Sputum mediator profiling in severe asthma: relationships with clinical phenotypes, airway inflammation and morphometry in stable disease and at exacerbations

By

Dhananjay Desai MBBS MRCP

Submitted for the degree of PhD

Abstract

Sputum mediator profiling in severe asthma: relationships with clinical phenotypes, airway inflammation and morphometry in stable disease and at exacerbations

Dhananjay Desai

Asthma is characterised by typical symptoms or cough, breathlessness and wheeze with variable airflow obstruction. It is severe in about 10% of asthmatics with persistent symptoms and frequent exacerbations. There is increasing recognition that asthma, and to a greater extent severe asthma, is a heterogeneous disease with respect to its aetiology, inflammatory profile, and clinical expression and treatment responses. However, our understanding of the relationship between cellular and cytokine profiles in severe asthma with symptoms, physiology, airway morphometry and environmental exposure to pathogens in stable disease and at exacerbations is poorly understood. I hypothesized that sputum mediator profiling in severe asthma would identify clinically important biological phenotypes in stable disease and provide insights into relationships with airway remodelling and reveal key dynamic changes in airway inflammation at exacerbation. I undertook a multivariate analysis of sputum mediators from severe asthmatics in stable state and found distinct biological phenotypes associated with clinical features and moreover differential sputum cell counts. Interestingly, in obese asthmatics sputum IL-5 was elevated in spite of a low sputum eosinophil count whereas bronchial submucosal eosinophils were increased suggesting eosinophil trafficking is altered in obese asthmatics. Sputum mediator profiles were weakly associated with airway morphometry. At exacerbation there were marked differences in sputum mediator profiles with upregulation of Th1 cytokines, TNF-R1 and IL-1β in those with evidence of bacterial colonisation and with IL-6R most strongly associated with severe exacerbations of asthma. In summary, this thesis has explored sputum mediator profiles in severe asthma; informed our understanding of the mechanisms underpinning the heterogeneity of disease and identified biomarkers of exacerbations.

Acknowledgements

I am immensely grateful to the patients who participated in the research studies; indeed nothing would be possible without their selflessness.

There is a significant body of people that helped my study or simply kept me sane. The nursing staff- Maria Shelley, Bev Hargadon, Amisha Singapuri, Michelle Bourne, deserve thanks for all their networking with the severe asthmatics and their help on the shop floor. The lab staff- Mitesh, Vijay, Nisha and William for handling all the patient samples and counts. The statisticians Chris Newby and Mike Ghebre made sense of many the numbers and helped with derivation of complex statistics. Ellen and Joe Goldie for data entry and sputum sample tracking.

The research registrars (now all established consultants); Pranab, Mona, Josh and Abdul were a constant source of ideas, sounding boards, peer reviews, clustering, hacked computer programs and general bonhomie.

The senior professors in the department Andy Wardlaw, Pete Bradding and Ian Pavord I am very grateful for their guidance and support.

Richard May at Medimmune deserves thanks for being more than a collaborator; his inquisitiveness and scientific mind have helped shape many aspects my thesis. I also thank Suzanne Cohen, Jo Woods and Athula Herath at Medimmune for their work with mediator analysis and statistics.

I realise how fortunate I was to work at Leicester. This body of work was only made possible the guidance of my research supervisor Chris Brightling. His knowledge, vision, efforts and enduring patience with me have been in equal measure; humbling and inspirational. He is the man with the plan.

Finally, to my family who had to endure this chapter in my life a lot more than I did, thank you for keeping your faith in me.

Publications arising directly from work on this thesis

1: **Desai D**, Brightling CE. Elevated sputum IL-5 and submucosal eosinophilia in obese individuals with severe asthma. Am J Respir Crit Care Med. 2014; 189(10):1285-6

2: Brightling CE, **Desai D**. Role of primary necrosis/lysis of submucosal eosinophils in obese individuals with asthma. Am J Respir Crit Care Med. 2013 15; 188(12):1468-9

3: **Desai D**, Newby C, Symon FA, Haldar P, Shah S, Gupta S, Bafadhel M, Singapuri A, Siddiqui S, Woods J, Herath A, Anderson IK, Bradding P, Green R, Kulkarni N, Pavord I, Marshall RP, Sousa AR, May RD, Wardlaw AJ, Brightling CE. Elevated sputum interleukin-5 and submucosal eosinophilia in obese individuals with severe asthma. Am J Respir Crit Care Med. 2013;188(6):657-63.

4: **Desai D**, Gupta S, Siddiqui S, Singapuri A, Monteiro W, Entwisle J, Visvanathan S, Parmar H, Kajekar R, Brightling CE. Sputum mediator profiling and relationship to airway wall geometry imaging in severe asthma. Respir Res. 2013;(11)14:17.

5: **Desai D**, Brightling C. Cytokines and cytokine-specific therapy in asthma. Adv Clin Chem. 2012;57:57-97.

6: **Desai D**, Siddiqui S, Brightling C. Can inhaled corticosteroids prevent asthma exacerbations? Curr Opin Pulm Med. 2011;17(1):16-22.

7: **Desai D**, Brightling C. TNF-alpha antagonism in severe asthma? Recent Pat Inflamm Allergy Drug Discov. 2010;4(3):193-200 20807193.

8: Brightling CE, **Desai D**, Siddiqui S. Severe asthma: a consequence of over exuberant repair? Clin Exp Allergy. 2009;39(11):1630-2.

9: **Desai D**, Brightling C. Cough due to asthma, cough-variant asthma and non-asthmatic eosinophilic bronchitis. Otolaryngol Clin North Am. 2010 Feb;43(1):123-30

10: **Desai D**, Brightling C. Cytokine and anti-cytokine therapy in asthma: ready for the clinic? Clin Exp Immunol. 2009;158(1):10-19.

Publications arising from equal work contributed and not included in thesis

1. Biological clustering supports both "Dutch" and "British" hypotheses of asthma and chronic obstructive pulmonary disease.
Ghebre MA, Bafadhel M, **Desai D**, Cohen SE, Newbold P, Rapley L, Woods J, Rugman P, Pavord ID, Newby C, Burton PR, May RD, Brightling CE.
J Allergy Clin Immunol. 2015;135(1):63-72

During my time in research I collaborated and shared data with that was included several other studies and also was in charge of the ‘running’ of other studies. I have therefore been included as a co-author on nine other peer reviewed articles and indexed on Medline. I also co-authored a book chapter in Middleton’s textbook of allergy.

I have not included any conference abstracts or posters because they were piece-meal work from the above publications.

Statement of Work Personally Performed

My research required collaborative efforts with several other scientists and some of the data were drawn their own projects to which they contributed the majority of their work. Therefore parts of data in my thesis have been utilized in other publications but have never been analysed or interpreted in the manner I undertook for this thesis.

Sputum Mediator Profiling and Submucosal Eosinophilia and IL-5

MB, A.S, PH, S Siddiqui, and S Shah were involved in the recruitment of patients demographic data collection; these patients were not recruited specifically for my thesis. FAS and CEB were involved in the immunohistochemistry. NK and CEB were involved in the macrophage colour assessment; these patients were not recruited specifically for my study. RDM, JW, IA at Medimmune performed mediator measurement. I with CN, RM, PH and CEB was involved in statistical analyses and interpretation. I undertook further data collection, collation, analysis and led with publications arising from this work.

Airway Wall Geometry

SG, SS, JE were involved with the qualitative and quantitative computerised tomography analysis which included patient recruitment and these patients were not recruited specifically for my thesis. WM undertook the sputum cell count and sputum processing. SV, HP and RK were involved with arranging the mediator analysis. I with SG, RK and CEB was involved in the study design, data collection and collation and data analysis.

Exacerbations of Asthma

I was responsible for all patient recruitment specifically for thesis; I performed assessment at all visits including sputum, blood, and spirometry and questionnaire data collection. WM, VM and MP performed cell and CFU counts; sputum and serum processing. KH provided microbiomic data. SJ, TK provided the virology data. CP and JM performed fungal analysis. JW and RDM at Medimmune performed all the mediator analysis. I was responsible for all the coordination of the work performed at the collaborating facilities and collating data. MG, MB and I contributed equally to

manuscripts arising from this aspect of my work and data from this chapter will be included in MGs thesis.

Collaborators

MB- Mona Bafadhel

AS- Amisha Singapuri

PH- Pranabashis Haldar

SS- Salman Siddiqui

SS- Sachil Shah

FAS- Fiona Symon

CN- Chris Newby

RDM- Richard May

JW- Jo Woods

IA- Ian Anderson

SG- Sumit Gupta

JE- James Entwisle

WM- William Monteiro

IDP- Ian Pavord

AJW- Andrew Wardlaw

RK- Radhika Kajekar

VM- Vijay Mistry

MP- Mitesh Pancholi

KH- Koriobi Haldar

TK- Tatiana Kebadze

SJ- Sebastian Johnston

MG- Michael Ghebre

	List of Abbreviations
ACQ	Asthma Control Questionnaire
AHR	Airway Hyperresponsiveness
ASM	Airway Smooth Muscle
BAL	Bronchoalveolar Lavage
BHR	Bronchial Hyperresponsiveness
BM	Bone Marrow
CD	Cluster of Differentiation
CSF	Colony Stimulating Factor
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPR	Effector Cell Protease Receptor
FEV1	Forced Expiratory Vital Capacity in 1 second
FGF	Fibroblast Growth Factor
GINA	Global Initiative for Asthma
GMCSF	Granulocyte Monocyte Colony Stimulating Factor
gp130	Glycoprotein 130
GWAS	Genome Wide Association Study
ICS	Inhaled Corticosteroids
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
IL-1RAP	Interleukin-1 Receptor Accessory Protein
INH	Inhibin
JAK	Janus Kinase
LAF	Lymphocyte Activating Factor
LT	Leukotriene
NFκB	Nuclear Factor Kappa B
NGF	Nerve Growth Factor
NK	Natural Killer Cells
PDGF	Platelet Derived Growth Factor
PEFR	Peak Expiratory Flow Rate
PG	Prostaglandin
PR3	Serine Protease 3
SNP	Single Nucleotide Polymorphism
STAT	Signal Transducer and Activator of Transcription
TGF	Transforming Growth Factor
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TNFSF	Tumour Necrosis Factor Super Family
TSLP	Thymic Stromal Lymphopoietin
TYK	Tyrosine Kinase
VEGF	Vascular Endothelial Growth Factor

List of tables

List of figures

Contents

Chapter 1

1.1 Introduction

Asthma is a complex inflammatory, chronic disease of the lower airways that is characterized by variable airflow obstruction and intermittently punctuated by exacerbations which are difficult to predict.. Of most concern is the group with 'severe' disease which affects only a small minority of patients, but is responsible for the greatest healthcare utilization, morbidity and mortality associated with asthma. These patients represent an unmet need and require newer therapies.

Research has focussed mainly on the allergy driven Th2 pathways with more recent recognition of Th1/Th17 pathways in severe asthma. The heterogeneity in severe asthma is likely to reflect on its aetiopathogenesis; making detailed characterization of asthma a necessity in order to accurately tailor therapy in an era of personalized medicine. Future studies based on integrating genetics, cellular and molecular mechanisms and clinical outcomes will bring together scalar domains of asthma and further understand the natural history of the condition.

1.2.1 Current definition of asthma

The modern definition of asthma is in many ways overlaps the historic definitions and the Global Initiative for Asthma (GINA) states that *"it is a chronic inflammatory disorder of the airways with airway hyper responsiveness; giving rise to the symptoms of wheezing, cough and breathlessness. The hallmark is variable airflow obstruction that is often reversible, either spontaneously or with treatment*". [www.ginaasthma.org]

Traditionally asthmatic lung physiology includes the presence of airway hyperresponsiveness, an airflow limitation and an airflow obstruction that is reversible. Airway hyperresponsiveness is defined by a PC20 <8 mg/ml; PC20 is the concentration of methacholine causing a 20% reduction in FEV_1. An airflow limitation is defined by <80% FEV_1 predicted normal and <70% FEV_1/FVC (forced vital capacity) predicted normal. A reversibility of 12% of the baseline FEV_1 after bronchodilation, using a bronchodilator, such as salbutamol indicates a reversible

lung function. An immunopathological definition of asthma does not exist; however airway inflammation, cellular infiltration, epithelial damage, mucus hypersecretion and remodelling are many of the features seen and may need to be incorporated into future definitions.

1.2.2 Severe asthma- definition

It is estimated that up to 5-10% of patients with asthma have severe or refractory disease. This group of patients experience poor control, frequent exacerbations, hospitalizations and are responsible for a majority of the mortality and morbidity associated with asthma. They disproportionately utilise healthcare resources and are more likely to suffer from iatrogenic sequlae and hence represent an unmet clinical need. It is estimated that these patients are responsible for over 60% of asthma related healthcare costs.[Krishnan 2012]. Most worryingly, the mortality in this group is very high, with half the asthma deaths occuring in patients with a prior history of severe asthma [O'Neill 2015]. The definition is as below [Chung 2014]

Asthma which requires treatment with guidelines suggested medications for GINA steps 4–5 asthma (high dose ICS and LABA or leukotriene modifier/theophylline) for the previous year or systemic CS for over 50% of the previous year to prevent it from becoming ''uncontrolled'' or which remains ''uncontrolled'' despite this therapy.

Uncontrolled asthma defined as at least one of the following:

1) Poor symptom control: ACQ consistently >1.5, ACT <20 (or ''not well controlled'' by NAEPP/GINA guidelines)

2) Frequent severe exacerbations: two or more bursts of systemic CS (.3 days each) in the previous year

3) Serious exacerbations: at least one hospitalisation, ICU stay or mechanical ventilation in the previous year

4) Airflow limitation: after appropriate bronchodilator withhold FEV_1 ,80% predicted (in the face of reduced FEV1/FVC defined as less than the lower limit of normal)

Controlled asthma that worsens on tapering of these high doses of ICS or systemic CS (or additional biologics)

It is important to differentiate difficult asthma from severe asthma as the former may be as a result of inadequate diagnosis, treatment and addressing the common comorbidties that contribute to poor asthma control [Strek 2012]. Non-adherence to therapy is one of the most common problems contributing to patients with difficult or severe asthma [Heaney 2010, Murphy 2012] and can be difficult to elicit.

1.3 Cytokines in the pathophysiology of severe asthma

Cytokines and their networks are implicated in the innate and adaptive immune responses driving airway inflammation in asthma and are modulated by host-environment interactions. Asthma is a complex heterogeneous disease and the paradigm of Th2 cytokine mediated eosinophilic inflammation as a consequence of allergic sensitisation has been challenged and probably represents a subgroup of asthma., There is increasing recognition of inflammatory sub-phenotypes [Haldar 2008] that are likely to be driven by different cytokine networks. Interestingly these networks may be specific to aspects of clinical expression as well as inflammatory cell profiles and therefore present novel phenotype-specific therapeutic strategies.

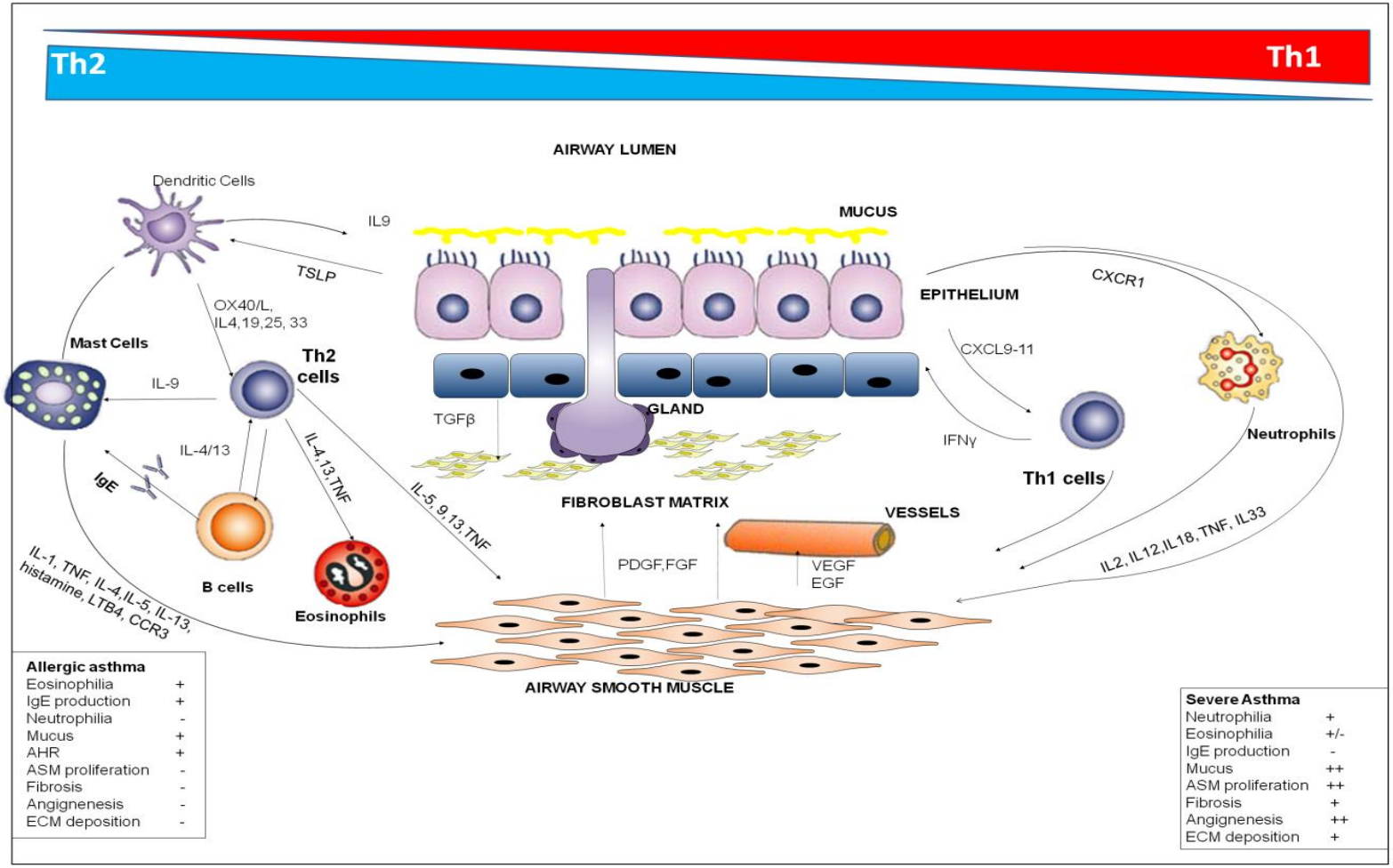
The generic term cytokine was coined a few years after the discovery in the late 1980s of Lymphocyte Activating Factor (LAF) which proved that macrophages released a mitogenic factor that promoted T-cell proliferation in the absence of other growth factors or antigens [Moore 1980, Smith1980]. More cytokines are being discovered as the field of immunology expands.

In asthma the onset and course of the disease are affected by complex host-environment interactions. Several hundred studies have been published that link increased frequency or severity of asthma to cytokine gene or cytokine signalling gene polymorphisms including IL-4 and IL-4RA [Wenzel 2007, Kabesch 2006, Chen 2004, Beghe 2003, Bashore 2004], IL-13 [Risma 2002, Noguchi 2001, Howard 2003, van Der Pouw Kraan 1999, Heinzmann 2000, Graves 2000], TNF [Kim 2008, Noguchi 2002, Witte 2002, Li Kam Wa 1999, Chagani 1999, Albuquerque 1998,], IL-18 and IL-18R [Harada 2009, Zhu 2008], IL-2RB, IL-1RL1 [Moffatt 2010] IL-33 [Moffatt 2010] and TSLP [He 2009, Harada 2010]. The evidence is most consistent for the IL-4/13 axis and large genome wide association studies have in particular implicated IL-18R and

Table1.1 Interleukins, receptors and their functions

Interleukin	Sources	Receptors	Predominant Function(s)
IL-1α, IL-1β	B cells, T cells, Macrophages, Fibroblasts, Neutrophils, ASM	IL-1RI IL-1RII, ILRN, IL-1RAP, ST2	Pro and anti inflammatory
IL-2	Th0 cells,CD4+ CD8+ cells, Dendritic cells	IL-2Rα, IL-2Rβ, IL-2Rγ	Eosinophilic recruitment, activation and inflammation
IL-3	Th2 cells, Mast cells, Eosinophils	IL-3R	Eosinophilic recruitment, activation and inflammation
IL-4	T cells, Mast cells, Eosinophils, Basophils	IL-4Rα	Eosinophilic recruitment, activation and inflammation
IL-5	T cells, Mast cells, Eosinophils	IL-5Rα	Eosinophilic recruitment, activation and inflammation
IL-6	B Cells, T cells, Fibroblasts, Airway Epithelial cells	IL-6RA, IL-6RB	Pro and anti inflammatory
IL-7	Endothelial cells, Dendritic cells	IL-7Rα	Cellular stimulation
IL-8	Endothelial cells, Macrophages, ASM, Eosinophils	CXCR1, CXCR2	Neutrophil recruitment
IL-9	CD4+ cells, Epithelial cells, Eosinophils	IL-9R	Eosinophilic recruitment, activation and inflammation
IL-10	T cells, CD8+ cells, Monocytes, macrophages	IL-10RA, IL-10RB	Anti inflammatory and Immunomodulation
IL-11	Fibroblasts, Eosinophils, Airway Epithelial cells, ASM	IL-11Rα	Proinflammatory
IL-12	B cells, T cells, macrophages, Dendritic cells, Eosinophils	IL-12RA, IL-12Rβ1, IL-12Rβ2	Anti inflammatory
IL-13	CD4+ cells, Basophils, Eosinophils	IL-13Rα1, IL-13Rα2	Eosinophilic recruitment, Proinflammatory
IL-14	B cells, T cells	IL-14RA	BM Progenitor maturation
IL-15	T Cells, Monocytes, Lung Fibroblasts	IL-15Rα IL-2Rβ IL-2Rγ complex	BM Progenitor maturation
IL-16	CD8+ Cells, Eosinophils, Mast Cells, Epithelial cells	CD4	BM Progenitor maturation, Proinflammatory
IL-17	CD4+cells, γδ T cells, Th17 Cells	IL-17 RA,RB,RC,RE,RF	Proinflammatory
IL-18	T cells, Dendritic Cells, Monocytes, Airway epithelium	IL-18R1, IL-18RAP	Proinflammatory
IL-19	Monocytes, Airway epithelium	IL-20RA	Proinflammatory
IL-20	Monocytes, Dendritic Cells	IL-20RA, IL-20RB	BM Progenitor maturation, Proinflammatory
IL-21	CD3+, CD4+, NK cells	IL-21R, IL-21RG	Antiviral
IL-22	Th17 cells	IL-22RA1, IL-22RA2	Proinflammatory
IL-23	Monocytes, Dendritic cells, Macrophages	IL-23R	Proinflammatory, Th 17 promoting
IL-24	Monocytes, Th2 cells	IL-20RA/IL-20RB and IL-22RA1/IL-20R2 complex	Cytotoxicity
IL-25/IL-17E	Epithelial cells, CD4+cells	IL-17RB	Profinflammatory
IL-26	Th1, Th17 cells	IL-26R	Profinflammatory
IL-27	Dendritic cells, T cells	IL-27RA	Immunomodulatory, T cell differentiation
IL-28	Dendritic Cells	IL-28R, IL-28RA, IL-28B	Proinflammatory
IL-29	Monocytes , Dendritic Cells	IL-28RA	Antiviral, Proinflammatory
IL-30/IL-27	Dendritic cells, T cells	IL-27RA	Immunomodulatory
IL-31	T cells, Mast cells	IL-31R	Proinflammatory
IL-32β	T cells, NK cells, Airway epithelium	PR3	Proinflammatory
IL-33	T cells, Mast cells, Endothelium, Fibroblasts Airway epithelium	ST2	Proinflammatory, Eosinophil recruitment
IL-34	Monocytes	CSF-1R	BM Progenitor maturation
IL-35	T_{reg}	IL-12R	Anti-inflammatory
IL-36	T cells	IL-36R/IL-1R subfamily	Pro inflammatory
IL-37	Macrophages	IL-18R	Anti inflammatory, regulatory
IL-38	T Cells??	IL-1-R??	Proinflammatory??

Figure 1.1 Cytokine effects on various airway components with a Th1/Th2 imbalance in mild and severe disease



IL-33. The typical symptoms of asthma and disordered airway function occur against a background of airway inflammation and remodelling. This microlocalisation with the airway smooth muscle is a consistent finding and is closely related to the degree of airway hyperresponsiveness [Brightling 2002, Siddiqui 2008]. In the asthmatic airway mast cells are in an activated state and are an important source of cytokines, chemokines, autocoid mediators, proteases and histamine [Brightling 2003, Saha 2008, Amin 2005, and Siddiqui 2007]. Importantly these cells can be activated via both IgE and non-IgE mechanisms and have been shown to affect airway smooth contractility directly [Tliba 2003, Laporte 2001, Grunstein 2002] and indirectly by up regulation of airway smooth muscle transforming growth factor-β, which in turn drives the airway smooth muscle into a more contractile phenotype via an autocrine activation [Woodman 2008]. Mast cells and neutrophils are also localised to the mucus glands and have been implicated in goblet cell and mucus gland hyperplasia and mucus plugging.

In severe disease neutrophils are also increased [Haldar 2007, Wenzel 2005, Kamath 2005] and have been implicated in disease [Idris 2009] but whether they play a key role in disease progression or are a consequence of corticosteroid therapy in unclear [Barnes 2008]. Structural cells within the airway including epithelial cells, fibroblasts, myofibroblasts, fibrocytes and airway smooth muscle are also important sources of chemokines and growth factors and indeed are likely to play a role in the inflammatory response. Importantly these structural cells are increased in number in severe disease and contribute to the remodelling process, which leads onto progressive disease and persistent airflow obstruction [Benayoun 2003]. The cytokines affecting particular pathophysiological aspects of asthma are summarized in **Table 1.2**

Cytokines are only one aspect of the complex paradigm of asthma and are part of multiple ‘scales or levels’ in disease evolution. The interaction between one scalar level and another in shaping a ‘phenotype’ or a characteristic of asthma is only possible by attempting to studying asthma at multiple levels and attempting to link the findings using both *a priori* and hypothesis free approaches.

Corticosteroids are the mainstay of therapy for asthma for several decades now [Smith 1983, Barnes 1993]. In particular benefits are observed in patients with eosinophilic disease. However, in refractory asthma this effect is inadequate, as described [Barnes 2008]. Therefore anti-cytokine therapy presents an important alternative or adjunct to current therapy. It is therefore important that the specific roles of cytokines implicated in the pathogenesis of asthma are understood as the 'one size fits all' approach does not necessarily apply for different asthma sub-phenotypes.

Table 1.2 Cytokines associated with pathophysiological features of asthma

Features	Interleukin	Chemokine/Receptor	Growth Factor
Airway Hyperresponsiveness	IL-2, IL-4,IL-5, IL-9, IL-13, IL-18, IL-33,TNFα	CCR7	
IgE Production	IL-4,IL-9, IL-13, IL-18		
Goblet Cell Metaplasia	IL-4, IL-13		
Mucin Hypersecretion	IL-9		EGF, TGFβ
Mastocytosis	IL-9		
Mast cell degranulation/migration	IL-9, IL-33	CCR3, CXCR1,3,4	
Eosinophilia	IL-4, IL-5, IL-9, IL-13, IL-17, TNFα	CCR1, CCR3	
Neutrophilia	IL-1, IL-2,IL-18, IL-33,TNFα, IFNγ	CXCR1, CXCR2	
Th2 induction	IL-4, IL-5, IL-9, IL-13, IL-25, IL-33		
Airway Smooth Muscle Hypertrophy	IL-13, IL-33	CCR7, CXCR4	TGFα, TGFβ, PDGF, EGF
Remodelling-Epithelial Damage/Repair	IL-5, IL-9, IL-18, IL-33		VEGF,EGFR,TGFα
Extracellular Matrix Collagen deposition			TGFβ,PDGF
Subepithelial Fibrosis	IL-13,IL-33	CCR7, CXCR4	TGFβ, FGF
Exacerbations	IL-4, IL-5, IL-6, IL-8, TNFα		

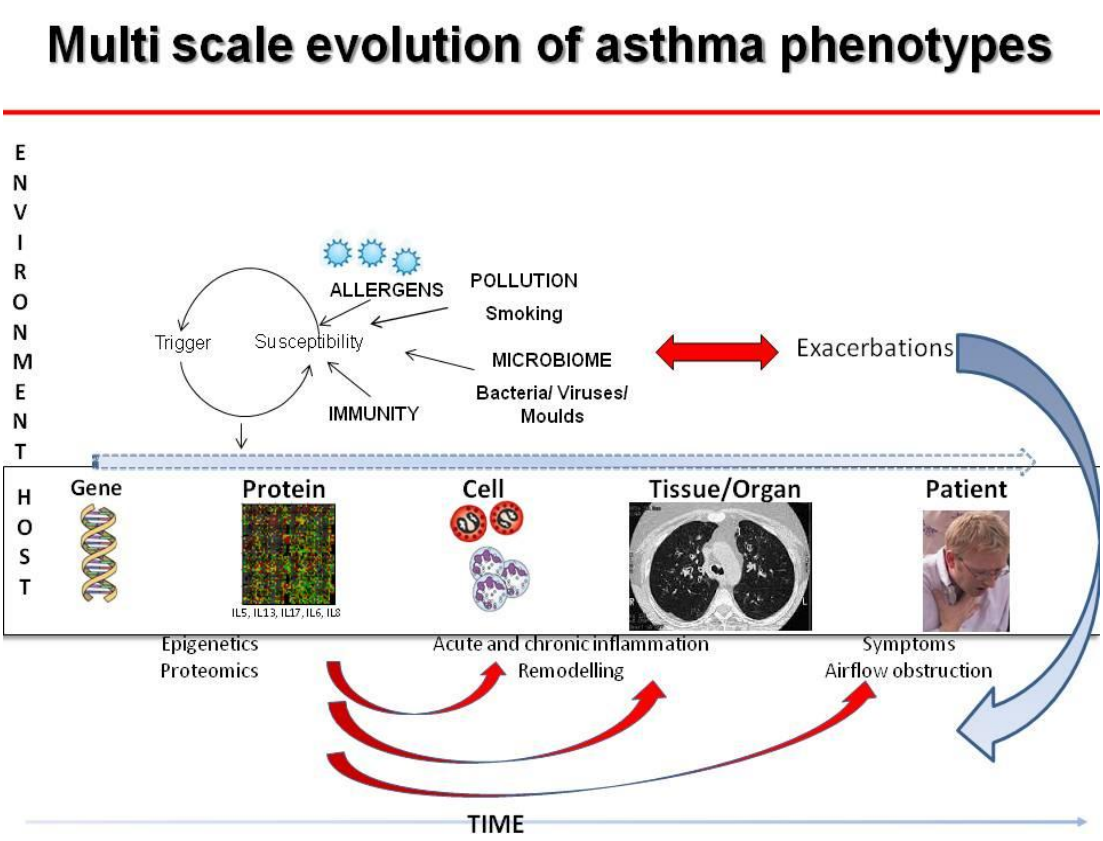


Figure 1.2 Asthma pathogenesis occurs due to host environment interactions at different spatial and over temporal scales

1.4 Scalar evolution of asthma

The concept that a chronic inflammatory disease evolves over multiple scales is not new; but with the evolution of genetic and transcriptomic studies this effect becomes apparent. Figure 1.2 shows how a phenotype of asthma is influenced by several complex underlying networks. These are discussed briefly further.

1.4.1 Gene to protein

Many large scale genome wide association studies have identified and replicated a number of genes or single nucleotide polymorphisms that have multiple effects, from susceptibility to disease severity [Moffatt Li 2012, Forno 2012]. Genetic variations may further interact and thereby influence outcomes like lung function and severity [Li 2013]. SNPs in the IL-4RA are associated with airways inflammation and severe asthma exacerbations [Slager 2012] while an IL-6R polymorphism was associated with lower lung function and severe asthma [Hawkins 2012]. Corticosteroid

sensitivity and smooth muscle regulation has been linked with gene function and this particularly important for therapy. For the future pharmacogenetics and understanding functional biology of gene variants may help identifying biomarkers or newer targeted therapies.

Table 1.3 Candidate genes (loci or SNPs not shown) linked to asthma and the intermediate phenotypes

Gene	Author	Trait or association
IL-33	Moffatt	Severe asthma, atopy, reduced lung function
IL-18R1	Moffatt	Severe asthma, AHR
IL-1R1	Forno	Severe asthma, AHR
IL-4/IL-4RA	Slager	Severe asthma, Atopy, Rhinitis
IL-6RA	Hawkins	Severe asthma, reduced lung function
TNF	Li	Severe asthma

1.4.2 Cell to tissue: Airway Remodelling

Persistent airway inflammation, despite full treatment is one of the hallmarks of severe asthma. However, there is no clear pathological definition of severe asthma. [Brightling, Gupta, Hollins 2011]. Allergic asthma airway inflammation is orchestrated by dendritic cell-Th2 cell interactions leading to mast cell activation and eosinophil recruitment. Evidence is emerging, particularly in severe asthma, that non Th2 pathways with activation of neutrophils may play a role [Doe 2012]. A consequence of this inflammation is epithelial damage and ciliary dysfunction goblet cell hyperplasia and mucus gland enlargement and mucus hypersecretion. Activated epithelium releases various growth factors like TGF β [Postma 2006] and pro-angiogenic factors VEGF which in turn causes matricellular proliferation [Holgate 2003]. Fibrocytes traffic to the airway and induce local proliferation of increase in airway smooth muscle (ASM) mass which causes airflow obstruction [Woodman 2008]. In addition to the pathogenesis of persistent disease, recurrent exacerbations

are an important component of severe disease and are often associated with pathogens, suggesting abnormalities in innate/adaptive immunity. Bacterial colonisation [Hilty 2010] and fungal sensitisation is seen in subjects with severe disease [Denning 2006] .

1.4.3 Tissue to organ: Imaging function linking inflammation

High-resolution computed tomography (HRCT) is an emerging tool allowing for assessment of the structure of airways and parenchyma not obtainable by any other in vivo methods. It has been used to measure various parameters of the airway lumen airway wall including thickness, diameter and area. The following studies summarise radiologic features of asthma as determined with cross sectional imaging.

Table 1.4 Image function linking studies

Author	Clinical features	Imaging features
Gupta	Eosinophilic asthma	Proximal bronchial thickening
Busacker	Neutrophilic asthma, atopy	Air trapping
Singh	Remodelling, ASM hyperplasia	Bronchial wall thickening
Haldar	Treatment effect of Mepolizumab	Reduction in RB1 wall area

Gupta [2010] and colleagues brought together airway inflammation, airway wall geometry and lung function, linking the presence of neutrophilic inflammation and increased bronchial wall thickening to poorer lung function. Airway wall geometry has been related to structural changes observed in endobronchial biopsies [Aysola 2009]. Both these studies linked image-function and inflammation. There is a paucity of studies that have attempted linking protein mediator data to image function data or encompassing genomic studies.

U-BIOPRED is attempting high-dimensional analyses to be integrated into a "handprint" of biomarkers that will be derived from staged sifting of molecular 'omics', histological, imaging and clinical data, aimed at accelerating discovery of novel diagnostic and therapeutic targets and findings from future studies like this are eagerly awaited.

1.4.4 Temporal scales of asthma pathogenesis

Current concepts suggest that remodelling from airway inflammation occurs over different time scales, probably, sequentially over several years. This is mainly from paediatric studies documenting 'atopic march' wherein allergic sensitization [Sporik 1990] and viral respiratory infection were much more likely to be associated with development of persistent asthma into adulthood. While most adult severe asthma develops in the earlier decades of life there are a well-recognised group of patients in whom the disease presents in the latter decades- the 'late onset asthma' [Miranda 2004]. Whether the pathogenesis began later in life or whether it was unmasked or provoked into presentation is also unclear based on current knowledge. Severe asthma may be a separate disease entity developing over short course of time and we have limited understanding of the natural history of asthma [Wenzel 2012].

1.5 Asthma classification- current methods

Asthma control is a measure that has traditionally been based on intensity of clinical symptoms and the frequency of exacerbations. However it is a patient's own subjective perception of symptoms that vary hugely from one asthmatic to another. It also incorporates the physician's perception from their monitoring of the individuals condition. Both these observations about control are hence subject to considerable bias and variability. Well validated questionnaires like the Asthma Control Test [Nathan 2004] and the Asthma Control Questionnaire (ACQ) [Juniper 1999] attempt to address these issues by standardizing the assessment and addressing the domains of asthma control. There are several studies that suggest considerable discordance exists both in symptom perception and severity of disease [Haldar 2008].

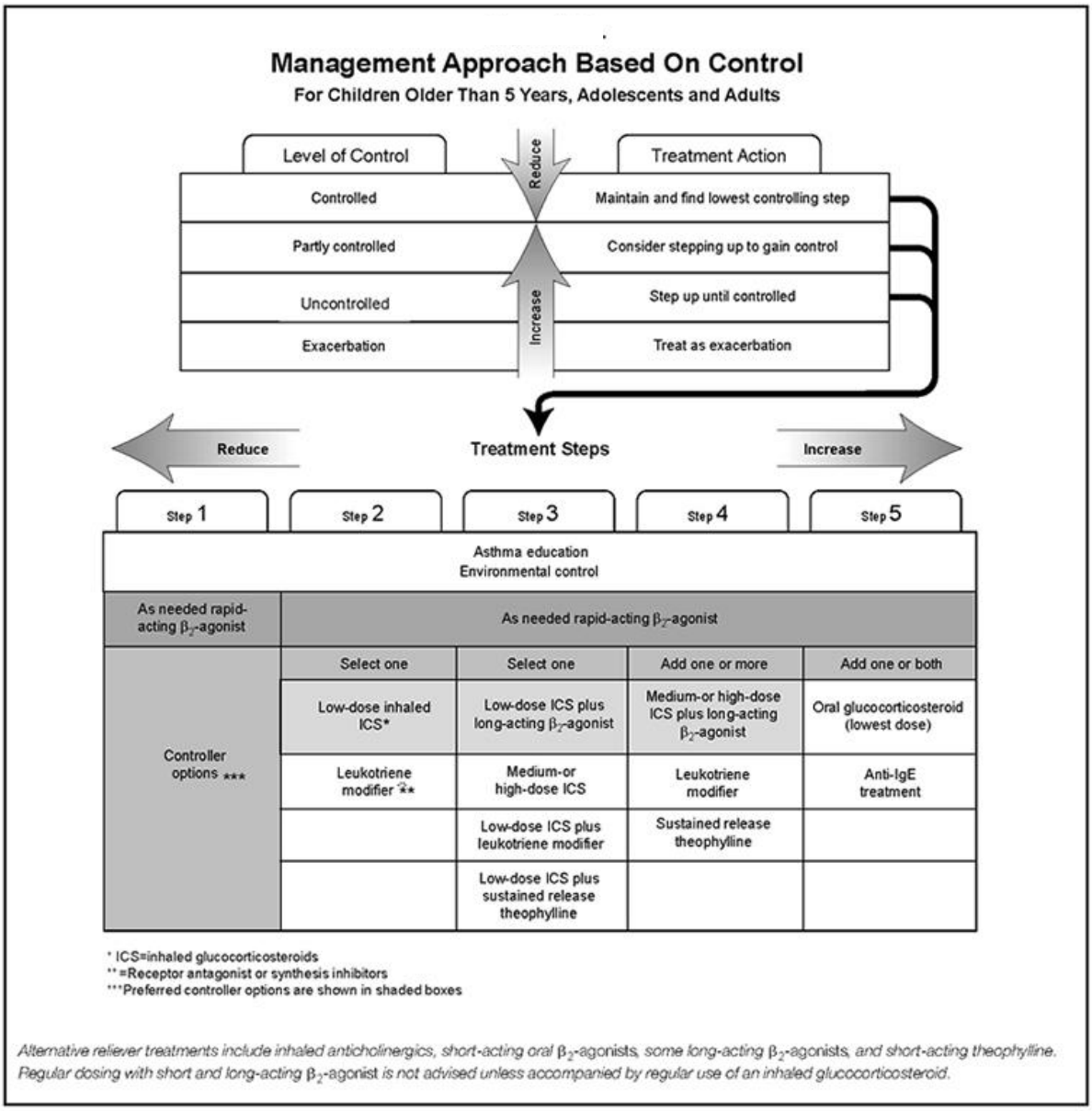


Figure 1.3 GINA classification of asthma severity and treatment steps

1.6 Phenotyping asthma

1.6.1 Definitions

Several recent studies have shown that severe asthma is not only different to other, milder forms of the disease, but there is also considerable variation with the population of severe asthmatics with regards to clinical and physiological characteristics, airway inflammation, response to treatment and outcomes [Barnes 2008, Wenzel 2012].

The concept of phenotyping asthma has evolved with several cross sectional and longitudinal analysis of this population. The definition of a phenotype is a contraction of endophenotype- is a subtype of disease defined functionally and pathologically by a molecular mechanism or by treatment response [Anderson 2008]. A phenotypic classification combines clinical characteristics with identifiable mechanistic pathways and recent attempts include multidimensional integration utilizing molecular, cellular, morphological and physiological features.

Broadly there are two strategies that attempt phenotypic classification- using hypothesis driven approach. A systems biology approach has gained considerable interest the past decade as it allows examining 'scales of disease' [Auffray 2009]. Hierarchical clustering, data reduction tools like factor analysis or discriminant analysis utilise a huge number of variables incorporating genomic, transcriptomic, inflammatory and clinico-physiological parameters the results of which can be used to generate a hypothesis.

1.6.2 Phenotyping using clinical characteristics and inflammation

One of the first [Haldar 2008] was the first to show the group of asthmatics with mild to severe disease in a mixed primary and secondary care population. He performed hierarchical clustering using clinical characteristics and sputum eosinophil counts revealing five clusters of severe asthma. There were differing levels of sputum eosinophilia between some but not all clusters which led to the conclusion of Th2 predominant asthma in the most eosinophilic subset. The study however did not undertake any inflammatory protein analysis and therefore unable to answer the question of whether the clinical clusters are shaped by different molecular mechanisms especially in the clusters that did not differ in their levels of cellular

inflammation. Similarly another clinical cluster study [Moore 2010] used only clinical characteristics to classify disease; not utilizing inflammation as a variable. The crystallized clusters did reveal differences but with significant overlap in the levels of airway inflammation between groups. The study offered no insight into molecular biology to account for the similarities or differences. This method however, to a clinician may remain the most important technique purely by being clinically based.

1.6.3 Molecular phenotyping

Clinical phenotyping is been followed by molecular phenotyping using mediator data in sputum or BAL and more recently, airway mRNA expression. Brasier performed an unbiased hierarchical clustering or cart analysis of sputum cytokines revealed significantly distinct patterns of inflammatory pathways. There were no distinguishing characteristics on sputum inflammometry; few clinical features based on lung function and AHR. The cytokine patterns were informative of disease phenotypes but did not necessarily provide further mechanistic insight into the underlying inflammatory pathways.

Hastie [2010] performed protein microarray screening of asthmatic sputa from SARP and stratified patients into four groups based on sputum granulocyte counts. The results of this study showed a Th1 component to inflammation and identified novel proteins less well recognized for participation in asthma. The levels of these mediators were generally higher in subjects with severe asthma but showed a stronger association with neutrophils than with eosinophils. Most importantly it showed stratification by granulocytes identified significant differences in lung function, asthma control, health care use, and symptoms.

A study performed a multivariate analysis on sputum cytokines mRNA expression revealing discrete patterns of Th2 high and Th1 high disease [Seyes 2013]. Their findings associated worse symptoms scores and poor lung function to a group with high IL-5, IL-17 and IL-25. This study was useful as steroid resistance is linked with IL-25 and may identify a group of patients that may be unresponsive to traditional therapies. The above studies show that mediator measurement has introduced an added layer of complexity-and may help identify specific therapies.

1.6.4 Phenotypic biomarkers and phenotype specific therapy

The National Institute of Health biomarker definitions working group [Atkinson, 2001] proposed that a biomarker should be defined as '*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.*

In asthma sputum eosinophils have been used as biomarkers to characterise disease; predict future loss of control [Jatakanon 2000] and tailor response to therapy with steroids [Green 2000, Chulmsky 2006]. Omalizumab has successfully addressed atopy driven asthma being identified simply by a serum IgE cut-off [Busse 2008]. Woodruff demonstrated Th2 high and low subsets of asthma that were otherwise clinically indistinguishable by performing mRNA transcriptomic analysis. The findings from this study led to identification of periostin as a marker of Th2 high asthma with high IL-4, IL-5 and IL-13 expression. Periostin also correlated with presence of peripheral blood eosinophilia. In the trial of lebrikizumab [Corren 2011], an initial assignment of Th2 high or low status was based according to eosinophils and IgE levels. Following the analysis of the study (post hoc), however, the subgroup of patients with a high periostin had the most beneficial effects with antibody treatment. This group would not have been identified ordinarily without measurement of periostin. In a study of TNFα antagonism with infliximab [Berry 2006] though was not an overall positive study, there was a subgroup of severe asthmatics that had elevated TNFα activity based on peripheral flow cytometry. This subgroup appeared to benefit most from infliximab therapy.

The findings from clinical phenotyping studies show that there is probably no clear cut delineation of phenotype there are literally several hundreds of characteristics that could be used to define a subtype, indeed the SARP study began with data reduction of 600+ variables [Moore 2010]. However, using a single discriminator can only very superficially classify asthma as there are so many domains to encompass. Almost all studies were cross sectional analysis which could not address the potential problem of longitudinal phenotypic stability incorporating the complexity of time [Boudier 2012].

Clinical classifications systems are likely to remain therefore most easily available and of most value to clinicians. Allergy or atopy is particularly important as it suggests classic Th2 pathways and guides towards anti allergic therapy like anti IgE antibody omalizumab. The age of onset is linked to differences in allergic status, lung function and eosinophilic inflammation. The presence of obesity suggests presence of co morbidities and possibly non eosinophilic asthma [Gibeon 2012]. Consequently there are unlikely to be standardized definitions of asthma phenotypes especially those that incorporate multiple domains; molecular profiling techniques are only available in research studies and are probably several years away from becoming clinically useful.

Thus there is the need for studies encompassing complex phenotyping using both clinical and molecular mechanisms and these require validation in different asthma cohorts and over longer periods of time.

1.7 Exacerbations of asthma

1.7.1 Definitions

Asthma exacerbations pose a unique risk to patients with substantial morbidity and mortality. However these mechanisms that drive exacerbations are complex and therefore there is considerable heterogeneity in the aetiopathology of exacerbations. Previously definitions of an asthma exacerbation consisted of increased symptoms and /or healthcare utilization and interpretations were variable both from the patient and clinicians perspective. Incorporating these as endpoints in clinical studies further led to significant discrepancy in defining an exacerbation.

In order to overcome these disparities an ATS/ERS consensus report aiming to standardise definitions of exacerbations for clinical trials has defined severe exacerbations as events that usually require hospitalisation/emergency room visits and/or the use of systemic corticosteroids for at least 3 days. An exacerbation is a worsening of asthma requiring the use of systemic corticosteroids (or for patients on a stable maintenance dose, an increase in the use of systemic corticosteroids) to prevent a serious outcome. [Fuhlbrigge 2012].In contrast a moderate exacerbation has been defined as an event that leads to an increase in existing asthma therapy

not including systemic corticosteroids or a change in asthma symptoms/lung function over a period of at least 2 days, not warranting hospital admission [Reddel 2009].

1.7.2 Airway inflammation during exacerbations

A study of asthmatic sputum at exacerbation was undertaken [Fahy 1995] with predominant neutrophilia and high levels of tryptase and IL-8. In a paediatric population [Norzila 2000] and stable and exacerbation values of sputum Il-8 and IL-5 with significantly higher values during exacerbation accompanied by raised total sputum count and neutrophilia. Johnston and several other researchers [Gern 2000] have performed experimental rhinoviral infection in asthmatic subjects and detected high levels of sputum IL-8, CXCL10, and IFNγ. A study of patient s in status asthmaticus [Lamblin 1998] revealed multiple cytokines namely IL-1β, IL-6, TNFα had significant elevation in BAL or sputum levels compared to groups of matched controls. In a group of mild paediatric asthmatics showed predominant airway eosinophilia and elevation of sputum ECP; there was no significant difference of sputum IL-8 at exacerbation [Ordonez 2000]. There are several studies of airway inflammation during exacerbations many studies had either a healthy cohort for comparison or performed mediator assessments only at exacerbation, and could not explore the dynamic changes in the same cohort of subjects.

1.7.3 Allergic exacerbations

The role of allergens in triggering exacerbations is probably the best understood mechanism. The known presence of atopy and history of exposure to allergens in an asthmatic mainly relying on history and clinical features may lead to the clinicians' assumption of allergy driven exacerbation. The success of anti IgE therapy like Omalizumab demonstrates the role of allergy driven exacerbations. Upon exposure, is a predominant IgE mediated Th2 cytokine response in allergic airway with IL-5 and IL-13 and IL-4 being raised after allergen challenge. However studies have also showed a concomitant Th1 and Th2 response. [Liu 2004]. There have been several decades and studies of human allergic asthma, yet, there is no clear definition of what constitutes an allergic exacerbation and how to differentiate from viral exacerbations.

1.7.4 Viral exacerbations

Viruses are likely to be identified as the most common causes of asthma exacerbations. For example, a French study of children hospitalized for a severe AAE, PCR detected a viral agent in about 82% of the cases, while more traditional methods (virus culture and immunofluorescence) identified only 34% [Papadopoulos 2010]. The prevalence in adults is less, but still in the range of 41–78% [Tan 2003]. However these studies relied on virus detection using PCR assays. Prior to the development of PCR technology, the aetiology of respiratory infections associated with exacerbations was established by cultures, which are difficult to perform and often did not support virus growth.

An observation on the inherent differences in the way that asthmatic subjects respond to respiratory tract viral infections was proved to be a deficiency in production of IFN-β. An in vitro study of airway basal epithelial cells showed deficient production of this antiviral cytokine in both steroid naïve and steroid treated individuals [Wark 2005], and exogenous replacement of IFN-β conferred protective effects by reducing viral replication. In addition, protective cytokine levels like IFN-λ are deficient in asthmatic airway cells [Contoli 2006] and inflammatory cytokines like TGF-β are present in relative excess in asthmatic airways. This may mediate enhanced rhinovirus replication, probably through suppressive actions on host type I and type III interferon responses [Thomas 2009].

Viral infections can trigger exacerbations by promoting airway inflammation via the influx of neutrophils, eosinophils, activated lymphocytes and increase secretion of pro inflammatory cytokines including IL-1b, IL-6, IL-8, G-CSF and RANTES [Corne 2001]. There is a predominant Th1 cascade in response to viral triggers, and while some studies have found a Th2 signal [Papadopoulos 2002] with IL-4 and IL-5 this is presumed to be due to inflammatory cell recruitment as opposed to a Th2 driven pathway. High IL-10 levels found during virus induced exacerbations are postulated to be negative feedback loop towards the Th1 response generated by viral infection. [Grissell 2005]. CXCL10 is induced by viral infection in bronchial epithelial cells. A study showed the utility of CXCL10 as a highly specific, but less sensitive biomarker of viral triggered asthma exacerbations [Wark 2007].

A study showed viral exacerbations of asthma were not only identified clinically but also by levels of sputum CXCL10 and treatment with recombinant IFN-β resulted in a reduction in symptom scores [Djukanovic 2014]. It was an important study as a proof of concept- that of identifying exacerbation aetiology and the protein biomarkers associated with it and finally therapeutic intervention with recombinant protein.
In a COPD exacerbation study, sputum CXCL10 again emerged as distinct marker of viral exacerbation. Interestingly, in COPD which is similar to acute asthma, the detection of new virus during exacerbations was seen but there was also a smaller proportion of subjects that had virus detected at both stable visits and during exacerbations [Bafadhel 2011]. This is particularly important as the asthma studies performed earlier did not aim to detect the presence of virus at study enrolment or stable visits, suggesting there may be a role for viral colonization.

1.7.5 Bacterial exacerbations

Traditionally bacterial exacerbations or 'bronchitis with asthma' have depended on the presence of purulent sputum, fever and the sputum culture positivity of bacterial pathogens. The lower airways were traditionally believed to be sterile but bacterial presence is being more recognised. In conditions that co-exist with asthma – airway wall thickening and bronchiectasis studies have identified correlations with sputum culture positivity for pathogens [Angrill 2001, 2002]. These pathogens can be isolated both during exacerbations, but also during stable visits.

One study showed. 27% of asthmatic patients presenting with an exacerbation of asthma had bacteria in sputum with *Streptococcus pneumonia, Streptococcus pyogenes, Staphylococcus aureus, Moraxella catarrhalis* and *Haemophilus influenzae* being the most common isolates [Cazzola 1991] A significant load of potentially pathogenic bacteria (> 10^6 cfu/mL) was cultured from the sputum of 15% subjects with stable asthma and was associated with higher total cell counts, proportion and number of neutrophils, sputum IL-8 and 8-isoprostane concentrations [Wood 2010] and neutrophilic asthma is being increasingly linked to bacterial colonization [Essilfie 2012]. Bacterial pathogens were thought to be rarely the cause of asthma exacerbations alone and a recent paediatric study suggests a viral-bacterial synergism- as rhinovirus infection enhanced detection of H *influenzae*, M *catarrhalis* and S pneumonia [Kloepfer2014]. An increased inflammatory response in

the presence of infection and the recognition of novel anti inflammatory mechanisms of macrolide antibiotic have led to successful trials in COPD [Albert 2011] in COPD to prevent exacerbations.

1.8 Benefits of phenotyping the heterogeneity of asthma exacerbations.

In COPD, Bafadhel validated serum procalcitonin as a marker of bacterial infection; in a further study showed sputum IL-6 as having a high sensitivity and specificity in differentiating bacterial exacerbation of COPD. She also demonstrated peripheral blood eosinophilia as marker of steroid responsiveness in COPD exacerbations and treatment could be specifically tailored during exacerbations with the phenotype targeted eosinophilic subtype demonstrated better outcomes.

The above studies and earlier sections show that clinical features during an exacerbation however do not point to the underlying aetiology and therefore may lead to standardised therapeutic approaches utilizing steroids or antibiotics. It has, however, become clear that no single phenotype can explain all severe refractory asthma and no single treatment approach will improve asthma control in all patients with severe disease. This highlights the need to accurately understand the inflammatory biology and aetiology of exacerbations.

Hypothesis

I hypothesized that sputum mediator profiling in severe asthma would: I) identify clinically important biological phenotypes in stable disease and ii) provide insights into relationships with airway remodelling and iii) reveal key dynamic changes in airway inflammation at exacerbation.

To address these hypotheses I had four integrated aims:

Aim 1. Assess sputum profiles in severe asthma and undertake multivariate and clustering techniques to both clinical and mediator data to identify biological phenotypes and relationships between airway inflammation and clinical expression of stable disease.

Aim 2. Validate novel observations in AIM 1 in bronchial submucosal biopsies. Specifically novel relationships between sputum IL-5 and sputum eosinophilic were observed in obese severe asthmatics that were further investigated to define eosinophilic inflammation in different compartments in obese asthmatics.

Aim 3. Define the relationship between sputum mediators and airway morphometry determined by quantitative computed tomography.

Aim 4. Determine the dynamic changes of sputum mediators in severe asthmatics at exacerbations compared to stable disease to identify relationship with exacerbation phenotypes, including presence of pathogens, and to reveal potential exacerbation biomarkers.

Chapter 2

Methods

2.1 Subject recruitment

All patients/subjects were recruited from Difficult Asthma Clinic, Glenfield Hospital, Leicester. All studies had local ethics committee approval. Their data was utilized in various sub studies that make up the chapters in my thesis.

For Chapters 3, 4 and5
Subjects were recruited between January 2007 and December 2008 from a single centre, 'Difficult Asthma Clinic', Leicester, UK. Asthma was defined according to Global Initiative for Asthma (GINA) guidelines or based on a physician's diagnosis and severity stratified according to the GINA treatment steps. Written informed consent was obtained from all subjects and the study was approved by the local research ethics (Leicestershire, Northamptonshire and Rutland) committee. Additionally these patients participated in further sub studies depending on their suitability. Specifically the macrophage phagocytosis subjects were recruited from this study and with separate ethics approval.

For chapter 6
Subjects were recruited between 2009 and 2010 and due to the longitudinal nature of the trial were followed up till 2012.
The patient population met the definition of severe asthma as defined by the 2011 ATS/ERS criteria. Adherence to therapy was not specifically checked prior to enrolment in my studies. It is therefore plausible that some of the patients were difficult asthmatics due to non adherence to therapy as opposed to having a genuinely severe asthma.

2.2 Study Criteria

2.2.1 Inclusion criteria for asthma patients

For inclusion in the studies patients had to fulfill all of the following criteria:

1. Provision of informed consent.

2. Male or female.

3. Aged 18 years or over.

4. Diagnosis of asthma and specifically severe asthma (see below)

2.2.2 Exclusion criteria for asthma patients

Any of the following is regarded as a criterion for exclusion from the studies

1. A history of or current active respiratory tuberculosis.

2. Upon questioning the patient known HIV infection or positive hepatitis B or C.

3. Clinically relevant disease or disorder (past or present) which in the opinion of the investigator may either put the subject at risk because of participating in the study or may influence the results of the study or the subject's ability to participate in the study.

4. Any clinically relevant lung disease other than asthma.

5. Donation of blood within 3 months or during the study (for other than study purpose).

6. Pregnancy or lactation.

7. Participation in an interventional clinical study within 3 months of Visit 1 or participation in a study using an investigational medicinal product in the previous 4 months (or 5 half-lives whichever is longer)

8. Patients being treated presently or in the last 3 months (or 5 half-lives whichever is longer) with anti-Tumour Necrosis Factor therapies or Anakinra.

2.2.3 Definition of Severe Asthma for inclusion in study

2.2.3.1 Major

1. Treatment with continuous or near continuous (>50% of year) with oral corticosteroids

2. Requirement of high dose inhaled corticosteroids (µg/day)

a. Beclomethasone Propionate >1260µg b. Budesonide >1200µg

c. Fluticasone >880µg d. Triamcinolone >2000µg

2.2.3.2 Minor

1. Additional controller medication

a. LABA b. Theophylline c. LTRA

2. Asthma symptoms requiring daily or near daily SABA

3. Airflow obstruction

a. FEV <80% b. PEF variability >20%

4. One or more urgent care visits for asthma per year

5. Three or more short oral steroid courses

6. Prompt deterioration with <25% reduction in oral or ICS dose

7. Near fatal asthma event in the past

The patients had either 1 major or 2 minor criteria

Treatments with oral corticosteroids or oral corticosteroids in combination with antibiotics and hospital admissions had to be a minimum 4 weeks apart to be considered separate exacerbations. Frequent exacerbations were defined as 2 or more exacerbations less than 1 year apart. A history of frequent exacerbations was defined as 2 or more exacerbations in the year before examination. For each participant, exacerbations were recorded 1 year prior to the examination date and forward until the end of follow-up

2.3 Protocols

Broadly, I followed a similar protocol- an initial contact, screening and recruitment. History taking, clinical examination, allergy testing, PFTs and sputum/blood sample collection. All visits (except those in chapter 6) were undertaken 8 weeks free from exacerbation of asthma.

2.3.1 Lung function

Lung function was performed in accordance with the joint American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines [Brusasco 2005]. Spirometry was performed on all visits with recordings obtained pre and post 400µg salbutamol bronchodilation. The best out of three consecutive blows to record the FEV_1 and the FVC was then used.

2.3.2 Methacholine Challenge Testing

Was carried out in accordance with ATS 1999 criteria; subjects were given increasing concentrations of methacholine to measure a 20% drop in their baseline FEV_1 and measurement of dyspnoea on a Borg scale. A value of >16µg/ml was representative of no airway hyperresponsiveness; for lower concentrations that induced a fall the corresponding value was noted.

2.3.3 Atopy testing

Skin prick testing was used to assess atopy to the aeroallergens *dermatophagoides pteronyssinus*, dog, cat, and grass pollen; and a comprehensive assessment of fungal atopy to *Alternaria alternata, Aspergillus fumigatus, Botrytis cinerea, Cladosporium herbarum* and *Penicillium chrysogenum* (Alk-Abello, Denmark), with negative and histamine controls. A positive response to an allergen was recorded when there was a wheal > 3mm bigger than the negative control. Total IgE levels (assay detection limits 2-5000 kU/L, normal reference range 0–114) and allergen specific IgE antibody levels to cat, dog, timothy grass, *dermatophagoides pteronyssinus, Aspergillus fumigatus* (assay detection limits 0.01-100 kU/L, normal reference range 0–0.34) and *Aspergillus fumigatus*-IgG levels (assay detection limits 0.02-200mg/L, normal reference range 0–40) were measured using the ImmunoCap 250 system (Phadia, UK).

2.4 Symptom Scores

2.4.1 Visual Analogue Scale

The visual analogue scale (VAS) for the domains of a) cough b) breathlessness and c) wheeze was used to record symptoms (Brightling, 2001). Each subject was asked to draw on a 100mm line with ‘no symptoms’ at one end and ‘the worst symptoms

ever' at the other for each symptom domain. A total score for VAS symptoms was taken as the cumulative of the individual domain.

2.4.2 Asthma Control Questionnaire

Asthma Control Questionnaire (ACQ) is a well validated tool measures both the adequacy of asthma control and change in asthma control, which occurs either spontaneously or as a result of treatment [Juniper 1999]. In my study the ACQ was shortened to be representative of symptoms alone by excluding scores obtained for the pre bronchodilator FEV_1 and dividing the total score obtained for the other domains by six [Juniper 2005]

2.5 Sputum induction

Spontaneous or induced sputum was collected from subjects during visits throughout the study. Both methods of sample collection have been shown to be similar for the differential cell counts [Bhowmik 1998].

In those subjects who were unable to spontaneously expectorate sputum, the following sputum induction protocol was performed. The procedure was fully explained with the following instructions:

Guidance on posture: sit upright during the nebulisation procedure and lean forward during expectoration.

Guidance on effective expectoration: instructions for coughing and moving sputum successfully into specimen container

Guidance on contamination reduction: instructions to blow nose and to rinse mouth prior to expectoration.

The procedure requires all subjects to have FEV1 measured before and after pre-treatment with 400µg inhaled salbutamol to minimise bronchoconstriction. Nebulised saline (5mL at 3, 4, and 5%) was given in sequence via an ultra-sonic nebuliser (UltraNeb, DeVilbiss, Sunrise Medical, USA) for 5 minutes. After each inhalation, subjects were asked to blow their nose and rinse their mouth prior to coughing and expectoration of sputum. FEV1 was measured after each inhalation to assess for bronchoconstriction and to assess safety for procedure continuation. The process

was terminated if there was a greater than 20% drop in FEV1, significant symptoms or successful sputum expectoration. The sputum induction protocol used is shown in figure 2.1. In COPD subjects, sputum induction has been shown to be safe in subjects with a FEV1 of ≥ 0.5 L (Brightling, 2001). All sputum samples were processed within 2 hours of collection in a Class II biological safety cabinet.

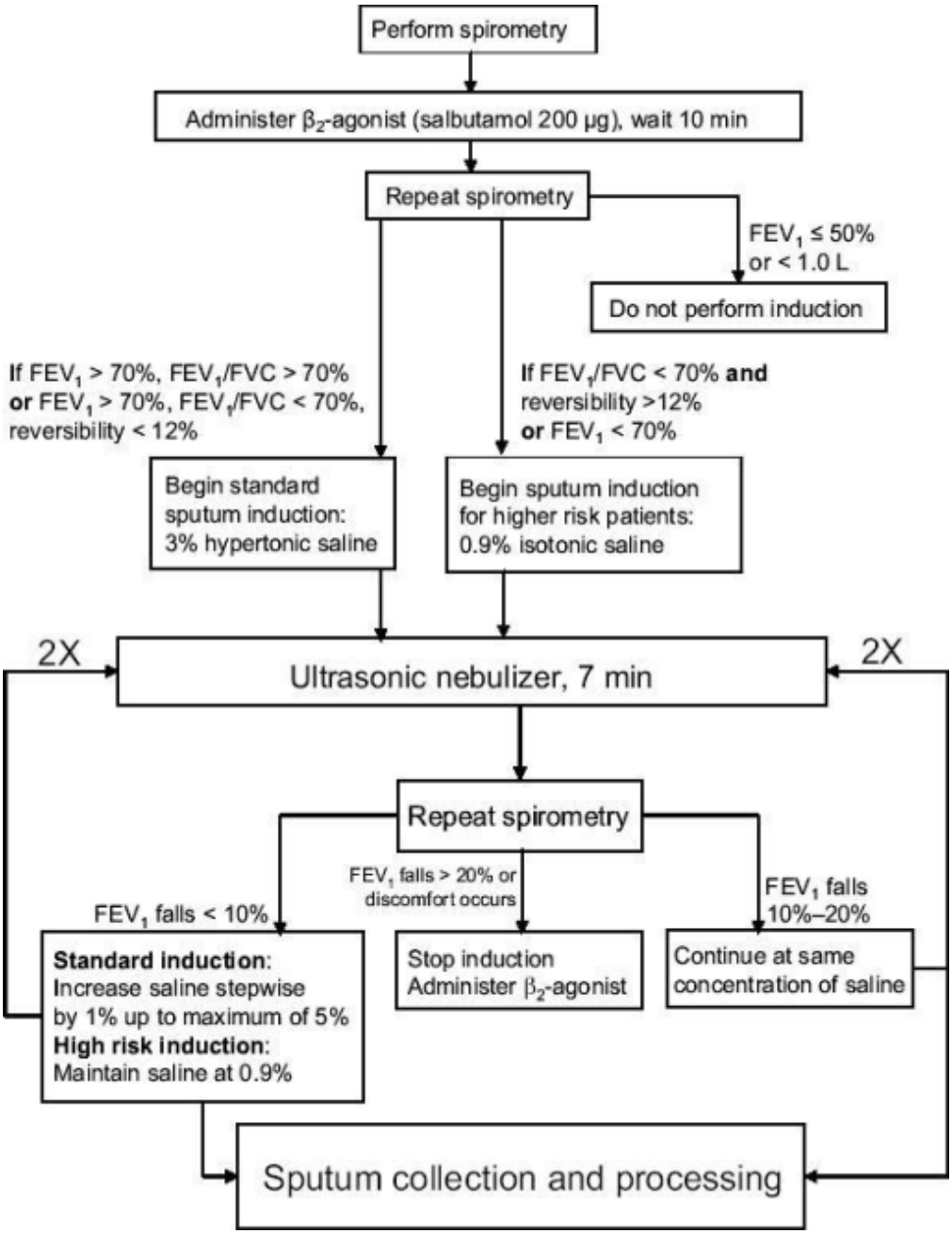


Figure 2.1 Sputum induction protocol

2.6 Sputum processing

The collected sputum sample was emptied into a petridish and placed on a dark background to aid visualisation of sputum plugs. Sputum plugs were selected from the saliva and were then gathered into a large condensed mass in small circular movements. Sputum plugs were then removed for analysis of bacteria and virus.

The remainder of the selected sputum was weighed and incubated with 8 times the volume/weight of Dulbecco's phosphate buffered saline (D-PBS) (Sigma, Poole, Dorset). The sputum sample was dispersed by gentle aspiration into a Pasteur pipette and placed onto a bench rocker for 15 minutes on ice and then centrifuged at 790g for 10 minutes at 4 ^{o} C. This was followed by removal of 4 times the volume/weight of this D-PBS supernatant with storage in 300µL aliquots at -80 ^{o} C for further mediator analysis. The remainder of the D-PBS sputum suspension was incubated with 0.2% dithiothreitol (DTT) (Sigma, Poole, Dorset), placed on a bench rocker on ice for 15 minutes and filtered through pre-wet 48µm gauze. 100 µL of this filtrate was removed for quantification of colony forming units and an additional 500 µL was removed for bacteria quantitative real time PCR (qPCR) analysis. A further 10µL of the filtrate was removed to assess the total cell count and cell viability using a Neubauer haemocytometer. The haemocytometer was flooded with 10µL of the filtrate mixed with 10µL of 0.4% trypan blue (Sigma, Poole, Dorset) and all cells were counted in the four corner squares of the haemocytometer to include viable, non-viable and squamous cells.

The total number of cells and total cell counts were calculated using the following formula:

Total number of cells (x106) = [mean number of cells counted/square × 2 × filtrate volume (mL)] / 100

Total cell count (x106/g sputum) = [mean number of cells counted/square × 2 × filtrate volume (mL)] / 100 × selected sputum weight

The remainder of the filtrate was then further centrifuged for 10 minutes at 790g at 4^{o} C. The DTT supernatant was removed into 300µL aliquots and stored at -80 ^{o} C for further mediator analysis.

Following DTT supernatant removal, the cell pellet was re-suspended in a small volume of D-PBS and adjusted to make a cell suspension of 0.50 – 0.75 x106 cell/mL with D-PBS for cytospin preparation. 75 µL of cell suspension was placed in cytocentrifuge cups and spun at 450 rpm for 6 minutes. The slides were then air dried for 15 minutes at room temperature and stained with Rowanowski stain (0.5g Eosin, 1.5g Azure-B-thiocyanate, 10nM HEPES buffer pH7.2, DMSO). A differential cell count was obtained by counting > 400 non squamous cells on the prepared slide. The sputum processing pathway design is demonstrated in figure 2.2.

2.7 Blood collection and processing

A volume of 10mL of venous blood was collected by venepuncture and collected into serum gel activator (coated with silica particles to enable clotting) and EDTA plasma (coated with K2 to prevent clotting) prepared containers. These were left to stand upright for 1 hour and then centrifuged at 1700rpm for 10mins at room temperature. Serum and plasma was then separated and following a further centrifugation step (2300rpm for 10mins at room temperature) were divided into serum and plasma aliquots for biomarker analysis. Venous blood was also taken to measure full blood count, differential cell count and CRP.

2.8 Analyte measurement-

2.8.1 Meso Scale Discovery Platform

Although similar to a traditional ELISA, Meso Scale Discovery Electrochemiluminsence (MSD-ECLU) uses non-radioactive electrochemiluminescent labels for ultra-sensitive detection. The Meso Scale Discovery MSD assay platform utilizes Ruthenium (II) tris-bipyridine-(4-methylsulfone) [Ru(bpy)3] that, once conjugated to the analyte, serves as the tracer in competitive assays.

ECL labels generate light when stimulated by electricity in the appropriate chemical environment. High binding carbon electrodes in the bottom of microplates allow for easy attachment of biological reagents. MSD assays use ECL labels that are conjugated to detection antibodies. Electricity is applied to the plate electrodes by an MSD instrument leading to light emission by labels. Light intensity is then measured to quantify analytes in the sample [www.mesoscale.com]

Serum and sputum samples were analysed using the MSD platform according to the manufacturer's instructions. In brief, 25µL of the cytokine assay diluents was added to the plate and incubated for 30 minutes. This was followed by the addition of 25µL of serum or sputum D-PBS supernatant and incubated for 2 hours. The plate was then washed 3 times with diluted wash buffer and 25µL of detection antibody was added. After a further incubation period of 1 hour and a repeated wash step, 150µL of read buffer was added and the plate was read.

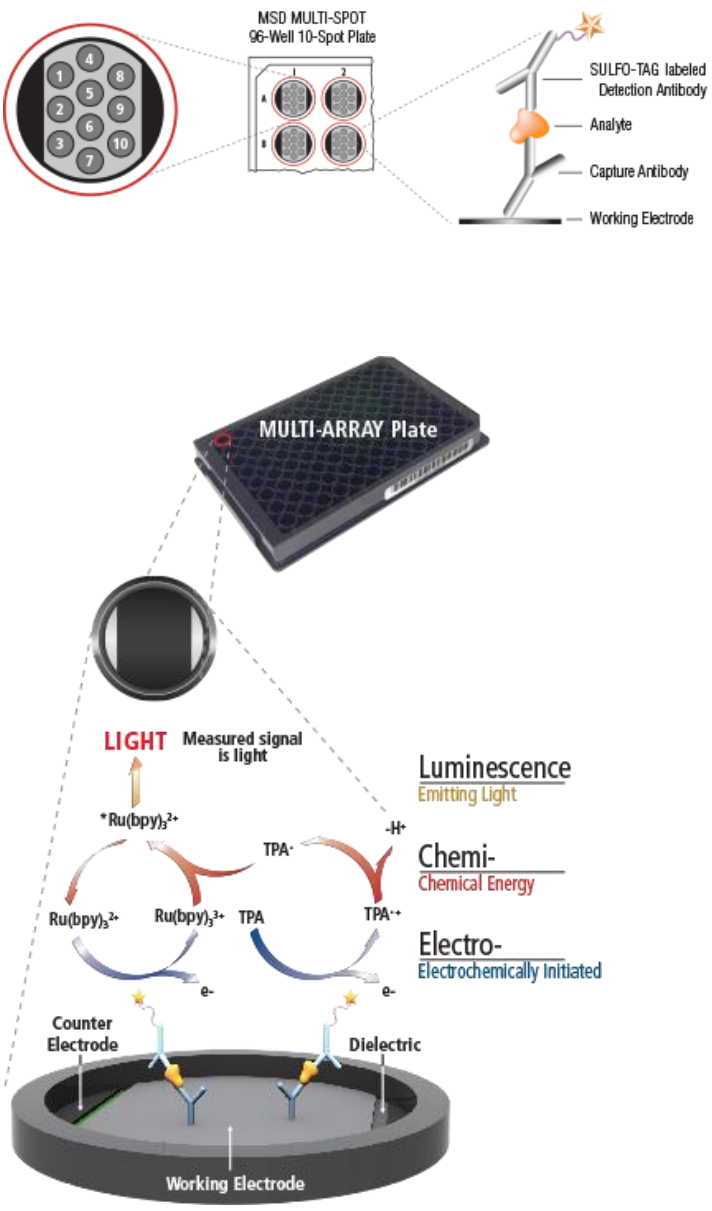


Figure 2.2 MSD platforms showing the layout of multiwall detectors and ECL technology (figure downloaded from www.mesoscale.com)

2.8.2 Analyte measurement- Myriad Luminex Platform

This platform was exclusively for sputum mediator measurement in the imaging chapter. It was not possible to compare the performance of MSD versus Luminex platforms on the same samples. A small volume from each of the sample plate wells is added to reaction wells containing capture beads. These microspheres are typically conjugated to antibodies and are encoded with a unique fluorescent signature that is specific to the analyte of interest. The beads are allowed time to incubate with the sample and the antigens of interest bind to their targets before the other reagents are added. There is a further addition of a cocktail of analyte specific, biotinylated detection reagents to the microsphere mixture, followed by the addition of a fluorescent reporter molecule. Lastly the multiplex is washed to remove unbound detection reagents and read on a Luminex machine. The Luminex instruments operate similarly to a flow cytometer, using the principle of hydrodynamic focusing to pass the microspheres, one at a time, along a path that is interrogated by two lasers. The excitation beams measure the unique fluorescent signature of each bead, in addition to the amount of fluorescence generated in proportion to the analyte concentration in the sample. Data are acquired and reported in real-time, giving the ability to measure an analyte's concentration [Rules Based Medicine].

2.8.3 Spike Recovery Experiment

The aim of this was to measure the rate of recovery of recombinant proteins added to sputum plugs which are then processed by the standard procedure. This method is used to determine whether a systematic shift occurs in the analytical signal of an analyte due to matrix effects. To determine the percent recovery of a spike, the sample is split into two portions and a known amount of a standard solution of analyte is added to one portion. The concentration of the analyte is determined for both; the spiked, F, and un-spiked portions, I, and the percent recovery, %R, is calculated as follows (where A is the concentration of analyte added to the spiked portion) %R= 100x (F-I/A). Because many parameters are not found in tests, it has

become a common practice to carry out two matrix spikes if adequate material is available. This procedure is called a matrix spike/matrix spike duplicate. This procedure allows determination of both accuracy and precision since the recovery and concentration are known and precision between the two results can be compared.

Spiking experiments were performed in duplicate according to current recommendations [Kelly 2002]. Briefly, known concentration of cytokine and chemokine mediators were spiked into selected sputum plugs processed with DTT or D-PBS and compared to unprocessed DTT or buffer (unprocessed buffer set as the spiking standard control experiment). Samples for the spiking experiments were obtained from 5 subjects with a physician diagnosis of asthma. Sputum was collected from asthma subjects following sputum induction as described earlier.

A regression of the ECLU of the standard curve samples versus concentrations was performed using Watson LIMS software and the concentrations of the analytes in the samples determined. The kit included calibration standards (CS).Lower limits of quantification (LLOQ), were based on results with CS in buffer.

The lower limit of quantification (LLOQ) and detection (LLOD) for each kit were determined from the respective standard curves for each cytokine. The LLOQ was defined as the lowest concentration on the standard curve which yielded a) a measured concentration within 25% of the nominal value, and b) a coefficient of variation (%CV) less than 25% [Chowdhury 2009]. The LLOD was defined as the lowest concentration on the standard curve whose readout was greater than 2.5 standard deviations above that of the blank. The LLOD is less stringent than the LLOQ as it is determined exclusively from the absolute value of the blank.

Sputum is a complex mixture of mucus, DNA, degraded cells and their products, secreted proteases and binding proteins; this heterogeneous make-up may result in inconsistencies in the measurement of soluble compounds. At exacerbation it is likely that there is increased complexity in the sputum. High levels of viscid DNA and proteases have been detected in patients with infected bronchiectasis and cystic fibrosis [Linnane 1998].

DTT and D-PBS processing of sputum and the subsequent recovery of the analytes have been compared and studied both at stable state and during exacerbations of COPD [Bafadhel 2009, 2011]. DTT has been shown to effect recovery of certain sputum cytokines and chemokines compared with D-PBS and may be attributable to physical mediator losses reflecting non-specific binding of cytokine to sites such as plastic containers or filters. The D-PBS sputum processing method improves the recovery of measureable cytokines and chemokines from COPD subjects using the MSD platform and does not affect the cytospin preparation.

D-PBS processed sputum was utilized only in chapter 7 the remainder of the samples were all processed with DTT.

In my study mediators that had recovery >80% thereby satisfying ERS recommendations were subsequently used in the analysis. GMCSF and IFNγ were excluded as they did not have recovery of even 30%. IL-4, IL-10, IL-13 and IL-17 had recovery of >40% but <60%.

For the samples that were below the LLOQ, a value of the (LLOD/2) was assigned; whilst such data imputation may not be acceptable for publication in peer reviewed journals I have used it for the purpose of this thesis.

2.9 Cross sectional imaging

HRCT was performed using Siemens Sensation 16 scanner. Scans were acquired using standard HRCT protocol (sequential scanning at 10-mm increments with 1-mm collimation) from the apex of the lung to the diaphragm [Gupta 2010]. Patients were scanned in the supine position at maximal inspiration (adequate breath holding rehearsed prior to scan), with their arms held over their heads. Images were reconstructed using a high spatial frequency algorithm, through a 512X512 matrix, with a small field of view targeted to image only pulmonary areas. Scanning time ranged from 30-45s with a voltage of 120kVp and peak effective tube current (dose modulation based on size and attenuation profile of the region scanned used to minimise radiation dose) of 140mAs (range 65-140mAs).

2.9.1 Qualitative and quantitative airway analysis

CT scans were analysed qualitatively by a single observer and the presence or absence of bronchiectasis and/or bronchial wall thickening was reported as described previously [Gupta 2009]. An automated program Emphylyx-J V 1.00.01 [Nakano 2005, Siddiqui 2009] using the full-width at half-maximum (FWHM) technique was used to determine the airway cross-sectional geometry. Image data were transferred from the CT workstation to a personal computer in DICOM 3.0 format. After identifying the RB1 the operator placed a seed point in the airway lumen from which 64-128 rays were cast across the airway wall. The boundaries of the wall were defined by the midpoint of the profile of CT numbers across each ray. Lumen Area (LA), wall area (WA), maximum and minimum airway diameter were measured. High spatial frequency algorithm was used for reconstruction of images as it results in reduced blurring and, as demonstrated by phantom studies, [Saba 2003] is associated with reduced errors in airway wall estimation using FWHM method. Correction equations for both size dependent error using the FWHM method and oblique orientation of airways were applied as previously described [Gupta 2010] LA and WA were corrected for body surface area (BSA). Total area (TA) and percentage WA (%WA) were derived from LA and WA (TA=LA+WA; %WA= WA/TA x100).

2.10 Microbiological Methods

2.10.1Bacterial routine culture

Selected whole sputum for routine bacterial culture was processed according to the Health Protection Agency National Standard Method BSOP 57 (Health Protection Agency, 2009). Sputum was viewed macroscopically and described as mucoid, mucopurulent or purulent. An equal volume of 0.1% of DTT was added to the sputum and after gentle agitation was incubated for 15 minutes at 37^0C to assist in homogenisation. A further dilution step of the homogenised sputum, whereby 10 µL was added to 5mL of sterile distilled water was performed. Using a sterile loop, 10µL (therefore at a dilution of 10-3) was inoculated using the streaking method to a chocolate and blood agar media plate. The chocolate and blood agar plates were pre-prepared with a bacitracin and optochin disc respectively. Each plate was incubated in 10% CO2 for 48 hours and read daily. Reporting for all samples was standardised as follows: No growth; No significant growth; and qualitative reporting for clinically significant pathogens as light, moderate and heavy growth for each dominant organism grown (*Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*).

2.10.2 Colony forming units

Semi-quantitative bacterial analysis was performed by CFU estimation in accordance to previously described methods [Pye 1995]. 900µL of sterile D-PBS solution was placed in 5 sterile eppendorfs labelled as 10^1, 10^2, 10^3, 10^4, 10^5 and serial dilutions of the 100µL DTT filtrate removed during the sputum processing procedure were made. Three 20µL drops were placed from each serial dilution onto chocolate and blood agar media. Each plate was then incubated for 24 hours in 5% CO2 at 37^0C. After incubation, counts were made from the dilution with <100 CFU and averaged for each of the droplets to determine a total CFU load.

2.10.3 Bacterial quantitative real time polymerase chain reaction assay

Total bacterial DNA was extracted from 500µL of homogenised sputum using the commercial QIAmp DNA Mini Kit assay (QIAGEN Ltd, Hilden Germany). Briefly DNA extraction was performed using a 2-step process. Firstly this involved lysis with

20mg/mL lysozyme followed by incubation at 37°C for 30 minutes. Further lysis was performed with Proteinase K (600mAU/mL solution) at 55°C for 30 minutes and 95°C for 15 minutes. The lysate obtained was buffered and added to the QIAamp spin column and centrifuged briefly for optimal adsorption of the DNA to the column's silica gel membrane. This was followed by three wash steps. Finally DNA was eluted in 200µL of DNAse, RNAse free distilled water and stored at -20°C.

DNA standards for RT-PCR were prepared from pure culture DNA extracted from overnight cultures of *E. coli, H. influenzae, M. catarrhalis, S. aureus* and *S. pneumoniae*. 10-fold dilutions were prepared ranging from 10^2 to 10^7.

Quantification of the total bacterial load, *H. influenzae* and *S. aureus* was performed using the SYBR Green assay (PE Applied Biosystems, Warrington, UK), which employs fluorescent binding to the minor groove of the DNA double helix following polymerisation. The TaqMan (Applied BioSystems, UK) assay was used to quantify *M. catarrhalis* and *S. pneumoniae*. Each assay run was performed with two negative controls (distilled RNAse DNAse water) and all sputum samples were examined in duplicate.

Table 2.1 Target genes and primers for each of the qPCR bacteria detection assays

Target organism	Target gene	Primers	Sequence
Total bacteria	16S rDNA	338F	5' ACTCCTACGGGNGGCNGCA 3'
		515R	5' GTATTACCGCNNCTGCTGGCAC 3'
S. pneumoniae	Pneumolysin	S.pneumoniae F (Greiner, 2001)	5' AGCGATAGCTTTCTCCAAGTGG-3'
		S.pneumoniae R	5' TTAGCCAACAAATCGTTTACCG 3'
		S.pneumoniae probe	5**'Cy5**ACCCCAGCAATTCAAGTGTTCGCG-**BHQ2** 3'
H. influenzae	Outer membrane protein P6	P6 F (Stralin, 2005)	5' TTGGCGGWTACTCTGTTGCT 3'
		P6 R	5' TGCAGGTTTTTCTTCACCGT 3'
S. aureus	Thermonuclease	S.aureus (nuc) F (Fang, 2003)	5' GCGATTGATGGTGATACGGTT 3'
		S.aureus (nuc) R	5' AGCCAAGCCTTGACGAACTAAAGC 3'
M. catarrhalis	Outer membrane protein CopB	M. catarrhalis F (Greiner, 2003)	5' GTGAGTGCCGCTTTACAACC 3'
		M. catarrhalis R	5' TGTATCGCCTGCCAAGACAA 3'
		M. catarrhalis probe	5'**JOE**TGCTTTTGCAGCTGTTAGCCAGCCTAA-**TAMRA** 3'
M. pneumoniae	16S rRNA	Myco-1	5'AAGGACCTGCAAGGGTTCGT 3'
		Myco-2	5' CTCTAGCCATTACCTGCTAA 3'
C. pneumoniae	Outer membrane protein ompA	APNOL	5'ATTAAGAAGCTCTGAGCATA 3'
		APNOU	5'AATTCTCTGTAAACAAACCC 3'
		APN 1	5'AGCCTAACATGTAGACTCTGAT 3'
		APN 2	5' TGCCAACAGACGCTGGCGT 3'

2.10.4 Detection of virus

Viral RNA for PCR analysis was extracted from sputum, using in-house assays for examination of the following viruses; rhinovirus, respiratory syncytial virus, human parainfluenza virus 1-3, adenovirus, influenza virus A and B, coronavirus 229E and OC43, human metapneumovirus and human bocavirus. Picorna virus analysis was performed by a reverse transcription method followed by PCR.

2.11 Data presentation

Throughout this thesis, parametric data is presented as mean (standard error of the mean) unless mean (range) or mean (95% confidence interval) is more appropriate. Log transformed data is presented as geometric mean (95% confidence interval). Non parametric data is presented as median (interquartile range); the interquartile range is presented as the difference between the 75th and 25th centile. Differences between groups are presented as mean difference (95% confidence interval of mean difference), fold difference (95% confidence interval of fold difference) and median difference (interquartile range of difference) as appropriate for parametric, log transformed and non-parametric data respectively. A p-value of <0.05 was taken as the threshold of significance for all statistical analyses.

2.12 Statistical Analysis

For comparison of unpaired or paired parametric or non-parametric groups, the Student T-test, Paired T-test, Mann-Whitney test and Wilcoxon matched pairs test was used respectively. For comparison of three groups or more for parametric and non-parametric variables the one-way analysis of variance (ANOVA) or Kruskal-Wallis test was used and the $\chi 2$ test for proportions. Correlations were assessed by the Pearson correlation coefficient (r) and Spearman rank correlation coefficient (rs) for parametric and non-parametric data.

Longitudinal and follow up data was analysed using the Cohen Kappa coefficient (κ), and intra class coefficient (ICC). The κ coefficient measures inter-observer agreement for categorical data, taking into account the agreement that may occur by

chance (in contrast to simple percentage agreement). The ICC closely resembles the Pearson correlation coefficient but differs in that data is centred and scaled for a pooled mean and standard deviation and agreement of 1 does not confer a linear association. Logistic and multiple regression analysis were used to assess relationships of independent variables on dependant categorical and continuous variables respectively.

Any specific statistical techniques have been described in each chapter

Chapter 3

Mediator profiling of severe asthma subphenotypes

3.1 Introduction

Defining the phenotypic heterogeneity of severe asthma is likely to shed light upon its immunopathogenesis and direct therapy.I sought to determine the relationship between phenotypes of severe asthma and sputum mediator profiles

My study aims were (i) identify clinical subgroups determined by cluster analysis of clinical parameters and sputum cell counts (ii) characterize the pattern of sputum mediator expression quantified by factor analysis and (iii) define the relationship between these biological factors and clinical sub-groups.

3.2 Subjects and Protocol

203 subjects were recruited from Difficult Asthma Clinic and had provided sputum samples. 164 of these subjects had adequate (>150 μl aliquot) of sputum supernatant and were included in the analysis. . Longitudinal visits were undertaken on all subjects that were being followed up in clinic with 106 subjects included for a 2^{nd} and 66 for a 3^{rd} visit with lung function and sputum assessments.

3.2.1 Sputum processing and cytokine assessment

Measurements for 23 validated mediators [Bafadhel 2009] were performed using the Meso Scale Discovery Platform (MSD) Two mediators (IFN-γ and GM-CSF) were undetectable in >80% of samples and excluded from the statistical analysis)

3.2.2 Statistical Analysis

Statistical analysis was performed using PRISM version 4 (GraphPad Software, San Diego, CA), and SPSS version 16 (SPSS, Inc. Chicago, IL). Repeatability was

assessed by kappa statistic and intra-class correlation. Data representation and analysis was carried out as describe in the methods chapter. There were some specific statistical techniques described below

Clinical Cluster Analysis

The clinical parameters used in the cluster analysis were determined from our earlier work and were representative of the various facets of asthma. These were i) age of onset, ii) body mass index (BMI), iii) gender, iv) atopy, v) bronchodilator reversibility, vi) ACQ and vii) log normalized sputum eosinophil and neutrophil count [Haldar 2008]. In the first step, hierarchical cluster analysis using Ward's method generated a dendrogram for estimation of the number of likely clusters within the studied population. Cuts made at points of large change between successive fusion levels were used to define likely cluster boundaries. This estimate was then pre-specified in a k-means cluster analysis that was used as the principal clustering technique and a cluster membership being assigned for four discrete groups.

Biological Factor Analysis

Mediator measurements with levels below level of quantification were substituted with half the lowest limit of quantification for that mediator. All mediator data was log-normalized and analysed in a principal components version of factor analysis as described previously [Bafadhel 2011]. Briefly, factor analysis takes a large number of outcome variables, in this case individual mediators and returns a smaller number of variables called factors that account for the variance seen in the larger number of outcome variables. Each factor is associated with each outcome variable to different degrees. The correlations between factor and outcome variables are quantified as factor loading. The factor scores are a patient's individual score on each of the factors. The factors can be seen as dimensional reduction variables that describe similar patterns of variation seen in certain subsets of outcome variables. In our analysis factors with an Eigenvalue ≥1 were extracted after orthogonal varimax rotation; coefficients with scores ≥0.5 only were included. The factors were saved as regression scores and utilized in the subsequent analysis.

The ‘mean factor score’ is the arithmetic mean of the values for each factor that is derived from the analysis. This is best represented graphically with a positive value indicating a higher reading relative to those with a negative value.

For each of the three time points analysed factor analysis was appropriate with Bartlett’s test of sphericity being significant ($p<0.0005$ at all time points) and the Kaiser-Meyer-Olkein test of sampling adequacy having high values (0.87, 0.79 and 0.78, respectively). The distribution of the factor scores for mediator expression across the clinical clusters, and between subjects stratified by asthma control, exacerbation frequency, treatment and sputum cell counts (eosinophilic ≥3% [6, 10, 13], neutrophilic ≥61%, paucigranulocytic <3% eosinophils and <61% neutrophils, mixed granulocytic ≥61% neutrophils and ≥3% eosinophils) [Simpson 2006] was assessed by ANOVA.

Biological Cluster Analysis

Mediator measurements with levels below level of quantification were substituted with half the lowest limit of quantification for that mediator. All mediator data was log-normalized and analysed by a hierarchical cluster analysis followed by k-means clustering analysis.

3.3 Results

3.3.1 Clinical characteristics of severe asthma clusters

164 out of the 203 patients recruited had adequate sputum supernatants and were included in the analysis. We identified 4 clinical clusters. The characteristics of the whole group and the individual clusters are as shown **(table 3.1)**. These clusters were significantly different in most attributes including age of onset of disease, sex, body mass index (BMI), ACQ, bronchodilator reversibility and airway inflammation. Individual sputum mediator levels were significantly different between clinical clusters (**table 3.2**). The factorial pattern of mediators (**table 3.3**) was significantly different between two clusters (**figure 3.1**) when comparing the mean factor scores. The largest group (n=67) consists of a group with early onset disease, atopic (58%) and highest symptoms (ACQ 2.96 [0.13]). They had most airflow obstruction (post bronchodilator FEV_1/FVC ratio 0.66) and predominantly neutrophilic airway

inflammation (73.4 [68.3-78.7]). The 'inflammation predominant' consists of late onset asthmatics with a higher proportion of males (55%). They are the only group with a mixed granulocytic sputum inflammation (eosinophils 4.6 [3.2-6.9] %; neutrophils 61.1[55.9-66.7] %) and despite this are the most discordant by expressing the least symptoms (ACQ 1.44[0.14]). An 'early onset atopic asthma' group consists of largely, females with early onset disease at 16(3.2) years, with a higher proportion of atopic individuals (57%) with high symptom expression (ACQ 2.71 [0.40]) and the most bronchodilator reversibility (9.1 [2.45] % change in FEV_1 and the most sputum eosinophilia (12.0 [5.4-26.9] %). The 'Obese, non eosinophilic' group was predominantly female (75%) with highest BMI (42.5 [1.21] kg/m^2) and highest symptom expression (ACQ 3.38 [0.20]). They had the highest number of severe exacerbations (5 [1]) and a sputum neutrophilia (66.2 [57.2-76.4] %).

3.3.2 Factor scores of the asthma clusters

Mean factor scores were derived as described earlier. These scores were mapped on to the clinical clusters (**Figure 3.1**). The inflammation predominant group showed a modest increase in all factors except the one expressing IL4, IL10 and IL17. The 'obese noneosinophilic' group group interestingly had the highest levels of IL-5, despite having low sputum eosinophils (1.8[1.0-3.2] %). They showed highest expression of the cytokine factor consisting of IL-5, IL-13 and CCL26 and also had the highest levels of chemokines CCL2, CCL5, CCL11 and CCL13. Despite being only modestly neutrophilic they also had highest levels of IL-6. The 'early onset atopic' group showed expressed the highest mean factor scores for IL13, IL5 and CCL26. The mean factor scores were not statistically significantly different when comparing between and within groups across the clinical clusters. The 'early onset symptom predominant' group showed low levels of the factor IL5, IL13 and CCL26 in addition to low level of factor IL6, CCL5, CXCL11,TNF and CCL2.

The mean factor scores were distinct and significantly different between subjects with and without a sputum eosinophilia (**figure 3.2A**) or sputum neutrophilia (**figure 3.2B**) and across the groups eosinophilic, neutrophilic, mixed granulocytic and paucigranulocytic (**figure 3.2C**).

Similarly the mean factor scores stratified for persistent airflow obstruction was not significantly different (**figure 3.3A**). The mean factor scores were significantly different in subjects with frequent versus infrequent exacerbation groups (online **figure 3.3B**), good versus poor control (**figure 3.3C**) and GINA 4 versus 5 (**figure** 3**.3D**).

The tables showing levels for the individual mediators between subjects with and without frequent exacerbations persistent airflow obstruction (post-bronchodilator FEV_1<80% and FEV_1/FVC <70%; poor control (ACQ>1.5); GINA 4 versus GINA 5, a sputum eosinophilia or a sputum neutrophilia **are not included in this thesis.**

3.3.3 Longitudinal measurements of clinical characteristics and sputum mediators

The repeatability of the clinical parameters, cell counts and mediator profiles was assessed in paired samples in 106 subjects and in three samples in 66 subjects (table 4). The median (interquartile range) time between visits was 142 (165) days. Within subject repeatability at 2 time points in 106 subjects and at 3 time points in 66 subjects was excellent for lung function and ACQ (κ >0.8, p<0.001), moderate for sputum cell counts (κ= 0.3-0.6), and mostly moderate for individual sputum mediator levels (κ=0.1-0.5). The repeatability of subject's assignment was good for an eosinophilic phenotype between visits 1 to 2 and 2 to 3 (κ=0.46, p<0.001; κ=0.46, p<0.001) and moderate to poor for neutrophilic phenotype (κ=0.38, p=0.016; κ=0.18, p=0.33). The biological factors were remarkably similar between visits with mediators distributed in consistent association (**tables not shown**).

The distribution of the factor scores across the different sputum cellular profiles was consistent with a strong association with factor 4, consisting of IL-5, IL-13 and CCL26, tracking positively with a sputum eosinophilia and reciprocally with a sputum neutrophilia (**figures 3.4A-C, 3.5A-C**).

3.3.4 Characteristics of K means biological or cytokine derived clusters

This identified 4 clusters that were distinct with sputum mediator measurements being significantly different in all 4 clusters but there was little biological plausibility for mediator data. The clusters could not be classified into predominantly Th2 or Th1 high/low categories as per classifications used [Woodruff 2009] mainly due to lack of a healthy control group. The clinical characteristics for these cytokine clusters were indistinguishable. This suggests that clustering of biological data was not associated with any identifiable clinical trait, did not link to clinical outcomes or to airway inflammation.

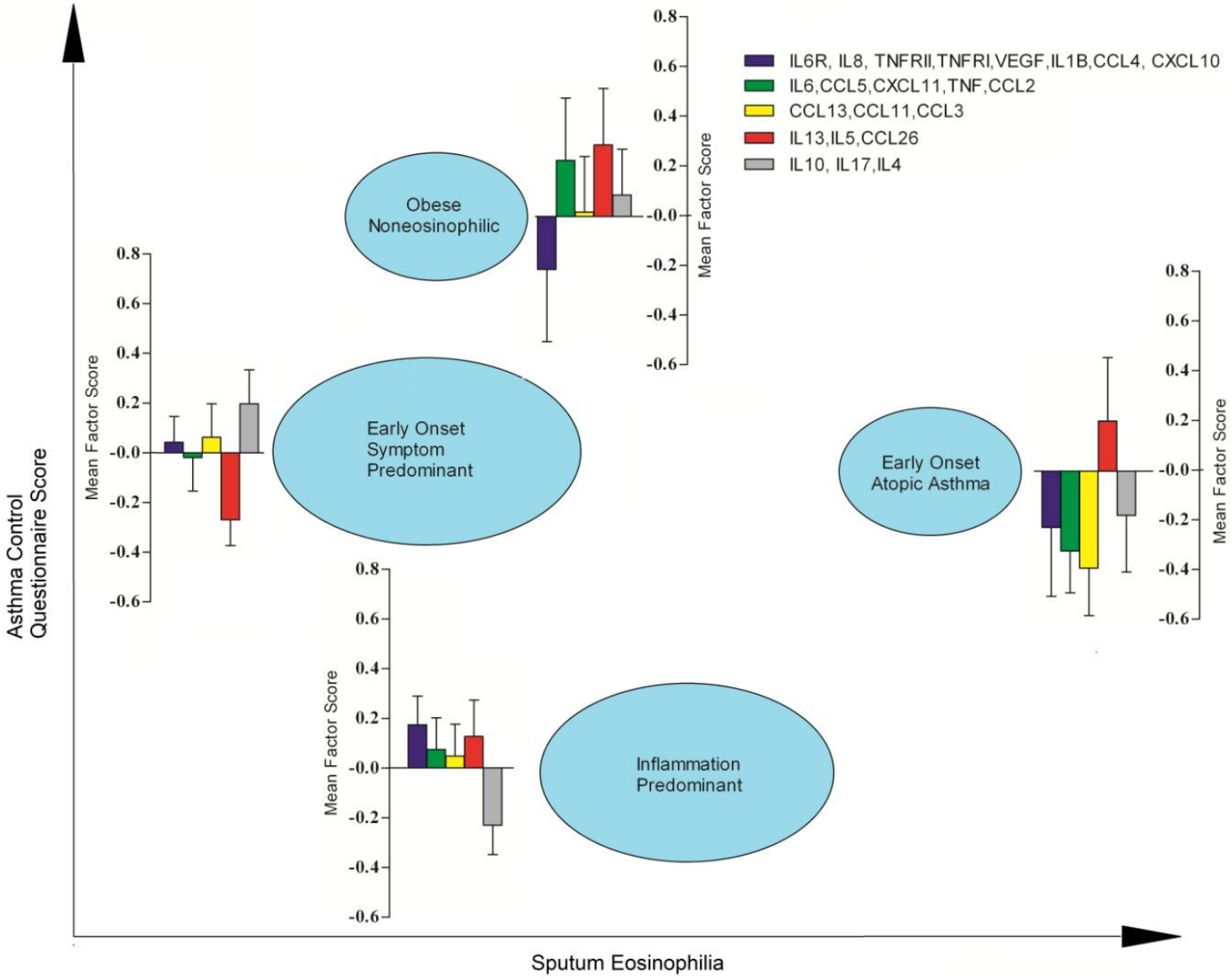
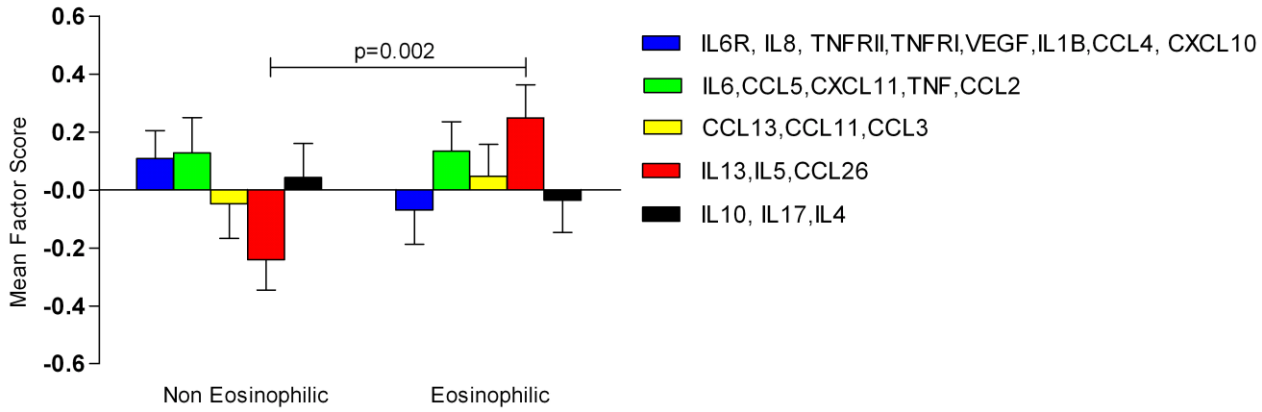

IL6R, IL8, TNFRII,TNFRI,VEGF,IL1B,CCL4, CXCL10
IL6,CCL5,CXCL11,TNF,CCL2
CCL13,CCL11,CCL3
IL13,IL5,CCL26
IL10, IL17,IL4
Obese Noneosinophilic
Early Onset Symptom Predominant
Early Onset Atopic Asthma
Inflammation Predominant
Mean Factor Score
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
Asthma Control Questionnaire Score
Sputum Eosinophilia

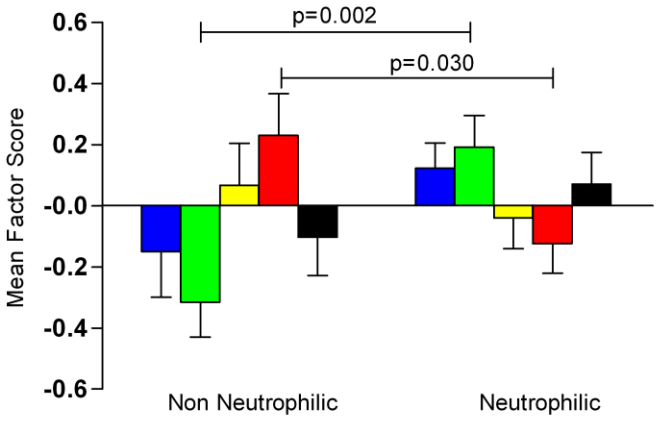
Figure 3.1 Relationship between clinical clusters and biological factors

The clinical clusters are plotted in two dimensions with airway inflammation on the x axis and asthma control (ACQ) on the y axis. The size of the ellipsoid represents the number of subjects within each clinical cluster. The distribution of the five biological factors for each cluster is shown as the mean (SEM) factor scores.

A


0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
Mean Factor Score
p=0.002
Non Eosinophilic
Eosinophilic
IL6R, IL8, TNFRII,TNFRI,VEGF,IL1B,CCL4, CXCL10
IL6,CCL5,CXCL11,TNF,CCL2
CCL13,CCL11,CCL3
IL13,IL5,CCL26
IL10, IL17,IL4

B


0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
Mean Factor Score
p=0.002
p=0.030
Non Neutrophilic
Neutrophilic

C

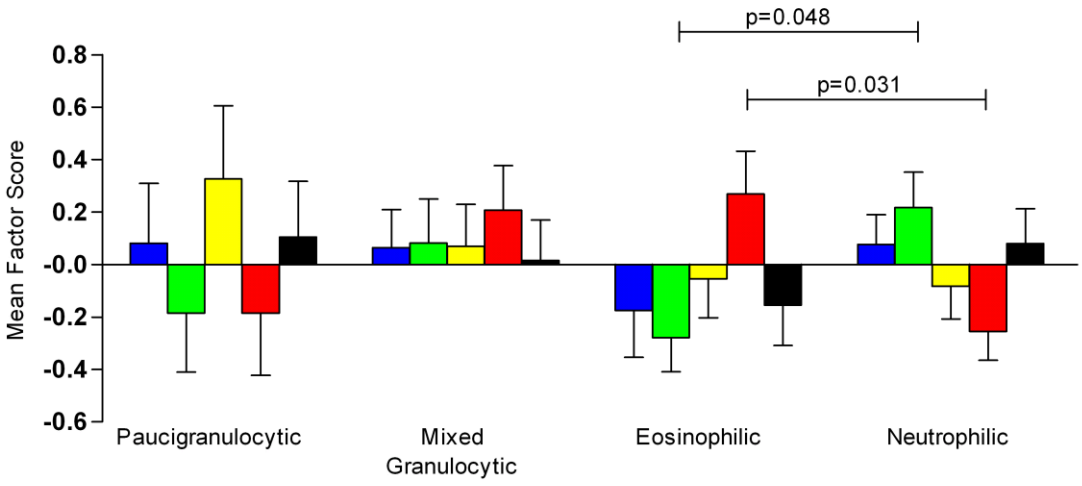

0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
Mean Factor Score
p=0.048
p=0.031
Paucigranulocytic
Mixed Granulocytic
Eosinophilic
Neutrophilic

Figure 3.2 Relationship between sputum cellular inflammation and biological factors. Mean factor score (SEM) between subjects with

a) sputum eosinophilia (≥3%) versus no eosinophilia (<3%),

b) sputum neutrophilia (≥61%) versus no neutrophilia (<61%), and

c) paucigranulocytic (sputum eosinophils <3% and neutrophils <61%), mixed granulocytic (eosinophils ≥3% and neutrophils >61%), eosinophilic (≥3% eosinophils and neutrophils <61%) or neutrophilic (≥61% neutrophils and <3% eosinophils) sputum.

Table 3.1 Characteristics of subjects in clinical clusters

Cluster	All subjects	Inflammation Predominant	Early Onset Symptom Predominant	Early Onset Atopic Asthma	Obese, Non Eosinophilic	p value**
Number	165	58	67	21	19	
Current Age	50(1)	57 (1.4)	47(1.8)	43 (2.4)	50.47(2.67)	**<0.005**
Male, n (%)*	68 (41)	32 (55)	25 (37)	6 (30)	5 (25)	**0.041**
Age of onset*	25 (1.6)	42(2.4)	13 (1.8)	16 (3.2)	28.42(4.11)	**<0.005**
BMI, kg/m^2*	29.8(0.52)	28.1(0.58)	28.3(0.60)	28.6(1.25)	42.5 (1.21)	**<0.005**
Pack years history	4(1)	4.70(1.40)	3.15(0.94)	2.60(1.23)	5.56(2.97)	0.601
Severe Exacerbations	3 (0.25)	2 (0.38)	3(0.35)	4 (0.65)	5 (0.98)	**0.014**
GINA step 5 treatment n	88 (66)	33 (58)	33 (50)	12 (60)	11 (60)	0.767
ACQ$_6$ score*	2.44(0.11)	1.44(0.14)	2.96 (0.13)	2.71 (0.40)	3.38(0.20)	**<0.005**
Prednisolone dose mg	6 (0.6)	6(1)	5.5 (1)	6 (1.5)	9 (2)	0.497
ICS dose‡	1600	1600	1600	1600	2000	0.582
Atopy present, n (%)*	74 (45)	17 (29)	39 (58)	12 (57)	6 (32)	**0.004**
Total IgE, IU/L‖	170 [134-	158 [106-233]	183 [124-270]	254 [120-538]	103 [48-217]	0.451
Pre BD FEV$_1$	2.09 (0.06)	2.25 (0.1)	2.01 (0.08)	2.08 (0.16)	1.89(0.14)	0.153
Pre BD FEV$_1$ % predicted	71.1(1.6)	79.6 (3)	66.6 (2.5)	66.22 (4.3)	67.04(4.04)	**0.003**
Pre BD FEV$_1$/FVC ratio	0.68 (1)	0.70 (1.3)	0.64 (1.9)	0.69 (2.9)	0.74(2.05)	**0.01**
BD Reversibility %FEV$_1$*	5.4(0.52)	4.1 (0.55)	5.7 (0.81)	9.1 (2.45)	4.5(1.29)	**0.025**
Post BD FEV$_1$ %	76.1	82.9	72.0	74.8	71.0	**0.026**
Post BD FEV$_1$/FVC ratio	0.69	0.71	0.66	0.72	0.73	**0.034**
Eosinophils%*	3.0	4.6 [3.2-6.9]	1.7 [1.2-2.3]	12.0 [5.4-26.9]	1.8 [1.0-3.2]	**<0.005**
Neutrophils%*	55.6	61.1[55.9-66.7]	73.4 [68.3-78.7]	15.3 [11.3-20.9]	66.2 [57.2-76.4]	**<0.005**
Total Cell Count	1.28	1.15	1.54	0.84	1.47	0.360
Absolute Eosinophils	3.8	5.4 [3.2-9.1]	2.4[1.4-4.1]	8.4 [2.9-23.9]	2.2 [0.7-6.2]	**<0.001**
Absolute Neutrophils	71.16	70.3 [48.8-101.2]	113 [71.7-180.5]	13.1 [7.6-22.8]	97 [44.7-210.1]	**<0.001**

Data expressed as Mean (SEM) unless otherwise stated. ∗Variables included in cluster analysis. † Asthma Control Questionnaire Score was adjusted to remove effect of FEV$_1$% predicted domain. ‡Doses of all inhaled corticosteroids were converted to the equivalent dose of Beclomethasone dipropionate and expressed here as median dose. ‖ Data expressed as Geometric Mean [95%CI]. ∗∗Comparison between clusters using analysis of variance for continuous variables.

Table 3.2 Sputum mediator levels in the clinical cluster groups

Mediators	All Subjects	Inflammation Predominant	Early Onset Symptom Predominant	Early Onset Atopic Asthma	Obese, Non Eosinophilic	p value
Cytokines/Receptor						
IL-1	96.1 [72.5-127.4]	98.2 [65.7-146.9]	122.7 [77.4-194]	55.1 [21.4-141.3]	92.01 [34.11-248.2]	0.362
IL-4	2.53 [2.21-2.89]	2.14 [1.74-2.64]	2.95 [2.32-3.75]	2.18 [1.53-3.11]	2.58 [1.77-3.76]	0.195
IL-5	1.09 [0.904-1.332]	1.33 [0.90-1.96]	0.819 [0.64-1.04]	1.048 [0.604-1.81]	1.77 [0.921-3.40]	**0.055**
IL-6	59.4 [46.1-76.5]	65.9[45.8-94.9]	68. [43.9-107.4]	26 [13.8-50]	71.7 [28.9-178]	0.11
IL-6R	257 [188-351]	371 [240-571]	287 [186-443]	129 [401-408]	187 [53-655]	0.158
IL-8	2318 [1568-3426]	3593 [2073-6225]	2714 [1524-4833]	859 [219-3367]	1739 [381-7923]	0.129
IL-10	2.02 [1.63-2.49]	1.62 [1.13-2.31]	2.59 [1.86-3.61]	1.71 [0.79-3.7]	1.86 [1.09-3.16]	0.266
IL-13	6.51[5.43-7.79]	7.42 [5.33-10.33]	5.31 [4.12-6.86]	7.08 [3.86-12.99]	7.73 [4.29-13.93]	0.365
IL-17	1.10 [0.925-1.31]	0.98 [0.711-1.37]	1.22 [0.93-1.62]	0.84 [0.53-1.34]	1.34 [0.78-2.31]	0.427
TNF	3.11 [2.37-4.06]	2.86 [1.87-4.36]	3.77 [2.28-6.24]	2.09 [1.13-3.85]	3.20 [1.48-6.91]	0.581
TNFRI	233 [176-309]	305 [206-451]	253 [167-383]	129 [51-326]	213 [67-672]	0.287
TNFRII	144 [103-201]	213 [133-341.6]	161 [96-267]	61[20-186]	113 [32-402]	0.119
Chemokines						
CCL2	246 [211.9-285.7]	285 [230-353]	232[179-300]	183 [124-272]	286 [156-523]	0.275
CCL3	8.94[6.71-11.92]	8.64 [5.39-13.87]	11.3 [7.08-18.1]	6.92 [3.02-15.88]	5.36 [1.94-14.82]	0.411
CCL4	158[111-225]	285[177-459]	129 [70-239]	133.5 [46-379]	89 [25-315]	0.362
CCL5	7.26[5.94-8.88]	8.34 [6.05-11.5]	7.31 [5.14-10.4]	3.62 [2.348-5.58]	10.38 [5.36-21]	**0.048**
CCL11	11.35 [9.08-14.17]	11.08 [7.804-15.73]	11.9[8.062-17.55]	6.42 [3.56-11.58]	18.17 [9.05-36.47]	0.15
CCL13	15.4 [13.05-18.18]	19.76 [15.19-25.7]	14.24 [10.72-18.91]	7.85 [5.35-11.52]	19.83 [11.68-33.68]	**0.005**
CCL17	18.47 [14.75-23.12]	22.58 [15.22-33.48]	16.72[11.46-24.41]	16.47 [8.85-30.63]	16.35 [8.57-31.19]	0.657
CCL26	5.07 [4.21-6.11]	4.87 [3.61-6.59]	4.124 [3.03-5.60]	6.37 [3.55-11.42]	8.68 [4.93-15.29]	*0.094*
CXCL10	372[270-513]	563 [339-934]	395 [237-660]	157 [65-380]	320 [104-983]	0.266
CXCL11	21.1 [15.8-28]	27.6 [16.9-45.1]	20.1 [12.7-34.4]	9.54 [5.5-16.3]	24.3 [10.5-58.1]	0.163
Growth Factors						
VEGF	1375 [1138-1661]	1674[1263-2219]	1432 [1077-1904]	1057 [538-2076]	1108 [572-2145]	0.368

All data represented as Geometric Mean (95%CI), all values are in pg/ml. *p value based on analysis of variance for continuous variables

Table 3.3 Factor analysis of sputum mediators at baseline (n=164 patients)

Mediator	FACTOR 1	FACTOR 2	FACTOR 3	FACTOR 4	FACTOR 5
IL-6R	.965				
IL-8	.956				
TNFRII	.931				
TNFRI	.918				
VEGF	.874				
IL-1β	.821				
CCL4	.780				
CXCL10	.731				
IL-6		.784			
CCL5		.739			
CXCL11		.700			
TNF-α		.614			
CCL2		.580			
CCL13			.805		
CCL11			.698		
CCL3			.653		
IL-13				.905	
IL-5				.798	
CCL26				.667	
IL-10					.760
IL-17					.740
IL-4					.708

Principal Component Analysis. Coefficients with values of 0.5 or above only are included.

Table 3.4 Repeatability of Biological and Clinical Data (ICC: Intraclass Correlation κ)

Mediators	ICC 2 visits n=106	p value	ICC 3 visits n=66	p value
IL-6-R	.332	.026	.492	0.02
IL-8	.508	<0.001	.649	<0.001
TNFRII	.350	.020	.637	<0.001
TNFRI	.332	.027	.654	<0.001
VEGF	.102	.303	.615	<0.001
IL-1β	.475	.001	.462	.004
CCL4	.250	.083	.525	.001
CXCL10	.397	.008	.696	<0.001
IL-6	.484	<0.001	.721	<0.001
CCL5	.457	.001	.690	<0.001
CXCL11	.459	.001	.724	<0.001
TNF-α	.458	.001	.564	<0.001
CCL2	.353	.013	.728	<0.001
CCL13	.384	.009	.454	.004
CCL11	.375	.008	.660	<0.001
CCL3	.386	.008	.483	.002
IL-13	.132	.234	.231	0.104
IL-5	.102	.291	.348	0.02
CCL26	.397	0.005	.564	<0.001
CCL17	.529	<0.001	.716	<0.001
IL-10	.100	.563	.337	.035
IL-17	.223	.107	.166	.21
IL-4	.571	<0.001	.525	<0.001
Eosinophils	.605	<0.001	.620	<0.001
Neutrophils	.310	.041	.491	<0.001
ACQ	.819	<0.001	.858	<0.001
Pre BD FEV_1 %	.958	<0.001	.976	<0.001
Pre BD FEV_1/FVC	.834	<0.001	.866	<0.001
Post BD FEV_1 %	.959	<0.001	.974	<0.001
Post BD FEV_1/FVC	.866	<0.001	.922	<0.001

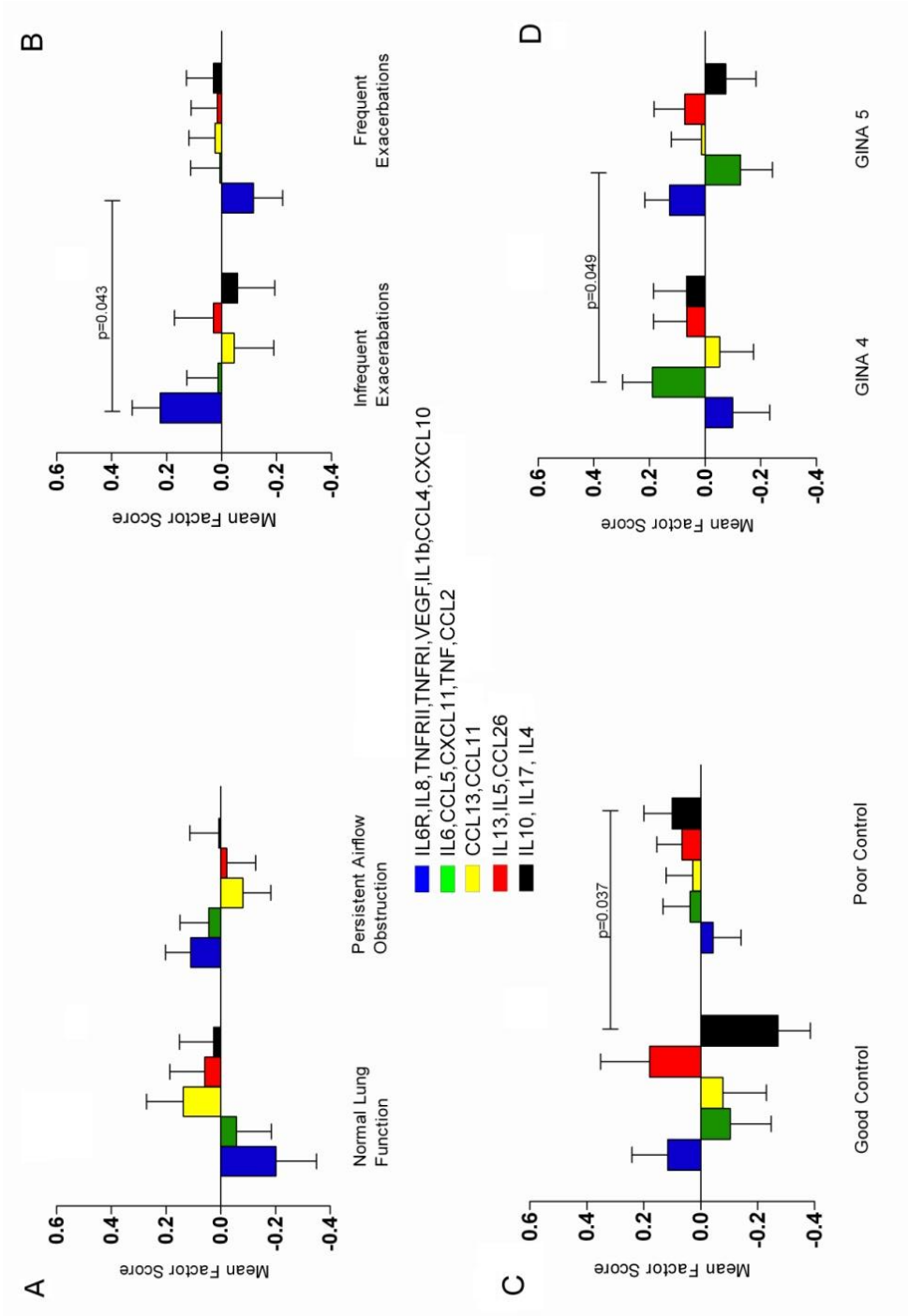


Figure 3.3 Relationship between clinical characteristics and biological factors.

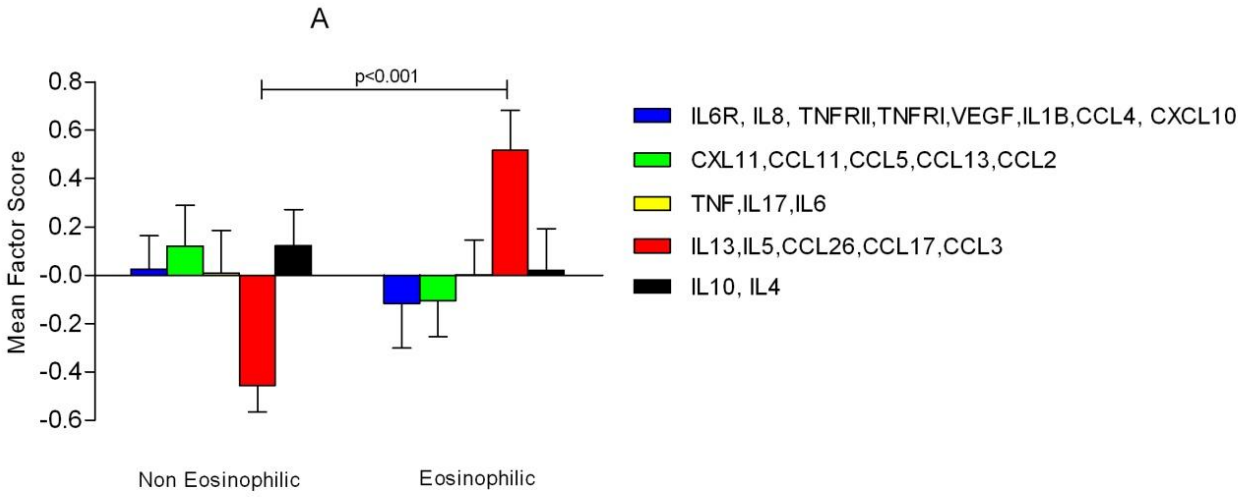
Mean factor score (SEM) between subjects

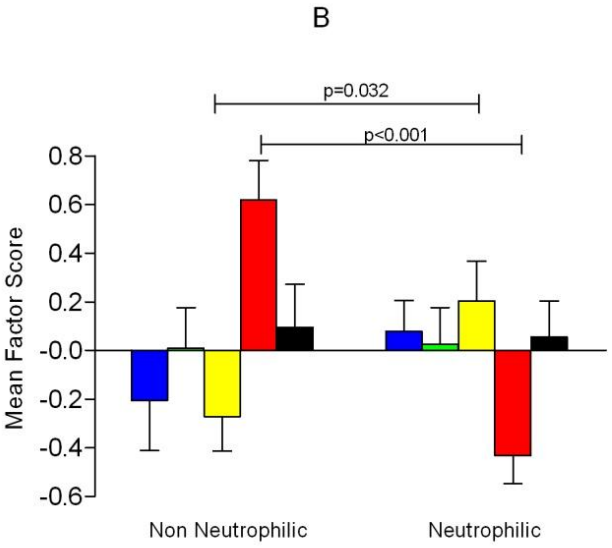
a) with or without persistent airflow obstruction,

b) with infrequent (<2/yr) versus frequent exacerbations (≥3/yr),

c) with and without poor control (ACQ>1.5) or good control (ACQ<1.5)

d) with GINA step 4 or GINA step 5 treatment.


A
p<0.001
Mean Factor Score
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
Non Eosinophilic
Eosinophilic
IL6R, IL8, TNFRII,TNFRI,VEGF,IL1B,CCL4, CXCL10
CXL11,CCL11,CCL5,CCL13,CCL2
TNF,IL17,IL6
IL13,IL5,CCL26,CCL17,CCL3
IL10, IL4


B
p=0.032
p<0.001
Mean Factor Score
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
Non Neutrophilic
Neutrophilic

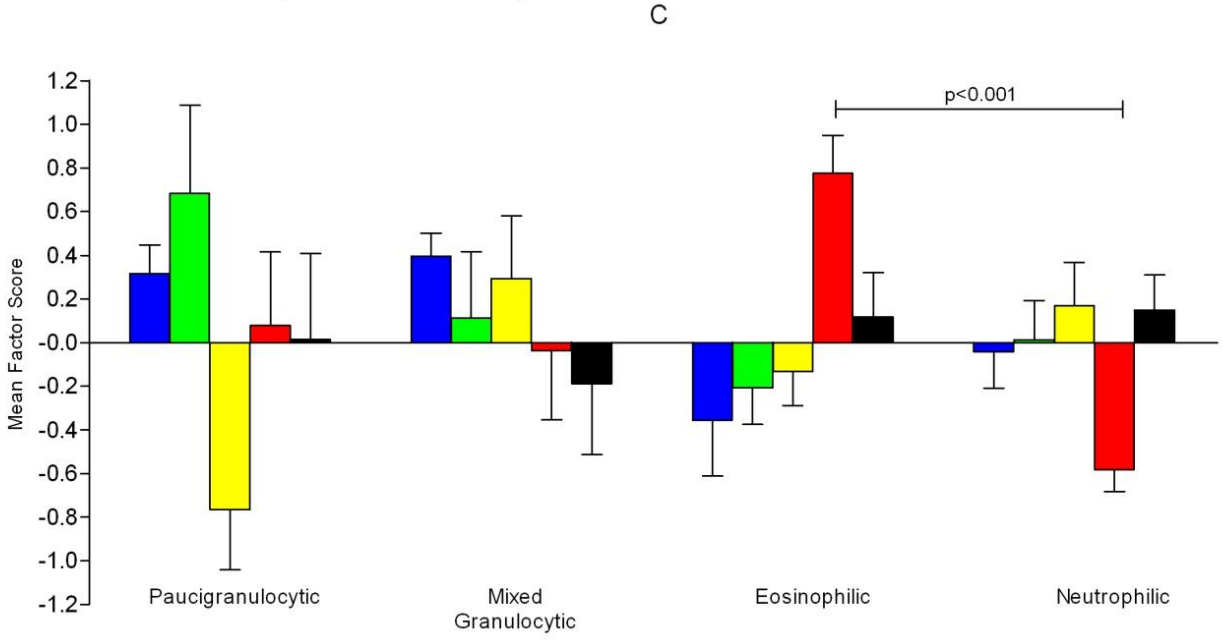

C
p<0.001
Mean Factor Score
1.2
1.0
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
Paucigranulocytic
Mixed Granulocytic
Eosinophilic
Neutrophilic

Figure 3.4 Relationship between sputum inflammation and biological factors 2nd visit
Mean factor score (SEM) between subjects at visit 2 with
a) sputum eosinophilia (≥3%) versus no eosinophilia (<3%),
b) sputum neutrophilia (≥61%) versus no neutrophilia (<61%), and
c) paucigranulocytic (sputum eosinophils <3% and neutrophils <61%), mixed granulocytic (eosinophils ≥3% and neutrophils >61%), eosinophilic (≥3% eosinophils and neutrophils <61%) or neutrophilic (≥61% neutrophils and <3% eosinophils) sputum.

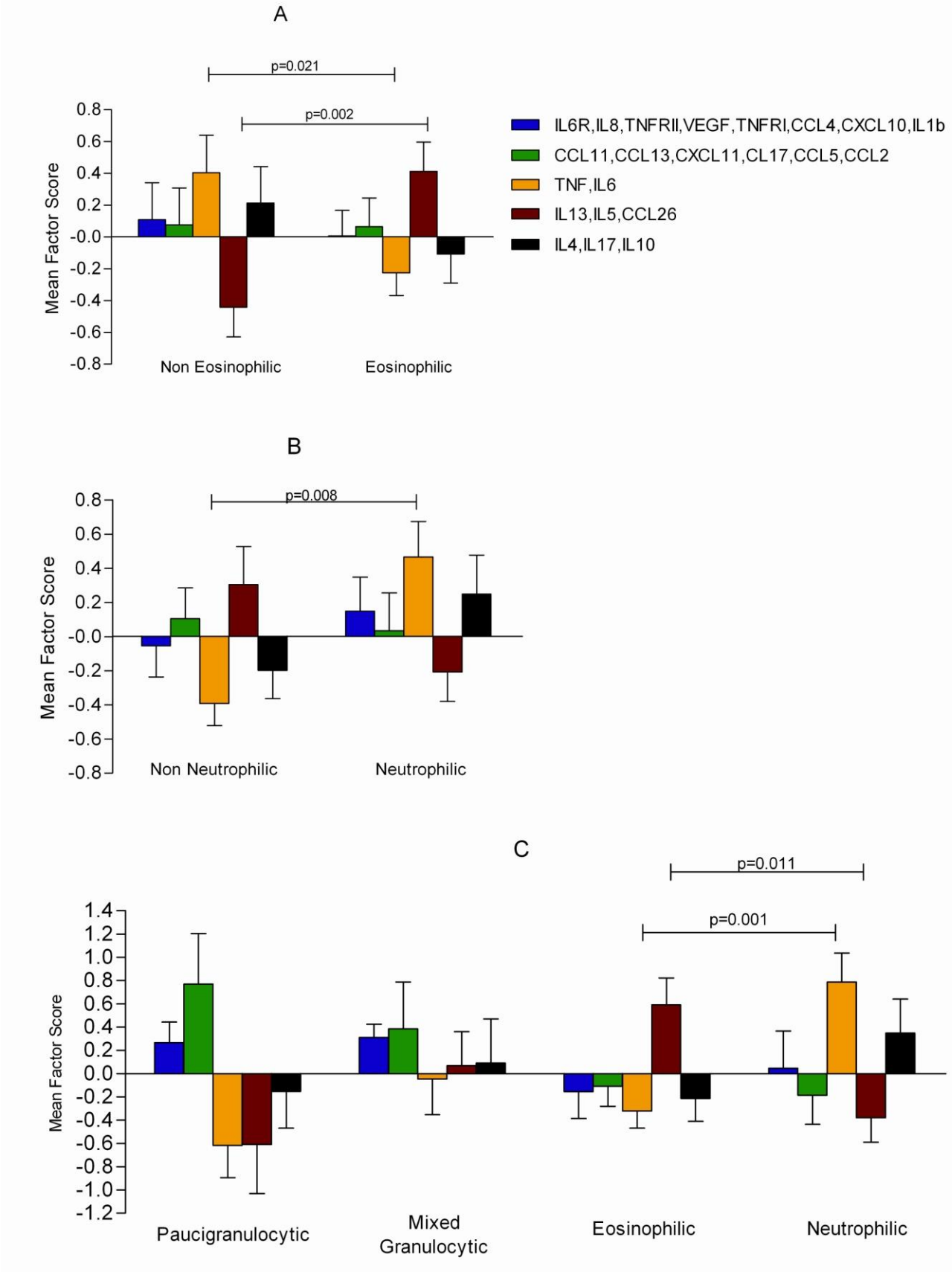

A
p=0.021
p=0.002
Mean Factor Score
0.8
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
Non Eosinophilic
Eosinophilic
IL6R,IL8,TNFRII,VEGF,TNFRI,CCL4,CXCL10,IL1b
CCL11,CCL13,CXCL11,CL17,CCL5,CCL2
TNF,IL6
IL13,IL5,CCL26
IL4,IL17,IL10
B
p=0.008
Mean Factor Score
Non Neutrophilic
Neutrophilic
C
p=0.011
p=0.001
Mean Factor Score
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
Paucigranulocytic
Mixed Granulocytic
Eosinophilic
Neutrophilic

Figure 3.5 Relationship between sputum inflammation and biological factors 3rd visit
Mean factor score (SEM) between subjects at visit 3 with
a) sputum eosinophilia (≥3%) versus no eosinophilia (<3%),
b) sputum neutrophilia (≥61%) versus no neutrophilia (<61%), and
c) paucigranulocytic (sputum eosinophils <3% and neutrophils <61%), mixed granulocytic (eosinophils ≥3% and neutrophils >61%), eosinophilic (≥3% eosinophils and neutrophils <61%) or neutrophilic (≥61% neutrophils and <3% eosinophils)

3.4 DISCUSSION

In this severe asthma population I performed complex phenotyping using cluster and factor analysis; wherein I identified 4 clinical clusters derived from clinical parameters and 5 biological factors derived from mediator data. The factors were differentially expressed between the clinical cluster groups and in subjects stratified by sputum cell counts, asthma control and exacerbation frequency. The within subject repeatability of the mediator profiles was moderate and the association between factors and cellular profiles was consistent suggesting that there are consistent biological processes underpinning the observed sputum cellular profiles. This underscores the importance of the combination of clinical and biological phenotyping in severe asthma over time to further understand the complexity of the disease.

I and others have applied data reduction and clustering techniques to identify severe asthma clusters [Haldar 2008, Moore 2010]. Here I undertook a cluster analysis of the clinical data using the criteria we had previously reported and found that the group was best described with 4 clusters. These clusters were broadly similar to our previous descriptions [Haldar 2008]. However the profiles of the individual mediators in subjects with a sputum eosinophilia or neutrophilia were significantly different. This is analogous with a report associating Th1 and Th2 cytokines with a neutrophilia and eosinophilia respectively [Simpson 2007]. Similarly, cytokine profiles in bronchoalveolar lavage in children were distinct in asthma versus controls [Fitzpatrick 2011] and in adults, differential transcriptional profiles in eosinophilic versus non-eosinophilic asthma have been reported [Baines 2011].

Using factor analysis I derived five biological factors. These factors shared many similarities with those described for chronic obstructive pulmonary disease suggesting that the two conditions may share common immunopathological pathways [Bafadhel 2011]. These biological factors did provide different mean factor scores on each of the severe asthma clinical clusters. Similarly, the mean factor scores were not different when the severe asthmatics were dichotomised based upon the finding of persistent airflow obstruction. However, a significant difference was observed in the mean factor scores in subjects with poor control, frequent exacerbations or those that required oral corticosteroid therapy i.e. GINA 4 versus 5

treatment steps. Interestingly, there was marked differential expression of biological factors in severe asthmatics stratified by their sputum cell counts. This suggests severe asthmatics with a sputum eosinophilia or neutrophilia are associated with distinct mediator profiles.

Taking the example, elevated IL-6 and polymorphisms in IL-6 signalling are associated with lung dysfunction in asthma; it remains unknown if elevated IL-6 levels are associated with a specific cellular inflammatory phenotype. Patients undergoing exacerbations of asthma were phenotyped according to their airway inflammatory characteristics using the Multiplex platform in a recent study [Chu 2015].This observed high levels of IL-6 in the sputum of patients with mixed eosinophilic/neutrophilic airway inflammation and that this was associated with worse pulmonary function similar to our findings. The study also showed patients with isolated neutrophilic bronchitis had elevated levels of sputum IL-1β.

In addition to cross-sectional analyses I was also able to determine the repeatability of the mediator expression across 3 independent visits. The repeatability of the clinical parameters was excellent; sputum differentials were good whereas most individual mediators were moderate. Interestingly, the pattern of the biological factors was highly consistent and was strongly associated with the sputum differential cell count. These data suggest that the cellular and sputum mediator profile track within an individual with good phenotypic stability. Adherence to therapy was not systematically assessed [Gamble 2009] and therefore cannot completely exclude the possibility of an effect on the repeatability of cellular and mediator profiles due to variable treatment usage. The ability to carefully assess the dynamic relationships between the clinical, physiological, cellular and sputum mediator profiles is limited by the number of subjects and moreover the frequency and number of visits and warrants further study. Similarly, the findings would have been strengthened by further increasing the granularity of the assessments to include additional mediators, molecular phenotyping such as genomic, epigenetic and transcriptomic analysis [Woodruff 2009, Siddiqui 2009]. I have not assessed fully the environmental exposure to allergens or pathogens. The study also only includes measures at stable visits and does not include assessments at exacerbations. The integration of data

across these multiple length scales, across time and at times of instability will provide further insights into the phenotypic complexity of severe asthma.
Biological ‘factors’ and clinical ‘clusters’ are not directly comparable. I have shown that the pattern of biological factor expression differs in each clinical cluster, which is exactly what you would expect if it is hypothesised that clinical patterns of disease expression have an underlying biological basis Having characterised patterns of mediator expression in asthma, a reasonable next step would be to include candidate mediators, representative of each biological factor to the clustering process, and see how resulting clusters map to outcome. Biological data is not in competition with clinical data, it is another layer of information that has become available for us to exploit in asthma classification.
The data showed that the presence or absence of sputum eosinophils was closely associated with a single biological factor from a panel of sputum cytokines. As a corollary, the other cytokine factors are therefore providing additional biological information, not obtained by studying sputum cell profiles alone.

The cluster with the obese noneosinophilic patients have relatively low levels of cellular inflammation, yet expressed highest levels of Th-2 cytokines IL-5 and IL-17 and several chemokines derived from eosinophils. The reasons for this effect are unclear, one possible explanation is that the source of mediators is lung adipocytes, or that in this group there is cellular chemotaxis to the lung parenchyma; accounting for low numbers of sputum eosinophils; but the cytokine secretion into airway lumen is not affected. We observed that there is a significant difference in IL-5 levels between subjects with a normal and high BMI. This could not be accounted for by differences in either systemic or inhaled corticosteroid therapy; as treatment level and disease severity was similar across both groups.

In conclusion, I have undertaken a comprehensive analysis of the clinical, cellular and mediator profiles of a group of severe asthmatics. Sputum mediator profiling in severe asthma revealed consistent biological factors that are closely associated with cellular profiles over time; these factors were moderately repeatable within an individual. These data provide evidence that the mediator profiles of severe asthma can be determined and that attempts to satisfy a stratified medicine agenda for

targeted therapy may be improved by the combination of clinical parameters and detailed assessment of sputum mediator profiles over time.

This chapter was an exploratory analysis and led to further work into the understanding of the novel observation of whether IL-5 levels were influenced by obesity and laid the basis for the next chapter.

Chapter 4

Elevated sputum interleukin-5 and submucosal eosinophilia in obese severe asthmatics

4.1 Introduction

The association of obesity and asthma has been evident from epidemiological studies, including case control studies and cross-sectional studies that have shown an increase risk of asthma in obese individuals based on the body mass index (BMI) [Sin 2002, Young 2001, Ford 2005]. Emerging evidence suggests that obesity might represent a distinct severe asthma phenotype [Gibeon 2012] with changes in exhaled nitric oxide metabolism [Holguin 2013], and macrophage function [Fernandez-Bovanapalli 2012, Lugogo 2012]. Its association with eosinophilic inflammation is contentious with some reports suggesting no association with sputum [Bafadhel 2010] or blood cell counts [Gibeon 2012] and others suggesting there is a non-eosinophilic obese phenotype [Haldar 2008].

The relationship between airway inflammation and obesity in severe asthma is poorly understood. I sought to determine the relationship between sputum mediator profiles and the distribution of eosinophilic inflammation and obesity in severe asthmatics.

I hypothesised that sputum mediator profiles and eosinophilic inflammation are differentially expressed between severe asthmatics categorised into normal, overweight and obese subgroups. To test the hypothesis I measured in two independent groups of severe asthmatics stratified by their BMI: i) sputum mediators and ii) eosinophilic inflammation in the peripheral blood, bronchial submucosa and sputum.

4.2 Subjects and Protocol

I recruited three independent subject groups.
a. The severe asthma 'sputum cytokine profiling' group (n=131) had a clinician's diagnosis of asthma requiring treatment step 4 or 5 according to GINA guidelines.
b. A second group, the 'bronchoscopy' group included subjects with severe asthma (n=45) and healthy controls (n=19). These subjects also participated in the macrophage studies.
c. Sputum cytokine data from a group of chronic obstructive pulmonary disease (COPD) patients described previously (n=34) was used as a disease control group or validation cohort [Bafadhel 2009].

The first group (a) consisted of the subjects that were included in chapter 3 and were recruited speicifically for mediator studies. Both the latter two groups of subjects (b) and (c) were participating in other research trials at our centre and were not recruited by me. I expressly obtained permission with regards to the ethics ,data sharing and intellectual property rights with the PIs of the other studies.

4.2.1 Protocol

As per the methods chapter 2. In addition: the 'bronchoscopy' group subjects underwent bronchoscopy in accordance with the British Thoracic Society guidelines within 1 week of their baseline assessment.

Sputum mediator measurements were performed using the MSD platform for 8 mediators that we had previously established to be valid following spiking experiments [Bafadhel 2008]. In half of the bronchoscopy subjects (n=23) eosinophil proteins in sputum macrophages were assessed by quantifying the red hue of sputum macrophages on Romanovski stained cytospins as described previously [Kulkarni 2010].

4.2.2Statistical analysis

Subjects were classified by their BMI (kg/m^2) into lean (<24.9), overweight (≥25-29.9) and obese (≥30). Statistical analysis was then carried out as per methods chapter.

4.3 Results

The patients in 'sputm cytokine profiling group' were a group of 50 (1) year old severe asthmatics with asthma age of onset being 26 (2) years. There were 58% female subjects. The subjects had 3.5 (0.3) severe asthma exacerbations in the previous 12 months prior to their enrolment in the study. They had a high ACQ score of 2.4(0.13) and 53% of the population were prescribed oral corticosteroids. They were also prescribed by a clinician a dose equivalent 1600 µg ranging [1000-2000 µg] Beclomethasone Dipropipoinate inhaled corticosteroid therapy. Adherence to therapy was not specifically assessed and the medication doses described above were based on what was prescribed to a apatient. The group had 45% subjects with atopy. Their lung function demonstrated impairment with 70 (1.9) FEV1% predicted and an obstructive spirometric ratio of 68 (1.2) and demonstrated insignificant 8.8(1.2)% reversibility or improvement in post bronchodilator FEV1.Airway inflammation demonstrated an eosinophilia[3.1 (2.3-4.2)]% in the sputum differential.

When dichotomised into different groups based upon their BMI the clinical characteristics of the 'lean', 'overweight' and 'obese' groups were not significantly different except for a small difference in airflow obstruction, which was less evident in the obese group **(table 4.1)**. The sputum cytokine profiles were similar across the groups except for sputum IL-5 **(table 4.2)** without a significant difference in the sputum eosinophil or neutrophil counts **(table 4.2)**. Sputum interleukin (IL)-5 geometric mean [95% confidence interval] (pg/ml) was elevated in the obese (1.8 [1.2-2.6]) compared to overweight (1.1 [0.8-1.3]; p=0.025) and lean (0.9 [0.6-1.2]; p=0.018) asthmatics (ANOVA across groups p=0.011) **(table 4. 2 and figure 4.1a).** Sputum IL-5 was correlated with BMI (r=0.29, p<0.001; **figure 4.1b**). There was no association between sputum IL-5 and other clinical parameters including age, gender, atopy or medication.

Figure 4.1a

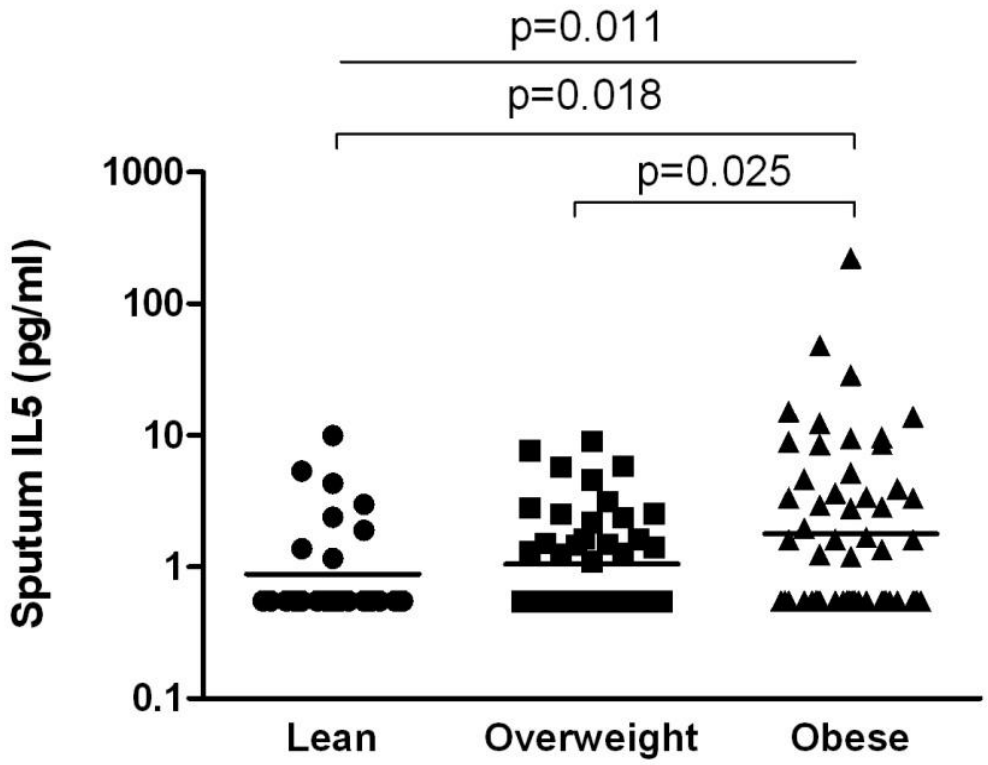


Figure 4.1 b

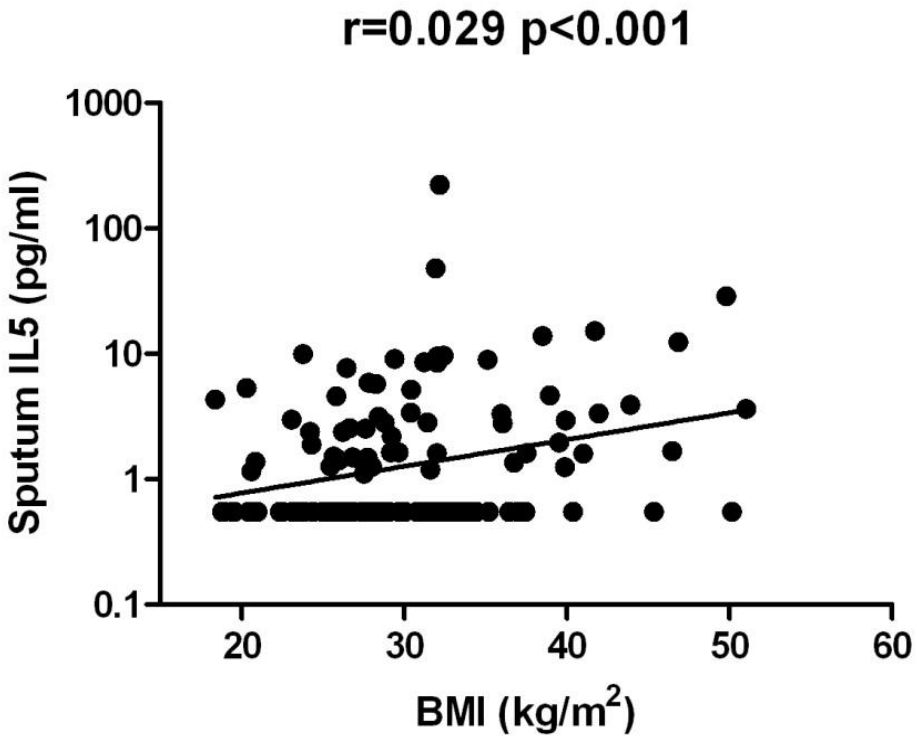


Figure 4.1 Relationship of sputum IL-5 and obesity in severe asthma

a) Subjects are classified by their BMI (kg/m^2) into lean (<24.9), overweight (≥25-29.9) and obese (≥30). The bar represents the geometric mean sputum IL-5 concentration (pg/ ml). P values are for the across groups and pairwise comparisons.

b) Spearmans correlation between sputum IL-5 and BMI.

We then considered whether the elevated sputum IL-5 in the overweight and obese severe asthmatics, in the absence of an increased sputum eosinophil count, might reflect eosinophilic inflammation in another compartment such as the bronchial submucosa. We therefore stratified an independent population of severe asthmatics that underwent bronchoscopy (n=45) into 3 groups based upon their BMI and assessed the burden of eosinophilic inflammation in these groups and healthy controls (n=19) in i) bronchial submucosa, ii) peripheral blood, iii) sputum and iv) red hue of eosin stained sputum macrophages as a marker of eosinophil clearance.

The 'bronchoscopy group' had been previously recruited for a severe asthma biopsy study from the Difficult Asthma Clinic. This group consisted of asthmatics 49(1) years old with a 56% proportion of females. The mean BMI was 29.7 (0.9)kg/m2 and presence of atopy was 45%. The median ICS dose was 1600µg,(range [640-4000] beclomethasone propionate equivalent) and 30% subjects were prescribed oral corticosteroids. The lung function showed impairment with mean 80 (3.1) FEV_1 % predicted and obstructive ratio of 72 (0.3). The sputum inflammation showed a (geometric mean [95%CI) of (4[1.3-17]) % eosinophils.

There were no significant differences between the 'bronchoscopy group' and the cytokine profiling groups where contemporaneous data was compared.

There were no significant differences in the clinical characteristics between the asthma groups **(Table 4.3)**. The median (interquartile range) number of submucosal eosinophils was increased in severe asthma compared to healthy controls as a whole (**Table 4.3**). A representative example of submucosal eosinophil staining in an obese asthmatic is as shown (photomicrograph at end of chapter)

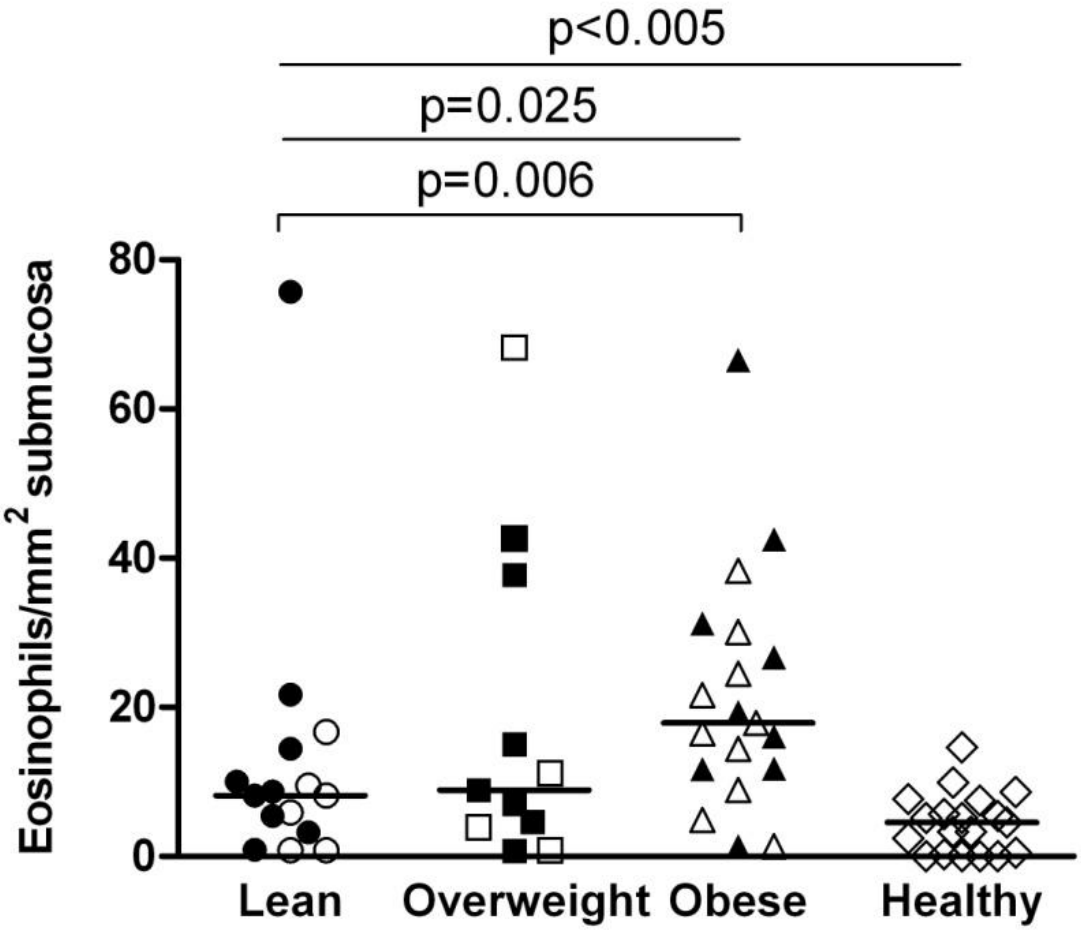


Figure 4.2 Bronchial submucosal eosinophil count in severe asthmatics stratified by BMI Asthmatic subjects are classified by their BMI (kg/m^2) into lean (<24.9), overweight (≥25-29.9) and obese (≥30) and compared with healthy controls. The horizontal bar is the median. Open symbols denote subjects with sputum eosinophilia <3%; closed symbols denote subjects with sputum eosinophils ≥3%. P values for across and between group comparisons are as shown.

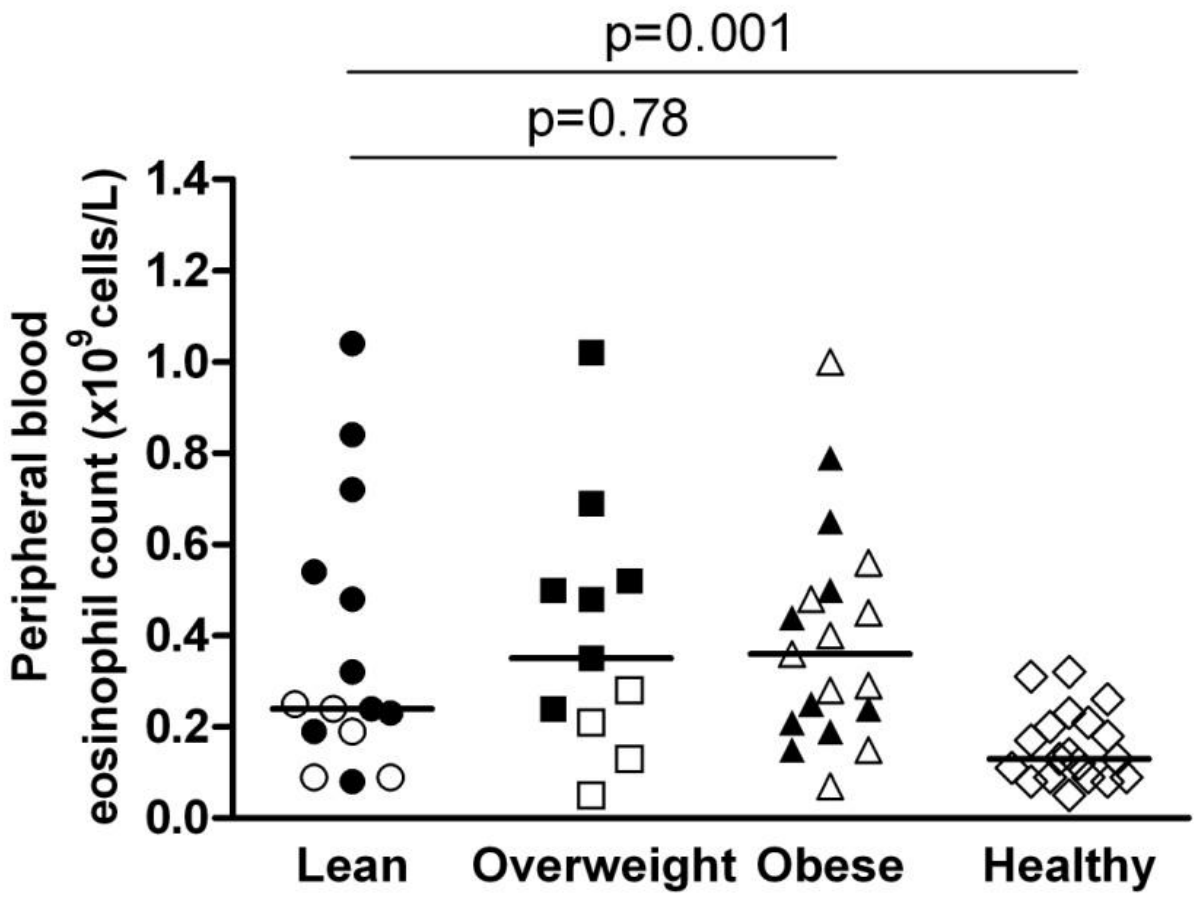


Figure 4.3 Peripheral blood eosinophil count in severe asthmatics stratified by BMI Asthmatic subjects are classified by their BMI (kg/m^2) into lean (<24.9), overweight (≥25-29.9) and obese (≥30) and compared with healthy controls. The horizontal bar is the median. Open symbols denote subjects with sputum eosinophilia <3%; closed symbols denote subjects with sputum eosinophils ≥3%. P values for across and between group comparisons are as shown.

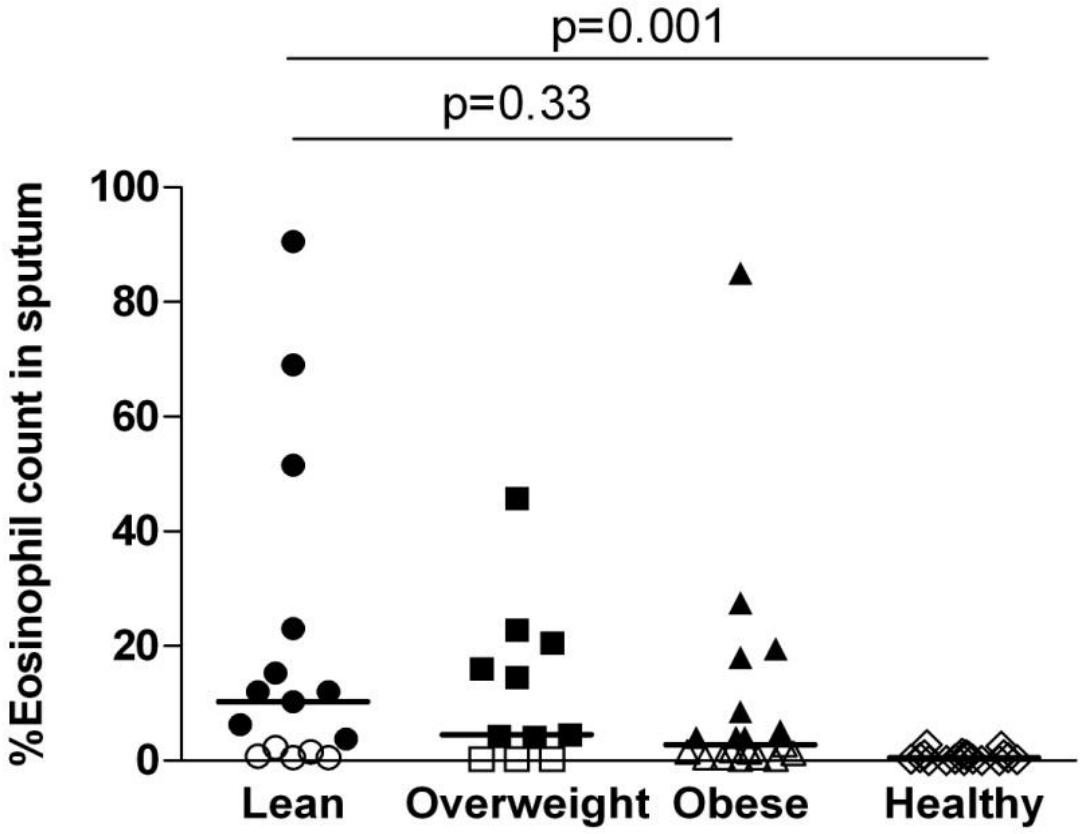


Figure 4.4 Sputum eosinophil count in severe asthmatics stratified by BMI

Asthmatic subjects are classified by their BMI (kg/m^2) into lean (<24.9), overweight (≥25-29.9) and obese (≥30) and compared with healthy controls. The horizontal bar is the median. Open symbols denote subjects with sputum eosinophilia <3%; closed symbols denote subjects with sputum eosinophils ≥3%. P values for across and between group comparisons are as shown.

Submucosal eosinophil number were increased in the obese (19.4 [11.8-31.2]) cells/mm^2 versus lean asthmatics (8.2 [5.4-14.6]) (p=0.006) (**Figure 4.2**). The submucosal eosinophil number in the asthmatic subjects was correlated with BMI (Spearman's rank correlation r_s=0.38, p=0.013; **table 4.4**). Likewise the peripheral blood eosinophil (**Figure 4.3**) count and sputum eosinophil count (**Figure 4.4**) were increased in severe asthma compared to healthy controls as a whole and in all 3 BMI-defined groups, but were not significantly different between the lean, overweight and obese severe asthma groups. BMI was correlated with peripheral blood (r_s=0.32, p=0.040), but not sputum eosinophil count (r_s=-0.17, p=0.30) (**table 4.4**).

Similarly, in COPD the sputum IL-5 concentration was correlated with BMI (r=0.55, p<0.001; (**figure 4.5**) and significantly increased in those overweight and obese subjects compared with those with lean BMI **(figure 4.5).**

A smaller number of patients (n=23) had eosinophilic protein measurement in their airway macrophages and there was no significant difference between groups, although the red hue was lowest in the obese severe asthma group **(figure 4.6).** In the healthy controls there were no significant correlations between the eosinophil number and BMI in any of the compartments (data not shown).

Table 4.1 Characteristics of asthmatic subjects classified by their BMI (kg/m^2)

	All subjects (n=131)	Lean (n=28)	Overweight (n=48)	Obese (n=55)	p value
Current Age	50 (1)	51 (3)	49 (2)	51 (2)	0.63
Male, n (%)	58 (44)	12 (43)	26 (54)	20 (36)	0.19
Age of onset	26 (2)	24 (4)	23 (3)	29 (3)	0.23
BMI, kg/m^2	30 (0.6)	22.0 (0.4)	27.5 (0.2)	36.2 (0.8)	<0.001
Pack year history, current and ex	5.1 (1.3)	7.4 (2.1)	2.9 (1.4)	5.9 (2.7)	0.42
Severe Exacerbations	3.5(0.3)	3.6 (0.5)	3.3 (0.4)	3.6 (0.5)	0.87
ACQ_6 score*	2.4 (0.13)	2.6 (0.26)	2.4 (0.21)	2.3 (0.22)	0.82
Oral corticosteroid use, n (%)	70 (53)	15 (54)	27 (56)	28 (51)	0.75
Daily prednisolone dose, mg	6.0 (0.7)	5.7 (1.2)	6.6 (1.3)	5.6 (1.0)	0.78
ICS dose†	1600 [1000-2000]	1600 [1000-2000]	1600 [1265 - 2000]	2000 [1200-2000]	0.37
Atopy present, n (%)	59 (45)	11 (39)	23 (48)	25 (46)	0.76
Total IgE, IU/L‡	156 [121-201]	168 [84-339]	172 [116-256]	137 [95-200]	0.77
Pre BD FEV_1	2.1 (0.1)	2.2 (0.2)	2.0 (0.1)	2.0 (0.1)	0.54
Pre BD FEV_1 % predicted	70.0 (1.9)	76.2 (4.1)	64.6 (3.3)	71.6 (2.7)	0.06
Pre BD FEV_1/FVC ratio	68.1(1.2)	65.6 (3.4)	64.5 (2.0)	72.3 (1.3)	0.007
BD Reversibility %FEV_1	8.8 (1.2)	7.1(2.5)	11 (2.2)	7.8 (1.8)	0.39
Post BD FEV_1 % predicted	75.3 (1.9)	81.1(4.3)	70.9(3.4)	76.2 (2.7)	0.14
Eosinophils % ‡	3.1 [2.3-4.2]	2.5 [1.3-4.8]	3.0 [1.8-5.0]	3.6 [2.3-5.7]	0.70
Neutrophils % ‡	55.6 [49.2-62.8]	57.0 [44.0-74.0]	58.3 [47.2-72.0]	52.8 [43.4-64.2]	0.77
Total Cell Count (10^6/g sputum) ‡	1.6 [1.2-2.1]	1.2 [0.6-2.5]	1.5 [0.9-2.5]	1.9 [1.4-2.6]	0.47

Data expressed as Mean (SEM) unless otherwise stated. ∗ Asthma Control Questionnaire Score was adjusted to remove effect of FEV_1% predicted domain.
† Doses of all inhaled corticosteroids were converted to the equivalent dose of beclomethasone dipropionate and expressed here as median dose [IQR].
‡Data expressed as Geometric Mean [95%CI]. p value based on analysis of variance for continuous variables.

Table 4.2 Sputum mediator levels in the groups classified by BMI (kg/m^2)

Mediators	All Subjects (n=131)	Lean (n=28)	Overweight (n=48)	Obese (n=55)	p value*
Cytokines/Receptors					
IL-1β	127 [94-171]	128 [83-199]	159 [88-288]	105 [67-165]	0.46
IL-5	1.3 [1.0-1.5]	0.9 [0.6-1.2]	1.1 [0.8-1.3]	1.8 [1.2-2.6]	0.011
IL-6	63 [48-84]	68 [39-120]	65 [39-108]	60 [38-94]	0.93
IL-8	3318 [2280-4830]	4945 [3066-7974]	3658 [2028-6596]	2525 [1260-5063]	0.39
TNF-α	4.0 [2.9-5.3]	3.5 [2.1-6.0]	5.4 [2.9-9.8]	3.2 [2.2-4.8]	0.31
Chemokines					
CCL4	210 [150-293]	215 [109-423]	272 [156-474]	167 [97-289]	0.44
CXCL10	481 [342-677]	610 [302-1234]	481 [278-832]	429 [241-766]	0.75
Growth Factors					
VEGF	1521 [1258 - 1840]	1670 [1086-2568]	1787 [1334-2394]	1272 [929-1740]	0.26

All data represented as Geometric Mean [95%CI], all values are in pg/ml. *p value based on analysis of variance for continuous variables on log normalized data

Table 4.3 Characteristics of bronchial biopsy asthmatic and healthy control subjects. Asthmatic subjects stratified by their BMI

	Healthy Controls (n=19)	All asthma subjects (n=45)	p value	Lean (n=15)	Overweight (n=11)	Obese (n=19)	p value
Current Age	40 (4)	49 (1)	0.012	49(1)	48(1)	50(1)	0.9
Male, n%	11 (57)	20 (44)	0.36	4(26)	7(63)	9 (47)	0.29
BMI, kg/m^2	25.5 (0.9)	29.7 (0.9)	0.014	23.1(0.4)	27.1(0.4)	35.7 (1.1)	<0.001
Pack year history	0	1.5(2)	-	1(0.5)	3(2)	0.5(1.5)	0.44
Oral corticosteroid use, n (%)	-	14 (30)	-	2 (15)	5 (45)	7 (20)	0.28
Daily prednisolone dose, mg	-	11.5 (2)	-	6.5(1.5)	13.5 (4.5)	13 (3)	0.39
ICS dose*	-	1600 [640-4000]	-	1440 [1000-4000]	1600 [1000-4000]	1600 [640-4000]	0.90
Atopy present, n (%)	4 (21)	21(45)	0.06	6 (40)	5 (45)	10 (52)	0.80
Total IgE, IU/L†	-	163 [86-310]	-	185 [80- 427]	203 [31-1306]	128 [53-284]	0.43
Pre BD FEV_1	3.3 (0.2)	2.37 (0.11)	<0.005	2.4 (0.2)	2.4 (0.2)	2.3 (0.2)	0.83
Pre BD FEV_1 % predicted	99.8 (3.6)	80.8 (3.1)	<0.005	87 (5.5)	78 (7.4)	77(4.4)	0.43
Pre BD FEV_1/FVC ratio	79 (0.01)	72(0.3)	<0.005	78 (0.6)	64(0.8)	71 (0.5)	0.37
Total cell count (10^6/g sputum)	1.3[0.8-2.2]	2.3[1.5-3.4]	0.79	2.5[0.8-5.4]	1.6 [0.5-4.4]	2.7[1.6-4.6]	0.60
Sputum eosinophils, %‡	0.5[0-1]	4 [1.35-17]	0.001	10.25 [1.5-23]	4.2 [0.25-20.5]	3 [1.25-8.5]	0.33
Sputum neutrophils, %	56.6[40.7-74.3]	48.1 [37.6-61.5]	0.43	40.5 [27.4-65.1]	51[36.3-71.7]	59.3 [46.3-75.8]	0.26
Eosinophils/mm^2 submucosa‡	4.6 [0.5-7.5]	11.7 [6-25.6]	<0.005	8.2 [5.4-14.6]	8.8 [3.9-37.7]	19.4 [11.8-31.2]	0.025
Peripheral blood eosinophil count ($x10^9$cells/Lt) ‡	0.13 [0.09-0.23]	0.29 [0.2-0.51]	0.001	0.24 [0.19-0.54]	0.35 [0.21-0.52]	0.36 [0.21-0.5]	0.78

Data expressed as Mean (SEM) unless otherwise stated. * Doses of all inhaled corticosteroids were converted to the equivalent dose of beclomethasone dipropionate and expressed here as median dose [range]. †Data expressed as Geometric Mean [95% CI]. ‡Data expressed as median[IQR]

Table 4.4 Spearman correlations of BMI with eosinophils in body compartments in asthma

	Blood	Tissue	Sputum
BMI	r=0.319 (p=0.04)	r=0.380 (p=0.01)	r=-0.174 (p=0.25)
Blood	-	r=0.383 (p=0.01)	r=0.097 (p=0.507)
Tissue	-	-	r=0.077 (p=0.61)

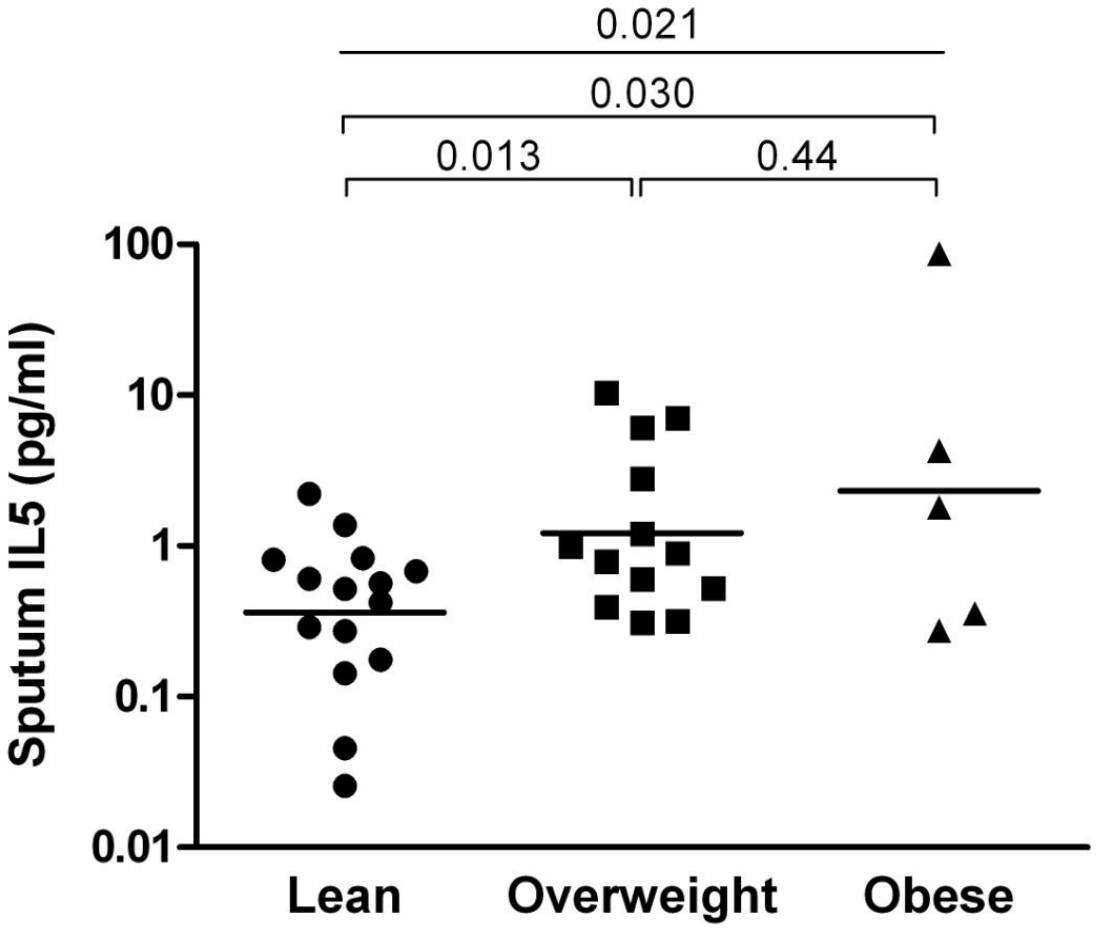


Figure 4.5 Relationship between BMI and sputum IL-5 levels in COPD

Subjects are classified by their BMI (kg/m^2) into lean (<24.9), overweight (≥25-29.9) and obese (≥30). The bar represents the geometric mean sputum IL-5 concentration (pg/ ml). p values are for the across groups and pairwise comparisons. b) Correlation between sputum IL-5 and BMI.

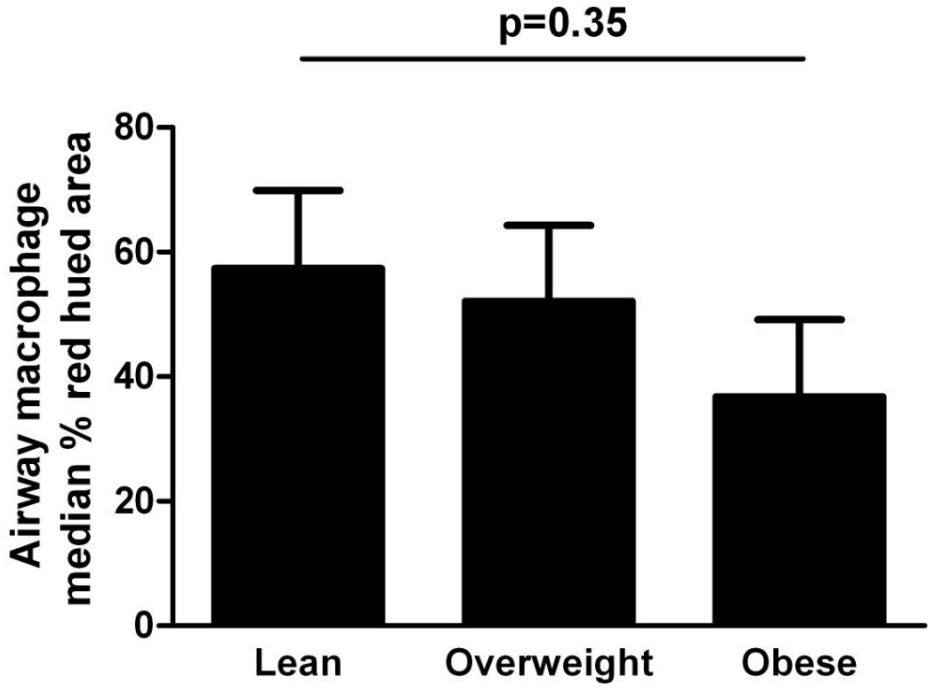


Figure 4.6 Eosinophil phagocytosis by airway macrophages

Eosinophil phagocytosis by airway macrophages determined by median percentage red hue area. Subjects were classified by their BMI (kg/m^2) into three groups, normal, overweight and obese (≤24.9, 25-29.9 and ≥30). p value represents ANOVA for across group comparisons.

4.4 Discussion

Here I have identified for the first time that obesity in severe asthma is associated with an elevated bronchial submucosal eosinophil number, and sputum IL-5 in the absence of an increased sputum eosinophil count. The findings suggest that eosinophilic inflammation may play an important role in a group of obese asthmatics that hitherto have been labelled as non-eosinophilic. This underscores the importance of the combination of clinical and biological phenotyping with the inclusion of inflammatory profiles in different compartments in severe asthma to further understand the complexity of the disease.

In the sputum mediator profiling the most striking observation was the surprising finding that the overweight and obese severe asthmatic groups was paradoxically associated with the highest sputum IL-5 concentration without a significant increase in the sputum eosinophil count. I feel this observation is robust and unlikely to be due to chance as in addition to elevated sputum IL-5 in the obese severe asthmatics we found it was also elevated in a disease control group of obese COPD subjects, notwithstanding the heterogeneity in the sputum IL-5 concentration and relatively small numbers in each subgroup. Interestingly, an association between sputum Th2 cytokines and obesity in asthma has been challenged [Sutherland 2008]. However, in this earlier study sputum IL-5 was increased in obese asthmatics and controls combined compared to those subjects with a lean BMI and demonstrated a non-significant increase in sputum IL-5 in the obese asthmatics versus those with a lean BMI ($p=0.052$) [Sutherland 2008]. Similarly, serum eotaxin levels were correlated with both obesity and weight loss following bariatric surgery [Vasudevan 2006]. In a recent 10-week intervention study of 38 asthmatics that underwent either weight reduction or exercise alone or in combination with weight reduction, subjects in the exercise only arm had a significant reduction in sputum eosinophils [Scott 2013]. Taken together these small studies do begin to suggest a relationship between changes in body habitus and eosinophilic airway inflammation. The findings might appear contrary to the current dogma of an association between obesity in asthma and non-eosinophilic inflammation. However, studies have reported in asthma both a decrease [van Veen 2008, Todd 2007] and no difference in the proportion of

asthmatics that have a sputum eosinophilia [Bafadhel 2010, Todd 2007] between those with and without obesity. Here we again show that a sputum and peripheral blood eosinophil count is not elevated in obese severe asthmatics, but for the first time report an increase in submucosal eosinophils and sputum IL-5 in obese asthmatics. This apparent anomaly between an increased Th2 sputum cytokine profile in the absence of a sputum eosinophilia suggests that either eosinophil function is altered in obesity, such that response to CCR3 chemokines and Th2 cytokines is impaired, or that eosinophils are retained in the airway wall and possibly have an altered survival or adhesion within the airway wall.

To test this hypothesis we enumerated the number of eosinophils in the peripheral blood, bronchial submucosa, sputum and eosinophil uptake by sputum macrophages in an independent group of severe asthmatics. Indeed, the number of submucosal eosinophils was increased in the obese severe asthmatics. This was associated with a trend to a reduction in the eosinophil clearance by macrophages, although this did not reach statistical significance. This increase in tissue, but not luminal airway eosinophilia, in obesity is entirely consistent with animal models. Murine models of asthma have shown that CC chemokines are up-regulated in obesity [Calixto 2010] with increased eosinophilia in lung tissue, but not bronchoalveolar lavage. Whether these tissue eosinophils are activated in obese asthmatics and contribute to disease needs to be further investigated.

Non-eosinophilic asthma defined by sputum cell counts is associated with a poor response to corticosteroids and obesity itself is associated with corticosteroid insensitivity. Therefore, it is recognised that this group respond poorly to inhaled or systemic corticosteroid therapy. This questions the clinical importance of a bronchial submucosal eosinophilia in obese asthma. However, following corticosteroid therapy and specific anti-IL-5 monoclonal therapy submucosal eosinophils are incompletely attenuated [Flood-Page 2003] suggesting that this important immunopathological feature of asthma is refractory to current therapy. Given the presence of airway eosinophilia has convincingly been shown to drive exacerbation frequency further randomised controlled trials are indicated to assess whether the target population for novel eosinophil-targeted therapies is much greater than first anticipated. Thus, it remains a possibility that the tissue eosinophilia in this group is clinically important

and their reduction may translate into meaningful clinical outcomes. Whether alternative strategies to reduce eosinophilic inflammation by targeting the IL-5 receptor [Kolbeck 2010], CRTh2 [Barnes 2012] or important eosinophil-derived cytokines such as IL-13 [Corren 2011, Piper 2013] have efficacy in obese asthmatics needs to be tested.

Our findings also underscore the importance of obesity as a driver for the production of numerous pro-inflammatory cytokines and suggest that obesity may contribute to the inflammatory burden in asthma as well as causing increased work of breathing, as a consequence of extra-thoracic restriction. Therefore, strategies targeted at diet and lifestyle to reduce obesity including bariatric surgery may indeed have an anti-inflammatory role beyond simply weight reduction and may contribute to the benefits in lung function observed following successful weight reduction [Haselkorn 2009, Boulet 2012]

4.4.1 Asthma and obesity are both proinflammatory conditions

Asthma and obesity are conditions that are mostly centred in westernised populations with the overall worldwide prevalence increasing steadily. The risk of asthma is increased nearly twofold in patients that are overweight and obese. There are several studies that have identified obese asthma as a distinct entity and it is the combination of two disorders which results in a condition with multiple clinical and pathobiological problems and behaves differently to the Th2 driven asthma model. These patients are consequently more difficult to treat with inhaled steroids and are in need of alternative therapies. There is come consensus that the obese asthmatic should be recognised as a distinct phenotype, and evalution of these patients needs to be tailored differently. This may be by measuring BMI longitudinally and by using non invasive techniques or biomarkers to guide disease activity and treatment response.

There are studies showing that obesity may actually precede the onset of asthma and suggesting that obesity has a causal role in asthma with it being a clear risk factor when other factors are accounted for- and this is in a stepwise fashion, with obese patients at most risk than overweight. A previous report that this association is

higher in females remains subject to debate. [Sutherland 2008, Ronmark 2005]. Obesity is associated with both childhood and adult onset severe asthma, but the impact of obesity may differ by age at onset and degree of allergic inflammation. In addition to the effects of allergic inflammation, both cellular mediators of immunity and pro inflammatory signalling molecules are afflicted in obesity leading to a state of chronic, low-grade systemic inflammation. The macrophage-lymphocyte population residing within adipose tissue are responsible for secreting large variety of inflammatory molecules such as TNFα, IL-6, PAI-1, and MCP-1 and complement [Weisberg 2003]. Adipocytes themselves also produce a number of hormones, including leptin and adiponectin which are opposite in action to each other. Leptin is homologous in structure to IL-6 and though it binds specifically to a membrane bound receptor, the receptor is similarly homologous to IL-6 receptor. Leptin can induce proliferation of naive T cells and modulate cytokine production toward production of Th1 cytokines.

Murine models showed reduced eosinophilic inflammation in the obese mice [Johnston 2007]. In human asthma studies there are varying findings in subjects stratified by BMI and asthma severity. One study showed that airway inflammation was of paucigranulocytic type [Sutherland 2008] whilst another showed that obese subjects had no difference in total or differential counts compared with non obese subjects adjusting for both presence and absence of asthma and treatment effect. This led to the conclusion there was no association between BMI and airway inflammation measured by sputum cell counts [Todd 2007]. Other studies showed a negative correlation between BMI and sputum cell counts [van Veen 2008, Lessard 2008]. Finally, cluster analysis has shown differing results with two studies identifying obese asthmatics with predominantly noneosinophilic inflammation. However, a more recent analysis identifies clusters of obese patients with high level of BAL and peripheral eosinophilia. [Wenzel 2013 JACI]. This suggests that inflammatory pathways must behave differently in the presence of obesity.

It must also be stressed that the absence of intraluminal airway inflammation does not imply a total lack of inflammation in other compartments or at a genomic level. A gene expression study in obese asthmatics identified several genes associated with systemic inflammation and obesity correlated with BMI. One of these genes

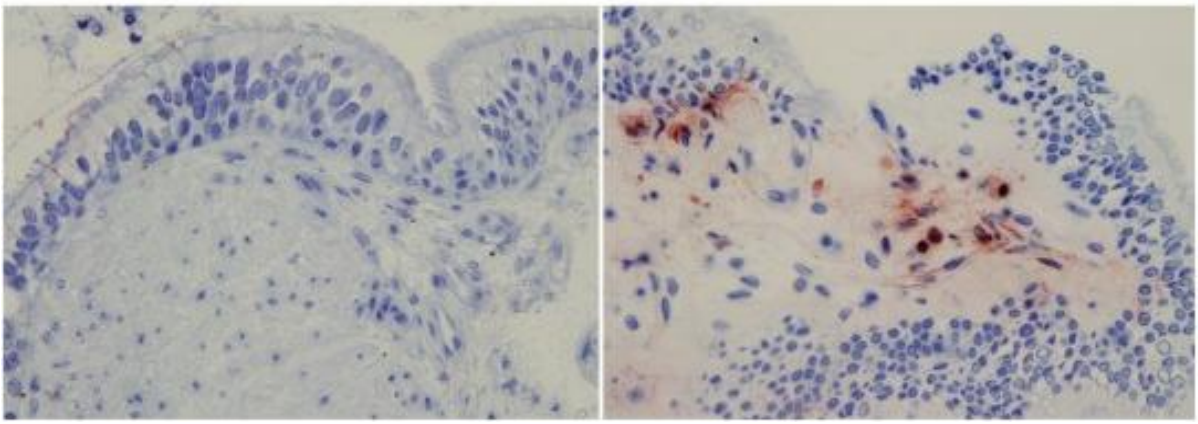
L-selectin is linked to neutrophilic airway inflammation and was upregulated; another gene TFF3 associated with epithelial integrity was down-regulated suggesting increased risk of epithelial injury [Fu 2013].

It is therefore important we identify the subset of severe asthmatics with obesity that have concomitant inflammation that may not be expressed in the airway or measurable by sputum or peripheral counts. These patients may be classified as having non inflammatory asthma which may lead to reduction of inhaled steroids leading to potentially worse outcomes. In the future therapy will also need to incorporate weight loss, dietary modification regimes and only the advent of large scale studies will prove if the above interventions result in meaningful change to both asthma and obesity.

Our study has a number of potential limitations. We have focussed upon severe asthma as this group represents the patients with the greatest unmet need. Whether the findings are consistent across disease severity needs to be studied. Adherence to therapy was not systematically assessed [Gamble 2009] and therefore we cannot completely exclude the possibility that some of the differences observed between the groups stratified by BMI are in part due to differences in adherence to therapy. We have not assessed fully the environmental exposure to allergens, occupational sensitizers and irritants, pollutants or pathogens. Our study also only includes measures at stable visits and does not include assessments at exacerbations. The integration of transcriptomic, proteomic and cellular data, longitudinally and at times of instability will provide further insights into the phenotypic complexity of severe asthma. The specificity of BMI as a measure of fat free mass has been questioned, but we have previously reported that BMI and fat free mass index are very closely related in severe asthma [Bafadhel 2010]. Recent studies have suggested that a fatty diet might promote neutrophilic inflammation [Scott 2011]. The subjects' diets were not formally assessed in this study and future studies should relate body habitus and diet to airway inflammation.

In conclusion, we have undertaken a comprehensive analysis of the clinical, cellular, endobronchial and sputum mediator profiles of severe asthma. My most important observation was that in the absence of a sputum eosinophilia obese severe

asthmatics have elevated sputum IL-5 and eosinophils in their airway wall. This has important implications in our understanding of the impact of obesity in the immunopathology of asthma. It questions our current view on the choice of current and future biological therapy in this group; highlights the importance of strategies to improve diet and lifestyle that may provide benefits in terms of weight reduction, reduced risk of co-morbidities and as suggested here eosinophilic airway inflammation.



Photomicrograph of a bronchial biopsy from an obese severe asthmatic showing isotype control (left panel) and major basic protein stained eosinophils (right panel) (x200)

Chapter 5

Sputum mediator profiling and relationship to airway wall geometry imaging in severe asthma

5.1 Introduction

Understanding the underlying pathobiology of severe asthma phenotypes mainly those with Th2 high [Woodruff 2009] or eosinophilic airway inflammation [Haldar 2008] has translated into recent success with anti IL-5 [Haldar 2009] and anti IL-13 therapy [Corren 2011, Piper 2012]. In particular, anti-IL-5 led to improvements in exacerbation frequency and airway remodelling in terms of sub-epithelial matrix deposition and airway wall geometry determined by CT analysis [Haldar 2009]. Understanding the relationship between the inflammatory profile in the airway and airway geometry may help to identify patient phenotypes that may be more amenable to strategies to modulate airway remodelling and might prioritise potential targets within stratified populations for specific highly targeted therapies.

We and others have used quantitative and qualitative CT image analysis to define the airway geometry in asthma [Gupta 2009, Gupta 2010, Castro 2011]. In a previous report, we found that airway wall remodelling defined by CT was most closely associated with neutrophilic inflammation and airflow obstruction but was poorly associated with patient reported outcomes [Gupta 2010]. This association between persistent neutrophilic inflammation and lung function decline is a consistent observation in asthma and COPD [Stanescu 1996, Shaw 2007]. Sputum and bronchoalveolar lavage cytokine profiling by analyses of protein and transcriptomics in asthma and chronic obstructive pulmonary disease has demonstrated that patterns of inflammatory mediators are associated with cellular profiles and clinical outcomes [Baines 2011, Hastie 2010, Bafadhel 2012]. I hypothesised that there is differential sputum mediator profiles in severe asthmatics stratified by airway geometry determined by CT analysis. To test our hypothesis we measured sputum mediators in patients that had undergone CT analysis and i)

dichotomised subjects by the median right upper lobe apical bronchus (RB1) luminal area or the median RB1 total area and ii) stratified by the median sputum neutrophil count and airway geometry.

5.2 Subjects and Protocol

59 subjects out of the 92 included in a previous quantitative CT analysis [Gupta 2009] between 2004 to 2008 were included in this study if they had adequate sputum samples for mediator analysis (>150µL supernatant volume). Sputum was obtained at the routine clinical visit immediately before or after the CT scan (+/-30 days). All assessments were undertaken at stable visits at least 8 weeks after an exacerbation.

Please see chapter 2 for details on imaging techniques and protocols

5.2.1 Statistical Analysis

Subjects were dichotomised into high and low luminal area, total area and wall area using the median values as the cut-offs (RB1 luminal area LA/ BSA (≤5 mm^2/m^2 versus >5 mm^2/m^2) or total area TA/BSA (≤16 mm^2/m^2 versus >16 mm^2/m^2) or wall area WA/BSA (≤11.5 mm^2/m^2 versus >11.5 mm^2/m^2). Spearman correlation coefficient was used to determine the relationship between RB1 dimensions (LA/BSA, WA/BSA, TA/BSA and %WA) and clinical indices and sputum mediator data.

Our previous study highlighted the relationship between CT-determined remodelling and neutrophilic inflammation [Gupta 2010]. Therefore, we further stratified the population into four groups using the medians as cut-offs for the differential sputum neutrophil counts (≤66% versus >66%) and the RB1 total area or luminal area. Comparisons across groups were made by Kruskal-Wallis test with post hoc Dunn's pair-wise comparisons.

5.3 Results

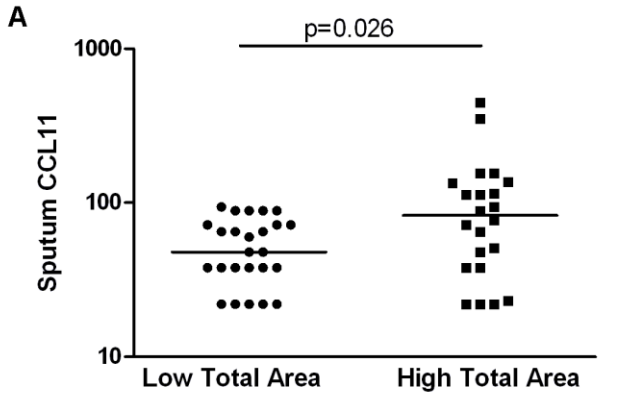
The clinical and CT imaging characteristics and sputum mediator concentrations for the whole population and dichotomised by the median RB1 total and luminal areas are as shown (**Table 5.1 and 5.2**). Subjects with a low total area and luminal area were older with a later age of onset of disease, higher body mass index, more bronchiectasis and more frequent exacerbations (**Table 5.1**). There was marked heterogeneity in mediator concentrations within and between the groups of subjects and only the sputum CCL11 concentration was significantly different in the groups stratified by median RB1 total area ([48.3 versus 76.3 pg/ml], p=0.026). Further differences were observed between subjects stratified by RB1 luminal area (**Figure 5.1, Table 5. 2**) for sputum CCL11 ([49.1 versus 74.9 pg/ml], p=0.043), IL-1α ([38 versus 19 pg/ml], p=0.043) and CCL4 ([349 versus 611 pg/ml] p=0.036). RB1 luminal area correlated with sputum CCL11 (r=0.31, p=0.03) but there were no significant correlations between RB1 total or luminal area and other mediators. There were no differences in cytokine concentrations between those with high or low wall area.

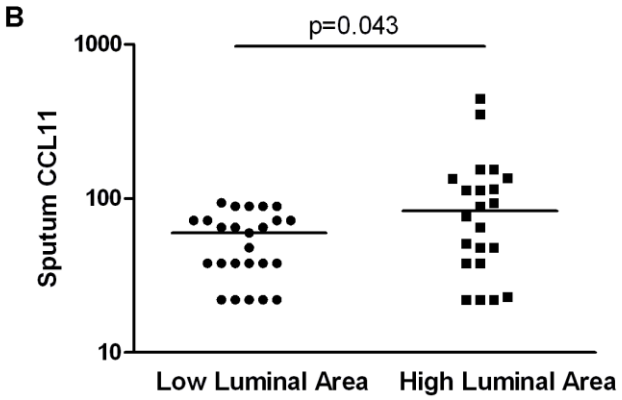
We further stratified the population by the sputum neutrophil count as we had previously reported relationships between CT parameters and neutrophilic inflammation. The clinical and CT imaging characteristics and sputum mediator concentrations for subjects stratified by differential sputum neutrophil count and the median RB1 total and luminal areas are as shown (**Table 5.3-5.6**). Subjects in the high neutrophil, high RB1 total or luminal area groups had significantly the highest number of severe exacerbations in the previous year and the highest proportion of subjects with qualitative CT evidence of bronchiectasis. Those in the low neutrophil and high RB1 total or luminal area groups had significantly the lowest body mass index (BMI). No other significant differences in clinical characteristics were observed (**Tables 5.3 and 5.5**).

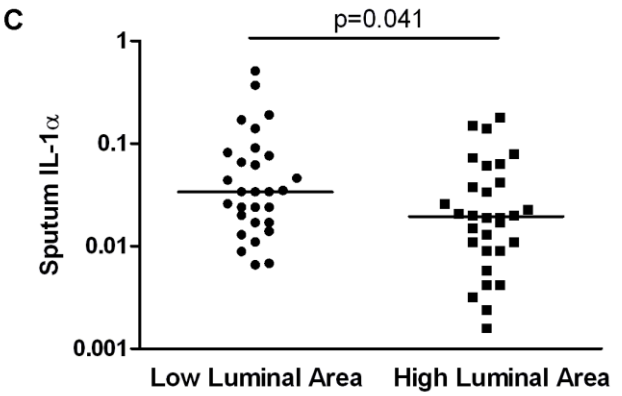
The patterns in sputum mediator concentrations were very different between these groups. The sputum CCL11 concentration was elevated in those with a low neutrophil count and either a high RB1 total (p=0.005) or luminal area (p=0.004)

(**Figures 5.2 and 5.3**). Reciprocally, these were the groups with the lowest sputum fibrinogen concentrations (p=0.002 and p=0.013 respectively). Similarly, sputum MMP9 was decreased in the low neutrophil group with high RB1 luminal area. Sputum ICAM was elevated in the groups with a low neutrophil count in both those with high or low RB1 total or luminal area whereas in contrast sputum IL-1β was elevated in those with a high neutrophil count irrespective of airway geometry. Sputum IL-1α was markedly increased in those with a high neutrophil count and a low RB1 total area (p<0.001) (**Figure 5.2**).

Similar differences between groups were observed if patients were stratified by sputum eosinophil count (≤3% versus >3%) and median RB1 total and luminal areas with sputum CCL11 concentration greatest in those subjects with elevated sputum eosinophil count and high RB1 total or lumen area and in the same group the lowest sputum fibrinogen, and IL-1β concentrations (data not shown).


A
p=0.026
1000
100
10
Sputum CCL11
Low Total Area
High Total Area


B
p=0.043
1000
100
10
Sputum CCL11
Low Luminal Area
High Luminal Area


C
p=0.041
1
0.1
0.01
0.001
Sputum IL-1α
Low Luminal Area
High Luminal Area

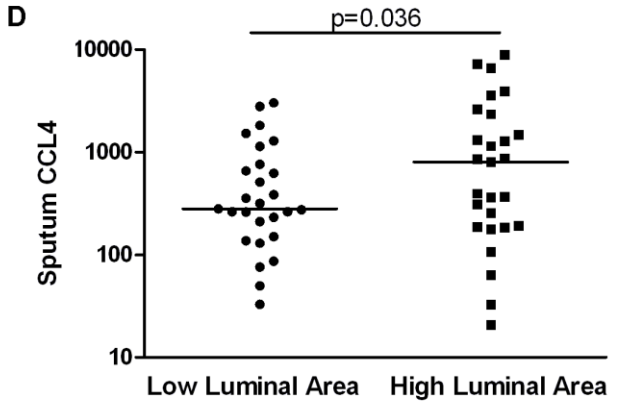
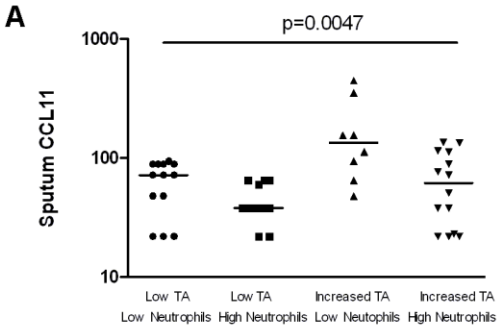
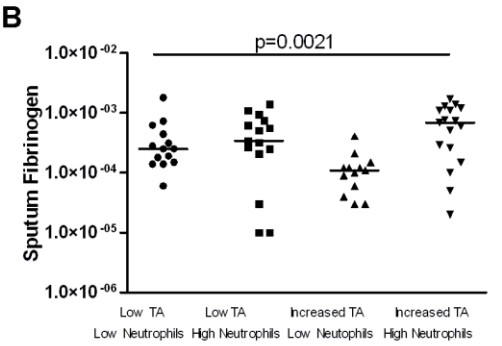
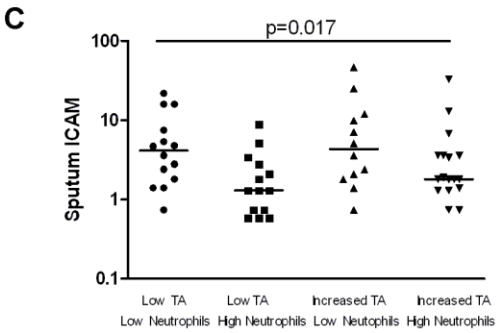
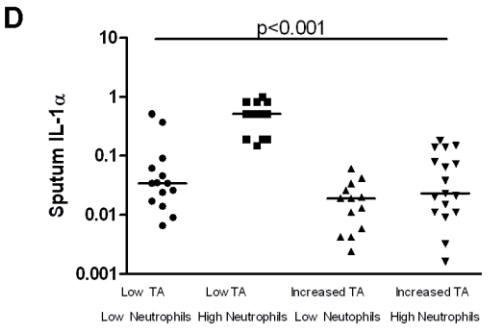

D
p=0.036
10000
1000
100
10
Sputum CCL4
Low Luminal Area
High Luminal Area

Figure 5.1. a) Subjects stratified by low or high RB1 total area on the X axis, their corresponding sputum CCL11 levels (pg/ml) on the log transformed Y axis. Bar represents median value, p value by Mann Whitney U test, and subjects stratified by low or high RB1 luminal area on the X axis, their corresponding b) sputum CCL11 (pg/ml), c) sputum IL-1α (pg/ml) and d) sputum CCL4 (pg/ ml) on the log transformed Y axis. Bar represents median value, p value by Mann Whitney U test.


A
p=0.0047
Sputum CCL11
1000
100
10
Low TA Low Neutrophils
Low TA High Neutrophils
Increased TA Low Neutophils
Increased TA High Neutrophils


B
p=0.0021
Sputum Fibrinogen
1.0×10-02
1.0×10-03
1.0×10-04
1.0×10-05
1.0×10-06
Low TA Low Neutrophils
Low TA High Neutrophils
Increased TA Low Neutophils
Increased TA High Neutrophils


C
p=0.017
Sputum ICAM
100
10
1
0.1
Low TA Low Neutrophils
Low TA High Neutrophils
Increased TA Low Neutophils
Increased TA High Neutrophils


D
p<0.001
Sputum IL-1α
10
1
0.1
0.01
0.001
Low TA Low Neutrophils
Low TA High Neutrophils
Increased TA Low Neutophils
Increased TA High Neutrophils

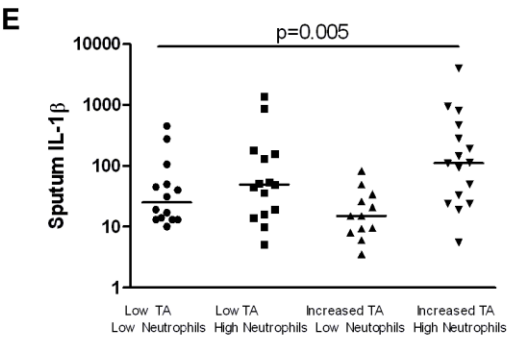
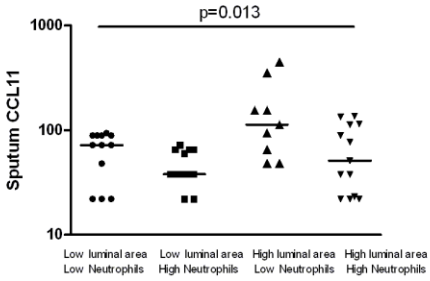
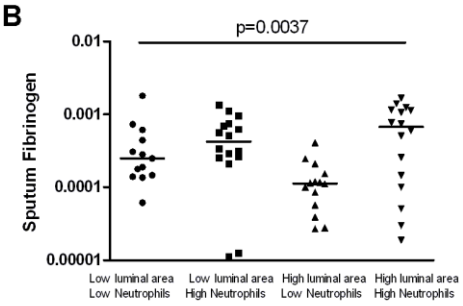

E
p=0.005
Sputum IL-1β
10000
1000
100
10
1
Low TA Low Neutrophils
Low TA High Neutrophils
Increased TA Low Neutophils
Increased TA High Neutrophils

Figure 5.2 Subjects stratified into four groups using the median sputum neutrophil count (≤66% versus >66%) and the RB1 total area (≤16 mm^2/m^2 versus >16 mm^2/m^2) on the X axis, their corresponding sputum levels of a) CCL11 (pg/ml), b) fibrinogen (ng/ml), c) ICAM (pg/ml) d) IL-1α (pg/ml) and e) IL-1β (pg/ml) on the log transformed Y axis. Bar represents median value, p value by Kruskal Wallis ANOVA

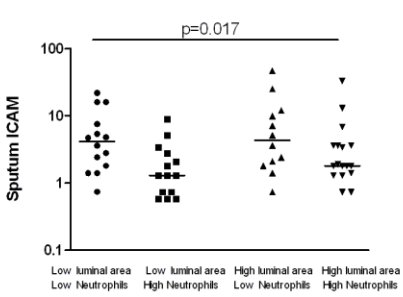
A


p=0.013
Sputum CCL11
1000
100
10
Low luminal area Low Neutrophils
Low luminal area High Neutrophils
High luminal area Low Neutrophils
High luminal area High Neutrophils

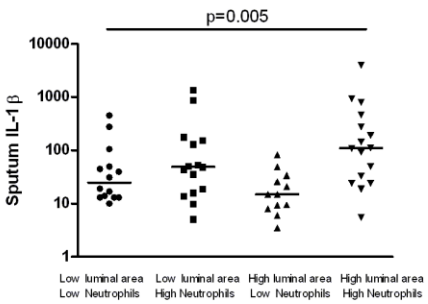
B


p=0.0037
Sputum Fibrinogen
0.01
0.001
0.0001
0.00001
Low luminal area Low Neutrophils
Low luminal area High Neutrophils
High luminal area Low Neutrophils
High luminal area High Neutrophils

C


p=0.017
Sputum ICAM
100
10
1
0.1
Low luminal area Low Neutrophils
Low luminal area High Neutrophils
High luminal area Low Neutrophils
High luminal area High Neutrophils

D


p=0.005
Sputum IL-1 β
10000
1000
100
10
1
Low luminal area Low Neutrophils
Low luminal area High Neutrophils
High luminal area Low Neutrophils
High luminal area High Neutrophils

E

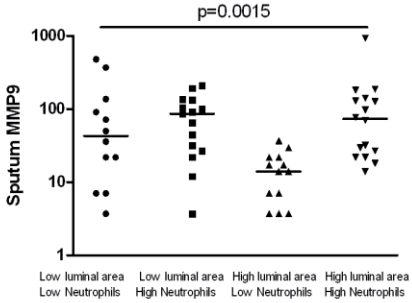

p=0.0015
Sputum MMP9
1000
100
10
1
Low luminal area Low Neutrophils
Low luminal area High Neutrophils
High luminal area Low Neutrophils
High luminal area High Neutrophils

Figure 5.3. Subjects stratified into four groups using the median sputum neutrophil count (≤66% versus >66%) and the RB1 luminal area (≤ 5 mm^2/m^2 versus >5 mm^2/m^2) on the X axis, their corresponding sputum levels of a) CCL11 (pg/ml), b) fibrinogen (ng/ml), c) ICAM (pg/ml) d) IL-1α (pg/ml) and e) MMP-9 (ng/ml) on the log transformed Y axis. Bar represents median value, p value by Kruskal Wallis ANOVA

Table 5.1. Clinical features of subjects stratified by the median RB1 total area and luminal area

	Total	Low total area	High total area	p value	Low luminal area	High luminal area	p value
N	59	30	29		30	29	
Male, n	25	12	13	0.37	12	13	0.37
Current age	49(2)	53 (2)	45 (2)	**0.02**	53 (2)	45(2)	**0.024**
Age of onset	28(2.5)	34 (3.5)	21 (3)	**0.037**	32(3.5)	22(3)	**0.041**
BMI, kg/m^2	29.8(0.8)	32.3 (1.2)	27.4(1.1)	**0.003**	32.6 (1.1)	27.1(1.1)	**0.001**
Habitual smokers and ex smokers with >10 py	13	10	3	0.16	9	4	0.3
Pack years history	8(2.5)	11(5)	6 (2.5)	0.36	11 (5)	6 (2.5)	0.23
Severe Exacerbations	2	2 (0.3)	3 (0.4)	**0.03**	2 (0.3)	3 (0.4)	**0.03**
IgE	153 [97-241]	145[74-183]	162[84-313]	0.87	143[82-325]	167[76-270]	0.89
ACQ_6	2.18(0.13)	2.2 (0.17)	2.14(0.23)	0.74	2.21 (0.17)	2.15 (0.23)	0.76
Inhaled Corticosteroid dose	2000[400-4000]	2000 [400-4000]	2000 [640-4000]	0.28	2000[400-4000]	2000[640-4000]	0.43
Subjects taking oral corticosteroids	25	15	10	0.72	15	10	0.72
Daily prednisolone dose, mg	4.5(0.7)	6(1.2)	2.5(0.7)	0.54	5.5(1.5)	3.5(0.8)	0.83
Pre Bronchodilator FEV_1	2.26(0.12)	2.24 (0.17)	2.2 (0.16)	0.9	2.18 (0.17)	2.34(0.15)	0.49
Pre Bronchodilator FEV_1 % predicted	2.42(0.12)	2.4 (0.18)	2.4 (0.16)	0.98	2.33 (0.18)	2.5 (0.15)	0.48
BD reversibility, ml	0.23(0.07)	0.31 (0.14)	0.13 (0.03)	0.23	0.29 (0.13)	0.15 (0.03)	0.36
Pre Bronchodilator FVC	3.26(0.1)	3.22 (0.21)	3.29 (0.20)	0.87	3.1 (0.22)	3.3 (0.18)	0.39
Pre Bronchodilator FVC	3.35(0.1)	3.36 (0.21)	3.32 (0.20)	0.88	3.27 (0.21)	3.42 (0.18)	0.61
Pre Bronchodilator FEV_1 % of predicted	75.2(3)	76 (4)	74.3 (3.6)	0.79	74.3 (5)	76 (3.4)	0.77
Post Bronchodilator FEV_1% of predicted	80.8(2.9)	80.8 (4)	80.8 (3.4)	0.99	78.4 (5)	83.1(3.2)	0.42
Bronchodilator Reversibility %FEV1	5.9(0.97)	5.4 (1.4)	6.4 (1.7)	0.58	4.7 (0.8)	7.1 (1.7)	0.22
Pre Bronchodilator FEV_1/FVC ratio	69.5(1.8)	69 (2.6)	69.3 (2.7)	0.82	69.6 (2.4	69.4 (2.5)	0.95
Post Bronchodilator FEV_1/FVC ratio	72.1(1.7)	78 (2.5)	72.8 (2.5)	0.62	70.7 (2.4)	73.4 (2.4)	0.44
Total Cells	0.5[0.4-0.8]	0.6[0.3-1.1]	0.5[0.3-0.8]	0.61	0.6[0.3-1]	0.5[0.3-0.8]	0.77
Neutrophils %	58.3[48-69.5]	55.6[40.7-76.3]	60 [50.1-72.6]	0.97	56.2 [47-77]	59.9[49.5-72.5]	0.76
Eosinophils %	2.2[1.5-3.4]	2.5[1.3-4.5]	2 [1.1-3.7]	0.43	2.2[1.1-4.2]	2.2[1.2-4]	0.80
Bronchiectasis present,%	23 (39)	7(30)	16(69)	**0.02**	9(39)	14(60)	0.16
Bronchial Wall Thickening present,%	39 (67)	21(53)	18(46)	0.66	22(56)	17(43)	0.22
Lumen area/Body Surface Area (mm^2/m^2)	5.3(0.3)	3.4 (0.2)	7.2 (0.42)	**0.001**	3.2 (0.2)	7.3 (0.4)	**0.001**
Total area/Body Surface Area (mm^2/m^2)	17.8(0.8)	12.7 (0.6)	22.8 (1)	**0.001**	13.2 (0.8)	22.3 (0.9)	**0.001**

Table 5.2. Sputum mediator concentrations for subjects stratified by the median RB1 total area and luminal area

	Total	Low total area	High total area	p	Low luminal area	High luminal area	p
Factor VII*	2.15[1.89-2.43]	2.03[1.69-2.44]	2.27[1.89-2.72]	0.40	2.01[1.68-2.41]	2.30[1.91-2.77]	0.25
Fibrinogen*	240[172-335]	260[161-421]	223[137-364]	0.48	291[184-461]	200[121-330]	0.16
Ferritin*	2.21 [1.54-3.17]	2.56[1.62-4.06]	1.94[1.10-3.42]	0.38	2.21[1.35-3.61]	2.21[1.27-3.85]	0.9
IL-1 α	27 [19-37]	35[23-54]	20 [12-33]	0.13	38[25-58]	19[12-30]	**0.041**
IL-1 β	47 [31-71]	44[26-76]	49[25-96]	0.40	45.4[26.72-77.14]	48.8[25-95.4]	0.40
IL-1RA	6951 [5168-9350]	8147[5120-12964]	5962[4033-8815]	0.59	9108[5752-14421]	5353[3662-7824]	0.19
IL-6	41 [30-56]	32[20-53]	52[35-76]	0.14	33.3[20.5-54.1]	51.8[35.3-76.1]	0.16
IL-8	3608 [2462-5287]	2846[1599-5064]	4537[2686-7665]	0.37	3062[1790-5237]	4227[2393-7469]	0.43
IL-15	440 [370-520]	410[320-540]	460[360-600]	0.60	410[320-540]	460[360-600]	0.60
IL-18	40.2[32.2-50.2]	37.2[27-51.3]	43.3[31.2-6]	0.31	39.2[27.7-55.5]	41.2[30.5-55.6]	0.53
TNF-α	6.46 [4.58-9.12]	4.66[3.45-7.30]	9.35[3.91-17.8]	0.09	4.71[3.48-6.36]	9.25[4.84-17.6]	0.06
TNFR2	330 [230-490]	280[160-510]	390[220-670]	0.46	290[160-500]	390[220-680]	0.39
CCL2	100 [75-134]	96[62-150]	105[71-156]	0.35	100[65-153]	101[67-153]	0.98
CCL4	462 [313-682]	380[241-600]	554[291-1052]	0.42	349[220-555]	611[322-1158]	**0.036**
CCL11	59.8 [48.4-73.9]	48.3[39.2-59.6]	76.3[52.4-111]	**0.026**	49.1[39.7-60.7]	74.9[51.3-109]	**0.043**
MMP-3*	0.25 [0.19-0.33]	0.27[0.18-0.40]	0.23[0.16-0.33]	0.51	0.26[0.18-0.39]	0.24[0.16-0.34]	0.56
MMP-9*	38.1 [26.7-54.3]	48.8[29.1-81.8]	30.3[18.3-50]	0.11	50.8[31.6-81.7]	29.1[17.1-49.5]	0.09
TIMP-1*	108 [78.6-14]	93[56-155]	124[82-188]	0.33	98[60-159]	118[75-185]	0.40
ICAM-1	2900 [2170-3870]	2490[1650-3740]	3370[2200-5150]	0.32	2400[1620-3550]	3530[2280-5460]	0.19
VCAM-1	1130[850-1490]	1240[830-1860]	1003[690-1560]	0.80	1200[820-1760]	1060[690-1630]	0.78
VEGF	837 [678-1033]	793[592-1061]	882[640-1214]	0.51	823[617-1098]	851[616-1175]	0.83

All units are pg/ml except *denoting ng/ml, data represented as Geometric mean [95%CI]

Table 5.3. Clinical features of subjects stratified by the median neutrophil count (%) and RB1 total area

	Low neutrophil, Low total area	High neutrophil, Low total area	Low neutrophil, High total area	High neutrophil, High total area	p value
N	14	15	13	17	
Male, n	5	9	3	8	0.24
Current age	52 (3)	54 (3)	44 (4)	47 (3)	0.15
Age of onset	36 (5)	31 (5)	24 (5)	20 (4)	0.08
BMI, kg/m^2	32.7 (1.6)	32 (2)	24.4 (1.4)	29.7 (0.8)	**0.002**
Habitual and ex smokers with >10 pack year history	4	5	2	2	0.95
Pack years history	6.5(3)	14.5 (9)	7 (4)	5 (3)	0.59
Severe Exacerbations	1.5 (0.5)	2.5 (0.4)	2.5 (0.6)	4 (0.6)	**0.01**
IgE	208	221	270	119	0.28
ACQ_6	2.2 (0.25)	2.2 (0.26)	1.9 (0.31)	2.3 (0.27)	0.68
Subjects taking oral corticosteroids, n	7	8	4	6	0.87
Daily Predinsolone dose, mg	6 (1.9)	5.5 (1.6)	3 (1.5)	2 (0.7)	0.14
Pre Bronchodilator FEV_1	2.21 (0.3)	2.29 (0.2)	2.24 (0.2)	2.31 (0.25)	0.99
Post Bronchodilator FEV_1	2.36 (0.3)	2.48 (0.2)	2.41 (0.2)	2.42 (0.25)	0.98
Bronchodilatorreversibility, mls	0.15 (0.03)	0.46 (0.2)	0.17 (0.06)	0.11 (0.04)	0.28
Pre Bronchodilator FVC	3.12 (0.3)	3.31 (0.2)	3.33 (0.2)	3.26 (0.32)	0.96
Post Bronchodilator FVC	3.24 (0.3)	3.49 (0.2)	3.39 (0.2)	3.28 (0.32)	0.92
Pre Bronchodilator FEV_1 % of predicted	74.4 (8.2)	77.6 (5.6)	76.7 (4.9)	74.1	0.93
Post Bronchodilator FEV_1 % of predicted	79.4 (7.8)	82.1 (6.2)	83.4 (4.3)	83.8	0.94
Bronchodilator Reversibility % FEV_1	5.03 (1.1)	5.8 (1.3)	6.7 (1.9)	5.5 (1.6)	0.94
Pre Bronchodilator FEV_1/ FVC ratio	69.8 (4.1)	69.8 (3.5)	68 (3.3)	70.3 (4.1)	0.97
Post Bronchodilator BD FEV_1/ FVC ratio	72.1 (4)	70.7 (3.1)	72.9	73.2 (4)	0.96
Total Cells	0.53[0.2-1.43]	0.7 [0.41-1.4]	0.31 [0.15-0.63]	0.82[0.4-1.4]	0.79
Neutrophils%	35[19.6-62.5]	86 [80.5-91.9]	39.5 [29.5-52.5]	83.8[78.4-88.6]	**<0.001**
Eosinophils%	2.8 [1.1-7]	2.2 [0.9-9.3]	3.7[1.4-10.8]	1.2 [0.2-2.7]	0.16
Bronchiectasis present n (%)	2 (14)	5 (33)	5 (38)	11(65)	**0.03**
Bronchial Wall Thickening present	10 (70)	11(73)	8 (62)	10 (63)	0.87
Lumen area/Body Surface Area (mm^2/m^2)	3.6 (0.26)	3.3 (0.35)	7.1 (0.66)	7.2 (0.58)	**<0.001**
Total area/Body Surface Area (mm^2/m^2)	13.3 (0.54)	12.1 (0.67)	21.8 (1.64)	23.6 (1.27)	**<0.001**

Table 5.4. Sputum mediator concentrations for subjects stratified by the median neutrophil count (%) and RB1 total area

	Low neutrophil, Low total area	High neutrophil, Low total area	Low neutrophil, High total area	High neutrophil, High total area	p value
Factor VII*	2.19[1.59-3.01]	1.88[1.5-2.37]	2.06[1.51-2.83]	2.41[1.89-3.07]	0.61
Fibrinogen*	271[165-442]	249[103-602]	94[60-148]	430[220-830]	**0.002**
Ferritin*	2.28[1.06-4.88]	2.93[1.63-5.26]	1.74[0.99-3.07]	2.11[0.79-5.66]	0.5
IL-1 α	38 [18-79]	43 [29-64]	13 [7-24]	28 [14-58]	**0.001**
IL-1 β	34.6[17.4-69]	56.6[23.7-135]	15.8[8.87-28.2]	117[47-290]	**0.005**
IL-1RA	8249[3792-17942]	8053[4281-15148]	4341[2370-7952]	7599[4441-13002]	0.39
IL-6	40.7[20.1-82.6]	26.1[12.3-55.5]	41[23.3-72.3]	62.7[35.1-112]	0.27
IL-8	3469[1790-6726]	2365[865-6646]	3857[2088-7124]	5138[2186-12076]	0.54
IL-15	390[260-600]	430[290-670]	410[250-670]	500[360-700]	0.43
IL-18	35[22-57]	38[23-64]	34.7[22.6-53.5]	50.3[30.8-82]	0.32
TNF-α	4.64[3.47-6.2]	4.7[2.21-9.99]	5.8[3.74-9]	10.5[4.67-23.7]	0.07
TNFR2	340[140-830]	240[100-560]	240[130-460]	540[230-1230]	0.54
CCL2	153[92-256]	62.2[31-124]	104[53-205]	106[62-181]	0.079
CCL4	380[225-640]	380[165-877]	866[299-2508]	414[174-983]	0.096
CCL11	56[39.5-79.4]	41.2[32.3-52.4]	137[72-261]	54.6[36.1-82.5]	**0.0047**
MMP-3*	0.29[0.14-0.57]	0.25[0.14-0.42]	0.20[0.11-0.36]	0.26[0.16-0.43]	0.58
MMP-9*	41.3[15.5-110]	55.7[29.9-103]	11.6[7.16-19]	65.8[36-120]	0.17
TIMP-1*	153[104-225]	58[23-145]	139[74-226]	114[62-210]	0.25
ICAM-1	4050[2250-7280]	1520[930-2510]	4800[2220-10600]	2600[1570-4320]	**0.017**
VCAM-1	1400[690-2820]	1080[670-1750]	1020[440-2350]	1040[670-1630]	0.47
VEGF	1109[799-1539]	579[370-907]	903[551-1481]	865[542-1381]	0.061

All units are pg/ml except *denoting ng/ml, data represented as Geometric mean [95%CI]

Table 5.5 Clinical features of subjects stratified by the median neutrophil count (%) and RB1 luminal area

	Low neutrophil, Low luminal area	High neutrophil, Low luminal area	Low neutrophil, High luminal area	High neutrophil, High luminal area	p value
N	13	16	14	16	
Male	4	10	4	7	0.22
Current age	52(3)	54(3)	44(3)	46(3)	0.14
Age of onset	35(4)	31(4)	25(4)	20(4)	0.16
BMI, kg/m^2	32.8(1.7)	32.4(1.8)	24.9(1.4)	29.1(0.8)	**<0.001**
Habitual smokers and ex smokers with >10 py	4	6	2	1	0.86
Pack years history	7(3)	15(8)	6.5(4)	4(3)	0.48
Severe Exacerbations	1.5	2	2	4	**0.05**
IgE	266	212	174	141	0.38
ACQ_6	2.11(0.24)	2.29(0.24)	1.98(0.30)	2.29(0.28)	0.82
Subjects taking oral corticosteroids	7	7	4	7	0.84
Daily prednisolone dose, mg	6.9(2)	3.9(1.2)	3.0(1.4)	3.4(1.3)	0.30
Pre Bronchodilator FEV_1	2.15(0.32)	2.21(0.2)	2.29(0.19)	2.39(0.25)	0.90
Pre Bronchodilator FEV_1 % predicted	2.29(0.31)	2.37(0.22)	2.47(0.2)	2.53(0.24)	0.90
Bronchodilator reversibility, ml	0.14(0.02)	0.42(0.25)	0.18(0.05)	0.14(0.04)	0.44
Pre Bronchodilator FVC	3.07(0.37)	3.18(0.27)	3.37(0.2)	3.40(0.31)	0.84
Pre Bronchodilator FVC	3.17(0.37)	3.36(0.28)	3.44(0.2)	3.41(0.31)	0.92
Pre Bronchodilator FEV_1 % of predicted	74.8(8.9)	73.9(5.7)	76.1(4.5)	75.9(5.3)	0.99
Post Bronchodilator FEV_1% of predicted	79.7(8.4)	77.4(6.2)	82.8(4)	83.4(4.9)	0.87
BD Bronchodilator Reversibility %FEV1	4.8(1.1)	4.7(1.1)	6.7(1.8)	7.4(2.8)	0.68
Pre BronchodilatorFEV_1/FVC ratio	69.2(4.4)	70(3.3)	68.7(3.2)	70.1(4.3)	0.99
Post Bronchodilator FEV_1/FVC ratio	71.4(4.3)	70.1(2.8)	72.9(2.8)	74(4.2)	0.87
Total Cells	0.45 [0.16-1.2]	0.80 [0.45-1.4]	0.37[0.17-0.80]	0.8 [0.4-1.5]	0.74
Neutrophils %	33.6 [18-62.7]	85.2 [79-91.1]	40.7 [30.9-53.6]	83.9 [78.9-89.6]	**<0.001**
Eosinophils %	2.7 [1-7.4]	1.9 [0.7-4.5]	3.7 [1.5-9.3]	1.4 [0.6-3.2]	0.32
Bronchiectasis present	2(15)	7(44)	5 (36)	9(60)	0.15
Bronchial Wall Thickening present	10 (77)	12 (75)	8 (57)	9(60)	0.59
Lumen area/Body Surface Area (mm^2/m^2)	3.4(0.2)	3.1(0.2)	7.0(0.6)	7.6(0.4)	**<0.001**
Total area/Body Surface Area (mm^2/m^2)	13.1(0.5)	13.31(1.1)	21.3(1.5)	23.1(1.4)	**<0.001**

Table 5.6. Sputum mediator concentrations for subjects stratified by the median neutrophil count (%) and RB1 luminal area

	Low neutrophil, Low luminal area	High neutrophil, Low luminal area	Low neutrophil, High luminal area	High neutrophil, High luminal area	p value
Factor VII*	2.23 [1.58-3.16]	1.85 [1.51-2.26]	2.03 [1.53-2.69]	2.54 [1.95-3.31]	0.36
Fibrinogen*	270[160-460]	306[141-662]	101[65-157]	363[166-793]	**0.037**
Ferritin	2280 [990-5220]	2150 [1100-4200]	1780 [1050-2990]	2750 [950-7900]	0.6
IL-1 α	43[20-90]	34 [19-61]	13 [7-23]	26 [12-54]	0.14
IL-1 β	34.3 [16.2-72.6]	57[25.1-127]	17 [9.81-29.4]	121 [45.8-323]	**0.005**
IL-1RA	9021[3972-20484]	9180 [5058-16662]	4183 [2386-7332]	6642 [3826-11532]	0.054
IL-6	40.5[18.5-88.7]	28.5 [14.2-57.2]	41.2 [24.6-68.9]	65.3 [35.3-120]	0.20
IL-8	3567 [1739-7316]	2705 [1155-6335]	3730 [2114-6581]	4716 [1710-13009]	0.49
IL-15	400 [250-640]	420 [290-610]	409 [269-623]	520 [360-740]	0.54
IL-18	37.3 [22.5-61.9]	41.1 [23.9-70.6]	33.4 [22.5-49.8]	49.2 [30.8-78.7]	0.19
TNF-α	4.62 [3.32-6.43]	4.81 [2.55-9.05]	5.53[4.2-7.28]	11.1 [4.58-27.1]	0.19
TNFR2	350[130-920]	250 [120-510]	250[140-440]	550 [210-1410]	0.59
CCL2	158 [91-275]	69.1[37.4-127]	104 [56.3-194]	98.7 [52.7-184]	0.38
CCL4	357 [205-621]	343[159-742]	861 [330-2245]	464 [181-1189]	0.10
CCL11	56.7 [38.8-83.1]	43 [33.8-54.6]	122 [65.9-226]	53.4 [34.2-83.6]	**0.013**
MMP-3*	0.30 [0.14-0.62]	0.24[0.15-0.38]	0.20 [0.12-0.34]	0.27 [0.16-0.47]	0.49
MMP-9*	37 [13-106]	63.2 [40.1-99.5]	13.9 [7.73-25]	58.2 [27.3-124]	**0.0015**
TIMP-1*	163[110-241]	65 [29-145]	132[73.5-239]	107 [51.7-221]	0.14
ICAM-1	3960[2100-7493]	1600 [1030-2490]	4890 [2400-9970]	2660[1500-4720]	**0.017**
VCAM-1	1270 [617-2620]	1140 [734-1790]	1140[510-2560]	990[610-1580]	0.54
VEGF	1192 [871-1632]	609 [398-932]	857[537-136]	845[512-1393]	0.14

All units are pg/ml except *denoting ng/ml, data represented as Geometric mean [95%CI]

5.4 Discussion

We report here differential sputum mediator profiles between groups of severe asthmatic patients stratified by airway wall geometry, with more marked differences between groups when patients were stratified further by sputum neutrophil counts. Some of the differences were closely related to the differential sputum cell counts such as sputum MMP9 and IL-1β, which were elevated in those groups with a high neutrophil count and sputum ICAM-1 was elevated in those subjects with a low sputum neutrophil count independent of airway geometry [Pazdrak 2008]. However, other differences were observed that were dependent upon both stratification by the sputum neutrophil count and airway geometry namely CCL11, IL-1αnd fibrinogen. Sputum CCL11 concentration was elevated in subjects with a low sputum neutrophil count and high luminal and total area, whereas sputum IL-1β was increased in subjects with a high sputum neutrophil count and low total area. Sputum fibrinogen was elevated in those subjects with luminal narrowing independent of sputum cellular differential, but was also increased in subjects with neutrophilic inflammation without luminal narrowing. These findings support the view that there are differential sputum mediator profiles related to airway geometry.

Several sputum mediators were associated more with neutrophilic inflammation than the pattern of airway geometry. Two of these mediators IL-1β and MMP-9 are observed in subjects with COPD and are reported to be associated with airway bacterial colonisation and neutrophilic inflammation. Interestingly, Matsumoto *et al* observed an inverse relationship between sputum MMP-9 and %WA which was consistent with our observation of a low MMP-9 in the high luminal area, non-neutrophilic group. In contrast, sputum MMP9 was elevated in our study in those with neutrophilic inflammation independent of luminal area [Matsumoto 2005]. IL-1β and MMP-9 promote subepithelial fibrosis, subepithelial collagen deposition and elastin fibre disruption in asthma and in COPD are associated with many of the histopathological features of emphysema [Lappalainen 2005, Maclay 2012, Kumagi 1999, Hoshino 1998]. This may suggest a phenotype of remodelling associated more with ‘scaffold destruction’ leading to airway dilatation from loss of airway wall integrity as opposed to a predominant airway thickening. Indeed, the group with high total and luminal area were associated with increased qualitative evidence of

bronchiectasis and frequent exacerbations. Although this process has relevance to those subjects with luminal dilatation, these mediators were also increased in subjects with neutrophilic inflammation and luminal narrowing. This suggests either that in these subjects an alternative inflammatory process is driving the airway narrowing or that there were changes that occurred earlier in the lung development of these subjects that led to either airway narrowing in early development or a predisposition to airway narrowing in the natural history of the disease.

Fibrinogen was also elevated predominately in those with neutrophilic inflammation, but was increased in subjects without neutrophilic inflammation with luminal narrowing and low RB1 total area. Fibrinogen is an acute phase soluble plasma glycoprotein, synthesised primarily in the liver and converted by thrombin into fibrin during blood coagulation. It is emerging as a promising systemic biomarker in COPD that shows relationships with disease severity, progression and mortality [Duvoix 2012]. There are studies that link its presence to microvascular leakage from plasma exudation, which occurs during remodelling or neurogenic inflammation in asthma [Brims 2009, Van Rensen 2002] and may contribute to pathophysiological features of airway hyperresponsiveness [Wagers 2004]. Fibrinogen leakage exudes into the extravascular compartment and interacts with neutrophils causing their sequential activation and promoting their survival by reducing apoptosis [Rubel 2001]. Sputum fibrinogen is therefore likely to be increased in subjects with airway wall oedema, which may contribute to the luminal narrowing in subjects independent of sputum cell differential. Interestingly, it was not increased in subjects without a sputum neutrophilia and without high RB1 suggesting that in this group airway wall oedema and increased vascularity may not play a prominent role. This needs to be confirmed by examination of the bronchial wall in this group of subjects. Intriguingly, this group also had elevated sputum CCL11 and the highest sputum eosinophil count, albeit non-significant, suggesting that although eosinophilic inflammation may be important in driving remodelling and luminal narrowing in some subjects it is also associated with normal airway geometry. Whether this represents a group of patients protected from airway remodelling or at an earlier stage of the natural history needs to be further explored.

In contrast to sputum IL-1β, sputum IL-1α was not closely related to neutrophilic inflammation but was increased in those subjects with low RB1 luminal area increased and in those subjects with both low RB1 total area and neutrophilic inflammation. Recent evidence has implicated IL-1α as an important link between the innate immune system and allergic sensitisation TLR4 activation induces IL-1α release from epithelial cells, which acts in an autocrine manner to release GM-CSF and IL-33 [Willart 2012]. IL-1α is a potent activator of fibroblasts [Ingram 2004] and might play a role in fibroblast activation and hyperplasia in asthma. In atherosclerosis [Alexander 2012] IL-1α has also been implicated in the plaque stability and outward vessel remodelling. Whether IL-1α plays a similarly important role in differential changes in airway geometry warrants further study.

One major criticism of our study is the cross-sectional design, which limits our interpretation of the dynamic relationship between the mediator profiles and airway remodelling. For example, it is uncertain whether the changes observed by CT imaging reflect progressive remodelling over many years as a consequence of airway inflammation or represent changes in airway geometry that were established in early lung development [Simpson 2009]. For example, there were small differences in the age of onset of disease and current age, albeit not disease duration, in subjects in the CT defined subgroups. This effect of time is particularly important when considering differential mediator profiles between subjects in whom the airway wall has remodelled towards the airway lumen versus those that have either maintained normal luminal patency or have remodelled away from the lumen and have airway dilatation. To address this question either longitudinal studies of the natural history of remodelling are required or studies of interventions that might impact in the short term upon remodelling such as bronchial thermoplasty [Castro 2010, Wahidi 2012]. Importantly, luminal area in the severe asthma group is decreased compared to controls such that even the high luminal area group described here do not represent bronchial dilatation compared to health. A further criticism is that sputum analysis reflects a composite measure of the proximal airways rather than a specific lobe whereas the CT imaging here focussed upon the dimensions of the RB1. Although a more comprehensive volumetric analyses the entire proximal airway tree would be informative we and others have reported that the RB1 geometry has good correlations with other proximal airways [Aysola 2008].

Therefore, we are confident that this is not a major limitation of this study. The sputum samples were processed with the mucolytic DTT, which affects the recovery of a number of mediators in particular Th2 cytokines. The scope of the mediators measured here are therefore hampered by this shortcoming, but none the less we have measured a comprehensive panel of biomarkers and are confident this reflects many aspects of the inflammatory profile in the airway of these subjects. Sputum analysis is restricted to sampling to the airway lumen and does not necessarily reflect airway inflammation in the airway wall. Large studies of the relationship between airway wall remodelling determined by bronchial biopsies and CT imaging are underway and the findings are eagerly awaited.

In conclusion, severe asthma is a complex heterogeneous disease and the interactions between airway inflammation and remodelling are poorly understood. Here we have demonstrated that sputum mediator profiling reveals a number of associations between airway geometry and sputum differential cell counts and mediator concentrations. Whether these findings reflect specific and consistent underlying biological phenotypes with predictable natural histories and the potential for stratified medicine approaches needs to be further investigated.

Chapter 6

Sputum mediators and the aetiology of exacerbations in severe asthma

6.1 Introduction

There is increasing recognition that severe asthma is a heterogeneous condition [Haldar 2008, SARP 2009] and this may be reflected during exacerbations. Several comorbidities are associated with recurrent exacerbations and adherence is also a key to achieving good control [Heaney 2012]. Yet, it remains difficult to characterise not only between poorly controlled disease and exacerbations but also the severity of exacerbations, mainly from a lack of standardized definitions. There are a number of chronic inflammatory conditions like rheumatoid arthritis where mediators like IL-6 are used as a disease activity marker and also serve as therapeutic targets [Nature paper]. Biomarkers in asthma have been used to phenotype disease and also guide therapy by indicating steroid responsiveness, and risk of future exacerbation with sputum eosinophilia being best studied [Green 2002]. Recognising the importance of the eosinophil led to development of monoclonal antibodies namely Mepolizumab [Haldar 2009, Pavord 2012]. There is need to better understand the aetiology and molecular mechanisms of exacerbations and tailor therapy which has been recently demonstrated in studies with recombinant beta interferon in asthma [Djukanovic 2014] and steroid therapy guided by peripheral eosinophilia in COPD [Bafadhel 2011].

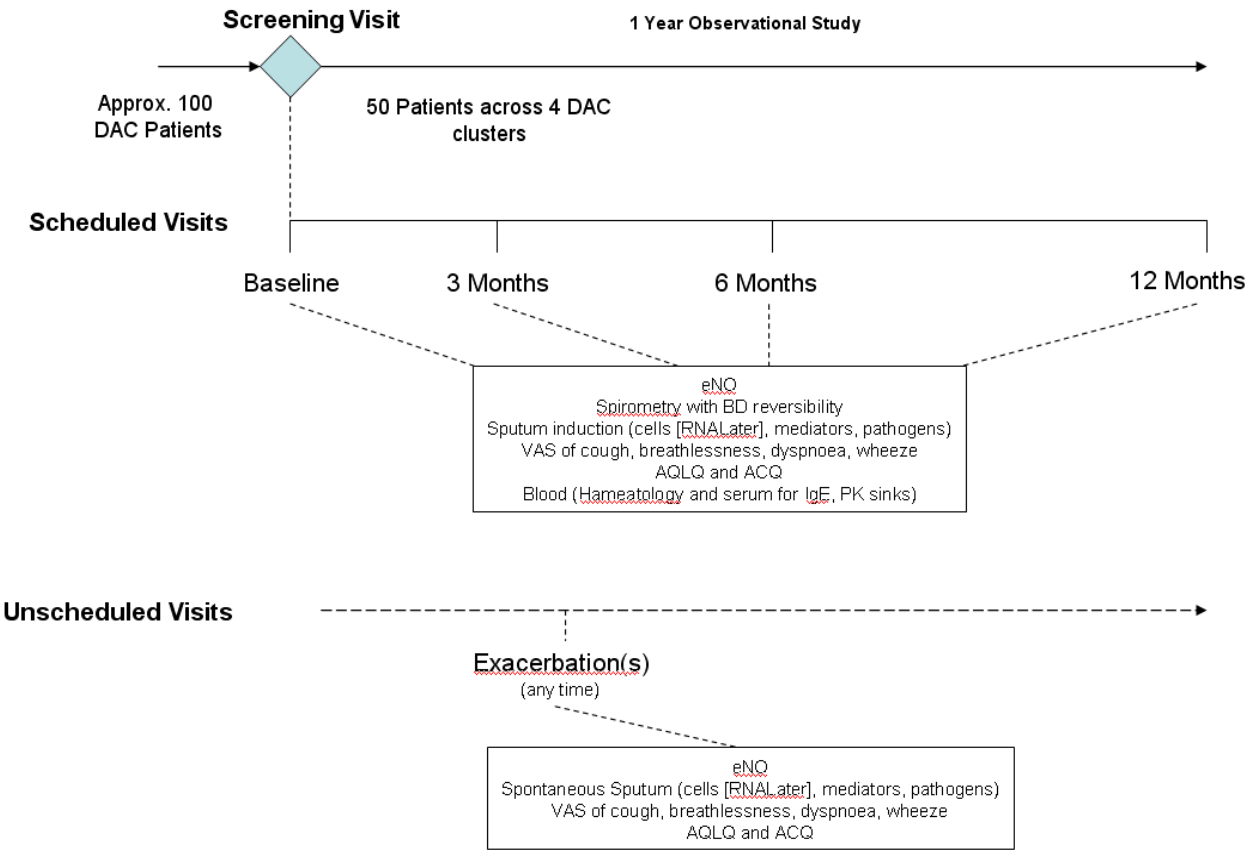
Currently markers that define based on a composite outcome of domains encompassing symptoms and lung function, and suffer from poor specificity. Measures like fraction of exhaled nitric oxide (FeNO) may have a role in defining risk of future exacerbations[Gelb] but it is probably applicable only to an eosinophilic subset of disease that suffer with [Djukanovic 2014] eosinophilic exacerbations. The role of viruses is well recognised aetiology in milder disease and especially in the paediatric population [Johnston] but much less is known of the role of bacteria.

We hypothesized that characterizing severe asthmatics at time of stable disease and during exacerbations would inform the changes in airway and systemic inflammation and try to define the aetiology of these events.

6.2 Subjects and Protocol

This was a prospective observational study of severe asthmatics seen at stable state and during exacerbations for the duration of 1 year **(figure6.1**). Stable visits including the baseline visits were 8 weeks free from an exacerbation. For exacerbation visits data recording and sampling were only performed in patients who were able to attend within 72 hours of when their exacerbation manifest, deemed by symptom change or from the point where patients themselves commenced rescue oral steroid therapy according to their self-management plan. Patients that had not commenced rescue oral steroids at time of assessment were then treated according to guidelines by the clinician and commenced on a course of oral Prednisolone (30 mg/day for 10 days).

Figure 6.1A Study outline



6.2.1 Measurements

See methods section: in addition, sputum was collected and analyzed for bacteria (using standard routine culture, CFU, and real-time quantitative polymerase chain reaction [qPCR]), for viruses by PCR, and processed to produce cytospins for cell differential and supernatant for fluid phase measurements. Venous blood was collected for differential counts and serology. Inflammatory mediators were measured in sputum supernatants and serum using the MSD platform.

6.2.2 Statistical analysis

Mediators which significantly discriminated between stable versus exacerbation were identified using receiver operating characteristic (ROC) curves. Subjects had 'paired samples' wherein the baseline data was compared with the 1st exacerbation data from that individual. Unpaired samples were subject data with more than one exacerbation (i.e. repeated individual) compared with their baseline data. Subjects were classified into bacterially colonised and non-colonised subgroups using the colony forming unit 10^7cutoff. Sputum cytokines which discriminated these subgroups significantly were identified using ROC curve. Repeatability of mediators was assessed across stable visits by intra-class correlations. I have previously described cluster methodology in Chapter 4. I performed a similar K means cluster analysis on the sputum mediator data only in order to replicate previous work done by Bafadhel [2012]. I used cytokine data was from all the exacerbation visits with log normalized and z score transformed mediator data; performed a hierarchical cluster analysis followed by a k-means clustering with Wards method and pre specified 4 clusters as were suggested by the previous hierarchical analysis.

6.3 Results

Table 6.1 shows the baseline clinical characteristics. The subjects consist of severe asthmatics with late onset disease. They demonstrate impaired lung function with a degree of fixed airflow obstruction, and on high dose of ICS. Sputum examination reveals a mild neutrophilia and there is no evidence of a peripheral blood eosinophilia.

The group consist of males [n (%) 50(48)] with a mean age of 53 years. The mean age of onset (SEM) is 25.9(1.8) years. Thus this group has long standing asthma spanning on an average two decades. The mean BMI (SEM) was 30(0.7) kg/m^2 and is therefore an obese group. They had self reported up a mean (SEM) of 3 (0.2) exacerbations of asthma needing oral corticosteroid use with or without antibiotics in the 12 months prior to enrolment in the study.

The group had 37.5% current or ex smokers and the average pack year history for the whole group of patients averages 6 pack years. The majority of the subjects had GINA stage 4/5 asthma 46/53% respectively. The subjects described symptoms consistent with poorly controlled asthma with mean (SEM) ACQ6 score of 2(0.1) and AQLQ score of 4.7 (0.13). The presence of atopy as defined in the methods section was 48% and they had elevated Age of 143.2 IU/L. Of the patients requiring maintenance steroid use the dose (range) of Prednisolone was 10 (7.5 - 12.5) mg. Subjects were also on high dose inhaled therapy with median daily ICS dose (range) 1600 (1000 - 2000) µg beclomethasone dipropionate equivalent. The subjects showed evidence of impaired lung function with a mean (SEM) FEV_1 of 74.9 (2.1) % predicted and evidence of obstructive spirometry with mean (SEM) FEV_1/FVC ratio of 68.2 (1.3). Sputum analysis revealed a predominantly neutrophilic inflammation with mean (SEM) sputum neutrophil (%) 62.4 (2.4) and sputum eosinophils (%) 1.9 (1.3 - 2.8).

A higher proportion of subjects (n=67) did not experience exacerbations during the study versus those that experienced at least one or more than one exacerbation (n=37). (**Table 6.1) P**atients with exacerbations had a higher mean (SEM) BMI [31.8 (1.1) vs. 28.9(0.8); p=0.031)] kg/m^2 in the non exacerbation group. The proportion

with GINA stage 5 disease in the exacerbations was [73% vs. 26 %;(p=0.001)] in the non exacerbations reflecting more severe disease. There was a higher proportion of current or ex smokers in the non exacerbations n (%) [31 (46) vs. 8 (22) ;(p=0.013)] Compared with the exacerbation group there was a higher incidence of cough as measured on the VAS scale in the non exacerbations with mean (SEM) [36.9 (3.2)vs. 26.6 (3.7); p=0.045)]mm compared with exacerbations There were no other demographic or sputum or blood differential that could distinguish between both the groups. The comparisons of mediator data of the above two groups of patients did not reveal any differences either in sputum or serum.

Clinical characteristics at exacerbation

We captured 37 events (including repeated exacerbations) from 28 individuals and show their paired comparison with baseline characteristics; there was a reduction in lung function at stable visit vs. exacerbation FEV1% predicted [78.2 vs. 71.2; ($p<0.001$)] and significant increase in symptom scores within all domains of the Visual Analogue Scale

Sputum and blood differential counts during stable and exacerbation visits.

Sputum neutrophils and eosinophils showed no significant change in the differential counts but the total cell count at exacerbation [3.6 (2-6.3)x 10^6 cells/mg sputum] was significantly higher than at stable visit [1.5 (1-2.3)], (p=0.03).

Systemic inflammation assessed by total and differential peripheral blood counts remained unchanged between stable state and exacerbations.

Figure 6.1A. Outline of study (stable visits)

a) Sample breakdown for stable visits

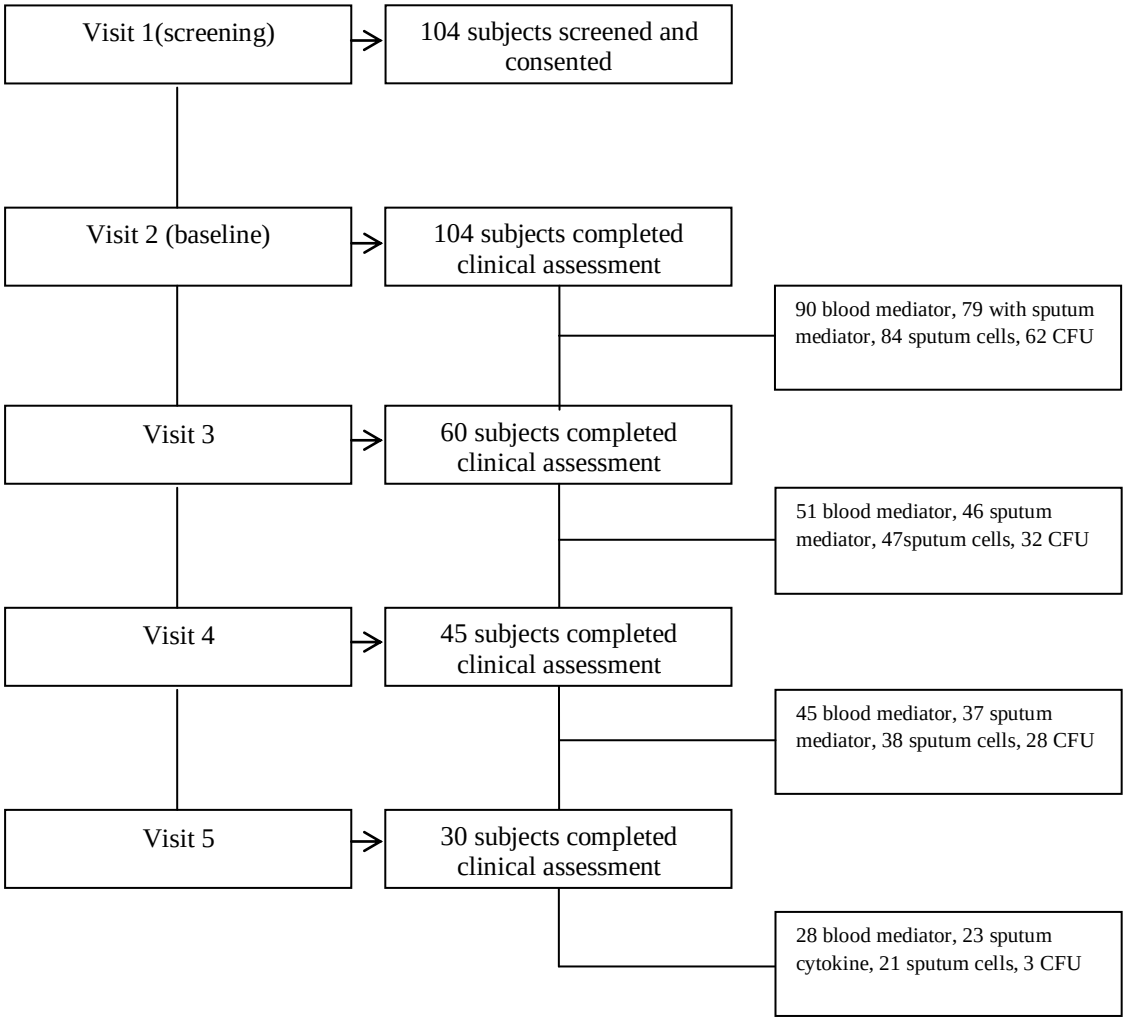


Figure 6.1B Study outline (exacerbation and recovery visits)

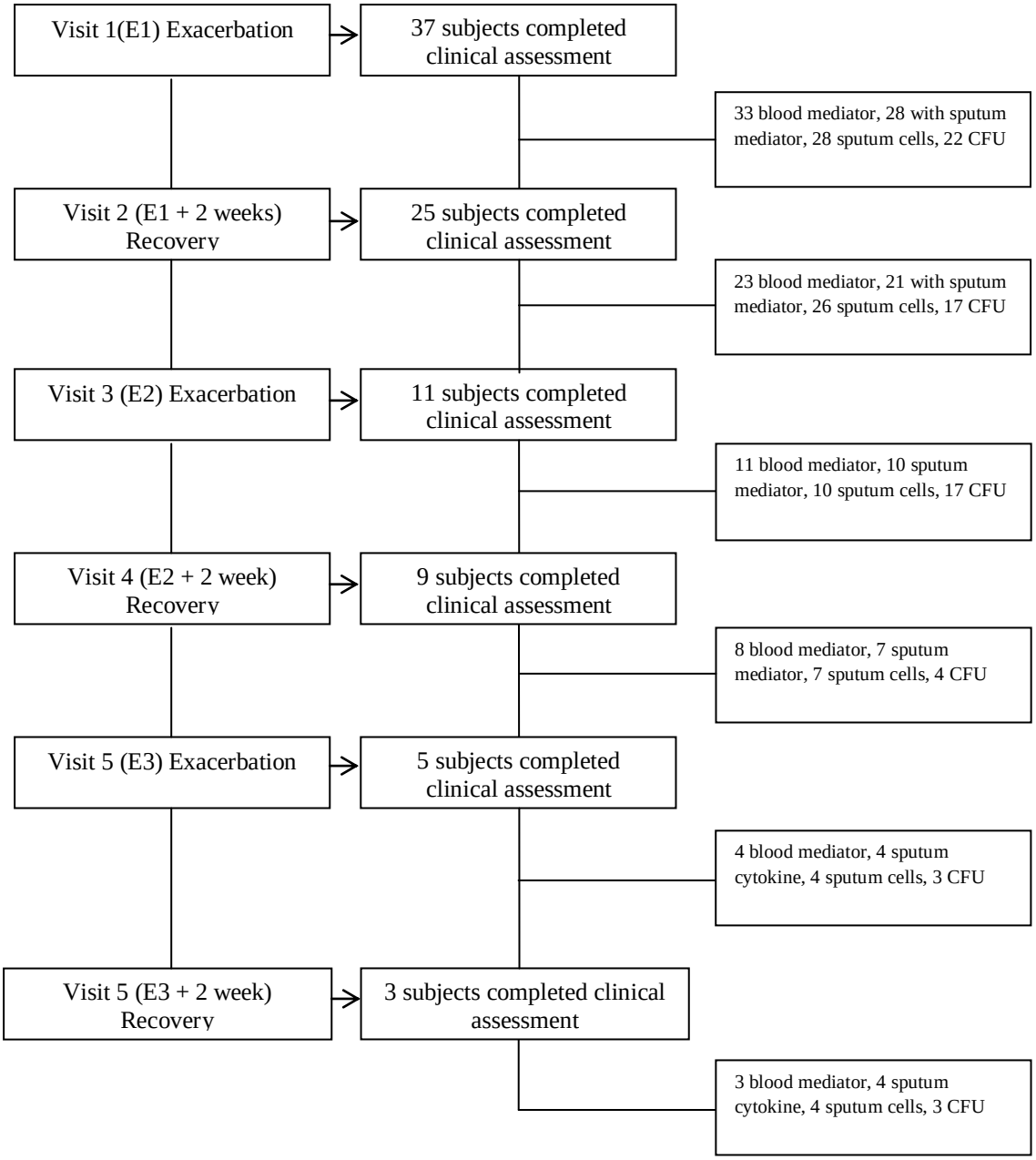


Table 6.1 Clinical characteristics at baseline and exacerbation for all subjects and stratified by exacerbation events

	All (n=104)	Not assessed at exacerbation (n=67)	Assessed at Exacerbation (n=37)	P-value	Exacerbation (n=37)	P-value**
Male [n (%)]	50(48.1)	34 (50.7)	16 (43.2)	0.46		
Current or Ex-smokers [n (%)]	39(37.5)	31 (46.3)	8 (21.6)	**0.013**		
Pack year history¥	6(3.7-9.7)	6.2 (3.6 - 10.7)	5.4 (1.9 - 15.4)	0.82		
Age (year)	53(1.2)	51.8 (1.6)	55.1 (1.8)	0.21		
Age of asthma onset (years old when diagnosed)	25.9(1.8)	24 (2.2)	29.5 (3.1)	0.15		
BMI (kg/m^2)	30(0.7)	28.9 (0.8)	31.8 (1.1)	**0.031**		
Exacerbation number of steroids in past 12 month	3(0.2)	2.8 (0.2)	3.4 (0.3)	0.13		
GINA Classification III/IV/V [n (%)]	5(4.8)/46(44.2)/53(51)	3(4.5)/38(56.7)/26(38.8)	2(5.4)/8(21.6)/27(73)	**0.001**		
Asthma Control Questionnaire (ACQ6)	2 (0.1)	2 (0.2)	2 (0.2)	0.9		
Asthma Quality of life Questionnaire (AQLQ)	4.7(0.13)	4.7 (0.2)	4.8 (0.2)	0.65		
IgE (IU/L)$^+$	143.2 (106.5 - 192.4)	142.9 (97.7 - 209)	143.7 (89.5 - 230.6)	0.99		
Daily Prednisolone dose [n (%)]	52 (50)	26 (38.8)	26 (70.3)	**0.002**		
Daily Prednisolone dose mg,*±	10 (7.5 - 12.5)	10 (5 - 10)	10 (7.5 - 12.5)	0.99		
Daily ICS dose, µg*	1600 (1000 - 2000)	1600 (1000 - 2000)	1600 (800 - 2000)	0.46		
Atopy [n (%)]	50 (48.1)	30 (44.8)	20 (54.0)	0.36		
Pre FEV_1 (L)	2.2 (0.1)	2.2 (0.1)	2.1 (0.1)	0.21	1.9 (0.2)	**<0.001**
pre FEV_1 percentage predicted	74.9 (2.1)	75.7 (2.4)	73.4 (4.1)	0.6	67.9 (4.6)	**0.01**
Pre FEV_1-FVC ratio	68.2 (1.3)	68.7 (1.8)	67.4 (1.9)	0.66	70.4 (2.4)	**0.046**
Post FEV_1 (L)	2.3 (0.1)	2.4 (0.1)	2.2 (0.1)	0.22	2 (0.2)	**<0.001**
Post FEV_1 percentage predicted (%)	79.8 (2.2)	81.5 (2.5)	76.8 (4)	0.3	71.2 (5)	**<0.001**
VAS cough (mm)	33.2 (2.5)	36.9 (3.2)	26.6 (3.7)	**0.045**	64.3 (3.6)	**<0.0001**
VAS dyspnoea (mm)	37.6 (2.6)	38.9 (3.2)	35.2 (4.3)	0.49	64.5 (3.4)	**<0.0001**
VAS wheeze (mm)	27.4 (2.7)	28.7 (3.5)	25.2 (4.4)	0.54	49.4 (4.2)	**<0.0001**
Sputum neutrophil (%)	62.4 (2.4)	60.3 (3.1)	66.3 (3.8)	0.23	61.7 (5.1)	0.68
Sputum eosinophils (%)$^+$	1.9 (1.3 - 2.8)	2.5 (1.6 - 4)	1.2 (0.6 - 2.2)	0.061	1.2 (0.6 - 2.6)	0.9
Sputum macrophage (%)$^+$	18.3 (14.9 - 22.5)	19.3 (15.4 - 24.2)	16.6 (11.1 - 24.9)	0.49	15.6 (10.3 - 23.8)	0.63
Sputum total cell count (10^6/mg sputum)$^+$	1.5 (1.2 - 1.9)	1.5 (1.1 - 2)	1.5 (1 - 2.2)	0.97	3.6 (2 - 6.3)	**0.03**
Blood neutrophilic cell count (x10^9 cells/L)	5.9 (0.2)	5.7 (0.3)	6.2 (0.4)	0.27	6.5 (0.4)	0.56
Blood eosinophils cell count (x10^9 cells/L)$^+$	0.2 (0.2 - 0.3)	0.3 (0.2 - 0.3)	0.2 (0.1 - 0.3)	0.095	0.2 (0.1 - 0.2)	0.99
Fraction of exhaled Nitric Oxide (FeNO) (ppb)$^+$	24 (20.6 - 27.9)	22.7 (19 - 27.1)	26.6 (20.1 - 35)	0.33	34.7 (25.2 - 47.8)	0.12

Data presented as mean (standard error of mean (SEM) unless stated; $^+$ geometric mean (95% CI); *median (first and 3rd quartile); ¥pack year history for current and ex-smokers; ± dose of Prednisolone in those receiving Prednisolone; **p-value for paired comparison between stable and exacerbation visit. Definition of abbreviations: VAS= Visual analogue score; BMI= Body Mass Index; FEV_1=Forced Expiratory Volume in the First Second; FVC=Forced Vital Capacity

Table 6.2 Sputum and serum values comparing first stable and paired exacerbations (all values are in pg/ml)

	Sputum Stable (n=30)	Sputum Exacerbation (n=30)	P-value	Serum Stable (n=34)	Serum Exacerbation (n=34)	P-value
IL-1α	49.1 (33 - 73.3)	57.3 (36.4 - 90.2)	0.49	1.6 (1.6 - 1.7)	1.6 (1.6 - 1.6)	0.2
IL-1β	75.5 (49.2 - 116)	137.2 (63.9 - 294.7)	0.08	6.1 (4.4 - 8.4)	3.2 (2.2 - 4.7)	0.002
IL -2	1 (0.6 - 1.5)	1.5 (0.9 - 2.6)	0.2	1.7 (1.6 - 1.8)	1.6 (1.6 - 1.7)	0.11
IL-4	1.6 (1.6 - 1.7)	1.6 (1.6 - 1.6)	0.37	1.7 (1.5 - 2)	1.8 (1.6 - 2.1)	0.4
IL-5	4.3 (2.6 - 7.3)	2.8 (1.5 - 5.2)	0.23	10.9 (7.9 - 15)	5.9 (4.3 - 8.1)	0.002
IL-6	53.7 (30.9 - 93.5)	102.7 (55.5 - 190)	0.07	3.1 (2.3 - 4.1)	2.5 (2.1 - 3)	0.18
IL-6R	330.5 (225.8 - 483.8)	1067.4 (658.5 - 1730.2)	<0.0001	21578.8 (16667.6 - 27937.1)	50912.3 (37508.3 - 69106.5)	<0.001
IL-8	3526.1 (2238 - 5555.7)	4868.6 (3079.2 - 7698)	0.15	13.2 (11.3 - 15.5)	12.5 (9.9 - 15.8)	0.68
IL-9	8.7 (7.7 - 9.9)	8.4 (7.9 - 8.8)	0.45	40 (40 - 40)	41.6 (38.6 - 44.8)	0.32
IL-10	2.4 (1.8 - 3.3)	9.9 (4.6 - 21.4)	<0.001	2.5 (2 - 3.2)	2.2 (1.8 - 2.7)	0.29
IL-13	12.8 (9.8 - 16.7)	12.8 (9.9 - 16.6)	0.98	50.2 (39.7 - 63.3)	44.5 (37.1 - 53.5)	0.45
IL-15	2.4 (2 - 2.9)	3.4 (2.5 - 4.7)	0.05	2.8 (2.4 - 3.1)	2.6 (2.3 - 3)	0.34
IL-17	12.9 (9.1 - 18.3)	12.6 (8.8 - 18.3)	0.91	9.9 (8.1 - 12)	9.8 (8 - 12.1)	0.94
IL-18	31.3 (21.8 - 44.9)	55.1 (31.8 - 95.4)	0.015	397.8 (337.7 - 468.6)	385.5 (342.9 - 433.4)	0.63
IL-23	202.9 (197.3 - 208.6)	201.3 (198.7 - 203.9)	0.63	1000.7 (999.3 - 1002.1)	1000 (1000 - 1000)	0.33
CXCL-9	630.5 (310.5 - 1280.3)	1990 (1038.2 - 3814.1)	0.002	74.5 (62.8 - 88.4)	76.2 (61.7 - 94)	0.83
CXCL-10	763.9 (432.7 - 1348.5)	1633.6 (900.2 - 2964.7)	0.04	139.4 (115.4 - 168.3)	163 (129.9 - 204.5)	0.17
CXCL-11	57.7 (31.1 - 107.1)	85.3 (31.5 - 230.9)	0.5	216.8 (171 - 274.8)	240.7 (187.2 - 309.6)	0.43
CCL-2	337.4 (211.2 - 539)	457.1 (287.8 - 726)	0.31	660.9 (582.7 - 749.7)	576.8 (498.6 - 667.3)	0.01
CCL-3	40.2 (26.1 - 62)	42.1 (25.4 - 69.9)	0.87	40 (40 - 40)	40.4 (39.8 - 41)	0.16
CCL-4	425.8 (252.7 - 717.6)	660.4 (352.3 - 1238.3)	0.2	198 (168.1 - 233.1)	185.9 (149.5 - 231.2)	0.51
CCL-5	9.8 (6.3 - 15.2)	21.9 (12.8 - 37.4)	0.007	18867.3 (17403.6 - 20454.2)	19439.8 (18386.9 - 20553)	0.56
CCL-11	62 (42 - 91.5)	48 (31.2 - 73.9)	0.32	1001.7 (819.6 - 1224.3)	829.9 (675.1 - 1020.1)	0.07
CCL-13	25.8 (18.5 - 35.9)	15.4 (11.9 - 20)	0.008	624.7 (546.4 - 714.2)	571.8 (494 - 662)	0.23
CCL-17	26.6 (16.2 - 43.7)	14 (7.4 - 26.4)	0.05	1001.7 (664 - 1511.3)	658.4 (480.5 - 902)	0.023
CCL-26	11.3 (7 - 18.3)	8.9 (5 - 15.9)	0.45	23.1 (15.3 - 35)	16.5 (9.6 - 28.2)	0.15
TNF-α	4.3 (2.6 - 7.2)	13 (5.5 - 30.5)	0.02	8 (5.6 - 11.5)	6.6 (4.7 - 9.2)	0.34
TNF-R1	626.5 (411 - 955.1)	902.8 (549.7 - 1482.5)	0.08	4296.2 (3956.7 - 4664.7)	3944.8 (3546.3 - 4388)	0.07
TNF-R2	322.4 (195.7 - 531)	666.5 (381.3 - 1165)	0.009	5711.4 (5153.7 - 6329.5)	5296.9 (4650.7 - 6032.8)	0.11
VEGF	1531.1 (1162.2 - 2017)	1377.3 (1082.4 - 1752.7)	0.44	958.5 (746.7 - 1230.4)	815.7 (624.1 - 1066)	0.27
GMCSF	0.4 (0.3 - 0.4)	0.4 (0.3 - 0.5)	0.28	1.9 (1.6 - 2.3)	1.7 (1.5 - 1.8)	0.21
IFNγ	0.6 (0.3 - 0.9)	2.5 (1 - 6.6)	0.008	0.5 (0.4 - 0.6)	0.5 (0.4 - 0.7)	0.34
CHTR	139863.2 (75682 - 258472.5)	194483 (97984.7 - 386015.8)	0.27	135419 (100783 - 181959)	118882.4 (90582.3 - 156024.1)	0.06
YKL-40	1057301 (608640.4 - 1836692.7)	1000449 (571907 - 1750109)	0.85	1474825.2 (1186231 - 1833630)	890207.6 (660391 - 1199999.7)	0.0002
PAI-1	50592.6 (20427.4 - 125303)	4876.8 (2576.5 - 9230.5)	<0.0001	99902 (71160.8 - 140250)	260045 (187091.8 - 361445)	0.0002

Table 6.3 Geometric mean (95% CI) sputum and serum mediator concentrations (pg/ml) for unpaired baseline and all exacerbation samples

	Sputum stable (n=88)	Sputum Exacerbation (n=31)	P-value	Serum stable (n=96)	Serum exacerbation (n=35)	P-value
IL-1α	53.6 (43.1 - 66.7)	60 (38.3 - 94)	0.63	1.6 (1.6 - 1.7)	1.6 (1.6 - 1.6)	0.18
IL-1β	69.5 (52.8 - 91.5)	135.3 (64.5 - 283.6)	0.04	6.1 (5.1 - 7.3)	3.3 (2.3 - 4.7)	0.001
IL -2	0.9 (0.7 - 1.1)	1.5 (0.9 - 2.5)	0.03	1.7 (1.6 - 1.7)	1.6 (1.6 - 1.7)	0.25
IL-4	1.6 (1.6 - 1.7)	1.6 (1.6 - 1.6)	0.55	1.7 (1.6 - 1.8)	1.8 (1.6 - 2.1)	0.17
IL-5	2.8 (2 - 3.9)	2.9 (1.6 - 5.2)	0.95	9.5 (8 - 11.3)	5.9 (4.4 - 8)	0.007
IL-6	43.2 (30.6 - 60.8)	101.8 (56.1 - 184.6)	0.01	2.9 (2.4 - 3.5)	2.7 (2.2 - 3.3)	0.58
IL-6R	255.7 (205 - 318.9)	1051.9 (658.7 - 1679.8)	<0.0001	24451 (20597 - 29027)	51194 (38044 - 68889)	<0.0001
IL-8	3052 (2287.5 - 4071.8)	4679.4 (2984.1 - 7337.9)	0.13	13.4 (12 - 15)	12.8 (10.2 - 16.1)	0.7
IL-9	8.5 (8.1 - 9)	8.4 (7.9 - 8.8)	0.62	40.6 (39.4 - 41.8)	41.5 (38.6 - 44.6)	0.52
IL-10	2.4 (2 - 2.8)	10.1 (4.8 - 21.3)	<0.0001	2.3 (2 - 2.7)	2.2 (1.8 - 2.7)	0.76
IL-13	12.6 (10.9 - 14.6)	12.6 (9.8 - 16.2)	0.99	50.2 (43.2 - 58.3)	44.4 (37.1 - 53)	0.37
IL-15	2.4 (2.1 - 2.6)	3.4 (2.5 - 4.6)	0.01	2.4 (2.3 - 2.6)	2.7 (2.4 - 3)	0.17
IL-17	9.7 (8.5 - 11.1)	12.6 (8.8 - 17.9)	0.1	8.7 (8.1 - 9.4)	9.8 (8 - 11.9)	0.18
IL-18	26.9 (22 - 32.8)	54.8 (32.3 - 93.2)	0.003	343.3 (313.3 - 376.1)	382.1 (340.6 - 428.7)	0.2
CXCL-9	557.3 (386.7 - 803.1)	1988.8 (1060 - 3731.5)	0.001	59.2 (52.9 - 66.2)	78.3 (63.4 - 96.7)	0.02
CXCL-10	690.6 (502.4 - 949.4)	1677.1 (940.2 - 2991.4)	0.007	122.7 (110 - 136.9)	171 (134.6 - 217.2)	0.006
CXCL-11	52.9 (36.8 - 76)	92.1 (34.8 - 244)	0.2	191.2 (164.4 - 222.5)	254.6 (194.8 - 332.9)	0.06
CCL-2	271.6 (217.5 - 339)	450.5 (287.7 - 705.3)	0.03	646.9 (588.3 - 711.3)	574.8 (498.9 - 662.3)	0.19
CCL-3	32.6 (24.9 - 42.6)	41 (25 - 67)	0.41	41.5 (38.8 - 44.3)	40.4 (39.8 - 41)	0.65
CCL-4	374.9 (269.2 - 522)	642.3 (348.8 - 1182.6)	0.11	196 (173 - 222.1)	182.4 (147.1 - 226.1)	0.56
CCL-5	8.5 (6.8 - 10.6)	21.6 (12.9 - 36.3)	<0.0001	19578.2 (19003.4 - 20170.5)	19456 (18431 - 20537)	**0.83**
CCL-11	57.2 (45.8 - 71.5)	47.4 (31.2 - 71.9)	0.41	923.4 (818.3 - 1042)	826.5 (676.3 - 1010.1)	0.35
CCL-13	22.3 (18.4 - 27.2)	16 (12.3 - 20.8)	0.07	550.7 (503.3 - 602.6)	564.4 (488.5 - 652.1)	0.78
CCL-17	25.7 (19.3 - 34.1)	13.5 (7.3 - 25)	0.04	776.3 (619.4 - 973)	664.9 (489.4 - 903.2)	0.46
CCL-26	10.8 (7.9 - 14.7)	8.9 (5.1 - 15.7)	0.55	19.7 (15.2 - 25.6)	16.3 (9.7 - 27.5)	0.49
TNF-α	3.3 (2.4 - 4.5)	12.5 (5.4 - 28.7)	<0.0001	7.7 (6 - 9.9)	6.6 (4.8 - 9.2)	0.5
TNF-R1	480.1 (381.6 - 604)	904.8 (560 - 1461.8)	0.01	4082.2 (3810.3 - 4373.4)	3893.3 (3499.8 - 4331.1)	0.48
TNF-R2	223.3 (167.7 - 297.2)	664 (386.9 - 1139.7)	<0.0001	5523.9 (5139.5 - 5937.1)	5233.5 (4602.4 - 5951.2)	0.46
VEGF	1414.3 (1207.1 - 1657.2)	1392.2 (1101.7 - 1759.3)	0.92	972.9 (851 - 1112.2)	778.6 (591.1 - 1025.5)	0.12
GMCSF	0.4 (0.3 - 0.4)	0.4 (0.3 - 0.5)	0.04	2 (1.7 - 2.3)	1.7 (1.6 - 1.8)	0.11
IFNγ	0.5 (0.4 - 0.6)	2.6 (1 - 6.5)	<0.0001	0.4 (0.4 - 0.4)	0.6 (0.4 - 0.8)	0.006
CHTR	92138 (63418 - 133864)	203461 (104388 - 396564)	0.04	136133 (113071 - 163899)	120344 (92366 - 156796)	0.49
YKL-40	712211 (528765 - 959301)	1053365 (608362 - 1823877)	0.21	1214096 (1009443 - 1460238)	877358 (655950 - 1173499)	0.07
PAI-1	27909.7 (17323 - 44965)	52458.4 (2784 - 9882)	<0.0001	104226 (88379.9 - 122914)	259787.7 (188698 - 357659)	<0.0001
MMP-9	4140181 (3110854 - 5510094)	6007625 (3306862 - 10914140)	0.23	2357117 (2121701 - 2618655)	2250246 (1714305 - 2953737)	0.7

Inflammatory mediators associated with exacerbations

Sputum mediators from paired samples

Table 6.2 shows mediators that were significantly elevated at exacerbation compared with their paired baseline samples were [geometric mean] (95%CI)

IL-6R 330.5 (225.8 - 483.8) vs.1067.4 (658.5 - 1730.2)p<0.0001]pg/mL,

IL-10 [2.4 (1.8 - 3.3) vs. 9.9 (4.6 - 21.4) p<0.001] pg/mL

IL-18 [31.3 (21.8 - 44.9) vs.55.1 (31.8 - 95.4)p=0.015]pg/mL,

CXCL-9 [630.5 (310.5 - 1280.3) vs.1990 (1038.2 - 3814.1)p=0.002]pg/mL

CXCL-10 [763.9 (432.7 - 1348.5) vs.1633.6 (900.2 - 2964.7)p=0.04]pg/mL

CCL-5 [9.8 (6.3 - 15.2) vs.21.9 (12.8 - 37.4)p=0.007]pg/mL

TNF-α [4.3 (2.6 - 7.2) vs.13 (5.5 - 30.5)p=0.02]pg/mL

TNF-R1 [626.5 (411 - 955.1) vs.902.8 (549.7 -1482.5)p=0.08]pg/mL

TNF-R2 [322.4 (195.7 - 531) vs.666.5 (381.3 -1165) p=0.009] pg/mL

IFNγ [0.6 (0.3 - 0.9) vs.2.5 (1 - 6.6)p=0.008]pg/mL

PAI-1 [50592.6 (20427.4 - 125303) vs.4876.8 (2576.5 - 9230.5)p<0.0001]pg/mL

Serum mediators from paired samples

Table 6.2 also shows serum mediators that were significantly elevated at exacerbation compared with their paired baseline [geometric mean (95%CI)]

IL-6R [21578.8(16667.6 - 27937.1) vs.50912.3 (37508.3 - 69106.5) p<0.001] pg/mL

YKL-40 [1474825.2(1186231-1833630) vs.890207.6 (660391 - 1199999.7) p=0.0002] pg/mL

PAI-1 [99902 (71160.8 - 140250) vs.260045 (187091.8 - 361445) p=0.0002] pg/mL

Sputum Mediators from unpaired baseline and exacerbation samples

Table 6.3 shows mediators that were significantly raised from at exacerbation compared with their baseline [geometric mean] (95%CI)

IL-6R 255.7 (205 - 318.9) vs.1051.9 (658.7 - 1679.8)p<0.0001]pg/mL,

IL-10 [2.4 (2 - 2.8) vs.10.1 (4.8 - 21.3)p<0.0001]pg/mL,

IL-18 [26.9 (22 - 32.8) vs.54.8 (32.3 - 93.2) p=0.003] pg/mL,

CCL5 [8.5 (6.8 - 10.6) vs.21.6 (12.9 - 36.3)p<0.0001]pg/mL,

CXCL10 [690.6 (502.4 - 949.4) vs.1677.1 (940.2 - 2991.4)p=0.007]pg/mL,

CXCL9 [557.3 (386.7 - 803.1) vs.1988.8 (1060 - 3731.5)p=0.001]pg/mL,

TNF-R2 [223.3 (167.7 - 297.2) vs.664 (386.9 - 1139.7) p<0.0001] pg/mL

TNFα [3.3 (2.4 - 4.5) vs.12.5 (5.4 - 28.7) p<0.0001] pg/mL

IFNγ [0.5 (0.4 - 0.6) vs.2.6 (1 - 6.5) p<0.0001] pg/mL

PAI-1 [27909.7(17323 - 44965) vs. 52458.4 (2784 - 9882) p<0.0001] pg/mL

Serum mediators from unpaired samples

Table 6.3 also shows serum mediators from unpaired baseline and exacerbation samples and the following showed significant rise at exacerbation [geometric mean] (95%CI)

IL-6R [24451 (20597 - 29027) vs.51194 (38044 - 68889) p<0.0001] pg/mL,

CXCL10 [122.7 (110 - 136.9) vs.171 (134.6 - 217.2) p=0.006] pg/mL,

IFN γ [0.4 (0.4 - 0.4) vs.0.6 (0.4 - 0.8) p=0.006] pg/mL,

PAI-1 [104226 (88379.9 - 122914) vs.259787.7 (188698 - 357659) p<0.0001] pg/mL

Table 6.4A shows ROC curves for sputum mediators in patients who exacerbated at least once 'first exacerbation' but did not include repeat exacerbation data from the same individual. Sputum mediator ROC curve data comparing all exacerbations to stable visits (including multiple exacerbations from same individuals) is shown in **Table 6.4B**.

Sputum IL-6R had the highest receiver operator characteristics to distinguish exacerbation [AUC=0.79(0.68-0.89)] followed by sputum IL-10 had similar sensitivity [AUC=0.78(0.68-0.88)], followed by sputum TNF-R2 [AUC=0.73(0.61-0.85)]; **figure 6.2.** Based on the ROC data, the paired and unpaired t test data and the fold change, sputum IL-6R was the best performing sputum biomarker to distinguish stable state from exacerbation.

Table 6.5A shows serum mediator ROC curve data comparing first exacerbations to first stable visits, table 4B shows all exacerbations and stable visits (including repeats from same individuals). The most sensitive mediators were PAI-1[AUC= 0.76 (0.65 - 0.87) p<0.0001] followed by IL-6R [AUC=0.69 (0.58 - 0.8) p<0.0001] and CXCL9 [AUC=0.64 (0.52 - 0.75) p=0.02]
Table 6.5B shows ROC analysis for serum mediator data for all exacerbations (including multiple exacerbations from the same individual) compared with all stable visits. In this comparison the best mediators are CXCL-11 [AUC=0.64(0.55-0.73) p=0.001] followed by IL-18 [AUC=0.62(0.53-0.7) p=0.01].

Serum Plasminogen Activator Inhibitor (PAI) emerged as the best serum biomarker of exacerbation [AUC=0.75(0.63-0.88)]; from various comparison; firstly its AUC value, secondly the fold change compared with baseline and thirdly based on paired and unpaired t testing with baseline. Other serum mediators performed poorly except serum IL-6R in parallel with sputum values showed a doubling from stable visits compared to exacerbations.

Mediators associated with stable asthma

The majority of Th2 serum mediators namely serum IL-5, IL-13 and YKL-40 were notable for their higher levels during stable visits compared with exacerbation and in

parallel with similar observations of their levels in sputum and can be seen in both paired and unpaired sample data in **tables 6.2 and 6.3.**

Viral and bacterial detection

Virus detection as determined by PCR was positive in 14% (6/41) of sputum samples during stable visits, and in 34% (8/23) samples during exacerbation. Using pooled data from all visits in the study **(data table not shown),** serum CCL3 (AUC=0.81 p<0.001) was the biomarker that best associated with virus positivity followed by serum periostin (AUC=0.79 p<0.001) and serum CXCL10 (AUC=0.75 p<0.001). Virus induced exacerbations were notable for their association with elevated sputum IFNγ in parallel with serum IFNγ. Sputum CXCL10 was elevated at exacerbation in both paired and unpaired comparisons. This difference was in parallel to serum CXCL10 levels.

Bacterial pathogen positivity was defined by a CFU count of $>10^7$ colonies/ml of sputum or culture positivity and was seen. We dichotomised baseline visits according to sputum CFU cut-off as seen in **table 6.6** and also generated ROC data based on the above cut-off in **table 6.7**. The best marker associating with a high CFU at exacerbation was sputum TNFR1 [AUC=0.79 (0.65-0.87)] followed by IL-1β [AUC=0.74 (0.62-0.82)]. There was a high correlation between sputum CFU counts and bacterial total qPCR (rs=0.547 p<0.001)

Mediator data from paired samples

Table 6.8 shows fold change between stable visits for both sputum and serum mediators which demonstrates that there is significant variability in the levels even during stable state.

Table 6.9A and B shows ROC data between stable and exacerbation visits for paired samples. The following mediators showed highest sensitivity in sputum
IL-6R [AUC=0.74 (0.61 - 0.87) p=0.002]
PAI-1[AUC= 0.73 (0.1 - 0.35) p=0.001]
IL-10 [AUC=0.71 (0.58 - 0.83) p=0.008]
For serum mediators in paired samples, the most sensitive were
IL-6R [AUC= 0.73 (0.6 - 0.85) p<0.0001]

PAI-1[AUC= 0.75 (0.62 - 0.87) p=0.001]. This data is similar to the unpaired samples seen in tables **6.4 A and B**

Data (or tables) not shown in this chapter of thesis

1. There was an increase in total bacterial 16S DNA as measured by qPCR at exacerbation this was non-significant by all comparisons and similar results for individual species of bacteria as measured by PCR.
2. Repeatability of the entire panel of mediators at stable visits comparing a combination of 2 visits or 3 visits showed moderate to poor kappa values as seen in chapter 5
3. Recovery visits were also compared to exacerbation and baseline data in a series of statistical analysis. The overall picture was of sputum and serum mediator levels being somewhere between stable and exacerbation state. ROC curve analysis showed very weak trends; indeed no parameter barring improving clinical symptoms could distinguish a recovery visit from an exacerbation.

Cluster analysis of sputum mediator exacerbations (data not shown as too bulky)

This revealed mediator clusters that were distinctly different to each other, clinical characteristics of the cytokine clusters were compared to identify any distinguishing features.

The bacterial exacerbation cluster had the highest CFU counts and bacterial 16S levels. 40% of subjects had new virus detected at the time of exacerbation. This group had the highest levels of IL-1α, IL-1b, IL-6R, TNFR1 and TNFR2 which have previously been associated with bacterial colonization.
The neutrophilic cluster had the highest level of sputum neutrophils and lowest eosinophils. There was no virus detected and the sputum CFU count was the second highest compared with other clusters. This group predominantly had elevated levels of IL-8.

The viral cluster had the highest level of virus detection with 80%positivity at exacerbation. Their clinical characteristics revealed this group had the lowest lung

function (FEV1% predicted) and had the worst VAS symptom score for cough. Their sputum was predominantly paucigranulocytic and had the lowest sputum CFU count compared with other clusters. This group had highest levels of CXCL9, CXCL10, CXCL11 and IFNγ. They also had the highest IL-13 and IL-5 levels and the highest IL-6 and TNF levels.

The eosinophilic cluster was distinctive clinically with the highest levels of FeNO though not statistically significant. They had the highest sputum eosinophils and lowest viral detection rate along with the lowest CFU count and 16S DNA levels compared with the other groups. Their cytokine profile was non distinct having only the highest levels of CCL17.

Table 6.4A.ROC area under the curve (AUC) (95% CI) for sputum mediators between first stable and first exacerbation samples

	Sputum (stable=88, exacerbation=31)	p value
IL-1α	0.52 (0.4 - 0.65)	0.62
IL-1β	0.58 (0.45 - 0.71)	0.05
IL 2	0.62 (0.51 - 0.73)	0.04
IL-4	0.5 (0.46 - 0.53)	0.65
IL-5	0.51 (0.38 - 0.63)	0.95
IL-6	0.63 (0.52 - 0.75)	0.02
IL-6R	0.79 (0.69 - 0.89)	<0.0001
IL-8	0.58 (0.47 - 0.7)	0.13
IL9	0.5 (0.43 - 0.57)	0.62
IL-10	0.72 (0.62 - 0.83)	<0.0001
IL-13	0.48 (0.37 - 0.59)	0.99
IL-15	0.6 (0.48 - 0.72)	0.01
IL-17	0.58 (0.49 - 0.68)	0.13
IL-18	0.63 (0.49 - 0.76)	0.005
CXCL9	0.69 (0.58 - 0.8)	0.001
CXCL10	0.65 (0.53 - 0.77)	0.009
CXCL11	0.55 (0.42 - 0.69)	0.2
CCL2	0.6 (0.49 - 0.72)	0.04
CCL3	0.55 (0.43 - 0.67)	0.4
CCL4	0.58 (0.46 - 0.71)	0.12
CCL5	0.69 (0.57 - 0.81)	0.001
CCL11	0.44 (0.32 - 0.56)	0.41
CCL13	0.4 (0.29 - 0.51)	0.08
CCL17	0.38 (0.25 - 0.51)	0.04
CCL26	0.45 (0.33 - 0.57)	0.55
TNFα	0.66 (0.54 - 0.78)	0.001
TNFR1	0.63 (0.51 - 0.76)	0.01
TNFR2	0.71 (0.59 - 0.82)	0.001
VEGF	0.49 (0.37 - 0.61)	0.92
GMCSF	0.55 (0.46 - 0.63)	0.06
IFNγ	0.72 (0.62 - 0.82)	<0.0001
YKL40	0.59 (0.46 - 0.72)	0.21
PAI1	0.29 (0.18 - 0.39)	0.001
MMP9	0.57 (0.44 - 0.71)	0.23

Table 6.4B ROC AUC (95% CI) for sputum between all stable and all exacerbation samples

	Sputum (stable=185; exacerbation=42)	p value
IL-1α	0.53 (0.43 - 0.64)	0.47
IL-1β	0.59 (0.48 - 0.7)	0.01
IL -2	0.62 (0.52 - 0.71)	0.003
IL-4	0.5 (0.48 - 0.53)	0.7
IL-5	0.53 (0.44 - 0.62)	0.67
IL-6	0.62 (0.53 - 0.72)	0.003
IL-6R	0.72 (0.64 - 0.81)	<0.0001
IL-8	0.59 (0.5 - 0.68)	0.05
IL-9	0.5 (0.45 - 0.55)	0.84
IL-10	0.7 (0.61 - 0.79)	<0.0001
IL-13	0.51 (0.42 - 0.6)	0.79
IL-15	0.59 (0.49 - 0.69)	0.005
IL-17	0.6 (0.52 - 0.67)	0.01
IL-18	0.63 (0.52 - 0.74)	0.002
CXCL-9	0.66 (0.57 - 0.76)	0.001
CXCL-10	0.63 (0.53 - 0.73)	0.005
CXCL-11	0.55 (0.44 - 0.66)	0.08
CCL-2	0.59 (0.49 - 0.68)	0.03
CCL-3	0.55 (0.45 - 0.64)	0.29
CCL-4	0.58 (0.48 - 0.68)	0.07
CCL-5	0.68 (0.58 - 0.78)	<0.0001
CCL-11	0.46 (0.36 - 0.56)	0.55
CCL-13	0.44 (0.35 - 0.53)	0.22
CCL-17	0.42 (0.32 - 0.52)	0.06
CCL-26	0.51 (0.42 - 0.61)	0.7
TNF-α	0.64 (0.54 - 0.74)	<0.0001
TNF-R1	0.63 (0.53 - 0.73)	0.002
TNF-R2	0.7 (0.6 - 0.79)	<0.0001
VEGF	0.51 (0.41 - 0.62)	0.91
GMCSF	0.54 (0.47 - 0.61)	0.04
IFNγ	0.7 (0.61 - 0.78)	<0.0001
YKL-40	0.63 (0.52 - 0.73)	0.01
PAI-1	0.45 (0.36 - 0.54)	0.13
MMP-9	0.6 (0.49 - 0.72)	0.02

Table 6.5A. ROC area under the curve (AUC) (95% CI) serum mediators between first stable and first exacerbation samples

	Serum (stable=96; exacerbation=35)	p value
IL-1α	0.47 (0.44 - 0.49)	-
IL-1β	0.33 (0.21 - 0.44)	0.002
IL 2	0.47 (0.42 - 0.51)	0.3
IL-4	0.52 (0.46 - 0.57)	0.19
IL-5	0.34 (0.23 - 0.45)	0.009
IL-6	0.49 (0.38 - 0.59)	0.58
IL-6R	0.69 (0.58 - 0.8)	<0.0001
IL-8	0.5 (0.38 - 0.62)	0.7
IL9	0.51 (0.48 - 0.54)	0.53
IL-10	0.53 (0.44 - 0.63)	0.76
IL-13	0.44 (0.38 - 0.5)	0.39
IL-15	0.56 (0.44 - 0.68)	0.18
IL-17	0.57 (0.49 - 0.65)	0.21
IL-18	0.58 (0.48 - 0.69)	0.2
CXCL9	0.64 (0.52 - 0.75)	0.02
CXCL10	0.63 (0.51 - 0.74)	0.008
CXCL11	0.59 (0.48 - 0.71)	0.06
CCL2	0.45 (0.33 - 0.57)	0.19
CCL3	0.51 (0.47 - 0.56)	0.68
CCL4	0.49 (0.37 - 0.61)	0.56
CCL5	0.5 (0.46 - 0.53)	0.83
CCL11	0.45 (0.34 - 0.57)	0.35
CCL13	0.52 (0.4 - 0.64)	0.78
CCL17	0.45 (0.34 - 0.56)	0.46
CCL26	0.44 (0.31 - 0.56)	0.49
TNFα	0.48 (0.37 - 0.59)	0.5
TNFR1	0.45 (0.34 - 0.57)	0.47
TNFR2	0.46 (0.34 - 0.58)	0.45
VEGF	0.43 (0.32 - 0.55)	0.12
GMCSF	0.45 (0.41 - 0.49)	0.17
IFNγ	0.61 (0.5 - 0.71)	0.01
YKL40	0.4 (0.29 - 0.52)	0.08
PAI1	0.76 (0.65 - 0.87)	<0.0001
MMP9	0.52 (0.39 - 0.65)	0.7

Table 6.5B ROC AUC (95% CI) for serum mediators between all stable and all exacerbation samples

	Serum (stable=214; exacerbation=49)	p value
IL-1α	0.48 (0.46 - 0.49)	-
IL-1β	0.42 (0.33 - 0.51)	0.14
IL -2	0.48 (0.45 - 0.51)	0.39
IL-4	0.49 (0.45 - 0.53)	0.96
IL-5	0.49 (0.4 - 0.58)	0.91
IL-6	0.52 (0.43 - 0.6)	0.34
IL-6R	0.6 (0.51 - 0.69)	0.046
IL-8	0.52 (0.42 - 0.62)	0.96
IL-9	0.5 (0.48 - 0.52)	0.75
IL-10	0.54 (0.46 - 0.61)	0.92
IL-13	0.47 (0.44 - 0.51)	0.56
IL-15	0.53 (0.43 - 0.62)	0.45
IL-17	0.54 (0.48 - 0.61)	0.87
IL-18	0.62 (0.53 - 0.7)	0.01
CXCL-9	0.6 (0.51 - 0.69)	0.02
CXCL-10	0.6 (0.5 - 0.7)	0.002
CXCL-11	0.64 (0.55 - 0.73)	0.001
CCL-2	0.45 (0.35 - 0.55)	0.12
CCL-3	0.5 (0.46 - 0.53)	0.57
CCL-4	0.48 (0.39 - 0.58)	0.38
CCL-5	0.48 (0.45 - 0.51)	0.16
CCL-11	0.5 (0.4 - 0.6)	0.8
CCL-13	0.54 (0.45 - 0.64)	0.24
CCL-17	0.52 (0.43 - 0.61)	0.93
CCL-26	0.54 (0.43 - 0.64)	0.21
TNF-α	0.53 (0.44 - 0.63)	0.68
TNF-R1	0.51 (0.42 - 0.6)	0.91
TNF-R2	0.53 (0.43 - 0.62)	0.54
VEGF	0.49 (0.4 - 0.58)	0.55
GMCSF	0.46 (0.43 - 0.49)	0.16
IFNγ	0.58 (0.49 - 0.67)	0.007
YKL-40	0.53 (0.44 - 0.63)	0.66
PAI-1	0.6 (0.51 - 0.7)	0.04
MMP-9	0.52 (0.42 - 0.62)	0.8

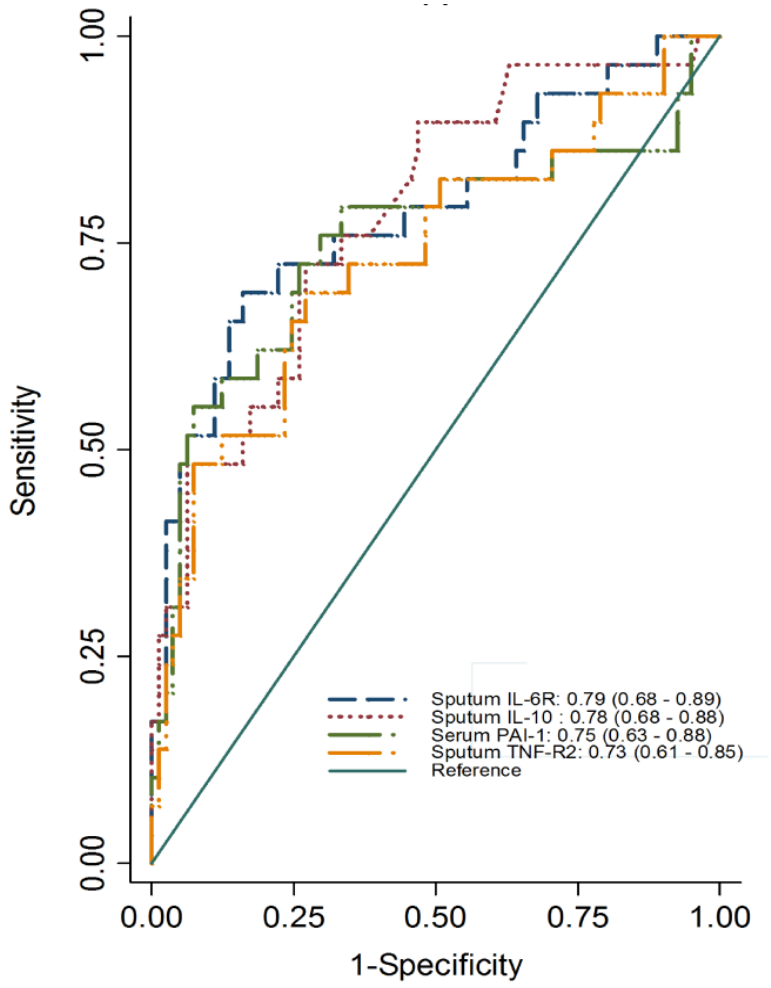


Figure 6.2

ROC curve for sputum and mediators that best distinguished between stable state and exacerbations (only the 4 highest performing mediators are selected).

Table 6.6 Sputum mediators dichotomised using sputum CFU cutoffs to detect bacterial colonisation

	Sputum CFU <=10^7/ ml or –ve culture (n=61)	Sputum CFU >10^7/ ml or +ve culture (n=27)	P-value
IL-1α	43.5 (35.3 - 53.5)	86 (52.3 - 141.3)	0.004
IL-1β	49.9 (36.5 - 68.3)	146.7 (94.7 - 227.1)	<0.0001
IL-2	0.7 (0.5 - 0.9)	0.8 (0.5 - 1.5)	0.44
IL-4	0.3 (0.3 - 0.4)	0.3 (0.3 - 0.4)	0.94
IL-5	1.8 (1.2 - 2.8)	5.2 (2.7 - 10)	0.009
IL-6	32.2 (21.1 - 49.2)	83.7 (50.5 - 138.5)	0.011
IL-6r	214.4 (162.4 - 283.1)	380.6 (279.2 - 518.8)	0.018
IL-8	2186.4 (1541.1 - 3101.7)	6484 (4414.2 - 9524.4)	<0.0001
IL9	2.8 (2.3 - 3.5)	3.9 (3.1 - 4.9)	0.066
IL-10	0.5 (0.4 - 0.8)	1.5 (0.7 - 3)	0.009
IL-13	6.9 (5.3 - 9)	10.2 (6.6 - 15.8)	0.12
IL-15	1.4 (1.1 - 1.8)	1.9 (1.3 - 2.9)	0.2
IL-17	2.6 (1.9 - 3.5)	3.4 (2 - 5.9)	0.34
IL-18	16.2 (11.8 - 22.4)	45.6 (32.8 - 63.2)	<0.0001
CCL2	214.9 (167.3 - 276.1)	460.4 (312.6 - 678.2)	0.002
CCL3	22 (15.7 - 30.8)	54.5 (31.8 - 93.6)	0.005
CCL4	252.7 (160.6 - 397.8)	743.8 (422.9 - 1308.2)	0.008
CCL5	6.5 (5 - 8.4)	14.8 (9.9 - 22)	0.001
CCL11	42.9 (31 - 59.3)	82.4 (54.4 - 124.9)	0.025
CCL13	15.7 (11.5 - 21.3)	23.9 (14.4 - 39.7)	0.15
CCL17	20.4 (15.1 - 27.7)	42.9 (23.8 - 77.5)	0.018
CCL26	7.4 (4.7 - 11.5)	13.9 (7.3 - 26.3)	0.12
CXCL9	390.2 (255.6 - 595.6)	1246.8 (670.4 - 2318.9)	0.003
CXCL10	501 (340.3 - 737.5)	1426.4 (899.6 - 2261.6)	0.002
CXCL11	39.4 (26.3 - 59)	102.6 (50.9 - 206.7)	0.016
TNFα	2.2 (1.6 - 3.2)	6.8 (3.5 - 13.2)	0.002
TNFR1	356.1 (271.9 - 466.3)	943.1 (689.5 - 1290)	<0.0001
TNFR2	159.8 (113.8 - 224.5)	475 (314.6 - 717.2)	<0.0001
VEGF	1183.5 (992.4 - 1411.4)	2113.9 (1598.5 - 2795.5)	0.001
GMCSF	0.2 (0.2 - 0.2)	0.2 (0.2 - 0.3)	0.039
IFNγ	0.2 (0.1 - 0.2)	0.3 (0.1 - 0.6)	0.12
CHTR	65468 (42479 - 100896)	196891 (102970 - 376479)	0.007
YKL-40	503555 (353858 - 716582)	1538810 (994448 - 2381156)	<0.0001
PAI-1	20166 (11342 - 35852)	54468.6 (23034.4 - 128799.8)	0.064
MMP9	3238432 (2298162 - 4563405)	7146476.5 (4500775 - 11347405)	0.011

All values are in pg/ml

Table 6.7 ROC AUC (95% CI) for sputum mediators between bacterial colonised and not-colonised

	Sputum (stable=61; exacerbation =27)	P-value
IL-1α	0.71 (0.59 - 0.84)	0.006
IL-1β	0.76 (0.65 - 0.87)	0.001
IL -2	0.52 (0.39 - 0.65)	0.42
IL-4	0.48 (0.45 - 0.5)	-
IL-5	0.68 (0.56 - 0.8)	0.01
IL-6	0.66 (0.54 - 0.78)	0.01
IL-6R	0.67 (0.56 - 0.79)	0.02
IL-8	0.75 (0.64 - 0.85)	0.001
IL-9	0.53 (0.45 - 0.61)	0.61
IL-10	0.66 (0.54 - 0.77)	0.03
IL-12p70	0.57 (0.47 - 0.67)	0.13
IL-13	0.57 (0.45 - 0.7)	0.18
IL-15	0.58 (0.45 - 0.71)	0.08
IL-17	0.47 (0.39 - 0.55)	0.6
IL-18	0.75 (0.65 - 0.86)	0.001
IL-23	0.53 (0.48 - 0.58)	0.4
CXCL-9	0.69 (0.57 - 0.8)	0.006
CXCL-10	0.7 (0.58 - 0.81)	0.004
CXCL-11	0.65 (0.53 - 0.78)	0.02
CCL-2	0.73 (0.61 - 0.86)	0.004
CCL-3	0.66 (0.54 - 0.79)	0.009
CCL-4	0.67 (0.55 - 0.79)	0.01
CCL-5	0.73 (0.62 - 0.84)	0.002
CCL-11	0.64 (0.51 - 0.77)	0.03
CCL-13	0.6 (0.47 - 0.73)	0.08
CCL-17	0.64 (0.51 - 0.77)	0.02
CCL-26	0.6 (0.46 - 0.73)	0.17
TNF-α	0.71 (0.59 - 0.83)	0.004
TNF-R1	0.79 (0.69 - 0.88)	0.001
TNF-R2	0.74 (0.63 - 0.85)	0.001
VEGF	0.7 (0.58 - 0.83)	0.002
GMCSF	0.58 (0.49 - 0.66)	0.14
IFNγ	0.56 (0.46 - 0.66)	0.09
EGF	0.73 (0.6 - 0.86)	0.001
SCF	0.66 (0.55 - 0.77)	0.007
ST2	0.6 (0.49 - 0.7)	0.03
CHTR	0.72 (0.6 - 0.83)	0.01
YKL-40	0.74 (0.63 - 0.85)	0.001
PAI-1	0.62 (0.49 - 0.75)	0.07
MMP-9	0.67 (0.55 - 0.78)	0.01

Table 6.8 Sputum and serum mediators fold change (95% CI) between first and second stable visits

	Sputum (n=37)	Serum (n=50)
IL-1α	0.69 (0.41 - 1.19)	1.05 (0.97 - 1.14)
IL-1β	0.58 (0.29 - 1.18)	0.48 (0.35 - 0.65)
IL -2	0.63 (0.42 - 0.95)	0.98 (0.94 - 1.03)
IL-4	0.97 (0.93 - 1.02)	1.15 (1.02 - 1.3)
IL-5	0.39 (0.22 - 0.68)	0.5 (0.38 - 0.66)
IL-6	0.42 (0.24 - 0.74)	1.04 (0.82 - 1.33)
IL-6R	1.45 (0.92 - 2.3)	2.27 (1.68 - 3.07)
IL-8	0.64 (0.39 - 1.04)	0.93 (0.79 - 1.09)
IL-9	0.91 (0.81 - 1.02)	1.04 (0.95 - 1.13)
IL-10	0.77 (0.53 - 1.11)	0.92 (0.75 - 1.13)
IL-12p70	0.86 (0.74 - 1)	0.99 (0.95 - 1.04)
IL-13	0.76 (0.61 - 0.95)	0.89 (0.79 - 1)
IL-15	0.8 (0.65 - 0.97)	1.05 (0.96 - 1.14)
IL-17	0.8 (0.6 - 1.07)	1.04 (0.99 - 1.08)
IL-18	0.68 (0.4 - 1.14)	0.91 (0.82 - 1.02)
CXCL-9	0.63 (0.34 - 1.17)	1.18 (1.01 - 1.38)
CXCL-10	0.64 (0.39 - 1.06)	1.02 (0.89 - 1.17)
CXCL-11	0.39 (0.21 - 0.73)	0.82 (0.68 - 0.98)
CCL-2	0.67 (0.49 - 0.92)	0.91 (0.83 - 1)
CCL-3	0.49 (0.3 - 0.8)	0.98 (0.96 - 1.01)
CCL-4	0.63 (0.34 - 1.16)	0.93 (0.85 - 1.02)
CCL-11	0.57 (0.39 - 0.81)	0.75 (0.67 - 0.85)
CCL-13	0.52 (0.4 - 0.69)	0.87 (0.81 - 0.93)
CCL-17	0.78 (0.51 - 1.2)	0.62 (0.48 - 0.81)
CCL-26	0.5 (0.3 - 0.83)	0.42 (0.31 - 0.57)
TNF-α	0.41 (0.22 - 0.77)	0.56 (0.39 - 0.8)
TNF-R1	0.55 (0.36 - 0.85)	0.97 (0.89 - 1.06)
TNF-R2	0.54 (0.31 - 0.94)	0.95 (0.86 - 1.05)
VEGF	0.74 (0.55 - 1.01)	0.81 (0.68 - 0.95)
GMCSF	0.89 (0.75 - 1.06)	0.93 (0.78 - 1.12)
IFNγ	0.8 (0.48 - 1.36)	1.2 (0.97 - 1.5)
CHTR	0.42 (0.21 - 0.83)	1 (0.86 - 1.17)
YKL-40	0.23 (0.12 - 0.46)	0.63 (0.5 - 0.8)
PAI-1	0.04 (0.01 - 0.08)	1.91 (1.45 - 2.51)
MMP-9	0.3 (0.16 - 0.58)	0.89 (0.76 - 1.04)

Reference is one; and the data should be interpreted as follows: e.g., 0.69 means the mediator decreased at second stable by 31%; however, if the 95% CI included **one** then that change isn't significant. The same interpretation applied if the change is above one, but it is increasing rather than decreasing.

Table 6.9A Receiver operator (ROC) area under the curve (AUC) (95% CI) for sputum mediators between paired stable and exacerbation samples

	Sputum (n=30)	**p-value**
IL-1α	0.53 (0.38 - 0.68)	0.61
IL-1β	0.57 (0.41 - 0.72)	0.19
IL -2	0.59 (0.45 - 0.74)	0.23
IL-4	0.5 (0.45 - 0.55)	0.52
IL-5	0.43 (0.28 - 0.58)	0.29
IL-6	0.6 (0.45 - 0.74)	0.13
IL-6R	0.74 (0.61 - 0.87)	0.002
IL-8	0.55 (0.4 - 0.7)	0.33
IL-9	0.51 (0.44 - 0.59)	0.51
IL-10	0.71 (0.58 - 0.83)	0.008
IL-13	0.49 (0.36 - 0.63)	0.98
IL-15	0.59 (0.46 - 0.73)	0.08
IL-17	0.5 (0.38 - 0.62)	0.94
IL-18	0.59 (0.44 - 0.75)	0.1
IL-23	0.5 (0.45 - 0.55)	0.63
CXCL-9	0.65 (0.52 - 0.79)	0.03
CXCL-10	0.63 (0.49 - 0.77)	0.08
CXCL-11	0.53 (0.37 - 0.68)	0.51
CCL-2	0.55 (0.4 - 0.7)	0.36
CCL-3	0.5 (0.35 - 0.65)	0.89
CCL-4	0.59 (0.44 - 0.73)	0.29
CCL-5	0.66 (0.52 - 0.8)	0.03
CCL-11	0.43 (0.28 - 0.58)	0.4
CCL-13	0.33 (0.19 - 0.47)	0.02
CCL-17	0.38 (0.23 - 0.53)	0.12
CCL-26	0.42 (0.27 - 0.57)	0.53
TNF-α	0.63 (0.48 - 0.78)	0.04
TNF-R1	0.57 (0.42 - 0.72)	0.27
TNF-R2	0.64 (0.49 - 0.78)	0.07
VEGF	0.44 (0.29 - 0.59)	0.57
GMCSF	0.54 (0.44 - 0.63)	0.34
IFNγ	0.67 (0.55 - 0.8)	0.02
CHTR	0.54 (0.37 - 0.7)	0.48
YKL-40	0.5 (0.33 - 0.66)	0.89
PAI-1	0.73 (0.1 - 0.35)	0.001
MMP-9	0.48 (0.32 - 0.65)	0.84

Table 6.9B Receiver operator (ROC) area under the curve (AUC) (95% CI) for serum mediators between paired stable and exacerbation samples

	Serum (n=34)	P-value
IL-1α	0.47 (0.43 - 0.51)	0.81
IL-1β	0.33 (0.19 - 0.46)	0.02
IL -2	0.44 (0.37 - 0.51)	0.22
IL-4	0.51 (0.45 - 0.58)	0.6
IL-5	0.32 (0.19 - 0.45)	0.01
IL-6	0.46 (0.32 - 0.59)	0.23
IL-6R	0.73 (0.6 - 0.85)	<0.0001
IL-8	0.51 (0.37 - 0.66)	0.7
IL-9	0.51 (0.49 - 0.54)	-
IL-10	0.48 (0.35 - 0.6)	0.45
IL-13	0.43 (0.35 - 0.51)	0.44
IL-15	0.47 (0.32 - 0.61)	0.56
IL-17	0.52 (0.42 - 0.62)	0.98
IL-18	0.48 (0.34 - 0.62)	0.76
IL-33	0.5 (0.46 - 0.54)	0.49
CXCL-9	0.53 (0.39 - 0.67)	0.87
CXCL-10	0.56 (0.42 - 0.7)	0.3
CXCL-11	0.54 (0.4 - 0.68)	0.55
CCL-2	0.42 (0.28 - 0.56)	0.17
CCL-4	0.47 (0.33 - 0.61)	0.65
CCL-5	0.51 (0.47 - 0.56)	0.55
CCL-11	0.42 (0.28 - 0.55)	0.2
CCL-13	0.46 (0.32 - 0.6)	0.38
CCL-17	0.38 (0.24 - 0.52)	0.12
CCL-26	0.41 (0.27 - 0.55)	0.32
TNF-α	0.45 (0.31 - 0.59)	0.43
TNF-R1	0.41 (0.27 - 0.54)	0.22
TNF-R2	0.41 (0.27 - 0.56)	0.37
VEGF	0.44 (0.31 - 0.58)	0.39
GMCSF	0.44 (0.37 - 0.51)	0.25
IFNγ	0.56 (0.43 - 0.69)	0.35
CHTR	0.46 (0.31 - 0.61)	0.52
YKL-40	0.34 (0.2 - 0.48)	0.01
PAI-1	0.75 (0.62 - 0.87)	0.001
MMP-9	0.49 (0.34 - 0.64)	0.44

6.4 Discussion

In this observational longitudinal study of severe asthmatics we studied the clinical characteristics of the population during exacerbations and have identified multiple inflammatory mediators associated with these events. There were no clinically distinguishing features other than increasing severity of disease was associated with likelihood of exacerbations. Measures of airway inflammation like FeNO and sputum differential were not informative and sputum eosinophilia was not a predominant pattern in this population. During exacerbations there was a modest fall in lung function, a marked change in symptom scores but little difference in inflammatory indices in sputum or blood.

A rise in total sputum cell count was the most significant difference at exacerbation as overall differential count as whole did not change to make the overall exacerbations predominantly eosinophilic or neutrophilic. This does suggest that inflammation increased predominantly in the airway compartment. The subjects at baseline were neutrophilic predominantly, and at exacerbations a similar neutrophilic pattern was seen. This is in keeping with findings from another study of asthma exacerbations, the nature of airway inflammation can change between consecutive exacerbations [D'Silva 2007]. They also showed that the pattern of airway inflammation during consecutive exacerbations was different in half the study population of patients and the same in the remaining half of patients. In my study, patients that were eosinophilic at baseline were more likely to have eosinophilic exacerbations (and similarly with neutrophilia at baseline) but the small number of exacerbations did not allow to study any change from one type of cellular inflammation to another. The change in the nature of sputum inflammation is of particular importance as there may be a need to offer a different type of therapy-eosinophilia at baseline may not necessarily mean eosinophilia at exacerbation [Green 2002]. This points to the need for accurately assessing airway inflammation not only during stable visits.

Inflammation appeared to be localised mainly to the airway, with no significant changes in peripheral blood differential profile. When serum mediator profiling was undertaken there were marked changes in multiple chemokines and cytokines which

were not reflected in the peripheral differential inflammation. The mediator with the highest fold increase at exacerbation was IL-6R a receptor complex of the pleiotropic cytokine IL-6 which is associated with a wide variety of inflammatory conditions. In conditions like rheumatoid arthritis it correlates with disease activity and in asthma, IL-6R polymorphisms been identified in GWAS studies as a marker of asthma severity and are associated with poor lung function [Morjaria 2010]. In another study, circulating IL-6R levels during exacerbations were elevated in response several fold compared to baseline [Yokoyama 1997].We found there is an increase in both sputum and serum IL-6R levels suggesting a generalised inflammatory cascade but more localized to the airway. My findings are in keeping with a study of asthma exacerbations [Chu 2015] which demonstrated elevated IL-6 and IL-β levels in sputum but this study did not compare exacerbations to any stable visits but does prove the presence of IL-6 and the correlation with severe exacerbations.

I found the next best discriminant of an exacerbation was sputum IL-10 which is intriguing, mainly due to the association of IL-10 in asthma with viruses. The levels of IL-10 during a virus associated exacerbation increase according to one study [Grissel 2005] whilst this may suggest that our study did not detect viruses sufficiently highly and therefore elevated IL-10 in the study was due to undetected viremia; it is equally plausible that IL-10 elevation was induced by the other cytokines as has been postulated by another study [Grissel 2005]. Another study implicated that deficient production of IL-10 may be due to the effects of rhinovirus and this study showed a higher Th2 response at time of exacerbation with elevated sputum IL-4 and IL-5 [Message 2008]. The findings from the last study suggest that the lack of a fall in IL-10 levels and elevation in other Th2 cytokines suggests that in this group of severe asthmatics, exacerbations may not be driven by viruses. IL-10 is a counter regulatory cytokine that aims to establish synergy between Th1 and Th2 mechanisms, and in my study it may be a reflection of counteracting Th1 responses.

Another cytokine which was elevated significantly during exacerbations in both sputum and serum was Plasminogen Activator Inhibitor. PAI-1 is thought to promote airway remodelling is inhibits both the fibrinolytic system and the matrix metalloproteinase (MMP) system [Ma 2009].

A recent study report that common colds are associated with increased PAI-1 production in airways of asthmatic subjects [Cho 2014]. The study involved sputum and nasal fluid mediator analysis during exacerbations of asthma precipitated by Human Rhinovirus. The study found the common cold increased PAI-1 levels in upper and lower airways of asthmatics, but not in control subjects. Lastly, in vitro, HRV induced epithelial production of PAI-1. In another experimental study of rhinovirus infection [Majoor 2014] HRV-16 challenge led to increased PAI-1 levels in plasma in patients with asthma. HRV-16 load in BAL showed a linear correlation with the fold change in PAI-1. The observations from these studies suggest that virus infection was most possibly the aetiology of exacerbation in my study, though the pickup rate for detection remained low. The utility of serum measurement makes it more attractive as a biomarker to try and phenotype a viral exacerbation. However, the levels will need to be measured in exacerbations from other aetiologies in order to derive a 95%CI or any reference ranges; indeed if it is going to be utilised as a useful biomarker tool.

In the study, I could attribute only a small number of exacerbation events due to microbial aetiology. The lack of expected virus associated exacerbations may be simply due to the lack of adequately sensitive detection methods, though the PCR techniques perform better than traditional virus culture and similar PCR techniques were used in other studies [Bafadhel 2011] and have widely been accepted. The other possibility is the virus shedding from the airway was completed by the time the patients presented with an exacerbation for study assessment. A 72 hour cut off from onset of exacerbation was applied to all study subjects and those presenting later than this window did not have their data included in the study. Interestingly, the exacerbation recovery visits (data not shown for any recovery visits that were timed 2 weeks after exacerbation) when pooled together along with exacerbation visits did increase the overall rate of virus detection. Virus-induced IFN-γ responses in blood mononuclear cells were inversely associated with viral shedding after inoculation, [Gern 2000] and stronger T helper-1-like responses in sputum cells (higher IFN-γ/IL-5 mRNA ratio) during induced colds were associated with milder cold symptoms and more rapid clearance of the virus [Parry 2000].

My results could also suggest the aetiology of exacerbations was spontaneous in nature, in a group of patients who had ongoing airway and to a lesser extent, systemic inflammation in the background- a worsening in the disequilibrium of the homeostasis. Th2 pathway upregulation has been described during exacerbations but in my study, Th2 mediator levels did not change significantly and levels of IL-5 and IL-13 fell compared to stable periods. We found an upregulation of the majority of Th1 mediators differentially associated with neutrophilia, apoptosis and epithelial damage but Th2 mediators were higher during stable periods, suggesting Th2 pathways undergo a rapid switch to Th1 pathways during exacerbations.

There are limitations to my observations. There were only a relatively small number of exacerbations that were recorded, and only a small number of exacerbations were missed. Whether this low number of exacerbations was because subjects had regular access to an experienced asthma physician and was while on the study cannot be elucidated. Patients that self-report symptoms and have easy access to non-specialist or primary healthcare when asthma control is poor may be wrongly classified as having exacerbations of asthma hence leading to an over diagnosis of acute illness. Dissecting out these differences and accurately classifying a true exacerbation by performing a detailed examination, lung function and inflammation testing will allow reducing risks from overtreatment.

However, dissecting periods of poor control from mild exacerbations may be difficult using physiological and quality of life measures. In my study there was a significant worsening in symptoms based on VAS, yet the ACQ value did not change significantly. Similarly the fall in lung function was modest with most subjects reporting a fall in their personal best peak flows and acting on their personalised asthma management plan.

Grading severity of exacerbations traditionally uses peak flow measurement and is entirely valid in a population with milder disease. However the same cut offs cannot be used in our population of severe asthmatics for reasons of safety. At time of presentation, all subjects had significantly increased their background medication use and were using increased β2 agonist. A worsening or non-improvement in symptoms and lung function despite the increased medication triggered need for oral

Prednisolone treatment, but this tipping point was naturally prone to bias by subject variability and symptom perception. However the results from my study would indicate that patients presented with genuine symptoms related to worsening inflammation.

Viral exacerbations have been implicated in other studies in up to 70% of asthma exacerbations [Wark 2006]. In my study viral detection rate remained low during exacerbations, which was surprising given the high sensitivity of our assay and its performance in other studies in COPD exacerbation [Bafadhel 2011]. But, viral implication is suggested due to the relatively low number of non-eosinophilic exacerbations combined with an increase in levels of CXCL9, CXCL10 and IFNγ which are all virus biomarkers. I did not assess if patients were experiencing a cold or flu like illness and the length of time that may have precipitated the exacerbation and is conceivable that at time of presentation, the virus replication had already taken place and was therefore undetectable by PCR analysis, however the cytopathic effects of virus leading to damaged airway epithelium and activation of innate immune pathways was reflected in mediator expression.

Though small volumes of sputum affected sputum culture rates, it did not affect CFU counts but we did not find significantly increased CFUs compared with baseline during exacerbations. Furthermore, levels of sputum IL-1β which from previous work [Bafadhel 2011] are highly correlated with bacterial presence rose only modestly at exacerbation in this study. There may be a few explanations for these findings. It is possible this group of severe asthmatics had sputum colonisation at the outset and exacerbations being predominantly viral or inflammatory in origin, there were truly no bacterial exacerbations and consequently no significant rise in CFU or IL-1β. We also undertook 16S DNA analysis on the sputum and the total 16S copies did increase compared to baseline but again did not reach statistical significance. The 16S data therefore strengthens our inferences from CFU counts and is complimentary to cytokine data. However the results from my study also suggest that the airways in asthma are far from sterile. It appears highly implausible that this effect is specific to severe asthma, but future studies similar to mine could incorporate a spectrum of asthma severity and have healthy controls and subjects

with other airways disease namely COPD as comparator subgroups. When I analysed the clinical characteristics of patients with sputum CFU above and below the 10^7cutoff; patients with higher CFU had lower lung volumes and a predominant mixed granulocytic sputum inflammation. This group also had significantly higher levels of inflammatory mediators including IL-1α, IL-β, and TNFα and of Th2 cytokines including IL-5 and IL-13.

Whether the presence of this type of inflammation in the airways is attributable only to the presence of bacteria or whether it is being driven by immune processes leading to poor lung function and subsequent bacterial colonization is unclear. It is probable that there is bronchiectasis or bronchial wall thickening in these subjects though imaging studies were not carried out. This group may benefit from the combination of an antibiotic and anti-inflammatory like azithromycin but anti neutrophilic agents may also help with breaking the inflammatory cycle.

The cluster data is impeded mainly the number of exacerbation events recorded was low and therefore a larger study with more captured events would be useful to define aetiopathology and biomarkers. However, the proportion of events and cluster wise distribution of patients was not dissimilar to Bafadhel, suggesting that the airway does react in similar fashion to the same triggering stimuli in COPD or asthma- a unifying airway theory.

The bacterial cluster had the highest levels of IL-1β and IL-6R which are associated with bacterial colonization. The neutrophilic cluster is very similar in cytokine and clinical description to the previous cluster and is probably one and the same group as this group had only a slightly lower CFU and 16S count compared to the previous cluster.

The viral cluster had the highest levels of the CXC chemokines and IFN γ all of which have been described with strong viral association. The elevated IL-5, IL-13 and IL-6 levels may suggest virus induced eosinophil/neutrophil degranulation in the airway- as this group was paucigranulocytic. The eosinophilic group had the lowest levels of measured mediators compared with all groups and the lack of elevation in

the eosinophil derived cytokines may suggest that degranulation may occur in the future- and detection may be possible by measuring these at a later time point in the exacerbation.

The clusters with their differing cytokine levels raise questions for future studies. If no clinical characteristics can help identify exacerbations with different inflammatory patterns then measurement of the mediators may guide towards therapy. For example a cluster with elevated IL-1α, IL-β could be associated with bacterial infection and therefore be treated with antibiotics and not with steroids. The main difficulty is to identify a cut off value for a high or low mediator level which will require measurements in all grades of severity in asthma and comparison with a healthy group.

I have shown in this study that there are many sputum mediators that undergo a several fold increase at the time of exacerbation these inflammatory changes may not be reflected in the traditional measures of airway inflammation. Whilst there is significant inter visit variability of mediator measurements during stable visits, the fold change during exacerbations suggests that it is a genuine mechanism with activation of an inflammatory cascade. Sputum analysis is not easily performed at exacerbation but serum markers performed more poorly than serum mediators at ROC analysis. The balance between utility and accuracy is important to clinicians and serum markers are more easily obtained. It may therefore be useful to have a panel of serum markers and these could be in combination used to rule in or rule out an exacerbation.

Desired characteristics of a clinically useful asthma biomarker include: it should accurately reflect airway inflammation, it should be mechanistically linked to the therapeutic target, it should be measured noninvasively, and it should be easily measured on robust standardized assay platforms. Ideally, detection of the therapeutic target would be the most direct approach to identifying patients most likely to benefit from treatment. Studies have associated clinical factors like early initiation of therapy, atopy, male gender, presence of bronchodilator reversibility and non-smokers to be better responders to steroids. There remains the need to develop

validated biomarkers for personalized therapy. An example is of serum periostin as the best single predictor of airway eosinophilia, and subsequently, as a biomarker to guide therapy. In my study sputum IL-6R performed best to differentiate exacerbations from stable state thus serving as an objective biomarker. Further work will need studying differences of IL-6 across the spectrum of severity of asthma, health and other comparable airways disease mainly COPD. It would be easy to say that targeting IL-6 pathway may result in reduction of exacerbation frequency but a similar thought process was applied to anti TNF therapy in asthma which turned out to be an unsuccessful venture. Indeed we need to establish whether the IL-6 upregulation at exacerbation is a cause or effect

In summary we have shown that for a population of subjects with severe asthma there are a number of inflammatory pathways that are upregulated even during quiescent periods, these undergo a switch at exacerbation resulting in large increases in mediators both in the airway and systemically. A viral aetiology to exacerbations is implicated yet it is possible that for this subgroup increases in underlying inflammation resulted in these events.

I look forward to work being done in the future on this subject with larger numbers of subjects and crucially capturing more exacerbation events. Finally no data to define a biomarker would be complete without comparisons with validation cohorts, requiring collaborative research and data sharing from other studies in the future.

Chapter 7

Final discussion and scope of future work.

In this thesis I have studied the inflammatory profiling of cytokines and the relationship at scalar levels with cell, tissue and organ in severe asthma.

Firstly, I performed exploratory biased and unbiased multivariate cluster and factor analysis. This led to the novel observation that a unique cytokine pattern underlies clinical profiles of patients. The temporal patterns of inflammation when comparing cellular inflammation was broadly similar suggesting that there is a good degree of correlation to the cellular profile. Clinical distinction using cluster or dichotomised outcomes highlighted several differences in the inflammatory mediators. However when a reverse approach using cluster analysis of mediators were performed there were no distinguishing clinical characteristics that could suggest the underlying pathobiology. This may suggest that measuring mediators to identify 'high' or 'low' groups may identify the best potentials for targeted therapies. One drawback from my analysis was that I had no healthy controls to derive a 'cut-off' as demonstrated by Fahy and Woodruff using a 'gene mean' analysis. The exploratory chapter led to the novel observation that IL-5 levels were elevated in a subset of obese asthmatics. Future work will entail linking large datasets to other datasets- be it GWAS trait association or imaging studies and applying similar systems biology approaches.

In order to further explore the effects of obesity in asthma I made the novel discovery that the effects of IL-5 elevation could not simply be explained by airway inflammation. In order to confirm these findings I made similar comparisons in a subset of patients with COPD. For the first time, I describe a subset of obese asthmatics that have submucosal eosinophilia. Our findings since the publication of the data have been replicated in other cohorts of patients.

Fahy and colleagues [ATS 2014 abstract] compared sputum mRNA levels of IL-4, IL-5 and IL-13 signatures across a group of patients with mild to moderate asthma and healthy controls in order to identify a gene-mean cut-off between health and disease and thereby identify Th2 high disease. They compared the prevalence of this Th2 high signature between obese and lean asthmatics and found similar proportions of

obese asthmatics had high Th2 signatures as the lean group (46% vs. 41%). The obese group however had significantly higher exacerbations and lower lung function compared with the lean group.

A Belgian group [Marijsse 2014] looked at data from their previously published study measuring sputum cell counts and sputum mRNA in asthmatics. They detected no significant difference in the sputum IL-4 and IL-13 mRNA levels between the BMI classes; however sputum IL-5 mRNA levels were different between the different BMI classes with a significantly higher IL-5 mRNA level in the sputum of obese asthmatics as compared to the lean group. They also found a significant correlation (r_s=0.18, P=0.03) between sputum IL-5 mRNA levels and BMI. Sputum eosinophils were not significantly different between groups, but obese asthmatics had significantly higher neutrophils. The lack of any differences of IL-4 and IL-13 levels between BMI groups argues against the predominance of classical Th2 airway inflammation in obese asthmatics. They found that sputum IL-17 and IL-25 levels were elevated in the obese groups [Marijsse 2014] suggesting the raised mediator levels along with the raised sputum neutrophils are indicative of the presence of an inflammatory phenotype as also reported by Telenga [2012]. In my study there was no increased neutrophil count nor did we find a difference in sputum IL-17 between the different BMI classes.

A Dutch group [van der Weil 2014] looked retrospectively at previous studies where they had investigated a large cohort of 147 predominantly mild-to-moderate asthma with assessment of eosinophilic inflammation in blood, sputum and bronchial wall biopsies. They found obese patients had significantly higher numbers of submucosal eosinophils and lower sputum eosinophil percentages than non-obese patients. Using conventional criteria, sputum or blood eosinophilia was rarely found in the group of obese subjects, despite the higher number of submucosal eosinophils. In obese subjects there were significant positive associations between numbers of submucosal eosinophils and blood eosinophils (r=0.515, p=0.010) and between blood eosinophils and sputum eosinophils (r=0.716, p<0.001). However, they found no significant association between the number of submucosal eosinophils and the percentage of sputum eosinophils in obese subjects (r=0.276, p=0.268).

Comparing asthma patients with and without submucosal eosinophils (>0.00 and 0.00 EPX+ eosinophils respectively), they found that the presence of submucosal eosinophils was associated with a higher BMI, never smoking and fewer pack-years smoking. Further in a multivariate regression analysis adjusting for the above parameters, a higher BMI was still positively associated with a higher number of EPX+ eosinophils (b=0.095, p=0.009). Thus, the association of obesity with higher bronchial eosinophil numbers in asthma appears to be independent of gender, smoking, pack-years and atopy. In non-obese subjects, there were significant but weak positive associations for numbers of submucosal eosinophils and blood eosinophils (r=0.232, p=0.030, between number of submucosal eosinophils and percentage of sputum eosinophils (r=0.396, p=0.001), and a stronger positive association between number of blood eosinophils and percentage of sputum eosinophils. Thus the findings of submucosal eosinophilia is replicated in the group of obese patients with albeit milder asthma, but the difference in severity of asthmatic subjects may explain the discrepancy of significantly lower sputum eosinophilia in obese asthmatics.

The study of dupilumab an anti IL-4/IL-13 receptor antagonist enrolled patients with moderate to severe disease (GINA 4 and below), the average BMI was high 31.4 kg/m^2 and they demonstrated elevated peripheral blood eosinophilia. The difference in severity of asthma in these subjects may again explain why the above group had peripheral eosinophilia as compared to my findings.

These findings from these studies provide further evidence that the relationship between blood, tissue and luminal eosinophilic inflammation is different in asthmatics with and without obesity. We described in severe asthma elevated sputum IL-5 and bronchial eosinophilic inflammation in obese asthmatics without an associated increase in blood or sputum eosinophils. In these and previous studies asthma associated with obesity is consistently characterised by an absence of a sputum eosinophilia. This apparent paradox between tissue and luminal eosinophilic inflammation remains inadequately explained in all of these studies. In our study, we were unable to demonstrate increased macrophage uptake of luminal eosinophils suggesting that the increased number in the tissue is due to increased retention in the lumen wall rather than more eosinophils trafficking from blood through the airway wall into the lumen with increased phago/efferocytosis of luminal eosinophils.

Increased retention in the airway wall might be a consequence of increased adhesion in the bronchial submucosa in obese asthmatics but may also reflect altered survival or clearance in the tissue-the mechanism is unclear. The role of the eosinophil is unclear despite the correlations we have described. From our own findings the focus is on location and role of eosinophils in obese asthmatics. There is a possibility that examination of the adipose tissue would help determine whether eosinophil numbers were different in groups stratified according to BMI and presence of asthma.

In contrast, to the consistency of the observation between studies of increased tissue eosinophilia and an absence of a sputum eosinophilia our study suggested the degree of sputum eosinophilia and neutrophilia was not different in asthmatics with and without obesity, whereas in the other studies there was a reduced sputum eosinophilia or the presence of elevated sputum neutrophilia. This difference in the pattern of inflammation in the airway lumen perhaps reflects differential expression of important cytokines between the studies.

What remains unclear is whether the increase in sputum IL-5 and a tissue eosinophilia in obese asthmatics is clinically important. A sputum eosinophilia is associated with a good response to corticosteroids and Mepolizumab. Therefore, whether obese asthmatics with a tissue eosinophilia without a sputum eosinophilia represent a group of asthmatics that respond to therapy is unknown. Furthermore, whether obese asthmatics with a tissue eosinophilia are at particular risk of exacerbations or lung function decline is also unknown. Our study then does present a consistent message that the inflammatory profile is different between asthmatics with and without obesity with increased sputum IL-5 and a tissue eosinophilia in obese asthmatics. Future studies need to explore the underlying mechanisms and clinical importance of these differences in inflammatory profiles. It also asks questions about drug therapy like Mepolizumab would be successful by affecting eosinophil recruitment and activation and whether anti eosinophil therapy like CRTh2 receptor blockade may also be successful.

Airway wall geometry is related to airway remodelling and cellular inflammation. The observation that mediator profiling is related to airway wall thickness is described for the first time. Measurement of the airway thickness or the lack of it in conjunction

with airway inflammation may inform of the underlying inflammation. The cytokines associated with the inflamed remodelled airway are not unique, but their role in the complex pathogenesis may probably have become clearer. One limitation is that it was not a dynamic longitudinal assessment and whether a cross sectional analysis would be enough to draw conclusions on a process which probably evolves over long periods can only be understood by studying the natural history of the disease. It is plausible to follow up a cohort of patients for several years or even decades with regular interval CT scanning and detailed attention to clinical features and concomitant measurement of airway wall thickness.

Exacerbations have traditionally been defined only by symptoms and diminishing lung function, it remains difficult to dissect periods of poor control. The number of genuine exacerbations was relatively low for what has been historically described in this group. I have shown that measurement of sputum IL-6R may allow objectively differentiating stable disease from exacerbation.

Indeed we need to establish whether the IL-6 upregulation at exacerbation is a cause or effect and in my study I have been unable to prove if these raised levels are causal to an exacerbation or an effect of an inflammatory cascade. IL-6 pathway blockade may theoretically prevent this inflammatory cascade and thus prevent exacerbations and indeed anti IL-6 therapies are being trialled in condition causing chronic inflammation like rheumatoid arthritis. A similar thought process was applied in trials of anti TNF therapy in severe asthma but turned out to be unsuccessful with numerous adverse events and no appreciable benefit. However, it may be possible that heterogeneity exists within these patients and those with the highest levels of upregulation may benefit from pathway antagonism. There may be a subset of patients more suited for the above intervention than others.

The bacterial detection was targeted towards the four bacteria seen in the airways of patients with severe asthma and COPD. However this did not include *Chlamydia* or *Mycoplasma Pneumoniae* in the specific qPCR probes. Wark [2002] observed a fourfold increase in *Chlamydia Pneumonaie* IgG or IgA titers in 38% of patients with acute asthma. In the Telicast study, IgM for *Chlamydia Pneumoniae* was positive in around 60% of cases (Johnston 2002) In an study from Israel, screening for 12 respiratory tract pathogens, including five bacterial species, by serology, *Chlamydia*

Pneumoniae was the only bacterial agent significantly more frequent (18%) in 100 hospitalized asthmatic adults than in matched controls; *Mycoplasma Pneumoniae* prevalence in the former group was lower (8%) (Leiberman 2003]. The possibility therefore in my study is that the pathogens did exist but they I never set out to measure them. A future study should therefore include a wider variety of pathogens and especially *Chlamydia* and *Mycoplasma*.

In my study I performed sputum culture, CFU count and 16S amplification. Each of these techniques answers different questions. Sputum culture rate was very low-less than 5 percent of all samples had sufficient sputum for culture. This could have been maximised by attempting sputum induction on all comers. The 16S amplification was only targeted towards 4 bacterial species. I could have included gram negative bacteria like *Pseudomonas* and *Klebsiella* in order to broaden my pick up rate. Another shortcoming is that bacterial detection was only in one compartment namely sputum. It may be of further value to perform serological testing that is contemporaneous to sputum tests. Finally there is little or no data about how different specimen collection techniques perform against one another. Whether a bronchoalveolar lavage is necessary to be more certain of picking up lower respiratory tract bacteria is going to be only answered by future studies. Observational studies of the pickup rates of BAL versus induced sputum versus oropharyngeal aspirates are necessary in order to again inform us about the investigation of choice in order to maximise pick up.

The aetiology of exacerbations is diverse and includes medical comorbidities like GORD and obesity amongst a few. I have not taken this into account mainly due to the low numbers of exacerbations recorded. The statistical analysis ideally should allow for correction against these factors but was underpowered. Another aspect that was not recorded was the presence of the ‘seasonal flu’ vaccination and pneumovax immunisation in my cohort of patients. Being under the care of the DAC usually means that their uptake with vaccination is higher than the rest of the population and may have an influence. Each patient visit at DAC runs through a checklist including vaccination; reminders are sent to their primary care providers. It may not be possible to measure the influence of immunisation as it would be ethically injustifiable to withhold such an intervention for the purposes of a study trial. However a trial constructed in a manner that included subjects with a first

presentation with an exacerbation may allow the measure of this effect as an incidence prevalence ratio.

The role of adherence in severe asthma is well recognised. A study from DAC Glenfield was performed where prescription data was audited and identified <80% adherence in upto 65% of patients on inhalers- either ICS alone or in combination [Murphy 2012]. This is the same population from which my study population is drawn from. Patient adherence can be difficult to quantify and during my studies I did not rely upon any objective evidence to assess adherence. At each visit the subject was asked the question as to whether they were taking their medication as prescribed; this was more relevant during exacerbation visits but I could not subjectively attribute the lack of adherence leading to an exacerbation. However my approach was not sufficient to pick up non compliance and should have included compliance checking.

I also did not attempt to measure whether and how medication was actually taken. Inhaler competence, the skill to inhale correctly, is particularly relevant for asthma medication, as inhaling of drugs requires considerable skill and practice [Price 2013]. Even if medication is taken daily, deposition in the lungs will be low with incorrect inhalation technique. I am however confident that inhaler technique did not have a factor to play in my study mainly because the DAC population was supported by a team of asthma nurses whose role included inhaler education.

In the last chapter, I have also shown that a majority of exacerbations in severe asthma have a predominant Th1 pattern of inflammation as opposed to Th2 biology. It remains to be seen if this is unique to severe asthma and whether mild or moderate asthmatics have a different exacerbation profile, more leaning towards Th2. This can only be done by using larger datasets and capturing more numbers of these events. Finally I identified biomarkers of viral and bacterial presence and results are similar to findings from previous published work. The presence of inflammatory exacerbations in my group of severe asthmatics suggests the need for more anti-inflammatory strategies above and beyond steroids but most importantly attempting to accurately characterise inflammation in severe asthma.
In the time it has taken for me to write up this thesis fully, drugs like Mepolizumab appear close to being accepted for patient treatment and selection is being based on peripheral blood eosinophil count as opposed to sputum inflammation- I have shown

how obesity affects these relationships- and this may allow a subgroup of patients access to novel therapy. Near patient tests to detect presence of eosinophilia or markers of neutrophilic inflammation are in development, allowing phenotyping of exacerbations of asthma. Several consortia are being set up at both local and international level (AIRPROM) and the data and results from several chapters are being fed into a massive computational model and will allow validation of my work and future studies being based on their background.

I have, in my thesis achieved the aims based on my hypothesis. I sincerely hope that findings of my work may help clinicians to unlock the paradigm of severe asthma for the benefit of patients.

References

Agache, I., Akdis, C., Jutel, M., & Virchow, J. C. (2012). Untangling asthma phenotypes and endotypes. Allergy, 67(7), 835-846.

Akbari, O., Faul, J. L., Hoyte, E. G., Berry, G. J., Wahlstrom, J., Kronenberg, M., et al. (2006). CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. The New England Journal of Medicine, 354(11), 1117-1129.

Albert, R. K., Connett, J., Bailey, W. C., Casaburi, R., Cooper, J. A.,Jr, Criner, G. J., et al. (2011). Azithromycin for prevention of exacerbations of COPD. The New England Journal of Medicine, 365(8), 689-698.

Alexander, M. R., Moehle, C. W., Johnson, J. L., Yang, Z., Lee, J. K., Jackson, C. L., et al. (2012). Genetic inactivation of IL-1 signaling enhances atherosclerotic plaque instability and reduces outward vessel remodeling in advanced atherosclerosis in mice. The Journal of Clinical Investigation, 122(1), 70-79.

Amin, K., Janson, C., Boman, G., & Venge, P. (2005). The extracellular deposition of mast cell products is increased in hypertrophic airways smooth muscles in allergic asthma but not in nonallergic asthma. Allergy, 60(10), 1241-1247.

Amin, K., Ludviksdottir, D., Janson, C., Nettelbladt, O., Bjornsson, E., Roomans, G. M., et al. (2000). Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR group. American Journal of Respiratory and Critical Care Medicine, 162(6), 2295-2301.

Amrani, Y., Panettieri, R. A.,Jr, Frossard, N., & Bronner, C. (1996). Activation of the TNF alpha-p55 receptor induces myocyte proliferation and modulates agonist-evoked calcium transients in cultured human tracheal smooth muscle cells. American Journal of Respiratory Cell and Molecular Biology, 15(1), 55-63.

Anderson, G. P. (2008). Endotyping asthma: New insights into key pathogenic mechanisms in a complex, heterogeneous disease. Lancet, 372(9643), 1107-1119..

Angrill, J., Agusti, C., De Celis, R., Filella, X., Rano, A., Elena, M., et al. (2001). Bronchial inflammation and colonization in patients with clinically stable bronchiectasis. American Journal of Respiratory and Critical Care Medicine, 164(9), 1628-1632.

Angrill, J., Agusti, C., de Celis, R., Rano, A., Gonzalez, J., Sole, T., et al. (2002). Bacterial colonisation in patients with bronchiectasis: Microbiological pattern and risk factors. Thorax, 57(1), 15-19.

Auffray, C., Chen, Z., & Hood, L. (2009). Systems medicine: The future of medical genomics and healthcare. Genome Medicine, 1(1), 2.

Aysola, R. S., Hoffman, E. A., Gierada, D., Wenzel, S., Cook-Granroth, J., Tarsi, J., et al. (2008). Airway remodeling measured by multidetector CT is increased in severe asthma and correlates with pathology. Chest, 134(6), 1183-1191.

Bafadhel, M., McCormick, M., Saha, S., McKenna, S., Shelley, M., Hargadon, B., et al. (2012). Profiling of sputum inflammatory mediators in asthma and chronic obstructive pulmonary disease. Respiration; International Review of Thoracic Diseases, 83(1), 36-44.

Bafadhel, M., McKenna, S., Terry, S., Mistry, V., Pancholi, M., Venge, P., et al. (2012). Blood eosinophils to direct corticosteroid treatment of exacerbations of chronic obstructive pulmonary disease: A randomized placebo-controlled trial. American Journal of Respiratory and Critical Care Medicine, 186(1), 48-55.

Bafadhel, M., McKenna, S., Terry, S., Mistry, V., Reid, C., Haldar, P., et al. (2011). Acute exacerbations of chronic obstructive pulmonary disease: Identification of biologic clusters and their biomarkers. American Journal of Respiratory and Critical Care Medicine, 184(6), 662-671.

Bafadhel, M., Saha, S., Siva, R., McCormick, M., Monteiro, W., Rugman, P., et al. (2009). Sputum IL-5 concentration is associated with a sputum eosinophilia and attenuated by corticosteroid therapy in COPD. Respiration; International Review of Thoracic Diseases, 78(3), 256-262.

Bai, T. R., Vonk, J. M., Postma, D. S., & Boezen, H. M. (2007). Severe exacerbations predict excess lung function decline in asthma. The European Respiratory Journal, 30(3), 452-456.

Baines, K. J., Simpson, J. L., Wood, L. G., Scott, R. J., Fibbens, N. L., Powell, H., et al. (2014). Sputum gene expression signature of 6 biomarkers discriminates asthma inflammatory phenotypes. The Journal of Allergy and Clinical Immunology, 133(4), 997-1007.

Baines, K. J., Simpson, J. L., Wood, L. G., Scott, R. J., & Gibson, P. G. (2011). Transcriptional phenotypes of asthma defined by gene expression profiling of induced sputum samples. The Journal of Allergy and Clinical Immunology, 127(1), 153-60, 160.e1-9.

Barker, B. L., & Brightling, C. E. (2013). Phenotyping the heterogeneity of chronic obstructive pulmonary disease. Clinical Science (London, England : 1979), 124(6), 371-387.

Barker, B. L., McKenna, S., Mistry, V., Pancholi, M., Patel, H., Haldar, K., et al. (2014). Systemic and pulmonary inflammation is independent of skeletal muscle changes in patients with chronic obstructive pulmonary disease. International Journal of Chronic Obstructive Pulmonary Disease, 9, 975-981.

Barnes, P. J., Pedersen S. (1993).Efficacy and safety of inhaled corticosteroids in asthma. American Review of Respiratory Disease, Vol. 148, Supplement: Efficacy and Safety of Inhaled Corticosteroids in Asthma. pp. S1-S26.

Barnes, P. J. (2008). The cytokine network in asthma and chronic obstructive pulmonary disease. The Journal of Clinical Investigation, 118(11), 3546-3556.

Barnes, P. J. (2010). Mechanisms and resistance in glucocorticoid control of inflammation. The Journal of Steroid Biochemistry and Molecular Biology, 120(2-3), 76-85.

Basehore, M. J., Howard, T. D., Lange, L. A., Moore, W. C., Hawkins, G. A., Marshik, P. L., et al. (2004). A comprehensive evaluation of IL4 variants in ethnically diverse populations: Association of total serum IgE levels and asthma in white subjects. The Journal of Allergy and Clinical Immunology, 114(1), 80-87.

Bel, E. H., Sousa, A., Fleming, L., Bush, A., Chung, K. F., Versnel, J., et al. (2011). Diagnosis and definition of severe refractory asthma: An international consensus statement from the innovative medicine initiative (IMI). Thorax, 66(10), 910-917.

Benayoun, L., Druilhe, A., Dombret, M. C., Aubier, M., & Pretolani, M. (2003). Airway structural alterations selectively associated with severe asthma. American Journal of Respiratory and Critical Care Medicine, 167(10), 1360-1368.

Berger, P., Girodet, P. O., Begueret, H., Ousova, O., Perng, D. W., Marthan, R., et al. (2003). Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 17(14), 2139-2141.

Berry, M., Morgan, A., Shaw, D. E., Parker, D., Green, R., Brightling, C., et al. (2007). Pathological features and inhaled corticosteroid response of eosinophilic and non-eosinophilic asthma. Thorax, 62(12), 1043-1049.

Berry, M. A., Hargadon, B., Shelley, M., Parker, D., Shaw, D. E., Green, R. H., et al. (2006). Evidence of a role of tumor necrosis factor alpha in refractory asthma. The New England Journal of Medicine, 354(7), 697-708.

Beuther, D. A., & Sutherland, E. R. (2007). Overweight, obesity, and incident asthma: A meta-analysis of prospective epidemiologic studies. American Journal of Respiratory and Critical Care Medicine, 175(7), 661-666.

Birring, S. S., Brightling, C. E., Bradding, P., Entwisle, J. J., Vara, D. D., Grigg, J., et al. (2002). Clinical, radiologic, and induced sputum features of chronic obstructive pulmonary disease in nonsmokers: A descriptive study. American Journal of Respiratory and Critical Care Medicine, 166(8), 1078-1083.

Borish, L. C., Nelson, H. S., Corren, J., Bensch, G., Busse, W. W., Whitmore, J. B., et al. (2001). Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. The Journal of Allergy and Clinical Immunology, 107(6), 963-970.

Borish, L. C., Nelson, H. S., Lanz, M. J., Claussen, L., Whitmore, J. B., Agosti, J. M., et al. (1999). Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial. American Journal of Respiratory and Critical Care Medicine, 160(6), 1816-1823.

Boudier, A., Curjuric, I., Basagana, X., Hazgui, H., Anto, J. M., Bousquet, J., et al. (2013). Ten-year follow-up of cluster-based asthma phenotypes in adults. A pooled analysis of three cohorts. American Journal of Respiratory and Critical Care Medicine, 188(5), 550-560.

Bousquet, J., Anto, J. M., Sterk, P. J., Adcock, I. M., Chung, K. F., Roca, J., et al. (2011). Systems medicine and integrated care to combat chronic noncommunicable diseases. Genome Medicine, 3(7), 43.

Bradding, P., Roberts, J. A., Britten, K. M., Montefort, S., Djukanovic, R., Mueller, R., et al. (1994). Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: Evidence for the human mast cell as a source of these cytokines. American Journal of Respiratory Cell and Molecular Biology, 10(5), 471-480.

Braman, S. S. (2006). The global burden of asthma. Chest, 130(1 Suppl), 4S-12S.

Brasier, A. R., Victor, S., Ju, H., Busse, W. W., Curran-Everett, D., Bleecker, E., et al. (2010). Predicting intermediate phenotypes in asthma using bronchoalveolar lavage-derived cytokines. Clinical and Translational Science, 3(4), 147-157.

Brightling, C. E. (2006). Clinical applications of induced sputum. Chest, 129(5), 1344-1348.

Brightling, C. E., Bleecker, E. R., Panettieri, R. A.,Jr, Bafadhel, M., She, D., Ward, C. K., et al. (2014). Benralizumab for chronic obstructive pulmonary disease and sputum eosinophilia: A randomised, double-blind, placebo-controlled, phase 2a study. The Lancet.Respiratory Medicine, 2(11), 891-901.

Brightling, C. E., & Desai, D. (2013). Reply: Role of primary necrosis/lysis of submucosal eosinophils in obese individuals with asthma. American Journal of Respiratory and Critical Care Medicine, 188(12), 1468-1469.

Brightling, C. E., McKenna, S., Hargadon, B., Birring, S., Green, R., Siva, R., et al. (2005). Sputum eosinophilia and the short term response to inhaled mometasone in chronic obstructive pulmonary disease. Thorax, 60(3), 193-198.

Brightling, C. E., Monteiro, W., Ward, R., Parker, D., Morgan, M. D., Wardlaw, A. J., et al. (2000). Sputum eosinophilia and short-term response to prednisolone in chronic obstructive pulmonary disease: A randomised controlled trial. Lancet, 356(9240), 1480-1485.

Brightling, C. E., Symon, F. A., Birring, S. S., Bradding, P., Pavord, I. D., & Wardlaw, A. J. (2002). TH2 cytokine expression in bronchoalveolar lavage fluid T lymphocytes and bronchial submucosa is a feature of asthma and eosinophilic bronchitis. The Journal of Allergy and Clinical Immunology, 110(6), 899-905.

Brightling, C. E., Ward, R., Woltmann, G., Bradding, P., Sheller, J. R., Dworski, R., et al. (2000). Induced sputum inflammatory mediator concentrations in eosinophilic bronchitis and asthma. American Journal of Respiratory and Critical Care Medicine, 162(3 Pt 1), 878-882.

Brims, F. J., Chauhan, A. J., Higgins, B., & Shute, J. K. (2009). Coagulation factors in the airways in moderate and severe asthma and the effect of inhaled steroids. Thorax, 64(12), 1037-1043.

Brusselle, G. G., Vanderstichele, C., Jordens, P., Deman, R., Slabbynck, H., Ringoet, V., et al. (2013). Azithromycin for prevention of exacerbations in severe asthma (AZISAST): A multicentre randomised double-blind placebo-controlled trial. Thorax, 68(4), 322-329.

Burrowes, K. S., De Backer, J., Smallwood, R., Sterk, P. J., Gut, I., Wirix-Speetjens, R., et al. (2013). Multi-scale computational models of the airways to unravel the pathophysiological mechanisms in asthma and chronic obstructive pulmonary disease (AirPROM). Interface Focus, 3(2), 20120057.

Busacker, A., Newell, J. D.,Jr, Keefe, T., Hoffman, E. A., Granroth, J. C., Castro, M., et al. (2009). A multivariate analysis of risk factors for the air-trapping asthmatic phenotype as measured by quantitative CT analysis. Chest, 135(1), 48-56.

Busse, W., Corren, J., Lanier, B. Q., McAlary, M., Fowler-Taylor, A., Cioppa, G. D., et al. (2001). Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma. The Journal of Allergy and Clinical Immunology, 108(2), 184-190.

Busse, W. W., Israel, E., Nelson, H. S., Baker, J. W., Charous, B. L., Young, D. Y., et al. (2008). Daclizumab improves asthma control in patients with moderate to severe persistent asthma: A randomized, controlled trial. American Journal of Respiratory and Critical Care Medicine, 178(10), 1002-1008.

Calhoun, W. J., Ameredes, B. T., King, T. S., Icitovic, N., Bleecker, E. R., Castro, M., et al. (2012). Comparison of physician-, biomarker-, and symptom-based strategies for adjustment of inhaled corticosteroid therapy in adults with asthma: The BASALT randomized controlled trial. JAMA : The Journal of the American Medical Association, 308(10), 987-997.

Carroll, N. G., Mutavdzic, S., & James, A. L. (2002). Distribution and degranulation of airway mast cells in normal and asthmatic subjects. The European Respiratory Journal, 19(5), 879-885.

Castro, M., Fain, S. B., Hoffman, E. A., Gierada, D. S., Erzurum, S. C., Wenzel, S., et al. (2011). Lung imaging in asthmatic patients: The picture is clearer. The Journal of Allergy and Clinical Immunology, 128(3), 467-478.

Castro, M., Rubin, A. S., Laviolette, M., Fiterman, J., De Andrade Lima, M., Shah, P. L., et al. (2010). Effectiveness and safety of bronchial thermoplasty in the treatment of severe asthma: A

multicenter, randomized, double-blind, sham-controlled clinical trial. American Journal of Respiratory and Critical Care Medicine, 181(2), 116-124.

Cazzola, M., Matera, M. G., & Rossi, F. (1991). Bronchial hyperresponsiveness and bacterial respiratory infections. Clinical Therapeutics, 13(1), 157-171.

Chagani, T., Pare, P. D., Zhu, S., Weir, T. D., Bai, T. R., Behbehani, N. A., et al. (1999). Prevalence of tumor necrosis factor-alpha and angiotensin converting enzyme polymorphisms in mild/moderate and fatal/near-fatal asthma. American Journal of Respiratory and Critical Care Medicine, 160(1), 278-282.

Chanez, P., Wenzel, S. E., Anderson, G. P., Anto, J. M., Bel, E. H., Boulet, L. P., et al. (2007). Severe asthma in adults: What are the important questions? The Journal of Allergy and Clinical Immunology, 119(6), 1337-1348.

Chapman, R. W., Phillips, J. E., Hipkin, R. W., Curran, A. K., Lundell, D., & Fine, J. S. (2009). CXCR2 antagonists for the treatment of pulmonary disease. Pharmacology & Therapeutics, 121(1), 55-68.

Chen, W., Ericksen, M. B., Levin, L. S., & Khurana Hershey, G. K. (2004). Functional effect of the R110Q IL13 genetic variant alone and in combination with IL4RA genetic variants. The Journal of Allergy and Clinical Immunology, 114(3), 553-560.

Cheng, G., Arima, M., Honda, K., Hirata, H., Eda, F., Yoshida, N., et al. (2002). Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. American Journal of Respiratory and Critical Care Medicine, 166(3), 409-416.

Chipps, B. E., Zeiger, R. S., Borish, L., Wenzel, S. E., Yegin, A., Hayden, M. L., et al. (2012). Key findings and clinical implications from the epidemiology and natural history of asthma: Outcomes and treatment regimens (TENOR) study. The Journal of Allergy and Clinical Immunology, 130(2), 332-42.e10.

Chlumsky, J., Striz, I., Terl, M., & Vondracek, J. (2006). Strategy aimed at reduction of sputum eosinophils decreases exacerbation rate in patients with asthma. The Journal of International Medical Research, 34(2), 129-139.

Cho, S.H., Hong, S.J., Chen, H., Habib, A., Cho, D., Lee, S.H., Kang, J., Ward, T., Boushey, H.A., Schleimer, R.P. and Avila, P.C., 2014. Plasminogen activator inhibitor-1 in sputum and nasal lavage fluids increases in asthmatic patients during common colds. The Journal of allergy and clinical immunology, 133(5), pp.1465-7.

Choy, E. H., & Panayi, G. S. (2001). Cytokine pathways and joint inflammation in rheumatoid arthritis. The New England Journal of Medicine, 344(12), 907-916.

Chu, D. K., Al-Garawi, A., Llop-Guevara, A., Pillai, R. A., Radford, K., Shen, P., et al. (2015). Therapeutic potential of anti-IL-6 therapies for granulocytic airway inflammation in asthma. Allergy, Asthma, and Clinical Immunology : Official Journal of the Canadian Society of Allergy and Clinical Immunology, 11(1), 14.

Chu, H. W., Honour, J. M., Rawlinson, C. A., Harbeck, R. J., & Martin, R. J. (2003). Effects of respiratory mycoplasma pneumoniae infection on allergen-induced bronchial hyperresponsiveness and lung inflammation in mice. Infection and Immunity, 71(3), 1520-1526.

Chung, K. F., Wenzel, S. E., Brozek, J. L., Bush, A., Castro, M., Sterk, P. J., et al. (2014). International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. The European Respiratory Journal, 43(2), 343-373.

Churg, A., Zhou, S., Wang, X., Wang, R., & Wright, J. L. (2009). The role of interleukin-1beta in murine cigarette smoke-induced emphysema and small airway remodeling. American Journal of Respiratory Cell and Molecular Biology, 40(4), 482-490.

Commins, S. P., Borish, L., & Steinke, J. W. (2010). Immunologic messenger molecules: Cytokines, interferons, and chemokines. The Journal of Allergy and Clinical Immunology, 125(2 Suppl 2), S53-72.

Contoli, M., Message, S. D., Laza-Stanca, V., Edwards, M. R., Wark, P. A., Bartlett, N. W., et al. (2006). Role of deficient type III interferon-lambda production in asthma exacerbations. Nature Medicine, 12(9), 1023-1026.

Corne, J. M., Lau, L., Scott, S. J., Davies, R., Johnston, S. L., & Howarth, P. H. (2001). The relationship between atopic status and IL-10 nasal lavage levels in the acute and persistent inflammatory response to upper respiratory tract infection. American Journal of Respiratory and Critical Care Medicine, 163(5), 1101-1107.

Corren, J., Busse, W., Meltzer, E. O., Mansfield, L., Bensch, G., Fahrenholz, J., et al. (2010). A randomized, controlled, phase 2 study of AMG 317, an IL-4Ralpha antagonist, in patients with asthma. American Journal of Respiratory and Critical Care Medicine, 181(8), 788-796.

Corren, J., Lemanske, R. F., Hanania, N. A., Korenblat, P. E., Parsey, M. V., Arron, J. R., et al. (2011). Lebrikizumab treatment in adults with asthma. The New England Journal of Medicine, 365(12), 1088-1098.

De Boever, E. H., Ashman, C., Cahn, A. P., Locantore, N. W., Overend, P., Pouliquen, I. J., et al. (2014). Efficacy and safety of an anti-IL-13 mAb in patients with severe asthma: A randomized trial. The Journal of Allergy and Clinical Immunology, 133(4), 989-996.

Demoulin, J. B., Van Roost, E., Stevens, M., Groner, B., & Renauld, J. C. (1999). Distinct roles for STAT1, STAT3, and STAT5 in differentiation gene induction and apoptosis inhibition by interleukin-9. The Journal of Biological Chemistry, 274(36), 25855-25861

Denning D, O'Driscoll B.R, Hogaboam C.R, Boweyer P, Niven R.M. (2006) The link between fungi and severe asthma: a summary of the evidence. European Respiratory Journal ; 27: 615–626

Desai, D., Gupta, S., Siddiqui, S., Singapuri, A., Monteiro, W., Entwisle, J., et al. (2013). Sputum mediator profiling and relationship to airway wall geometry imaging in severe asthma. Respiratory Research, 14, 17-9921-14-17.

Desai, D., Newby, C., Symon, F. A., Haldar, P., Shah, S., Gupta, S., et al. (2013). Elevated sputum interleukin-5 and submucosal eosinophilia in obese individuals with severe asthma. American Journal of Respiratory and Critical Care Medicine, 188(6), 657-663.

Desmouliere, A., Geinoz, A., Gabbiani, F., & Gabbiani, G. (1993). Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. The Journal of Cell Biology, 122(1), 103-111.

Deykin, A., Lazarus, S. C., Fahy, J. V., Wechsler, M. E., Boushey, H. A., Chinchilli, V. M., et al. (2005). Sputum eosinophil counts predict asthma control after discontinuation of inhaled corticosteroids. The Journal of Allergy and Clinical Immunology, 115(4), 720-727.

Dinarello, C. A. (1999). IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. The Journal of Allergy and Clinical Immunology, 103(1 Pt 1), 11-24.

Djukanovic, R., Harrison, T., Johnston, S. L., Gabbay, F., Wark, P., Thomson, N. C., et al. (2014). The effect of inhaled IFN-beta on worsening of asthma symptoms caused by viral infections. A randomized trial. American Journal of Respiratory and Critical Care Medicine, 190(2), 145-154.

Djukanovic, R., Wilson, S. J., Kraft, M., Jarjour, N. N., Steel, M., Chung, K. F., et al. (2004). Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma. American Journal of Respiratory and Critical Care Medicine, 170(6), 583-593.

Doe, C., Bafadhel, M., Siddiqui, S., Desai, D., Mistry, V., Rugman, P., et al. (2010). Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. Chest, 138(5), 1140-1147.

Dougherty, R. H., Sidhu, S. S., Raman, K., Solon, M., Solberg, O. D., Caughey, G. H., et al. (2010). Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma. The Journal of Allergy and Clinical Immunology, 125(5), 1046-1053.e8.

Durrant, D. M., Gaffen, S. L., Riesenfeld, E. P., Irvin, C. G., & Metzger, D. W. (2009). Development of allergen-induced airway inflammation in the absence of T-bet regulation is dependent on IL-17. Journal of Immunology (Baltimore, Md.: 1950), 183(8), 5293-5300.

Duvoix, A., Dickens, J., Haq, I., Mannino, D., Miller, B., Tal-Singer, R., et al. (2013). Blood fibrinogen as a biomarker of chronic obstructive pulmonary disease. Thorax, 68(7), 670-676.

Dweik, R. A., Sorkness, R. L., Wenzel, S., Hammel, J., Curran-Everett, D., Comhair, S. A., et al. (2010). Use of exhaled nitric oxide measurement to identify a reactive, at-risk phenotype among patients with asthma. American Journal of Respiratory and Critical Care Medicine, 181(10), 1033-1041.

Elias, J. A., Zhu, Z., Chupp, G., & Homer, R. J. (1999). Airway remodeling in asthma. The Journal of Clinical Investigation, 104(8), 1001-1006.

Erin, E. M., Leaker, B. R., Nicholson, G. C., Tan, A. J., Green, L. M., Neighbour, H., et al. (2006). The effects of a monoclonal antibody directed against tumor necrosis factor-alpha in asthma. American Journal of Respiratory and Critical Care Medicine, 174(7), 753-762.

Erpenbeck, V. J., Hohlfeld, J. M., Volkmann, B., Hagenberg, A., Geldmacher, H., Braun, A., et al. (2003). Segmental allergen challenge in patients with atopic asthma leads to increased IL-9 expression in bronchoalveolar lavage fluid lymphocytes. The Journal of Allergy and Clinical Immunology, 111(6), 1319-1327.

Essilfie, A. T., Simpson, J. L., Dunkley, M. L., Morgan, L. C., Oliver, B. G., Gibson, P. G., et al. (2012). Combined haemophilus influenzae respiratory infection and allergic airways disease drives chronic infection and features of neutrophilic asthma. Thorax, 67(7), 588-599.

Eustace, A., Smyth, L. J., Mitchell, L., Williamson, K., Plumb, J., & Singh, D. (2011). Identification of cells expressing IL-17A and IL-17F in the lungs of patients with COPD. Chest, 139(5), 1089-1100.

Fahy, J. V., Boushey, H. A., Lazarus, S. C., Mauger, E. A., Cherniack, R. M., Chinchilli, V. M., et al. (2001). Safety and reproducibility of sputum induction in asthmatic subjects in a multicenter study. American Journal of Respiratory and Critical Care Medicine, 163(6), 1470-1475.

Fahy, J. V., Kim, K. W., Liu, J., & Boushey, H. A. (1995). Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. The Journal of Allergy and Clinical Immunology, 95(4), 843-852.

Fahy, J. V., & Locksley, R. M. (2011). The airway epithelium as a regulator of Th2 responses in asthma. American Journal of Respiratory and Critical Care Medicine, 184(4), 390-392.

Fahy, J. V., Wong, H., Liu, J., & Boushey, H. A. (1995). Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. American Journal of Respiratory and Critical Care Medicine, 152(1), 53-58.

Fantuzzi, G., & Dinarello, C. A. (1999). Interleukin-18 and interleukin-1 beta: Two cytokine substrates for ICE (caspase-1). Journal of Clinical Immunology, 19(1), 1-11.

Fang, H. and G. Hedin. 2003. Rapid screening and identification of methicillin-resistant Staphylococcus aureus from clinical samples by selective-broth and real-time PCR assay. *J.Clin.Microbiol.* 41:2894-2899

Ferhani, N., Letuve, S., Kozhich, A., Thibaudeau, O., Grandsaigne, M., Maret, M., et al. (2010). Expression of high-mobility group box 1 and of receptor for advanced glycation end products in chronic obstructive pulmonary disease. American Journal of Respiratory and Critical Care Medicine, 181(9), 917-927.

Fernandez-Boyanapalli, R., Goleva, E., Kolakowski, C., Min, E., Day, B., Leung, D. Y., et al. (2013). Obesity impairs apoptotic cell clearance in asthma. The Journal of Allergy and Clinical Immunology, 131(4), 1041-7, 1047.e1-3.

Fitzpatrick, A. M., Teague, W. G., Meyers, D. A., Peters, S. P., Li, X., Li, H., et al. (2011). Heterogeneity of severe asthma in childhood: Confirmation by cluster analysis of children in the national institutes of Health/National heart, lung, and blood institute severe asthma research program. The Journal of Allergy and Clinical Immunology, 127(2), 382-389.e1-13.

Fleming, L., & Bush, A. (2012). Use of sputum eosinophil counts to guide management in children with severe asthma. Thorax, 67(11), 1015-6; author reply 1016.

Flood-Page, P., Menzies-Gow, A., Phipps, S., Ying, S., Wangoo, A., Ludwig, M. S., et al. (2003). Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. The Journal of Clinical Investigation, 112(7), 1029-1036.

Flood-Page, P., Swenson, C., Faiferman, I., Matthews, J., Williams, M., Brannick, L., et al. (2007). A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. American Journal of Respiratory and Critical Care Medicine, 176(11), 1062-1071.

Flood-Page, P. T., Menzies-Gow, A. N., Kay, A. B., & Robinson, D. S. (2003). Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. American Journal of Respiratory and Critical Care Medicine, 167(2), 199-204.

Ford, J. G., Rennick, D., Donaldson, D. D., Venkayya, R., McArthur, C., Hansell, E., et al. (2001). Il-13 and IFN-gamma: Interactions in lung inflammation. Journal of Immunology (Baltimore, Md.: 1950), 167(3), 1769-1777.

Forno, E., Lasky-Su, J., Himes, B., Howrylak, J., Ramsey, C., Brehm, J., et al. (2012). Genome-wide association study of the age of onset of childhood asthma. The Journal of Allergy and Clinical Immunology, 130(1), 83-90.e4.

Fu, J. J., Baines, K. J., Wood, L. G., & Gibson, P. G. (2013). Systemic inflammation is associated with differential gene expression and airway neutrophilia in asthma. Omics : A Journal of Integrative Biology, 17(4), 187-199.

Fuhlbrigge, A., Peden, D., Apter, A. J., Boushey, H. A., Camargo, C. A.,Jr, Gern, J., et al. (2012). Asthma outcomes: Exacerbations. The Journal of Allergy and Clinical Immunology, 129(3 Suppl), S34-48.

Gern, J. E., Vrtis, R., Grindle, K. A., Swenson, C., & Busse, W. W. (2000). Relationship of upper and lower airway cytokines to outcome of experimental rhinovirus infection. American Journal of Respiratory and Critical Care Medicine, 162(6), 2226-2231.

Gibeon, D., Batuwita, K., Osmond, M., Heaney, L. G., Brightling, C. E., Niven, R., et al. (2013). Obesity-associated severe asthma represents a distinct clinical phenotype: Analysis of the british thoracic society difficult asthma registry patient cohort according to BMI. Chest, 143(2), 406-414.

Global Initative for Asthma. www.ginaasthma.org. Accessed 2015.

Goh, K. I., Cusick, M. E., Valle, D., Childs, B., Vidal, M., & Barabasi, A. L. (2007). The human disease network. Proceedings of the National Academy of Sciences of the United States of America, 104(21), 8685-8690.

Graves, P. E., Kabesch, M., Halonen, M., Holberg, C. J., Baldini, M., Fritzsch, C., et al. (2000). A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children. The Journal of Allergy and Clinical Immunology, 105(3), 506-513.

Green, R. H., Brightling, C. E., McKenna, S., Hargadon, B., Parker, D., Bradding, P., et al. (2002). Asthma exacerbations and sputum eosinophil counts: A randomised controlled trial. Lancet, 360(9347), 1715-1721.

Green, R. H., Brightling, C. E., Woltmann, G., Parker, D., Wardlaw, A. J., & Pavord, I. D. (2002). Analysis of induced sputum in adults with asthma: Identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. Thorax, 57(10), 875-879.

Greiner, O., P. J. Day, M. Altwegg, and D. Nadal. 2003. Quantitative detection of Moraxella catarrhalis in nasopharyngeal secretions by real-time PCR. *J.Clin.Microbiol.* 41:1386-1390.

Grissell, T. V., Powell, H., Shafren, D. R., Boyle, M. J., Hensley, M. J., Jones, P. D., et al. (2005). Interleukin-10 gene expression in acute virus-induced asthma. American Journal of Respiratory and Critical Care Medicine, 172(4), 433-439.

Gupta, S., Hartley, R., Khan, U. T., Singapuri, A., Hargadon, B., Monteiro, W., et al. (2014). Quantitative computed tomography-derived clusters: Redefining airway remodeling in asthmatic patients. The Journal of Allergy and Clinical Immunology, 133(3), 729-38.e18.

Gupta, S., Siddiqui, S., Haldar, P., Entwisle, J. J., Mawby, D., Wardlaw, A. J., et al. (2010). Quantitative analysis of high-resolution computed tomography scans in severe asthma subphenotypes. Thorax, 65(9), 775-781.

Haldar, P., Brightling, C. E., Hargadon, B., Gupta, S., Monteiro, W., Sousa, A., et al. (2009). Mepolizumab and exacerbations of refractory eosinophilic asthma. The New England Journal of Medicine, 360(10), 973-984.

Haldar, P., Brightling, C. E., Singapuri, A., Hargadon, B., Gupta, S., Monteiro, W., et al. (2014). Outcomes after cessation of mepolizumab therapy in severe eosinophilic asthma: A 12-month follow-up analysis. The Journal of Allergy and Clinical Immunology, 133(3), 921-923.

Haldar, P., Pavord, I. D., Shaw, D. E., Berry, M. A., Thomas, M., Brightling, C. E., et al. (2008). Cluster analysis and clinical asthma phenotypes. American Journal of Respiratory and Critical Care Medicine, 178(3), 218-224.

Hansel, T. T., Johnston, S. L., & Openshaw, P. J. (2013). Microbes and mucosal immune responses in asthma. The Lancet, 381(9869), 861-873.

Harada, M., Hirota, T., Jodo, A. I., Hitomi, Y., Sakashita, M., Tsunoda, T., et al. (2011). Thymic stromal lymphopoietin gene promoter polymorphisms are associated with susceptibility to bronchial asthma. American Journal of Respiratory Cell and Molecular Biology, 44(6), 787-793.

Haselkorn, T., Fish, J. E., Zeiger, R. S., Szefler, S. J., Miller, D. P., Chipps, B. E., et al. (2009). Consistently very poorly controlled asthma, as defined by the impairment domain of the expert panel report 3 guidelines, increases risk for future severe asthma exacerbations in the epidemiology and natural history of asthma: Outcomes and treatment regimens (TENOR) study. The Journal of Allergy and Clinical Immunology, 124(5), 895-902.e1-4.

Haselkorn, T., Zeiger, R. S., Chipps, B. E., Mink, D. R., Szefler, S. J., Simons, F. E., et al. (2009). Recent asthma exacerbations predict future exacerbations in children with severe or difficult-to-treat asthma. The Journal of Allergy and Clinical Immunology, 124(5), 921-927.

Hastie, A. T., Moore, W. C., Meyers, D. A., Vestal, P. L., Li, H., Peters, S. P., et al. (2010). Analyses of asthma severity phenotypes and inflammatory proteins in subjects stratified by sputum granulocytes. The Journal of Allergy and Clinical Immunology, 125(5), 1028-1036.e13.

Hauk, P. J., Wenzel, S. E., Trumble, A. E., Szefler, S. J., & Leung, D. Y. (1999). Increased T-cell receptor vbeta8+ T cells in bronchoalveolar lavage fluid of subjects with poorly controlled asthma: A potential role for microbial superantigens. The Journal of Allergy and Clinical Immunology, 104(1), 37-45.

Hawkins, G. A., Robinson, M. B., Hastie, A. T., Li, X., Li, H., Moore, W. C., et al. (2012). The IL6R variation asp(358)ala is a potential modifier of lung function in subjects with asthma. The Journal of Allergy and Clinical Immunology, 130(2), 510-5.e1.

He, J. Q., Hallstrand, T. S., Knight, D., Chan-Yeung, M., Sandford, A., Tripp, B., et al. (2009). A thymic stromal lymphopoietin gene variant is associated with asthma and airway hyperresponsiveness. The Journal of Allergy and Clinical Immunology, 124(2), 222-229.

Heaney, L. G., Brightling, C. E., Menzies-Gow, A., Stevenson, M., Niven, R. M., & British Thoracic Society Difficult Asthma Network. (2010). Refractory asthma in the UK: Cross-sectional findings from a UK multicentre registry. Thorax, 65(9), 787-794.

Heinzmann, A., Mao, X. Q., Akaiwa, M., Kreomer, R. T., Gao, P. S., Ohshima, K., et al. (2000). Genetic variants of IL-13 signalling and human asthma and atopy. Human Molecular Genetics, 9(4), 549-559.

Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, Davies J, Ervine A, Poulter L, Pachter L, Moffatt MF, Cookson WO. (2010). PLoS One 5;5(1):e8578.

Holguin, F., Bleecker, E. R., Busse, W. W., Calhoun, W. J., Castro, M., Erzurum, S. C., et al. (2011). Obesity and asthma: An association modified by age of asthma onset. The Journal of Allergy and Clinical Immunology, 127(6), 1486-93.e2.

Holgate S.T. D. E. Davies, S. Puddicombe, A. Richter, P. Lackie, J. Lordan, P. Howarth. (2003). Mechanisms of airway epithelial damage: epithelial-mesenchymal interactions in the pathogenesis of asthma. European Respiratory Journal. 22: S44, 24s–29s

Hoshino, K., Tsutsui, H., Kawai, T., Takeda, K., Nakanishi, K., Takeda, Y., et al. (1999). Cutting edge: Generation of IL-18 receptor-deficient mice: Evidence for IL-1 receptor-related protein as an essential IL-18 binding receptor. Journal of Immunology (Baltimore, Md.: 1950), 162(9), 5041-5044.

Hoshino, M., Nakamura, Y., Sim, J., Shimojo, J., & Isogai, S. (1998). Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. The Journal of Allergy and Clinical Immunology, 102(5), 783-788.

Huang, Y. J., Nelson, C. E., Brodie, E. L., Desantis, T. Z., Baek, M. S., Liu, J., et al. (2011). Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. The Journal of Allergy and Clinical Immunology, 127(2), 372-381.e1-3.

Huber, M., Beutler, B., & Keppler, D. (1988). Tumor necrosis factor alpha stimulates leukotriene production in vivo. European Journal of Immunology, 18(12), 2085-2088.

Humbert, M., Durham, S. R., Ying, S., Kimmitt, P., Barkans, J., Assoufi, B., et al. (1996). IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: Evidence against "intrinsic" asthma being a distinct immunopathologic entity. American Journal of Respiratory and Critical Care Medicine, 154(5), 1497-1504.

Humbles, A. A., Lloyd, C. M., McMillan, S. J., Friend, D. S., Xanthou, G., McKenna, E. E., et al. (2004). A critical role for eosinophils in allergic airways remodeling. Science (New York, N.Y.), 305(5691), 1776-1779.

Hutson, P. A., Church, M. K., Clay, T. P., Miller, P., & Holgate, S. T. (1988). Early and late-phase bronchoconstriction after allergen challenge of nonanesthetized guinea pigs. I. the association of disordered airway physiology to leukocyte infiltration. The American Review of Respiratory Disease, 137(3), 548-557.

Imaoka, H., Hoshino, T., Takei, S., Kinoshita, T., Okamoto, M., Kawayama, T., et al. (2008). Interleukin-18 production and pulmonary function in COPD. The European Respiratory Journal, 31(2), 287-297.

Imboden, M., Bouzigon, E., Curjuric, I., Ramasamy, A., Kumar, A., Hancock, D. B., et al. (2012). Genome-wide association study of lung function decline in adults with and without asthma. The Journal of Allergy and Clinical Immunology, 129(5), 1218-1228.

Ingram, J. L., Rice, A. B., Geisenhoffer, K., Madtes, D. K., & Bonner, J. C. (2004). IL-13 and IL-1beta promote lung fibroblast growth through coordinated up-regulation of PDGF-AA and PDGF-ralpha. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 18(10), 1132-1134.

in't Veen, J. C., Smits, H. H., Hiemstra, P. S., Zwinderman, A. E., Sterk, P. J., & Bel, E. H. (1999). Lung function and sputum characteristics of patients with severe asthma during an induced exacerbation by double-blind steroid withdrawal. American Journal of Respiratory and Critical Care Medicine, 160(1), 93-99.

Jackson, D. J., & Johnston, S. L. (2010). The role of viruses in acute exacerbations of asthma. The Journal of Allergy and Clinical Immunology, 125(6), 1178-87; quiz 1188-9.

Jafri, H. S., Chavez-Bueno, S., Mejias, A., Gomez, A. M., Rios, A. M., Nassi, S. S., et al. (2004). Respiratory syncytial virus induces pneumonia, cytokine response, airway obstruction, and chronic inflammatory infiltrates associated with long-term airway hyperresponsiveness in mice. The Journal of Infectious Diseases, 189(10), 1856-1865.

Jarjour, N. N., Erzurum, S. C., Bleecker, E. R., Calhoun, W. J., Castro, M., Comhair, S. A., et al. (2012). Severe asthma: Lessons learned from the national heart, lung, and blood institute severe asthma research program. American Journal of Respiratory and Critical Care Medicine, 185(4), 356-362.

Jatakanon, A., Lim, S., & Barnes, P. J. (2000). Changes in sputum eosinophils predict loss of asthma control. American Journal of Respiratory and Critical Care Medicine, 161(1), 64-72.

Jayaram, L., Pizzichini, M. M., Cook, R. J., Boulet, L. P., Lemiere, C., Pizzichini, E., et al. (2006). Determining asthma treatment by monitoring sputum cell counts: Effect on exacerbations. The European Respiratory Journal, 27(3), 483-494.

Jensen, M. E., Gibson, P. G., Collins, C. E., & Wood, L. G. (2013). Airway and systemic inflammation in obese children with asthma. The European Respiratory Journal, 42(4), 1012-1019.

Jessup, H. K., Brewer, A. W., Omori, M., Rickel, E. A., Budelsky, A. L., Yoon, B. R., et al. (2008). Intradermal administration of thymic stromal lymphopoietin induces a T cell- and eosinophil-dependent systemic Th2 inflammatory response. Journal of Immunology (Baltimore, Md.: 1950), 181(6), 4311-4319.

Jia, G., Erickson, R. W., Choy, D. F., Mosesova, S., Wu, L. C., Solberg, O. D., et al. (2012). Periostin is a systemic biomarker of eosinophilic airway inflammation in asthmatic patients. The Journal of Allergy and Clinical Immunology, 130(3), 647-654.e10.

Johnston, R. A., Zhu, M., Rivera-Sanchez, Y. M., Lu, F. L., Theman, T. A., Flynt, L., et al. (2007). Allergic airway responses in obese mice. American Journal of Respiratory and Critical Care Medicine, 176(7), 650-658.

Johnston SL, Blasi F, Black PN, Martin RJ, Farrell DJ, Nieman RB. (2002) The effect of telithromycin in acute exacerbations of asthma. N Engl J Med 35

4:1589–1600.

Jones, T. G., Hallgren, J., Humbles, A., Burwell, T., Finkelman, F. D., Alcaide, P., et al. (2009). Antigen-induced increases in pulmonary mast cell progenitor numbers depend on IL-9 and CD1d-restricted NKT cells. Journal of Immunology (Baltimore, Md.: 1950), 183(8), 5251-5260.

Juniper, E. F., O'Byrne, P. M., Guyatt, G. H., Ferrie, P. J., & King, D. R. (1999). Development and validation of a questionnaire to measure asthma control. The European Respiratory Journal, 14(4), 902-907.

Juniper, E. F., Svensson, K., Mork, A. C., & Stahl, E. (2004). Measuring health-related quality of life in adults during an acute asthma exacerbation. Chest, 125(1), 93-97.

Juniper, E. F., Svensson, K., Mork, A. C., & Stahl, E. (2005). Measurement properties and interpretation of three shortened versions of the asthma control questionnaire. Respiratory Medicine, 99(5), 553-558.

Kabesch, M., Schedel, M., Carr, D., Woitsch, B., Fritzsch, C., Weiland, S. K., et al. (2006). IL-4/IL-13 pathway genetics strongly influence serum IgE levels and childhood asthma. The Journal of Allergy and Clinical Immunology, 117(2), 269-274.

Kamath, A. V., Pavord, I. D., Ruparelia, P. R., & Chilvers, E. R. (2005). Is the neutrophil the key effector cell in severe asthma? Thorax, 60(7), 529-530.

Kawaguchi, M., Kokubu, F., Kuga, H., Tomita, T., Matsukura, S., Kadokura, M., et al. (2000). Expression of eotaxin by normal airway epithelial cells after influenza virus A infection. International Archives of Allergy and Immunology, 122 Suppl 1, 44-49.

Keatings, V. M., Evans, D. J., O'Connor, B. J., & Barnes, P. J. (1997). Cellular profiles in asthmatic airways: A comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid. Thorax, 52(4), 372-374.

Kelly, M., Leigh, R., & Hargreave, F. E. (2000). Validation of assays for inflammatory mediators in sputum. The European Respiratory Journal, 16(6), 1208-1209.

Kelly, M. M., Keatings, V., Leigh, R., Peterson, C., Shute, J., Venge, P., et al. (2002). Analysis of fluid-phase mediators. The European Respiratory Journal.Supplement, 37, 24s-39s.

Kim, W. K., Kwon, J. W., Seo, J. H., Kim, H. Y., Yu, J., Kim, B. J., et al. (2012). Interaction between IL13 genotype and environmental factors in the risk for allergic rhinitis in korean children. The Journal of Allergy and Clinical Immunology, 130(2), 421-6.e5.

Kloepfer, K. M., Lee, W. M., Pappas, T. E., Kang, T. J., Vrtis, R. F., Evans, M. D., et al. (2014). Detection of pathogenic bacteria during rhinovirus infection is associated with increased respiratory symptoms and asthma exacerbations. The Journal of Allergy and Clinical Immunology, 133(5), 1301-7, 1307.e1-3.

Kriegler, M., Perez, C., DeFay, K., Albert, I., & Lu, S. D. (1988). A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. Cell, 53(1), 45-53.

Krishnan, J. A., Lemanske, R. F.,Jr, Canino, G. J., Elward, K. S., Kattan, M., Matsui, E. C., et al. (2012). Asthma outcomes: Symptoms. The Journal of Allergy and Clinical Immunology, 129(3 Suppl), S124-35.

Krishnan, V., Diette, G. B., Rand, C. S., Bilderback, A. L., Merriman, B., Hansel, N. N., et al. (2006). Mortality in patients hospitalized for asthma exacerbations in the united states. American Journal of Respiratory and Critical Care Medicine, 174(6), 633-638.

Kulkarni, N. S., Hollins, F., Sutcliffe, A., Saunders, R., Shah, S., Siddiqui, S., et al. (2010). Eosinophil protein in airway macrophages: A novel biomarker of eosinophilic inflammation in patients with asthma. The Journal of Allergy and Clinical Immunology, 126(1), 61-9.e3.

Kumagai, K., Ohno, I., Imai, K., Nawata, J., Hayashi, K., Okada, S., et al. (2002). The involvement of matrix metalloproteinases in basement membrane injury in a murine model of acute allergic airway inflammation. Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 32(10), 1527-1534.

Lamblin, C., Gosset, P., Tillie-Leblond, I., Saulnier, F., Marquette, C. H., Wallaert, B., et al. (1998). Bronchial neutrophilia in patients with noninfectious status asthmaticus. American Journal of Respiratory and Critical Care Medicine, 157(2), 394-402.

Lambrecht, B. N., & Hammad, H. (2012). The airway epithelium in asthma. Nature Medicine, 18(5), 684-692.

Lappalainen, U., Whitsett, J. A., Wert, S. E., Tichelaar, J. W., & Bry, K. (2005). Interleukin-1beta causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung. American Journal of Respiratory Cell and Molecular Biology, 32(4), 311-318.

Leckie, M. J., ten Brinke, A., Khan, J., Diamant, Z., O'Connor, B. J., Walls, C. M., et al. (2000). Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet, 356(9248), 2144-2148.

Lieberman, D., Lieberman, D., Printz, S., Ben-Yaakov, M., Lazarovich, Z., Ohana, B., Friedman, M.G., Dvoskin, B., Leinonen, M. and Boldur, I., 2003. Atypical pathogen infection in adults with acute exacerbation of bronchial asthma. American journal of respiratory and critical care medicine, 167(3), pp.406-410.

Lessard, A., St-Laurent, J., Turcotte, H., & Boulet, L. P. (2011). Leptin and adiponectin in obese and non-obese subjects with asthma. Biomarkers : Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals, 16(3), 271-273.

Lessard, A., Turcotte, H., Cormier, Y., & Boulet, L. P. (2008). Obesity and asthma: A specific phenotype? Chest, 134(2), 317-323.

Levanen, B., Bhakta, N. R., Torregrosa Paredes, P., Barbeau, R., Hiltbrunner, S., Pollack, J. L., et al. (2013). Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients. The Journal of Allergy and Clinical Immunology, 131(3), 894-903.

Lewis, C. C., Aronow, B., Hutton, J., Santeliz, J., Dienger, K., Herman, N., et al. (2009). Unique and overlapping gene expression patterns driven by IL-4 and IL-13 in the mouse lung. The Journal of Allergy and Clinical Immunology, 123(4), 795-804.e8.

Li Kam Wa, T. C., Mansur, A. H., Britton, J., Williams, G., Pavord, I., Richards, K., et al. (1999). Association between - 308 tumour necrosis factor promoter polymorphism and bronchial hyperreactivity in asthma. Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 29(9), 1204-1208.

Li, X., Ampleford, E. J., Howard, T. D., Moore, W. C., Torgerson, D. G., Li, H., et al. (2012). Genome-wide association studies of asthma indicate opposite immunopathogenesis direction from autoimmune diseases. The Journal of Allergy and Clinical Immunology, 130(4), 861-8.e7.

Li, X., Hawkins, G. A., Ampleford, E. J., Moore, W. C., Li, H., Hastie, A. T., et al. (2013). Genome-wide association study identifies TH1 pathway genes associated with lung function in asthmatic patients. The Journal of Allergy and Clinical Immunology, 132(2), 313-20.e15.

Lim, K. G., Wan, H. C., Bozza, P. T., Resnick, M. B., Wong, D. T., Cruikshank, W. W., et al. (1996). Human eosinophils elaborate the lymphocyte chemoattractants. IL-16 (lymphocyte chemoattractant factor) and RANTES. Journal of Immunology (Baltimore, Md.: 1950), 156(7), 2566-2570.

Linnane, S. J., Keatings, V. M., Costello, C. M., Moynihan, J. B., O'Connor, C. M., Fitzgerald, M. X., et al. (1998). Total sputum nitrate plus nitrite is raised during acute pulmonary infection in cystic fibrosis. American Journal of Respiratory and Critical Care Medicine, 158(1), 207-212.

Liu, L., Jarjour, N. N., Busse, W. W., & Kelly, E. A. (2004). Enhanced generation of helper T type 1 and 2 chemokines in allergen-induced asthma. American Journal of Respiratory and Critical Care Medicine, 169(10), 1118-1124.

Lukacs, N. W., Strieter, R. M., Chensue, S. W., Widmer, M., & Kunkel, S. L. (1995). TNF-alpha mediates recruitment of neutrophils and eosinophils during airway inflammation. Journal of Immunology (Baltimore, Md.: 1950), 154(10), 5411-5417.

Ma Z, Paek D, Oh CK. Plasminogen activator inhibitor-1 and asthma: role in the pathogenesis and molecular regulation.(2009). Clinical and Experimental Allergy. 39(8):1136–44

Maclay, J. D., McAllister, D. A., Rabinovich, R., Haq, I., Maxwell, S., Hartland, S., et al. (2012). Systemic elastin degradation in chronic obstructive pulmonary disease. Thorax, 67(7), 606-612.

Majoor, C.J., van de Pol, M.A., Kamphuisen, P.W., Meijers, J.C., Molenkamp, R., Wolthers, K.C., van der Poll, T., Nieuwland, R., Johnston, S.L., Sterk, P.J. and Bel, E.H., 2014. Evaluation of coagulation activation after Rhinovirus infection in patients with asthma and healthy control subjects: an observational study. Respiratory research, 15(1), p.14.

Margulis, A., Nocka, K. H., Brennan, A. M., Deng, B., Fleming, M., Goldman, S. J., et al. (2009). Mast cell-dependent contraction of human airway smooth muscle cell-containing collagen gels: Influence of cytokines, matrix metalloproteases, and serine proteases. Journal of Immunology (Baltimore, Md.: 1950), 183(3), 1739-1750.

Marijsse GS, Seys SF, Schelpe AS, Dilissen E, Goeminne P, Dupont LJ, Ceuppens JL, Bullens DM. (2004) Obese individuals with asthma preferentially have a high IL-5/IL-17A/IL-25 sputum inflammatory pattern .American Journal Respiratory Critical Care Medicine.;189(10):1284-5.

Martin, R. J., Boguniewicz, M., Henson, J. E., Celniker, A. C., Williams, M., Giorno, R. C., et al. (1993). The effects of inhaled interferon gamma in normal human airways. The American Review of Respiratory Disease, 148(6 Pt 1), 1677-1682.

Martin, R. J., Chu, H. W., Honour, J. M., & Harbeck, R. J. (2001). Airway inflammation and bronchial hyperresponsiveness after mycoplasma pneumoniae infection in a murine model. American Journal of Respiratory Cell and Molecular Biology, 24(5), 577-582.

Matsumoto, H., Niimi, A., Takemura, M., Ueda, T., Minakuchi, M., Tabuena, R., et al. (2005). Relationship of airway wall thickening to an imbalance between matrix metalloproteinase-9 and its inhibitor in asthma. Thorax, 60(4), 277-281.

McGrath, K. W., Icitovic, N., Boushey, H. A., Lazarus, S. C., Sutherland, E. R., Chinchilli, V. M., et al. (2012). A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. American Journal of Respiratory and Critical Care Medicine, 185(6), 612-619.

Melen, E., Granell, R., Kogevinas, M., Strachan, D., Gonzalez, J. R., Wjst, M., et al. (2013). Genome-wide association study of body mass index in 23 000 individuals with and without asthma. Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 43(4), 463-474.

Message, S. D., Laza-Stanca, V., Mallia, P., Parker, H. L., Zhu, J., Kebadze, T., et al. (2008). Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. Proceedings of the National Academy of Sciences of the United States of America, 105(36), 13562-13567.

Meyts, I., Hellings, P. W., Hens, G., Vanaudenaerde, B. M., Verbinnen, B., Heremans, H., et al. (2006). IL-12 contributes to allergen-induced airway inflammation in experimental asthma. Journal of Immunology (Baltimore, Md.: 1950), 177(9), 6460-6470.

Milburn, M. V., Hassell, A. M., Lambert, M. H., Jordan, S. R., Proudfoot, A. E., Graber, P., et al. (1993). A novel dimer configuration revealed by the crystal structure at 2.4 A resolution of human interleukin-5. Nature, 363(6425), 172-176.

Miranda, C., Busacker, A., Balzar, S., Trudeau, J., & Wenzel, S. E. (2004). Distinguishing severe asthma phenotypes: Role of age at onset and eosinophilic inflammation. The Journal of Allergy and Clinical Immunology, 113(1), 101-108.

Moffatt, M. F., Gut, I. G., Demenais, F., Strachan, D. P., Bouzigon, E., Heath, S., et al. (2010). A large-scale, consortium-based genomewide association study of asthma. The New England Journal of Medicine, 363(13), 1211-1221.

Molet, S., Hamid, Q., Davoine, F., Nutku, E., Taha, R., Page, N., et al. (2001). IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. The Journal of Allergy and Clinical Immunology, 108(3), 430-438.

Moller-Larsen, S., Nyegaard, M., Haagerup, A., Vestbo, J., Kruse, T. A., & Borglum, A. D. (2008). Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. Thorax, 63(12), 1064-1069.

Moore RN, Oppenheim JJ, Farrar JJ, Carter CS, Waheed A, Shadduck RK. Production of lymphocyte-activating factor (Interleukin 1) by macrophages activated with colony-stimulating factors. The Journal of Immunology. 1980 Sep 1;125(3):1302-5.

Moore, W. C., Bleecker, E. R., Curran-Everett, D., Erzurum, S. C., Ameredes, B. T., Bacharier, L., et al. (2007). Characterization of the severe asthma phenotype by the national heart, lung, and blood institute's severe asthma research program. The Journal of Allergy and Clinical Immunology, 119(2), 405-413.

Moore, W. C., Hastie, A. T., Li, X., Li, H., Busse, W. W., Jarjour, N. N., et al. (2013). Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. The Journal of Allergy and Clinical Immunology,

Moore, W. C., Meyers, D. A., Wenzel, S. E., Teague, W. G., Li, H., Li, X., et al. (2010). Identification of asthma phenotypes using cluster analysis in the severe asthma research program. American Journal of Respiratory and Critical Care Medicine, 181(4), 315-323.

Morjaria, J. B., Babu, K. S., Vijayanand, P., Chauhan, A. J., Davies, D. E., & Holgate, S. T. (2011). Sputum IL-6 concentrations in severe asthma and its relationship with FEV1. Thorax, 66(6), 537.

Morjaria, J. B., Chauhan, A. J., Babu, K. S., Polosa, R., Davies, D. E., & Holgate, S. T. (2008). The role of a soluble TNFalpha receptor fusion protein (etanercept) in corticosteroid refractory asthma: A double blind, randomised, placebo controlled trial. Thorax, 63(7), 584-591.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., & Coffman, R. L. (2005). Two types of murine helper T cell clone. I. definition according to profiles of lymphokine activities and secreted proteins. 1986. Journal of Immunology (Baltimore, Md.: 1950), 175(1), 5-14.

Mukaida, N., Mahe, Y., & Matsushima, K. (1990). Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. The Journal of Biological Chemistry, 265(34), 21128-21133.

Murphy, A. C., Proeschal, A., Brightling, C. E., Wardlaw, A. J., Pavord, I., Bradding, P., et al. (2012). The relationship between clinical outcomes and medication adherence in difficult-to-control asthma. Thorax, 67(8), 751-753.

Murray, C. S., Simpson, A., & Custovic, A. (2004). Allergens, viruses, and asthma exacerbations. Proceedings of the American Thoracic Society, 1(2), 99-104.

Nair, P., Gaga, M., Zervas, E., Alagha, K., Hargreave, F. E., O'Byrne, P. M., et al. (2012). Safety and efficacy of a CXCR2 antagonist in patients with severe asthma and sputum neutrophils: A randomized, placebo-controlled clinical trial. Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 42(7), 1097-1103.

Nakamura, Y., Russell, S. M., Mess, S. A., Friedmann, M., Erdos, M., Francois, C., et al. (1994). Heterodimerization of the IL-2 receptor beta- and gamma-chain cytoplasmic domains is required for signalling. Nature, 369(6478), 330-333.

Nakano, Y., Wong, J. C., de Jong, P. A., Buzatu, L., Nagao, T., Coxson, H. O., et al. (2005). The prediction of small airway dimensions using computed tomography. American Journal of Respiratory and Critical Care Medicine, 171(2), 142-146.

Nathan, R. A., Sorkness, C. A., Kosinski, M., Schatz, M., Li, J. T., Marcus, P., et al. (2004). Development of the asthma control test: A survey for assessing asthma control. The Journal of Allergy and Clinical Immunology, 113(1), 59-65.

Nelson, B. H., Lord, J. D., & Greenberg, P. D. (1994). Cytoplasmic domains of the interleukin-2 receptor beta and gamma chains mediate the signal for T-cell proliferation. Nature, 369(6478), 333-336.

Newby, C., Heaney, L. G., Menzies-Gow, A., Niven, R. M., Mansur, A., Bucknall, C., et al. (2014). Statistical cluster analysis of the british thoracic society severe refractory asthma registry: Clinical outcomes and phenotype stability. PloS One, 9(7), e102987.

Newcomb, D. C., & Peebles, R. S.,Jr. (2009). Bugs and asthma: A different disease? Proceedings of the American Thoracic Society, 6(3), 266-271.

Noguchi, E., Yokouchi, Y., Shibasaki, M., Inudou, M., Nakahara, S., Nogami, T., et al. (2002). Association between TNFA polymorphism and the development of asthma in the japanese population. American Journal of Respiratory and Critical Care Medicine, 166(1), 43-46.

Norzila, M. Z., Fakes, K., Henry, R. L., Simpson, J., & Gibson, P. G. (2000). Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. American Journal of Respiratory and Critical Care Medicine, 161(3 Pt 1), 769-774.

Oliver, B. G., Lim, S., Wark, P., Laza-Stanca, V., King, N., Black, J. L., et al. (2008). Rhinovirus exposure impairs immune responses to bacterial products in human alveolar macrophages. Thorax, 63(6), 519-525.

O'Neill, S., Sweeney, J., Patterson, C. C., Menzies-Gow, A., Niven, R., Mansur, A. H., et al. (2015). The cost of treating severe refractory asthma in the UK: An economic analysis from the british thoracic society difficult asthma registry. Thorax, 70(4), 376-378.

Ordonez, C. L., Shaughnessy, T. E., Matthay, M. A., & Fahy, J. V. (2000). Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: Clinical and biologic significance. American Journal of Respiratory and Critical Care Medicine, 161(4 Pt 1), 1185-1190.

Palmqvist, C., Wardlaw, A. J., & Bradding, P. (2007). Chemokines and their receptors as potential targets for the treatment of asthma. British Journal of Pharmacology, 151(6), 725-736.

Papadopoulos, N. G., Stanciu, L. A., Papi, A., Holgate, S. T., & Johnston, S. L. (2002). A defective type 1 response to rhinovirus in atopic asthma. Thorax, 57(4), 328-332.

Parry, D. E., Busse, W. W., Sukow, K. A., Dick, C. R., Swenson, C., & Gern, J. E. (2000). Rhinovirus-induced PBMC responses and outcome of experimental infection in allergic subjects. The Journal of Allergy and Clinical Immunology, 105(4), 692-698.

Pauwels, N. S., Bracke, K. R., Dupont, L. L., Van Pottelberge, G. R., Provoost, S., Vanden Berghe, T., et al. (2011). Role of IL-1alpha and the Nlrp3/caspase-1/IL-1beta axis in cigarette smoke-induced pulmonary inflammation and COPD. The European Respiratory Journal, 38(5), 1019-1028.

Pavord, I. D., Birring, S. S., Berry, M., Green, R. H., Brightling, C. E., & Wardlaw, A. J. (2006). Multiple inflammatory hits and the pathogenesis of severe airway disease. The European Respiratory Journal, 27(5), 884-888.

Pavord, I. D., Brightling, C. E., Woltmann, G., & Wardlaw, A. J. (1999). Non-eosinophilic corticosteroid unresponsive asthma. Lancet, 353(9171), 2213-2214.

Pazdrak, K., Young, T. W., Stafford, S., Olszewska-Pazdrak, B., Straub, C., Starosta, V., et al. (2008). Cross-talk between ICAM-1 and granulocyte-macrophage colony-stimulating factor receptor signaling modulates eosinophil survival and activation. Journal of Immunology (Baltimore, Md.: 1950), 180(6), 4182-4190.

Pepe, C., Foley, S., Shannon, J., Lemiere, C., Olivenstein, R., Ernst, P., et al. (2005). Differences in airway remodeling between subjects with severe and moderate asthma. The Journal of Allergy and Clinical Immunology, 116(3), 544-549.

Peters, M. C., & Fahy, J. V. (2013). Type 2 immune responses in obese individuals with asthma. American Journal of Respiratory and Critical Care Medicine, 188(6), 633-634.

Peters, M. C., Mekonnen, Z. K., Yuan, S., Bhakta, N. R., Woodruff, P. G., & Fahy, J. V. (2014). Measures of gene expression in sputum cells can identify TH2-high and TH2-low subtypes of asthma. The Journal of Allergy and Clinical Immunology, 133(2), 388-394.

Petersen, A. M., Penkowa, M., Iversen, M., Frydelund-Larsen, L., Andersen, J. L., Mortensen, J., et al. (2007). Elevated levels of IL-18 in plasma and skeletal muscle in chronic obstructive pulmonary disease. Lung, 185(3), 161-171.

Piper, E., Brightling, C., Niven, R., Oh, C., Faggioni, R., Poon, K., et al. (2013). A phase II placebo-controlled study of tralokinumab in moderate-to-severe asthma. The European Respiratory Journal, 41(2), 330-338.

Pizzichini, E., Pizzichini, M. M., Efthimiadis, A., Dolovich, J., & Hargreave, F. E. (1997). Measuring airway inflammation in asthma: Eosinophils and eosinophilic cationic protein in induced sputum compared with peripheral blood. The Journal of Allergy and Clinical Immunology, 99(4), 539-544.

Pizzichini, E., Pizzichini, M. M., Efthimiadis, A., Dolovich, J., & Hargreave, F. E. (1997). Measuring airway inflammation in asthma: Eosinophils and eosinophilic cationic protein in induced sputum compared with peripheral blood. The Journal of Allergy and Clinical Immunology, 99(4), 539-544.

Pizzichini, M. M., Popov, T. A., Efthimiadis, A., Hussack, P., Evans, S., Pizzichini, E., et al. (1996). Spontaneous and induced sputum to measure indices of airway inflammation in asthma. American Journal of Respiratory and Critical Care Medicine, 154(4 Pt 1), 866-869.

Poole, A., Urbanek, C., Eng, C., Schageman, J., Jacobson, S., O'Connor, B. P., et al. (2014). Dissecting childhood asthma with nasal transcriptomics distinguishes subphenotypes of disease. The Journal of Allergy and Clinical Immunology, 133(3), 670-8.e12.

Postma D.E., Timens W. (2006) Remodeling in Asthma and Chronic Obstructive Pulmonary Disease", Proceedings of the American Thoracic Society, 3(5) pp. 434-439.

Prefontaine, D., Lajoie-Kadoch, S., Foley, S., Audusseau, S., Olivenstein, R., Halayko, A. J., et al. (2009). Increased expression of IL-33 in severe asthma: Evidence of expression by airway smooth muscle cells. Journal of Immunology (Baltimore, Md.: 1950), 183(8), 5094-5103.

Price, D., Bosnic-Anticevich, S., Briggs, A., Chrystyn, H., Rand, C., Scheuch, G., Bousquet, J. and Inhaler Error Steering Committee, 2013. Inhaler competence in asthma: common errors, barriers to use and recommended solutions. Respiratory medicine, 107(1), pp.37-46.

Reddel, H. K., Taylor, D. R., Bateman, E. D., Boulet, L. P., Boushey, H. A., Busse, W. W., et al. (2009). An official american thoracic Society/European respiratory society statement: Asthma control and exacerbations: Standardizing endpoints for clinical asthma trials and clinical practice. American Journal of Respiratory and Critical Care Medicine, 180(1), 59-99.

Reijmerink, N. E., Postma, D. S., Bruinenberg, M., Nolte, I. M., Meyers, D. A., Bleecker, E. R., et al. (2008). Association of IL1RL1, IL18R1, and IL18RAP gene cluster polymorphisms with asthma and atopy. The Journal of Allergy and Clinical Immunology, 122(3), 651-4.e8.

Renauld, J. C., Druez, C., Kermouni, A., Houssiau, F., Uyttenhove, C., Van Roost, E., et al. (1992). Expression cloning of the murine and human interleukin 9 receptor cDNAs. Proceedings of the National Academy of Sciences of the United States of America, 89(12), 5690-5694.

Robinson, D. S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A. M., et al. (1992). Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. The New England Journal of Medicine, 326(5), 298-304.

Rochman, Y., Spolski, R., & Leonard, W. J. (2009). New insights into the regulation of T cells by gamma(c) family cytokines. Nature Reviews.Immunology, 9(7), 480-490.

Rohde, G., Gevaert, P., Holtappels, G., Fransen, L., Borg, I., Wiethege, A., et al. (2004). Soluble interleukin-5 receptor alpha is increased in acute exacerbation of chronic obstructive pulmonary disease. International Archives of Allergy and Immunology, 135(1), 54-61.

Ronmark, E., Andersson, C., Nystrom, L., Forsberg, B., Jarvholm, B., & Lundback, B. (2005). Obesity increases the risk of incident asthma among adults. The European Respiratory Journal, 25(2), 282-288.

Rouhani, F. N., Meitin, C. A., Kaler, M., Miskinis-Hilligoss, D., Stylianou, M., & Levine, S. J. (2005). Effect of tumor necrosis factor antagonism on allergen-mediated asthmatic airway inflammation. Respiratory Medicine, 99(9), 1175-1182.

Rovina, N., Dima, E., Gerassimou, C., Kollintza, A., Gratziou, C., & Roussos, C. (2009). Interleukin-18 in induced sputum: Association with lung function in chronic obstructive pulmonary disease. Respiratory Medicine, 103(7), 1056-1062.

Rubel, C., Fernandez, G. C., Dran, G., Bompadre, M. B., Isturiz, M. A., & Palermo, M. S. (2001). Fibrinogen promotes neutrophil activation and delays apoptosis. Journal of Immunology (Baltimore, Md.: 1950), 166(3), 2002-2010.

Saba, O. I., Hoffman, E. A., & Reinhardt, J. M. (2003). Maximizing quantitative accuracy of lung airway lumen and wall measures obtained from X-ray CT imaging. Journal of Applied Physiology (Bethesda, Md.: 1985), 95(3), 1063-1075.

Saha, S., Doe, C., Mistry, V., Siddiqui, S., Parker, D., Sleeman, M., et al. (2009). Granulocyte-macrophage colony-stimulating factor expression in induced sputum and bronchial mucosa in asthma and COPD. Thorax, 64(8), 671-676.

Saha, S. K., Berry, M. A., Parker, D., Siddiqui, S., Morgan, A., May, R., et al. (2008). Increased sputum and bronchial biopsy IL-13 expression in severe asthma. The Journal of Allergy and Clinical Immunology, 121(3), 685-691.

Sandford, A. J., Chagani, T., Zhu, S., Weir, T. D., Bai, T. R., Spinelli, J. J., et al. (2000). Polymorphisms in the IL4, IL4RA, and FCERIB genes and asthma severity. The Journal of Allergy and Clinical Immunology, 106(1 Pt 1), 135-140.

Scheurich, P., Thoma, B., Ucer, U., & Pfizenmaier, K. (1987). Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-alpha: Induction of TNF receptors on human T cells and TNF-alpha-mediated enhancement of T cell responses. Journal of Immunology (Baltimore, Md.: 1950), 138(6), 1786-1790.

Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T. K., et al. (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity, 23(5), 479-490.

Schroth, M. K., Grimm, E., Frindt, P., Galagan, D. M., Konno, S. I., Love, R., et al. (1999). Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. American Journal of Respiratory Cell and Molecular Biology, 20(6), 1220-1228.

Scott, H. A., Gibson, P. G., Garg, M. L., Pretto, J. J., Morgan, P. J., Callister, R., et al. (2012). Relationship between body composition, inflammation and lung function in overweight and obese asthma. Respiratory Research, 13, 10-9921-13-10.

Seys, S. F., Grabowski, M., Adriaensen, W., Decraene, A., Dilissen, E., Vanoirbeek, J. A., et al. (2013). Sputum cytokine mapping reveals an 'IL-5, IL-17A, IL-25-high' pattern associated with poorly controlled asthma. Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 43(9), 1009-1017.

Shaw, D. E., Berry, M. A., Hargadon, B., McKenna, S., Shelley, M. J., Green, R. H., et al. (2007). Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. Chest, 132(6), 1871-1875.

Shaw, D. E., Berry, M. A., Thomas, M., Green, R. H., Brightling, C. E., Wardlaw, A. J., et al. (2007). The use of exhaled nitric oxide to guide asthma management: A randomized controlled trial. American Journal of Respiratory and Critical Care Medicine, 176(3), 231-237.

Sia, D. Y., & Parish, C. R. (1980). Anti-self receptors. II. demonstration of H-2L region-restricted receptors on subpopulations of peripheral T and B lymphocytes. Journal of Immunology (Baltimore, Md.: 1950), 124(5), 2366-2372.

Sia, D. Y., Rylatt, D. B., & Parish, C. R. (1982). Anti-self receptors. V. properties of a mouse serum factor that blocks autorosetting receptors on lymphocytes. Immunology, 45(2), 207-216.

Siddiqui, S., Gupta, S., Cruse, G., Haldar, P., Entwisle, J., Mcdonald, S., et al. (2009). Airway wall geometry in asthma and nonasthmatic eosinophilic bronchitis. Allergy, 64(6), 951-958.

Siddiqui, S., Hollins, F., Saha, S., & Brightling, C. E. (2007). Inflammatory cell microlocalisation and airway dysfunction: Cause and effect? The European Respiratory Journal, 30(6), 1043-1056.

Siddiqui, S., Mistry, V., Doe, C., Roach, K., Morgan, A., Wardlaw, A., et al. (2008). Airway hyperresponsiveness is dissociated from airway wall structural remodeling. The Journal of Allergy and Clinical Immunology, 122(2), 335-41, 341.e1-3.

Siddiqui, S., Mistry, V., Doe, C., Stinson, S., Foster, M., & Brightling, C. (2010). Airway wall expression of OX40/OX40L and interleukin-4 in asthma. Chest, 137(4), 797-804.

Simon, H. U., Seelbach, H., Ehmann, R., & Schmitz, M. (2003). Clinical and immunological effects of low-dose IFN-alpha treatment in patients with corticosteroid-resistant asthma. Allergy, 58(12), 1250-1255.

Simpson, J. L., Gibson, P. G., Yang, I. A., Upham, J., James, A., Reynolds, P. N., et al. (2013). Impaired macrophage phagocytosis in non-eosinophilic asthma. Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 43(1), 29-35.

Simpson, J. L., Milne, D. G., & Gibson, P. G. (2009). Neutrophilic asthma has different radiographic features to COPD and smokers. Respiratory Medicine, 103(6), 881-887.

Simpson, J. L., Scott, R., Boyle, M. J., & Gibson, P. G. (2006). Inflammatory subtypes in asthma: Assessment and identification using induced sputum. Respirology (Carlton, Vic.), 11(1), 54-61.

Sin, D. D., & Sutherland, E. R. (2008). Obesity and the lung: 4. obesity and asthma. Thorax, 63(11), 1018-1023.

Singh, B., Arora, S., & Khanna, V. (2010). Association of severity of COPD with IgE and interleukin-1 beta. Monaldi Archives for Chest Disease = Archivio Monaldi Per Le Malattie Del Torace / Fondazione Clinica Del Lavoro, IRCCS [and] Istituto Di Clinica Tisiologica e Malattie Apparato Respiratorio, Universita Di Napoli, Secondo Ateneo, 73(2), 86-87.

Siva, R., Green, R. H., Brightling, C. E., Shelley, M., Hargadon, B., McKenna, S., et al. (2007). Eosinophilic airway inflammation and exacerbations of COPD: A randomised controlled trial. The European Respiratory Journal, 29(5), 906-913.

Slager, R. E., Otulana, B. A., Hawkins, G. A., Yen, Y. P., Peters, S. P., Wenzel, S. E., et al. (2012). IL-4 receptor polymorphisms predict reduction in asthma exacerbations during response to an anti-IL-4 receptor alpha antagonist. The Journal of Allergy and Clinical Immunology, 130(2), 516-22.e4.

Slats, A. M., Janssen, K., van Schadewijk, A., van der Plas, D. T., Schot, R., van den Aardweg, J. G., et al. (2007). Bronchial inflammation and airway responses to deep inspiration in asthma and chronic obstructive pulmonary disease. American Journal of Respiratory and Critical Care Medicine, 176(2), 121-128.

Smith, K.A., Lachman, L.B., Oppenheim, J.J. and Favata, M.F., 1980. The functional relationship of the interleukins. The Journal of experimental medicine, 151(6), pp.1551-1556.

Smith, M.J, Hodson M. (1983). High Dose Beclamethasone Inhaler in the treatment of Asthma. The Lancet 321(8319), 265-169

Solberg, O. D., Ostrin, E. J., Love, M. I., Peng, J. C., Bhakta, N. R., Hou, L., et al. (2012). Airway epithelial miRNA expression is altered in asthma. American Journal of Respiratory and Critical Care Medicine, 186(10), 965-974.

Sporik R, Holgate ST, Platts-Mills TA, Cogswell JJ. Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. (1990) New England Journal of Medicine. 23;323(8):502-7

Stanescu, D., Sanna, A., Veriter, C., Kostianev, S., Calcagni, P. G., Fabbri, L. M., et al. (1996). Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. Thorax, 51(3), 267-271.

Stream, A. R., & Sutherland, E. R. (2012). Obesity and asthma disease phenotypes. Current Opinion in Allergy and Clinical Immunology, 12(1), 76-81.

Stralin, K., A. Backman, H. Holmberg, H. Fredlund, and P. Olcen. 2005. Design of a multiplex PCR for Streptococcus pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae and Chlamydophila pneumoniae to be used on sputum samples. *APMIS* 113:99-111.

Strek, M. E. (2006). Difficult asthma. Proceedings of the American Thoracic Society, 3(1), 116-123.

Su, Y. C., Rolph, M. S., Hansbro, N. G., Mackay, C. R., & Sewell, W. A. (2008). Granulocyte-macrophage colony-stimulating factor is required for bronchial eosinophilia in a murine model of allergic airway inflammation. Journal of Immunology (Baltimore, Md.: 1950), 180(4), 2600-2607.

Sutherland, E. R. (2008). Obesity and asthma. Immunology and Allergy Clinics of North America, 28(3), 589-602, ix.

Sutherland, E. R., Goleva, E., King, T. S., Lehman, E., Stevens, A. D., Jackson, L. P., et al. (2012). Cluster analysis of obesity and asthma phenotypes. PloS One, 7(5), e36631.

Sutherland, E. R., Goleva, E., Strand, M., Beuther, D. A., & Leung, D. Y. (2008). Body mass and glucocorticoid response in asthma. American Journal of Respiratory and Critical Care Medicine, 178(7), 682-687.

Sutherland, E. R., Lehman, E. B., Teodorescu, M., Wechsler, M. E., & National Heart, Lung, and Blood Institute's Asthma Clinical Research Network. (2009). Body mass index and phenotype in subjects with mild-to-moderate persistent asthma. The Journal of Allergy and Clinical Immunology, 123(6), 1328-34.e1.

Sutherland, E. R., & Martin, R. J. (2007). Asthma and atypical bacterial infection. Chest, 132(6), 1962-1966.

Sutherland, T. J., Cowan, J. O., Young, S., Goulding, A., Grant, A. M., Williamson, A., et al. (2008). The association between obesity and asthma: Interactions between systemic and airway inflammation. American Journal of Respiratory and Critical Care Medicine, 178(5), 469-475.

Tan, W. C., Xiang, X., Qiu, D., Ng, T. P., Lam, S. F., & Hegele, R. G. (2003). Epidemiology of respiratory viruses in patients hospitalized with near-fatal asthma, acute exacerbations of asthma, or chronic obstructive pulmonary disease. The American Journal of Medicine, 115(4), 272-277.

Tanaka, H., Miyazaki, N., Oashi, K., Tanaka, S., Ohmichi, M., & Abe, S. (2000). Sputum matrix metalloproteinase-9: Tissue inhibitor of metalloproteinase-1 ratio in acute asthma. The Journal of Allergy and Clinical Immunology, 105(5), 900-905.

Tanaka, H., Miyazaki, N., Oashi, K., Teramoto, S., Shiratori, M., Hashimoto, M., et al. (2001). IL-18 might reflect disease activity in mild and moderate asthma exacerbation. The Journal of Allergy and Clinical Immunology, 107(2), 331-336.

Telenga ED, Tideman SW, Kerstjens HA, Hacken NH, Timens W, Postma DS, van den Berge M. Obesity in asthma: more neutrophilic inflammation as a possible explanation for a reduced treatment response. Allergy. 2012;67(8):1060-8

ten Brinke, A., Sterk, P. J., Masclee, A. A., Spinhoven, P., Schmidt, J. T., Zwinderman, A. H., et al. (2005). Risk factors of frequent exacerbations in difficult-to-treat asthma. The European Respiratory Journal, 26(5), 812-818.

Thamrin, C., Zindel, J., Nydegger, R., Reddel, H. K., Chanez, P., Wenzel, S. E., et al. (2011). Predicting future risk of asthma exacerbations using individual conditional probabilities. The Journal of Allergy and Clinical Immunology, 127(6), 1494-502.e3.

Thomas, B. J., Lindsay, M., Dagher, H., Freezer, N. J., Li, D., Ghildyal, R., et al. (2009). Transforming growth factor-beta enhances rhinovirus infection by diminishing early innate responses. American Journal of Respiratory Cell and Molecular Biology, 41(3), 339-347.

Thomas, P. S., Yates, D. H., & Barnes, P. J. (1995). Tumor necrosis factor-alpha increases airway responsiveness and sputum neutrophilia in normal human subjects. American Journal of Respiratory and Critical Care Medicine, 152(1), 76-80.

Thomas, S. Y., Banerji, A., Medoff, B. D., Lilly, C. M., & Luster, A. D. (2007). Multiple chemokine receptors, including CCR6 and CXCR3, regulate antigen-induced T cell homing to the human asthmatic airway. Journal of Immunology (Baltimore, Md.: 1950), 179(3), 1901-1912.

Thomsen, M., Ingebrigtsen, T. S., Marott, J. L., Dahl, M., Lange, P., Vestbo, J., et al. (2013). Inflammatory biomarkers and exacerbations in chronic obstructive pulmonary disease. JAMA : The Journal of the American Medical Association, 309(22), 2353-2361.

Tliba, O., Deshpande, D., Chen, H., Van Besien, C., Kannan, M., Panettieri, R. A.,Jr, et al. (2003). IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle. British Journal of Pharmacology, 140(7), 1159-1162.

Todd, D. C., Armstrong, S., D'Silva, L., Allen, C. J., Hargreave, F. E., & Parameswaran, K. (2007). Effect of obesity on airway inflammation: A cross-sectional analysis of body mass index and sputum cell counts. Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 37(7), 1049-1054.

Torigoe, K., Ushio, S., Okura, T., Kobayashi, S., Taniai, M., Kunikata, T., et al. (1997). Purification and characterization of the human interleukin-18 receptor. The Journal of Biological Chemistry, 272(41), 25737-25742.

Townsend, J. M., Fallon, G. P., Matthews, J. D., Smith, P., Jolin, E. H., & McKenzie, N. A. (2000). IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. Immunity, 13(4), 573-583.

Truyen, E., Coteur, L., Dilissen, E., Overbergh, L., Dupont, L. J., Ceuppens, J. L., et al. (2006). Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. Thorax, 61(3), 202-208.

van der Wiel E, Ten Hacken NH, van den Berge M, Timens W, Reddel HK, Postma DS. Eosinophilic inflammation in subjects with mild-to-moderate asthma with and without obesity: disparity between sputum and biopsies. Am J Respir Crit Care Med. 2014;189(10):1281-4

van Leeuwen, B. H., Martinson, M. E., Webb, G. C., & Young, I. G. (1989). Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5. Blood, 73(5), 1142-1148.

Van Rensen, E. L., Hiemstra, P. S., Rabe, K. F., & Sterk, P. J. (2002). Assessment of microvascular leakage via sputum induction: The role of substance P and neurokinin A in patients with asthma. American Journal of Respiratory and Critical Care Medicine, 165(9), 1275-1279.

van Veen, I. H., Ten Brinke, A., Sterk, P. J., Rabe, K. F., & Bel, E. H. (2008). Airway inflammation in obese and nonobese patients with difficult-to-treat asthma. Allergy, 63(5), 570-574.

Venkayya, R., Lam, M., Willkom, M., Grunig, G., Corry, D. B., & Erle, D. J. (2002). The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyperresponsiveness through direct effects on resident airway cells. American Journal of Respiratory Cell and Molecular Biology, 26(2), 202-208.

Verbanck, S., Schuermans, D., & Vincken, W. (2010). Inflammation and airway function in the lung periphery of patients with stable asthma. The Journal of Allergy and Clinical Immunology, 125(3), 611-616.

Vernooy, J. H., Kucukaycan, M., Jacobs, J. A., Chavannes, N. H., Buurman, W. A., Dentener, M. A., et al. (2002). Local and systemic inflammation in patients with chronic obstructive pulmonary disease: Soluble tumor necrosis factor receptors are increased in sputum. American Journal of Respiratory and Critical Care Medicine, 166(9), 1218-1224.

Verrills, N. M., Irwin, J. A., He, X. Y., Wood, L. G., Powell, H., Simpson, J. L., et al. (2011). Identification of novel diagnostic biomarkers for asthma and chronic obstructive pulmonary disease. American Journal of Respiratory and Critical Care Medicine, 183(12), 1633-1643.

Wagers, S. S., Norton, R. J., Rinaldi, L. M., Bates, J. H., Sobel, B. E., & Irvin, C. G. (2004). Extravascular fibrin, plasminogen activator, plasminogen activator inhibitors, and airway hyperresponsiveness. The Journal of Clinical Investigation, 114(1), 104-111.

Wahidi, M. M., & Kraft, M. (2012). Bronchial thermoplasty for severe asthma. American Journal of Respiratory and Critical Care Medicine, 185(7), 709-714.

Walker, C., Bauer, W., Braun, R. K., Menz, G., Braun, P., Schwarz, F., et al. (1994). Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. American Journal of Respiratory and Critical Care Medicine, 150(4), 1038-1048.

Walker, C., Kaegi, M. K., Braun, P., & Blaser, K. (1991). Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. The Journal of Allergy and Clinical Immunology, 88(6), 935-942.

Walker, S., Monteil, M., Phelan, K., Lasserson, T. J., & Walters, E. H. (2006). Anti-IgE for chronic asthma in adults and children. The Cochrane Database of Systematic Reviews, (2)(2), CD003559.

Walter, D. M., McIntire, J. J., Berry, G., McKenzie, A. N., Donaldson, D. D., DeKruyff, R. H., et al. (2001). Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. Journal of Immunology (Baltimore, Md.: 1950), 167(8), 4668-4675.

Wan, Y. I., Shrine, N. R., Soler Artigas, M., Wain, L. V., Blakey, J. D., Moffatt, M. F., et al. (2012). Genome-wide association study to identify genetic determinants of severe asthma. Thorax, 67(9), 762-768.

Wang, F., He, X. Y., Baines, K. J., Gunawardhana, L. P., Simpson, J. L., Li, F., et al. (2011). Different inflammatory phenotypes in adults and children with acute asthma. The European Respiratory Journal, 38(3), 567-574.

Wark PA, Johnston SL, Simpson JL, Hensley MJ, Gibson PG.(2002). Chlamydia pneumonia immunoglobulin A reactivation and airway inflammation in acute asthma. Eur Respir J 20:834–840.

Wark, P. A., Johnston, S. L., Bucchieri, F., Powell, R., Puddicombe, S., Laza-Stanca, V., et al. (2005). Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. The Journal of Experimental Medicine, 201(6), 937-947.

Warren, H. S., Kinnear, B. F., Phillips, J. H., & Lanier, L. L. (1995). Production of IL-5 by human NK cells and regulation of IL-5 secretion by IL-4, IL-10, and IL-12. Journal of Immunology (Baltimore, Md.: 1950), 154(10), 5144-5152.

Warwick, G., Thomas, P. S., & Yates, D. H. (2013). Non-invasive biomarkers in exacerbations of obstructive lung disease. Respirology (Carlton, Vic.), 18(5), 874-884.

Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., & Ferrante, A. W.,Jr. (2003). Obesity is associated with macrophage accumulation in adipose tissue. The Journal of Clinical Investigation, 112(12), 1796-1808.

Wenzel, S. E., Balzar, S., Ampleford, E., Hawkins, G. A., Busse, W. W., Calhoun, W. J., et al. (2007). IL4R alpha mutations are associated with asthma exacerbations and mast cell/IgE expression. American Journal of Respiratory and Critical Care Medicine, 175(6), 570-576.

Wenzel, S. E., Barnes, P. J., Bleecker, E. R., Bousquet, J., Busse, W., Dahlen, S. E., et al. (2009). A randomized, double-blind, placebo-controlled study of tumor necrosis factor-alpha blockade in severe persistent asthma. American Journal of Respiratory and Critical Care Medicine, 179(7), 549-558.

Wenzel, S. E., Schwartz, L. B., Langmack, E. L., Halliday, J. L., Trudeau, J. B., Gibbs, R. L., et al. (1999). Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. American Journal of Respiratory and Critical Care Medicine, 160(3), 1001-1008.

Wenzel, S. E. (2012). Asthma phenotypes: the evolution from clinical to molecular approaches. Nature Medicine 18(5), 716-725

Willart, M. A., Deswarte, K., Pouliot, P., Braun, H., Beyaert, R., Lambrecht, B. N., et al. (2012). Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. The Journal of Experimental Medicine, 209(8), 1505-1517.

Wood, L. G., Baines, K. J., Fu, J., Scott, H. A., & Gibson, P. G. (2012). The neutrophilic inflammatory phenotype is associated with systemic inflammation in asthma. Chest, 142(1), 86-93.

Wood, L. G., Garg, M. L., & Gibson, P. G. (2011). A high-fat challenge increases airway inflammation and impairs bronchodilator recovery in asthma. The Journal of Allergy and Clinical Immunology, 127(5), 1133-1140.

Wood, L. G., Simpson, J. L., Hansbro, P. M., & Gibson, P. G. (2010). Potentially pathogenic bacteria cultured from the sputum of stable asthmatics are associated with increased 8-isoprostane and airway neutrophilia. Free Radical Research, 44(2), 146-154.

Woodman, L., Siddiqui, S., Cruse, G., Sutcliffe, A., Saunders, R., Kaur, D., et al. (2008). Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-beta 1. Journal of Immunology (Baltimore, Md.: 1950), 181(7), 5001-5007.

Woodruff, P. G., Boushey, H. A., Dolganov, G. M., Barker, C. S., Yang, Y. H., Donnelly, S., et al. (2007). Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. Proceedings of the National Academy of Sciences of the United States of America, 104(40), 15858-15863.

Woodruff, P. G., Dolganov, G. M., Ferrando, R. E., Donnelly, S., Hays, S. R., Solberg, O. D., et al. (2004). Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. American Journal of Respiratory and Critical Care Medicine, 169(9), 1001-1006.

Woodruff, P. G., Modrek, B., Choy, D. F., Jia, G., Abbas, A. R., Ellwanger, A., et al. (2009). T-helper type 2-driven inflammation defines major subphenotypes of asthma. American Journal of Respiratory and Critical Care Medicine, 180(5), 388-395.

Wu, W., Bleecker, E., Moore, W., Busse, W. W., Castro, M., Chung, K. F., et al. (2014). Unsupervised phenotyping of severe asthma research program participants using expanded lung data. The Journal of Allergy and Clinical Immunology, 133(5), 1280-1288.

Yamaguchi, Y., Suda, T., Shiozaki, H., Miura, Y., Hitoshi, Y., Tominaga, A., et al. (1990). Role of IL-5 in IL-2-induced eosinophilia. in vivo and in vitro expression of IL-5 mRNA by IL-2. Journal of Immunology (Baltimore, Md.: 1950), 145(3), 873-877.

Ying, S., Humbert, M., Barkans, J., Corrigan, C. J., Pfister, R., Menz, G., et al. (1997). Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. Journal of Immunology (Baltimore, Md.: 1950), 158(7), 3539-3544.

Ying, S., Robinson, D. S., Varney, V., Meng, Q., Tsicopoulos, A., Moqbel, R., et al. (1991). TNF alpha mRNA expression in allergic inflammation. Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 21(6), 745-750.

Yokoyama, A., Kohno, N., Sakai, K., Kondo, K.I., Hirasawa, Y. and Hiwada, K., 1997. Circulating levels of soluble interleukin-6 receptor in patients with bronchial asthma. American journal of respiratory and critical care medicine, 156(5), pp.1688-1691.

Zeiger, R. S., Chipps, B. E., Haselkorn, T., Rasouliyan, L., Simons, F. E., & Fish, J. E. (2009). Comparison of asthma exacerbations in pediatric and adult patients with severe or difficult-to-treat asthma. The Journal of Allergy and Clinical Immunology, 124(5), 1106-1108.

Zheng, T., Zhu, Z., Wang, Z., Homer, R. J., Ma, B., Riese, R. J.,Jr, et al. (2000). Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. The Journal of Clinical Investigation, 106(9), 1081-1093.

Zhu, G., Whyte, M. K., Vestbo, J., Carlsen, K., Carlsen, K. H., Lenney, W., et al. (2008). Interleukin 18 receptor 1 gene polymorphisms are associated with asthma. European Journal of Human Genetics : EJHG, 16(9), 1083-1090.