnant at the time of enrolment.

Individuals were recruited to the study as controls if they reported no history of AR, tested negative to dust mites, grass and tree pollen, and were free from chronic disease. Participants were excluded from the control group if they consumed probiotics in the previous 8-12 weeks, had taken antibiotics within

the previous 30 days, used anti-inflammatory or immune-modulating medications, had existing respiratory disease, immune dysfunction or gastrointestinal disease or disorder, were ill or had an infectious disease at the time of enrolment or were pregnant at the time of enrolment.

Symptom analysis

The severity of AR symptoms was evaluated using a collection of self-reported symptom surveys completed prior to sample collection. The AR-specific symptom severity surveys included the mini Rhinoconjuctivitis Quality of Life Questionnaire (mRQLQ), Total nasal symptom score (TNSS) survey, Total Ocular Symptom Score (TOSS) survey, The Other Allergic Rhinitis Symptom Score (OARSS), and overall symptom severity measured using a 100 mm Visual Analogue Scale. The mRQLQ consists of 14 questions separated into five domains: activities, practical problems, nose symptoms, eye symptoms and other symptoms. All items on the questionnaire were rated on a 7-point likert scale (0-6) with each item averaged to give a maximum overall score of six. The TNSS consisted of nasal congestion, runny nose, itchy nose and sneezing. Symptoms were scored on a four-point scale: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms; such that the maximum daily TNSS score was 12. The TOSS consisted of itchy eyes, watery eyes, eye redness, scored on the same four-point scale as the TNSS such that the maximum daily TOSS was 9. The other symptom score consisted of post-nasal drip, unrefreshed sleep, itchy throat/palate or ears and sinus pain, scored on the same four-point scale, such that the daily maximum score was 12.

Sample collection and laboratory analysis

Venous blood samples were collected for full blood count including white cell differential and specific IgE for dust mites and grass pollen mix. In addition, erythrocyte sedimentation rate (ESR) over one hour was measured using fresh blood samples collected in sodium citrate tubes and using commercially available Vacuette ESR pipettes (Greiner Bio-One, Kremsmünster, Austria) as per the Westergren method (14).

Nasal washing, brushing and whole blood samples were collected as described previously (15). Briefly, nasal wash samples were collected by instilling 100 ml of PBS in each nostril. Expelled fluid was collected in a sterile container and supplemented with 20 ml of RPMI. Nasal brushings were collected with a brush placed between the nasal septum and inferior turbinate of each nostril. Harvested nasal mucosal cells were shaken from nasal brushes into 3.5 ml RPMI to release cells. Nasal wash and nasal brushing samples were combined, and cellular material concentrated via centrifugation with subsequent lysis using a commercially available RLT lysis buffer (Qiagen, Hilden, Germany). For whole blood samples, RNA was extracted from PAXgene tubes using a Maxwell® RSC automated RNA extraction instrument using commercially available Maxwell® RSC simplyRNA Tissue Kit (Promega Corporation, Wisconsin, USA). The quality and quantity of extracted RNA was assessed with the

NanoDrop 1000 UV-Vis spectrophotometer (ThermoScientific, Massachusettes, United States) and by capillary electrophoresis (LapChip GXII Touch HT, Perkin Elmer, Massachusetts, USA).

Gene expression analysis

Immune gene expression analysis of nasal cell lysate and extracted RNA from blood was performed using a commercially available Nanostring nCounter PanCancer Immune Profiling panel (NanoString Technologies, Seattle, WA, USA) as described previously (15). This panel contained 40 reference (housekeeping) genes and 730 immune genes, and was used in combination with the nCounter panel plus probe set which contained an additional 30 immune genes (760 immune genes in total) (15). Gene expression data underwent imaging quality control and normalisation checks prior to analysis and interpretation of data. Raw NanoString gene expression data was first normalised against a set of eight negative controls to account for background noise and platform associated variation by subtracting the mean + 2 standard deviations of the negative control counts from each sample. The data was then normalised against the geometric mean of six positive control samples. Samples that did not meet the quality control parameters where removed from further analysis. Genes were removed from further analysis if they had under 20 raw counts in greater than 50% of samples from either group. The GeNorm Algorithm was used to select the most stable housekeeping genes to use for reference normalisation. In total 34 housekeeping genes were used for reference normalisation of the blood samples and 29 housekeeping genes were used to perform reference normalisation of the nasal lysate samples.

Pathway analysis

Functional gene annotation and pathway analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) using differentially expressed genes ($p<0.05$ padjust) with significant pathway enrichment accepted at $p < 0.05$ Bonferroni padjust.

Protein-Protein interaction (PPI) network

The differentially expressed genes were analysed with Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org/). STRING is an online database for predicting functional interactions between proteins. The minimum required interaction score was defined at 0.40.

Statistical analysis

Differences in demographic and clinical measures between groups was assessed with an independent t-test for continuous variables and a Chi-squared test for categorical variables. Differential gene expression analysis was performed on normalised data using the nSolver Advanced analysis software version 4.0 (NanoString technologies, Seattle, WA) with the Bonferroni Hochberg p-value correction (padjust) with significance accepted at $p<0.05$.

Correlations between clinical features and differentially expressed gene counts were performed using the Pearson correlation coefficient with significance accepted at $p<0.05$.

Results:

Study participants

The demographic and clinical characteristics of the cohort are included in Table 1. The groups were matched in key physical attributes. However consistent with diagnosis of atopic conditions, the AR group had significantly higher white blood cell, lymphocyte, eosinophil and basophil counts compared to the control group (CG). The AR group had moderate symptoms based on the symptom severity questionnaires, with the majority sensitised to both plant pollens and dust mites (Table 2). Several participants also reported allergies other than AR; a total of 49% of the cohort reported a history of skin allergies (eczema, hand dermatitis, urticaria and itchy rash), 27% also reported a history of food allergy, and 11% also reported a history of drug allergy (including Codeine (opiod), antibiotics and metoclopramide (dopamine D_2 receptor antagonist/5-HT_3 receptor antagonist/5-HT_4 receptor agonist). Nasal lysate samples from 37 AR participants and 21 CG participants met the quality control guidelines and were included in gene expression analysis. The demographic and clinical characteristics of the participants whose nasal lysate samples were included in the gene expression analysis were similar to that of the entire study cohort and are included in Supplementary Tables 1 and 2.

Differentially expressed genes between AR and non-AR controls

Blood

Of the 760 immune genes tested on the NanoString arrays, 466 were expressed above background noise and were included in the subsequent analyses. Gene expression changes in all genes are summarised in Figure 1 (left panel). In total 175 genes were differentially expressed between the AR and CG cohorts based on $p<0.05$ and 113 genes were differentially expressed after controlling for FDR (padjust) (Supplementary Table 3). The top 20 differentially expressed genes according to p value are shown in Table 3. Of the 113 differentially expressed genes, 35 genes were upregulated (1.73 Log2 FC to 0.195 Log2 FC) and 78 genes were downregulated compared to the control group (-0.674 log2 to -0.119 Log2 FC).

Nasal lysate

A total of 474 immune genes were expressed above background noise and were included in the subsequent analyses. Gene expression changes in all genes are summarised in Figure 1 (right panel). In total 63 genes were differentially expressed between the AR and CG cohorts based on $p<0.05$ and 14 genes were differentially expressed after controlling for false discovery rate (Supplementary Table 4). The top 20 differentially expressed genes according to p value are shown in Table 3. Of the 14

differentially expressed genes, 12 genes were upregulated (1.13 Log2 FC to 3.41 Log2 FC) and 2 genes were downregulated compared to the control group (-1.65 log2 to -0.568 Log2 FC).

Enrichment of the differentially expressed genes into pathways

Blood

The differentially expressed genes were significantly enriched into 66 Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways. The top four KEGG pathways were toll-like receptor signalling pathway, cytokine-cytokine receptor interaction, osteoclast differentiation and chagas disease (American trypanosomiasis) (Table 4). The differentially expressed genes were also enriched into 189 DAVID genetic association database (GAD) disease pathways. The top four GAD disease pathways include type 2 diabetes/edema/rosiglitazone, respiratory syncytial virus bronchiolitis, asthma/bronchiolitis, viral/respiratory syncytial virus infections and bronchiolitis, viral/respiratory syncytial virus infections (Table 5).

Nasal lysate

The differentially expressed genes were significantly enriched into 3 KEGG pathways. These KEGG pathways include cytokine-cytokine receptor interaction, hematopoietic cell lineage and chemokine signalling pathway (Table 4). The differentially expressed genes were also enriched into 17 DAVID GAD pathways. The top four GAD disease pathways include asthma, asthma/bronchiolitis, viral/respiratory syncytial virus infections, respiratory syncytial virus bronchiolitis and bronchiolitis, viral/respiratory syncytial virus infections (Table 5).

Investigation of the differentially expressed genes with Protein-Protein Interaction networks

Blood

The differentially expressed gene list was submitted to the STRING data base to provide a better understanding of the biological relationship between the DEGs. A total of 112 genes were included in the PPI network with an average node degree of 4.98. As shown in Figure 2, the PPI is very complex and shows a high level of interaction between the DEGs. The top 20 nodes are listed in Table 6.

Nasal lysate

A total of 14 genes were included in the PPI network (Figure 3) with an average node degree of 0.286. Limited interactions between the differentially expressed genes was observed. As shown in Figure 3, a single interaction pathway between PPBP and PTGDR2 and CCL17 was detected.

Investigation of the relationship between differentially expressed genes and clinical markers

Blood

The relationship between clinical measures and the differentially expressed genes were explored. As shown in Figure 4, eosinophils were on average moderately correlated with the DEGs (mean R=0.30). Whole blood cell counts of total white cells, eosinophils, neutrophils and lymphocytes as well as specific IgE and IgG4 were on average weakly (mean R=0.1 to 3) associated with the DEG counts. In contrast, self-reported symptom severity was very weakly (mean R = 0 to 1) associated with the DEGs. The top 10 individual correlations are shown in Supplementary Figure 2.

Nasal lysate

Correlation analysis between the clinical factors and the nasal lysate DEGs was also performed. As shown in Figure 5, gene expression in the nasal lysate samples was on average moderately correlated with eosinophils (mean R =0.45) and weakly correlated with total white cells (R=0.22), lymphocytes (R=0.22), and basophils (R=0.22). In addition, the DEGs in nasal lysate samples were on average weakly correlated with specific IgE for dust mites (R=0.28 and 0.29) and IgG4 for dust mites (R=0.25 and 0.26). None or very weak (R= 0 to 0.1) correlations between BMI, neutrophils, monocytes, ESR and self-reported symptom severity and DEG counts was observed. The top 10 individual correlations are shown in Figure 6.

Discussion:

The results presented here provide further insights into the pathophysiology of AR at both the local site of the allergic response (nasal mucosa) and the systemic immune system. In this study, genes and pathways that separate AR samples from controls were identified and can be explored as potential biomarkers of disease. This study provides new information about the pathophysiology of AR through the identification of genes that have not been previously associated with AR or atopy. In addition, this study supports the findings of previous studies and confirms the role of specific genes encoding prostaglandin receptors, chemokines TARC and eotaxin, and tryptase in the pathophysiology of AR. Evidence for interactions between the mucosal and systemic immune system during active disease is also provided in this study.

A key finding of this study was the large proportion of differentially expressed genes in blood samples from AR sufferers compared with non-AR controls. A total of 113 DEGs were identified in blood samples which represents over 24% of genes that were expressed above background, thereby indicating that the gene-expression profiles of blood samples from AR sufferers is vastly different from that of the control samples. In contrast, only 14 DEGs (approx. 3% of genes expressed above background) were observed between the groups in the nasal mucosal samples. The relatively low number of DEGs in nasal lysate samples compared to blood samples is likely due to the large variability in mRNA expression across nasal lysate samples. The total mRNA quantity, quality and numbers of infiltrating immune cells varied across samples and this occurrence has been previously reported using this methodology (15). The nasal lavage and nasal brushing technique used in our study was self-administered by the participants and as a result can introduce heterogeneity in the sample collection. In addition, the nasal mucosa unlike the circulatory system, is an open-system and is more responsive to environmental stimulus (e.g. allergen exposure, temperature changes, pollutants, airborne microbes) which could drive sporadic changes in gene expression. As such, gene expression profiles in the nose are likely to vary depending on the individual and time of sample collection. Collectively, the self-administered sample collection technique and responsive/shifting nature of the nasal mucosa has the potential to induce large variability in mRNA expression between samples.

Diagnosis of AR is typically formed based on patient history and physical examination. The skin prick test or allergen specific IgE tests are employed to identify the specific allergens that trigger AR symptoms. Diagnosis of AR is confounded by the common occurrence of positive allergen-specific IgE or skin prick test to allergens in non-symptomatic patients (16) and the other chronic respiratory diseases/rhinitis endotypes that share similar symptoms and clinical presentation, but have different underlying pathophysiology (i.e. non-allergic rhinitis, rhinosinusitis, nasal polyposis, and infectious rhinitis). Biological markers are useful in providing objective measures of disease phenotypes. Gene expression profiling provides a useful means to investigate the molecular mechanisms underpinning

allergic disease and the identification of disease specific biomarkers. As such, the DEGs were explored as potential AR-specific biomarkers.

Genes that separate AR participants from non-AR participants the greatest were further explored. The top three DEGs in the blood samples include Mitogen-Activated Protein Kinase 1 (MAPK1), TANK binding kinase 1 (TBK1) and the Prostaglandin D2 receptor (PTGDR2). Both MAPK1 and TBK1 genes were downregulated in blood samples from AR participants compared with the controls. The MAPK1 gene encodes MAP kinases, which are involved in a wide range of cellular processes including proliferation, differentiation and regulation of transcription. In the context of allergy, MAPK1 is an integral component of IL-33 signalling, an epithelial produced cytokine known for its role in activating the Th2 response (16, 17). Similar to MAPK1, the TBK1 gene is also involved in gene transcription. TBK1 is associated with the activation and nuclear translocation of the NFκB transcription factor complex (17). The NFκB transcription factor is responsible for the transcription of many of pro-inflammatory genes and therefore the downregulation of TBK1 in AR samples was an unexpected result. The PTGDR2 gene was significantly upregulated in both blood and nasal lysate samples. Interaction of PGD2 with PTGDR2 stimulates the activation and chemotaxis of key inflammatory cells pertinent to the allergic response including eosinophils, basophils and Th2 cells (18). Increased numbers of effector cells in the nasal mucosa would likely lead to worsened symptoms. The top three DEGs in nasal lysate samples included two CC-chemokines (CCL17 and CCL26) and TPSAB1. These genes have been previously associated with allergic disease. CCL17 (also known as TARC) and CCL26 (also known as Eotaxin-3) are involved in the chemotaxis of Th2 cells, and eosinophils and basophils, respectively (19, 20). The upregulation of CCL17 and CCL26 gene expression in this study is consistent with increased protein production of these chemokines reported in atopic cohorts (21, 22). Increased CCL17 levels have been previously reported in sputum and serum samples of asthma sufferers (comorbidity of AR) compared with non-allergic controls (21). Similarly, increased levels of CCL26 have been reported in nasal secretions and nasal lavage samples in seasonal AR participants during the pollen season and following nasal allergen challenge (22). Higher levels of CCL26 were also reported in seasonal AR sufferers compared with non-allergic controls (22) which is consistent with the findings in our current study. The TPSAB1 gene encodes tryptase (alpha-1 and beta-1) enzymes which are released from mast cells and basophils upon activation. Increased tryptase levels have been reported in nasal lavage samples from seasonal AR sufferers following nasal allergen challenge (23). Notably, increased tryptase levels were not observed in the control group following allergen challenge.

In this study we identified DEGs that were unique to both nasal lysate samples and the blood samples and genes that were differentially expressed in both sample types (Supplementary Figure 1). A large proportion of the nasal lysate DEGs (6 of 8, 75%) including RUNX3, IL1RL1, PTGDR2, FLT3LG, CTSH and PPBP were also significantly differentially expressed in blood samples. The common DEGs

in both sample types, are involved in the differentiation of multiple cell lineages and receptors/mediators involved in the chemotaxis of effector cells involved in the allergic response. The DEGs unique to nasal lysate samples are related to mediator release from mast cells, antigen presentation to T cells and chemokine release to induce chemotaxis of inflammatory cells. The DEGs unique to blood samples are primarily involved in cytokine signalling, toll-like receptor cascades and neutrophil degranulation. Collectively, these findings indicate that the nasal mucosal immune system and systemic immune system potentially have both individual and shared roles in the pathogenesis of AR.

The DEGs in blood and nasal lysate samples were further analysed for enrichment into KEGG and GAD pathways. The DEGs in blood were enriched into 66 KEGG pathways. Of these enriched KEGG pathways, the top four pathways included cytokine-cytokine receptor interaction, toll-like receptor signalling, Chagas disease and osteoclast differentiation. These results were not entirely unexpected given that atopic diseases have been previously associated with cytokine-cytokine receptor interaction, toll-like receptor signalling, osteoclast differentiation and pathways related to parasitic infections (24-27). Interestingly, the DEGs from the nasal lysate samples were enriched into similar immune pathways as the blood samples including cytokine-cytokine receptor interaction and chemokine-chemokine signalling. The DEGs from the nasal lysate samples were also enriched into the hematopoietic cell lineage pathway which is consistent with the role of the nasal olfactory epithelium as a source of progenitor cells (28) and the airway allergic response stimulates the production of effector cells. Interestingly, the GAD pathway analysis revealed that both the DEGs from the blood samples and nasal lysate samples were enriched in disease pathways primarily affecting the respiratory system. The top four enriched disease pathways for both the blood and nasal lysate samples included asthma, bronchiolitis and viral respiratory disease. This data suggests that both sample types can be used as markers to identify respiratory allergic disease as well as other conditions affecting the airways.

Protein-Protein Interaction networks predict direct and indirect functional interactions between expressed proteins and provide a system-wide understanding of cellular function under selected conditions. The differentially expressed genes in nasal lysate and blood samples were analysed with the STRING database to identify the key genes as drivers of the allergic response. The top three genes in the protein-protein interaction network for blood samples according to degree where UBC, MAPK1 and APP, and have been previously associated with atopic disease. The metalloproteinase 33 enzyme encoded by the ADAM33 gene is a recognised allergy candidate gene and is known to cleave peptides of the amyloid precursor protein (APP). Similarly, the UBC and MAPK1 genes have been linked with asthma, which is a common comorbidity of allergic rhinitis (29, 30). The PPI network of the nasal lysate DEGs had much fewer interactions (and average node degree) than the blood DGE network. A single interaction between pro-platelet basic protein (PPBP) and prostaglandin D2 receptor 2 (PTGDR2) and

chemokine (C-C motif) ligand (CCL17) was observed. The role of PTGDR2 and CCL17 (TARC) in the pathogenesis of allergic disease is well-recognised (31, 32). The PPBP gene is a known chemoattractant and activator of neutrophils (33) and its downregulation in the nasal mucosa of AR participants has not been previously reported.

The relationship between clinical measures, including white cell differential, specific IgE and IgG4, ESR, demographic features and symptom severity, and the DEG counts in blood and nasal lysate samples were explored to provide a greater understanding of the role of these genes in the pathogenesis of AR. None of the patient-reported symptom severity measures (mRQLQ, VAS, TNSS, TOSS and OARSS) correlated with counts of the DEGs in both blood and nasal lysate samples. Response bias in self-reported data is well-recognised event known to confound data interpretation (34) and may explain the lack of correlation observed between the subjective symptom severity and gene expression markers. Indeed, self-reported symptoms and quality of life have been shown to have poor associations with objective measures of disease severity in chronic rhinosinusitis (35, 36) which is a co-morbid condition of AR.

While no relationship between the symptom severity measures and DEG counts was reported, moderate correlations between the DEG counts in both blood and nasal lysate samples and other clinical markers including peripheral blood immune cell counts and serum specific IgE and IgG4 levels were observed. Overall, the association between clinical markers in blood and counts of DEGs in the nasal mucosa, provide greater support for an interaction between the systemic and mucosal immune system in the pathogenesis of AR. Indeed, changes in the activation of peripheral blood leukocytes and peripheral immune gene signatures have been observed following local nasal allergen challenge, indicating that allergen exposure at the local mucosal site can promote changes to at the systemic immune level (8, 37). This data further provides evidence that the blood and nasal mucosa function as separate immune compartments with shared roles in the pathogenesis of AR.

The top 10 specific associations between clinical markers measured in blood and DEG counts in nasal mucosal samples were further explored. Peripheral blood eosinophil counts were positively correlated with PTGDR2, IL1RL1, FLT3LG, RUNX3, PTGS1 and CCND3 gene expression counts in nasal lysate samples. The PTGS1, PTGDR2 and IL1RL1 gene have been previously associated with eosinophils (38-40). However, the relationship between eosinophils and the FLT3LG, RUNX3 and CCND3 genes are more complex and provides greater insight into the subsidiary interactions occurring in the pathophysiology of AR. The FLT3LG, RUNX3 and CCND3 genes were upregulated in nasal mucosal samples and share roles in the differentiation of multiple immune cell lineages (41-46), including eosinophils. Increased eosinophil production may occur as a direct effect of these genes or as an indirect effect through the production of other effector cells (Th2 cells, dendritic cells, ILC2 cells, neutrophils)

whose products then interact with eosinophils. The CCND3 and IL18R1 genes were weakly positively correlated with dust mite IgE levels. The IL1R1 gene has been directly associated with total IgE levels (47), and have been previously linked with asthma and atopic phenotypes (48, 49). The relationship between CCND3 and IgE levels is not as clear but may be related to B cell maturation by CCND3 and the production of IgE antibodies by plasma B cells.

This study identified DEGs in nasal lysate samples and blood samples that could serve as disease specific biomarkers. The genetic biomarkers identified in this study could form the basis for a cost-effective specialised reverse-transcription PCR based gene expression panel to refine the diagnosis of AR. Large scale studies are needed to determine normative gene expression values for rhinitis endotypes. In future, non-invasive gene expression testing may be used as an additional means to diagnose AR.

Overall, investigation of the peripheral blood and nasal mucosa with the NanoString nCounter system revealed distinct gene expression profiles in our AR cohort compared with controls. Notably, the peripheral blood DEGs represented a large proportion of the total immune genes, indicating the unique nature of the systemic immune system in AR compared with controls. The shared DEGs in the nasal lysate and blood samples and the associations between clinical markers in blood and gene expression in the nasal mucosa, points to a strong interaction between these immune compartments in the pathogenesis of AR. The AR specific genes and pathways identified in this study may be used as biological markers in the diagnosis of AR, as potential new drug targets for reduction of AR symptoms, and as markers to evaluate the effectiveness of systemic and topical AR drugs.

Acknowledgments

The authors acknowledge the support of the Queensland Allergy Services Clinic for providing clinic access and for performing the skin prick tests

References

1. Bjorksten B, Clayton T, Ellwood P, Stewart A, Strachan D, Group IPIS. Worldwide time trends for symptoms of rhinitis and conjunctivitis: Phase III of the International Study of Asthma and Allergies in Childhood. *Pediatr Allergy Immunol* 2008;**19**(2):110-124.
2. Fokkens WJ, Hellings PW. Rhinology in the forefront of European political attention. *Rhinology* 2015;**53**(4):289.
3. Australian Institute of Health and Welfare 2011. Allergic rhinitis ('hay fever') in Australia. Cat. no. ACM 23. Canberra: AIHW
4. WAO white book on allergy: update 2013 executive summary. Milwaukee (WI): WAO; 2013.
5. Zuberbier T, Lotvall J, Simoens S, Subramanian SV, Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2) LEN review. *Allergy* 2014;**69**(10):1275-1279.
6. Cook M, Douglass J, Mallon D, Mullins R, Smith J, M W. The ecconomic impact of Allergic Disease in Australia: Not to Be Sneezed At. *Australian Society of Clinical Immunology and Allergy* 2007.
7. Marcellusi A, Viti R, Incorvaia C, Mennini FS. Direct and indirect costs associated with respiratory allergic diseases in Italy. A probabilistic cost of illness study. *Recenti Prog Med* 2015;**106**(10):517-527.
8. Shamji M, Bellido V, Scadding G, Layhadi J, Cheung D, Calderon M, et al. Effector cell signature in peripheral blood following nasal allergen challenge in grass pollen allergic individuals. *Allergy* 2015;**70**(2):171-179.
9. Benson M, Svensson PA, Carlsson B, Jernås M, Reinholdt J, Cardell LO, et al. DNA microarrays to study gene expression in allergic airways. *Clin Exp Allergy* 2002;**32**(2):301-308.
10. Zhang RX, Yu SQ, Jiang JZ, Liu GJ. Complementary DNA microarray analysis of chemokines and their receptors in allergic rhinitis. *J Investig Allergol Clin Immunol* 2007;**17**(5):329-336.
11. Benson M, Jansson L, Adner M, Luts A, Uddman R, Cardell LO. Gene profiling reveals decreased expression of uteroglobin and other anti-inflammatory genes in nasal fluid cells from patients with intermittent allergic rhinitis. *Clin Exp Allergy* 2005;**35**(4):473-478.
12. Council M. Australian guidelines to reduce health risks from drinking alcohol. *NHMRC Canberra* 2009.
13. Bouchery EE, Harwood HJ, Sacks JJ, Simon CJ, Brewer RD. Economic Costs of Excessive Alcohol Consumption in the U.S., 2006. *Am J Prev Med.* 2011;**41**(5):516-524.
14. K. G. Von Boroviczeny LEB, B. S. Bull, A. Chattas, J. B. Dawson, K. Fukutake, F. W. Gunz, J. A. Koepke, S. M. Lewis, E. Rewald, M. Roubicek, G. Ruthenstroth-Bauer. Recommendation of measurement of erythrocyte sedimentation rate of human blood. *Am J Clin Pathol* 1977;**68**(4):505-507.
15. Watts AM, West NP, Cripps AW, Smith PK, Cox AJ. Distinct Gene Expression Patterns between Nasal Mucosal Cells and Blood Collected from Allergic Rhinitis Sufferers. *Int Arch Allergy Immunol* 2018;**177**(1):29-34.
16. Bousquet J, Anto JM, Bachert C, Bousquet PJ, Colombo P, Crameri R, et al. Factors responsible for differences between asymptomatic subjects and patients presenting an IgE sensitization to allergens. A GA2LEN project. *Allergy* 2006;**61**(6):671-680.
17. Liu T, Zhang L, Joo D, Sun S-C. NF-κB signaling in inflammation. *Signal Transduct Target Ther* 2017;**2**:17023.
18. Strasser DS, Farine H, Holdener M, Zisowsky J, Roscher R, Hoerner J, et al. Development of a decision-making biomarker for CRTH2 antagonism in clinical studies. *New Horiz Transl Med* 2015;**2**(4-5):118-125.
19. Lambrecht BN, Hammad H. The role of dendritic and epithelial cells as master regulators of allergic airway inflammation. *Lancet* 2010;**376**(9743):835-843.
20. Kitaura M, Suzuki N, Imai T, Takagi S, Suzuki R, Nakajima T, et al. Molecular cloning of a novel human CC chemokine (Eotaxin-3) that is a functional ligand of CC chemokine receptor 3. *J Biol Chem* 1999;**274**(39):27975-27980.
21. Sekiya T, Yamada H, Yamaguchi M, Yamamoto K, Ishii A, Yoshie O, et al. Increased levels of a TH2-type CC chemokine thymus and activation-regulated chemokine (TARC) in serum and induced sputum of asthmatics. *Allergy* 2002;**57**(2):173-177.

22. Baumann R, Rabaszowski M, Stenin I, Tilgner L, Scheckenbach K, Wiltfang J, et al. Comparison of the Nasal Release of IL-4, IL-10, IL-17, CCL13/MCP-4, and CCL26/eotaxin-3 in Allergic Rhinitis during Season and after Allergen Challenge. *Am J Rhinol Allergy* 2013;**27**(4):266-272.
23. Juliusson S, Holmberg K, Baumgarten CR, Olsson M, Enander I, Pipkorn U. Tryptase in nasal lavage fluid after local allergen challenge: Relationship to histamine levels and TAME-esterase activity. *Allergy* 1991;**46**(6):459-465.
24. Pawankar R, Hayashi M, Yamanishi S, Igarashi T. The paradigm of cytokine networks in allergic airway inflammation. *Curr Opin Allergy Clin Immunol* 2015;**15**(1):41-48.
25. Fransson M, Adner M, Erjefält J, Jansson L, Uddman R, Cardell L-O. Up-regulation of Toll-like receptors 2, 3 and 4 in allergic rhinitis. *Respir Res* 2005;**6**(1):100.
26. Persson H, Kwon AT, Ramilowski JA, Silberberg G, Söderhäll C, Orsmark-Pietras C, et al. Transcriptome analysis of controlled and therapy-resistant childhood asthma reveals distinct gene expression profiles. *J Allergy Clin Immunol* 2015;**136**(3):638-648.
27. Amoah AS, Boakye DA, Yazdanbakhsh M, van Ree R. Influence of Parasitic Worm Infections on Allergy Diagnosis in Sub-Saharan Africa. *Curr Allergy Asthma Rep* 2017;**17**(10):65-65.
28. Winstead W, Marshall CT, Lu CL, Klueber KM, Roisen FJ. Endoscopic biopsy of human olfactory epithelium as a source of progenitor cells. *Am J Rhinol* 2005;**19**(1):83-90.
29. Celedón JC, Soto-Quiros ME, Avila L, Lake SL, Liang C, Fournier E, et al. Significant linkage to airway responsiveness on chromosome 12q24 in families of children with asthma in Costa Rica. *Human Genet* 2007;**120**(5):691-699.
30. Hwang S, Son S-W, Kim SC, Kim YJ, Jeong H, Lee D. A protein interaction network associated with asthma. *J Theor Biol* 2008;**252**(4):722-731.
31. Sandoval-López G, Teran LM. TARC: novel mediator of allergic inflammation. *Clin Exp Allergy* 2001;**31**(12):1809-1812.
32. Marone G, Galdiero MR, Pecoraro A, Pucino V, Criscuolo G, Triassi M, et al. Prostaglandin D2 receptor antagonists in allergic disorders: safety, efficacy, and future perspectives. *Expert Opin Investig Drugs* 2019;**28**(1):73-84.
33. Di Stefano A, Caramori G, Gnemmi I, Contoli M, Bristot L, Capelli A, et al. Association of increased CCL5 and CXCL7 chemokine expression with neutrophil activation in severe stable COPD. *Thorax* 2009;**64**(11):968-975.
34. Rosenman R, Tennekoon V, Hill LG. Measuring bias in self-reported data. *Int J Behav Healthc Res* 2011;**2**(4):320.
35. Lam M, Hull L, McLachlan R, Snidvongs K, Chin D, Pratt E, et al. Clinical severity and epithelial endotypes in chronic rhinosinusitis. In: International forum of allergy & rhinology; 2013: Wiley Online Library; 2013. p. 121-128.
36. Stewart MG, Smith TL. Objective versus subjective outcomes assessment in rhinology. *Am J Rhinol* 2005;**19**(5):529-535.
37. Kim YW, Singh A, Shannon CP, Thiele J, Steacy LM, Ellis AK, et al. Investigating Immune Gene Signatures in Peripheral Blood from Subjects with Allergic Rhinitis Undergoing Nasal Allergen Challenge. *J Immunol* 2017;**199**(10):3395-3405.
38. Savenije OE, Kerkhof M, Reijmerink NE, Brunekreef B, de Jongste JC, Smit HA, et al. Interleukin-1 receptor–like 1 polymorphisms are associated with serum IL1RL1-a, eosinophils, and asthma in childhood. *J Allergy Clin Immunol* 2011;**127**(3):750-756. e755.
39. Schratl P, Royer JF, Kostenis E, Ulven T, Sturm EM, Waldhoer M, et al. The role of the prostaglandin D2 receptor, DP, in eosinophil trafficking. *J Immunol* 2007;**179**(7):4792-4799.
40. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* 2001;**193**(2):255-261.
41. Hannum C, Culpepper J, Campbell D, McClanahan T, Zurawski S, Kastelein R, et al. Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. *Nature* 1994;**368**:643.
42. Lyman SD, Jacobsen SEW. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood* 1998;**91**(4):1101-1134.

549 43. Diener KR, Moldenhauer LM, Lyons AB, Brown MP, Hayball JD. Human Flt-3-ligand-
550 mobilized dendritic cells require additional activation to drive effective immune responses. *Exp*
551 *Hematol* 2008;**36**(1):51-60.
552 44. Yu Y, Wang L, Gu G. The correlation between Runx3 and bronchial asthma. *Clinica Chimica*
553 *Acta* 2018;**487**:75-79.
554 45. Sankaran VG, Ludwig LS, Sicinska E, Xu J, Bauer DE, Eng JC, et al. Cyclin D3 coordinates
555 the cell cycle during differentiation to regulate erythrocyte size and number. *Genes Dev*
556 2012;**26**(18):2075-2087.
557 46. Cooper AB, Sawai CM, Sicinska E, Powers SE, Sicinski P, Clark MR, et al. A unique function
558 for cyclin D3 in early B cell development. *Nat Immunol* 2006;**7**(5):489-497.
559 47. Mete F, Ozkaya E, Aras S, Koksal V, Etlik O, Baris I. Association between gene
560 polymorphisms in TIM1, TSLP, IL18R1 and childhood asthma in Turkish population. *Int J Clin Exp*
561 *Med* 2014;**7**(4):1071-1077.
562 48. Zhu G, Whyte MK, Vestbo J, Carlsen K, Carlsen K-H, Lenney W, et al. Interleukin 18 receptor
563 1 gene polymorphisms are associated with asthma. *Eur J Hum Genet* 2008;**16**(9):1083.
564 49. Reijmerink NE, Postma DS, Bruinenberg M, Nolte IM, Meyers DA, Bleecker ER, et al.
565 Association of IL1RL1, IL18R1, and IL18RAP gene cluster polymorphisms with asthma and atopy. *J*
566 *Allergy Clin Immunol* 2008;**122**(3):651.

567

568

569 **Tables**

570

571 Table 1: Clinical and demographic features of the study cohort

	AR mean ± SD	**CG mean ± SD**	**P value**
n	45	24	-
Age (years)	37.58 ± 12.82	36.57 ± 10.22	0.74
Sex (M/F)	16/29 (64% F)	11/13 (54% F)	0.405
Height (cm)	171.30 ± 9.33	175.11 ± 9.52	0.113
Weight (kg)	74.40 ± 15.11	75.51 ± 19.40	0.808
BMI (kg/m^2)	25.18 ± 3.73	24.34 ± 4.63	0.411
Ethnicity (% Caucasian)	78%	96%	0.051
Immune measures			
White cell count ($x10^9/L$)	6.60 ± 1.82	5.58 ± 1.27	0.017
Lymphocytes ($x10^9/L$)	2.18 ± 0.73	1.83 ± 0.59	0.045
Eosinophils ($x10^9/L$)	0.41 ± 0.30	0.11 ± 0.08	<0.00001
Neutrophils ($x10^9/L$)	3.45 ± 1.15	3.15 ± 0.97	0.273
Basophils ($x10^9/L$)	0.06 ± 0.04	0.04 ± 0.03	0.025
ESR (mm/hr)	9.11 ± 9.11	7.38 ± 7.64	0.429

572

573 n, number; AR, allergic rhinitis; CG, Control Group; BMI, Body Mass Index; M, Male; F, Female;
574 cm, centimetre; kg, kilogram; m, metre; %, percentage; L, litre; mm, millimetre; hr, hour

575

576 Table 2: Disease characteristics of the primary AR cohort (blood samples)

Disease characteristic	**AR (mean ± SD)**
Allergen sensitivity	
Co-allergy to dust mites and pollen (%)	60%
Dust mite only (%)	40%
IgE *D. pteronyssinus* (kU/L)	24.05 ± 31.94
IgE *D. farinae* (kU/L)	19.90 ± 29.21
IgE grass pollen mix (kU/L)	7.30 ± 20.33
IgG4 *D. pteronyssinus* (kU/L)	0.46 ± 0.46
IgG4 *D. farinae* (kU/L)	0.37 ± 0.35
IgG4 grass pollen mix (kU/L)	0.86 ± 0.72
Symptom severity	
Total Nasal Symptom Score (0-12 U)	5.4 ± 3.26
Total Ocular Symptom Score (0-9 U)	2.71 ± 2.40
Mini rhinoconjunctivitis quality of life score (0-6 U)	2.8 ± 1.06
Other Allergic Rhinitis Symptom Score (0-12 U)	4.04 ± 3.27
Visual Analogue Scale (0-100 mm)	52.47 ± 27.94

577

578 AR, allergic rhinitis; %, percentage; kU, kilounit; L, Litre; U, unit; mm, millimetre

579

580 Table 3. Top 20 differentially expressed genes for blood and nasal lysate samples

Gene	Log2 fold change	Linear fold change	Lower confidence limit (log2)	Upper confidence limit (log2)	P value	P adjust
Blood						
MAP2K1	-0.417	0.749	-0.512	-0.322	1.84E-12	9.86E-10
TBK1	-0.608	0.656	-0.769	-0.447	2.90E-10	5.18E-08
PTGDR2	1.730	3.310	1.270	2.180	2.78E-10	5.18E-08
CD83	0.979	1.970	0.681	1.280	1.53E-08	1.65E-06
CD164	-0.203	0.869	-0.266	-0.140	2.47E-08	2.21E-06
CD24	1.320	2.500	0.903	1.740	4.22E-08	3.24E-06
IL2RA	0.946	1.930	0.601	1.290	1.06E-06	7.12E-05
MICA	0.711	1.640	0.441	0.980	2.31E-06	1.38E-04
IL12RB1	0.527	1.440	0.318	0.736	5.31E-06	2.85E-04
ABCB1	-0.544	0.686	-0.764	-0.325	7.21E-06	3.52E-04
LILRA1	-0.484	0.715	-0.681	-0.287	8.71E-06	3.90E-04
HMGB1	-0.177	0.884	-0.250	-0.104	1.02E-05	4.23E-04
NCR1	-0.674	0.627	-0.958	-0.391	1.51E-05	5.79E-04
IFNGR1	-0.350	0.785	-0.500	-0.200	2.20E-05	7.40E-04
IRF3	0.527	1.440	0.300	0.753	2.20E-05	7.40E-04
TNFAIP3	-0.275	0.827	-0.393	-0.156	2.42E-05	7.65E-04
HRH4	0.896	1.860	0.507	1.280	2.64E-05	7.88E-04
IKBKG	-0.303	0.811	-0.436	-0.171	2.94E-05	8.31E-04
APP	-0.536	0.690	-0.774	-0.298	3.68E-05	9.08E-04
PSEN1	-0.292	0.817	-0.422	-0.163	3.61E-05	9.08E-04
Nasal lysate						
CCL17	2.84	7.14	2.03	3.64	4.41E-09	2.51E-06
CCL26	2.72	6.61	1.82	3.63	2.39E-07	5.13E-05
TPSAB1	3.08	8.45	2.05	4.11	2.71E-07	5.13E-05
PTGS1	2.41	5.30	1.49	3.33	4.01E-06	4.57E-04
IL1RL1	3.41	10.70	1.97	4.86	2.14E-05	2.02E-03
CD1A	2.46	5.52	1.40	3.53	3.05E-05	2.48E-03
CCND3	1.13	2.19	0.57	1.69	2.36E-04	1.68E-02
PPBP	-1.65	0.32	-2.51	-0.78	4.60E-04	2.91E-02
IL18R1	1.96	3.89	0.88	3.04	7.53E-04	3.89E-02
CD1C	1.56	2.96	0.68	2.44	9.70E-04	4.04E-02
CTSH	-0.57	0.68	-0.89	-0.25	1.01E-03	4.04E-02
FLT3LG	1.16	2.23	0.50	1.81	1.06E-03	4.04E-02
RUNX3	1.68	3.21	0.74	2.63	9.63E-04	4.04E-02
PTGDR2	2.44	5.43	1.04	3.84	1.17E-03	4.15E-02
IL13	1.61	3.06	0.63	2.59	2.11E-03	6.66E-02
JUN	-0.83	0.56	-1.34	-0.31	2.63E-03	7.14E-02
TXNIP	0.89	1.85	0.34	1.44	2.45E-03	7.14E-02
KLRB1	-1.47	0.36	-2.39	-0.55	2.78E-03	7.20E-02

CXCR3	1.38	2.60	0.51	2.25	3.06E-03	7.58E-02
CARD9	1.29	2.45	0.46	2.12	3.50E-03	8.31E-02

581

582

583 Table 4: enriched KEGG pathways blood and nasal lysate samples

KEGG Pathway	Count	Percentage	P value	Adjust. P value (Benjamini)
Blood				
Cytokine-cytokine receptor interaction	23	20.4	4.3E-12	3.5EE10
Osteoclast differentiation	18	15.9	4.9E-12	2.7E-10
Toll-like receptor signaling pathway	17	15.0	2.0E-12	3.3E-10
Chagas disease (American trypanosomiasis)	16	14.2	2.1E-11	8.5E-10
Nasal lysate				
Cytokine-cytokine receptor interaction	4	28.6	3.1E-3	6.6E-2
Hematopoietic cell lineage	3	21.4	5.4E-3	5.8E-2
Chemokine signalling pathway	3	21.4	2.3E-2	1.6E-1

584

585 Table 5: GAD disease pathways blood and nasal lysate samples

GAD Disease pathways	Count	Percentage	P value	Adjust P value (Benjamini)
Blood				
Type 2 Diabetes| edema | rosiglitazone	56	49.6	2.1E-16	6.3E-14
respiratory syncytial virus bronchiolitis	28	24.8	1.8E-25	2.1E-22
Asthma|Bronchiolitis, Viral|Respiratory Syncytial Virus Infections	27	23.9	4.3E-24	2.4E-21
Bronchiolitis, Viral|Respiratory Syncytial Virus Infections	27	23.9	1.0E-23	2.4E-21
Nasal lysate				
Asthma	5	35.7	8.6E-4	5.3E-2
Asthma/Bronchiolitis, Viral/Respiratory Syncytial Virus Infections	4	28.6	9.8E-4	4.0E-2
Respiratory syncytial virus bronchiolitis	4	28.6	9.8E-4	4.0E-2
Bronchiolitis, Viral/Respiratory Syncytial Virus infection	4	28.6	1.1E-3	3.3E-2

586

587

588 Table 6 – Top 20 genes in the Protein Protein Interaction network by degree (blood samples)

Gene	Interactions/degree
UBC	19
MAPK1	15
APP	14
EP300	14
JUN	14
CHUK	13
NFKBIA	13
STAT3	13
FYN	12
IL2RA	12
IRF3	12
ITGB2	12
CCL5	11
HLA-E	11
HMGB1	11
IKBKG	11
PF4	10
PPBP	10
SYK	10
TNFAIP3	10

589

590

Figure Legends

Figure 1. Volcano plot summarising the differential gene expression. The blood samples are on the left panel and the nasal lysate samples are on the right panel. Fold changes greater than zero indicate increased gene expression in AR samples compared to the control group. Dotted horizonal line indicates significance at p value 0.05.

Figure 2. The protein-protein interaction network of differentially expressed genes. The line thickness of the network edges indicates the strength of confidence in the interaction (the thicker the line, the greater the confidence in the interaction). Filled nodes; some 3D structure is known or predicted. Empty nodes; proteins of unknown 3D structure. The top 20 nodes are shown by a red box outlining the gene name.

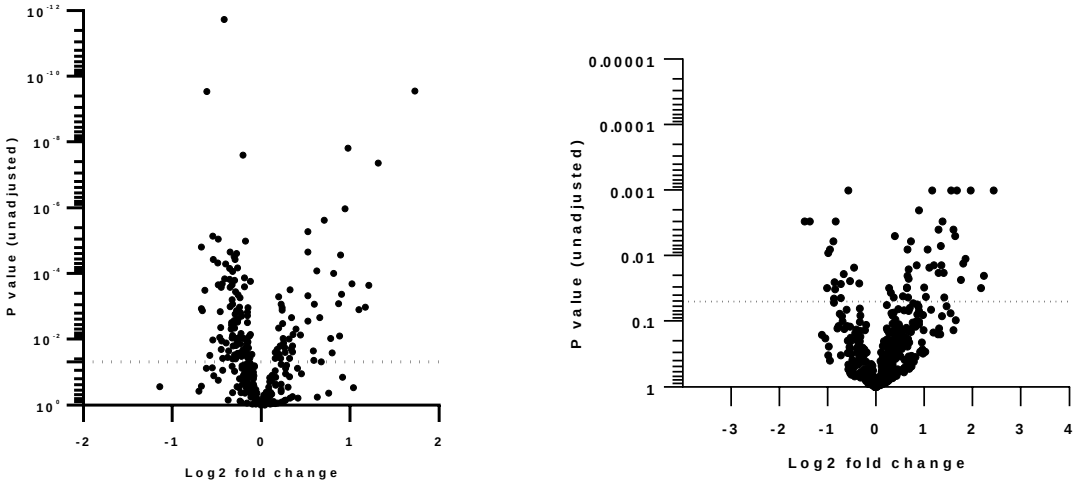
Figure 3: The protein-protein interaction network of differentially expressed genes (nasal lysate). The line thickness of the network edges indicates the strength of confidence in the interaction (the thicker the line, the greater the confidence in the interaction). Filled nodes; some 3D structure is known or predicted. Empty nodes; proteins of unknown 3D structure.

Figure 4: Heat map of the Pearson correlation values for clinical markers versus DEGs in blood samples. BMI, Body Mass Index; WCC, white cell count; NEUT, neutrophil count; MONO, monocyte count; EOSINO, eosinophil count; BASO, basophil count; ESR, erythrocyte sedimentation rate; DP *Dermatophagoides pternyssinus;* DF, *Dermatophagoides farinae;* GP, Grass pollen mix; TNSS, total nasal symptom score; MRQLQ, mini rhinoconjunctivitis quality of life questionnaire; OARSS, other allergic rhinitis symptom score; TOSS, total ocular symptom score; VAS, visual analogue scale.

Figure 5: Heat map of the Pearson correlation values for clinical markers versus DEGs in nasal lysate samples. BMI, Body Mass Index; WCC, white cell count; NEUT, neutrophil count; MONO, monocyte count; EOSINO, eosinophil count; BASO, basophil count; ESR, erythrocyte sedimentation rate; DP *Dermatophagoides pternyssinus;* DF, *Dermatophagoides farinae;* GP, Grass pollen mix; TNSS, total nasal symptom score; MRQLQ, mini rhinoconjunctivitis quality of life questionnaire; OARSS, other allergic rhinitis symptom score; TOSS, total ocular symptom score; VAS, visual analogue scale.

Figure 6: Top 10 individual correlation plots for the differentially expressed genes in nasal lysate samples versus clinical factors. Each correlation was significant ($p<0.05$). Correlations for IgE *D.farinae* and IgE *D. pteronyssinus* were conducted on AR samples only (n=37). The remaining correlations were performed with the inclusion of the control group samples (n=58 total).

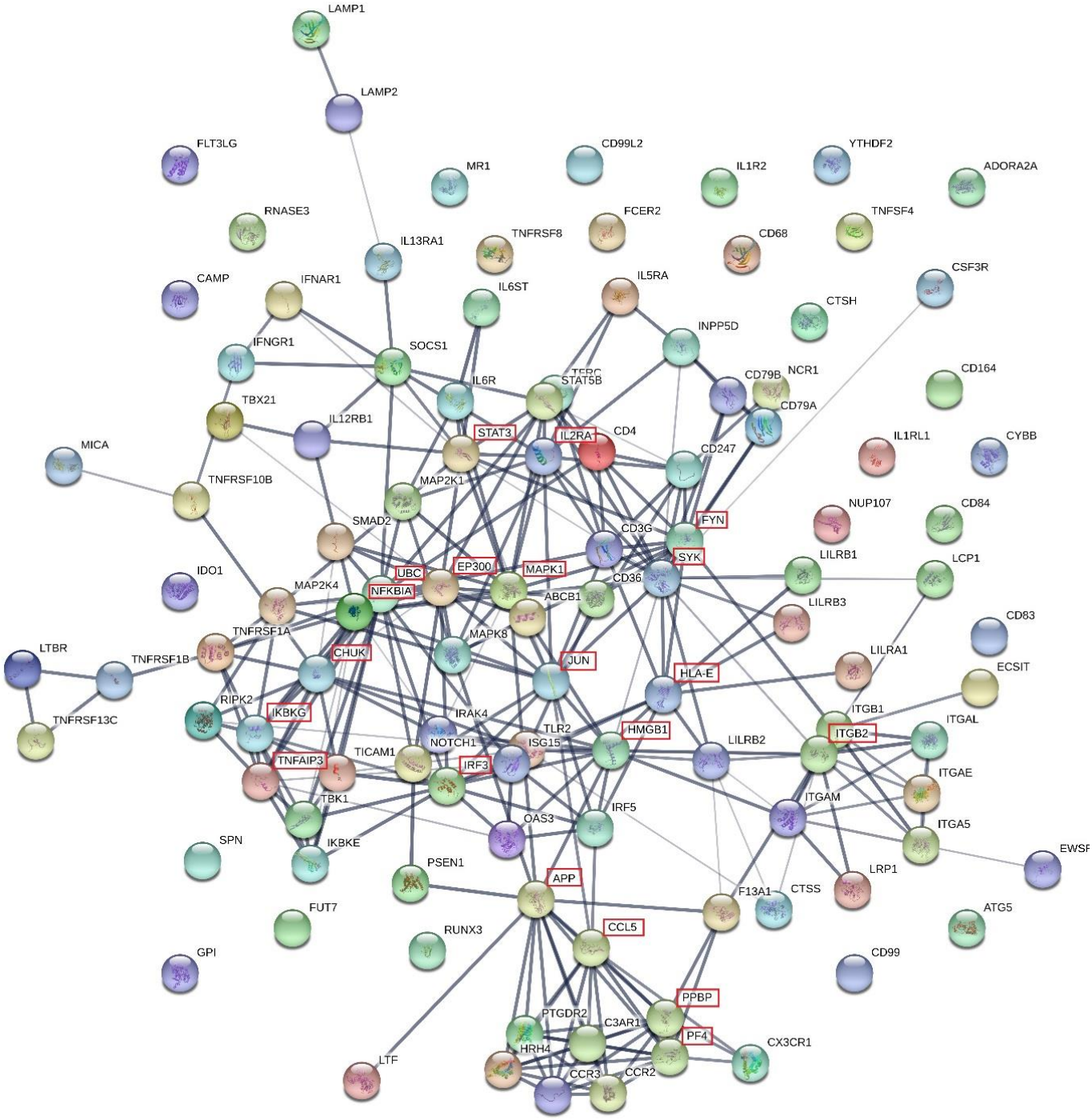
631 **Figure 1**



632

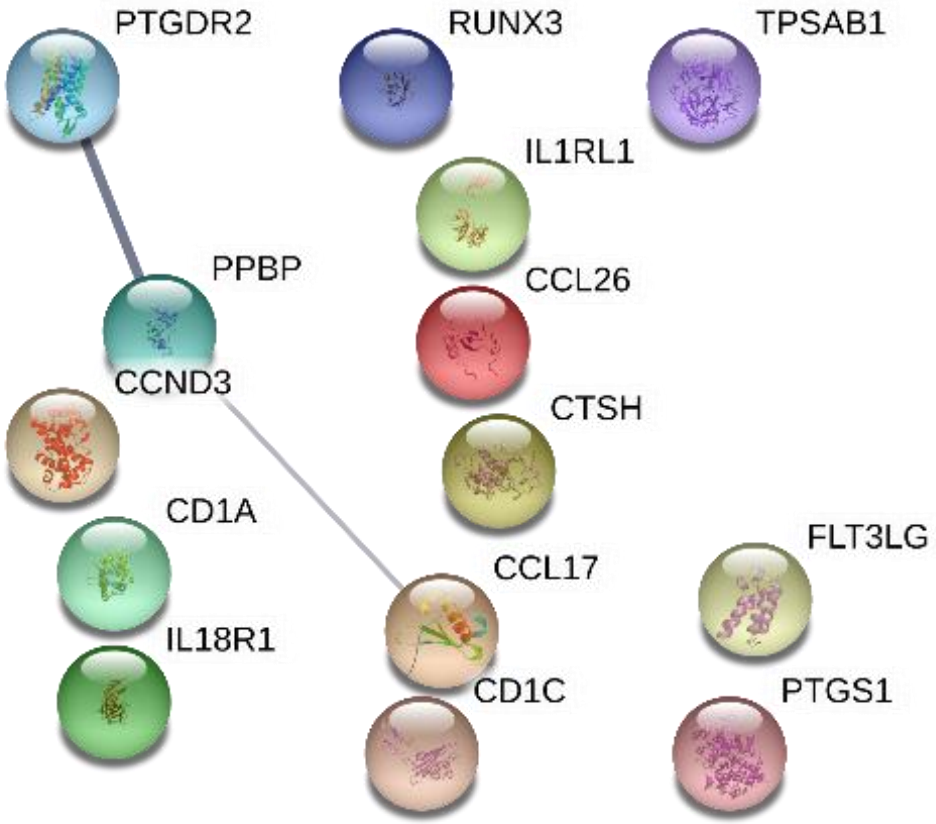
633 **Figure 2**

634



635 **Figure 3**

636



637

638 **Figure 4**

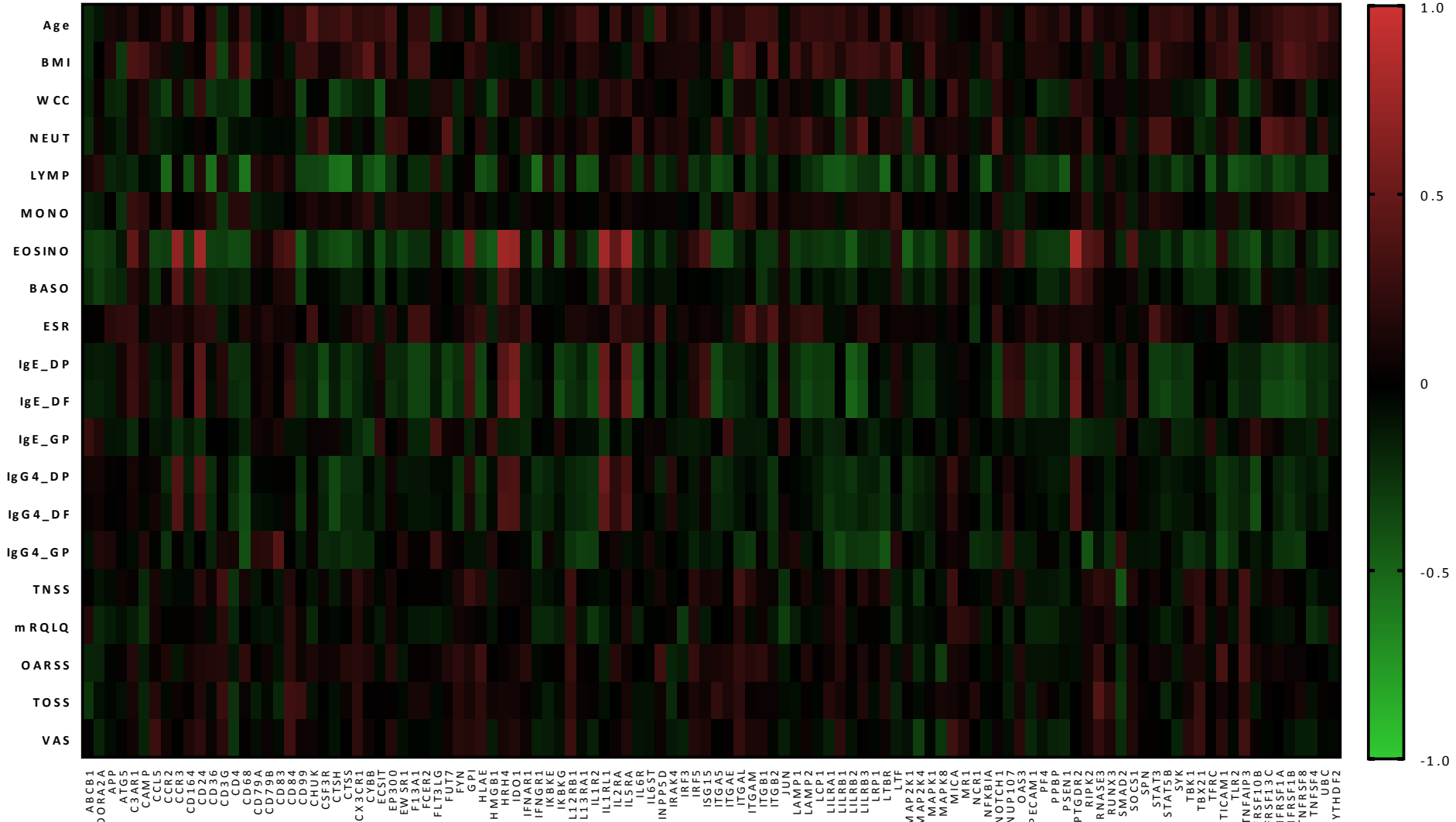
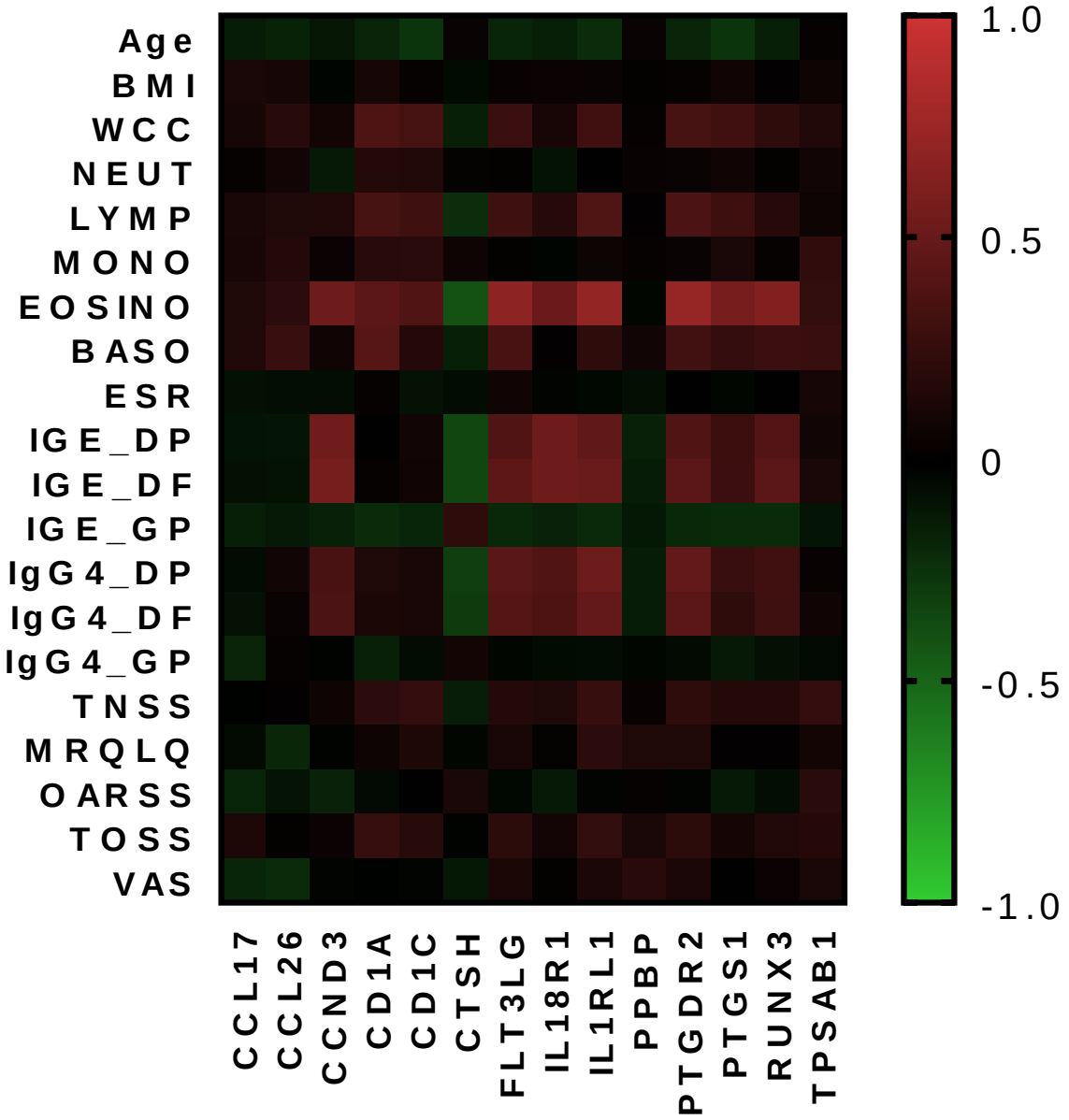

Age
BMI
WCC
NEUT
LYMP
MONO
EOSINO
BASO
ESR
IgE_DP
IgE_DF
IgE_GP
IgG4_DP
IgG4_DF
IgG4_GP
TNSS
mRQLQ
OARSS
TOSS
VAS
ABCB1
ADORA2A
APP
ATG5
C3AR1
CAMP
CCL5
CCR2
CCR3
CD164
CD24
CD36
CD3G
CD4
CD68
CD79A
CD79B
CD83
CD84
CD99
CHUK
CSF3R
CTSH
CTSS
CX3CR1
CYBB
ECSIT
EP300
EWSR1
F13A1
FCER2
FLT3LG
FUT7
FYN
GPI
HLAE
HMGB1
HRH4
IDO1
IFNAR1
IFNGR1
IKBKE
IKBKG
IL12RB1
IL13RA1
IL1R2
IL1RL1
IL2RA
IL5RA
IL6R
IL6ST
INPP5D
IRAK4
IRF3
IRF5
ISG15
ITGA5
ITGAE
ITGAL
ITGAM
ITGB1
ITGB2
JUN
LAMP1
LAMP2
LCP1
LILRA1
LILRB1
LILRB2
LILRB3
LRP1
LTBR
LTF
MAP2K1
MAP2K4
MAPK1
MAPK8
MICA
MR1
NCR1
NFKBIA
NOTCH1
NUP107
OAS3
PECAM1
PF4
PPBP
PSEN1
PTGDR2
RIPK2
RNASE3
RUNX3
SMAD2
SOCS1
SPN
STAT3
STAT5B
SYK
TBK1
TBX21
TFRC
TICAM1
TLR2
TNFAIP3
TNFRSF10B
TNFRSF13C
TNFRSF1A
TNFRSF1B
TNFSF8
TNFSF4
UBC
YTHDF2
1.0
0.5
0
-0.5
-1.0

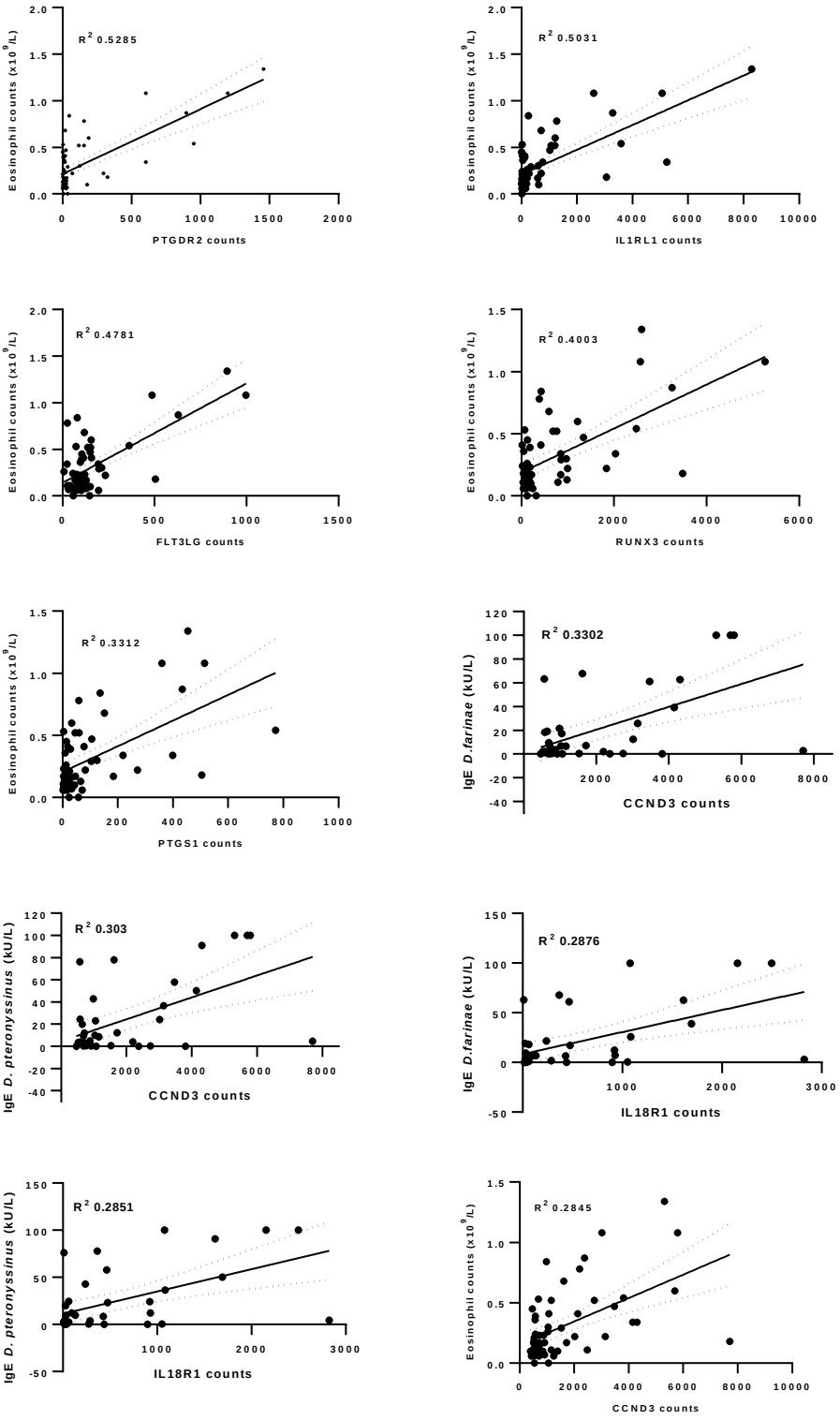
Figure 5


Age
BMI
WCC
NEUT
LYMP
MONO
EOSINO
BASO
ESR
IGE_DP
IGE_DF
IGE_GP
IgG4_DP
IgG4_DF
IgG4_GP
TNSS
MRQLQ
OARSS
TOSS
VAS
CCL17
CCL26
CCND3
CD1A
CD1C
CTSH
FLT3LG
IL18R1
IL1RL1
PPBP
PTGDR2
PTGS1
RUNX3
TPSAB1
1.0
0.5
0
-0.5
-1.0

643 **Figure 6**

644

645



CHAPTER SEVEN

Study six: Comparison of immune gene expression profiles following treatment with the combination nasal spray containing azelastine hydrochloride and fluticasone propionate compared to monotherapy with either azelastine hydrochloride or fluticasone propionate: a randomized, double-blind trial in adults with persistent allergic rhinitis

COMPARISON OF IMMUNE GENE EXPRESSION PROFILES FOLLOWING TREATMENT WITH THE COMBINATION NASAL SPRAY CONTAINING AZELASTINE HYDROCHLORIDE AND FLUTICASONE PROPIONATE COMPARED TO MONOTHERAPY WITH EITHER AZELASTINE HYDROCHLORIDE OR FLUTICASONE PROPIONATE: A RANDOMIZED, DOUBLE-BLIND TRIAL IN ADULTS WITH PERSISTENT ALLERGIC RHINITIS

This Chapter is formatted in a style suitable for publication in Clinical and Experimental Allergy. *Supplementary material for the Chapter is provided in Appendix 4.*

My contribution to the Chapter involved:

Recruitment of study participants and management of clinical trial, RNA extraction, quantification and gene expression experiments, analysis of gene expression data, demographic, clinical, efficacy, compliance and adverse events data, preparation of figures and tables, and primary writer of manuscript.

_____7th May 2019

Annabelle Monica Watts

_____7th May 2019

Supervisor: Professor Allan Cripps

Abstract

Background: The combination of the antihistamine azelastine hydrochloride (AZE) with the corticosteroid fluticasone propionate (FP) in a single spray, has been reported to be significantly more effective at reducing allergic rhinitis symptoms than treatment with either corticosteroid or antihistamine monotherapy However, the biological basis for enhanced symptom relief of the combination spray is not known and warrants further investigation

Objective: To compare immune gene expression profiles in nasal mucosa and peripheral blood samples following application of nasal sprays containing either an antihistamine, corticosteroid or combination of antihistamine and corticosteroid.

Methods: In a double-blind parallel group design, moderate/severe persistent AR sufferers with a confirmed dust mite allergy were randomised to treatment with either an AZE (125 ug / spray) nasal spray (n=16 participants) or FP (50 ug/spray) nasal spray (n=14 participants) or combination spray AZE/FP (125 ug AZE / 50 ug FP/ spray) (n=14 participants) for seven days, twice daily. Prior to the treatment period, all participants completed a washout of topical nasal sprays for 14 days. Gene expression analysis was performed using a panel of 760 immune genes with the NanoString nCounter on purified RNA from peripheral blood and cell lysates prepared from combined nasal lavage and nasal brushing. Treatment efficacy was assessed with self-reported symptom questionnaires completed daily for the entire duration of the study.

Results: Immune gene changes in peripheral blood samples following each treatment were minimal. In nasal mucosal samples, distinct immune gene expression patterns across treatments were observed. The FP nasal spray had the greatest effect on immune gene expression in nasal mucosal samples compared with the other treatments. A total of 206 genes were significantly differentially expressed following treatment with FP. Of these genes, 182 were downregulated (-2.57 to -0.45 Log2 FC) and 24 genes were upregulated (0.49 to 1.40 Log2 FC). A total of 16 genes were significantly differentially expressed in the AZE/FP group following treatment. Of these genes, 10 genes were downregulated (-1.53 to -0.58 Log2 FC) and six genes were upregulated (1.07 to 1.62 Log2 FC). The AZE group had the least effect on gene expression. A total of five immune genes were significantly differentially expressed following treatment. Of these genes, one gene was downregulated (-1.68 Log2 FC) and four genes were upregulated (0.59 to 1.19 Log2 FC) following treatment. The combination spray AZE/FP and FP spray had comparable effects on symptom reduction while the AZE spray reduced symptoms the least

Conclusion: AZE/FP and FP had comparable effects on symptom reduction but diverse effects on immune gene expression profiles in nasal mucosa samples. The moderate number of genes modulated by AZE/FP sufficiently reduces AR symptoms whilst avoiding extensive local immune suppression.

Introduction

Allergic rhinitis (AR) is estimated to affect between 10 – 30% of the global population (1-4) and is associated with significant medical and economic burden (5-7). The primary symptoms of AR include nasal congestion, rhinorrhoea, itchy nose and sneezing. Symptoms of post nasal drip, itchy/red eyes also occur in some sufferers. Intranasal antihistamines and corticosteroids are first-line treatments for AR management. Corticosteroids suppress many stages of the allergic inflammatory reaction via modifying the transcription of anti- and pro- inflammatory genes. Intranasal steroids are considered the most effective treatment for AR. However, these drugs may take some time to reach peak efficacy (8). Antihistamines bind with histamine 1 (H1) receptors to alter the activity of the H1 receptor towards an inactivate state (9, 10). Intranasal antihistamines are therefore effective at reducing histamine mediated symptoms such as sneezing, itching and rhinorrhoea (11). In comparison to intranasal corticosteroids, intranasal antihistamines have a more rapid onset of action, but are less effective at reducing symptoms, especially nasal congestion (12, 13).

In a survey conducted in the United Kingdom, approximately 70% of moderate-to-severe AR sufferers required multiple therapies to achieve symptomatic relief during the pollen season (14). On this basis, a combination spray containing an antihistamine and corticosteroid was developed to meet the demands for better coverage of symptoms in the convenient form of a single spray. In randomised, placebo-controlled studies with AR cohorts, head-to-head comparisons of each active agent (antihistamine and corticosteroid) versus the newly developed combination therapy, revealed that the combination spray was more effective than either monotherapy at reducing AR symptoms (12, 15-18). Antihistamines and corticosteroids have a distinct mechanism of action and therefore potential additive or synergistic effects may contribute to the enhanced symptomatic relief observed. However, experimental studies examining the mechanism of action behind these enhanced effects are limited.

Multiplex gene expression analysis is an effective means to identify genes involved in the pathophysiology of chronic diseases and further characterise the molecular mechanisms of action of therapeutic agents. Gene expression experiments investigating the effects of topical treatments on the site of action (nasal mucosa) have traditionally utilised microarray analysis and have relied on nasal biopsies to obtain enough sample material for analysis. Non-invasive methods such as nasal brushing and nasal lavage allow for recurrent and cost-effective collection of nasal mucosal cells. However, the RNA yields from these methods are typically of insufficient quantity for multiplex gene expression analysis. In a pioneering study, we have effectively used the NanoString nCounter to measure gene expression of 800 immune genes from cellular material collected via nasal lavage and nasal brushing techniques (19)

The purpose of this study is to investigate the potential mechanisms through which antihistamine and corticosteroid nasal sprays provide relief from AR symptoms and to determine if combining an antihistamine and corticosteroid provides any synergistic effects on gene expression profiles in nasal mucosa and blood samples. In a parallel group design, a total of 48 AR sufferers were assigned to either an antihistamine nasal spray (azelastine hydrochloride), a steroid spray (fluticasone propionate) or a combination spray (azelastine hydrochloride/fluticasone propionate) to administer twice daily for seven days. Gene expression profiles were measured before and after administering the nasal spray in blood and nasal lavage/ brushing samples with the NanoString nCounter system.

Methods

Study design

This study was a randomised, double-blind, three-armed parallel-group study with an active control group to characterise the effects of combination therapy versus monotherapy with an intranasal antihistamine and intranasal steroid on gene expression profiles in the nasal mucosa and in blood samples. Clinical assessments were conducted at the Queensland Allergy Services Clinic (Gold Coast, Australia) and the Clinical Trial Unit at Griffith University (Gold Coast, Australia) from November 2016 to May 2018. Participants attended a screening visit (day -14) at QLD Allergy Services Clinic for evaluation of allergen sensitivities and provision of blood samples. Following screening, eligible participants were instructed to complete a 14-day washout period and cease use of all intranasal and immune modulating medications. Participants were advised to use the following as rescue medication in the event of a considerable symptomatic episode: (1) nasal irrigation with saline solution, (2) oral decongestants, (3) oral antihistamines. Participants were requested not to take any allergy medications in the 48 hours prior to the screening (day -14) and baseline (day 0).

At the baseline visit (day 0), participants provided nasal lavage/brush and blood samples for gene expression analysis and were randomised to one of three treatment groups (1) intranasal antihistamine, (2) intranasal steroid, or (3) combined intranasal antihistamine and intranasal steroid. On provision of the nasal spray, participants were instructed to administer the allocated nasal spray 1 spray per nostril, twice daily, for seven days. Participants were discouraged from using any allergy medications other than the study medication during the treatment period. Following the treatment period, participants returned for the final visit (day 7) for provision of nasal lavage/brushing and blood samples. Participants were instructed to evaluate symptoms and record medication usage in a symptom and medication diary for the entire duration of the study. Participants also completed the mini Rhinoconjuctivitis Quality of Life Questionnaire at all study visits (day -14, day 0 and day 7).

This study was approved by the Griffith University Human Research Ethics Committee (Ref:2016/279) and was registered with the Australian and New Zealand Clinical Trial Registry (ACTRN12616001439437) prior to commencement. All participants provided written and informed consent prior to participation.

Participant selection - Inclusion and Exclusion Criteria

Participants were both male and female aged between 18 to 65 years of age with a more than two year history of AR. Participants had persistent AR and moderate-to-severe symptoms as defined by the Allergic Rhinitis and Its Impact on Asthma guidelines (ARIA) with symptoms occurring for more than four days per week and more than four weeks in a row and one or more of the following conditions present: (1) sleep disturbance, (2) impairment of daily activities, (3) impairment of school or work, or

(4) troublesome symptoms. Participants were also required to have a Total Nasal Symptom Score (TNSS) of at least six and a score of at least 50 mm on a Visual Analogue Scale (VAS) for overall symptom severity in the previous 24 hours. Participants had a positive allergic response to dust mites determined with a skin prick test and/ or serum specific IgE radioallergosorbent test (RAST) (QML Pathology, Murarrie, Queensland, Australia) to *Dermatophagoides pteronyssinus* or *D. farinae*. Participants were also tested for against a panel of pollen allergens and specific IgE RAST for grass pollen mix, for randomisation and characterisation of the cohort.

Participants were excluded from the study if they suffered from non-allergic rhinitis, consumed probiotics in the previous 12 weeks, were treated with oral corticosteroids within the previous six months or antibiotics within the previous 30 days, used anti-inflammatory or immune-modulating medications, had existing respiratory disease including asthma, nasal polyposis, or chronic obstructive pulmonary disorder, had existing immune dysfunction (other than allergies), had recent nasal surgery or nasal trauma that could affect sampling and deposition of study medication, were ill or had infectious disease at time of enrolment, reported hepatic impairment or excessive alcohol consumption as per the NHMRC alcohol guidelines Australia (20) and Bouchery et al. 2011 (21), had known hypersensitivity to steroids or antihistamines, were pregnant at the time of enrolment.

Study medications

Participants were randomised to one of the following three treatment groups: (1) Azep® nasal spray [Mylan Health Pty Ltd], azelastine hydrochloride (AZE) 125 µg / spray, (2) Flixonase® nasal spray [Mylan Health Pty Ltd], fluticasone propionate (FP) 50 µg / spray, or (3) Dymista® nasal spray [Mylan Health Pty Ltd], 125 µg of azelastine hydrochloride / 50 µg fluticasone propionate (AZE/FP). The total daily dose of the active ingredients, administered as one spray per nostril twice daily, was 500 µg AZE and 200 µg of FP. Compliance was assessed based on self-report of the number of doses missed and was also estimated by measuring the amount of study medication remaining (by weight) relative to the amount (weight) before dispensing.

Blinding and randomisation

Participants were assigned in a counter-balanced manner to study medication using a block randomisation method stratified by allergen sensitivity (dust mite only or dust mite and grass allergy) and sex. Participants who met all the inclusion/exclusion criteria and completed the washout period received the next available randomisation number in the appropriate sequence. The study medications were supplied in commercial packaging with all labels removed and replaced with a single label with the blinded code (Group A, Group B or Group C). The study medications were provided in sealed envelopes so that study investigators were blinded to each participants treatment. In addition, all

participants were instructed to not to discuss the appearance of their assigned treatment with the investigators.

Symptom analyses

The symptom and medication diary was completed for the entire duration of the study and consisted of consisted of three symptom questionnaires; the Total Nasal Symptom Score (TNSS), total ocular symptom score (TOSS) and Other Allergic Rhinitis Symptom Score (OARSS), in addition to a 100 mm Visual Analogue Scale for overall symptom severity. Full details of the symptom questionnaires used are described in Chapter 6. Use of allergy and non-allergy related medications were also recorded in the diary and consisted of questions on type and name of medication/supplement, date and time medication was taken, and dose of medication. Participants were requested to complete the diary each day reflecting on the previous 24-hour period.

Sample collection and laboratory analysis

Venous blood samples were collected at the day -14 visit for analysis of full blood count, white cell differential, erythrocyte sedimentation rate (ESR), and specific IgE to dust mites (*D. pteronyssinus* and *D. farinae*.) and grass pollen mix (Bermuda, Timothy, Meadow, Johnson, Rye and Paspalum) (QML Pathology). ESR over one hour was measured using fresh blood samples collected in sodium citrate tubes and using commercially available Vacuette ESR pipettes (Greiner Bio-One, Kremsmünster, Austria) as per the Westergren method (22). Nasal washing, brushing and whole blood samples were collected at day 0 and day 7 visits as previously described in Watts et al. 2018 (19) and Chapter 6.

Gene expression analysis

Immune gene expression analysis of nasal cell lysate and extracted RNA from blood was performed using a commercially available NanoString nCounter PanCancer Immune Profiling panel (NanoString Technologies, Seattle, WA, USA) as described previously in Watts et al. 2018 and Chapter 6. This panel contained 40 reference (housekeeping) genes and 730 immune genes and was used in combination with the nCounter panel plus probe set which contained an additional 30 immune genes relating to the allergic response and mechanism of action of steroids and antihistamines (760 immune genes in total) (19). Gene expression data underwent imaging quality control and normalisation checks prior to analysis and interpretation of data. Genes that were expressed at counts below 20 in 80% or more samples were excluded from further analysis. Reference (housekeeping) normalisation was performed using the GeNorm Algorithm where 20 out of 40 housekeeping genes were used for the nasal lysate samples and 33/40 housekeeping genes used for the peripheral blood samples.

Statistical analysis

Based on a standard deviation of gene expression intensity of 0.6, an α of 0.001 and at least two-fold difference in gene expression, a sample size of 16 patients per group was estimated to achieve 95% power. Differences in demographic and clinical measures between groups was assessed with a one-way ANOVA and a Chi-squared test for categorical variables. Variables were log transformed where appropriate to approximate a normal distribution. Change (pre-post) in symptom severity questionnaires was measured with a paired T test. Differences in absolute change in symptom severity questionnaires between groups was measured with an ANCOVA with baseline score as the covariate. Differentially expressed genes were identified using R package Limma (23), where moderated t tests were performed to compare the gene expression levels between groups. The significantly differentially expressed genes ($p<0.05$) in each treatment group were assessed for enrichment into Reactome pathways. Statistical significance of all clinical measures differentially expressed genes and pathway enrichment was accepted at $p<0.05$.

Results

Study cohort

A total of 48 participants were enrolled in the study and randomised to a treatment group. Two participants did not complete the treatment period and were withdrawn from the study. Reasons for withdrawal included developing a respiratory infection that required antibiotics for one participant and diagnosis of thyroid disease requiring administration of immune-modulating medications for another participant. Both events were deemed unlikely to be related to the study drug. A consort diagram to demonstrate flow and retention of study participants is shown in Figure 1. The per-protocol population consisted of a total of 46 participants, with n=14 participants in the FP group, n=16 participants in the AZE/FP group and n=16 participants in the AZE group. The demographic and baseline characteristics of the study group are given in Table 1. In general, the per-protocol population was comprised of middle-aged adults (38.1 ± 13.1 years), was largely female (67%), and mostly Caucasian (78%). The groups were considered matched at baseline on all key demographic, allergen sensitivity and symptom severity measures. Although, blood eosinophil counts were significantly different between the FP and AZE groups.

All three nasal sprays were well tolerated by participants. All adverse events were mild, having minimal impact on daily activities and were consistent with previously reported findings (8, 12). All reported adverse events are recorded in the Supplementary Table 1. The most common adverse event was disagreeable bitter/metallic taste, occurring in n=11 participants in the AZE/FP group, in three participants in AZE group and in one participant in FP group. Irritation of nasal passages shortly after application (i.e. sneezing, itching, running nose) was reported by four participants in the FP group, in four participants in the AZE group, and in no participants in the AZE/FP group. Tender/sore nasal passages were reported in two participants in the AZE group, one participant in the AZE/FP group and in no participants in the FP group. A total of two participants in the AZE/FP group reported worsened nasal congestion. Two participants in the FP group experienced itchy eyes during the administration period. No other adverse events were reported by more than one participant. Compliance to intervention was similar across treatment groups. Compliance was calculated as 96% ± 6% of total scheduled doses based on self-report for the FP group, 96% ± 6% for the AZE/FP group and 99% ± 3% for the AZE group. All participants administered ≥86 % of doses which is considered appropriate. Compliance was also calculated from weights of returned medication and reported as the proportion of participants who missed less than two doses (93% compliant). A total of 86% of participants from the FP group, 93% of participants from the AZE/FP group and 81% of participants from the AZE group missed less than two doses.

Differentially expressed genes

Nasal mucosa

Gene expression data from nasal lysate samples of 13 participants in the FP group, 11 participants in the AZE/FP group and 11 participants from the AZE group met the quality control guidelines and were included in the analysis. The demographic and clinical characteristics of the nasal lysate cohort were similar to the per-protocol population and are included in Supplementary Table 2. Of the 760 immune genes included in the NanoString nCounter panel, 588 genes were expressed above background noise and were included in the subsequent analyses. Gene expression changes in all genes are summarised in Figure 3. As shown in the heat map (Figure 3), FP had a strong down-regulatory effect on gene expression, while AZE/FP and AZE had a mostly up-regulatory effect on immune gene expression. The top 20 differentially expressed genes for all treatment groups are shown in Tables 2-4.

FP had the greatest effect on immune gene expression compared to the other nasal sprays, with 206 immune genes differentially expressed at $p<0.05$ following treatment. Of these 206 differentially expressed genes (DEGs), 24 immune genes were upregulated (0.49 to 1.40 Log2 FC), and 182 immune genes were downregulated (-2.57 to -0.45 Log2 FC) at $p<0.05$ following FP administration. A total of 16 immune genes were differentially expressed following administration with AZE/FP. Of these 16 DEGs, 10 genes were downregulated (-1.53 to -0.58 Log2 FC) and six genes were upregulated (1.07 to 1.62 Log2 FC) at $p<0.05$ following treatment. AZE had the smallest effect on immune gene expression compared with FP and AZE/FP. A total of five immune genes were differentially expressed following treatment with AZE. Of these five DEGs, one immune gene was downregulated (-1.68 Log2 FC) and four immune genes were upregulated (0.59 to 1.19 Log2 FC) at $p<0.05$ following treatment.

Blood

Blood samples could not be collected at day 0 and 7 for two participants and therefore peripheral blood gene expression experiments for these participants was not performed. In total, blood samples from 13 participants were included in the FP group, 16 participants in the AZE/FP group and 15 participants in the AZE group (Demographic and Baseline data, Supplementary Table 3). Of the 760 immune genes in the nCounter panel, 485 genes were expressed above the background threshold and were included in the analyses. The top 20 differentially expressed genes for each treatment group are shown in Tables 2-4. As shown in Figure 4, the magnitude of change in gene expression counts of the DEGs was much lower in the blood samples compared with the nasal lysate samples. Similar to the nasal lysate samples, treatment with FP had the greatest effect on immune gene expression compared with the other treatments, with 34 genes differentially expressed at $p<0.05$. Of these 34 DEGs, six were downregulated (-0.47 to -0.15 Log2 FC) and 24 genes were upregulated (0.16 to 0.50 Log2 FC) following treatment with FP. A total of 18 genes were differentially expressed following treatment with AZE/FP. Of these DEGs, nine genes were downregulated (-0.26 to -0.15 Log2 FC) and nine were upregulated (0.12 to

0.20 Log2 FC) following treatment with AZE/FP. A total of 20 genes were differentially expressed in the AZE group. Of these 20 genes, six were downregulated (-0.44 to -0.15 Log2 FC) and 14 genes were upregulated (0.13 to 0.43 Log2 FC). Given the small fold change values, additional analyses were not performed on the gene expression data from the blood samples.

Comparison of immune gene expression between treatment groups

As shown in Figure 5, four DEGs were in common between AZE/FP and FP. These genes included TPSAB1, NOS2, CD274 and TNFSF13 and were downregulated in both treatment groups (Table 5). There were no DEGs in common between AZE/FP and AZE, or between FP and AZE. The gene expression fold change following nasal spray administration for all genes was also compared between groups. The difference in fold change values between FP and AZE was the greatest, with 126 genes significantly different between these groups at $p<0.05$ (Supplementary Table 4). When comparing FP and AZE/FP, a total of 112 genes had significantly different FC values (Supplementary Table 4). AZE/FP and AZE were the most similar when comparing FC values, with only eight genes significantly different between groups (Supplementary Table 4).

Pathway enrichment

The DEGs in the FP group were significantly enriched into 186 Reactome Pathways. The top four Reactome Pathways include Immune System, Cytokine Signalling in Immune system, Signalling by Interleukins and Innate Immune System (Table 6). The DEGs in the AZE/FP group were significantly enriched into four Reactome Pathways (Table 6). The four Reactome Pathways in the AZE/FP group include Haemostasis, Immune System, Cytokine Signalling in Immune System and PI5P, PP2A and IER3 Regulate PI3K/AKT Signalling. These four enriched pathways were also significantly enriched in the FP group (data not shown). The DEGs in the AZE group were not enriched into any Reactome Pathways.

Symptom analysis

All symptom severity measures (TNSS, mRQLQ, TOSS, OARSS, overall symptom severity measured with a 100mm VAS) were significantly improved following treatment in all groups. As shown in Figure 2, the absolute mean improvement in mRQLQ from baseline was greatest in the AZE/FP group (1.94; 95% CI [1.26-2.22]) compared with FP (1.74; [1.50 – 2.40]) and AZE (1.12; [0.67 – 1.57]). The mean improvement in mRQLQ was significantly greater in the AZE/FP group compared with the AZE group ($p=0.014$). The absolute mean improvement in TNSS from baseline was greatest in the FP group (4.073; [2.75 – 5.40]) closely followed by the AZE/FP group (4.065; [2.80 – 5.36] and lowest in the AZE group (1.73; [0.45 – 3.01]). When comparing between groups, both FP ($p=0.013$) and AZE/FP ($p=0.016$) treatments had a significantly greater effect on TNSS improvement compared to AZE. The mean improvement in the OARSS was greatest in the FP group (3.20; [2.10 – 4.29]) followed by the AZE/FP

group (3.06; [2.01 – 4.11]) and AZE (1.51; [0.46 – 2.55]). Mean improvement in OARSS was significantly greater between the FP (p=0.029) and AZE/FP (p=0.044) groups in comparison to the AZE group. Mean change in overall symptom severity measured via a VAS, was greatest in the AZE/FP group (35.00; [23.69 – 46.31]) followed by the FP group (33.27; [21.28 – 45.27]) and the AZE group (15.98; [4.67 – 27.30]). Mean change in overall symptom severity between groups was significantly greater in the AZE/FP (p=0.022) and FP (p=0.040) groups when compared to AZE. Mean change in TOSS scores from baseline following treatment was greatest in the AZE/FP group (1.88; [1.08 – 2.67]) followed by the FP group (1.50; [0.66 – 2.34]) and AZE (1.13; [0.34 – 1.91]). There was no significant difference between groups in TOSS change scores.

Discussion

The combination of the antihistamine AZE with the corticosteroid FP in a single spray, has been reported to be significantly more effective at reducing self-reported AR symptoms than treatment with either monotherapy alone (8, 12). Antihistamines and corticosteroids work via separate mechanisms to induce anti-allergic effects. The biological basis for the enhanced AR symptom relief provided by the combination spray is not known and warrants further investigation. To this end, the current study used a parallel-group design to compare the immune gene expression profiles of nasal lysate samples from AR sufferers following administration with either the antihistamine spray (AZE), corticosteroid spray (FP) or the combination spray (AZE/FP). The results presented here demonstrate that FP and the combination spray AZE/FP are significantly more effective at reducing AR symptoms than AZE. The genes and pathways modulated by each nasal spray were also characterised in this project.

A key finding in this study was distinct gene expression patterns between groups in response to treatment. FP had a strong down-regulatory effect on gene expression, while most genes were upregulated following treatment with AZE/FP and AZE. Analyses of the DEGs between groups revealed that FP had the strongest effect on gene expression compared with AZE/FP and AZE. A total of 206 DEGs were identified in the FP group which represents over 44% of genes that were expressed above background. The majority of the DEGs were downregulated, which is consistent with the primary mechanism of action of corticosteroids (24). The top three DEGs in the FP group include AMICA1, GZMB and LTB which were all downregulated following FP treatment. AMICA1 also known as JAML is involved in leukocyte migration and antigen processing and presentation pathways (25). The downregulation of this gene following FP treatment supports the potential for FP to modulate the early stages of the allergic response. Indeed, the high affinity IgE receptor (FCER1A gene), which plays a key role in mast cell sensitisation, was also significantly downregulated by FP treatment. Downregulation of GZMB by corticosteroids has been previously reported (26, 27). Granzyme B is a serine protease encoded by the GZMB gene and expressed by CD8+ T cells, natural killer cells, mast cells, B cells and basophils (28) and is a known inducer of apoptosis (29). GZMB is also involved in extracellular matrix proteolysis and cytokine processing (30-32). The LTB gene was downregulated following FP treatment and encodes lymphotoxin beta which is a member of the TNF cytokine family. Binding of lymphotoxin β to the LTβ receptor induces activation of transcription factor NF-κB which is involved in the expression of many pro-inflammatory molecules pertinent to the allergic response (33, 34).

AZE had the smallest effect on gene expression compared with the other nasal sprays with only five genes differentially expressed (0.85% of total genes) following treatment. This finding is consistent with the known mechanism of antihistamines, whereby antihistamines act on a specific component of the allergic response (i.e. interaction of histamine with histamine receptors), rather than exhibiting broad

immune modulatory action as seen with steroids. The top three DEGs following treatment with AZE were APOE, TPTE and CAMP. Apolipoprotein E is encoded by the APOE gene and is involved in the capture and delivery of lipid antigens to antigen presenting cells (35). Downregulation of this gene may provide symptomatic relief through preventing enhanced antigen presentation and downstream allergic inflammation. The TPTE gene is involved in signal transduction pathways, however its specific role in allergic disease is not known. The CAMP gene encodes the cathelicidin-related antimicrobial peptides and was upregulated following treatment with AZE. Cathelicidin has anti-microbial and immunoregulatory functions (36, 37). Reduced levels of cathelicidin were observed in the nasal lavage fluid of children suffering AR compared with controls, indicating that cathelicidin may be involved in the pathogenesis of AR.

A total of 16 genes were differentially expressed following treatment with AZE/FP representing 7.8% of the total genes expressed above baseline. Of these 16 DEGs, some were upregulated (n=6) and others downregulated (n=10). The top three DEGs in the AZE/FP group included TNFSF10, NOS2A, and PPBP. TNFSF10 also known as TRAIL induces the activation of transcription factor NF-κB which is involved in the expression of many pro-inflammatory molecules pertinent to the allergic response. In a murine model it has been shown that TNFSF10 knock out animals had lessened airway hyperactivity and peribronchial eosinophilia and reduced levels of mast cells in the airways, compared with wild-type mice (38). As such, downregulation of this gene could contribute to the improvement of AR symptoms. Nitric oxide synthase is an enzyme encoded by the NOS2 gene. Upregulation of NOS2A has been identified in bronchial biopsy samples from allergic asthmatics compared with healthy controls and downregulation of this gene was reported following inhaled corticosteroids (39). Suppression of inducible nitric oxide expression has also been reported with AZE treatment in isolated mouse peritoneal macrophages (40). Therefore, the effects of FP and AZE treatment on NOS2A expression may provide additional modulation of this gene when these agents are combined. Interestingly, the average fold change for NOS2A was lowest in the AZE/FP group, compared with FP and AZE, although this was not statistically significant. Pro-Platelet Basic Protein (PPBP) is a powerful chemoattractant and activator for neutrophils and has been previously reported to be downregulated by glucocorticoids (41). Neutrophils contribute to allergic inflammation via release of reactive oxygen species and proteases which damage the nasal epithelium and promote migration of effector cells (42, 43). As such, reduced neutrophil infiltration in the nasal mucosa would likely reduce AR symptoms.

Limited effects on gene expression in peripheral blood samples were observed following use of FP, AZE/FP and AZE. Indeed, the maximum effect on gene expression was 0.50 Log2 FC in the FP group, -0.26 Log2 FC in the AZE/FP group and -0.44 Log2 FC in the AZE group. The translation of these small changes in gene expression to substantial protein production and meaningful clinical effects is generally considered unlikely. Indeed, second generation steroids such as FP have an estimated

systemic bioavailability of less than 1% and systemic adverse events are considered rare (44). Intranasal antihistamines have a similar systemic safety profile (45). Intranasal application of a single dose of FP and Rhinocort (Budesonide) at 200 µg and 800 µg had no significant effect on numbers of B cells, CD4+ and CD8+ lymphocytes or percentage of lymphocyte populations in peripheral blood samples of healthy individuals (46). The small gene expression changes in blood samples following nasal spray administration reported here, maybe be a follow-on effect from reduced inflammation in the nasal mucosa and which prevents the stimulation of inflammatory cell production in the bone marrow and thymus and subsequent release into circulation.

The DEGs in nasal lysate samples were further enriched into Reactome pathways. As was expected the DEGs in the FP group were enriched into a greater number of pathways than the DEGs in the AZE/FP and AZE groups. In contrast, the DEGs in the AZE group were not enriched into any Reactome pathways. This result indicates that AZE acts on a small number of distinct genes to exert its clinical effects rather than modulating many genes within a single pathway. The DEGs in the AZE/FP group were enriched into four Reactome pathways. These pathways relate to blood coagulation following injury, cytokine signalling of the adaptive and innate immune system and signal transduction. The reactome pathways enriched in the AZE/FP group were also significantly enriched in the FP group, indicating a shared mechanism of action between the AZE/FP and FP.

The DEGs were compared between groups to identify genes that were common between treatments. Four DEGs were shared between FP and AZE/FP. The genes included TPSAB1, NOS2, CD274 and TNFSF13 which were downregulated in both treatments. The FC values for NOS2, CD274 and TNFSF13 were lower in the AZE/FP group compared with FP and AZE, indicating possible synergistic effects, however the difference in FC values did not reach statistical significance. There were no DEGs shared between the FP and AZE group, indicating separate mechanisms of action of these two drugs. To gain further insight into the unique and shared patterns of gene expression between the three nasal sprays, the fold-change values pre- to post- treatment for all genes (n=588) were compared between groups. The greatest number of genes that were significantly expressed differently between groups, was between FP and AZE with 126 genes identified. This finding is not surprisingly given that these treatments contain two separate drug classes with different mechanisms of action. A total of 112 genes were significantly expressed differently between AZE/FP and FP. AZE/FP and AZE were the most similar when comparing fold change values with only eight genes significantly different between groups. The difference in modulation of these eight genes between AZE/FP and AZE may be the explain the enhanced clinical effects of AZE/FP compared to AZE.

Clinical responses to drug treatment were measured with self-reported symptom severity questionnaires including the TNSS, mRQLQ, TOSS, OARSS and overall symptom severity on a VAS scale. In general,

for each treatment, the pattern of reduction was similar for the different symptom domains. FP and AZE/FP had a comparable effect on symptom reduction, while AZE had the least effect on symptom reduction compared to the other treatments. While this study was not powered to detect differences in clinical outcome between treatments, the results presented here show a similar pattern of efficacy to previously published studies. Carr et al. 2012 performed a metanalysis of three randomised placebo-controlled studies (n=3398 total participants) and compared the efficacy of AZE, FP and AZE/FP on AR symptom reduction over a 14-day treatment period. AZE/FP had the greatest effect on reduction of TNSS scores, followed by FP, and lastly AZE. While treatment with AZE/FP had a significantly greater effect on the reduction of the TNSS compared with FP, the difference in the average TNSS between the two treatments was similar (-5.7 [5.3] AZE/FP 'Dymista®' vs -5.1 [4.9] FP 'Flixonase®'; average [standard deviation]).

In this report, application of AZE/FP and FP had comparable effects on symptom relief determined via self-reported symptom surveys. However, AZE/FP and FP had contrasting effects on immune-gene expression. FP had the strongest effect on immune gene expression with a substantial proportion of immune genes and pathways modulated by this treatment. AZE improved symptoms the least compared with the other treatments and this coincided with only a small number of genes modulated by AZE. The combination spray, AZE/FP modulated more genes and pathways than AZE but less than FP. It is possible that the degree of modulation of genes and pathways by AZE/FP is enough to reduce symptoms, whilst having a lessened immunosuppressive effect. Indeed corticosteroids have been shown *in vitro* to suppress the antimicrobial activity of human macrophages (47) In addition, use of intranasal corticosteroids has been linked with pharyngeal candidiasis in a small number of cases (48) and was hypothesized to be caused by the broad immunosuppressive action of corticosteroids.

The strengths in the current study lie in the power of the gene expression analyses to characterise changes in many immune genes in response to pharmaceutical treatment at the site of application. In addition, participants had very well characterised allergic disease based on the use of multiple categories of symptom questionnaires, skin prick testing and specific IgE and IgG4 testing. Nonetheless, there were several limitations of this study. Many of the DEGs identified in this study did not reach statistical significance when adjusted for false discovery rate and as such additional studies are needed to confirm the validity of the DEGs. In addition, the effect of the intranasal sprays on immune gene expression was evaluated at a single time point only (day 7), and greater resolution of biological pathways may be achieved with more frequent sampling. As this was a community-based study, the comparative effect of each nasal sprays on early-phase and late-phase allergic responses are not known and should be studied under conditions of controlled allergen exposure.

Overall, investigation of nasal mucosa samples of AR sufferers following intranasal application of FP, AZE/FP and AZE showed distinct gene expression patterns across treatments. The greatest distinction between gene expression profiles was between FP and AZE which is indicative of the different mechanism of action between corticosteroids and antihistamines. A compelling finding of this study was that FP and AZE/FP had comparable effects on symptom reduction, but had diverse effects on gene expression. FP had a strong downregulatory effect on gene expression compared with AZE/FP which had an intermediate effect on gene expression with a mix of downregulated and upregulated genes following treatment. The moderate number of genes modulated by AZE/FP appears to be sufficient to significantly reduce AR symptoms, whilst avoiding total suppression of the local mucosal immune system.

Conflict of Interest

This study was funded by Mylan N.V. The authors have no other conflicts of interest to declare.

Author contributions

Design: AMW, NPW, AJC, PKS; Data collection; AMW, AJC; Experiments: AMW; Data analysis: AMW, PZ, NPW; Drafting of manuscript: AMW; Manuscript revision: AMW, AJC, AWC, PZ, PKS.

Acknowledgements

The authors acknowledge the support of the Queensland Allergy Services Clinic for providing clinic access and for performing the skin prick tests.

References

1. Bjorksten B, Clayton T, Ellwood P, Stewart A, Strachan D, Group IPIS. Worldwide time trends for symptoms of rhinitis and conjunctivitis: Phase III of the International Study of Asthma and Allergies in Childhood. *Pediatr Allergy Immunol* 2008;**19**(2):110-124.
2. Fokkens WJ, Hellings PW. Rhinology in the forefront of European political attention. *Rhinology* 2015;**53**(4):289.
3. Australian Institute of Health and Welfare 2011. Allergic rhinitis ('hay fever') in Australia. Cat. no. ACM 23. Canberra: AIHW.
4. WAO white book on allergy: update 2013 executive summary. Milwaukee (WI): WAO; 2013.
5. Zuberbier T, Lotvall J, Simoens S, Subramanian SV, Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2) LEN review. *Allergy* 2014;**69**(10):1275-1279.
6. Cook M, Douglass J, Mallon D, Mullins R, Smith J, M W. The ecconomic impact of Allergic Disease in Australia: Not to Be Sneezed At. *Australian Society of Clinical Immunology and Allergy* 2007.
7. Marcellusi A, Viti R, Incorvaia C, Mennini FS. Direct and indirect costs associated with respiratory allergic diseases in Italy. A probabilistic cost of illness study. *Recenti Prog Med* 2015;**106**(10):517-527.
8. Carr W, Bernstein J, Lieberman P, Meltzer E, Bachert C, Price D, et al. A novel intranasal therapy of azelastine with fluticasone for the treatment of allergic rhinitis. *J Allergy Clin Immunol* 2012;**129**(5):1282-1289 e1210.
9. Simons FE, Simons KJ. Histamine and H1-antihistamines: celebrating a century of progress. *J Allergy Clin Immunol* 2011;**128**(6):1139-1150 e1134.
10. Mahdy AM, Webster NR. Histamine and antihistamines. *Anaesthesia & Intensive Care Medicine*;**12**(7):324-329.
11. Scadding G. Optimal management of nasal congestion caused by allergic rhinitis in children: safety and efficacy of medical treatments. *Paediatr Drugs* 2008;**10**(3):151-162.
12. Meltzer EO, LaForce C, Ratner P, Price D, Ginsberg D, Carr W. MP29-02 (a novel intranasal formulation of azelastine hydrochloride and fluticasone propionate) in the treatment of seasonal allergic rhinitis: a randomized, double-blind, placebo-controlled trial of efficacy and safety. *Allergy Asthma Proc* 2012;**33**(4):324-332.
13. Patel P, D'Andrea C, Sacks HJ. Onset of action of azelastine nasal spray compared with mometasone nasal spray and placebo in subjects with seasonal allergic rhinitis evaluated in an environmental exposure chamber. *Am J Rhinol* 2007;**21**(4):499-503.
14. Pitman R, Paracha N, Parker C, Acaster S, Bachert C, Bousquet J. Episode pattern and healthcare utilisation in patients with seasonal allergic rhinitis. *Allergy* 2012;**67**(Suppl 96):1-687.

15. Hampel FC, Ratner PH, Van Bavel J, Amar NJ, Daftary P, Wheeler W, et al. Double-blind, placebo-controlled study of azelastine and fluticasone in a single nasal spray delivery device. *Ann Allergy Asthma Immunol* 2010;**105**(2):168-173.

16. Ratner PH, Hampel F, Van Bavel J, Amar NJ, Daftary P, Wheeler W, et al. Combination therapy with azelastine hydrochloride nasal spray and fluticasone propionate nasal spray in the treatment of patients with seasonal allergic rhinitis. *Ann Allergy Asthma Immunol* 2008;**100**(1):74-81.

17. Fokkens W, Hellings P, Mullol J, Munzel U, Bachert C. A new allergic rhinitis therapy (MP29-02*) provides effective and rapid symptom relief for patients who suffer most from the bothersome symptoms of nasal congestion or ocular itch. *Clin Transl Allergy* 2015;**5**(Suppl 4):P33-P33.

18. Ratner P, Hampel F, Breazna A, Caracta C, Tantry S. Efficacy and safety of olopatadine/mometasone combination nasal spray for the treatment of seasonal allergic rhinitis. In: 2017; new york: elsevier science inc. p. S90-S90.

19. Watts AM, West NP, Cripps AW, Smith PK, Cox AJ. Distinct Gene Expression Patterns between Nasal Mucosal Cells and Blood Collected from Allergic Rhinitis Sufferers. *Int Arch Allergy Immunol* 2018;**177**(1):29-34.

20. Council M. Australian guidelines to reduce health risks from drinking alcohol. *NHMRC Canberra* 2009.

21. Bouchery EE, Harwood HJ, Sacks JJ, Simon CJ, Brewer RD. Economic Costs of Excessive Alcohol Consumption in the U.S., 2006. *Am J Prev Med* 2011;**41**(5):516-524.

22. K. G. Von Boroviczeny LEB, B. S. Bull, A. Chattas, J. B. Dawson, K. Fukutake, F. W. Gunz, J. A. Koepke, S. M. Lewis, E. Rewald, M. Roubicek, G. Ruthenstroth-Bauer. Recommendation of measurement of erythrocyte sedimentation rate of human blood. *Am J Clin Pathol* 1977;**68**(4):505-507.

23. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;**43**(7):e47-e47.

24. Watts AM, Cripps A, West N, Cox A. Modulation of allergic inflammation in the nasal mucosa of allergic rhinitis sufferers with topical pharmaceutical agents. *Front Pharmacol* 2019;**10**:294.

25. Thomas SY, Whitehead GS, Takaku M, Ward JM, Xu X, Nakano K, et al. MyD88-dependent dendritic and epithelial cell crosstalk orchestrates immune responses to allergens. *Mucosal Immunol* 2018;**11**(3):796-810.

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

27. Wang H, Barrenäs F, Bruhn S, Mobini R, Benson M. Increased IFN-[gamma] activity in seasonal allergic rhinitis is decreased by corticosteroid treatment. *J Allergy Clin Immunol* 2009;**124**(6):1360.

28. Salvi V, Vermi W, Cavani A, Lonardi S, Carbone T, Facchetti F, et al. IL-21 May Promote Granzyme B-Dependent NK/Plasmacytoid Dendritic Cell Functional Interaction in Cutaneous Lupus Erythematosus. *J Invest Dermatol* 2017;**137**(7):1493-1500.

29. Trapani JA. Granzymes: a family of lymphocyte granule serine proteases. *Genome Biol* 2001;**2**(12):REVIEWS3014.

30. Froelich CJ, Pardo J, Simon MM. Granule-associated serine proteases: granzymes might not just be killer proteases. *Trends Immunol* 2009;**30**(3):117-123.

31. Omoto Y, Yamanaka K, Tokime K, Kitano S, Kakeda M, Akeda T, et al. Granzyme B is a novel interleukin-18 converting enzyme. *J Dermatol Sci* 2010;**59**(2):129-135.

32. Afonina Inna S, Tynan Graham A, Logue Susan E, Cullen Sean P, Bots M, Lüthi Alexander U, et al. Granzyme B-Dependent Proteolysis Acts as a Switch to Enhance the Proinflammatory Activity of IL-1α. *Mol Cell* 2011;**44**(2):265-278.

33. VanArsdale TL, VanArsdale SL, Force WR, Walter BN, Mosialos G, Kieff E, et al. Lymphotoxin-β receptor signaling complex: role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor κB. *Proc Natl Acad Sci* 1997;**94**(6):2460-2465.

34. Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, Makris C, et al. The Lymphotoxin-β Receptor Induces Different Patterns of Gene Expression via Two NF-κB Pathways. *Immunity* 2002;**17**(4):525-535.

35. van den Elzen P, Garg S, Leon L, Brigl M, Leadbetter EA, Gumperz JE, et al. Apolipoprotein-mediated pathways of lipid antigen presentation. *Nature* 2005;**437**(7060):906-910.

36. Davidson DJ, Currie AJ, Reid GS, Bowdish DM, MacDonald KL, Ma RC, et al. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *J Immunol* 2004;**172**(2):1146-1156.

37. Bandurska K, Berdowska A, Barczyńska-Felusiak R, Krupa P. Unique features of human cathelicidin LL-37. *BioFactors* 2015;**41**(5):289-300.

38. Collison A, Li J, Pereira de Siqueira A, Zhang J, Toop HD, Morris JC, et al. Tumor necrosis factor-related apoptosis-inducing ligand regulates hallmark features of airways remodeling in allergic airways disease. *Am J Respir Cell Mol Biol* 2014;**51**(1):86-93.

39. Laprise C, Sladek R, Ponton A, Bernier M-C, Hudson TJ, Laviolette M. Functional classes of bronchial mucosa genes that are differentially expressed in asthma. *BMC genomics* 2004;**5**(1):21-21.

40. Yoneda K, Yamamoto T, Ueta E, Osaki T. Suppression by azelastine hydrochloride of NF-kappa B activation involved in generation of cytokines and nitric oxide. *Jpn J Pharmacol* 1997;**73**(2):145-153.

41. El-Gedaily A, Schoedon G, Schneemann M, Schaffner A. Constitutive and regulated expression of platelet basic protein in human monocytes. *J Leukoc Biol* 2004;**75**(3):495-503.

42. Fransson M, Benson M, Wennergren G, Cardell L-O. A role for neutrophils in intermittent allergic rhinitis. *Acta Otolaryngol* 2004;**124**(5):616-620.

43. Monteseirin J. Neutrophils and asthma. *J Investig Allergol Clin Immunol* 2009;**19**(5):340-354.
44. Gupta R, Fonacier LS. Adverse Effects of Nonsystemic Steroids (Inhaled, Intranasal, and Cutaneous): a Review of the Literature and Suggested Monitoring Tool. *Curr Allergy Asthma Rep* 2016;**16**(6):44.
45. Salib RJ, Howarth PH. Safety and tolerability profiles of intranasal antihistamines and intranasal corticosteroids in the treatment of allergic rhinitis. *Drug Saf* 2003;**26**(12):863-893.
46. Fokkens WJ, van de Merwe JP, Braat JP, Overbeek SE, Hooijkaas H. The effect of intranasal and inhaled corticosteroids in healthy volunteers on the number of circulating lymphocytes and lymphocyte subsets. *Allergy* 1999;**54(**2):158-164.
47. Schaffner A. Therapeutic concentrations of glucocorticoids suppress the antimicrobial activity of human macrophages without impairing their responsiveness to gamma interferon. *J Clin Invest.* 1985;**76**(5):1755-1764.
48. Kyrmizakisl DE, Papadakisz CE, Lohuiss PJ, Manolarakisl G, Karakostasl E, Amanakis Z. Acute candidiasis of the oro-and hypopharynx as the result of topical intranasal steroids. *Rhinology* 1999;**38**:87-89.

Tables

Table 1: Baseline demographic and clinical measures (per-protocol population)

	FP	AZE/FP	AZE	P value
n	14	16	16	-
Age (years)	37.63 ± 14.60	39.42 ± 10.03	37.26 ± 15.08	0.889
Sex F/M (% Female)	10/4 (71%)	10/6 (63%)	11/5 (69%)	0.864
Height (cm)	169.11 ± 9.35	171.03 ± 10.26	171.94 ± 8.63	0.710
Weight (kg)	76.69 ± 15.72	72.16 ± 15.33	73.46 ± 13.20	0.694
BMI (kg/m^2)	26.65 ± 3.95	24.45 ± 3.37	24.78 ± 3.64	0.226
Ethnicity (% Caucasian)	78.60%	68.80%	87.50%	0.437
Immune measures (day-14)				
White cell count ($x10^9/L$)	7.18 ± 1.70	6.98 ± 2.09	5.90 ± 1.50	0.112
Lymphocytes ($x10^9/L$)	2.32 ± 0.80	2.26 ± 0.73	1.98 ± 0.63	0.366
Eosinophils ($x10^9/L$)	0.53 ± 0.37	0.44 ± 0.30	0.26 ± 0.16	0.038
Neutrophils ($x10^9/L$)	3.74 ± 1.22	3.68 ± 1.33	3.18 ± 1.00	0.366
Basophils ($x10^9/L$)	0.06 ± 0.05	0.07 ± 0.03	0.04 ± 0.03	0.085
ESR (mm/hr)	14.50 ± 13.78	7.94 ± 6.17	7.94 ± 8.27	0.267
Allergen sensitivity (day-14)				
Co-allergy to dust mites and pollen (%)	50%	62.50%	62.50%	0.731
IgE *D. pteronyssinus* (kU/L)	39.28 ± 40.39	17.12 ± 21.60	11.37 ± 20.34	0.088
IgE *D. farinae* (kU/L)	35.11 ± 39.85	13.41 ± 18.31	8.56 ± 17.31	0.093
IgE grass pollen mix (kU/L)	2.13 ± 4.29	10.44 ± 25.84	6.20 ± 21.29	0.526
IgG4 *D. pteronyssinus* (kU/L)	0.50 + 0.54	0.47 ± 0.42	0.38 ± 0.43	0.772
IgG4 *D. farinae* (kU/L)	0.42 ± 0.42	0.39 ± 0.26	0.30 ± 0.38	0.617
IgG4 grass pollen mix (kU/L)	0.67 ± 0.35	1.07 ± 0.97	0.80 ± 0.66	0.469
Symptom severity (day 0)				
Total Nasal Symptom Score (0-12 U)	5.93 ± 3.95	4.00 ± 1.86	7.06 ± 3.64	0.299
Total Ocular Symptom Score (0-9 U)	3.57 ± 2.44	2.00 ± 2.00	3.19 ± 2.74	0.182
Mini rhinoconjunctivitis quality of life score (0-6 U)	2.90 ± 1.25	2.66 ± 0.79	3.00 ± 1.12	0.653
Other symptoms (0-12 U)	4.51 ± 3.89	3.07 ± 2.16	5.21 ± 3.47	0.865
Visual Analogue Scale (0-100 mm)	54.18 ± 33.51	48.16 ± 22.31	60.50 ± 30.10	0.485
Medication Usage (day-14 – day 0)				

Allergy medication use; % of total diary responses (washout period)	0.37 ± 0.29	0.25 ± 0.20	0.40 ± 0.31	0.237

622

623 n, number; L, Litre; hr, kU, Kilounit; U, unit; mm, millimetre; %, percentage; FP, fluticasone
624 propionate 'Flixonase ®' Group; AZE/FP, azelastine hydrochloride /fluticasone propionate 'Dymista
625 ®' group; AZE, azelastine hydrochloride 'Azep®' group.

626 Table 2: Top 20 differentially expressed genes in nasal lysate and blood samples in the FP group

Gene	Log2 fold change	Linear fold change	Lower confidence limit (log2)	Upper confidence limit (log2)	P value	P adjust
Nasal mucosa samples						
AMICA1	-2.46	0.18	-3.10	-1.83	3.16E-07	1.86E-04
GZMB	-2.54	0.17	-3.30	-1.78	2.37E-06	6.96E-04
LTB	-2.41	0.19	-3.20	-1.62	6.83E-06	1.03E-03
FCER1A	-2.55	0.17	-3.38	-1.71	7.03E-06	1.03E-03
SOCS1	-2.20	0.22	-3.01	-1.40	2.49E-05	2.92E-03
PTGDR2	-2.29	0.20	-3.20	-1.38	6.19E-05	5.43E-03
IL1RL1	-2.36	0.19	-3.33	-1.40	8.39E-05	5.43E-03
HLA-DRA	-1.75	0.30	-2.47	-1.03	9.22E-05	5.43E-03
CXCR3	-2.04	0.24	-2.89	-1.19	1.02E-04	5.43E-03
CD1C	-1.62	0.33	-2.30	-0.94	1.07E-04	5.43E-03
ITGAM	-1.72	0.30	-2.44	-1.00	1.15E-04	5.43E-03
CD96	-2.08	0.24	-2.95	-1.20	1.16E-04	5.43E-03
DUSP6	-1.58	0.33	-2.25	-0.91	1.25E-04	5.43E-03
ITGA4	-1.73	0.30	-2.46	-0.99	1.29E-04	5.43E-03
CSF2RB	-2.20	0.22	-3.14	-1.26	1.38E-04	5.43E-03
RUNX3	-1.90	0.27	-2.72	-1.08	1.56E-04	5.52E-03
IL16	-1.81	0.29	-2.59	-1.02	1.60E-04	5.52E-03
CKLF	-1.54	0.34	-2.21	-0.86	1.76E-04	5.76E-03
CEACAM1	-1.30	0.41	-1.89	-0.72	2.07E-04	5.84E-03
NCF4	-1.74	0.30	-2.52	-0.96	2.14E-04	5.84E-03
Peripheral blood samples						
IL3RA	-0.47	0.72	-0.65	-0.29	4.95E-05	2.40E-02
IL1B	0.33	1.26	0.15	0.51	1.20E-03	2.91E-01
MS4A2	-0.38	0.77	-0.62	-0.14	4.45E-03	5.14E-01
IL5RA	-0.33	0.79	-0.55	-0.12	5.08E-03	5.14E-01
NFKB1	0.22	1.16	0.07	0.37	6.29E-03	5.14E-01
RNASE3	-0.47	0.72	-0.79	-0.15	7.12E-03	5.14E-01
HRH4	-0.29	0.82	-0.49	-0.09	7.43E-03	5.14E-01
CD24	-0.45	0.73	-0.77	-0.13	9.39E-03	5.61E-01
SLPI	0.30	1.23	0.08	0.52	1.04E-02	5.61E-01
NFKB2	0.20	1.15	0.05	0.34	1.22E-02	5.91E-01

KLRD1	-0.21	0.86	-0.38	-0.04	1.63E-02	6.14E-01
PLAUR	0.17	1.12	0.03	0.31	1.96E-02	6.14E-01
SERPING1	0.50	1.41	0.09	0.91	2.05E-02	6.14E-01
CD7	0.21	1.16	0.04	0.39	2.16E-02	6.14E-01
OAS3	0.34	1.27	0.05	0.63	2.24E-02	6.14E-01
CD9	-0.23	0.85	-0.43	-0.04	2.25E-02	6.14E-01
IL1RAP	-0.18	0.88	-0.34	-0.03	2.42E-02	6.14E-01
BCL6	0.19	1.14	0.03	0.36	2.48E-02	6.14E-01
IFITM1	0.18	1.13	0.03	0.34	2.54E-02	6.14E-01
IFIT1	0.43	1.35	0.06	0.80	2.59E-02	6.14E-01

627

628 FP, fluticasone propionate ‘Flixonase ®’ group

629

630

631

632

633 Table 3: Top 20 differentially expressed genes in nasal lysate and blood samples in the AZE/FP group

Gene	Log2 fold change	Linear fold change	Lower confidence limit (log2)	Upper confidence limit (log2)	P value	P adjust
Nasal mucosa samples						
TNFSF10	-0.92	0.53	-1.48	-0.36	3.58E-03	9.85E-01
NOS2A	-1.53	0.35	-2.57	-0.49	7.43E-03	9.85E-01
PPBP	1.52	2.87	0.48	2.57	7.66E-03	9.85E-01
ABCB1	1.23	2.35	0.32	2.14	1.17E-02	9.85E-01
KIT	-0.98	0.51	-1.76	-0.21	1.66E-02	9.85E-01
IFNA8	1.40	2.64	0.25	2.55	2.10E-02	9.85E-01
IL33	-1.07	0.47	-1.97	-0.18	2.25E-02	9.85E-01
CD274	-0.98	0.51	-1.82	-0.14	2.62E-02	9.85E-01
TNFSF13	-0.83	0.56	-1.55	-0.11	2.65E-02	9.85E-01
TPSAB1	-1.12	0.46	-2.17	-0.07	3.81E-02	9.85E-01
NLRP3	1.62	3.06	0.09	3.15	4.01E-02	9.85E-01
EPCAM	-0.59	0.66	-1.17	-0.02	4.50E-02	9.85E-01
TSC22D3	1.07	2.10	0.02	2.11	4.59E-02	9.85E-01
C1QBP	-0.58	0.67	-1.15	-0.01	4.62E-02	9.85E-01
NOS2	-0.99	0.50	-1.97	-0.01	4.83E-02	9.85E-01
LRRN3	1.49	2.81	0.01	2.98	4.89E-02	9.85E-01
DEFB1	-0.62	0.65	-1.24	0.00	5.03E-02	9.85E-01
CT45A1	1.15	2.22	-0.06	2.35	6.00E-02	9.85E-01
CCL26	-0.96	0.51	-1.98	0.06	6.31E-02	9.85E-01
CX3CL1	-0.82	0.57	-1.70	0.06	6.45E-02	9.85E-01
Peripheral blood samples						
CXCR6	-0.26	0.84	-0.43	-0.08	7.35E-03	9.43E-01
CLEC4C	-0.25	0.84	-0.44	-0.06	1.12E-02	9.43E-01
LY9	0.17	1.12	0.04	0.30	1.33E-02	9.43E-01
ITK	0.17	1.12	0.03	0.30	1.78E-02	9.43E-01
PNMA1	-0.16	0.90	-0.29	-0.03	1.89E-02	9.43E-01
CSF1	-0.25	0.84	-0.46	-0.04	2.11E-02	9.43E-01
FAS	-0.23	0.85	-0.42	-0.04	2.13E-02	9.43E-01
PVR	-0.19	0.88	-0.36	-0.03	2.54E-02	9.43E-01
AKT3	0.12	1.09	0.01	0.23	3.34E-02	9.43E-01

CD59	-0.19	0.88	-0.36	-0.01	3.51E-02	9.43E-01
TNFSF8	0.12	1.09	0.01	0.23	3.56E-02	9.43E-01
CD27	0.14	1.10	0.01	0.27	3.67E-02	9.43E-01
LILRB2	-0.15	0.90	-0.30	-0.01	3.91E-02	9.43E-01
TXK	0.20	1.15	0.01	0.40	4.23E-02	9.43E-01
ICAM1	-0.19	0.88	-0.37	-0.01	4.36E-02	9.43E-01
ICAM2	0.12	1.09	0.00	0.24	4.45E-02	9.43E-01
LRRN3	0.17	1.13	0.00	0.34	4.59E-02	9.43E-01
LCK	0.12	1.09	0.00	0.24	4.98E-02	9.43E-01
TRAF2	0.19	1.14	0.00	0.39	5.10E-02	9.43E-01
TNFSF14	-0.16	0.90	-0.31	0.00	5.15E-02	9.43E-01

AZE/FP, azelastine hydrochloride / fluticasone propionate ‘Dymista ®’ group

637 Table 4: Top 20 differentially expressed genes in nasal lysate and blood samples in the AZE group

	Log2 fold change	Linear fold change	Lower confidence limit (log2)	Upper confidence limit (log2)	P value	P adjust
Nasal mucosa samples						
APOE	-1.68	0.31	-2.93	-0.43	1.19E-02	9.90E-01
TPTE	1.00	2.00	0.20	1.81	1.84E-02	9.90E-01
CAMP	1.19	2.28	0.12	2.26	3.15E-02	9.90E-01
CD27	0.59	1.51	0.03	1.16	3.94E-02	9.90E-01
IL23A	0.59	1.50	0.03	1.15	4.02E-02	9.90E-01
IL18RAP	1.13	2.19	-0.03	2.30	5.56E-02	9.90E-01
C4B	0.46	1.37	-0.04	0.95	6.71E-02	9.90E-01
CCR2	0.60	1.52	-0.08	1.28	7.84E-02	9.90E-01
CT45A1	0.70	1.62	-0.09	1.49	7.86E-02	9.90E-01
IL18	-0.43	0.74	-0.92	0.06	7.99E-02	9.90E-01
C1QB	-0.81	0.57	-1.79	0.16	9.57E-02	9.90E-01
IFIT1	1.13	2.19	-0.24	2.50	9.74E-02	9.90E-01
ARG1	0.72	1.64	-0.15	1.59	9.90E-02	9.90E-01
HRH4	0.88	1.84	-0.19	1.95	1.00E-01	9.90E-01
IL2RA	-0.57	0.67	-1.27	0.13	1.02E-01	9.90E-01
PAX5	0.76	1.69	-0.18	1.69	1.04E-01	9.90E-01
SELPLG	0.95	1.93	-0.23	2.13	1.06E-01	9.90E-01
CTLA4	0.78	1.72	-0.20	1.76	1.09E-01	9.90E-01
MICA	0.47	1.38	-0.13	1.06	1.15E-01	9.90E-01
TP53	-0.36	0.78	-0.81	0.10	1.16E-01	9.90E-01
Peripheral blood samples						
NT5E	0.39	1.31	0.15	0.62	2.66E-03	9.54E-01
SPN	0.40	1.32	0.11	0.69	1.02E-02	9.54E-01
ITGAL	0.17	1.13	0.04	0.30	1.07E-02	9.54E-01
CCR4	-0.35	0.78	-0.62	-0.08	1.34E-02	9.54E-01
IL2RB	0.25	1.19	0.05	0.45	1.53E-02	9.54E-01
CEACAM8	-0.44	0.74	-0.79	-0.09	1.60E-02	9.54E-01
FYN	0.13	1.09	0.03	0.23	1.68E-02	9.54E-01
OAS3	0.30	1.23	0.05	0.55	1.97E-02	9.54E-01
SLC11A1	-0.22	0.86	-0.40	-0.03	2.50E-02	9.54E-01
IL6ST	0.16	1.12	0.02	0.30	2.59E-02	9.54E-01

PIN1	-0.16	0.89	-0.31	-0.02	2.80E-02	9.54E-01
CX3CR1	0.21	1.15	0.02	0.39	2.88E-02	9.54E-01
NFATC2	0.16	1.12	0.01	0.30	3.53E-02	9.54E-01
SERPING1	0.43	1.35	0.03	0.84	3.59E-02	9.54E-01
IDO1	0.31	1.24	0.02	0.61	3.67E-02	9.54E-01
FCER1G	-0.15	0.90	-0.29	-0.01	3.68E-02	9.54E-01
STAT4	0.15	1.11	0.01	0.28	3.70E-02	9.54E-01
LAIR2	0.14	1.11	0.01	0.28	4.10E-02	9.54E-01
ETS1	0.18	1.13	0.01	0.34	4.25E-02	9.54E-01
ITGAX	-0.16	0.90	-0.31	0.00	4.40E-02	9.54E-01

638

639 AZE, azelastine hydrochloride ‘Azep®’ group.

640

Table 5: Differentially expressed genes ($p<0.05$) in common between FP and AZE/FP groups.

	FP		AZE/FP		AZE		Between groups
	FC	**p value**	**FC**	**P value**	**FC**	**P value**	**P value**
TPSAB1	-2.04 (1.69)	<0.000	-1.12 (1.69)	0.038	0.03 (1.75)	0.956	FP and AZE p=0.008
NOS2	-0.87 (1.21)	0.018	-0.99 (1.56)	0.048	0.25 (1.30)	0.517	AZE/FP and AZE p=0.057 FP and AZE p=0.04
CD274	-0.87 (1.57)	0.049	-0.98 (1.29)	0.026	0.37 (1.99)	0.503	n/a
TNFSF13	-0.70 (0.90)	0.042	-0.83 (1.04)	0.026	0.18 (0.62)	0.449	AZE/FP and AZE p=0.012 FP and AZE p=0.012

Average Fold Change (Standard Deviation)

FC, Fold Change; FP, fluticasone propionate 'Flixonase ®' group; AZE/FP, azelastine hydrochloride / fluticasone propionate 'Dymista ®' group.

Table 6: Top four Reactome pathways for FP and AZE/FP. The DEGs from the AZE group were not enriched into any Reactome pathways.

Pathway	Description	Count in gene set	False discovery rate
FP			
HSA-168256	Immune System	144 of 1925	1.02e-91
HSA-1280215	Cytokine Signaling in Immune system	79 of 654	3.69e-57
HSA-449147	Signaling by Interleukins	62 of 439	2.63e-47
HSA-449147	Innate Immune System	69 of 1012	3.14e-34
AZE/FP			
HSA-109582	Haemostasis	5 of 601	0.0059
HSA-168256	Immune System	7 of 1925	0.0129
HSA-1280215	Cytokine Signaling in Immune system	4 of 654	0.0389
HSA-6811558	PI5P, PP2A and IER3 Regulate PI3K/AKT Signaling	2 of 85	0.0449

FP, fluticasone propionate 'Flixonase ®' group; AZE/FP, azelastine hydrochloride / fluticasone propionate 'Dymista ®' group.

Figure legends

Figure 1. Consort diagram depicting flow and retention of study participants FP, fluticasone propionate 'Flixonase®' group; AZE/FP, azelastine hydrochloride/ fluticasone propionate 'Dymista®' group; and AZE, azelastine hydrochloride 'Azep®' group.

Figure 2: Clinical response to treatment. Dots and lines represent change in symptom scores for each participant from Pre- nasal spray application (day 0) to post- nasal spray application (day 7). The data shown in the unadjusted values. Asterisk (*) indicates significance at $p<0.05$ following nasal spray application. Hash (#) indicates raw change in symptoms scores (pre-post) was significant between groups (ANCOVA with baseline score as the covariate). FP, fluticasone propionate 'Flixonase®' group; AZE/FP, azelastine hydrochloride/ fluticasone propionate 'Dymista®' group; and AZE, azelastine hydrochloride 'Azep®' group.

Figure 3: Heat map of Log2 expression all genes in the nasal mucosa samples included in the analysis (n=588) by treatment group. Each row represents a gene and each column represents a sample. The Log2 gene expression counts are represented on a Z scale whereby blue indicates low expression (downregulation) and yellow indicates high expression (upregulation). FP, fluticasone propionate 'Flixonase®' group; AZE/FP, azelastine hydrochloride/ fluticasone propionate 'Dymista®' group; and AZE, azelastine hydrochloride 'Azep®' group.

Figure 4: Gene expression values (Log2 FC) of the differentially expressed genes per group for each sample type. Outer limits of each box represent the 25^{th} and 75^{th} percentiles, with the median shown as the line within the box. Whiskers (error bars) show the 5^{th} and 95^{th} percentiles, which filled circles showing the outliers. The dotted line indicates no change in gene expression following treatment. FP, fluticasone propionate 'Flixonase®' group; AZE/FP, azelastine hydrochloride/ fluticasone propionate 'Dymista®' group; and AZE, azelastine hydrochloride 'Azep®' group.

Figure 5: Venn diagram depicting shared DEGs in nasal mucosa samples between treatment groups

682 Figure 1.

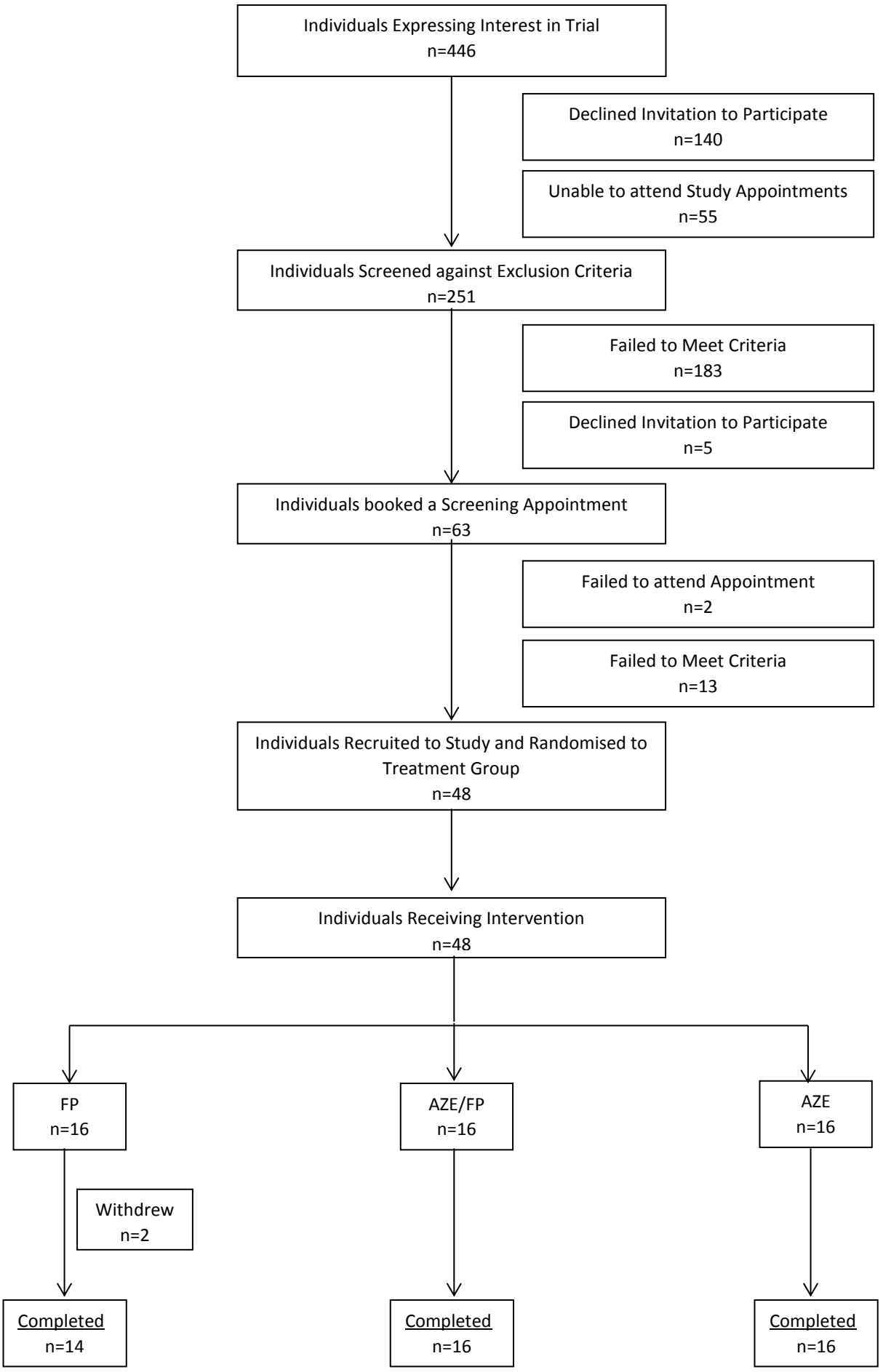

Individuals Expressing Interest in Trial
n=446
Declined Invitation to Participate
n=140
Unable to attend Study Appointments
n=55
Individuals Screened against Exclusion Criteria
n=251
Failed to Meet Criteria
n=183
Declined Invitation to Participate
n=5
Individuals booked a Screening Appointment
n=63
Failed to attend Appointment
n=2
Failed to Meet Criteria
n=13
Individuals Recruited to Study and Randomised to Treatment Group
n=48
Individuals Receiving Intervention
n=48
FP
n=16
AZE/FP
n=16
AZE
n=16
Withdrew
n=2
Completed
n=14
Completed
n=16
Completed
n=16

Figure 2.

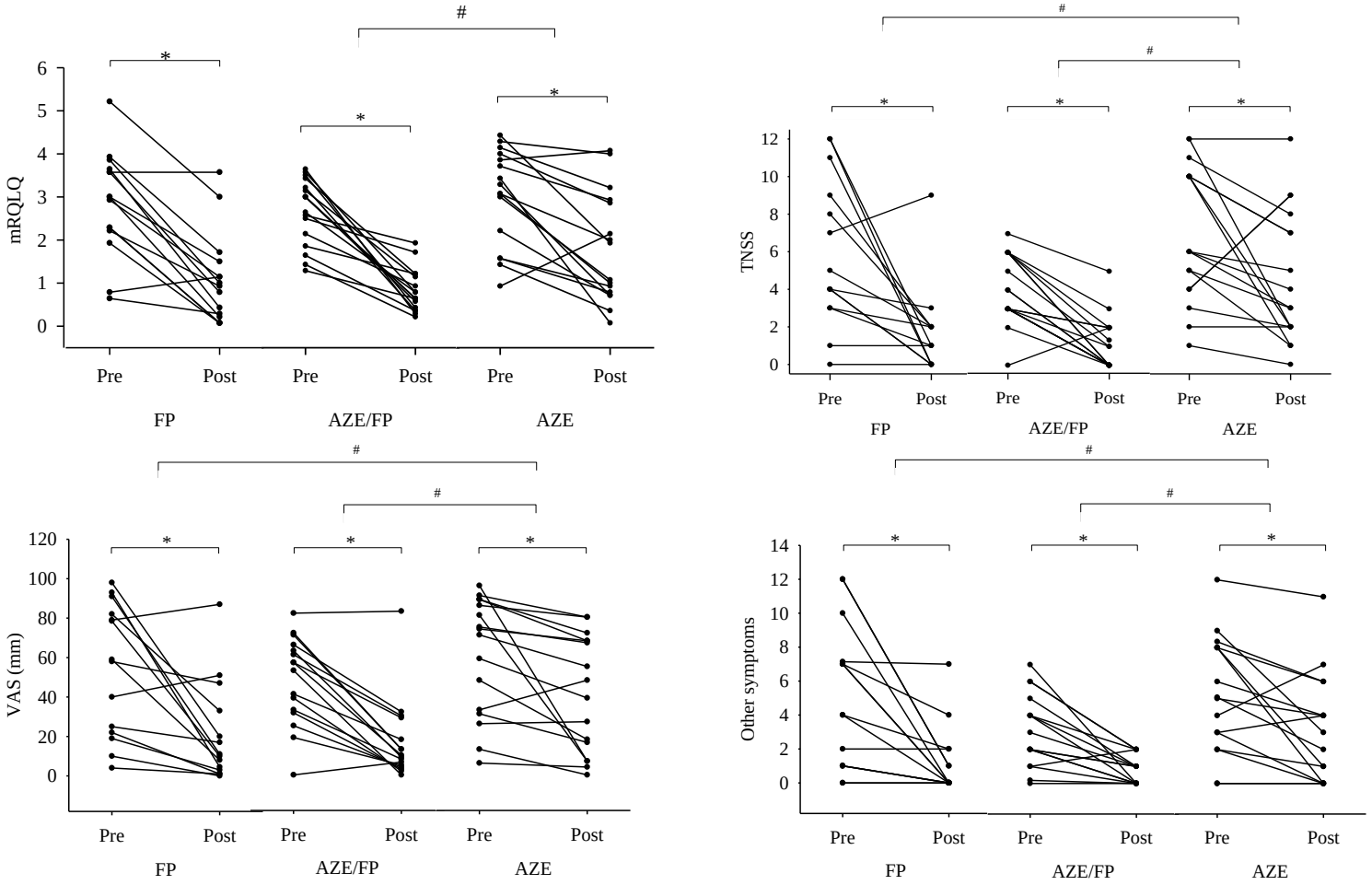


Figure 3.

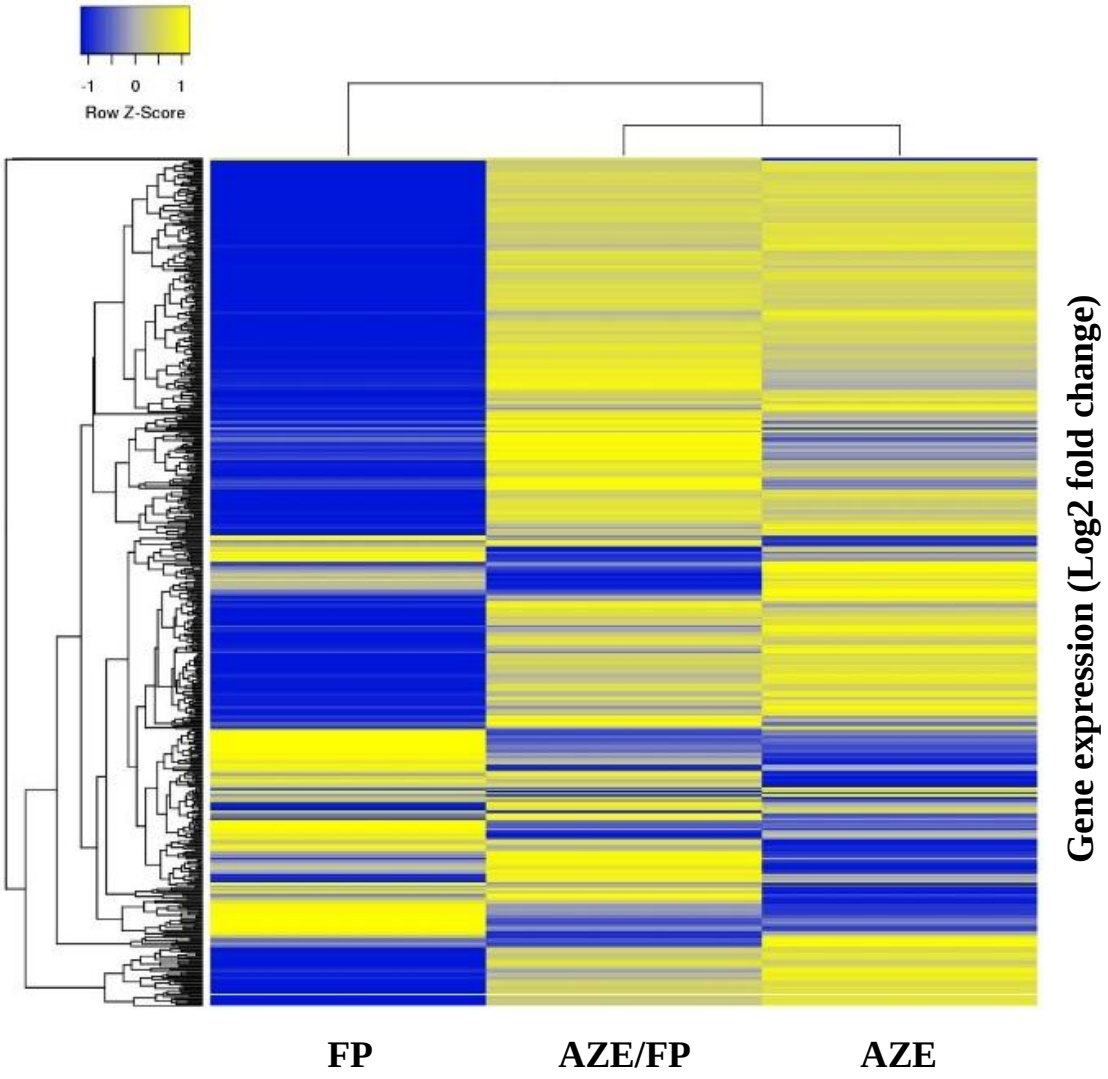

-1
0
1
Row Z-Score
Gene expression (Log2 fold change)
FP
AZE/FP
AZE

Figure 4.

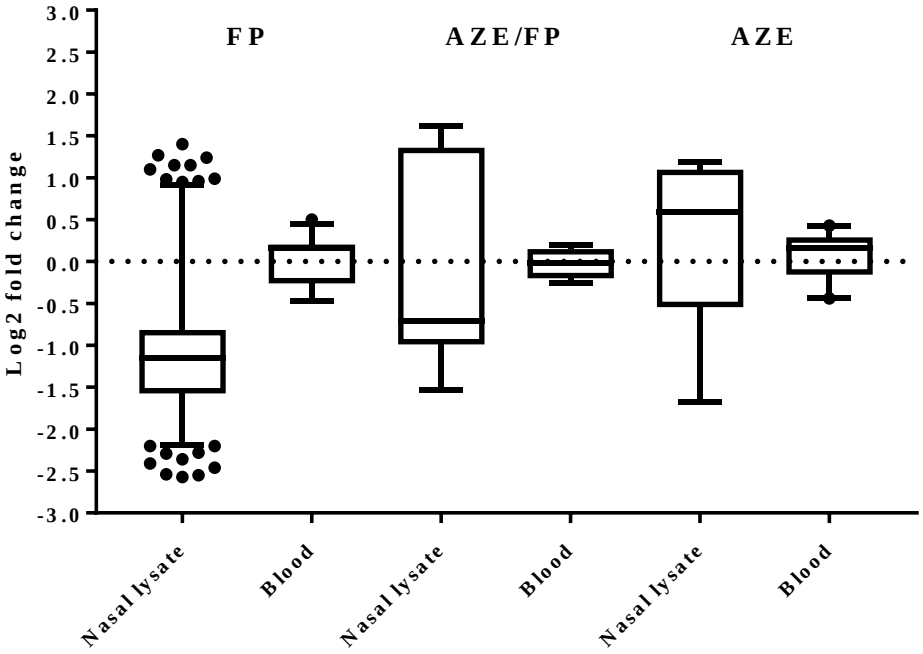
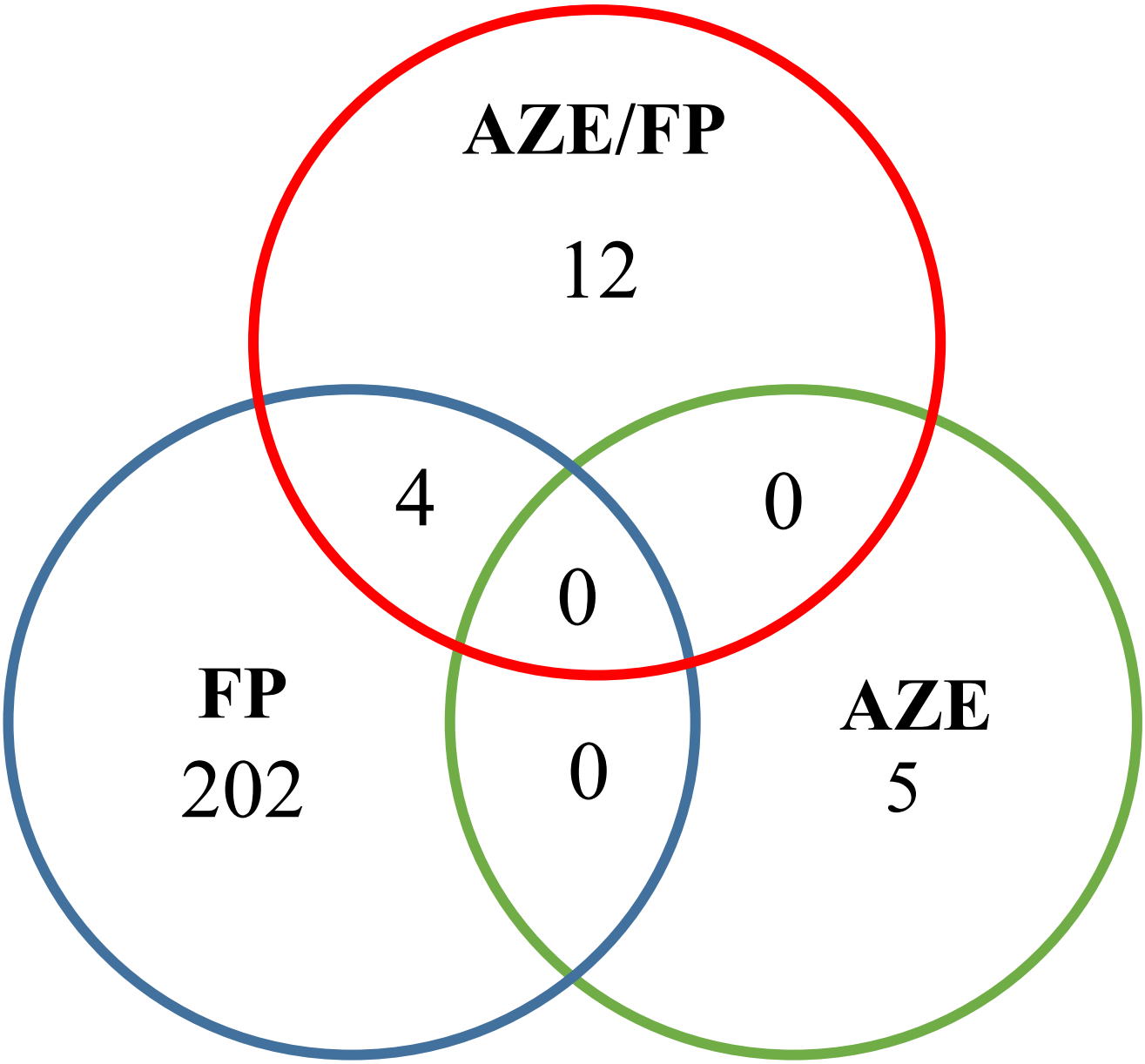

FP
AZE/FP
AZE
Log2 fold change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
Nasal lysate
Blood
Nasal lysate
Blood
Nasal lysate
Blood

Figure 5.



CHAPTER EIGHT

General Discussion

GENERAL DISCUSSION

In a series of six studies, this thesis investigated the pathophysiology and management of AR via immune and molecular phenotyping of the gut, nasal mucosa and peripheral blood. The multifactorial and complex nature of the disease hinders the accurate diagnosis and therapeutic management of AR. This thesis has identified unique features of the immune system in AR sufferers which improves our understanding of the disease and paves the way for disease modification strategies, drug development and biomarkers to diagnose and monitor the disease.

Prior to this thesis, no faecal microbiota studies utilising gene sequencing technologies have been conducted in adult AR sufferers. The findings presented in this thesis addresses this knowledge gap. Chapter 2 described the gut microbial composition of a moderate-sized cohort of adult AR sufferers (n=57) compared to a cohort of controls (n=23) of similar age and sex. Measures of microbial diversity in stool samples from allergic cohorts have yielded varying results in both adult and infant allergic cohorts (Table 1, Chapter 1). In this thesis, the Shannon alpha diversity index was significantly reduced in the AR group compared with the control group and trends for reduced diversity using other indices were also noted. Microbial alpha diversity is considered an indicator of the 'health' of the gut microbiome (113). However, no normative data exists to define 'healthy' versus 'unhealthy' microbial diversity. Large scale population studies are still needed to provide normative data to aid in the interpretation of diversity indices in specific disease states.

An altered abundance of certain bacterial taxa or specific bacterial species in the adult AR sufferers was also identified in this study. The increased abundance of Bacteroidetes (10, 114) and bifidobacteria (8) and reduced abundance of Clostridiales (10, 57, 115) identified in our AR cohort has also been observed in other studies of adult and paediatric atopic cohorts (e.g. asthma, atopic dermatitis). Collectively, these findings suggest that altered abundance of bifidobacteria, Bacteroidetes and Clostridiales could be microbiome markers of risk for allergic disease. A novel finding of this thesis was the reduced abundance of *Oxalobacter* in the AR cohort. Chapter 2 has progressed our understanding of the microbiome profile of adults with AR. However, prospective cohort longitudinal studies that manipulate diversity and specific species are needed to better understand the link between AR and the microbiome. In the future, disease modification may be possible through therapies or interventions that modulate the microbiome such as probiotics, prebiotics, dietary changes and faecal transplants.

Probiotic preparations have been considered as a treatment option for AR sufferers. The rationale for the use of probiotic supplementation in allergic disease stems from the aberrant microbial composition patterns previously reported in atopic conditions and in adult AR sufferers in this

thesis (Chapter 2), and the known immunomodulatory capacity of commensal bacteria. In this thesis (Chapter 3 and 4), an adapted Simon-Two stage design was utilised to determine if a specific probiotic supplement had a positive clinical outcome in a pre-specified proportion of the cohort to warrant further clinical investigation and provide preliminary data for designing phase II clinical trials. The probiotic supplement investigated in this thesis contained six bacterial strains from the genera *Lactobacillus*, *Bifidobacterium* and *Lactococcus*. The number of strains included in this supplement was greater than probiotic formulations that have been previously examined, with most research trials using probiotic preparations containing less than three strains (89, 116). The specific strains contained in this formulation were selected for their ability to suppress Th2 responses in cell-culture models (93, 94, 117).

A total of 40 adult seasonal AR sufferers completed the probiotic intervention study. Of these 40 participants, 25 (63%) showed a clinically defined improvement in mRQLQ scores. The proportion of participants who exhibited an improvement in quality of life metrics was considerably higher than the pre-specified threshold of 33% and as such the probiotic intervention was considered effective and worthy of further investigation in phase III trials. The findings of improved mRQLQ scores was further supported by improvement in overall symptom scores and reduced frequency of allergy medications taken during the supplementation period. Overall, Chapters 3 and 4 described a novel phase II trial protocol specific for studying interventions in AR and provided important evidence supporting the continued investigation of probiotics for the management of AR.

Multiplex gene expression analyses, including microarray experiments, are an effective means of gaining a global representation of the cellular mechanisms behind complex diseases and enables study of disease pathophysiology and response to pharmaceutical treatment. The third study in this thesis sought to develop a novel gene expression protocol with nasal lavage and nasal brushing samples using the NanoString nCounter system. The nasal lavage and nasal brushing method developed in this thesis yielded sufficient molecular material for analysis of 760 immune genes and circumvented the need for invasive sample collection. This technology was then applied to characterise immune gene expression profiles of the nasal mucosa and blood samples collected from 12 intermittent/seasonal AR sufferers. The blood and nasal mucosal samples exhibited highly distinct overall gene expression profiles, reflective of their individual anatomical and functional origins. Overall the gene expression protocol developed in Chapter 5, successfully quantified gene expression from both nasal mucosal and peripheral blood samples. Distinct gene expression profiles were observed between sample types which confirms the sensitivity of the gene expression protocol to distinguish between tissue types. This novel gene expression protocol paves the way for undertaking mechanistic studies investigating the pathophysiology of AR at the

local site, identifying novel therapeutic targets and studying responses to pharmaceutical interventions. As such, this gene expression protocol was utilised in Chapters 6 and 7 of this thesis examining AR pathophysiology and drug action.

The gene expression protocol designed in Chapter 5 was employed to identify biomarkers of AR in nasal mucosal and blood samples. In a cross-sectional study, immune gene expression profiles in nasal lysate samples and blood samples in adult perennial/persistent AR sufferers (n=45) were compared to otherwise healthy adults without AR (n=24). The gene expression profiles in blood samples collected from AR sufferers were vastly different from the control group samples. DEGs were identified between groups in both blood (n=133 DEGs) and nasal mucosa samples (n=14 DEGs). These DEGs were further explored as potential biomarkers of AR. Many of the DEGs identified in blood and nasal mucosal samples had been previously associated with atopy and are involved in immune cell chemotaxis, mast cell activity and inflammatory signalling. There were also DEGs identified in this study that have not been previously associated with atopy. The downregulation of the *TBKI* gene in blood samples in the AR group was unexpected given its role in activating the transcription factor NF-κB (118), which is involved in the transcription of many pro-inflammatory genes. Similarly, the PPBP gene identified in the nasal mucosal PPI network is involved in neutrophil chemotaxis (119), but not been previously associated with allergy. The identification of these novel biomarkers provides new insights into the pathogenesis of AR and the activity of these genes should be investigated further under controlled allergen conditions.

Diagnosis of AR is determined from patient history, physical examination and allergy sensitivity testing. Diagnosis of AR is confounded by the common occurrence of positive allergen-specific IgE or SPT to allergens in patients with other chronic respiratory diseases that share similar symptoms but have a different underlying aetiology. Indeed, recruitment of control participants free of allergen sensitivities was a major hurdle in this thesis. While approximately 43% of control participants reported no prior AR or allergy symptoms, these participants tested positive to at least one allergen. This indicates that the production of IgE antibodies to a specific allergen was not causing clinical manifestation of allergy. By extension, individuals with non-allergic rhinitis may test positive for specific IgE antibodies, however the nasal mucosal symptoms were instead caused by exposure to volatile nasal irritants such as smoke and perfumes. It has been suggested that some sensitised, but asymptomatic individuals, produce IgG_4 'blocking antibodies' which prevents manifestation of clinical allergy (120). Biological markers provide an objective measure of disease phenotypes and would greatly support accurate diagnosis of AR. The gene expression protocol used in this thesis effectively identified genetic biomarkers for prospective analysis in both nasal mucosal and peripheral blood samples of AR sufferers. In addition, the differentially expressed genes in both sample types were significantly enriched into asthma, bronchiolitis and

viral respiratory disease GAD pathways, thereby indicating that these genes can characterise diseases of the respiratory system.

Given the complexity of AR and the involvement of many cell types, cytokines and mediators, it is unlikely that a single biomarker will accurately distinguish AR from other rhinitis endotypes. The development of a biomarker panel containing a collection of well selected and characterised biomarkers may provide a more accurate method of delineating rhinitis endotypes. The genes identified in this study could be incorporated into a specialised gene expression biomarker panel and quantified with a cost-effective reverse-transcription PCR assay in a prospective investigation. Larger studies with hundreds of participants with different rhinitis endotypes are needed to provide reference ranges for the expression of these genes. Nasal brushing/lavage or blood samples could be collected from patients and the RT-qPCR panel could then be used to distinguish between disease endotypes, to provide a more accurate diagnosis of AR.

Following on from the biomarker study in Chapter 6, the final study utilised the same gene expression protocol used in Chapters 5 and 6 to compare changes in immune gene expression in samples collected from the nasal mucosa and peripheral blood in a cohort of adult AR sufferers in response to treatment with topical nasal sprays. The combination of the antihistamine AZE with the corticosteroid FP has been previously shown to be more effective at reducing nasal symptom scores than treatment with either therapy alone (121). Indeed, in a meta-analysis of three randomised placebo-controlled trials comparing treatment with antihistamine AZE and corticosteroid FP to the combination spray AZE/FP including a total of 3398 participants, showed that AZE/FP had a significantly greater effect on reducing TNSS compared with monotherapy with AZE or FP (121). However, both AZE/FP and FP had a similar reduction in TNSS and this small difference in TNSS scores may not translate to clinically superior effects. The change in TNSS scores pre- to post- nasal spray treatment, as shown in Chapter 7, differed from the results of the published meta-analysis. While both FP and AZE/FP had superior effects on TNSS reduction over AZE, there was no significant difference in TNSS change scores between AZE/FP and FP. The discrepancy between studies may be due to the difference in duration of treatment periods and/or sample size between studies. In Chapter 7, the treatment period was one week whereas the trials in the meta-analysis had a treatment period of two weeks. The difference in TNSS scores between FP and AZE/FP may become more apparent over a longer period of administration. In addition, change in symptoms scores were evaluated in a small cohort of participants (n=14-16 per group) compared with 200-450 participants per group that were included in larger efficacy studies (121, 122) and therefore this study was not powered to detect differences in symptom scores.

The biological mechanisms underpinning the enhanced effects of the combination spray as reported in the meta-analysis is not known. Antihistamines and corticosteroids work via different mechanisms of action to reduce symptoms of AR. It was hypothesised that the antihistamine and corticosteroid component of the combination spray would act synergistically on targets of the allergic response to enhance symptom reduction (123). Despite this, there is limited experimental data available to support this hypothesis. In-depth review of the literature on the mechanism of action of corticosteroids and antihistamines (Chapter 1), revealed that both steroids and antihistamines interfere with the transcription factor NF-κB which is involved in the transcription of many pro-inflammatory genes pertinent to the allergic response. In addition, review of the literature revealed that corticosteroids have a broad mechanism of action, downregulating transcription of many pro-inflammatory genes. As such, it was recommended in Chapter 1, that studies examining the mechanism of action of topical nasal sprays should use methods that examine a wide range of biomarkers. The gene expression protocol developed in this thesis effectively distinguished individuals with AR from otherwise-healthy controls (Chapter 6), and therefore was considered an appropriately sensitive method to detect changes relevant to AR pathophysiology by topical pharmaceutical treatments.

Unique gene expression profiles in the nasal mucosa were observed for all three treatment groups following the seven-day administration period. The greatest contrast in gene expression profiles was observed between FP and AZE, which is not surprising given these two nasal sprays contain drugs with separate mechanisms of action. A compelling finding of this study was that FP and AZE/FP had comparable effects on symptom reduction but had diverse effects on gene expression. FP treatment modulated the expression of many genes (n=206) and had a mostly downregulatory effect on the expression of these genes whereas AZE/FP modulated a fewer number of genes (n=16) and had both upregulatory and downregulatory effects on the expression of these genes. The findings of this study do not support the original hypothesis that the antihistamine and steroid components in the combination spray would synergistically downregulate transcription factors and/or pro-inflammatory genes involved in the allergic response. The findings of this study instead revealed that when the antihistamine and steroid are combined the downregulatory effect as seen in the steroid monotherapy is moderated. The proportion of genes modulated by AZE/FP is sufficient to markedly reduce AR symptoms, whilst in theory also preventing excessive suppression of the local immune system.

Conclusions

The key contributions of this thesis to further understanding the pathophysiology of AR and exploring knowledge gaps relevant to treatment and management of symptomatology include:

1) The microbiota of the gut in adult AR sufferers is different compared to non-AR sufferers and is characterised by a reduced microbial diversity and altered abundance of the major phyla Bacteroidetes, Actinobacteria and Firmicutes. The microbiota of AR sufferers also features an altered abundance of the taxa *Parabacteroides, Bifidobacterium, Oxalobacter* and Clostridiales.

2) The Simon-Two Stage phase II clinical trial design typically used in cancer trials can be effectively modified to preliminary evidence for treatments in other conditions, such as AR.

3) Supplementation with a specifically formulated probiotic supplement may reduce symptoms and improve quality of life in adults suffering intermittent/seasonal AR.

4) The gene expression protocol developed in this thesis detected sufficient mRNA signal in nasal mucosal samples collected via cost-effective and non-invasive means.

5) The gene expression protocol was sensitive enough to effectively distinguish between different sample types (nasal lysate and peripheral blood), different topical treatments, and disease (AR vs without-AR).

6) Rhinitis endotypes may be further defined using specifically designed gene expression panels containing candidate genes identified in this study.

7) Administration with steroid containing nasal sprays 'AZE/FP' and 'FP' reduced perennial/persistent AR symptoms more so than the antihistamine nasal spray 'AZE'.

8) FP treatment reduces symptoms of persistent/perennial AR through the downregulation of many immune genes in the nasal mucosa.

9) AZE/FP nasal spray effectively reduces persistent/perennial AR symptoms without causing extensive local immune suppression.

Recommendations

The outcomes presented in this thesis have provided direction for further research including:

1) The data generated from the many symptom severity questionnaires used in this thesis did not correlate closely together. This suggests that the symptom surveys do not measure AR symptomology equally. Therefore, studies examining the efficacy of AR treatments should utilise various symptom severity questionnaires to ensure thorough coverage of the multiple different symptom domains common in AR.

2) The skin prick test results and serum specific IgE test results from AR participants included in the thesis studies were not always consistent with each other. As such studies characterising the allergen sensitivity of AR participants should utilise both serum specific IgE and skin prick testing to avoid false positive and negative results.

3) A correlation between reduced microbial diversity and altered abundance of specific taxa in adults with AR compared with controls was reported in chapter two. However, this thesis did not address the causal effects of reduced microbial diversity and altered abundance of specific taxa on AR pathophysiology. As such, mechanistic studies with gnotobiotic mice and prospective cohort longitudinal studies should be conducted to clarify how the altered taxa identified in this thesis contribute to the pathogenesis of AR. Identification of the metabolites and specific bacterial components of the identified taxa and their interaction with the host immune system will improve the understanding of how the microbiome composition regulates immune homeostasis relevant to AR.

4) Data from the phase II probiotic intervention study provided evidence that probiotic supplementation may be effective at reducing AR symptoms. A large-scale randomised placebo-controlled study is needed to confirm these results. Analysis of the microbiome should be incorporated as an outcome measure to determine if (a) the probiotic supplement modulates the microbiome (b) if the baseline microbiome delineates ‘responders’ from ‘non-responders’ and (c) confirm compliance to supplementation.

5) Additional studies are needed to examine the mechanism of action of the probiotic supplement examined in this thesis. The effect of the probiotic mixture on immune parameters should be examined ideally in (a) mucosal tissue, via collection of gastrointestinal biopsies (b) systemically, via collection of peripheral blood samples and (c) in nasal mucosal tissues via collection of nasal cells through nasal washing/brushing

procedures. The metabolomic profile of the strains in the probiotic mixture are also worth investigating.

6) The gene expression protocol developed in this thesis provides opportunities to identify biomarkers in other rhinitis endotypes including non-allergic rhinitis, rhinosinusitis, nasal polyposis and gustatory rhinitis. The data generated from these studies could be used to develop a specialised gene expression panel containing a selection of genes which can accurately delineate between rhinitis endotypes. Large sample sizes will be required to generate reference norms. In future gene expression profiling of nasal washing and nasal brushing samples from patients may become a component of personalised patient management.

7) The combined nasal washing/brushing procedure employed in this thesis could be used to collect microbes colonised in the luminal space and attached to nasal mucosal surface. The collection of these samples would facilitate microbiome profiling of the nasal mucosa. The involvement of nasal mucosal microbes and the pathogenesis of AR was not explored in this thesis and is worthy of further consideration.

8) In this thesis the gut microbial composition profiles and gene expression profiles in the nasal mucosa and peripheral blood were examined in adult AR sufferers. However, the link between the gut microbiome composition and expression of immune genes systemically and locally (nasal mucosa) were not explored. The gene expression protocol developed in this thesis could be used to examine the relationship between the gut microbial composition and AR pathophysiology.

9) The differentially expressed genes identified in Chapter 7, should be confirmed in larger sample sizes. Sample size estimates should include compensation for participant withdrawal/drop-out and for samples not meeting quality control guidelines. Long-term studies comparing the adverse events of the combination spray compared with steroid monotherapy should be performed to determine if the ‘lessened immune suppressive effects’ of the combination spray observed in Chapter 7, translate to fewer adverse events clinically.

REFERENCES (CHAPTERS ONE AND EIGHT)

1. Alkanani AK, Hara N, Gottlieb PA, Ir D, Robertson CE, Wagner BD, et al. Alterations in Intestinal Microbiota Correlate With Susceptibility to Type 1 Diabetes. *Diabetes* 2015;**64**(10):3510-3520.

2. Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, et al. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. *BMC Medicine* 2013;**11**(1):46.

3. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. *Elife* 2013;**2**:e01202.

4. Vaahtovuo J, Munukka E, Korkeamaki M, Luukkainen R, Toivanen P. Fecal microbiota in early rheumatoid arthritis. *J Rheumatol* 2008;**35**(8):1500-1505.

5. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. *Gut* 2011;**60**(5):631-637.

6. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, et al. Low counts of Faecalibacterium prausnitzii in colitis microbiota. *Inflamm Bowel Dis* 2009;**15**(8):1183-1189.

7. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Paz Soldan MM, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep* 2016;**6**:28484.

8. Hevia A, Milani C, Lopez P, Donado CD, Cuervo A, Gonzalez S, et al. Allergic Patients with Long-Term Asthma Display Low Levels of Bifidobacterium adolescentis. *PLoS One* 2016;**11**(2):e0147809.

9. Watanabe S, Narisawa Y, Arase S, Okamatsu H, Ikenaga T, Tajiri Y, et al. Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. *J Allergy Clin Immunol* 2003;**111**(3):587-591.

10. Hua X, Goedert JJ, Pu A, Yu G, Shi J. Allergy associations with the adult fecal microbiota: Analysis of the American Gut Project. *EBioMedicine* 2016;**3**:172-179.

11. Melli LC, do Carmo-Rodrigues MS, Araujo-Filho HB, Sole D, de Morais MB. Intestinal microbiota and allergic diseases: A systematic review. *Allergol Immunopathol (Madr)* 2016;**44**(2):177-188.

12. Hormannsperger G, Clavel T, Haller D. Gut matters: microbe-host interactions in allergic diseases. *J Allergy Clin Immunol* 2012;**129**(6):1452-1459.

13. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. *Science* 2006;**312**(5778):1355-1359.

14. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010;**464**(7285):59-65.

15. Savage DC. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol* 1977;**31**:107-133.

16. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PloS Biol* 2016;**14**(8).

17. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. *Science* 2005;**308**(5728):1635-1638.

18. Fujimura KE, Lynch SV. Microbiota in allergy and asthma and the emerging relationship with the gut microbiome. *Cell Host Microbe* 2015;**17**(5):592-602.

19. Schroeder BO, Backhed F. Signals from the gut microbiota to distant organs in physiology and disease. *Nat Med* 2016;**22**(10):1079-1089.

20. McLoughlin RM, Mills KH. Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma. *J Allergy Clin Immunol* 2011;**127**(5):1097-1107; quiz 1108-1099.

21. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. *J Gastroenterol* 2009;**44**(1):26-46.

22. Ipci K, Altintoprak N, Muluk NB, Senturk M, Cingi C. The possible mechanisms of the human microbiome in allergic diseases. *Eur Arch Otorhinolaryngol* 2016.

23. Perez-Munoz ME, Arrieta M, Ramer-Tait AE, Walter J. A critical assessment of the "sterile womb" and "in utero colonization" hypothesis: implications for research on the pioneer infant microbiome. *Microbiome* 2017;**5**(48).

24. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. *Nature* 2012;**486**(7402):222-227.

25. Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* 2001;**108**(4):516-520.

26. Stokholm J, Thorsen J, Chawes BL, Schjorring S, Krogfelt KA, Bonnelykke K, et al. Cesarean section changes neonatal gut colonization. *J Allergy Clin Immunol* 2016;**138**(3):881-889 e882.

27. Backhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe* 2015;**17**(6):852.

28. Huang L, Chen Q, Zhao Y, Wang W, Fang F, Bao Y. Is elective cesarean section associated with a higher risk of asthma? A meta-analysis. *J Asthma* 2015;**52**(1):16-25.

29. Renz-Polster H, David MR, Buist AS, Vollmer WM, O'Connor EA, Frazier EA, et al. Caesarean section delivery and the risk of allergic disorders in childhood. *Clin Exp Allergy* 2005;**35**(11):1466-1472.

30. Fernandez L, Langa S, Martin V, Maldonado A, Jimenez E, Martin R, et al. The human milk microbiota: origin and potential roles in health and disease. *Pharmacol Res* 2013;**69**(1):1-10.

31. Hunt KM, Foster JA, Forney LJ, Schütte UME, Beck DL, Abdo Z, et al. Characterization of the Diversity and Temporal Stability of Bacterial Communities in Human Milk. *PLoS One* 2011;**6**(6):e21313.

32. Ege MJ, Mayer M, Normand AC, Genuneit J, Cookson WO, Braun-Fahrlander C, et al. Exposure to environmental microorganisms and childhood asthma. *N Engl J Med* 2011;**364**(8):701-709.

33. House JS, Wyss AB, Hoppin JA, Richards M, Long S, Umbach DM, et al. Early-life farm exposures and adult asthma and atopy in the Agricultural Lung Health Study. *J Allergy Clin Immunol* 2017;**140**(1):249-256.e214.

34. Bedolla-Barajas M, Javier Ramirez-Cervantes F, Morales-Romero J, Jesus Perez-Molina J, Meza-Lopez C, Delgado-Figueroa N. A rural environment does not protect against asthma or other allergic diseases amongst Mexican children. *Allergol Immunopathol (Madr)* 2018;**46**(1):31-38.

35. Pelucchi C, Galeone C, Bach JF, La Vecchia C, Chatenoud L. Pet exposure and risk of atopic dermatitis at the pediatric age: a meta-analysis of birth cohort studies. *J Allergy Clin Immunol* 2013;**132**(3):616-622 e617.

36. Strachan DP. Hay Fever, Hygiene, And Household Size. *BMJ: British Medical Journal* 1989;**299**(6710):1259-1260.

37. Wold AE. The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? *Allergy* 1998;**53**(46 Suppl):20-25.

38. Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nat Rev Immunol* 2001;**1**(1):69-75.

39. Bloomfield SF, Rook GA, Scott EA, Shanahan F, Stanwell-Smith R, Turner P. Time to abandon the hygiene hypothesis: new perspectives on allergic disease, the human microbiome, infectious disease prevention and the role of targeted hygiene. *Perspect Public Health* 2016;**136**(4):213-224.

40. Rook GAW, Brunet LR. Microbes, immunoregulation, and the gut. *Gut* 2005;**54**(3):317-320.

41. Rook GA, Raison CL, Lowry CA. Microbial 'old friends', immunoregulation and socioeconomic status. *Clin Exp Immunol* 2014;**177**(1):1-12.

42. Kalliomaki M, Antoine JM, Herz U, Rijkers GT, Wells JM, Mercenier A. Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of allergic diseases by probiotics. *J Nutr* 2010;**140**(3):713S-721S.

43. Matsumoto M, Ohishi H, Kakizoe K, Benno Y. Faecal microbiota and secretory immunogloblin a levels in adult patients with atopic dermatitis. *Microb Ecol Health Dis* 2004;**16**(1):13-17.

44. Bjorksten, Naaber, Sepp, Mikelsaar, Linköpings u, Institutionen för molekylär och klinisk m, et al. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy* 1999;**29**(3):342-346.

45. He F, Ouwehand AC, Isolauri E, Hashimoto H, Benno Y, Salminen S. Comparison of mucosal adhesion and species identification of bifidobacteria isolated from healthy and allergic infants. *FEMS Immunol Med Microbiol* 2001;**30**(1):43-47.

46. Kalliomäki M, Kirjavainen P, Eerola E, Kero P, Salminen S, Isolauri E. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* 2001;**107**(1):129-134.

47. Sepp E, Julge K, Mikelsaar M, Bjorksten B. Intestinal microbiota and immunoglobulin E responses in 5-year-old Estonian children. *Clin Exp Allergy* 2005;**35**(9):1141-1146.

48. Penders J, Stobberingh EE, Thijs C, Adams H, Vink C, van Ree R, et al. Molecular fingerprinting of the intestinal microbiota of infants in whom atopic eczema was or was not developing. *Clin Exp Allergy* 2006;**36**(12):1602-1608.

49. Adlerberth I, Strachan DP, Matricardi PM, Ahrne S, Orfei L, Aberg N, et al. Gut microbiota and development of atopic eczema in 3 European birth cohorts. *J Allergy Clin Immunol* 2007;**120**(2):343-350.

50. Songjinda P, Nakayama J, Tateyama A, Tanaka S, Tsubouchi M, Kiyohara C, et al. Differences in developing intestinal microbiota between allergic and non-allergic infants: a pilot study in Japan. *Biosci Biotechnol Biochem* 2007;**71**(9):2338-2342.

51. Suzuki S, Shimojo N, Tajiri Y, Kumemura M, Kohno Y. Differences in the composition of intestinal Bifidobacterium species and the development of allergic diseases in infants in rural Japan. *Clin Exp Allergy* 2007;**37**(4):506-511.

52. Sjogren YM, Jenmalm MC, Bottcher MF, Bjorksten B, Sverremark-Ekstrom E. Altered early infant gut microbiota in children developing allergy up to 5 years of age. *Clin Exp Allergy* 2009;**39**(4):518-526.

53. Stsepetova J, Sepp E, Julge K, Vaughan E, Mikelsaar M, de Vos WM. Molecularly assessed shifts of Bifidobacterium ssp. and less diverse microbial communities are characteristic of 5-year-old allergic children. *FEMS Immunol Med Microbiol* 2007;**51**(2):260-269.

54. Waligora-Dupriet AJ, Campeotto F, Romero K, Mangin I, Rouzaud G, Menard O, et al. Diversity of gut Bifidobacterium species is not altered between allergic and non-allergic French infants. *Anaerobe* 2011;**17**(3):91-96.

55. van Nimwegen FA, Penders J, Stobberingh EE, Postma DS, Koppelman GH, Kerkhof M, et al. Mode and place of delivery, gastrointestinal microbiota, and their influence on asthma and atopy. *J Allergy Clin Immunol* 2011;**128**(5):948-955 e941-943.

56. Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC. Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol* 2012;**129**(2):434-440, 440.e431-432.

57. Candela M, Rampelli S, Turroni S, Severgnini M, Consolandi C, De Bellis G, et al. Unbalance of intestinal microbiota in atopic children. *BMC microbiology* 2012;**12**(1):95.

58. Oyama N, Sudo N, Sogawa H, Kubo C. Antibiotic use during infancy promotes a shift in the T(H)1/T(H)2 balance toward T(H)2-dominant immunity in mice. *J Allergy Clin Immunol* 2001;**107**(1):153-159.

59. Sudo N, Yu XN, Aiba Y, Oyama N, Sonoda J, Koga Y, et al. An oral introduction of intestinal bacteria prevents the development of a long-term Th2-skewed immunological memory induced by neonatal antibiotic treatment in mice. *Clin Exp Allergy* 2002;**32**(7):1112-1116.

60. Tlaskalova-Hogenova H, Sterzl J, Stepankova R, Dlabac V, Veticka V, Rossmann P, et al. Development of immunological capacity under germfree and conventional conditions. *Ann N Y Acad Sci* 1983;**409**:96-113.

61. Rask C, Evertsson S, Telemo E, Wold AE. A full flora, but not monocolonization by Escherichia coli or lactobacilli, supports tolerogenic processing of a fed antigen. *Scand J Immunol* 2005;**61**(6):529-535.

62. Herbst T, Sichelstiel A, Schar C, Yadava K, Burki K, Cahenzli J, et al. Dysregulation of allergic airway inflammation in the absence of microbial colonization. *Am J Respir Crit Care Med* 2011;**184**(2):198-205.

63. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 2012;**336**(6080):489-493.

64. Russell SL, Gold MJ, Hartmann M, Willing BP, Thorson L, Wlodarska M, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep* 2012;**13**(5):440-447.

65. Chua HH, Chou HC, Tung YL, Chiang BL, Liao CC, Liu HH, et al. Intestinal Dysbiosis Featuring Abundance of Ruminococcus gnavus Associates With Allergic Diseases in Infants. *Gastroenterology* 2018;**154**(1):154-167.

66. Huang YJ, Boushey HA. The microbiome in asthma. *J Allergy Clin Immunol* 2015;**135**(1):25-30.

67. Benard A, Desreumeaux P, Huglo D, Hoorelbeke A, Tonnel AB, Wallaert B. Increased intestinal permeability in bronchial asthma. *J Allergy Clin Immunol* 1996;**97**(6):1173-1178.

68. Pires GV, Souza HS, Elia CC, Zaltman C, Carvalho AT, Tortori CJ, et al. Small bowel of patients with asthma and allergic rhinitis: absence of inflammation despite the presence of major cellular components of allergic inflammation. *Allergy Asthma Proc* 2004;**25**(4):253-259.

69. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. London, Ontario, Canada. April 30 and May 1, 2002.

70. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consenus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* 2014;**11**(8):506-514.

71. Fontana L, Bermudez-Brito M, Plaza-Diaz J, Munoz-Quezada S, Gil A. Sources, isolation, characterisation and evaluation of probiotics. *Br J Nutr* 2013;**109 Suppl 2**:S35-50.

72. Ozdemir O. Various effects of different probiotic strains in allergic disorders: an update from laboratory and clinical data. *Clin Exp Immunol* 2010;**160**(3):295-304.

73. Falagas M, Betsi GI, Athanasiou S. Probiotics for the treatment of women with bacterial vaginosis. *Clin Microbiol Infect* 2007;**13**(7):657-664.

74. de Milliano I, Tabbers MM, van der Post JA, Benninga MA. Is a multispecies probiotic mixture effective in constipation during pregnancy? 'A pilot study'. *Nutr J* 2012;**11**:80.

75. Kato K, Mizuno S, Umesaki Y, Ishii Y, Sugitani M, Imaoka A, et al. Randomized placebo-controlled trial assessing the effect of bifidobacteria-fermented milk on active ulcerative colitis. *Aliment Pharmacol Ther* 2004;**20**(10):1133-1141.

76. Bernaola Aponte G, Bada Mancilla CA, Carreazo NY, Rojas Galarza RA. Probiotics for treating persistent diarrhoea in children. *Cochrane Database Syst Rev* 2013(8):CD007401.

77. Falagas ME, Betsi GI, Tokas T, Athanasiou S. Probiotics for prevention of recurrent urinary tract infections in women: a review of the evidence from microbiological and clinical studies. *Drugs* 2006;**66**(9):1253-1261.

78. de Roos NM, Giezenaar CG, Rovers JM, Witteman BJ, Smits MG, van Hemert S. The effects of the multispecies probiotic mixture Ecologic(R)Barrier on migraine: results of an open-label pilot study. *Benef Microbes* 2015;**6**(5):641-646.

79. Persborn M, Gerritsen J, Wallon C, Carlsson A, Akkermans LM, Soderholm JD. The effects of probiotics on barrier function and mucosal pouch microbiota during maintenance treatment for severe pouchitis in patients with ulcerative colitis. *Aliment Pharmacol Ther* 2013;**38**(7):772-783.

80. Ejtahed HS, Mohtadi-Nia J, Homayouni-Rad A, Niafar M, Asghari-Jafarabadi M, Mofid V, et al. Effect of probiotic yogurt containing Lactobacillus acidophilus and Bifidobacterium lactis on lipid profile in individuals with type 2 diabetes mellitus. *J Dairy Sci* 2011;**94**(7):3288-3294.

81. Hao Q, Lu Z, Dong BR, Huang CQ, Wu T. Probiotics for preventing acute upper respiratory tract infections. *Cochrane Database Syst Rev* 2011(9):CD006895.

82. West NP, Horn PL, Pyne DB, Gebski VJ, Lahtinen SJ, Fricker PA, et al. Probiotic supplementation for respiratory and gastrointestinal illness symptoms in healthy physically active individuals. *Clin Nutr* 2014;**33**(4):581-587.

83. Kramer MF, Heath MD. Probiotics in the treatment of chronic rhinoconjunctivitis and chronic rhinosinusitis. *J Allergy (Cairo)* 2014;**2014**:983635.

84. Kim H-J, Kim HY, Lee S-Y, Seo J-H, Lee E, Hong S-J. Clinical efficacy and mechanism of probiotics in allergic diseases. *Korean J Pediatr* 2013;**56**(9):369-376.

85. Panduru M, Panduru NM, Salavastru CM, Tiplica GS. Probiotics and primary prevention of atopic dermatitis: a meta-analysis of randomized controlled studies. *J Eur Acad Dermatol Venereol* 2015;**29**(2):232-242.

86. Kim SO, Ah YM, Yu YM, Choi KH, Shin WG, Lee JY. Effects of probiotics for the treatment of atopic dermatitis: a meta-analysis of randomized controlled trials. *Ann Allergy Asthma Immunol* 2014;**113**(2):217-226.

87. Lin J, Zhang Y, He C, Dai J. Probiotics supplementation in children with asthma: A systematic review and meta-analysis. *J Paediatr Child Health* 2018;**54**(9):953-961.

88. Azad MB, Coneys JG, Kozyrskyj AL, Field CJ, Ramsey CD, Becker AB, et al. Probiotic supplementation during pregnancy or infancy for the prevention of asthma and wheeze: systematic review and meta-analysis. *BMJ* 2013;**347**:f6471.

89. Zajac AE, Adams AS, Turner JH. A systematic review and meta-analysis of probiotics for the treatment of allergic rhinitis. *Int Forum Allergy Rhinol* 2015;**5**(6):524-532.

90. de Roock S, van Elk M, van Dijk ME, Timmerman HM, Rijkers GT, Prakken BJ, et al. Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans. *Clin Exp Allergy* 2010;**40**(1):103-110.

91. de Roock S, van Elk M, Hoekstra MO, Prakken BJ, Rijkers GT, de Kleer IM. Gut derived lactic acid bacteria induce strain specific CD4(+) T cell responses in human PBMC. *Clin Nutr* 2011;**30**(6):845-851.

92. Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TM, et al. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J Allergy Clin Immunol* 2005;**115**(6):1260-1267.

93. Niers LE, Hoekstra MO, Timmerman HM, van Uden NO, de Graaf PM, Smits HH, et al. Selection of probiotic bacteria for prevention of allergic diseases: immunomodulation of neonatal dendritic cells. *Clin Exp Immunol* 2007;**149**(2):344-352.

94. Niers LE, Timmerman HM, Rijkers GT, van Bleek GM, van Uden NO, Knol EF, et al. Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. *Clin Exp Allergy* 2005;**35**(11):1481-1489.

95. Maassen CB, van Holten-Neelen C, Balk F, den Bak-Glashouwer MJ, Leer RJ, Laman JD, et al. Strain-dependent induction of cytokine profiles in the gut by orally administered Lactobacillus strains. *Vaccine* 2000;**18**(23):2613-2623.

96. Lyons A, O'Mahony D, O'Brien F, MacSharry J, Sheil B, Ceddia M, et al. Bacterial strain-specific induction of Foxp3+ T regulatory cells is protective in murine allergy models. *Clin Exp Allergy* 2010;**40**(5):811-819.

97. Rosenfeldt V, Benfeldt E, Valerius NH, Paerregaard A, Michaelsen KF. Effect of probiotics on gastrointestinal symptoms and small intestinal permeability in children with atopic dermatitis. *J Pediatr* 2004;**145**(5):612-616.

98. Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* 2001;**121**(3):580-591.

99. Patel RM, Myers LS, Kurundkar AR, Maheshwari A, Nusrat A, Lin PW. Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function. *Am J Pathol* 2012;**180**(2):626-635.

100. Takahashi N, Kitazawa H, Iwabuchi N, Xiao JZ, Miyaji K, Iwatsuki K, et al. Immunostimulatory oligodeoxynucleotide from Bifidobacterium longum suppresses Th2 immune responses in a murine model. *Clin Exp Immunol* 2006;**145**(1):130-138.

101. Kim JY, Choi YO, Ji GE. Effect of oral probiotics (Bifidobacterium lactis AD011 and Lactobacillus acidophilus AD031) administration on ovalbumin-induced food allergy mouse model. *J Microbiol Biotechnol* 2008;**18**(8):1393-1400.

102. Morita H, He F, Kawase M, Kubota A, Hiramatsu M, Kurisaki J-I, et al. Preliminary Human Study for Possible Alteration of Serum Immunoglobulin E Production in Perennial Allergic Rhinitis with Fermented Milk Prepared with Lactobacillus gasseri TMC0356. *Microbiol Immunol* 2006;**50**(9):701-706.

103. Ren C, Zhang Q, de Haan BJ, Zhang H, Faas MM, de Vos P. Identification of TLR2/TLR6 signalling lactic acid bacteria for supporting immune regulation. *Sci Rep* 2016;**6**:34561.

104. Forsythe P, Inman MD, Bienenstock J. Oral treatment with live Lactobacillus reuteri inhibits the allergic airway response in mice. *Am J Respir Crit Care Med* 2007;**175**(6):561-569.

105. Hoarau C, Lagaraine C, Martin L, Velge-Roussel F, Lebranchu Y. Supernatant of Bifidobacterium breve induces dendritic cell maturation, activation, and survival through a Toll-like receptor 2 pathway. *J Allergy Clin Immunol* 2006;**117**(3):696-702.

106. Hart AL, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P, et al. Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut* 2004;**53**(11):1602-1609.

107. Veckman V, Miettinen M, Pirhonen J, Siren J, Matikainen S, Julkunen I. Streptococcus pyogenes and Lactobacillus rhamnosus differentially induce maturation and production of Th1-type cytokines and chemokines in human monocyte-derived dendritic cells. *J Leukoc Biol* 2004;**75**(5):764-771.

108. Mohamadzadeh M, Olson S, Kalina WV, Ruthel G, Demmin GL, Warfield KL, et al. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. *Proc Natl Acad Sci U S A* 2005;**102**(8):2880-2885.

109. Torii A, Torii S, Fujiwara S, Tanaka H, Inagaki N, Nagai H. Lactobacillus Acidophilus strain L-92 regulates the production of Th1 cytokine as well as Th2 cytokines. *Allergol Int* 2007;**56**(3):293-301.

110. Feleszko W, Jaworska J, Rha RD, Steinhausen S, Avagyan A, Jaudszus A, et al. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. *Clin Exp Allergy* 2007;**37**(4):498-505.

111. Weise C, Zhu Y, Ernst D, Kuhl AA, Worm M. Oral administration of Escherichia coli Nissle 1917 prevents allergen-induced dermatitis in mice. *Exp Dermatol* 2011;**20**(10):805-809.

112. Karimi K, Inman MD, Bienenstock J, Forsythe P. Lactobacillus reuteri-induced regulatory T cells protect against an allergic airway response in mice. *Am J Respir Crit Care Med* 2009;**179**(3):186-193.

113. Bäckhed F, Fraser Claire M, Ringel Y, Sanders Mary E, Sartor RB, Sherman Philip M, et al. Defining a Healthy Human Gut Microbiome: Current Concepts, Future Directions, and Clinical Applications. *Cell Host & Microbe* 2012;**12**(5):611-622.

114. Songjinda P, Nakayama J, Tateyama A, Tanaka S, Tsubouchi M, Kiyohara C, et al. Differences in Developing Intestinal Microbiota between Allergic and Non-Allergic Infants: A Pilot Study in Japan. *Bioscience, Biotechnology, and Biochemistry* 2007;**71**(9):2338-2342.

115. Verhulst SL, Vael C, Beunckens C, Nelen V, Goossens H, Desager K. A longitudinal analysis on the association between antibiotic use, intestinal microflora, and wheezing during the first year of life. *J Asthma* 2008;**45**(9):828-832.

116. Watts AM, West NP, Smith PK, Cripps AW, Cox AJ. Probiotics and Allergic Rhinitis: A Simon Two-Stage Design to Determine Effectiveness. *J Altern Complement Med* 2016;**22**(12):1007-1012.

117. Besseling-van der Vaart I, Heath MD, Guagnini F, Kramer MF. In vitro evidence for efficacy in food intolerance for the multispecies probiotic formulation Ecologic(R) Tolerance (Syngut). *Benef Microbes* 2015:1-8.

118. Liu T, Zhang L, Joo D, Sun S-C. NF-κB signaling in inflammation. *Signal Transduct Target Ther* 2017;**2**:17023.

119. Di Stefano A, Caramori G, Gnemmi I, Contoli M, Bristot L, Capelli A, et al. Association of increased CCL5 and CXCL7 chemokine expression with neutrophil activation in severe stable COPD. *Thorax* 2009;**64**(11):968-975.

120. Bousquet J, Anto JM, Bachert C, Bousquet PJ, Colombo P, Crameri R, et al. Factors responsible for differences between asymptomatic subjects and patients presenting an IgE sensitization to allergens. A GA2LEN project. *Allergy* 2006;**61**(6):671-680.

121. Carr W, Bernstein J, Lieberman P, Meltzer E, Bachert C, Price D, et al. A novel intranasal therapy of azelastine with fluticasone for the treatment of allergic rhinitis. *J Allergy Clin Immunol* 2012;**129**(5):1282-1289 e1210.

122. Meltzer EO, LaForce C, Ratner P, Price D, Ginsberg D, Carr W. MP29-02 (a novel intranasal formulation of azelastine hydrochloride and fluticasone propionate) in the treatment of seasonal allergic rhinitis: a randomized, double-blind, placebo-controlled trial of efficacy and safety. *Allergy Asthma Proc* 2012;**33**(4):324-332.

123. Watts AM, Cripps A, West N, Cox A. Modulation of allergic inflammation in the nasal mucosa of allergic rhinitis sufferers with topical pharmaceutical agents. *Front Pharmacol* 2019;**10**:294.

124. Juniper EF, Thompson AK, Ferrie PJ, Roberts JN. Development and validation of the mini Rhinoconjunctivitis Quality of Life Questionnaire. *Clin Exp Allergy* 2000;**30**(1):132-140.

125. Von Boroviczeny KG, Böttiger LE, Bull BS, Chattas A, Dawson JB, Fukutake K, et al. Recommendation for Measurement of Erythrocyte Sedimentation Rate of Human Blood: International Committee For Standardization in Haematology. *Am J Clin Pathol* 1977;**68**(4):505-507.

APPENDICES

APPENDIX 1: SUPPLEMENTARY MATERIAL FROM CHAPTER TWO

Table 1. Prevalent and less prevalent Order detected in our AR and CG cohort

Prevalent taxa (>50% detected in either cohort)				
Order	**All**	**AR**	**CG**	**P value**
Unclassified	0.48 ± 2.66	0.60 ± 3.15%	0.20 ± 0.28	0.546
Actinobacteria unclassified	0.06 ± 0.08	0.05 ± 0.05	0.08 ± 0.13	0.265
Bifidobacteriales	0.69 ± 1.56	0.87 ± 1.81	0.24 ± 0.34	0.013
Bacteroidales	34.13 ± 11.59	36.32 ± 12.14	28.69 ± 7.97	0.007
Erysipelotrichales	0.77 ± 0.83	0.76 ± 0.88	0.79 ± 0.73	0.907
Lactobacillales	0.34 ± 0.53	0.30 ± 0.52	0.44 ± 0.58	0.297
Clostridiales	53.14 ± 14.26	50.47 ± 14.68	59.76 ± 10.82	0.008
Negativicutes unclassifed	4.31 ± 3.27	4.38 ± 3.18	4.15 ± 3.57	0.774
Burkholderiales	0.40 ± 0.60	0.40 ± 0.65	0.39 ± 0.44	0.936
Desulfovibrionales	0.28 ± 0.32	0.29 ± 0.31	0.26 ± 0.35	0.732
Gammaproteobacteria unclassified	0.65 ± 1.48	0.73 ± 1.64	0.45 ± 1.02	0.443
Verrucomicrobiales	3.76 ± 7.12	3.90 ± 7.86	3.41 ± 4.99	0.783
Not prevent taxa (<50% detected either cohort)				
Methanobacteriales	0.03 ± 0.06	0.03 ± 0.06	0.03 ± 0.05	0.886
Bacteria unclassified	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.494
Actinomycetales	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.403
Coriobacteriales	0.10 ± 0.15	0.11 ± 0.17	0.09 ± 0.10	0.692
Flavobacteriales	0.01 ± 0.02	<0.01 ± 0.02	0.01 ± 0.03	0.442
Oscillatoriales	<0.01 ± 0.00	<0.01 ± <0.01	<0.01 ± <0.01	0.347
Selenomonadales	0.58 ± 3.03	0.45 ± 2.31	0.91 ± 4.38	0.539
Fusobacteriales	0.19 ± 1.28	0.26 ± 1.51	<0.01 ±<0.01	0.410
Kiloniellales	<0.01 ± 0.01	<0.01± 0.01	0.01 ± 0.02	0.407
Rhodospirillales	0.03 ± 0.09	0.03 ± 0.10	0.04 ± 0.08	0.579
Campylobacterales	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.961
Pasteurellales	0.01 ± 0.02	0.01 ± 0.02	0.02 ± 0.03	0.124
Synergistales	0.02 ± 0.08	0.02 ± 0.09	0.02 ± 0.08	0.851
Acholeplasmatales	<0.01 ± 0.01	<0.01 ± <0.01	<0.01 ± 0.01	0.243

AR, allergic rhinitis; CG, control group. Data are presented as mean ± SD

Table 2. Prevalent and less prevalent Genre detected in our AR and CG cohort

Prevalent taxa (>50% detected in either cohort)				
Genre	**All**	**AR**	**CG**	**P value**
unclassified	0.48 ± 2.66	0.60 ± 3.15	0.20 ± 0.28	0.545
Actinobacteria unclassified	0.03 ± 0.04	0.03 ± 0.05	0.02 ± 0.03	0.284
Bifidobacterium	0.70 ± 1.56	0.87 ± 1.81	0.26 ± 0.35	0.017
Collinsella	0.10 ± 0.15	0.10 ± 0.17	0.08 ± 0.09	0.593
Bacteroidales unclassified	0.50 ± 1.69	0.50 ± 1.84	0.48 ± 1.28	0.959
Barnesiella	0.93 ± 1.24	0.79 ± 1.04	1.26 ± 1.62	0.216
Butyricimonas	0.10 ± 0.11	0.09 ± 0.11	0.11 ± 0.12	0.569
Odoribacter	0.22 ± 0.30	0.24 ± 0.35	0.16 ± 0.16	0.304
Parabacteroides	2.04 ± 2.29	2.40 ± 2.55	1.15 ± 1.03	0.003
Bacteroides	21.55 ± 14.63	23.42 ± 15.96	16.93 ± 9.40	0.028
Alistipes	4.15 ± 4.13	4.10 ± 3.84	4.25 ± 4.88	0.889
Erysipelotrichaceae unclassified	0.68 ± 0.77	0.67 ± 0.81	0.73 ± 0.70	0.745
Holdemania	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.03	0.751
Turicibacter	0.03 ± 0.05	0.03 ± 0.06	0.03 ± 0.05	0.747
Lactobacillus	0.11 ± 0.15	0.11 ± 0.12	0.12 ± 0.20	0.837
Streptococcus	0.24 ± 0.54	0.19 ± 0.51	0.38 ± 0.60	0.195
Clostridiales unclassified	3.19 ± 2.67	2.91 ± 2.50	3.90 ± 2.99	0.136
Gracilibacter	1.15 ± 1.91	1.12 ± 2.02	1.22 ± 1.64	0.834
Christensenella	0.32 ± 0.80	0.19 ± 0.51	0.63 ± 1.23	0.111
Caloramator	0.17 ± 0.17	0.16 ± 0.18	0.19 ± 0.17	0.602
Clostridium	0.10 ± 0.22	0.09 ± 0.20	0.12 ± 0.25	0.521
Lutispora	0.02 ± 0.04	0.03 ± 0.04	0.02 ± 0.01	0.219
Clostridiales Family XIII. *Incertae Sedis* unclassified	0.05 ± 0.07	0.04 ± 0.06	0.08 ± 0.10	0.143
Anaerovorax	0.05 ± 0.05	0.05 ± 0.06	0.05 ± 0.04	0.968
Eubacterium	2.32 ± 1.91	2.10 ± 1.46	2.86 ± 2.69	0.214
Lachnospiraceae unclassified	6.25 ± 3.73	5.88 ± 3.55	7.16 ± 4.08	0.164
Anaerostipes	0.17 ± 0.19	0.18 ± 0.20	0.15 ± 0.17	0.546
Blautia	2.46 ± 2.41	2.60 ± 2.70	2.13 1.47	0.437
Coprococcus	1.97 ± 2.59	1.56 ± 2.27	2.97 ± 3.08	0.056
Dorea	0.38 ± 0.35	0.36 ± 0.35	0.41 ± 0.37	0.593
Hespellia	0.05 ± 0.05	0.04 ± 0.05	0.05 ± 0.04	0.738
Lachnobacterium	0.11 ± 0.22	0.08 ± 0.12	0.19 ± 0.36	0.145
Lachnospira	0.05 ± 0.11	0.05 ± 0.12	0.07 ± 0.08	0.542
Pseudobutyrivibrio	0.02 ± 0.04	0.01 ± 0.03	0.02 ± 0.06	0.316
Roseburia	0.99 ± 1.17	1.08 ± 1.31	0.76 ± 0.68	0.156
Oscillibacter	3.51 ± 3.16	3.19 ± 2.47	4.30 ± 4.41	0.156
Desulfotomaculum	0.02 ± 0.03	0.02 ± 0.03	0.01 ± 0.02	0.075

Peptostreptococcaceae unclassified	0.11 ± 0.14	0.12 ± 0.15	0.09 ± 0.12	0.283
Ruminococcaceae unclassified	3.26 ± 2.98	3.09 ± 3.15	3.65 ± 2.53	0.417
Acetanaerobacterium	0.11 ± 0.32	0.11 ± 0.31	0.13 ± 0.34	0.176
Acetivibrio	0.12 ± 0.16	0.12 ± 0.17	0.12 ± 0.13	0.999
Anaerotruncus	0.12 ± 0.60	0.14 ± 0.71	0.05 ± 0.04	0.528
Faecalibacterium	20.03 ± 11.91	19.32 ± 12.10	21.77 ± 11.51	0.410
Gemmiger	2.91 ± 3.23	3.15 ± 3.65	2.31 ± 1.77	0.170
Ruminococcus	2.15 ± 2.71	2.12 ± 2.39	2.21 ± 3.43	0.900
Sporobacter	0.32 ± 0.70	0.34 ± 0.80	0.26 ± 0.35	0.629
Phascolarctobacterium	2.82 ± 3.24	3.19 ± 3.44	1.90 ± 2.54	0.109
Dialister	1.34 ± 2.72	0.90 ± 1.94	2.41 ± 3.90	0.088
Veillonella	0.03 ± 0.17	0.04 ± 0.20	0.01 ± 0.02	0.509
Oxalobacter	<0.01 ± 0.01	<0.01 ± <0.01	<0.01 ± 0.01	0.023
Sutterellaceae unclassified	0.31 ± 0.60	0.33 ± 0.65	0.26 ± 0.45	0.614
Desulfovibrio	0.28 ± 0.32	0.29 ± 0.31	0.27 ± 0.35	0.860
Escherichia	0.60 ± 1.45	0.70 ± 1.63	0.35 ± 0.84	0.210
Haemophilus	0.01 ± 0.03	0.01 ± 0.02	0.02 ± 0.04	0.114
Akkermansia	3.77 ± 7.12	3.90 ± 7.86	3.43 ± 4.99	0.790
Not prevent taxa (<50% detected either cohort)				
Methanobrevibacter	0.02 ± 0.06	0.03 ± 0.06	0.02 ± 0.04	0.687
Methanosphaera	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± 0.01	0.439
Vampirovibrio	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± <0.01	0.513
Adlercreutzia	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.346
Eggerthella	0.02 ± 0.07	0.01 ± 0.03	0.05 ± 0.13	0.228
Enterorhabdus	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.218
Slackia	0.01 ± 0.02	0.00 ± 0.01	0.01 ± 0.03	0.221
Rothia	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± 0.01	0.093
Actinomyces	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.404
Atopobium	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± 0.01	0.787
Olsenella	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± 0.01	0.500
Coriobacteriaceae unclassified	<0.01 ± 0.01	<0.01 ± <0.01	0.01 ± 0.01	0.053
Coenonia	0.01 ± 0.02	<0.01 ± 0.02	0.01 ± 0.03	0.442
Porphyromonas	0.10 ± 0.55	0.04 ± 0.20	0.27 ± 0.97	0.272
Prevotellaceae unclassified	0.73 ± 1.85	0.70 ± 2.00	0.81 ± 1.45	0.805
Paraprevotella	0.26 ± 0.74	0.25 ± 0.78	0.29 ± 0.64	0.812
Prevotella	3.71 ± 7.04	3.69 ± 7.69	3.75 ± 5.25	0.975
Rikenellaceae unclasssified	0.08 ± 0.27	0.06 ± 0.15	0.14 ± 0.45	0.412
Sporanaerobacter	<0.01 ± 0.02	<0.01 ± 0.02	<0.01 ± <0.01	0.485

Catenibacterium	0.04 ± 0.25	0.05 ± 0.30	0.01 ± 0.02	0.455
Solobacterium	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.410
Granulicatella	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.367
Enterococcus	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.387
Lactococcus	<0.01 ± 0.02	<0.01 ± 0.01	0.01 ± 0.03	0.391
Howardella	0.02 ± 0.04	0.01 ± 0.04	0.03 ± 0.05	0.132
Catabacter	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± 0.01	0.267
Butyricicoccus	<0.01 ± 0.02	<0.01 ± 0.01	<0.01 ± 0.02	0.809
Tindallia	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.529
Garciella	<0.01 ± 0.02	0.01 ± <0.01	0.01 ± 0.03	0.307
Anaerosporobacter	0.01 ± 0.02	0.01 ± 0.02	0.00 ± 0.01	0.328
Butyrivibrio	0.05 ± 0.25	0.03 ± 0.22	0.08 ± 0.32	0.497
Oribacterium	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.680
Parasporobacterium	0.06 ± 0.30	0.07 ± 0.35	0.02 ± 0.02	0.442
Robinsoniella	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± <0.01	0.130
Desulfitobacterium	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.741
Desulfonispora	<0.01 ± 0.01	<0.01 ± <0.01	<0.01 ± 0.01	0.454
Desulfosporosinus	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.255
Peptococcus	0.01 ± 0.04	0.01 ± 0.04	0.02 ± 0.05	0.393
Ethanoligenens	0.04 ± 0.14	0.03 ± 0.15	0.05 ± 0.12	0.703
Hydrogenoanaerobacterium	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.182
Papillibacter	<0.01 ± 0.01	<0.01 ± <0.01	<0.01 ± 0.01	0.189
Subdoligranulum	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.914
Acidaminococcus	0.17 ± 0.73	0.24 ± 0.86	0.02 ± 0.10	0.068
Allisonella	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± <0.01	0.482
Megasphaera	0.01 ± 0.05	0.01 ± 0.06	<0.01 ± <0.01	0.402
Megamonas	0.60 ± 3.03	0.44 ± 2.31	1.01 ± 4.38	0.449
Mitsuokella	<0.01 ± 0.02	<0.01 ± 0.03	<0.01 ± <0.01	0.534
Fusobacterium	0.19 ± 1.28	0.26 ± 1.51	<0.01 ± <0.01	0.410
Kiloniella	<0.01 ± 0.01	<0.01 ± 0.01	0.01 ± 0.02	0.406
Novispirillum	0.02 ± 0.08	0.02 ± 0.10	<0.01 ± 0.01	0.379
Rhodospirillum	0.01 ± 0.04	0.00 ± 0.01	0.03 ± 0.07	0.121
Sutterella	0.09 ± 0.16	0.07 ± 0.13	0.14 ± 0.22	0.082
Campylobacter	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.963
Citrobacter	<0.01 ± 0.02	<0.01 ± 0.03	<0.01 ± <0.01	0.392
Cloacibacillus	0.02 ± 0.08	0.02 ± 0.08	0.02 ± 0.08	0.824

Acholeplasma	0.00 ± 0.01	<0.01 ± <0.01	<0.01 ± 0.01	0.198
Fucophilus	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.619

AR, allergic rhinitis; CG, control group. Data are presented as mean ± SD

APPENDIX 2: SUPPLEMENTARY MATERIAL FROM CHAPTER FIVE PUBLICATION

Pages 214 - 262 have been removed from the published version of this thesis. The published version of Appendix 2 can be accessed from International Archives of Allergy and Immunology using the following citation details:

Watts AM, West NP, Cripps AW, Smith PK, Cox AJ. Distinct Gene Expression Patterns between Nasal Mucosal Cells and Blood Collected from Allergic Rhinitis Sufferers. Int Arch Allergy Immunol 2018;177(1):29-34.

APPENDIX 3: SUPPLEMENTARY MATERIAL FROM CHAPTER SIX

Supplementary Table 1: Clinical and demographic features of the sub-group cohort (nasal lysate)

	All mean ± SD	**AR mean ± SD**	**CG mean ± SD**	**P value**
n	58	37	21	-
Age (years)	37.34 ± 12.12	38.19 ± 12.92	35.83 ± 10.71	0.481
Sex (M/F)	24/34 (59%)	14/23 (62%)	10/11 (52%)	0.467
Height (cm)	173.11 ± 9.39	171.38 ± 9.38	176.15 ± 8.82	0.062
Weight (kg)	76.12 ± 17.06	75.54 ± 15.75	77.14 ± 19.54	0.733
BMI (kg/m^2)	25.21 ± 4.30	25.54 ± 3.99	24.61 ± 4.83	0.430
Ethnicity (% Caucasian)	86%	81%	95%	0.133
Immune measures				
White cell count ($x10^9/L$)	6.31 ± 1.52	6.65 ± 1.58	5.73 ± 1.24	0.026
Lymphocytes ($x10^9/L$)	2.05 ± 0.67	2.16 ± 0.70	1.86 ± 0.58	0.093
Eosinophils ($x10^9/L$)	0.31 ± 0.29	0.42 ± 0.31	0.11 ± 0.09	<0.00001
Neutrophils ($x10^9/L$)	3.41 ± 1.00	3.49 ± 1.02	3.26 ± 0.98	0.411
Basophils ($x10^9/L$)	0.05 ± 0.04	0.06 ± 0.04	0.03 ± 0.03	0.032
ESR (mm/hr)	8.69 ± 8.87	9.22 ± 9.36	7.76 ± 8.08	0.533

n, number; AR, allergic rhinitis; CG, Control Group; M, Male; F, Female; cm, centimetre; kg, kilogram; m, metre; %, percentage; L, litre; mm, millimetre; hr, hour

Supplementary table 2: Disease characteristics of the sub-group cohort (nasal lysate)

Disease characteristic	**AR (mean ± SD)**
Allergen sensitivity	
Co-allergy to dust mites and pollen (%)	59%
Dust mite only (%)	41%
IgE *D. pteronyssinus* (kU/L)	24.50 ± 33.20
IgE *D. farinae* (kU/L)	20.62 ± 31.00
IgE grass pollen mix (kU/L)	8.39 ± 22.24
IgG4 D. pteronyssinus (kU/L)	0.48 ± 0.49
IgG4 D. farinae (kU/L)	0.39 ± 0.37
IgG4 grass pollen mix (kU/L)	0.86 ± 0.68
Symptom severity	
Total Nasal Symptom Score (0-12 U)	5.35 ± 3.16
Total Ocular Symptom Score (0-9 U)	2.59 ± 2.30
Mini rhinoconjunctivitis quality of life score (0-6 U)	2.77 ± 1.11
Other Allergic Rhinitis Symptom Score (0-12 U)	3.99 ± 3.23
Visual Analogue Scale (0-100 mm)	53.22 ± 27.46

AR, allergic rhinitis; %, percentage; kU, kilounit; L, Litre; U, unit; mm, millimetre

Supplementary Table 3. Differentially expressed genes in blood samples padjust <0.05

Gene	Log2 fold change	Linear fold change	Lower confidence limit (log2)	Upper confidence limit (log2)	P value	P adjust
MAP2K1	-0.417	0.749	-0.512	-0.322	1.84E-12	9.86E-10
TBK1	-0.608	0.656	-0.769	-0.447	2.90E-10	5.18E-08
PTGDR2	1.730	3.310	1.270	2.180	2.78E-10	5.18E-08
CD83	0.979	1.970	0.681	1.280	1.53E-08	1.65E-06
CD164	-0.203	0.869	-0.266	-0.140	2.47E-08	2.21E-06
CD24	1.320	2.500	0.903	1.740	4.22E-08	3.24E-06
IL2RA	0.946	1.930	0.601	1.290	1.06E-06	7.12E-05
MICA	0.711	1.640	0.441	0.980	2.31E-06	1.38E-04
IL12RB1	0.527	1.440	0.318	0.736	5.31E-06	2.85E-04
ABCB1	-0.544	0.686	-0.764	-0.325	7.21E-06	3.52E-04
LILRA1	-0.484	0.715	-0.681	-0.287	8.71E-06	3.90E-04
HMGB1	-0.177	0.884	-0.250	-0.104	1.02E-05	4.23E-04
NCR1	-0.674	0.627	-0.958	-0.391	1.51E-05	5.79E-04
IFNGR1	-0.350	0.785	-0.500	-0.200	2.20E-05	7.40E-04
IRF3	0.527	1.440	0.300	0.753	2.20E-05	7.40E-04
TNFAIP3	-0.275	0.827	-0.393	-0.156	2.42E-05	7.65E-04
HRH4	0.896	1.860	0.507	1.280	2.64E-05	7.88E-04
IKBKG	-0.303	0.811	-0.436	-0.171	2.94E-05	8.31E-04
APP	-0.536	0.690	-0.774	-0.298	3.68E-05	9.08E-04
PSEN1	-0.292	0.817	-0.422	-0.163	3.61E-05	9.08E-04
SPN	-0.486	0.714	-0.705	-0.267	4.69E-05	1.10E-03
MAPK1	-0.400	0.758	-0.581	-0.219	5.07E-05	1.13E-03
IL6R	-0.353	0.783	-0.515	-0.190	6.79E-05	1.40E-03
STAT5B	-0.267	0.831	-0.390	-0.144	6.60E-05	1.40E-03
TNFRSF13C	0.625	1.540	0.333	0.916	8.08E-05	1.61E-03
CD4	-0.322	0.800	-0.473	-0.171	8.55E-05	1.64E-03
FCER2	0.816	1.760	0.430	1.200	9.78E-05	1.81E-03
FYN	-0.187	0.878	-0.278	-0.097	1.31E-04	2.35E-03
ECSIT	-0.410	0.752	-0.610	-0.211	1.44E-04	2.50E-03
TNFRSF10B	-0.355	0.782	-0.528	-0.182	1.50E-04	2.52E-03
CTSS	-0.298	0.813	-0.445	-0.152	1.60E-04	2.61E-03
EWSR1	-0.119	0.921	-0.178	-0.061	1.67E-04	2.63E-03
CD36	-0.480	0.717	-0.720	-0.240	2.13E-04	3.02E-03
NOTCH1	-0.440	0.737	-0.660	-0.221	2.04E-04	3.02E-03
MAP2K4	-0.326	0.798	-0.489	-0.163	2.13E-04	3.02E-03

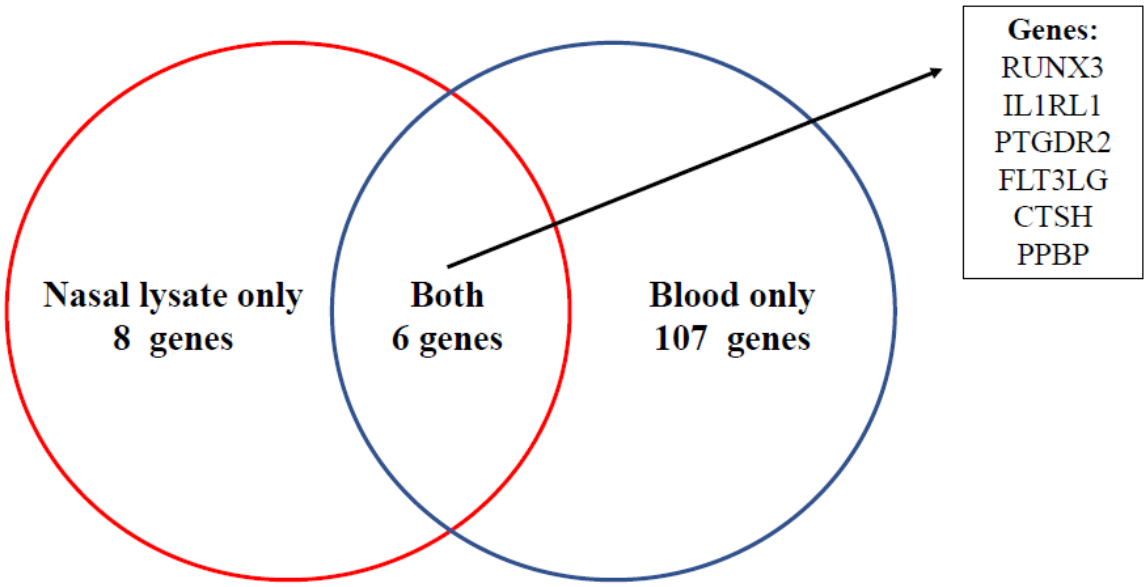
CAMP	1.020	2.020	0.509	1.520	2.04E-04	3.02E-03
IL5RA	1.210	2.310	0.601	1.820	2.26E-04	3.11E-03
TLR2	-0.448	0.733	-0.676	-0.221	2.59E-04	3.23E-03
MAPK8	-0.350	0.785	-0.527	-0.173	2.48E-04	3.23E-03
CYBB	-0.347	0.786	-0.522	-0.171	2.53E-04	3.23E-03
IRAK4	-0.189	0.877	-0.284	-0.093	2.48E-04	3.23E-03
TICAM1	0.327	1.250	0.159	0.496	3.04E-04	3.71E-03
LRP1	-0.634	0.644	-0.961	-0.306	3.22E-04	3.84E-03
LCP1	-0.286	0.820	-0.434	-0.137	3.51E-04	4.09E-03
CD99	-0.260	0.835	-0.397	-0.122	4.29E-04	4.80E-03
IL1RL1	0.908	1.880	0.428	1.390	4.26E-04	4.80E-03
CD79A	0.524	1.440	0.245	0.803	4.61E-04	5.05E-03
NUP107	0.198	1.150	0.092	0.304	4.94E-04	5.30E-03
ITGA5	-0.236	0.849	-0.364	-0.109	5.41E-04	5.69E-03
TNFRSF1B	-0.325	0.798	-0.502	-0.148	6.07E-04	6.27E-03
ITGB1	-0.321	0.800	-0.501	-0.141	8.40E-04	8.19E-03
IKBKE	0.225	1.170	0.099	0.351	8.54E-04	8.19E-03
C3AR1	0.601	1.520	0.264	0.938	8.37E-04	8.19E-03
IDO1	0.871	1.830	0.384	1.360	8.16E-04	8.19E-03
CD68	-0.346	0.787	-0.543	-0.149	1.01E-03	9.43E-03
ITGAE	-0.243	0.845	-0.382	-0.104	1.03E-03	9.43E-03
LTF	1.170	2.250	0.501	1.830	1.04E-03	9.43E-03
SMAD2	-0.147	0.903	-0.232	-0.063	1.09E-03	9.63E-03
CD84	0.237	1.180	0.101	0.373	1.08E-03	9.63E-03
F13A1	-0.671	0.628	-1.060	-0.284	1.14E-03	9.86E-03
RNASE3	1.100	2.140	0.461	1.740	1.24E-03	1.05E-02
PF4	-0.659	0.633	-1.040	-0.274	1.32E-03	1.06E-02
TBX21	-0.442	0.736	-0.701	-0.184	1.33E-03	1.06E-02
IL6ST	-0.278	0.825	-0.440	-0.115	1.33E-03	1.06E-02
FLT3LG	0.239	1.180	0.100	0.379	1.27E-03	1.06E-02
IL1R2	-0.462	0.726	-0.734	-0.190	1.40E-03	1.11E-02
TNFRSF1A	-0.254	0.839	-0.404	-0.104	1.47E-03	1.13E-02
NFKBIA	-0.232	0.852	-0.369	-0.095	1.50E-03	1.13E-02
INPP5D	-0.152	0.900	-0.242	-0.062	1.49E-03	1.13E-02
LAMP2	-0.290	0.818	-0.464	-0.116	1.69E-03	1.24E-02
UBC	-0.159	0.896	-0.254	-0.064	1.68E-03	1.24E-02
STAT3	-0.286	0.820	-0.459	-0.113	1.84E-03	1.33E-02
EP300	-0.153	0.899	-0.245	-0.060	1.87E-03	1.34E-02
CD247	-0.222	0.857	-0.357	-0.087	1.94E-03	1.37E-02
MR1	0.345	1.270	0.133	0.557	2.17E-03	1.49E-02
CCR3	0.659	1.580	0.254	1.060	2.15E-03	1.49E-02

ITGB2	-0.208	0.866	-0.336	-0.080	2.20E-03	1.50E-02
LAMP1	-0.196	0.873	-0.317	-0.075	2.29E-03	1.54E-02
PECAM1	-0.265	0.832	-0.430	-0.101	2.37E-03	1.57E-02
ITGAM	-0.257	0.837	-0.417	-0.097	2.42E-03	1.59E-02
LILRB2	-0.304	0.810	-0.495	-0.113	2.66E-03	1.72E-02
JUN	0.526	1.440	0.195	0.858	2.74E-03	1.75E-02
CCL5	-0.334	0.793	-0.547	-0.121	3.09E-03	1.87E-02
CTSH	-0.306	0.809	-0.501	-0.110	3.11E-03	1.87E-02
LILRB1	-0.292	0.817	-0.478	-0.106	3.08E-03	1.87E-02
CCR2	-0.264	0.833	-0.431	-0.096	2.95E-03	1.87E-02
HLA-E	-0.155	0.898	-0.254	-0.056	3.01E-03	1.87E-02
RIPK2	0.241	1.180	0.086	0.396	3.30E-03	1.97E-02
LILRB3	-0.334	0.793	-0.551	-0.117	3.57E-03	2.10E-02
TFRC	-0.452	0.731	-0.751	-0.152	4.27E-03	2.50E-02
IL13RA1	-0.298	0.813	-0.498	-0.099	4.53E-03	2.59E-02
GPI	0.195	1.140	0.065	0.325	4.52E-03	2.59E-02
IRF5	0.391	1.310	0.129	0.652	4.71E-03	2.66E-02
LTBR	-0.338	0.791	-0.565	-0.110	4.85E-03	2.71E-02
CD3G	-0.223	0.857	-0.379	-0.068	6.48E-03	3.59E-02
TNFRSF8	0.358	1.280	0.106	0.611	6.97E-03	3.82E-02
ATG5	-0.121	0.920	-0.206	-0.036	7.12E-03	3.86E-02
SOCS1	0.441	1.360	0.128	0.754	7.36E-03	3.95E-02
FUT7	-0.322	0.800	-0.551	-0.093	7.53E-03	4.00E-02
YTHDF2	-0.129	0.915	-0.221	-0.037	7.69E-03	4.04E-02
OAS3	0.883	1.840	0.253	1.510	7.76E-03	4.04E-02
TNFSF4	-0.467	0.723	-0.806	-0.128	8.71E-03	4.49E-02
CSF3R	-0.249	0.842	-0.429	-0.068	8.79E-03	4.49E-02
SYK	-0.227	0.854	-0.392	-0.062	8.92E-03	4.52E-02
ADORA2A	-0.326	0.798	-0.563	-0.088	9.11E-03	4.54E-02
IFNAR1	-0.272	0.828	-0.470	-0.073	9.17E-03	4.54E-02
CHUK	-0.193	0.875	-0.335	-0.052	9.38E-03	4.54E-02
RUNX3	0.247	1.190	0.066	0.428	9.38E-03	4.54E-02
ISG15	0.785	1.720	0.211	1.360	9.23E-03	4.54E-02
CD79B	0.312	1.240	0.083	0.541	9.62E-03	4.61E-02
ITGAL	-0.156	0.898	-0.270	-0.041	9.75E-03	4.63E-02
CX3CR1	-0.326	0.798	-0.567	-0.085	1.00E-02	4.72E-02
PPBP	-0.543	0.686	-0.946	-0.141	1.02E-02	4.76E-02

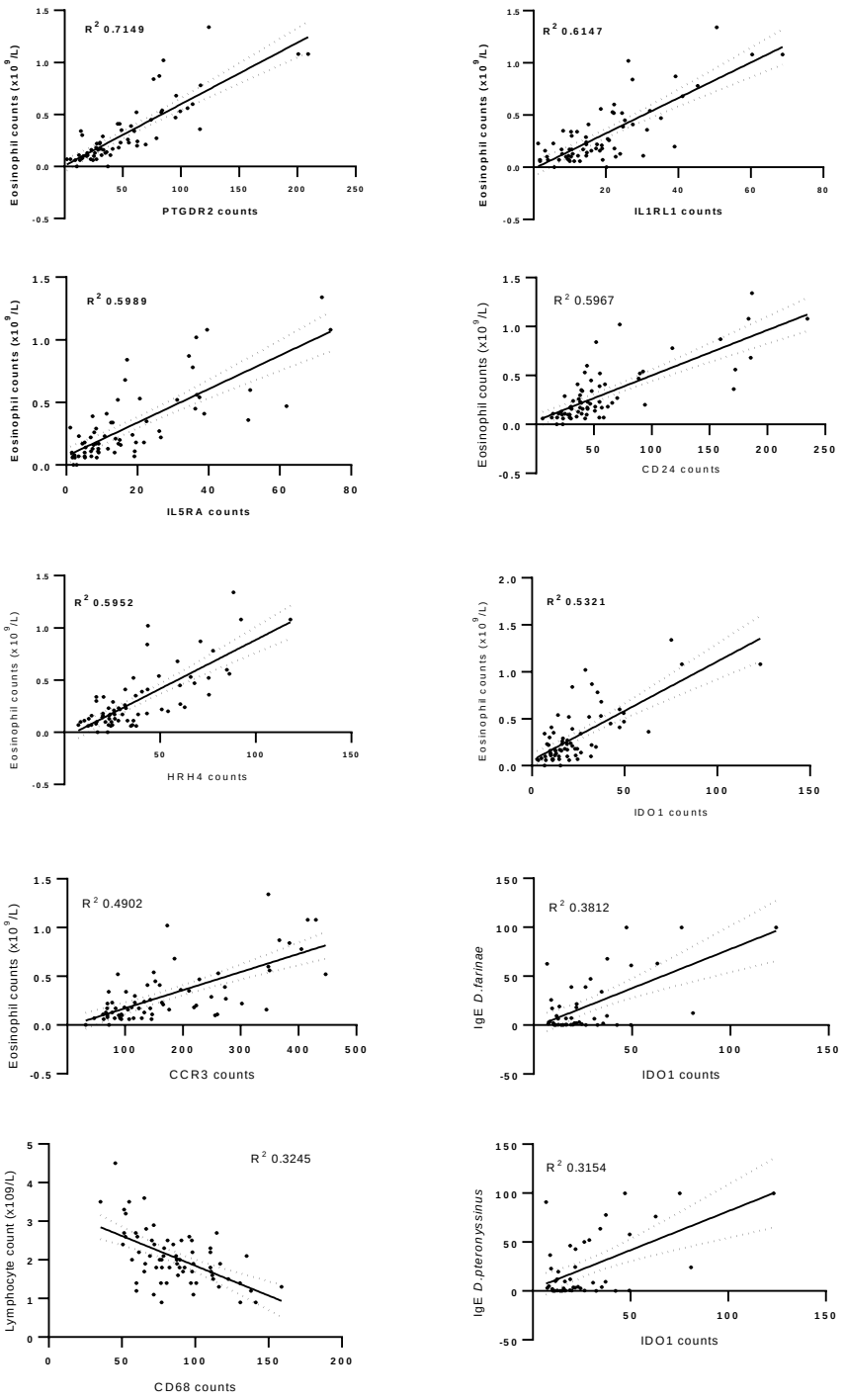
Supplementary Table 4: Differentially expressed genes in nasal lysate samples padjust <0.05

Gene	Log2 fold change	Linear fold change	Lower confidence limit (log2)	Upper confidence limit (log2)	P value	P adjust
CCL17	2.840	7.140	2.030	3.640	4.41E-09	2.51E-06
CCL26	2.720	6.610	1.820	3.630	2.39E-07	5.13E-05
TPSAB1	3.080	8.450	2.050	4.110	2.71E-07	5.13E-05
PTGS1	2.410	5.300	1.490	3.330	4.01E-06	4.57E-04
IL1RL1	3.410	10.700	1.970	4.860	2.14E-05	2.02E-03
CD1A	2.460	5.520	1.400	3.530	3.05E-05	2.48E-03
CCND3	1.130	2.190	0.570	1.690	2.36E-04	1.68E-02
PPBP	-1.650	0.320	-2.510	-0.780	4.60E-04	2.91E-02
IL18R1	1.960	3.890	0.880	3.040	7.53E-04	3.89E-02
CD1C	1.560	2.960	0.680	2.440	9.70E-04	4.04E-02
CTSH	-0.570	0.680	-0.890	-0.250	1.01E-03	4.04E-02
FLT3LG	1.160	2.230	0.500	1.810	1.06E-03	4.04E-02
RUNX3	1.680	3.210	0.740	2.630	9.63E-04	4.04E-02
PTGDR2	2.440	5.430	1.040	3.840	1.17E-03	4.15E-02
IL33	-1.370	0.390	-2.220	-0.520	2.61E-03	7.14E-02
JUN	-0.830	0.560	-1.340	-0.310	2.63E-03	7.14E-02
TXNIP	0.890	1.850	0.340	1.440	2.45E-03	7.14E-02
KLRB1	-1.470	0.360	-2.390	-0.550	2.78E-03	7.20E-02
CXCR3	1.380	2.600	0.510	2.250	3.06E-03	7.58E-02
CARD9	1.290	2.450	0.460	2.120	3.50E-03	8.31E-02
CYSLTR1	1.600	3.030	0.550	2.650	4.29E-03	9.39E-02
SOCS1	1.640	3.110	0.550	2.730	4.71E-03	9.92E-02
ATG16L1	0.390	1.310	0.130	0.650	5.09E-03	1.03E-01
IFI27	-0.880	0.540	-1.490	-0.280	5.72E-03	1.07E-01
TNFSF13	0.720	1.640	0.230	1.200	5.83E-03	1.07E-01
PF4	1.340	2.520	0.410	2.260	6.54E-03	1.16E-01
IRAK4	0.650	1.570	0.190	1.100	7.71E-03	1.33E-01
RRAD	-0.950	0.520	-1.620	-0.270	8.01E-03	1.34E-01
NFATC2	1.070	2.100	0.300	1.840	8.38E-03	1.36E-01
C3	-0.990	0.500	-1.720	-0.270	9.46E-03	1.50E-01
CCR7	1.850	3.600	0.480	3.220	1.07E-02	1.57E-01
MRC1	1.810	3.500	0.430	3.190	1.30E-02	1.76E-01
TFEB	0.840	1.790	0.190	1.480	1.36E-02	1.80E-01
PIK3CG	1.350	2.550	0.310	2.390	1.40E-02	1.81E-01
SMPD3	1.200	2.300	0.270	2.130	1.43E-02	1.81E-01
IKBKE	-0.450	0.730	-0.800	-0.100	1.47E-02	1.82E-01

TNFSF12	1.100	2.140	0.240	1.950	1.51E-02	1.82E-01
PAFAH2	0.680	1.610	0.140	1.230	1.64E-02	1.94E-01
IL16	1.300	2.460	0.260	2.340	1.79E-02	2.07E-01
RNASE3	1.400	2.630	0.270	2.520	1.82E-02	2.07E-01
IL3RA	2.230	4.690	0.410	4.050	1.96E-02	2.10E-01
TYK2	0.650	1.570	0.120	1.180	2.02E-02	2.13E-01
CD36	0.680	1.600	0.120	1.240	2.16E-02	2.20E-01
CLEC4C	1.760	3.390	0.290	3.240	2.29E-02	2.25E-01
LRP1	-0.540	0.690	-0.990	-0.080	2.42E-02	2.30E-01
SCGB1A1	-0.850	0.560	-1.570	-0.120	2.54E-02	2.36E-01
SMAD3	-0.350	0.790	-0.650	-0.050	2.57E-02	2.36E-01
CD40	-0.720	0.610	-1.340	-0.100	2.68E-02	2.42E-01
CCR3	2.170	4.510	0.250	4.100	3.11E-02	2.59E-01
CREBBP	0.640	1.550	0.080	1.200	3.03E-02	2.59E-01
CX3CL1	-1.010	0.500	-1.900	-0.120	3.10E-02	2.59E-01
NCAM1	1.000	2.000	0.120	1.880	3.01E-02	2.59E-01
REPS1	0.270	1.210	0.030	0.520	3.14E-02	2.59E-01
SERPINB2	0.640	1.550	0.070	1.200	3.11E-02	2.59E-01
TRAF3	-0.840	0.560	-1.590	-0.090	3.24E-02	2.63E-01
MAP2K2	0.310	1.240	0.030	0.590	3.62E-02	2.90E-01
CYLD	0.670	1.590	0.030	1.300	4.34E-02	3.21E-01
EGR1	1.040	2.050	0.060	2.010	4.19E-02	3.21E-01
GZMB	1.420	2.680	0.080	2.760	4.30E-02	3.21E-01
MAPK1	0.560	1.470	0.040	1.080	4.10E-02	3.21E-01
TRAF6	0.370	1.290	0.020	0.720	4.27E-02	3.21E-01
CD68	-0.720	0.610	-1.400	-0.030	4.42E-02	3.23E-01
VEGFA	-0.870	0.550	-1.690	-0.040	4.53E-02	3.24E-01



Supplementary Figure 1. Venn diagram displaying the number of DEGs unique to nasal lysate and blood samples and DEGs that were shared between both sample types.



Supplementary Figure 2: Top 10 Individual correlation plots for the differentially expressed genes in blood and clinical factors. Each point represents a sample (filled circles). Clinical factors are on the y axis and gene counts are on the x axis. Lines of best fit are plotted as a solid line with the 95% confidence bands as a dotted line for each correlation. Each correlation was significant ($p<0.05$) and the direction indicates a negative or positive correlation. Correlations for IgE *D.farinae* and IgE *D. pteronyssinus* were conducted on AR samples only (n=45). The remaining correlations were performed with the inclusion of the control group samples (n=69 total).

APPENDIX 4: SUPPLEMENTARY MATERIAL FROM CHAPTER SEVEN

Supplementary Table 1: Adverse events by treatment group assessed on the per-protocol population

	FP	AZE/FP	AZE
Event			
Sneezing after application	2	0	2
Itchy eyes	2	0	0
Bad scent	2	0	0
Runny nose after application	1	0	1
Itchy nose	1	0	1
Itchy eyes	2	0	0
Wheezing after application	1	0	0
Eye twitch	1	0	0
Bad/bitter/metallic/copper/funny taste	1	11	3
Dryness inside the nose	0	1	0
Feeling jittery	0	1	0
Worsened nasal congestion	0	2	0
Sinus pain	0	1	0
Soreness of nasal passages	0	1	2

FP, fluticasone propionate 'Flixonase ®' Group; AZE/FP, azelastine hydrochloride/ fluticasone propionate 'Dymista ®' group; AZE, azelastine hydrochloride 'Azep®' group.

Supplementary Table 2: Baseline demographic and clinical measures (nasal lysate cohort)

	FP	AZE/FP	AZE	P value
n	13	11	11	-
Age (years)	39.11 ± 14.08	40.68 ± 11.34	31.97 ± 11.82	0.234
Sex F/M (% Female)	9/4 (69%)	6/5 (55%)	8/3 (72%)	0.631
Height (cm)	169.04 ± 9.73	171.68 ± 9.46	171.59 ± 8.29	0.725
Weight (kg)	77.21 ± 16.24	75.15 ± 14.32	73.14 ± 13.65	0.801
BMI (kg/m^2)	26.83 ± 4.05	25.34 ± 3.38	24.80 ± 4.12	0.417
Ethnicity (% Caucasian)	76.92%	55%	100%	0.040
Immune measures				
White cell count ($x10^9/L$)	7.32 ± 1.67	6.74 ± 1.40	5.96 ± 1.69	0.132
Lymphocytes ($x10^9/L$)	2.31 ± 0.83	2.09 ± 0.51	2.06 ± 0.71	0.647
Eosinophils ($x10^9/L$)	0.55 ± 0.38	0.43 ± 0.28	0.26 ± 0.17	0.044
Neutrophils ($x10^9/L$)	3.87 ± 1.17	3.62 ± 1.00	3.15 ± 1.11	0.285
Basophils ($x10^9/L$)	0.06 ± 0.05	0.07 ± 0.02	0.04 ± 0.04	0.278
ESR (mm/hr)	14.15 ± 14.28	9.09 ± 7.08	6.27 ± 6.93	0.102
Allergen sensitivity				
Co-allergy to dust mites and pollen (%)				
IgE *D. pteronyssinus* (kU/L)	38.74 ± 41.98	8.26 ± 8.66	15.79 ± 23.46	0.055
IgE *D. farinae* (kU/L)	34.82 ± 41.47	5.67 ± 5.76	11.89 ± 20.24	0.059
IgE grass pollen mix (kU/L)	2.29 ± 4.43	10.37 ± 29.99	8.45 ± 25.71	0.648
IgG4 *D. pteronyssinus* (kU/L)	0.49 ± 0.57	0.46 ± 0.49	0.46 ± 0.48	0.988
IgG4 *D. farinae* (kU/L)	0.40 ± 0.43	0.38 ± 0.28	0.36 ± 0.42	0.964
IgG4 grass pollen mix (kU/L)	0.64 ± 0.35	0.78 ± 0.57	0.89 ± 0.72	0.946
Symptom severity (day 0)				
Total Nasal Symptom Score (0-12 U)	6.15 ± 4.02	3.82 ± 1.83	6.45 ± 3.78	0.064
Total Ocular Symptom Score (0-9 U)	3.85 ± 2.30	1.82 ± 2.09	2.18 ± 2.60	0.089
Mini rhinoconjunctivitis quality of life score (0-6 U)	2.90 ± 1.30	2.60 ± 0.73	2.73 ± 1.24	0.811
Other symptoms (0-12 U)	4.78 ± 3.90	3.02 ± 2.21	4.40 ± 3.43	0.411
Visual Analogue Scale (0-100 mm)	56.88 ± 33.25	46.23 ± 22.54	59.64 ± 32.04	0.542
Medication Usage				

Allergy medication use; % of total diary responses (washout period)	0.38 ± 0.30	0.22 ± 0.19	0.36 ± 0.31	0.351

n, number; L, Litre; kU, Kilounit; U, unit; mm, millimetre; %, percentage; FP, fluticasone propionate 'Flixonase ®' Group; AZE/FP, azelastine hydrochloride/ fluticasone propionate 'Dymista ®' group; AZE, azelastine hydrochloride 'Azep®' group.

Supplementary Table 3: Baseline demographic and clinical measures (peripheral blood samples)

	FP	AZE/FP	AZE	P value
n	13	16	15	-
Age (years)	38.47 ± 14.85	39.43 ± 10.03	35.67 ± 14.16	0.712
Sex F/M (% Female)	9/4 (69%)	10/6 (~63%)	10/5 (67%)	0.928
Height (cm)	169.85 ± 9.30	171.03 ± 10.26	172.27 ± 8.83	0.799
Weight (kg)	76.91 ± 16.34	72.16 ± 15.33	73.03 ± 13.55	0.679
BMI (kg/m^2)	26.46 ± 4.05	24.45 ± 3.37	24.52 ± 3.62	0.275
Ethnicity (% Caucasian)	76.92%	68.75%	86.67%	0.493
Immune measures				
White cell count ($x10^9/L$)	7.05 ± 1.69	6.98 ± 2.09	5.80 ± 1.50	0.116
Lymphocytes ($x10^9/L$)	2.31 ± 0.83	2.26 ± 0.73	1.97 ± 0.65	0.403
Eosinophils ($x10^9/L$)	0.55 ± 0.38	0.44 ± 0.30	0.26 ± 0.17	0.033
Neutrophils ($x10^9/L$)	3.59 ± 1.13	3.68 ± 1.33	3.10 ± 0.98	0.349
Basophils ($x10^9/L$)	0.07 ± 0.05	0.07 ± 0.03	0.04 ± 0.03	0.045
ESR (mm/hr)	12.85 ± 12.82	7.94 ± 6.17	7.07 ± 7.77	0.383
Allergen sensitivity				
Co-allergy to dust mites and pollen (%)				
IgE *D. pteronyssinus* (kU/L)	41.55 ± 41.10	17.12 ± 21.60	11.82 ± 20.97	0.087
IgE *D. farinae* (kU/L)	37.30 ± 40.60	13.41 ± 18.31	8.90 ± 17.87	0.091
IgE grass pollen mix (kU/L)	2.29 ± 4.42	10.44 ± 25.84	6.56 ± 21.99	0.567
IgG4 *D. pteronyssinus* (kU/L)	0.52 ± 0.56	0.47 ± 0.42	0.38 ± 0.43	0.735
IgG4 *D. farinae* (kU/L)	0.43 ± 0.43	0.39 ± 0.26	0.30 ± 0.38	0.581
IgG4 grass pollen mix (kU/L)	0.66 ± 0.36	1.07 ± 0.97	0.80 ± 0.66	0.303
Symptom severity (day 0)				
Total Nasal Symptom Score (0-12 U)	5.46 ± 3.69	4.00 ± 1.86	6.87 ± 3.68	0.301
Total Ocular Symptom Score (0-9 U)	3.38 ± 2.43	2.00 ± 2.00	3.00 ± 2.73	0.277
Mini rhinoconjunctivitis quality of life score (0-6 U)	2.85 ± 1.28	2.66 ± 0.78	2.95 ± 1.15	0.746
Other symptoms (0-12 U)	4.32 ± 3.97	3.07 ± 2.16	5.03 ± 3.51	0.248
Visual Analogue Scale (0-100 mm)	51.19 ± 32.88	48.16 ± 22.31	58.80 ± 30.35	0.573
Medication Usage				

Allergy medication use; % of total diary responses (washout period)	0.38 ± 0.30	0.25 ± 0.20	0.41 ± 0.32	0.221

n, number; L, Litre; kU, Kilounit; U, unit; mm, millimetre; %, percentage; FP, fluticasone propionate ‘Flixonase ®’ group; AZE/FP, azelastine hydrochloride/ fluticasone propionate ‘Dymista ®’ group; AZE, azelastine hydrochloride ‘Azep®’ group.

Supplementary Table 4: Fold Change values between groups.

FP – AZE/FP					
	log2 fold change	**Lower confidence limit (Log2)**	**Upper confidence limit (log2)**	**P value**	**P adjust**
CEACAM1	-1.50	-2.33	-0.66	0.001	0.161
FLT3LG	-1.42	-2.22	-0.61	0.001	0.161
PTGDR2	-2.47	-3.90	-1.03	0.001	0.161
FCER1A	-2.07	-3.30	-0.84	0.002	0.161
NFATC2	-1.59	-2.55	-0.63	0.002	0.161
CD96	-2.15	-3.45	-0.84	0.002	0.161
SOCS1	-2.02	-3.27	-0.76	0.002	0.161
SPN	-2.02	-3.29	-0.75	0.003	0.161
DUSP6	-1.71	-2.80	-0.62	0.003	0.161
LTB	-2.63	-4.31	-0.95	0.003	0.161
FYN	-1.52	-2.50	-0.54	0.003	0.161
IL1RL1	-2.06	-3.40	-0.73	0.003	0.161
CFD	-2.09	-3.45	-0.72	0.004	0.161
IL4	-1.87	-3.11	-0.64	0.004	0.161
INPP5D	-1.82	-3.03	-0.62	0.004	0.161
RUNX3	-1.58	-2.66	-0.51	0.005	0.172
TCF7	-1.24	-2.09	-0.39	0.005	0.172
ABCB1	-1.78	-3.00	-0.56	0.005	0.172
PTGS1	-1.95	-3.29	-0.61	0.006	0.172
C2	1.13	0.35	1.91	0.006	0.172
TARP	-2.31	-3.94	-0.69	0.007	0.184
MST1R	1.53	0.44	2.61	0.007	0.189
IL2RA	-2.11	-3.64	-0.59	0.008	0.199
TGFB1	-1.81	-3.12	-0.50	0.008	0.200
CD1A	-1.87	-3.24	-0.49	0.009	0.200
CXCR6	-1.59	-2.77	-0.42	0.009	0.200
CD1D	-1.70	-2.95	-0.44	0.009	0.200
CXCR3	-1.53	-2.66	-0.40	0.010	0.200
BTK	-1.96	-3.41	-0.50	0.010	0.201
CARD9	-1.25	-2.19	-0.31	0.011	0.201
GZMB	-1.63	-2.85	-0.40	0.011	0.201
PIK3CG	-1.70	-3.01	-0.40	0.012	0.205
ITGA4	-1.73	-3.06	-0.40	0.012	0.205
AMICA1	-2.33	-4.13	-0.53	0.013	0.205
BCL2	-1.32	-2.35	-0.29	0.013	0.205
CYSLTR2	-2.37	-4.22	-0.53	0.013	0.205
CCL28	1.51	0.33	2.68	0.014	0.205
SMPD3	-1.36	-2.43	-0.29	0.014	0.205

IFNA8	-1.68	-3.00	-0.36	0.014	0.205
MEF2C	-1.68	-3.01	-0.35	0.015	0.205
CD79A	-2.10	-3.76	-0.43	0.015	0.205
PAX5	-1.63	-2.94	-0.33	0.016	0.205
CD48	-2.14	-3.86	-0.43	0.016	0.205
ITK	-1.62	-2.92	-0.32	0.016	0.205
CCR3	-2.27	-4.09	-0.45	0.016	0.205
FCGR2B	-2.03	-3.66	-0.40	0.016	0.205
CCL23	-2.02	-3.67	-0.38	0.017	0.207
IRF4	-1.66	-3.00	-0.31	0.018	0.207
CCND3	-0.92	-1.68	-0.17	0.018	0.207
CD4	-1.67	-3.03	-0.30	0.018	0.207
CCR6	-1.27	-2.31	-0.23	0.018	0.207
IL2RB	-1.42	-2.58	-0.25	0.019	0.207
CD1C	-1.57	-2.87	-0.27	0.019	0.207
MS4A1	-2.19	-4.00	-0.38	0.019	0.207
CD84	-1.60	-2.93	-0.27	0.020	0.209
ITGB4	0.97	0.15	1.79	0.021	0.221
ENTPD1	-1.41	-2.61	-0.22	0.022	0.225
ETS1	-1.22	-2.26	-0.18	0.023	0.229
TYK2	-0.85	-1.57	-0.12	0.023	0.229
LILRB2	-2.08	-3.85	-0.30	0.023	0.229
CFP	-1.83	-3.41	-0.25	0.025	0.229
CD207	-1.69	-3.15	-0.22	0.025	0.229
IL32	-1.63	-3.04	-0.21	0.025	0.229
HLA-DOB	-1.26	-2.35	-0.16	0.026	0.229
ITGAL	-2.02	-3.78	-0.26	0.026	0.229
CD79B	-1.46	-2.73	-0.18	0.026	0.229
IL10RA	-1.78	-3.35	-0.22	0.027	0.229
ALOX 5	-1.77	-3.33	-0.22	0.027	0.229
RNASE3	-1.35	-2.53	-0.16	0.027	0.229
CSF2RB	-2.24	-4.21	-0.27	0.027	0.229
LILRA1	-1.83	-3.47	-0.18	0.030	0.248
IL12RB1	-1.46	-2.77	-0.15	0.030	0.248
IL2RG	-1.83	-3.49	-0.18	0.031	0.250
NCF4	-1.96	-3.74	-0.19	0.031	0.250
NLRC5	-0.91	-1.75	-0.08	0.033	0.255
CD27	-1.60	-3.06	-0.13	0.033	0.255
HRAS	0.96	0.08	1.84	0.034	0.255
CKLF	-1.49	-2.86	-0.11	0.035	0.255
CYSLTR1	-1.53	-2.94	-0.12	0.035	0.255
TFEB	-0.95	-1.83	-0.07	0.035	0.255
HLA-DRA	-1.19	-2.30	-0.08	0.036	0.255
APP	0.94	0.06	1.82	0.037	0.255
LAIR2	-1.63	-3.15	-0.10	0.037	0.255

EGR2	-1.84	-3.57	-0.11	0.037	0.255
FCGR1A	-1.65	-3.21	-0.09	0.038	0.255
IL13	-1.18	-2.29	-0.06	0.039	0.255
BTLA	-1.34	-2.60	-0.07	0.039	0.255
CASP8	-1.10	-2.14	-0.06	0.039	0.255
CYLD	-0.92	-1.80	-0.05	0.039	0.255
SYK	-1.09	-2.13	-0.06	0.040	0.255
IKBKE	0.60	0.03	1.18	0.040	0.255
RIPK2	-1.24	-2.42	-0.06	0.040	0.255
CCL17	-1.40	-2.73	-0.06	0.040	0.255
CREB5	-1.67	-3.26	-0.07	0.041	0.256
IL18R1	-1.59	-3.11	-0.06	0.042	0.257
ITGAM	-1.83	-3.58	-0.07	0.042	0.257
CD244	-1.86	-3.65	-0.07	0.042	0.257
CD37	-2.02	-3.98	-0.06	0.044	0.257
TBX21	-1.66	-3.29	-0.04	0.045	0.257
PSEN2	0.59	0.01	1.17	0.045	0.257
JAK2	-0.68	-1.35	-0.02	0.045	0.257
IL4R	-1.00	-1.98	-0.02	0.046	0.257
PIK3CD	-1.61	-3.18	-0.03	0.046	0.257
IL23A	-0.93	-1.85	-0.01	0.047	0.257
ITGB2	-1.62	-3.21	-0.02	0.047	0.257
NOTCH1	-1.16	-2.31	-0.01	0.048	0.257
KIT	1.10	0.01	2.20	0.048	0.257
CX3CL1	1.06	0.01	2.11	0.048	0.257
NOD1	-0.73	-1.46	0.00	0.049	0.257
FUT7	-1.76	-3.51	0.00	0.050	0.257
IL13RA1	-0.66	-1.32	0.00	0.050	0.257
POU2F2	-1.09	-2.19	0.00	0.050	0.257

FP, fluticasone propionate ‘Flixonase ®’ group; AZE/FP, azelastine hydrochloride / fluticasone propionate ‘Dymista ®’ group.

FP - AZE					
	Log2 fold change	**Lower confidence limit (Log2)**	**Upper confidence limit (log2)**	**P value**	**P adjust**
GZMB	-2.53	-3.75	-1.30	0.000	0.090
FCER1A	-2.41	-3.64	-1.18	0.000	0.090
SOCS1	-2.36	-3.61	-1.10	0.001	0.090
PTGDR2	-2.66	-4.10	-1.22	0.001	0.090
RUNX3	-1.90	-2.98	-0.83	0.001	0.115
LTB	-2.80	-4.48	-1.12	0.002	0.115
AMICA1	-3.00	-4.80	-1.20	0.002	0.115
PIK3CG	-2.15	-3.46	-0.85	0.002	0.115
CEACAM1	-1.35	-2.19	-0.51	0.002	0.115
CCL23	-2.65	-4.30	-1.00	0.002	0.115
IL1RL1	-2.13	-3.46	-0.80	0.003	0.115
IRF4	-2.14	-3.49	-0.80	0.003	0.115
CD96	-2.07	-3.37	-0.77	0.003	0.115
IFI35	-1.25	-2.04	-0.45	0.003	0.115
NOD1	-1.14	-1.87	-0.42	0.003	0.115
DUSP6	-1.66	-2.75	-0.57	0.004	0.118
HLA-DPA1	-1.93	-3.20	-0.66	0.004	0.118
TNFSF12	-1.81	-3.00	-0.62	0.004	0.118
SMPD3	-1.61	-2.68	-0.55	0.004	0.118
FCGR2B	-2.44	-4.06	-0.81	0.004	0.118
MS4A2	-2.10	-3.50	-0.70	0.004	0.118
TPSAB1	-2.07	-3.46	-0.69	0.004	0.118
ALCAM	1.33	0.44	2.22	0.005	0.118
SELPLG	-2.64	-4.43	-0.85	0.005	0.121
HLA-DRA	-1.63	-2.74	-0.52	0.005	0.121
PAX5	-1.91	-3.21	-0.60	0.005	0.121
CD1A	-2.00	-3.38	-0.63	0.006	0.121
CSF2RB	-2.80	-4.78	-0.83	0.007	0.126
TARP	-2.31	-3.93	-0.68	0.007	0.126
CD74	-1.50	-2.57	-0.44	0.007	0.126
SPN	-1.79	-3.06	-0.51	0.007	0.126
CXCR3	-1.59	-2.72	-0.45	0.007	0.126
CCR3	-2.54	-4.36	-0.72	0.008	0.126
CKLF	-1.91	-3.29	-0.54	0.008	0.126
PAFAH2	-1.09	-1.88	-0.31	0.008	0.126
INPP5D	-1.67	-2.88	-0.47	0.008	0.126
BTK	-2.02	-3.48	-0.56	0.008	0.126
CFD	-1.88	-3.25	-0.52	0.008	0.126
CYSLTR1	-1.92	-3.33	-0.50	0.009	0.137
IL16	-2.32	-4.03	-0.61	0.009	0.137
LGALS3	1.41	0.35	2.46	0.010	0.148

CYSLTR2	-2.44	-4.28	-0.59	0.011	0.154
C2	1.02	0.24	1.81	0.012	0.155
NCF4	-2.33	-4.10	-0.55	0.012	0.155
FLT3LG	-1.05	-1.85	-0.24	0.012	0.155
CD1C	-1.68	-2.97	-0.38	0.013	0.155
CASP8	-1.35	-2.39	-0.31	0.013	0.155
ICAM3	-2.07	-3.67	-0.47	0.013	0.155
PTGS1	-1.73	-3.07	-0.39	0.013	0.155
ITGA4	-1.71	-3.04	-0.38	0.013	0.155
SYK	-1.32	-2.35	-0.28	0.014	0.164
MUC1	1.58	0.31	2.84	0.016	0.177
ITGB2	-1.96	-3.55	-0.36	0.018	0.177
IL18	0.77	0.14	1.40	0.018	0.177
FYN	-1.20	-2.18	-0.22	0.018	0.177
ITGAM	-2.14	-3.90	-0.38	0.018	0.177
CTLA4	-2.09	-3.82	-0.37	0.018	0.177
IFIT1	-1.86	-3.40	-0.33	0.019	0.177
CD207	-1.77	-3.24	-0.31	0.019	0.177
ISG15	-1.33	-2.43	-0.23	0.019	0.177
CLEC4C	-2.04	-3.73	-0.35	0.020	0.177
CYLD	-1.05	-1.93	-0.18	0.020	0.177
ITGB4	0.98	0.16	1.79	0.020	0.177
ALOX 5	-1.86	-3.42	-0.31	0.020	0.177
JAK2	-0.79	-1.46	-0.13	0.021	0.177
TGFB1	-1.56	-2.87	-0.25	0.021	0.177
CCND3	-0.90	-1.65	-0.14	0.021	0.177
CLDN1	1.12	0.18	2.07	0.021	0.177
PIK3CD	-1.87	-3.44	-0.29	0.021	0.177
HLA-DRB3	-1.51	-2.79	-0.24	0.022	0.177
HLA-DMB	-1.06	-1.96	-0.16	0.022	0.177
IL13	-1.31	-2.43	-0.20	0.022	0.177
PSMB9	-1.12	-2.06	-0.17	0.022	0.177
NFATC2	-1.13	-2.09	-0.17	0.022	0.177
CDH1	1.18	0.18	2.18	0.023	0.177
TNFSF13	-0.88	-1.63	-0.12	0.024	0.184
TYK2	-0.84	-1.56	-0.11	0.024	0.184
APOE	1.69	0.23	3.15	0.025	0.184
PTPRC	-2.49	-4.65	-0.34	0.025	0.184
IRF7	-1.24	-2.31	-0.16	0.025	0.184
HLA-DPB1	-2.43	-4.55	-0.31	0.026	0.187
CD79A	-1.90	-3.57	-0.23	0.027	0.190
RNASE3	-1.34	-2.52	-0.15	0.028	0.194
CD163	1.50	0.17	2.82	0.028	0.194
IL17RA	-1.27	-2.40	-0.14	0.029	0.194
CCL13	-1.63	-3.08	-0.18	0.029	0.194

MEF2C	-1.49	-2.82	-0.16	0.029	0.194
CD244	-2.00	-3.79	-0.21	0.029	0.194
IL32	-1.58	-3.00	-0.17	0.030	0.194
SELL	-2.16	-4.11	-0.22	0.030	0.194
ITGAL	-1.96	-3.73	-0.20	0.030	0.194
ITGB1	0.70	0.07	1.32	0.030	0.194
OSM	-2.56	-4.88	-0.24	0.032	0.195
RIPK2	-1.30	-2.48	-0.12	0.032	0.195
MS4A1	-1.99	-3.80	-0.18	0.032	0.195
LCP1	-2.12	-4.04	-0.19	0.032	0.195
EGR1	-1.71	-3.27	-0.15	0.033	0.195
LILRB2	-1.95	-3.73	-0.17	0.033	0.195
IL2RB	-1.28	-2.44	-0.11	0.033	0.195
CFP	-1.72	-3.30	-0.14	0.033	0.197
NFATC3	-0.68	-1.30	-0.05	0.035	0.202
IL3RA	-1.59	-3.06	-0.12	0.035	0.202
TLR1	-1.49	-2.87	-0.10	0.036	0.204
IFIT2	-1.62	-3.13	-0.11	0.037	0.205
HCK	-2.05	-3.96	-0.13	0.037	0.205
TNFRSF10C	-2.24	-4.35	-0.13	0.038	0.206
CD37	-2.08	-4.04	-0.12	0.038	0.206
ENTPD1	-1.27	-2.46	-0.07	0.039	0.206
CD48	-1.81	-3.53	-0.10	0.039	0.206
IL23A	-0.97	-1.88	-0.05	0.039	0.206
F13A1	-1.56	-3.03	-0.08	0.039	0.206
EGR2	-1.82	-3.55	-0.09	0.040	0.206
IL5RA	-1.05	-2.06	-0.05	0.040	0.206
IL18R1	-1.60	-3.13	-0.08	0.040	0.206
CYBB	-2.17	-4.25	-0.09	0.042	0.212
JAK3	-1.69	-3.31	-0.06	0.042	0.212
IL10RA	-1.63	-3.19	-0.06	0.042	0.212
CD180	-1.19	-2.34	-0.04	0.043	0.212
IL4	-1.27	-2.50	-0.04	0.044	0.215
SMAD3	0.71	0.02	1.40	0.044	0.215
CR1	-1.96	-3.89	-0.03	0.047	0.226
FUT7	-1.78	-3.53	-0.02	0.047	0.226
PNMA1	0.72	0.01	1.43	0.047	0.226
PECAM1	-1.94	-3.87	-0.02	0.048	0.227
NOS2	-1.12	-2.23	-0.01	0.048	0.227
TAP1	-0.83	-1.65	0.00	0.049	0.229

FP, fluticasone propionate ‘Flixonase ®’ Group; AZE, azelastine hydrochloride ‘Azep®’ group.

AZE/FP - AZE					
	Log2 fold change	**Lower confidence limit (Log2)**	**Upper confidence limit (log2)**	**P value**	**P adjust**
TNFSF10	-1.02	-1.64	-0.39	0.002	0.997
NOS2A	-1.76	-3.03	-0.48	0.008	0.997
TNFSF13	-1.01	-1.80	-0.22	0.013	0.997
APOE	1.87	0.35	3.39	0.017	0.997
IL18	0.77	0.11	1.42	0.023	0.997
NOS2	-1.24	-2.40	-0.08	0.037	0.997
CD163	1.44	0.06	2.82	0.042	0.997
MARCO	1.47	0.04	2.89	0.044	0.997

AZE/FP, azelastine hydrochloride / fluticasone propionate ‘Dymista ®’ group; AZE, azelastine hydrochloride ‘Azep®’ group.

APPENDIX 5: SCHAMATIC DIAGRAM OF THESIS STUDIES AND PARTICIPANTS