The Clinical Implications of Eosinophilic Inflammation in Asthma

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TABLE OF CONTENTS

Contents: Figures and Tables

5. METHODS

6.1 CLUSTER ANALYSIS AND CLINICAL ASTHMA PHENOTYPES

Figures and tables 6.1.7

6.2 MEPOLIZUMAB AND EXACERBATIONS OF REFRACTORY EOSINOPHILIC ASTHMA

Figures and tables 6.2.6

6.3 CLINICAL AND PATHOLOGICAL OUTCOMES AFTER MEPOLIZUMAB THERAPY: A 12 MONTH WASHOUT ANALYSIS

Figures and tables 6.3.5

I. ABSTRACT

Title: The Clinical Implications of Eosinophilic Inflammation in Asthma

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Asthma is a complex and heterogeneous disorder, comprising domains of pathology (airway inflammation), physiology (disordered airway function) and clinical expression (symptoms and exacerbations) that are variably related. Within this network, the role of eosinophils remains uncertain. This thesis explores further the relationship between eosinophilic inflammation and clinical asthma by: 1. using multivariate statistical techniques to characterise and classify relationships between individual domains in the asthma population; 2. investigating clinical outcomes with mepolizumab (anti-IL 5) in a randomised placebo controlled trial for 12 months, in subjects with refractory eosinophilic asthma. In the first study I have used factor analysis techniques to formulate statistically independent domains of the clinical phenotype. These have been entered into a cluster analysis algorithm to identify subgroups that define a classification model based on expression patterns and composite relationships across the asthma domains. By applying this independently to populations of Primary and Secondary Care asthma, I have identified two secondary care predominant clusters, characterised by discordance between asthma symptoms and eosinophilic airway inflammation. I have shown that discordant clusters derive greatest clinical benefit with a management strategy directed at maintaining a normal sputum eosinophil count, supporting its implementation in secondary care. More specifically, I report a 10-fold reduction in severe exacerbations with inflammation guided therapy in discordant eosinophil-predominant asthma, supporting a specific role for eosinophils in these events. In the second study, I report a significant reduction in severe exacerbations with mepolizumab therapy, but no effect on other clinical outcome measures. These findings support a specific effector role for eosinophils in the pathogenesis of severe exacerbations; and dissociation of this endpoint and other clinical outcome measures. Finally, I report a 12 month washout analysis of mepolizumab treated subjects in which a significant increase in severe exacerbation frequency is observed that is temporally preceded by rising sputum eosinophils.

II. ACKNOWLEDGEMENTS

It goes without saying that the realisation of any research project is a product of the efforts of many people. To say the work described in this thesis was a team effort would be an understatement. I would like to begin with my thanks to Bev, Sue, Maria, Michelle and Amisha who kept everything, including me, under control. Thanks also to Hilary, who was there at the beginning, but sensibly moved on. I am indebted to Bev for taking the lead and tackling everything, including those morale-sapping piles of bureaucracy. The contributions of the research nurses went far beyond managing the day to day aspects of the clinical trial. The relationships they have built over years with all the patients attending the asthma clinic have provided us all with the luxury of recruitment to and retention of subjects for our research studies.

My thanks and gratitude to the patients. Without their participation, of course, there would be nothing. For some, to help was the only motive and an act of generosity I am not sure I could replicate. For others, it was in the hope of some relief with a possible 'wonder drug'. This alone is evidence enough that we need to do better.

Thanks to my fellow registrars in the office for stimulating discussion that was not always academic and for being there to keep things moving when I was away. I am grateful to Mike Berry and Dominic Shaw for showing me the ropes when I started and for teaching me how to design a database. Thanks to Will Monteiro for all his work with the sputum samples, for showing me how it should be done and for honouring the deadlines that I kept imposing for results. My thanks to Debbie Parker for supporting Will with this work. My thanks to Natalie Neale who agreed to take charge of early morning bronchoscopy samples and to Katy Roach for helping with processing and analysing those samples. Thanks also to my bowling colleagues in the lab for their accuracy on the alley; special thanks to Vijay whose low scores kept things competitive for the other teams.

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I end on a personal note, and take this fleeting opportunity to officially acknowledge the love and support of my family. I am grateful to my brother for being who he is. To my parents I say, any small achievement in my life is only made possible with your blessings. To my wife, your patience with me since the birth of Ayanna has been far more than I could have hoped or expected. One day, I will make it up to you….

III. STATEMENT OF WORK PERSONALLY PERFORMED

Study 1: Cluster analysis and clinical asthma phenotypes

Data for performing the analyses was obtained from the clinical database for patients attending the Glenfield Hospital Refractory Asthma Clinic; provided by Dr Dominic Shaw and Dr Mike Thomas for subjects participating in the Primary Care asthma studies; and provided by Dr Ruth Green for the post hoc analysis of her clinical trial. I devised and planned the methodology used for applying multivariate statistical techniques to construct phenotypes of asthma and performed all the analyses related to this study. I wrote the published manuscript.

Study 2: Mepolizumab and severe exacerbations in refractory eosinophilic asthma

I participated in the design of this study and co-wrote the study protocol. I personally obtained ethical approval for the study and was responsible for ensuring the trial fulfilled standards of research governance stipulated by the local research and ethics committee and MHRA. I personally designed all study related paperwork and created a secure and encrypted database for electronic data storage. I was responsible for subject recruitment to the study and obtained the consent of all subjects to the study. I performed all clinical assessments of subjects at their study visits and at the time of unscheduled visits for exacerbations. I personally performed approximately 20% of all clinical measurements at scheduled visits. I assisted with all bronchoscopies. Sputum processing and cell count measurements were performed by an experienced and skilled technician (William Monteiro). I was personally responsible for analysis and interpretation of all study data.

IV. PUBLICATIONS ARISING FROM THIS THESIS

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Ref Type: Abstract

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Ref Type: Abstract

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Ref Type: Abstract

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Ref Type: Abstract

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Ref Type: Abstract

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Ref Type: Abstract

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Ref Type: Abstract

1. OVERVIEW OF THESIS

1.1 Introduction

Asthma is a common disease of the lower airways that impacts significantly on the global burden of chronic disease. It is characterised by typically variable symptoms arising from a complex interplay between chronic airway inflammation and disordered airway function. Although effective therapies exist, a significant unmet clinical need remains. This is most apparent in the subgroup with difficult asthma, in which accepted treatment algorithms are largely ineffective. The socioeconomic impact of this minority group is disproportionately high, accounting for the majority of asthma related morbidity, mortality and healthcare costs. This has led to greater emphasis being placed on research seeking to understand factors associated with a poor response to therapy. It is increasingly apparent that reasons underlying this problem are complex and multi-factorial and illustrative of the complexity and heterogeneity of difficult asthma. Characterising this heterogeneity to better inform management and therapeutic strategies is currently a critical goal of asthma research.

In this chapter, I summarise some of the evolving concepts, key questions and challenges facing clinical asthma currently that form the background of work undertaken in this thesis. This is followed by a summary of the studies that have been performed.

1.2 Background – Evolving Concepts in Clinical Asthma

Although traditionally considered a disease of Western societies, the prevalence of asthma has risen rapidly across the world in recent decades and is now viewed more appropriately as a disease of global importance. In the UK, the economic burden of asthma includes NHS costs of £850 million and over 18 million lost working days per annum (1). Of the 1400 deaths each year due to asthma, one third occurs in people under the age of 65 and most are preventable. There is a clear need for more effective

medical intervention and it is fortunate that asthma attracts considerable research interest.

1.2.1 Defining Asthma – a composite of domains

Asthma derives its name from the greek verb *aazein*, meaning 'to pant' and one of the earliest references of its use in a medical context was by Hippocrates to describe ailments characterised by spasms of breathlessness occurring more frequently in anglers, tailors and metal workers (2). It is noteworthy that the description of asthma as a clinical syndrome of variable breathlessness and wheeze, associated with exposure to environmental material has changed little with time. On the one hand this suggests a condition that is characterised with little difficulty in clinical practice; however it is perhaps also indicative of the limited advances that have been achieved in our understanding of the aetiology and underlying pathological mechanisms of asthma. The current definition provided by the Global Initiative for Asthma (GINA, 2006) (3) states:

> *Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, chest tightness, breathlessness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment.*

This statement characterises asthma across three domains: clinical expression (symptoms); airway pathology (chronic inflammation); and disordered airway function (reversible airflow obstruction and airway hyperresponsiveness). Furthermore, a linear relationship is inferred between these domains: chronic inflammation predisposes to disordered airway function; airflow obstruction arising from disordered function in turn leads to clinical symptoms. Two implications of this model are that symptoms reliably inform underlying biological activity in asthma and treatment of airway inflammation is sufficient to control both the biological activity of asthma and clinical symptoms.

1.2.2 Clinical Outcomes in Asthma – understanding different endpoints

The management of clinical asthma is characterised by two major endpoints: control of fluctuating daily symptoms and treatment of exacerbations. Severe exacerbations are of considerable importance with short and longer term consequences. Acutely, they are a cause of significant morbidity and impairment in quality of life. In their severest form, exacerbations are the primary cause of asthma related mortality. In the United States severe exacerbations are reported to occur in only 20% of patients and these 'high cost' patients are responsible for 80% of asthma related costs (4).The economic burden of healthcare utilisation is compounded by the indirect cost of prolonged absence from work in a population that is comprised primarily of younger, working adults. In the longer term, there is evidence that frequent severe exacerbations are associated with accelerated decline of lung function (5). Preventing severe exacerbations is therefore an important therapeutic goal in asthma.

The relationship between symptoms and exacerbations is also considered to be linear, such that exacerbations occur as a consequence of poor asthma control, defined by worsening symptoms. Indeed, despite their clinical importance, exacerbations do not form part of the GINA definition of asthma, likely reflecting the opinion that they represent part of the continuum of symptoms, and not a distinct entity. Together, the relationships described form the basis of the stepwise approach to asthma therapy, in which symptoms are the central indicator of both biological activity and clinical risk of exacerbation [Figure 1]; anti-inflammatory therapy is therefore titrated with the primary aim of relieving symptoms (6).

However, the relationship between symptoms, disease activity and exacerbations is not straightforward. It is increasingly recognised that exacerbations may occur on a background of apparently good clinical asthma control. This idea is supported by the observation that a significant minority of asthma deaths occur in patients with a history of mild or moderate asthma (7). In the more controlled setting of a clinical trial, severe exacerbations were also reported for participants enrolled in the Gaining

Optimal Asthma control (GOAL) study with 'well controlled' or 'totally controlled' asthma (8).

One fundamental problem that undermines studies investigating asthma exacerbations is the absence of a clear, consistent and objective definition. Exacerbations are defined clinically by worsening symptoms beyond normal day-to-day variation that trigger a need for additional therapy. This definition is of value for guiding appropriate and safe clinical practice, but fails to provide either a scientific basis or an objective indicator for these events. The need for a course of oral glucocorticoids is commonly used in clinical trials as an objective measure of a severe exacerbation episode (9). However, it follows that a decision to initiate additional therapy is based on a *subjective* judgement made by either the self medicating patient or assessing clinician. Both the perception of their symptoms by an individual and how this information is communicated to healthcare providers will vary considerably within a population. The inclusion of more rigorous measurable criteria has failed to be reliable. In the Formoterol And Corticosteroids Establishing Therapy (FACET) study, a 30% fall in peak flow for two consecutive days was included in the definition of severe exacerbations (10). However, only 30% of severe exacerbations met this criterion and only 32% of episodes fulfilling the criterion were treated with oral glucocorticoids. Increasing symptoms at the time of exacerbation are therefore not always associated with a significant measurable decline in lung function. The differences or otherwise between exacerbations and poor asthma control remain a matter of debate. The traditional view that exacerbations represent one end of the spectrum of symptom expression would imply that the two states are clinically and pathologically synonymous. More recently, exacerbations and poor control have been distinguished by whether good clinical control was achieved prior to the onset of clinical deterioration. Using this criterion, Reddel and colleagues reported differences in diurnal peak flow variability between the two groups (11). From a scientific perspective this definition has merit as it restricts the labelling of exacerbations to events that are sporadic and unpredictable. Understanding mechanisms by which these events occur may better inform strategies for risk screening and prevention that is of clinical benefit. However, clinicians need to remain mindful of the risk of severe

‘exacerbations‘, occurring in patients with poor clinical control. Near fatal and fatal asthma exacerbations are most frequently reported in patients with poor day-to-day asthma control. In this context, a distinction between exacerbations and poor clinical control is not helpful.

The increasing awareness of dissociation between clinical endpoints of asthma is prompting reappraisal of traditional linear pathways of airway pathology, physiology and clinical disease expression. In response to the uncertainty of relationships that exist between these domains of asthma, a recent European Respiratory Society (ERS) and American Thoracic Society (ATS) expert panel recommended consideration of individual endpoints in clinical studies of drug efficacy as discrete measures of either current asthma control or future exacerbation risk (12).

1.2.3 Difficult and Refractory Asthma – challenges of overcoming unmet clinical need

In the UK, approximately 80% of asthma is managed satisfactorily in primary care using a stepwise algorithm. The remaining 20% require evaluation in secondary care and 50% of this group will have ‘difficult asthma’ that is managed in specialist clinics long term. Patients with difficult asthma either fail to achieve satisfactory control or require high doses of therapy to retain control (generally defined as step 4 or 5 of the British guideline) (13), with consequent risk of iatrogenic sequelae. In broad terms, the population with difficult asthma may be categorised into two groups. One group (refractory asthma) comprises patients with severe, treatment resistant disease, at risk of severe exacerbation events (14). In a minority, these exacerbation events are life threatening. It is estimated over half of all asthma related deaths occur in patients with a prior history of severe asthma (7); most such fatalities are likely to arise in this population. The second group consists of a heterogeneous population characterised by poor clinical control due to miscellaneous disorders that can mimic or aggravate asthma symptoms, independent of any effect on disease activity (15) [Table 1]. International bodies have sought to distinguish refractory asthma from the remainder of difficult-to-treat patients by specifying the absence of common confounders in the diagnosis of refractory asthma (14;16).

The population with difficult asthma represents a critically important subgroup for a number of reasons. From a health economic perspective, it is estimated that these patients are responsible for over 60% of asthma related healthcare costs. Much of this extra cost is attributable to a higher frequency of severe exacerbations in patients with refractory asthma (9). From a scientific viewpoint, the failure of stepwise treatment algorithms in difficult asthma is multifactorial with broad implications for asthma research generally. Dissociation between measures of clinical and disease activity is a key element. This is complicated further by heterogeneity in patterns of dissociation observed in subgroups of difficult asthma. In a proportion of patients, reported symptoms are due to co-morbidities, unrelated to the disease activity of asthma and therefore of little prognostic importance [Table 1]. In this setting, symptoms overestimate asthma severity and for reasons discussed earlier may lead to inaccurate labelling of patients as 'frequent exacerbators'. In contrast, paucity of symptoms leading to delay in accessing healthcare resources is a significant contributor to episodes of near fatal asthma. Poor perception of bronchoconstriction (17) and an abnormal chemoreceptor response to hypoxia (18) are factors that have been implicated in this group. Improving assessment by characterising clinical heterogeneity, to better understand relationships that exist between the different elements of disease, is an essential part of optimising care in difficult asthma (19).

In this context, the distinction between 'clinical control' that is a measure of symptoms alone, and 'asthma control', which refers to assessment of disease activity, is receiving increasing favour (20). In clinical practice, asthma control is considered the sum of two components: current clinical impairment and future exacerbation risk (12). It is a concept that has been made possible with the recent identification of two biomarkers of airway inflammation, fractional exhaled nitric oxide (FeNO) and sputum eosinophils, which can provide useful additional information of disease activity. FeNO is a marker of the likely response of symptoms and lung function to glucocorticoid therapy, (21). In clinical studies, the negative predictive value of a low FeNO has been utilised to guide safe down-titration of glucocorticoid dose in asthma (22). The sputum eosinophil count is the best available marker for predicting future exacerbation risk and several studies have demonstrated a significant reduction in exacerbation frequency with a

management strategy that incorporates the sputum eosinophil count to guide glucocorticoid therapy (23-25) [section 2.3.1].

1.2.4 Specific Molecular Therapies – use and abuse

Although improvements in disease assessment are of value for tailoring strategies to deliver available therapies effectively, there is a need to develop new agents for patients with refractory asthma in whom currently available therapies are either ineffective, or for drugs such as oral glucocorticoids and immunosuppressants, prohibitively toxic for long term use at an effective dose. Recent advances in immunology have identified several potential targets for pharmacological intervention that are directing new avenues of drug development. A number of novel and primarily engineered molecular therapies are at an advanced stage of clinical development (26); patient trials with these drugs are invaluable not only for informing clinical efficacy but for also providing a novel strategy to understand elements of asthma immunobiology *in vivo*.

While attractive, the narrow spectrum of biological activity of molecular drug targets hastens the need to better classify clinical and biological diversity in asthma. The full clinical potential of immunomodulation may only be realised in a highly selected subgroup of patients. Correctly identifying the right patients for a particular molecular target is a fundamental part of achieving success with these drugs. This is well recognised by the scientific community and illustrated by a change in emphasis from developing therapies appropriate for all patients to a 'phenotype specific' approach. It is likely that specific molecular therapies will inform the biological basis of disease and in this way, help characterise clinical and biological phenotypes of asthma.

1.3 Summary – an overview of studies performed

This thesis includes two original studies that explore distinct but related priority areas of asthma research:

Study 1. Characterisation of asthma heterogeneity using multi-variate statistical techniques.

Study 2. Evaluation of mepolizumab, a novel molecular therapy with highly specific anti-eosinophilic activity, for prevention of severe exacerbations in refractory eosinophilic asthma.

1.3.1 Characterising asthma heterogeneity

The importance of characterising heterogeneity in asthma to clinically inform management strategies and biologically inform the likely response to specific molecular therapies, has already been discussed. The objective of characterisation is to identify subgroups or phenotypes that form the basis of a model for classification. In chapter 3, I discuss in greater detail the limitations imposed by heterogeneity, the principles of classification using the taxonomy of organisms as a biological paradigm and the goals of classification that are of relevance to asthma. Complexity in the characterisation of asthma arises from the multidimensional nature of the disease. Each aspect or dimension of the disease is an important component of every phenotype. I present the case for using multi-variate statistical techniques to construct phenotypes that incorporate multiple dimensions in an unbiased manner.

Study 1 [section 6.1] examines the application of the multi-variate techniques factor and cluster analysis, to construct asthma phenotypes independently in two populations managed in primary care (predominantly mild to moderate asthma) and secondary care (predominantly refractory asthma) respectively. The clinical relevance of the phenotypes identified is then explored by comparing phenotype specific outcomes in a third population. Subjects in this group were participants of a randomised study comparing outcomes using treatment strategies guided by clinical control or the sputum eosinophil count (23).

1.3.2 Evaluation of mepolizumab therapy in refractory eosinophilic asthma

The recent development of several molecular therapies with specific and potent anti-eosinophil properties has renewed interest in the role of eosinophils in clinical asthma. As a drug target, the eosinophil is attractive because it is regulated primarily by a

single cytokine (interleukin 5) [section 2.2] and as a cell without a critical role in immune function, specific suppression of eosinophilic inflammation is considered to be safe. To date, the role of eosinophils in asthma remains a matter of debate. A synopsis of the conflicting evidence is presented in [section 2.3]. The utility of monitoring and treating eosinophilic inflammation for preventing severe exacerbations is part of a persuasive body of evidence that suggests an effector role for eosinophils in the pathogenesis of these events [section 2.3]. From a clinical perspective, this is a question of critical importance as the disproportionate costs associated with severe exacerbations mean that even highly expensive molecular therapies can be cost effective, if efficacious.

Study 2 [section 6.2] explores the hypothesis that eosinophils have an important effector role in the pathogenesis of severe exacerbations. Mepolizumab is a monoclonal antibody against interleukin-5 with specific anti-eosinophilic properties [section 2.4.3]. We have used this drug in a clinical trial to explore the hypothesis from two perspectives. Firstly, the frequency of severe exacerbations is compared in a randomised, double blind, placebo controlled, parallel group study using mepolizumab for 12 months, in subjects with documented evidence of eosinophilic airway inflammation and a history of recurrent severe exacerbations. The rationale for phenotypic characterisation has been discussed in a general context earlier in this chapter and presented more specifically in the context of previous clinical experience with mepolizumab in asthma and other eosinophilic disorders, in section 2.4.3.

After completion of the treatment phase, subjects were followed up for a further 12 months. This has provided an opportunity to observe and map changes in airway inflammation and clinical disease expression longitudinally after drug washout. Results of this observational study are presented in [section 6.3].

1.3.3 Figures and Tables

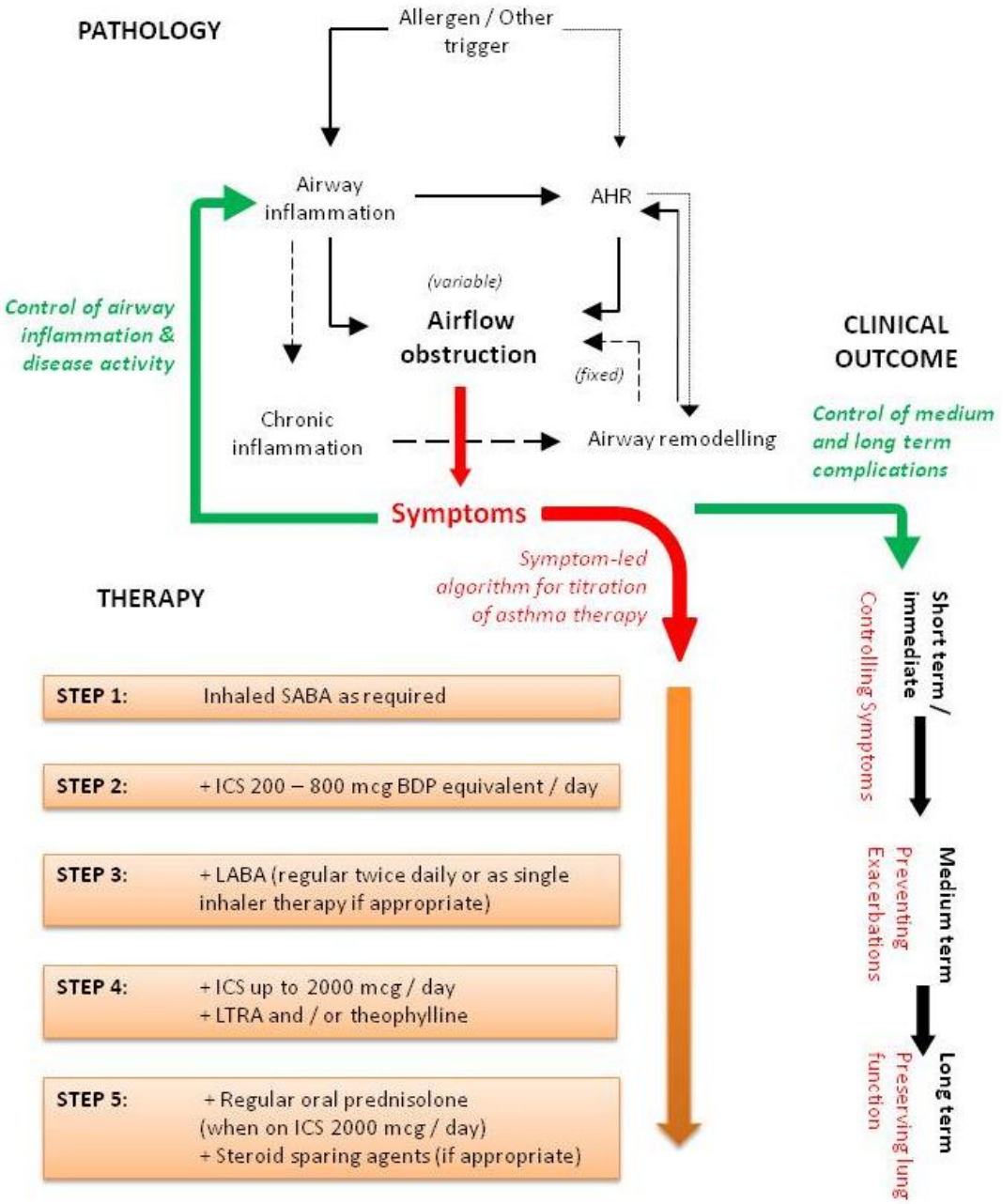


Figure 1: Linear paradigm of asthma

Three domains are described (pathology, clinical outcomes and therapy). Linear relationships exist both between and within domains. Clinical symptoms are at the centre of this model. They are an indicator of underlying disease activity and future clinical risk and the primary determinant of stepwise therapy titration.

Table 1: Common asthma aggravants – a differential diagnosis for asthma symptoms

Without airflow obstruction		With airflow obstruction
Normal spirometry	**Restrictive spirometry**	
Dysfunctional breathing *	Cardiac failure†	COPD*
Vocal cord dysfunction*	Pulmonary fibrosis	Bronchiectasis*† (including allergic bronchpulmonary aspergillosis)
Gastroesophageal reflux disease*		Churg Strauss Syndrome*
Pulmonary vascular disease		Inhaled foreign body†
Chronic Cough syndromes		Obliterative bronchiolitis
Psychological factors ^		Large airway stenosis
		Sarcoidosis†

The table summarises commonly recognised disorders that may mimic or aggravate clinical symptoms of asthma. Most disorders do not affect underlying disease activity in asthma and therefore are a cause of dissociation between clinical expression and underlying pathology.

* These conditions may coexist with asthma

† These conditions may be associated with normal spirometry

^ Psychological factors refer to a miscellany of disorders that are associated with an altered psychological state nad manifesting with increased asthma-like symptoms, often as a consequence of dysfunctional breathing. Psychological factors are also associated with behavioural traits that lead to increased disease activity in asthma. These include poor treatment adherence, failure to attend medical appointments and smoking. The precise relationship between these conditions and asthma symptoms can be difficult to measure as they may not be recognised; are not readily measurable with objective tools; and may arise primarily as a consequence of poor asthma control.

Table 2: American Thoracic Society clinical criteria for refractory asthma (2000)

Definition ^: ATS Workshop – Severe or Refractory Asthma	
Major criteria (must have at least one)	
1	Oral steroids for >50% of past year
2	Continuous high-dose inhaled steroids > 1260 µg BDP *
Minor criteria (must have at least two)	
1	Concurrent use of at least one other controller medication
2	Daily symptoms requiring a short-acting b-agonist
3	FEV_1 <80% predicted
4	One or more urgent-care visits in the past year
5	Three or more oral steroid bursts in the past year
6	Deterioration with decrease in steroid dose of 25%
7	History of a near-fatal event

^The definition stipulates that criteria are met after exclusion of common aggravants and patient adherence with therapy is deemed satisfactory.

* BDP = Beclomathasone dipropionate. Criteria are listed for other inhaled corticosteroids, although commonly applied BDP dose equivalents do not apply (14).

2. EOSINOPHILS AND ASTHMA

2.1 Introduction

The German scientist Paul Ehrlich established procedures to stain peripheral blood cells and in so doing discovered the eosinophil, a leucocyte so named due to a characteristic affinity for acid aniline dyes such as eosin (27). Since then, the role of eosinophils in disease pathology has remained a subject of considerable debate. An association of circulating and tissue eosinophilia with helminthic infections (28), asthma (29) and anaphylaxis (30) was reported soon afterward. The observation of pulmonary eosinophilia in guinea pigs surviving anaphylactic shock (31) led to the suggestion that eosinophils may beneficially modify this process. A protective role for eosinophils in allergic diseases remained a popular idea into the 1960's and several possible mechanistic pathways were proposed to explain this, including evidence for the release of an anti-histamine (32). A shift in scientific opinion occurred with evidence for proinflammatory and cytotoxic activity of eosinophils in schistosomal disease (33). This finding was coupled with isolation of the first protein products of eosinophil degranulation during the same period and initiated an era of eosinophil research directed at evaluating their role as immune effector cells in disease.

In this section I summarise the form, function and regulation of eosinophils and discuss their potential role in asthma.

2.2 Eosinophil Biology

2.2.1 Overview of events in eosinophil trafficking

The natural history of eosinophils *in vivo* may be summarised in 3 stages (34) [Figure 2]:

1. Development, maturation and release into the circulation – haemopoiesis
2. Migration from the circulation into specific tissues – homing
3. Tissue survival and apoptosis

Eosinophil haemopoiesis

Eosinophils are terminally differentiated pleiotropic granulocytes that originate from pluripotent common myeloid progenitor cells in the bone marrow and share their lineage with basophils. Differential patterns of cytokine receptor expression of progenitor cells are critical determinants of their differentiation and development. In broad terms, surface expression of CD34 is inversely related to the maturation level of eosinophils and other leucocytes [Fig 2]. However, the temporal and lineage specificity observed is a product of restricted expression of the different cytokine receptors rather than differences in effector function of the cytokines, which are closely related (35). Evidence of increased eosinophil haemopoiesis has been reported in human studies of atopy (36) and asthma (37) and in animals using an allergen challenge model (38).

Interleukin-5 (IL-5) mediates terminal differentiation and release of mature eosinophils into the circulation. This is supported by several lines of evidence. Early *in vitro* studies using recombinant human interleukin-5 (rhIL-5), in either liquid or semi-solid cultures, induced eosinophil production from normal human bone marrow, with no activity on other cell lineages (39). More recently, the kinetics of eosinophilia *in vivo* after administration of IL-5 both systemically and locally by inhalation has been evaluated in a small placebo-controlled crossover study of patients with mild asthma (40). The authors reported a significant 5-fold increase in circulating eosinophils at 24 hours with intravenous but not inhaled IL-5. Furthermore, the kinetics of this response

was non-linear. Rapid release of preformed bone marrow CD34+ cells (intermediate to late eosinophil progenitors) was followed by increasing numbers of mature eosinophils expressing high surface CCR3 levels. The results support a role for systemic IL-5 in bone marrow to initiate an acute eosinophilic response by release of preformed cells at an advanced stage of development and by accelerating differentiation of bone marrow CD34+ cells to maintain this response over time. The recent development of specific and efficacious molecular antagonists of IL-5 has opened a new avenue for research of IL-5 function. A recent study found treatment with mepolizumab, a specific anti IL-5 agent [section 2.4.3] in subjects with mild asthma was associated with a significant fall in numbers of bone marrow eosinophil progenitors, indicating accelerated apoptosis or maturational arrest in the absence of IL-5 (41).

Eosinophil homing

Eosinophils are predominantly tissue dwelling cells, spending only a brief period in circulation. At steady state, the ratio of tissue: blood eosinophils is usually 100:1 or more (42). The peripheral blood eosinophil count may therefore be a poor guide of tissue eosinophilia. This is supported by the variable occurrence of peripheral blood eosinophilia in different eosinophilic disorders (43).

In health, eosinophils migrate primarily to the gastrointestinal mucosa where they are thought to have a role in gut surveillance and provide host immunity against helminthic parasites (44). In disease, eosinophils typically migrate to the organ of involvement. Homing and accumulation of eosinophils in specific tissues is a multi-factorial and sequential process that is well co-ordinated (34); the following steps are recognised:

1) Interactions between circulating blood eosinophils and the vascular endothelium within the target organ leading to stepwise transformation of a free flowing cell into a stationary cell that is closely bound to adjacent endothelium.
2) Diapedesis or transmigration of eosinophils across the vascular endothelium and out of the vascular compartment.
3) Migration or chemotaxis of eosinophils from the interstitium to sites of inflammation

4) Prolonged survival and persistence within inflamed tissues

Each step in the process is regulated by factors that are eosinophil specific, promoting cumulative enrichment of these cells. In broad terms, steps 1 and 2 are mediated primarily by specific cellular interactions [Table 3]; steps 3 and 4 are a function of eosinophil specific chemokines. However, cytokines play an important permissive role in the first stages by activating cellular expression of ligands and adhesion molecules respectively. There is *in vitro* evidence for enhanced adhesion of eosinophils with human microvascular endothelial cells in the presence of IL-5 (45).

Arrest of circulating eosinophils within the vascular bed of target organs is mediated by several specific interactions between adhesion molecules expressed on vascular endothelial cells and counter-ligands expressed on eosinophils. Inflammatory cytokines expressed at the site of tissue damage promote expression of both endothelial adhesion molecules and surface ligands on eosinophils. The first interactions bring the eosinophil closer to the endothelium (tethering) and its motion is viewed as 'rolling' along the endothelial surface. This leads to eosinophil activation and greater expression of surface binding ligands that permit stronger interactions to form. These progressively slow the eosinophil to a halt ('firm arrest'), enabling transmigration to occur.

Eosinophil chemotaxis

Almost 60 years ago, in a series of elegant experiments with ovalbumin challenged guinea pigs, Samter proposed that *'an eosinotactic factor develops in the peribronchial tissue of guinea pigs subsequent to antigen-antibody reactions'* (31). Since then, over thirty different chemoattractant molecules or chemokines have been identified and characterised. Chemokines are soluble proteins that bind with receptors on the surface of leucocytes and trigger intracellular signalling cascades that direct cell locomotion along a path determined by the chemokine concentration gradient (chemotaxis). Several chemokines, both general and eosinophil specific, have been identified with varying efficacy for eosinophil chemotaxis and many different cell types can express chemokines at sites of inflammation.

Chemokine receptor (CCR) 3 is found most abundantly on the surface of eosinophils and binds members of the β-chemokine family including eotaxin 1-3; Regulated upon Activation, T-cell Expressed and Stimulated (RANTES); Monocyte Chemotactic Protein (MCP) 3 and 4; and Macrophage Inflammatory Protein (MIP) 5 (46). Specificity for eosinophil chemotaxis arises from a combination of highly polarised expression by eosinophils of CCR 3, together with specificity of chemokines for this receptor. Of the chemokines binding CCR 3 and therefore active in eosinophil chemotaxis, the eotaxins bind exclusively with CCR 3 alone and therefore exhibit functional eosinophil specificity. Studies demonstrate synergy in the action of eotaxin and IL-5 to promote eosinophilic inflammation (47). This is achieved in part by complementary effects of the two proteins on eosinophil trafficking. IL-5 acts predominantly through effects on the bone marrow to expand the circulating pool of eosinophils; eotaxins play a major role in directing these eosinophils to sites of tissue inflammation. There is also evidence that IL-5 has a priming effect on eosinophils, increasing their responsiveness to eotaxin and other chemokines (48). This is achieved in part by increasing the surface expression of CCR 3 (49).

The function of chemokine receptors is not limited to chemotaxis as studies indicate a role for these receptors in promoting cellular activation and modulating respiratory burst (46).

Eosinophil survival in tissues

Once established in the tissues, eosinophil survival is maintained through a combination of interactions with components of the tissue matrix and effects of a number of pro-inflammatory cytokines in the microenvironment (43). The life span of eosinophils *in vivo* is not known but is estimated to be several weeks in the presence of a favourable milieu (44). In asthma, there is evidence supporting prolonged survival and delayed apoptosis of eosinophils in circulating blood (50) and the bronchial submucosa (51).

The precise mechanisms for survival are complex and unclear. It is probable that a number of different intracellular signalling pathways are involved. Mechanistically, these pathways inhibit apoptosis by either delaying passive cell death (i.e. maintaining

the survival signal) or preventing active cell death (52). Activated eosinophils are themselves an important source of cytokines and therefore play a role in their own survival. Cytokines of eosinophil origin may act either directly in an autocrine or paracrine manner or may act indirectly to activate the release of survival cytokines by other cell types. The haemopoietins IL 3, GM-CSF and IL-5 are the major recognised survival factors for tissue eosinophils and all are secreted by eosinophils. Eosinophils also secrete IL-4, IL-13 and RANTES, which can activate the expression of survival cytokines, notably IL-5, by CD 4+ T-cells.

There is evidence that eosinophil adhesion to the matrix proteins fibronectin or laminin promotes release of GM-CSF by the cell and increased cell survival that is reversed by treatment with dexamethasone or antibodies to laminin and GM-CSF (53), implying synergy between tissue matrix factors and secreted cytokines in this process. This idea is also supported by the finding that IL-5 mediated survival and maturation of eosinophils into an activated, hypodense phenotype is facilitated in the presence of fibroblasts (54).

IL-5 is a critical survival factor for tissue eosinophils. One study performed in explanted tissue from eosinophilic nasal polyps identified high levels of IL-5 in lymphocytes, mast cells and eosinophils. Treatment with an anti IL-5 agent induced eosinophil apoptosis and significant reduction in tissue eosinophilia (55). Interestingly, one recent study has reported failure of glucocorticoid mediated eosinophil apoptosis in the presence of IL-5 (56), suggesting a role for anti-IL 5 therapies in glucocorticoid resistant eosinophilic disease.

The survival of tissue eosinophils is regulated by a complex network of numerous inter-related factors. It is therefore unlikely that targeting a single factor will achieve complete amelioration of tissue eosinophilia.

2.2.2 Interleukin 5

Introduction

Formally discovered in 1986 and originally labelled IL-4 (57), interleukin 5 (IL-5) belongs to a family of highly conserved and phylogenetically related growth factor cytokines that also includes IL-3, IL-4 and GM-CSF. Genes coding for these proteins are closely linked on chromosome 5q in humans (58).

In the context of eosinophil biology, IL-5 is uniquely positioned as the single most important cytokine modulating eosinophil trafficking. Haemopoiesis, homing and tissue survival are all dependent upon IL-5 [Fig 2]. There is consistent evidence in murine studies and more recently in humans of a pivotal role for IL-5 in eosinophilia. Overproduction of IL-5 in transgenic mice results in profound eosinophilia (59), while IL-5 knockouts have very few eosinophils in the lungs and circulation following allergen challenge in a murine model of asthma (60). Transgenic models allow a distinction to be made between constitutive eosinophilia and eosinophilia arising from immune activation. Life expectancy of transgenic mice with a constitutive eosinophilia is normal (59); implying that eosinophilia in the absence of cellular activation is not associated with pathology. In human subjects with mild asthma, systemic administration of recombinant IL-5 induces a peripheral blood eosinophilia (40). Finally, specific anti-IL-5 therapies are associated with a marked fall in circulating eosinophil levels and amelioration of the eosinophil response after antigen challenge in animal models (61-63) and humans (64). This is a class effect seen with all such molecules used.

IL-5 therefore represents a critical target for drug development strategies to control eosinophil associated disease. In keeping with this, diseases involving eosinophilia without increases in other blood-cell lineages are usually accompanied by an overproduction of IL-5 (65).

IL-5 Structure

In humans, functional IL-5 has a novel homodimeric structure. Each monomer is a 115 amino acid polypeptide arranged in a 4 α-helix bundle. In isolation, monomers have no biological activity. The dimeric conformation of IL-5 is formed by the association of monomers in anti-parallel. Each domain of this protein comprises a 4 α-helix bundle that is composed of 3 α-helices from one monomer and 1 α- helix from the other. Cys-Cys interactions form disulphide bridges that bind the domains and are critical for protein assembly (66). Secreted IL-5 has a molecular weight that is almost twice as high as the native protein due to extensive carbohydrate binding. The significance of this is uncertain as the carbohydrate appears unnecessary for biological activity of IL-5 *in vitro* (67).

To date, IL-5 has been isolated from six species (human, macaque, mangabay, murine, rat and bovine). The primary sequence of amino acids between species is highly conserved. The sequence for human IL-5 shows > 95% homology with other primates; 73% homology with murine and rat IL-5; and 66% homology with bovine IL-5. Species specificity of protein function is conferred by sequence differences at the C-terminal end. Studies evaluating functional cross reactivity between human and murine IL-5 have demonstrated comparable efficacy of molecules for human eosinophils. In contrast, the activity of human IL-5 for mouse eosinophils is 100-fold lower. The observed functional differences are attributed to 8 residues near the C-terminal end of human IL-5 (68). Differences may also exist in the species specific biological function of IL-5. In the mouse, IL-5 is a B-cell growth and differentiation factor (69); a property of the molecule that has not been identified in humans (70). This has implications for interpreting outcomes of IL-5 studies that are based on animal models.

IL-5 Production

IL-5 has been historically described as a T-cell product (65). However, three important sources of IL-5 are now recognised: CD4+ Th2 lymphocytes; mast cells; and eosinophils. There is conflicting evidence about the primary source of IL-5 *in vivo*. One study reported 70-80% of the IL-5 mRNA signal was located in CD3+ T-lymphocytes from bronchalveolar lavage fluid and bronchial biopsy specimens of patients with

asthma (71). In contrast, another study performed in a similar asthma population identified most detectable IL-5 protein in bronchial biopsy samples to be associated with mast cells (72). One explanation for these differences might be that both mast cells and eosinophils store preformed IL-5 protein within intracellular granules that is available for release during the inflammatory response. T-lymphocytes on the other hand have a primary role in orchestrating the immune response and rapidly use up any preformed cytokine to achieve this. Elevated mRNA levels reflect continuous synthesis of IL-5 protein in these cells.

A number of studies have examined pathways associated with T-cell regulation of IL-5 gene expression *in vitro*. Efficient production of IL-5 requires activation of both the T-cell receptor (TCR) and costimulatory ligand (CD 28) (73). Differences exist in the pathways leading to IL-5 gene expression following ligand binding of the two receptor domains, implying a complementary effect. Ligand binding of the TCR is associated with pathways that are activated by a rise in intracellular calcium and are inhibited by cyclosporin A. Pathways mediated through coupling of CD 28 are calcium independent and insensitive to cyclosporin A but sensitive to rapamycin (66). Both TCR and CD 28 mediated signalling share a final common pathway that involves activation and nuclear translocation of transcription factors and consequent upregulation of cytokine gene expression. A rise in intracellular calcium concentration is associated with activation of the protein kinase C (PKC) pathway and protein kinase A (PKA) pathway, via cyclic AMP. The PKC pathway exhibits glucocorticoid sensitivity and is associated with expression of AP-1, NFAT and NFkB, which together promote transcription of several cytokines, including IL-5, IL-4, IL-3 and GM-CSF. Cyclic AMP dependent activation of the PKA pathway is also associated with expression of NFAT and AP-1 but not NFkb. This pattern of transcription factors induces gene expression of IL-5 but not other cytokines. This is observed with specific activation of the cAMP dependent PKA pathway by histamine, mediated through binding with the H_2 receptor (74). Co-expression of IL-4 with IL-5 can promote B-cell isotype switching and Ig E production. The expression of NFkb may be an important determinant of eosinophilic inflammation, in the presence or absence of atopy (66).

IL-5 Receptor: Structure and function

The IL-5 receptor has been identified *in vitro* on mature eosinophils and basophils in man and additionally on B-lymphocytes in mice. This restricted pattern of receptor expression is the basis for biological specificity with IL-5.

Molecular cloning of the human IL-5 receptor was performed in 1992 (75). It is a type 1 cytokine receptor and shares similarity in tertiary structure with other receptors of this family; these are typically other haemopoietin and growth factor receptors. The receptor has a heterodimeric structure that consists of separate α and β subunits. The β subunit is common to IL-5, IL-3 and GM-CSF; the α-subunit confers cytokine specificity (76).

The interaction of IL-5 with its surface receptor is a sequential process. Multiple conformational changes occur that establish heterodimerisation and promote high affinity binding of IL-5 with 1:1 stoichiometry. These events induce further conformational changes leading to dimerisation of α-β receptor complexes via disulphide bridge (S-S) formation. Janus associated kinases (JAK) are bound to the cytoplasmic domain of the β subunit and catalyse tyrosine phosphorylation of the subunit on receptor dimerisation. Phosphorylation of the β subunit is a critical step in receptor activation that initiates intracellular signal transduction pathways. There is emerging evidence that some signalling pathways may not require receptor dimerisation. E12K is an IL-5 mutant protein that prevents the formation of S-S bonds needed for receptor dimerisation. In vitro studies with E12K indicate that although primarily an antagonist of IL-5 function, the molecule supports eosinophil survival (77).

The sequence of events leading to signal transduction with IL-3 and GM-CSF following binding of cytokines to their respective α-subunits is identical to those described for IL-5. Differences exist between the cytokines in their binding affinity for the α-β receptor complex. However this does not appear to be of functional significance and does not confer any cytokine specificity for intracellular processes. Indeed, one implication of signal transduction occurring primarily through phosphorylation of the common β-subunit is that all three cytokines share the same intracellular signalling pathways. This lack of specificity is perhaps unsurprising given their common phylogeny and biological

function as haemopoietins. The principal downstream intracellular pathways activated include Ras, PKC, Map kinase and pathways mediated by STAT 5 activation. Together these pathways promote cell cycle, improve cell survival and play a role in cell activation.

The α-subunit of the IL-5 receptor (IL-5Rα) exists in soluble and membrane bound isoforms. The soluble form lacks the cytoplasmic tail of the membrane bound form and is produced by differential splicing of IL-5 transcripts (78). Based on *in vitro* studies, it is estimated that 90% of IL-5Rα may exist in the soluble form. Functional studies indicate soluble IL-5Rα is not biologically active but can bind IL-5 with the same affinity as the membrane bound receptor isoform (75). Soluble IL-5Rα may therefore bind with and sequester free IL-5. The precise role of this isoform is not clear but is likely to represent a pathway for negative feedback control during eosinophilic inflammation. In this setting, soluble IL-5Rα may be protective in eosinophil associated disease. This hypothesis is supported by a study performed in asthma, which identified an inverse correlation between FEV_1 and levels of membrane bound IL-5Rα mRNA in bronchial biopsies but a positive correlation between FEV_1 and mRNA expression for the soluble receptor isoform (79).

2.2.3 Eosinophil Morphology Constituents and Function

Eosinophils are characterised morphologically by their typical bilobed nuclei and distinctive cytoplasmic granules, which are responsible for the specific tinctorial properties of these cells. Eosinophils are capable of synthesising, storing and releasing a diverse spectrum of molecules. The list of such eosinophil derived products is continuing to expand, emphasising the diverse immunological role of the eosinophil (46).

Four different populations of granules are recognised: crystalloid granules, primary granules, small granules and secretory vesicles. The largest and most distinct of these are the crystalloid granules which also store the majority of eosinophil granule proteins. Structurally, the granules have a central, electron dense crystalline core that is surrounded by non-crystalline matrix. There are four highly basic eosinophil granule proteins that are stored: major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP). Of these, MBP is located primarily in the crystalline core; the remainder are stored in the matrix. The four proteins have several cytotoxic and proinflammatory properties that are important for mediating the effector function of eosinophils [Table 4].

Primary granules are formed early in eosinophil maturation and are composed of Charcot Leyden Crystal (CLC) protein. This protein, also called galectin-10 (46), is found uniquely at high levels in eosinophils and to a lesser extent in basophils. Their precise role is unclear, but identification of Charcot-Leyden crystals in tissue is virtually pathognomonic of eosinophil degranulation. Finally, small granules and secretory vesicles are tightly packed in the cytoplasm and contain numerous preformed enzymes such as aryl sulphatase; the functions of these remain to be fully characterised.

Other eosinophil products imply a significant role for these cells in immunoregulation. In addition to granules, the cytoplasm of eosinophils contains variable amounts of lipid bodies. These are non-membrane bound lipid rich inclusions that can also be found in other cell types. Lipid bodies are a specialised store for products of arachidonic acid metabolism. In keeping with their pro-inflammatory function, lipid bodies are up-regulated during cellular activation. Eosinophils can synthesise and secrete over 35

different cytokines, including immunomodulatory cytokines, chemokines and growth factors (46). It is increasingly recognised that expression of growth factors by eosinophils may play an important role in tissue repair and healing (80). In this context, the association between chronic eosinophilic inflammation and some structural changes of airway remodelling in asthma may represent disordered repair. This is supported by a biopsy study of mepolizumab (an anti IL-5 agent) in asthma that reported 86% of all TGF_{β} producing cells in the bronchial mucosa are eosinophils (81); treatment with mepolizumab in this study was associated with a significant fall in the level of TGF_{β} that was accompanied by favourable changes in the protein composition of the extracellular matrix. In addition to their expression of cytokines, recent studies support a role for eosinophils as antigen presenting cells with MHC II restriction (82), implicating another potential mechanism of eosinophil mediated immunoregulation.

2.2.3 Figures and Tables

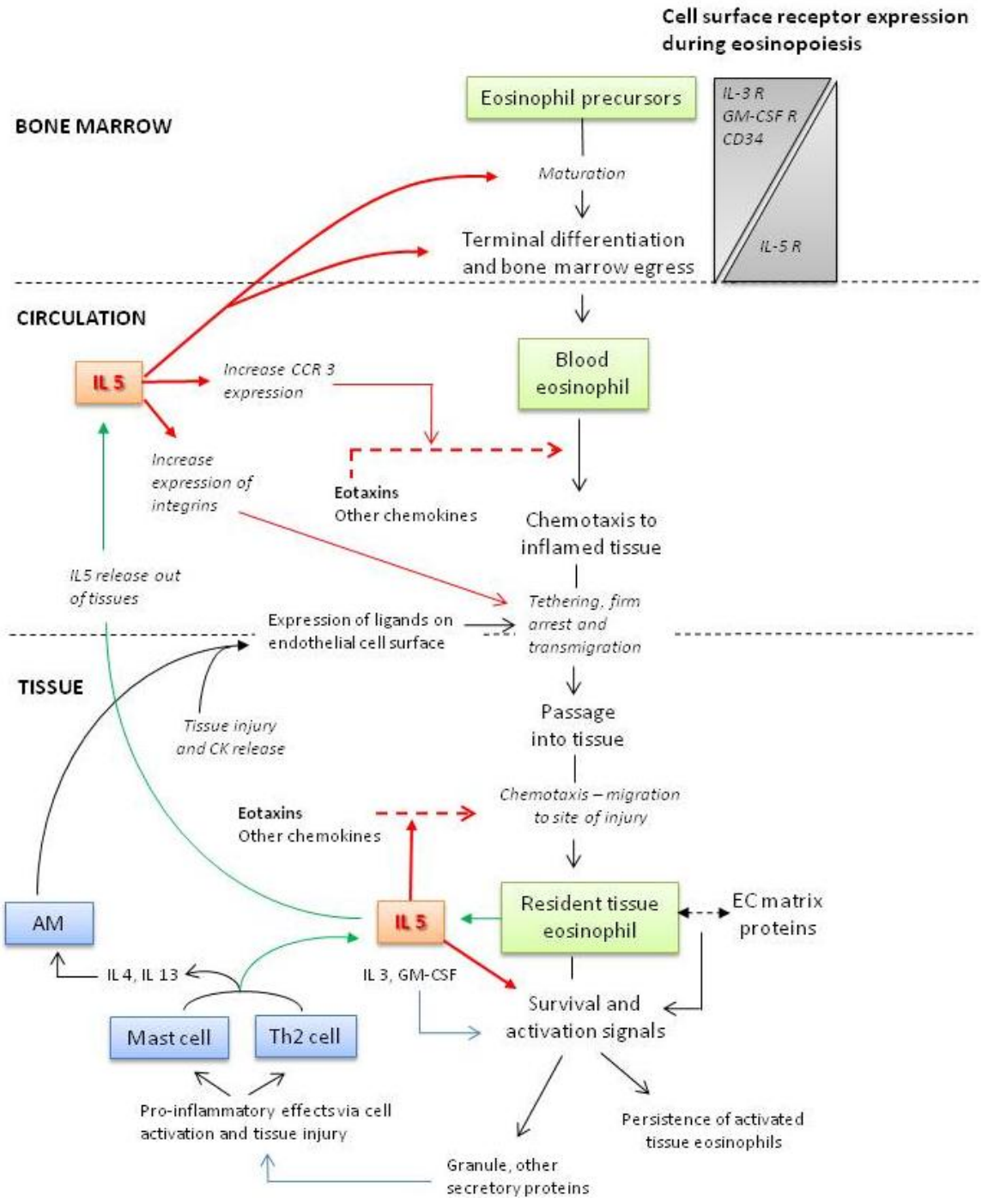


Figure 2: Eosinophil life cycle - Importance of IL 5

Schema of factors regulating eosinophilopoiesis, homing and tissue persistence in disorders of eosinophilic inflammation. Although IL 5 is a key determinant in all 3 compartments of this model, other factors have an important role, particularly in eosinophil chemotaxis and tissue persistence.

Abbreviations: AM= alveolar macrophage, EC (matrix) = extracellular

Table 3: Important ligand-receptor interactions in eosinophil homing

Eosinophil expression	Endothelial ligand
Integrin mediated	
αdβ2	**VCAM-1**
α4β7	**MAdCAM-1**
α4β1 (VLA-4)	**VCAM-1**
β2 (CD18) integrins	ICAM-1
Selectin mediated	
PSGL-1	**P-selectin**
L-selectin	MAdCAM-1, CD 34
Sialyl-Lewis X	E-selectin

Abbreviations: VCAM=Vascular cell adhesion molecule; ICAM = Intercellular cell adhesion molecule; MAdCAM = Mucosal addressin cell adhesion molecule; VLA = Very late antigen; PSGL = P-selectin specific glycoprotein ligand

In broad terms, tissue inflammation release circulating factors that mediate expression of eosinophil ligands; tissue factors act locally to mediate expression of receptors on the surface of endothelium [Fig 2].

Selectins are single chain transmembrane glycoproteins that mediate cell-cell interactions by binding with carbohydrate moieties. Integrins are heterodimeric cell surface glycoprotein receptors, comprising an alpha and beta subunit that mediate cell-cell interactions.

Interactions in bold are predominantly eosinophil-specific and lead to selective enrichment of eosinophils at sites of inflammation. Tissue specificity is mediated by the selective expression of endothelial receptors. MAdCAM-1 is a gut endothelial receptor that is expressed constitutively and accounts for the homing of eosinophils to the intestine, in health.

Table 4: Effector function of the major basic eosinophil granule proteins

Protein	Generic biological activity	Asthma related biological activity
Major basic protein (MBP)	Potent helminthotoxin and cytotoxin	Damages respiratory epithelium
	Bactericidal	Histamine release from basophils and mast cells
	C3b inhibitor	Inhibitor of M_2 muscarinic receptors
		Increases bronchial hyperresponsiveness
Eosinophil cationic protein (ECP)	Potent helminthotoxin and cytotoxin	Damages respiratory epithelium
	Bactericidal	Histamine release from mast cells
	Potent neurotoxin	
	Weak RNAase activity	
Eosinophil derived neurotoxin (EDN)	Potent neurotoxin	
	Potent RNAase activity	
	Weak helminthotoxin	
Eosinophil Peroxidase (EPO)	*In presence of H_2O_2:*	
	Cytotoxic	Histamine release from mast cells
	Kills Brugia microfilariae	Damages respiratory epithelium
	In absence of H_2O_2:	
	Helminthotoxic	Damages respiratory epithelium
		Increases bronchial hyper responsiveness
		Causes bronchospasm

Adapted from *Gleich and Adolphson* (65)

2.3 The Role of Eosinophils in Asthma

The role of eosinophils in asthma has been a subject of considerable debate for a century. This observation alone illustrates a balance of scientific evidence that does not provide clarity. A conclusive answer to this question has gained urgency as novel and specific anti-eosinophilic therapies reach a marketing stage of development. Here I present and discuss separately some of the key observations that respectively support (2.3.1) and refute (2.3.2) a role for eosinophils in asthma.

2.3.1 Evidence in favour of a role for eosinophils in the pathogenesis of asthma

A substantial body of evidence exists to support a role for the eosinophil in asthma.

1. Eosinophils are found at significantly higher levels in the peripheral blood, airways and peribronchial tissues of people with asthma but not in non-asthma healthy controls.
2. Products of eosinophil degranulation are detectable in the airways and bronchial submucosa, implying that the eosinophils present are of an activated phenotype.
3. Eosinophil granule proteins can reproduce *in vitro* some of the pathological features of asthma characterised *in vivo*.
4. Both the level of eosinophilic airway inflammation and titre of eosinophil degranulation products have been shown to correlate with objective markers clinical asthma and disease severity.
5. The burden of eosinophilic airway inflammation is associated with future risk of asthma exacerbation, implying a temporal relationship of eosinophil pathology with clinical disease severity.
6. Amelioration of eosinophilic inflammation with glucocorticoid therapy, administered either systemically or locally by inhalation, is associated with improvement in objective measures of clinical disease and risk of future asthma exacerbations.

While primarily circumstantial, the case favouring a role for eosinophils is persuasive for being multifaceted. These points are discussed further.

Eosinophilic airway inflammation in the pathology of asthma

Eosinophilic airway inflammation is one of the earliest recognised characteristics of asthma. It is the oldest 'pillar' of evidence and forms the basis of all studies that have followed, seeking to characterise the function of eosinophils in asthma. Gollasch reported evidence of eosinophils in sputum of patients with asthma (29). This finding was corroborated and extended by necropsy studies in patients dying of fatal asthma (83;84); identification of extensive eosinophilic inflammation and eosinophil degranulation (Charcot Leyden crystals) accompanied by mucus hypersecretion and plugging of the small airways is well characterised in several case series reports . These early studies were limited by the absence of non-asthma controls and the consideration of pathology only at the extreme of severity.

With the development of fibreoptic bronchoscopy as a technique to sample the lower airways, the study of two compartments (airway lumen and peribronchial tissue) could be extended to milder disease, more representative of asthma in the population. Early studies identified variable eosinophilia in bronchoalveolar lavage fluid (BAL) from subjects with asthma (85;86). This was accompanied by detection of eosinophil granule proteins and leukotrienes (87;88), suggesting that airway eosinophils were secreting mediators; an idea supported by the finding of a close correlation between the percentage eosinophil count and MBP titre in BAL (88). Furthermore, BAL eosinophils exhibited a pattern of cell surface receptor expression that was reproducible *in vitro* by treatment of cells with IL-5 and indicative of an activated phenotype (89). Together, these studies supported the observations at necropsy and developed the idea that eosinophils are an important effector component of inflammatory pathology in the airways of asthma.

Earlier necropsy studies described intense sub epithelial infiltration with eosinophils, pronounced reticular basement membrane thickening and shedding of normal ciliated bronchial epithelium with variable replacement stratified squamous epithelium (90). Although described in the context of fatally severe asthma, the same pattern of

changes were identified in biopsies of mild asthma (86;91). One study reported an increase in the number of eosinophils in airway submucosa without an increase in either neutrophils or mast cells (85). Immunohistochemical studies of biopsy material reported an association between CD25+ cells (representative of the CD4 T-lymphocyte population) and EG2+ cells (ECP secreting, activated eosinophils) (92). This finding supported the mechanistic hypothesis that activated CD4 T-cells mediate eosinophilic inflammation and eosinophil activation as part of a Th2 immune response in the airways of subjects with asthma. This hypothesis was strengthened by evidence for elevated levels of IL-5 mRNA expression (93;94) and IL-5 protein (94;95) in biopsy material and BAL fluid of subjects with asthma and the finding that 70-80% of IL-5 mRNA signal originated from CD3 T-cells (71).

Eosinophil granule proteins in the pathology of asthma

The four basic proteins stored within the crystalloid granules of eosinophils are potent cytotoxins and constitute the primary mechanism of eosinophil effector activity. A significant body of evidence exists characterising a role for these effector molecules in the pathology of asthma. Increased levels of these proteins are found in the airways of subjects with asthma. Elevated sputum MBP levels were reported in subjects with asthma prior to the bronchoscopic studies described above. Indeed sputum MBP was one of the first biomarkers characterised for the diagnosis of asthma (96). MBP levels are elevated at the time of asthma exacerbation suggesting an association with disease activity (96). This is supported by the findings of a immunohistochemical study that demonstrated subepithelial eosinophils at different stages of degranulation and extensive extracellular MBP staining in mucus plugs, on damaged epithelial surfaces and in necrotic areas below the basement membrane in post mortem specimens of patients that had died from fatal asthma and in patients with a history of severe asthma dying from other causes, but not controls without asthma (97).

In vitro studies performed using cultures of guinea pig respiratory epithelium have demonstrated ciliostasis and epithelial disruption that is typical of asthma, in the presence of MBP and EPO (98). The damage was dose dependent with MBP and occurred at protein concentrations measurable in sputum. In addition to its cytotoxic

effects, MBP has also been shown to trigger histamine release from purified populations of mast cells and basophils in a non-cytolytic manner (99). In moderate, persistent asthma, there is evidence to suggest ongoing eosinophil degranulation with elevated levels of BAL histamine that is absent in milder asymptomatic asthma and non-asthma controls (100).

Eosinophilic inflammation and clinical asthma

The association between eosinophilic inflammation and objective markers of clinical asthma have been explored since the 1970s. Early studies identified significant correlations between the total blood eosinophil count and a diagnosis of asthma, clinical asthma severity (101) and airway hyperresponsiveness (102;103); and an inverse correlation with airway conductance (101). Horn and colleagues first suggested the use of the blood eosinophil count as a marker of disease activity to guide glucocorticoid therapy (101). Bronchoscopic sampling of the lower airways extended the observations made in blood to measures of eosinophilic inflammation in the airway compartment. Wardlaw and colleagues identified a significant correlation between both percentage BAL eosinophils and BAL MBP levels with airway hyperresponsiveness (88). In this study the authors also reported higher BAL eosinophil counts and greater hyperresponsiveness in subjects with symptomatic asthma compared with subjects that were symptom free. Evidence for increased eosinophil degranulation and associated expression of mast cell products in subjects with asthma of greater clinical severity has also been described (100).

The individual observations of earlier studies were brought together in a landmark study by Bousquet and colleagues (104). In this cross-sectional study, clinical asthma control was defined by a scoring system that included symptom control and medication use. Eosinophilic inflammation was assessed in multiple biological compartments including peripheral blood, bronchial tissues (bronchial biopsies) and within the airway (BAL). The study found a moderate but statistically significant correlation between clinical asthma severity and both blood and lavage eosinophil counts, although considerable overlap existed between the severity groups. Biopsies demonstrated eosinophilic infiltration within the bronchial submucosa and eosinophil

degranulation with detectable extracellular ECP - all observations that were consistent with earlier studies. The association between eosinophilic inflammation and clinical severity in this study was evidence favouring the paradigm of a linear relationship between eosinophilic inflammation, disordered airway function and clinical symptoms.

Finally, an indirect marker of eosinophil activity is the rate of eosinophil apoptosis. Asthma is associated with a reduction in eosinophil apoptosis (51). The eosinophil apoptosis index describes the ratio of apoptotic eosinophils to the total eosinophil count. One study reported an inverse correlation between the apoptosis index of eosinophils in sputum and symptom scores, severity scores and age in subjects with asthma. A positive correlation was reported between the apoptosis index and lung function (105). These observations favour a causal association between clinical asthma and the persistence of activated eosinophils in the lung. In this context, the primacy of IL 5 for prolonging eosinophil survival (106) supports development of therapeutic targets against this cytokine.

Eosinophilic inflammation and asthma exacerbations

Exacerbations are sustained episodes of worsening asthma control that are beyond the individual's normal variability and require an escalation of therapy to re-establish control. The severity of an exacerbation is categorised by the level of therapeutic intervention needed to achieve control [section 1.2]; severe exacerbations are defined by the need for a course of high dose oral glucocorticoid therapy.

Fatal asthma represents one end of the spectrum of exacerbation severity and evidence of a role for eosinophils in necropsy studies of fatal asthma has been described above. In the context of the present discussion, severe exacerbations are an objective marker of temporal change in clinical asthma control. A criticism that is applicable to all the studies described so far has been their cross-sectional design. Sputum induction is a non-invasive technique for sampling the lower airways (107) [section 2.3.2], enabling serial measurement of airway inflammation and making it possible to examine longitudinal correlations between airway inflammation and the clinical expression of asthma. Associations have been reported between the sputum eosinophil count and change in asthma control both prior to the onset of an

exacerbation and during treatment for an exacerbation. One study reported a fall in circulating numbers of blood eosinophils in a study group with asthma exacerbations that was associated with clinical improvement after inhaled glucocorticoid therapy (108). In another study performing serial sputum inductions on patients treated for asthma exacerbations with oral prednisolone, the authors reported a significant inverse correlation between the change (fall) in sputum eosinophil count and lung function with therapy (109). Studies using a glucocorticoid withdrawal protocol to induce exacerbations have shown an association between the risk of exacerbation and both eosinophil counts and products of eosinophil degranulation in sputum that are either high at baseline and/or rise significantly with time and treatment withdrawal (110-114).

As a corollary to this observation, three randomised studies performed in adults have demonstrated benefit with a strategy of glucocorticoid therapy delivered to ameliorate eosinophilic airway inflammation in sputum, for lowering the rate of severe exacerbations, compared with usual clinical practice (23-25). Furthermore, in their study Jayaram and colleagues reported that the observed benefit was confined to eosinophilic exacerbations (25).

Together with the cross-sectional observations at post-mortem, these longitudinal associations strongly implicate an important role for the eosinophil in the clinical expression of asthma, with particular reference to the pathogenesis of severe exacerbations.

2.3.2 Evidence not consistent with a role for eosinophils in asthma

Despite the seemingly robust evidence presented in favour of eosinophils, a number of observations bring this evidence into question:

1. Eosinophilic lung diseases, such as eosinophilic pneumonia, may occur in the absence of any clinical or physiological features of asthma.
2. Eosinophilic inflammation is absent in a significant proportion of the asthma population with evidence of suboptimal control.

3. Non-eosinophilic asthma is clinically and physiologically indistinguishable from eosinophilic asthma. This implies that characteristic disordered airway physiology and clinical outcomes of asthma may occur in the absence of eosinophilic inflammation.
4. When present, eosinophilic inflammation in asthma does not occur in isolation; it is a product of one of many Th2 immune pathways. In studies that have identified an association between eosinophils and asthma, the evidence is not specific for the eosinophil and more appropriately viewed as supportive of a role for Th2 inflammation in asthma.

These points are discussed further.

Sputum induction and evaluation of airway inflammation

The contribution of sputum induction, as a non-invasive tool for the study of lower airway inflammation in asthma, cannot be overstated. The technique has enabled examination of airway inflammation in a broader context, including collection of data from larger and more representative populations of asthma, across the spectrum of severity; greater sampling from non-asthma controls; and mapping of longitudinal change in inflammatory cell kinetics with time and treatment. The results of sputum induction studies have been well validated with assessments of airway inflammation at bronchoscopy. Studies in patients with mild to moderate asthma have shown a close correlation between inflammatory cell counts in induced sputum and bronchial wash and a less close correlation with cell counts from bronchoalveolar lavage fluid (115). The relationship between induced sputum findings and cell counts in bronchial biopsy is more variable (116), probably because granulocytes are not resident cells and therefore not well represented in tissue. This is important as the absence of eosinophils demonstrated with this technique does not imply absence of eosinophils in lung tissue. Indeed, both inhaled and systemic glucocorticoids are effective suppressants of eosinophilic airway inflammation but have only a limited effect on tissue eosinophilia.

Non-eosinophilic asthma

The accepted criteria for diagnosing non-eosinophilic asthma include: i) presence of typical symptoms; ii) objective evidence of significant variable airflow obstruction or airway hyperresponsiveness; iii) consistent absence of sputum eosinophilia; and iv) exclusion of an alternative diagnosis (117).

A 'normal' sputum eosinophil count is difficult to define as data from studies in non-asthma healthy subjects is limited. There is evidence of variability with gender and atopic status, in the absence of clinical asthma (118). This has implications for the identification and prevalence estimates for the phenotype. The 90^{th} centile for the sputum eosinophil count in normal controls has been reported as 1.8% (118) and 1.9% (119) in two community based surveys; values in this region may be appropriate for use as guidance. A sputum eosinophil count of 3% is approximately 2 standard deviations above this upper limit of normal and has been the threshold used to categorise clinically significant eosinophilia in several studies of asthma.

The phenotype of non-eosinophilic asthma is confounded significantly by anti-inflammatory asthma therapy that leads to dissociation of eosinophilic inflammation between airway and tissue compartments, as described above. Cross-sectional population studies using sputum induction have reported normal sputum eosinophil counts (cut off <1.9%) in up to 25% of patients with *untreated* symptomatic asthma (120) and for over 50% of patients (cut off < 2.5%) treated with high doses of inhaled corticosteroids (121). In a survey of several asthma studies reporting inflammatory cell counts, using either induced sputum or bronchoscopic methods, 49% of participants with asthma had a non-eosinophilic (<2% eosinophils) phenotype (122). Non-eosinophilic asthma is frequent in patients with occupational asthma (123), in patients with refractory asthma (124) and in patients presenting at the time of mild (125;126) or more severe (127) exacerbations. Absence of eosinophilic inflammation has also been reported in post mortem studies of fatal asthma (128;129); suggesting that even the severest manifestation of asthma may be independent of eosinophil associated pathology. However, it is noteworthy that in many cases catastrophic mast cell

degranulation was reported, suggesting that anaphylaxis rather than asthma may have driven the terminal event.

A significant limitation of point prevalence figures for non-eosinophilic based on cross-sectional studies is the absence of information on longitudinal stability of the phenotype. As a disease typically characterised by variable severity, non-eosinophilic asthma may simply represent a period of good disease control. While plausible for a subset of this population, studies have identified the non-eosinophilic phenotype in subjects with symptomatic asthma, not receiving glucocorticoid therapy (130). Non-eosinophilic asthma has also been a stable finding during serial evaluation in subjects followed longitudinally, both during scheduled visits (131) and at the time of exacerbation (132).

It has been suggested that the different inflammatory profiles are due to different patterns of antigen exposure in the airways (133). Thus, eosinophilic disease is considered a consequence of allergen mediated activation of mast cells and T-cells in the airway with release of T_H 2 cytokines. In contrast, non-eosinophilic, neutrophil predominant inflammation is the product of innate and cell mediated immune responses. Numerous aetiological factors are believed to evoke responses along these immune pathways, particularly through the direct activation of macrophages (134). Important examples include endotoxin, viral and bacterial infection, constituents of cigarette smoke and many occupational agents. While useful, this model is likely to be an oversimplification. There is increasing recognition of considerable cross-talk between Th1 and Th2 pathways, with cytokines such as TNF_α playing an important role in the augmentation of both types of immunity. Although a viral aetiology is identified most often during asthma exacerbations (135;136), the pattern of airway inflammation (either eosinophilic or non-eosinophilic) is far more heterogeneous and is reported to exhibit within-subject consistency at successive exacerbation episodes (132). It is therefore more likely that eosinophilic and non-eosinophilic phenotypes differ not only in the pattern of exposure and susceptibility to specific antigens but also in the type of responses they evoke to a given antigen. This idea is supported *in vitro* by studies using endotoxin demonstrating heterogeneity in the inflammatory response to this antigen under different conditions (137). In one study, subjects with

ragweed sensitivity that underwent bronchial challenge using ragweed contaminated with endotoxin had a neutrophilic response whereas endotoxin free ragweed led to an eosinophilic response (138). In contrast, other studies have reported augmentation of Th2 responses with endotoxin, evidenced by enhanced nasal eosinophilia in atopic subjects (139) and greater skin test reactivity and Ig E mediated histamine release from mast cells and basophils (140).

In addition to the hypothesis driven differences in immunopathology, clear differences have been reported in airway structure of non-eosinophilic asthma at a tissue level (124;130), implying distinct pathways of remodelling likely arising from differences in the chronic inflammatory milieu. Together, the evidence presented supports the place of non-eosinophilic asthma as a distinct chronic and stable phenotype of asthma.

The relationship between eosinophilic airway inflammation and disordered airway physiology in asthma

The existence of non-eosinophilic asthma raises important questions about the role of eosinophils in asthma pathophysiology. A clear implication is that characteristic abnormalities of airway physiology in asthma are not a product of eosinophilic airway inflammation, an idea that is supported by several lines of evidence. Cross sectional studies have identified a poor correlation between paired measurements of airway hyperresponsiveness and the sputum eosinophil count (141). Factor analysis has identified loading of variables associated with sputum inflammation, variable airflow obstruction and lung function onto independent factors in subjects with asthma (142). Although not conclusive, the statistical independence between the variables described is highly suggestive of biological dissociation. Factor analysis is discussed more fully in section 3.10.2. Perhaps the strongest evidence to date of dissociation between eosinophilic airway inflammation and airway hyperresponsiveness is found with eosinophilic bronchitis; a condition characterised by chronic cough, eosinophilic airway inflammation and normal airway responsiveness to bronchoconstrictor challenge (143). Eosinophilic bronchitis has proved a useful control model for understanding the biological basis of airway hyperresponsiveness. Histological comparison of the disorder with asthma has revealed mast cell localisation to the airway smooth muscle layer and

elevated expression of IL-13 in sputum and bronchial submucosa as two distinctive features of asthma, likely to be associated with airway hyperresponsiveness (144). Although IL-13 is a Th2 cytokine, mast cell localisation to the airway smooth muscle has also been described in subjects with non-eosinophilic asthma (130). This observation supports a mechanism for airway hyperresponsiveness that is independent of Th2 immunity, lending further support to the biological feasibility of non-eosinophilic asthma.

The relationship between eosinophilic airway inflammation and the clinical expression of asthma

Symptoms and exacerbations comprise the major clinical manifestations of asthma. The relationship between eosinophilic airway inflammation, symptoms and exacerbations is complex and poorly understood. The biological basis of symptoms in asthma is airflow obstruction, which is typically variable and a consequence of airway hyperresponsiveness. In light of the evidence demonstrating dissociation between inflammation and airway hyperresponsiveness, a poor relationship between asthma associated symptoms and inflammation is not unexpected. This is compounded by symptoms in chronic asthma being multifactorial and often unrelated to disease activity.

Although defined clinically by symptoms, severe exacerbations are regarded as discrete pathological events characterised by upregulation of bronchial inflammation. Severe exacerbations may occur in the absence of a background of poor symptom control, implying that they are not simply one end of the symptoms continuum. A significant proportion of severe and near fatal asthma exacerbations occur in patients with a history of mild to moderate asthma (7;8). In keeping with this, a subgroup of patients with brittle asthma has been described that present with sudden and severe exacerbations on a background of good symptom control (145). Impaired perception of bronchoconstriction has been reported with severe eosinophilic airway inflammation in patients with a history of near fatal asthma (17) and may represent one mechanism for dissociation between symptoms and exacerbations.

A persuasive body of evidence supports an association between eosinophilic airway inflammation and exacerbation risk and benefit with glucocorticoid therapy titrated to maintain normal sputum eosinophil counts has previously been discussed [section 2.3.1]. Benefit of an inflammation guided strategy over clinical symptoms in these studies is in keeping with the idea that symptoms and exacerbation risk are not closely related. Jayaram and colleagues reported superiority of the sputum strategy over symptom directed clinical care was associated specifically with a reduction in the number of eosinophilic exacerbations and confined to subjects with moderate to severe asthma (25). In addition to supporting a role for eosinophilic inflammation in the pathogenesis of severe exacerbations, the observations suggest that dissociation between symptoms and exacerbation risk is greatest in these asthma subgroups. This is supported by increasing failure of symptom driven treatment algorithms and evidence of impaired perception of bronchoconstriction with eosinophilic airway inflammation in severe asthma [section1.2.3]. In the study of Green and colleagues (23), 80% of participants had refractory asthma and the observation that inflammation guided management led to a significant reduction in severe exacerbations, with no benefit on overall control of symptoms is entirely consistent with the idea of dissociation.

Despite this, studies report considerable heterogeneity of airway inflammation at the time of exacerbation, with absence of eosinophilia in a significant proportion (25;126). The importance of eosinophilic inflammation in the aetiology of exacerbations is therefore likely to be restricted to a subgroup of asthma. In keeping with this, there is evidence that the pattern of airway inflammation exhibits within-subject consistency at evaluation of serial exacerbation episodes (132), implying that when eosinophilia occurs at the time of one exacerbation, it is present at subsequent events. In eosinophilic patients, there may be subgroups at risk of frequent exacerbations. Chronic sino-pulmonary infection has been identified as a risk factor for frequent exacerbations (146). A recent study has reported a significant association between this condition and persistent sputum eosinophilia (147).

However, we should be mindful that different patterns of airway inflammation will represent a composite of distinct pathological processes, differing responses to anti-

inflammatory therapy and variable adherence to treatment. Some of the observations discussed here may be attributable to the effects of adherence with therapy; in this context, an association between eosinophilic airway inflammation and exacerbation risk may simply represent poor treatment adherence, leading to an inevitable loss of asthma control.

Eosinophilic inflammation and Th2 Immunity

To recognise non-eosinophilic asthma is to accept that eosinophils are not necessary for asthma. However, the role of eosinophils in asthma associated with eosinophilic inflammation (at least 50% of all asthma) remains topical. Within this subgroup, deciphering the role of eosinophils precisely remains problematic. Eosinophil accumulation is one product of a broader network of cellular activity that occurs as part of the Th2 immune response [Fig 3]. In asthma, IL-5 driven eosinophilic inflammation is accompanied by upregulation of other Th2 cytokines, classically IL-4 and IL-13. The former induces immunoglobulin isotype switching on B-cells to Ig E, the latter is associated with airway hyperresponsiveness and mucus cell hypersecretion. In the broader context, eosinophilic inflammation may therefore represent only a biomarker of Th2 immune activity. However this too may be over-simplistic as patterns of Th2 expression vary with different eosinophilic airways disease phenotypes; examples include low IL-13 expression in eosinophilic bronchitis and low Ig E expression in non-atopic eosinophilic asthma.

2.3.3 Conclusions

Establishing the role of eosinophils in asthma is fraught with difficulty, arising from the complexity and heterogeneity of disease expression. The primacy of eosinophils in the early literature has been superceded by recognition of redundancy in asthma pathology; a concept typified by non-eosinophilic asthma. In this context, the relevance of eosinophils is probably restricted to a subgroup of the asthma population.

Complexity is compounded by the poor correlation that exists between airway inflammation, disordered airway function and clinical disease expression. The contribution of eosinophils to each of these aspects of disease expression therefore

requires separate consideration. The present literature suggests eosinophilic inflammation is most closely related to severe exacerbation risk.

2.4 Anti-eosinophil therapy in asthma

2.4.1 Introduction

The clinical and pathological effects of therapy associated with amelioration of eosinophilic inflammation can help to inform further the role of eosinophils in asthma. Glucocorticoids are powerful suppressants of eosinophilic inflammation and have been the mainstay of anti-inflammatory therapy used in asthma for over 50 years. An extensive literature base exists for this class of agents; the benefit reported in clinical, pathological and physiological markers of asthma has been viewed as powerful evidence supporting a causal role for eosinophils. However, glucocorticoids are non-eosinophil specific suppressants of Th2 inflammation; more recently molecular therapies have been designed that specifically target eosinophilic inflammation. In conjunction with the observations of studies using glucocorticoid therapies, outcomes with these agents will help inform the role of eosinophils more specifically within a Th2 environment.

In this section I present the evidence gathered from glucocorticoid studies in asthma and describe outcomes to date using the specific anti-eosinophilic therapy mepolizumab.

2.4.2 Glucocorticoid therapy and asthma

Glucocorticoids are a class of steroid hormones that mediate their actions through binding at the glucocorticoid receptor. In health, glucocorticoids have a significant and extensive role in physiological homeostasis. This includes potent anti-inflammatory activity that is a critical component of the negative feedback response to stress. As anti-inflammatory agents, glucocorticoids exhibit potent anti-eosinophilic properties and are the most effective and widely used agents for the treatment of eosinophilic disorders (43).

Glucocorticoid therapy in asthma – a historical perspective

A therapeutic role for the adrenocorticotropin (ACTH) - cortisol axis was first identified for the treatment of rheumatoid arthritis in 1949 (148). Shortly after this, Boardley and colleagues reported benefit with ACTH therapy in patients with asthma (149). Efficacy of ACTH and cortisone was established in a series of studies performed in the 1950s. Even then, heterogeneity in the response to corticosteroids was recognised. A double blind randomised placebo controlled trial by the MRC concluded benefit with glucocorticoids for treatment of asthma exacerbations [MRC 1956a] but not chronic symptoms [MRC 1956b] (150). Other authors reported a lack of benefit in patients with concurrent bacterial infection or significant emphysema with an absence of significant reversible airflow obstruction. In a descriptive case series, Heyworth and colleagues described variability in the response to inhaled hydrocortisone and identified greatest benefit for the subgroup of patients with early onset atopic asthma with intermittent symptoms and least benefit for chronic and persistent wheezy bronchitis (151). Although crude, the study illustrated the importance of clinical phenotype to therapy response. This was followed shortly afterwards by a paper that reported an association between the response to glucocorticoid and presence of sputum eosinophils in both asthma and chronic bronchitis (152). While largely ignored at the time, the significance of Morrow-Brown's observations, together with efforts of early authors to describe phenotypic associations with glucocorticoid responsiveness, resonate strongly with asthma research today.

Anti-inflammatory mechanisms of glucocorticoids

Glucocorticoids are lipophilic molecules that diffuse easily across the cell membrane. Binding of the glucocorticoid receptor that is located within the cytoplasm promotes dissociation of molecular chaperones, leading to internalisation and translocation to the nucleus of the ligand-receptor complex. Within the nucleus, the complex exerts a number of different effects on gene transcription activity that are incompletely understood. In broad terms, glucocorticoid activity may be divided into three types (153;154); all are associated with alteration in the balance of pro and anti-inflammatory processes in favour of the latter:

i) Direct regulation of gene transcription (trans-activation).

The glucocorticoid-receptor complex binds as a homodimer to specific DNA sequences, termed glucocorticoid responsive elements (GREs) and regulates GRE specific gene transcription. The expression of a number of anti-inflammatory proteins (e.g. IκB, annexin-1, IL-10) and enzymes (e.g. MAPK phosphatase-1) is upregulated by this mechanism. GRE specific repressor genes also exist and are associated with negative-feedback pathways of endocrine and metabolic homeostasis. Non-physiological binding at these sites is associates with many of the recognised side effects of long term exogenous glucocorticoid therapy.

ii) Indirect regulation of gene transcription (trans-repression).

The glucocorticoid and receptor complex can interfere with and suppress the transcriptional activity of pro-inflammatory transcriptional factors, notably NFκB and activator protein 1 (AP-1). These transcriptional factors are activated by inflammatory cytokines and mediate expression of several cytokines, chemokines and adhesion molecules, as part of positive feedback pathways for upregulating inflammation. Trans-repression occurs at lower doses of glucocorticoid than transactivation and yields a broader anti-inflammatory effect. It is therefore considered to be the primary anti-inflammatory mechanism of glucocorticoids.

Trans-repressor activity is mediated primarily by interference with chromatin remodelling. Chromatin is a dynamic structural matrix that modulates DNA conformation and has an important physical role in transcriptional regulation. The structure of chromatin is regulated by the acetylation status of constituent histone proteins. Histone acetylation leads to reduction in electrostatic and ionic bonding that is associated with unravelling of chromatin and increased accessibility of underlying DNA for transcription; deacetylation reverses this process. The acetylation status of histone proteins is regulated by enzymes [histone acetyl transferases (HATs) and histone deacetylases (HDACs)]. Glucocorticoids alter the balance of enzyme activity in favour of histone deacetylation by recruitment of HDAC 2 and inhibition of HATs, leading to trans-repression.

iii) Non-genomic mechanisms.

This refers to several non-specific mechanisms that are not directly associated with gene transcription. Binding of intracellular glucocorticoid with signal transduction molecules and pro-inflammatory transcription factors can interfere with these processes. It has been reported that glucocorticoids promote instability of some pro-inflammatory mRNA transcripts such as vascular endothelial growth factor (VEGF) (155). Inhibition of stabilising proteins by glucocorticoids has been postulated as a mechanism. Interestingly, mRNA transcripts for GM-CSF and cyclo-oxygenase 2 are particularly susceptible to ribonuclease breakdown (153). However, it is uncertain how significant these mechanisms are *in vivo*.

The potent anti-eosinophil properties of glucocorticoids are mediated through effects on numerous cell types, leading to interference with eosinophil trafficking at multiple levels. Transcription of the canonical Th2 cytokines IL-4, IL-5 and IL-13 is NFκB dependent and sensitive to glucocorticoids. Inhibition of IL- 5 dependent pathways is therefore a dominant eosinopenic mechanism of these drugs. IL-4 and IL 13 transcription is induced by signal transduction pathways Additionally, transrepression is associated with reduced expression of adhesion molecules (ICAM-1, VCAM-1) and eosinophil chemokines (RANTES, eotaxin-1). Glucocorticoids impair eosinophil survival both *in vivo* (156;157) and *in vitro* (158); this is likely to be an important mechanism for amelioration of tissue eosinophilia with these agents. Effects on eosinophil survival are mediated dually by inhibition of haemopoietin mediated cell survival and activation of apoptotic pathways. Interestingly, the pro-apoptotic effects of glucocorticoids appear to be eosinophil specific as studies report an opposing anti-apoptotic effect in neutrophils (158).

The clinical and pathological response to glucocorticoids in asthma

The efficacy of glucocorticoids in asthma for suppressing eosinophilic inflammation in blood (109;159), airway (109;159-161) and bronchial submucosa (162) is well established. In the airways, inhaled glucocorticoid therapy is associated with a reduction in both eosinophil number (163;164) and activation, as evidenced by a fall in the concentration of ECP (87) and proportion of low density cells in BAL samples.

However, these changes do not occur in isolation and inhaled corticosteroid therapy is associated with a reduction in the number and activation of other inflammatory cells, notably activated CD4+ T-cells and mast cells in the bronchial mucosa (162). Prolonged therapy with inhaled glucocorticoid is associated with resolution of several structural airway abnormalities associated with asthma; supporting the hypothesis that airway remodelling arises as a consequence of chronic airway inflammation. In a randomised double blind parallel group study, treatment for 3 months with inhaled budesonide was associated with restoration of disrupted epithelium and normalisation of ciliated cell : goblet cell ratio (163). A similar 12 month study with inhaled fluticasone demonstrated reduction in inflammatory cell counts that continued for the first 3 months but significant regression in thickness of the subepithelial reticular basement membrane (RBM) that was evident only after 12 months of therapy (165). RBM thickness is an aspect of airway remodelling that may be attributable specifically to eosinophilic airway inflammation. Cross sectional studies performed in steroid naïve (130) and severe steroid dependent (124) cohorts respectively, comparing subgroups of eosinophilic and non-eosinophilic asthma, both reported significantly greater RBM thickness in eosinophilic patients. More recently, a randomised trial of 3 months treatment with the anti IL-5 agent mepolizumab was associated with a significant reduction in RBM thickness in subjects receiving therapy (81).

The clinical response to glucocorticoid therapy in asthma has been evaluated from a number of different perspectives. Studies have been performed in both mild and severe asthma and at stable and exacerbation states; the clinical response has been evaluated with short and longer courses of glucocorticoid therapy. Changes in symptom scores and lung function parameters have been used primarily to measure the short term response to therapy; studies of longer duration have evaluated the effect of treatment on exacerbation frequency. Although the precise relationship between the different study types is not clear, one study reported long term response to inhaled glucocorticoid therapy may be predicted by a positive response to a short term trial (166).

The longitudinal relationship between changes occurring concomitantly in clinical indices and airway pathology with glucocorticoid therapy has been explored in some

detail. A study performed in subjects at the time of exacerbation reported an inverse correlation between the change in FEV1 and sputum eosinophil count, measured serially over 3 weeks during treatment with prednisolone (109). A placebo controlled study of 2-weeks oral prednisolone in subjects with stable moderate asthma reported significant reduction in BAL eosinophils and cellular expression of IL-4 and IL-5 mRNA in the treatment subgroup. These pathological changes were accompanied by a fall in airway hyperresponsiveness (160). In the open study of Djukanovic and colleagues, 6-weeks of therapy with inhaled beclomethasone in subjects with asthma symptoms at baseline was associated with significant improvement in clinical symptoms and multiple objective indices of airway function including morning peak flow, peak flow variability, FEV1 and airway hyperresponsiveness (162). Together these studies would support a mechanistic association between amelioration of airway inflammation with glucocorticoid and clinical improvement. In the 12 month study of Ward and colleagues using fluticasone, the authors reported a significant independent correlation between improvement in airway hyperresponsiveness and regression of RBM thickness, after inflammatory cell counts stabilised at 3 months; suggesting an association between clinical markers and changes of chronic eosinophilic inflammation beyond the shorter term response of inflammatory cells (165).

Studies have reported an association between a severe exacerbation episode and increased risk of future exacerbations (167). It is proposed that upregulation of mediators promoting airway remodelling at the time of exacerbation may lead to alterations of airway structure and future increased susceptibility. The beneficial longer term effects of glucocorticoid therapy on airway structure may in part explain the observed efficacy of these agents for preventing exacerbations.

Heterogeneity in the clinical response to glucocorticoid therapy

The correlations observed between suppression of eosinophilic inflammation and clinical response to therapy are an important part of the framework of evidence supporting a role for eosinophils in asthma. The evidence is strengthened further by heterogeneity in the clinical response to glucocorticoids. A number of studies have reported the presence of underlying eosinophilic airway inflammation to be a critical

determinant of clinical responsiveness to glucocorticoid therapy (168;169). Moreover, this pattern of responsiveness has been reported in COPD (170;171) and also in subjects without a specific respiratory diagnosis, presenting with new symptoms of airways disease (21;172). The latter observations support the idea that clinical benefit with glucocorticoids is not disease specific but is instead closely associated with eosinophilic inflammation. This is entirely consistent with the dominant anti-eosinophilic properties of glucocorticoids and the reported temporal correlation between clinical improvement and resolution of eosinophilic airway inflammation with therapy.

However, the precise role of eosinophils in asthma remains speculative. The anti-eosinophilic effects of glucocorticoids are primarily mediated by blocking of upstream events that are not specific to the eosinophil pathway. Glucocorticoids are more appropriately viewed as suppressants of Th2 inflammation, generally. This is supported by the observation in bronchoscopy studies of reduction with therapy in CD4 T-cells and mast cells, as well as eosinophils and the downregulation of cellular expression of IL-4 mRNA as well as IL-5. In the same way that eosinophilic airway inflammation may be viewed as a 'Th2-ometer', the polarised clinical response to glucocorticoid therapy is a measure of benefit obtained with suppression of Th2 inflammation. This is in keeping with studies that report comparable performance with fractional exhaled nitric oxide (FeNO) for predicting glucocorticoid response in airways disease (21). This molecule is a product of Th2, but not eosinophilic, inflammation [Fig 3].

Studies using specific anti-eosinophilic agents are valuable for isolating the function of eosinophils from the remainder of the Th2 pathway. It may be hypothesised that similarities and differences in outcome between treatment with glucocorticoid and a specific anti-eosinophilic agent will respectively inform components of asthma that are eosinophil dependent or a function of other elements of the Th2 pathway.

2.4.3 Anti-IL5 strategies and mepolizumab

Introduction

The past decade has seen the rapid development of a number of monoclonal antibodies for pharmacological use in asthma. Each of these agents has been developed as a target to block the actions of a specific cytokine believed to play an important role in asthma pathogenesis. Therapeutic credentials apart, these drugs are a valuable research tool, providing a unique opportunity to examine the *in vivo* effects of cytokine blockade in humans.

Mepolizumab is a fully humanised neutralising Ig G1 antibody to IL-5 that sequesters the cytokine and prevents binding to the specific α-subunit on cell surface receptors. Of the specific anti-eosinophilic therapies in development, mepolizumab has been the most extensively studied in human eosinophilic disorders, including asthma. It has been awarded orphan drug designation by the FDA for use in the hypereosinophilic syndrome. In this section I present an overview of the clinical and biological experience to date with the use of mepolizumab in asthma and other eosinophilic disorders, together with the implications of what has been learnt for future clinical application.

Overview of strategies for targeted anti-eosinophilic therapies

The eosinophil is a unique and attractive focus for the design of targeted therapies. As previously described [section 2.2], the circulating level of eosinophils is regulated primarily by a single cytokine, interleukin-5 (IL-5). However chemokines, particularly members of the eotaxin family play an important role in eosinophil migration to tissues and other Th2 cytokines contribute to survival and persistence of these cells at sites of tissue inflammation. In light of the variably overlapping mechanisms described for eosinophil homing, strategies for specific amelioration of eosinophilic inflammation include:

i. Blockade of IL-5
ii. Antagonism of eotaxin
iii. Blockade of CCR 3

Blockade of IL-5 is an effective strategy for reducing the number of circulating eosinophils. The effectiveness of this strategy alone for significantly reducing tissue eosinophilia will be determined by the biological efficacy of IL-5 inhibition and strength of the underlying chemotactic signal. More effective inhibition of eosinophilic inflammation is likely with a strategy that combines blockade of IL-5 and chemotaxis.

Anti-IL 5 molecules and mepolizumab

Several avenues of drug development to achieve IL-5 blockade have been explored. These include:

i) **Neutralising antibodies.** In order to achieve adequate efficacy, the binding affinity of these antibodies are required to be significantly higher than the affinity of the cytokine to its native receptor. A number of this mechanistic class of drugs are at an advanced stage of development in humans, and include mepolizumab (SB240563) and SCH55700.

ii) **Soluble receptor.** Homeostatic regulation of the activity of a number of cytokines is in part achieved by cells expressing soluble variants of the receptor that sequester the free cytokine before cell surface binding and receptor activation. This is achieved through translation of alternatively spliced mRNA. A soluble form of the α-subunit of the IL-5 receptor is produced by cell types that express the receptor natively, though it is undetectable in physiological systems. Expression systems for the extracellular domain of the α-subunit have been developed and act as IL-5 antagonists in bioassays *in vitro.*

iii) **IL-5 mutant protein.** Systematic replacement of charged residues with alanine (scanning alanine mutagenesis) has led to the development of mutant IL-5 proteins. Effectiveness of this strategy requires development of a mutant construct that binds the receptor with wild type affinity but has no agonist activity. To date, mutant proteins have been developed with wild type receptor binding affinity but all retain some agonist properties. While not presently of therapeutic potential, they have generated research interest as one such protein, E12K is a full antagonist in assays of eosinophil

activation but is an agonist of eosinophil survival. This observation suggests that the multiple effects of IL-5 on eosinophil function may be mediated through varied interactions with the receptor.

Animal studies with mepolizumab

Experience with mepolizumab in animal models is limited by the species specificity of IL-5. In cynomolgus monkeys, the cytokine differs by two amino acids in protein sequence with human IL-5 and this does not appear to effect mepolizumab efficacy. Reported outcomes of *in vivo* studies with this animal model closely resemble the experience in humans. The key observations are summarised (63):

i) There was profound depletion of peripheral blood and lavage eosinophils that remained suppressed after sequential antigen challenge and was dose dependent. Doses used ranged from 0.5 mg/kg to 50 mg/kg and none were associated with toxicity.

ii) High dose mepolizumab failed to abolish circulating eosinophils. The drug had no significant effect on low basal counts of circulating eosinophils, suggesting IL-3 or GMCSF responsiveness in these cells.

iii) The blood eosinophil count remained significantly suppressed for 74 days in 2 dose studies of mepolizumab. The biological half life of the drug was therefore considerably longer than the pharmacological half life (13±2 days).

iv) No significant effect was seen in tissue eosinophil counts of either the lung or small intestine with high dose treatment. This suggested that peripheral blood eosinophil counts are a poor marker of the tissue response with mepolizumab.

v) Mepolizumab concentrations were found to be 500 fold lower in lavage fluid than in the bloodstream. This suggests poor tissue penetration, with the drug being retained primarily in the circulation.

vi) Bioavailability and drug pharmacokinetics with subcutaneous delivery were comparable to intravenous administration.

vii) No anti-mepolizumab antibodies were detected after 6 doses at monthly intervals, suggesting that the drug lacks significant antigenicity and tachyphylaxis may not be a major problem with chronic therapy.

viii) Mepolizumab therapy did not alter airway hyperresponsiveness following allergen challenge in ascaris sensitised monkeys. This observation was in keeping with the hypothesis that eosinophilic inflammation and airway dysfunction are independent processes that occur in parallel.

Studies of mepolizumab in humans

The experience with mepolizumab in humans now spans a decade. The studies that have been performed may be broadly categorised as:

i) Studies of immunobiological efficacy
ii) Studies of clinical efficacy in asthma
iii) Studies of clinical efficacy in eosinophilic disorders other than asthma

Brief details of these groups of studies are summarised in [Tables 5 & 6].

Immuno-biological effects of mepolizumab in human asthma

Much of the information gathered about the biological effects of mepolizumab in asthma has been drawn from a series of publications that are based on a single randomised, placebo controlled trial of 3 doses of mepolizumab given at monthly intervals to subjects with mild, corticosteroid naïve asthma (173)[Table 5].

Mepolizumab and eosinophil counts in different tissue compartments

The effect of mepolizumab on eosinophil numbers in asthma has been characterised in a number of different tissue compartments including the bone marrow, peripheral blood, proximal and distal airways and within the bronchial submucosa.

Within the bone marrow, 3 doses of mepolizumab achieved a 70% mean reduction in terminally differentiated bone marrow eosinophils, a 37% and 44% reduction in myelocytes and metamyelocytes respectively but had no effect on levels of early progenitors ($CD34^+$/$IL\text{-}5R^+$) or eosinophil/basophil colony forming units (41). The results suggest that IL-5 is important in the later stages of eosinophil development in

the bone marrow, correlating inversely with surface CD34 expression. Although IL-5R is expressed on early progenitors, the level of expression is low and accompanied by expression of other haemopoietin receptors (IL-3 and GM-CSF) that are more important at this stage of development.

Studies indicate a gradient of anti-eosinophil efficacy with mepolizumab on mature eosinophils across different tissues compartments. Eosinophil suppression is most complete in the peripheral blood (>95% in all studies). This suppression is specific and not accompanied by a fall in the count of other leucocytes. Following 3 infusions of mepolizumab at monthly intervals, Flood-Page et al reported progressively less efficacy in the airway (79% reduction of lavage eosinophil counts) and bronchial submucosa (55% reduction) respectively (173). The relative resistance of tissue eosinophils to mepolizumab is likely to be multifactorial. Poor tissue penetration of the drug may be important as the previously described studies in cynomolgus monkeys demonstrated a 500 fold lower mepolizumab concentration in lavage fluid than plasma. A second important reason is a lesser dependence of tissue eosinophils on IL-5. The trafficking of eosinophils to sites of tissue inflammation is mediated primarily by chemokines, notably eotaxin-1, with IL-5 acting as a cofactor. Tissue eosinophils express CCR3 and receptor expression levels are unaffected by either mepolizumab therapy (174), or IL-5 and other haematopoietins. In the presence of a powerful chemotactic drive, the fall in circulating eosinophil numbers achieved with mepolizumab therapy is unlikely to be sufficient to prevent ongoing tissue accumulation of eosinophils. Within the tissues, the other haematopoietins IL-3 and GM-CSF may have a greater role in promoting eosinophil maturation and survival. This is supported by evidence that mepolizumab has no effect on $CD34^{+}/IL\text{-}5R\alpha^{+}$ tissue eosinophil progenitors (41). Finally, one recent study in eosinophilic oesophagitis has reported a significant rise in circulating eotaxin levels after mepolizumab therapy (175). This suggests that there may even be upregulation in the chemotactic drive with anti-IL 5 treatment that maintains tissue eosinophilia.

Mepolizumab and airway structure

Reticular basement membrane (RBM) thickening is closely associated with eosinophilic inflammation and is ameliorated by regular inhaled corticosteroid use (165). Eosinophils are an important source of transforming growth factor beta (TGF_{β}), a potent regulator of cell proliferation with pro-fibrotic properties that are considered important in tissue repair [section 2.2.3]. It has therefore been hypothesised that persistent eosinophilic inflammation plays an important role in airway remodelling. Despite the modest effects on tissue eosinophilia, Flood-Page and colleagues showed that mepolizumab therapy is associated with a significant reduction in the extracellular matrix glycoproteins tenascin and lumican, together with a reduction in the thickness of the RBM. The clinical significance of these structural changes is uncertain as there was no associated improvement in either FEV1 or airway hyperresponsiveness to histamine in study participants (81). However, the results support a role for eosinophils in airway remodelling and indicate that the effect of mepolizumab on tissue eosinophils is sufficient to influence structural changes, either through quantitative suppression of eosinophilic inflammation alone or possibly through additional effects on eosinophil activation.

Other immunological effects of mepolizumab therapy

Data for the effects of mepolizumab on other aspects of immune function is derived from studies in both asthma and other eosinophilic disorders. However, some discordance exists in the reported outcomes. In a study of patients with moderate asthma (receiving a daily dose of inhaled corticosteroid ≤ 1000 μg beclomethasone dipropionate [BDP] equivalent), Buttner and colleagues reported no effect of 3 doses of mepolizumab on non-eosinophil leucocyte numbers, markers of T-cell activation, intracellular cytokine expression or cytokine receptor expression (176). In another study of mepolizumab therapy administered to a heterogeneous population of patients with eosinophilic disease, Stein and colleagues reported an increase in the intracellular content of IL-5 in T cells after mepolizumab therapy (177). A profound fall in peripheral blood eosinophil counts was observed in both studies; this was associated with a parallel fall in measured ECP levels by Buttner. However, no change

was observed in the expression of other markers of eosinophil activation (CD11b and CD69), suggesting the fall in ECP was due to reduced eosinophil numbers alone, with little additional effect of mepolizumab therapy on eosinophil activation. In contrast, Stein and colleagues reported a reduction in eosinophil shape change with eotaxin *in vitro,* suggesting impaired eosinophil activation after mepolizumab therapy. The authors also found an 18% increase in IL-5 R expression but no change in CCR3 expression with treatment. The increase in IL-5 R expression has not been corroborated in another study of mepolizumab in eosinophilic oesophagitis (178). In this double-blind placebo controlled study, 2 doses of mepolizumab 750 mg were administered 7 days apart, followed by 2 further doses of 1500 mg at 4-weekly intervals if there was evidence of persistent tissue eosinophilia (175). In addition to the expected fall in blood and tissue eosinophil counts, this study also reported no effect of mepolizumab therapy on the number of T-cells and tryptase positive mast cells in oesophageal biopsies. A number of noteworthy points arise from this discussion:

i) The absence of an effect with mepolizumab on CCR3 expression is in keeping with the observations of Flood-Page and colleagues and supports the hypothesis that blockade of this receptor is also needed to effectively ameliorate tissue eosinophilia in disease. However, the relative efficacy of mepolizumab may vary with the severity and type of eosinophilic disease. In an open-label study of mepolizumab therapy for eosinophilic esophagitis, Stein and colleagues have reported an impressive 9-fold reduction in tissue eosinophils after 3 doses. The greater efficacy of mepolizumab observed in this disease may suggest differences in the relative importance of IL-5 and eotaxin for eosinophil trafficking to different organs. In keeping with this, Mishra and colleagues have shown IL-5 to be necessary and sufficient for the development of eosinophilic oesphagitis in a mouse model (179).

ii) A risk of rebound eosinophilic inflammation exists following cessation of therapy due to up-regulated synthesis of IL-5 by Th2-cells; up-regulated expression of the IL-5R by eosinophils; elevated circulating levels of eotaxin (175) and the theoretical availability of a circulating store of IL-5 in complex with drug that may

have impaired clearance kinetics. Although this has not been reported to date with mepolizumab, one study observed rebound eosinophilic inflammation to supra-basal levels following therapy with another anti-IL 5 agent (SCH55700) in patients with hypereosinophilic syndrome (180).

iii) Although variability exists in the precise immunological outcomes after mepolizumab therapy, the evidence in the studies described consistently describes immunological specificity. In particular, the absence of effect on Th2-cells implies that mepolizumab therapy leads to uncoupling of eosinophil function from other processes of the Th2 pathway. This is pertinent when considering the comparative clinical effects of corticosteroids and mepolizumab.

Clinical studies of mepolizumab in asthma

Little data was available prior to 2007 examining the effect of mepolizumab on clinical measures of asthma. Two early studies with mepolizumab and one using an alternative anti-IL 5 agent (SCH55700), reported no effect of therapy on airway hyperresponsiveness to histamine challenge both in mild chronic asthma (173) and mild asthma after allergen challenge (64); the late response following allergen challenge; and FEV1. This was despite a significant reduction in blood and eosinophil counts after treatment in all the studies. The evidence favouring a primary role for mast cells in airway hyperresponsiveness has been previously discussed [section 2.3.2]. In this context, the observation that mepolizumab therapy did not have an effect on mast cell numbers in eosinophilic oesophagitis is noteworthy (175).

While favouring the hypothesis that eosinophilic inflammation and airway hyperresponsiveness represent distinct and dissociated processes in asthma, there has been caution expressed about interpreting the available evidence in this way as dosing regimens used in both mepolizumab studies may have been too short to detect a significant change and the drug did not effectively abolish tissue eosinophils. Given the observed airway structural changes achieved with 3 doses of mepolizumab, it is conceivable that a longer duration of therapy may have led to significant changes in airway wall geometry with consequent improvement of airway hyperresponsiveness.

However, the reduction in tissue eosinophils achieved with three doses of mepolizumab is comparable with the reported effect of high dose oral corticosteroid therapy (181), which does significantly improve airway hyperresponsiveness.

The absence of an effect of mepolizumab on FEV1 by Flood-Page and colleagues in mild asthma may have been attributable to the normal baseline lung function of the study cohort. However, in another study by Kips and colleagues, SCH55700 administered to patients with severe persistent asthma and persistent airflow limitation failed to demonstrate any improvement in FEV1 despite a marked fall in peripheral blood eosinophil counts (182). Taken together, the evidence is suggestive of dissociation between eosinophilic inflammation and lung function, although the caveats described above are equally applicable to the present discussion.

The first major clinical study of mepolizumab in asthma was a multi-centre, multi-national placebo controlled parallel group trial enrolling 362 participants with moderate persistent asthma (183). Subjects were taking ≤ 1000µg BDP equivalent inhaled corticosteroid per day and had persistent symptoms. The study included two active treatment groups that received 3 doses of mepolizumab (250 mg or 750 mg) at monthly intervals and follow up was performed for a further 8 weeks after the final dose of treatment (end of study week 20). The primary endpoint of the study was morning peak flow and a number of asthma related secondary outcomes measures were defined *apriori.* Of these, exacerbations were defined at 3 levels of severity: requiring an escalation in use of inhaled therapy (level 1); requiring a course of oral corticosteroids (level 2); and requiring hospitalisation (level 3). Exacerbation frequency was measured and compared between study groups for 3 predefined time periods: weeks 0-20 (the study period); weeks 0-12 (the treatment period); and weeks 12-20 (the follow up period). Subjects were withdrawn if they had greater than 2 exacerbations during the study and in any given time period, only the highest level exacerbation was recorded. Sputum induction was performed in 32 subjects (10%). 17 subjects (53%) had sputum eosinophilia at baseline and of these 3 subjects entered the higher dose active therapy arm of the study. The study failed to show benefit with either high or low dose mepolizumab therapy in any clinical outcome measures. Although clinically ineffective, the study made important contributions to how

mepolizumab may be taken forward in clinical practice. Some of the key observations are summarised:

i) The study was consistent with previous findings that mepolizumab therapy has no effect on measures of pulmonary function.

ii) A 50% reduction in severe exacerbations (level 2 and 3) was observed during the follow-up period that showed a trend toward significance (p=0.06). Given the recognised association between eosinophilic airway inflammation and exacerbation risk, this observation merits further study, particularly as the follow up period for recording exacerbations was short and the study was not powered to measure this endpoint.

iii) Based on the sputum data collected, it may be estimated that only half of the subjects were likely to have been eosinophilic and therefore have responded to anti-IL 5 therapy. This proportion is comparable to the estimated prevalence of eosinophilic inflammation in the general asthma population, and illustrates the poor discriminatory power of generally accepted inclusion criteria to clinical studies of asthma (that are primarily focussed on demonstrating variable airflow obstruction) for detecting / predicting underlying eosinophilic inflammation. Given the very narrow spectrum of activity of mepolizumab, appropriate patient selection using induced sputum eosinophils or another reliable marker of eosinophilic airway inflammation, is essential.

Clinical studies of mepolizumab in other eosinophilic disorders

In contrast to the disappointing results with asthma, mepolizumab has been used successfully as a therapeutic agent in other eosinophilic disorders. These include idiopathic hypereosinophilic syndrome (184), a heterogeneous condition characterised by moderate to severe peripheral blood eosinophilia and end organ damage associated with eosinophilic infiltration; and eosinophilic oesophagitis (174;175). Both groups of conditions have been managed traditionally with corticosteroid therapy. The success of mepolizumab in these disorders raises two important points that are valuable for our understanding of its appropriate use in asthma:

1. Although a heterogeneous group, these disorders are characterised by clinical disease due to end organ damage that is *directly* attributable to eosinophilic inflammation. This is a pertinent observation as it suggests that the anti-eosinophilic activity of mepolizumab is sufficient to be of clinical benefit *if* the measured outcome is eosinophil driven. Heterogeneity in asthma extends to the role of the eosinophil in the clinical expression of disease. Mepolizumab therapy therefore needs to be considered in subgroups of patients that have a clinical outcome which is likely to be eosinophil driven.
2. Rothenberg and colleagues have reported the use of mepolizumab to enable successful down-titration of glucocorticoid therapy in patients with hypereosinophilic syndrome (184). This highlights a potential role for mepolizumab as an effective steroid sparing agent. However, translating this to asthma is more complex because the role of glucocorticoids in asthma extends beyond their anti-eosinophilic properties. Thus, even in a subgroup of patients with an eosinophil driven clinical outcome, mepolizumab may only be a useful corticosteroid sparing agent for those individuals where the outcome is the dominant clinical feature.

2.4.4 Conclusion

In summary, mepolizumab is a specific and efficacious anti-eosinophilic agent with little additional biological effect. Clinical trials to date have demonstrated a favourable safety profile with few reported adverse effects attributable to the drug. These characteristics support further consideration for use in clinical practice.

Studies in asthma and other eosinophilic disorders have been valuable for understanding more clearly the role of eosinophils in asthma, by dissociating the cell from other effectors of the Th2 pathway. This may also contribute to understanding further the different anti-inflammatory mechanisms of corticosteroids that are important for improving specific clinical outcomes in asthma. Mepolizumab use has also underlined the difficulty of using a drug of high specificity in a very heterogeneous disease population. In this context, two important requirements should be met when conducting clinical trials with specific molecular therapies in asthma:

1. There is an *absolute* need for accurate patient selection and inclusion criteria should incorporate measures that are likely to identify potential treatment responders.
2. Careful consideration needs to be given *apriori* to the primary study endpoint as there is little association between different endpoints. In the case of mepolizumab and other anti-eosinophilic agents, endpoints chosen should at least be presumed to be eosinophil driven. The evidence presented suggests abnormalities of airway physiology are unlikely to be a function of eosinophilic inflammation; however eosinophils may be important effector cells in the pathogenesis of exacerbations in patients with eosinophilic asthma.

2.4.5 Figures and Tables

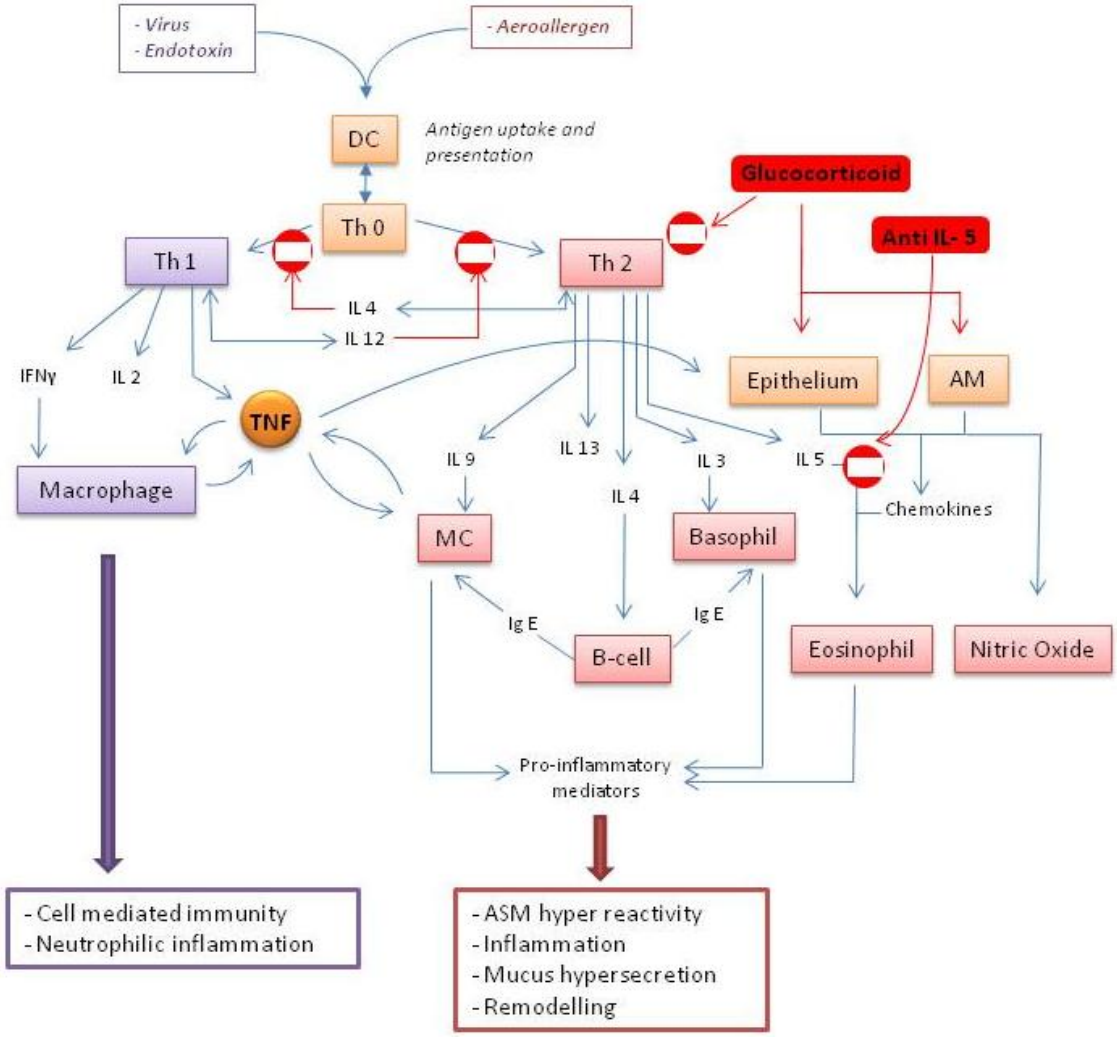


Figure 3: Adaptive immunity in asthma

Both Th2 and Th1 pathways are believed to play a role in asthma associated chronic airway inflammation. It is increasingly recognised that while either Th1 or Th2 processes may predominate, they are not exclusive. Key pro-inflammatory mediators such as TNFα have an important role in both pathways, enabling co-existence of inflammatory components from both pathways.

Glucocorticoids have potent and broad-spectrum anti-Th2 activity. The effects of anti IL-5 are more restricted. A comparison of clinical outcome between the two treatments offers the prospect of isolating the role of eosinophils from other Th2 factors.

Abbreviations: MC = Mast cell; DC = Dendritic cell; AM = Alveolar macrophage

Table 5: Mepolizumab studies in asthma

Study Reference	Study Design			Results	Comments
	Methods and objectives	Subjects	Measured Outcomes		
Leckie MJ et al 2000 (1)	3-centre double blind RPCT[5]. Single dose of 2.5 mg/kg or 10 mg/kg. Mechanistic, dose-response study.	N=24. Male subjects aged 18-45 years with mild allergic asthma	Late allergen response (LAR), blood eos, sputum eos and His PC20[5]	Dose dependent magnitude and duration of fall in blood and sputum eos. Significant fall in sputum eos achieved only with higher dose. No significant effect on LAR and His PC20	Biological effect of mepolizumab on eosinophilic inflammation is dose dependent. A single dose does not affect clinical measures of asthma.
Flood-Page P et al 2003* (2)	2-centre double blind RPCT. 3 x monthly dose 750 mg. Biological study on eosinophilic inflammation in different tissue compartments.	N=24. Aged 18-55 years with mild allergic asthma	Change in: bone marrow eos, blood eos, BAL eos, bronchial mucosal eos, other tissue inflammatory cells, FEV1 and His PC20	Complete suppression of blood eos until 9 weeks after final dose. Median reduction in eos: 79% for BAL, 55% for bronchial mucosa, 52% for bone marrow. No change in staining for MBP[6]. No change in FEV1 or His PC20.	Anti-eosinophil activity of mepolizumab varies between tissue compartments. No change in measures of airway physiology, as above.
Flood-Page P et al 2003* (3)	Biological study of effects on markers of airway remodelling	As above	Markers of airway remodelling: TGFβ level in BAL, TGFβ mRNA expression in Eos, expression of tenascin, lumican and procollagen III in peri-bronchial tissues	Postive baseline correlation between mucosal eos and tenascin density. Significant reduction with mepolizumab therapy in tenascin and lumican but not procollagen III, significant reduction in TGFβ protein in BAL and mRNA expression in eos	Eosinophilic inflammation is associated with remodelling in the bronchial tissues that is mediated by TGFβ and may be reversed by mepolizumab.
Menzies-Gow A et al 2003* (4)	Biological study of effects on eosinophilopoiesis in bone marrow	As above	Bone marrow expression of eosinophil precursors	Mean reduction with mepolizumab: 70% bone marrow eos, 37% eos myelocytes, 44% eos metamyelocytes. No change in: early progenitors (CD 34+, IL5 R+), eos/basophil CFUs.	Mepolizumab interferes with later stages of eosinophil development in the bone marrow.
Buttner C et al 2003 (5)	Multi-centre double blind RPCT. 3 x monthly dose of 250 mg or 750 mg. Biological study on eosinophil activation and effects on other immune cells.	N=19. Moderate asthma	Differential leucocyte count; ECP level; Lymphocyte subsets and their cytokine secretion profiles.	7-fold reduction in peripheral blood eosinophils and parallel fall in serum ECP. No change in the proportion or activity of T-cell subsets	The immunological effects of mepolizumab are highly selective and dissociate eosinophilic inflammation from other Th2 inflammation.

Flood-Page P et al 2007 (6)	Multi-centre double blind RPCT. 3 x monthly dose of 250 mg or 750 mg. Clinical study on measures of asthma control.	N=362. Aged 18-55 years with suboptimal control of moderate persistent asthma^.	Primary: Change in morning peak flow. Secondary: Change in FEV1, asthma symptoms, AQLQ, rescue bronchodilator use, exacerbations categorised at 3 levels of severity	34% of subjects had suppressed blood eosinophils 3 months after last dose of mepolizumab. Terminal half life of mepolizumab 21 days. No significant change in any clinical outcome measure. Trend toward reduction in level 2/3 exacerbations in the 8 week period after last dose of mepolizumab (p=0.06).	Largest study to date reinforcing the absence of benefit with mepolizumab on clinical outcome measures in asthma. Mepolizumab may reduce the frequency of severe exacerbations but study was not powered to evaluate this.
Nair P et al 2009 (7)	Double blind RPCT. 5 x monthly dose of 750 mg. Protocol for down-titration of maintenance oral prednisolone. Clinical study of steroid withdrawal.	N=20. Severe eosinophilic asthma on maintenance oral prednisolone (median dose 10 mg)	Exacerbation frequency expressed as proportion of subjects having an exacerbation. Prednisolone taper achieved, expressed as: % of maximum achievable (based on protocol), absolute % change in dose from baseline. Time to first exacerbation. Change in AQLQ.	Significant reduction in the proportion of exacerbations treated with prednisolone. However, exacerbations were only treated if eosinophilia identified in sputum. Significantly greater dose reduction of prednisolone achieved. Final dose of prednisolone in study groups was not significantly different.	Mepolizumab may be a useful steroid sparing agent in oral glucocorticoid dependent severe asthma

§ Abbreviations used: His PC20= bronchial challenge test with histamine; RPCT= randomised placebo controlled trial; HES= hypereosinophilic syndrome; EGID=eosinophilic gastrointestinal disorder; EE= eosinophilic esophagitis

* All part of same study

^ Suboptimal control was defined objectively by subject reported symptoms above a threshold on a validated questionnaire

1 Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM et al. Lancet 2000; 356(9248):2144-2148.

2 Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS. Am J Respir Crit Care Med 2003; 167(2):199-204.

3 Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS et al. J Clin Invest 2003; 112(7):1029-1036.

4 Menzies-Gow A, Flood-Page P, Sehmi R, Burman J, Hamid Q, Robinson DS et al. J Allergy Clin Immunol 2003; 111(4):714-719.

5 Buttner C, Lun A, Splettstoesser T, Kunkel G, Renz H. Eur Respir J 2003; 21(5):799-803.

6 Flood-Page P, Swenson C, Faiferman I, Matthews J, Williams M, Brannick L et al. Am J Respir Crit Care Med 2007; 176(11):1062-1071.

7 Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E et al. N Engl J Med 2009; 360(10):985-993.

Table 6: Mepolizumab studies in other eosinophilic disorders

Study Reference	Study Design			Results	Comments
	Methods and objectives	Subjects	Measured Outcomes		
Stein ML et al 2006 (1)	Open label study. 3 x monthly doses 750 mg. Primarily a phase I / early phase II study of safety, biological and clinical efficacy.	N=4. Longstanding EE[3]	Change from baseline in: blood eos, tissue eos from oesophageal biopsies, plasma IL 5, CCR 3 expression and quality of life	Significant reduction in blood eos and tissue eos (8.9 fold). No change in eos CCR 3 expression. Clinical improvement in dysphagia - no correlation with baseline IL 5 or eotaxin.	A larger effect of mepolizumab on tissue eos, compared with asthma studies. Suggestion of clinical benefit but study not powered for this endpoint.
Stein ML et al 2008 (2)	Open label study. 3 x monthly dose 750 mg. Prednisolone dose taper in 2 groups after 2nd dose. A biological study examining immunological effects of therapy.	N=25. Miscellaneous disorders associated with eosinophilia (HES, EGID, EE)[5]. Proportion of subjects on maintenance prednisolone.	Change from baseline in: plasma IL 5, eos cell surface expression of IL 5 R and CCR 3, eos activation, numbers and activity of lymphocyte subsets	Suppression of blood eosinophils consistent across disease groups and unrelated to baseline plasma IL 5. Significant increase in circulating IL 5 with therapy (in complex). Increase in cell surface IL 5 Rα expression but not CCR 3. Increase in intracellular IL 5 mRNA expression by T-cells. Reduced eotaxin induced shape change of eos.	Similar to findings of Buttner et al, with specificity of mepolizumab for eosinophilic inflammation. This study suggests an upregulation of the IL 5 axis with mepolizumab but rebound eosinophilia was not observed.
Rothenberg M et al 2008 (3)	Multi-centre double blind RPCT. 9 x monthly dose 750 mg. Protocol for down-titration of maintenance oral prednisolone. A clinical study of symptom control and achievable dose reduction of maintenance oral glucocorticoid.	N=85. HES without tyrosine kinase receptor mutation and treated with prednisolone monotherapy.	Proportion of subjects achieving maintenance dose ≤ 10 mg prednisolone for ≥ 8 weeks	Significantly greater proportion of subjects achieved effective suppression of blood eosinophils and prednisolone dose reduction to ≤ 10 mg with treatment.	Mepolizumab has an additive biological effect for suppressing blood eosinophils in subjects receiving long term glucocorticoid. This supports its consideration as a steroid sparing agent for eosinophilic disorders.
Straumann A et al 2009 (4)	Double blind RPCT. 2 x doses 750 mg 1 week apart + 2 x monthly doses 1500 mg (if inadequate tissue response). A mechanistic study of dose response for symptoms and biological response in tissues.	N=11. Symptomatic EE, despite maintenance glucocorticoid.	Change in: symptoms and aspects of tissue inflammation	Significant reduction in blood eos, tissue eos, EDN deposition (in proportion to eos reduction), TGFβ and tenascin expression. No change in tissue mast cells and T-cells. No change in IL 5R expression.	The effects of mepolizumab on tissue inflammatory cells and tissue architecture are consistent with other studies. This is the only clinical study to have used a 1500 mg dose. No increase in adverse events was reported, however significant additional biological effects were not observed

1 Stein ML, Collins MH, Villanueva JM, Kushner JP, Putnam PE, Buckmeier BK et al. J Allergy Clin Immunol 2006; 118(6):1312-1319.

2 Stein ML, Villanueva JM, Buckmeier BK, Yamada Y, Filipovich AH, Assa'ad AH et al. J Allergy Clin Immunol 2008.

3 Rothenberg ME, Klion AD, Roufosse FE, Kahn JE, Weller PF, Simon HU et al. N Engl J Med 2008; 358(12):1215-1228.

4 Straumann A, Conus S, Grzonka P, Kita H, Kephart G, Bussmann C et al. Gut 2010; 59(1):21-30.

3. ASTHMA HETEROGENEITY

3.1 Introduction

> *'Surely it is hard to believe that the wheeze which comes to the young school girl for a day or two in the middle of the ragweed season is the same disease as that which develops suddenly in the tired business man or in the harassed housewife and pushes them down to the depths of depletion and despair.*
> *The problem is still wide open: the approach to it is not at all clear.'*
>
> ***F. Rackemann 1948***

Heterogeneity in asthma is well recognised but poorly characterised. It encompasses all aspects of disease expression and represents the major intellectual limitation to progress at a time when research at the molecular and genetic level is gaining momentum. The prospect of such research offering new insights in asthma pathogenesis and susceptibility are unlikely to be realised until disease heterogeneity is understood and classified in a meaningful way. However the problem of heterogeneity is not confined to asthma and is integral to chronic airways disease as a whole. This is evident in the considerable overlap in expression of measurable characteristics that exists among different categories of chronic airways disease.

In this section, I summarise the factors associated with heterogeneity in asthma, the principles of meaningful classification and present the potential utility of multivariate mathematical techniques to tackle the problem.

3.2 Chronic airways disease and the 'Dutch Hypothesis'

Before considering heterogeneity in asthma, it is worth reviewing the place of asthma in the spectrum of chronic airway diseases. The overlap between different categories of chronic airways disease implies poor specificity of the diagnostic labels that are assigned for representing distinct disease entities. Although efforts to achieve clearer

separation that is clinically and pathologically meaningful have been ongoing for over 50 years (185), an appropriate model of classification remains elusive. The alternative view proposed in 1961 (186) and later known as the Dutch Hypothesis, holds that the various forms of airway obstruction are different expressions of a single disease entity with common genetic origins. The term chronic non specific lung disease (CNSLD) was introduced to describe this single disease. Support for the hypothesis is driven by the failure of modern medical science to achieve a model of classification that identifies distinct disease entities, together with the observation that the different airways diseases are considered to share a common pathogenic pathway [Fig 4]. Although validity of the Dutch hypothesis continues to be debated (187) it highlights the uncertainties that surround the characterisation of chronic airways disease. One consequence of this is the idea that current diagnostic labels are too imprecise and should be disregarded in favour of a new model that is free of the apriori bias inherent with the use of traditional labels (188;189). In this context, a model characterising heterogeneity in asthma alone may be viewed as a subset of a unifying model for chronic airways disease.

3.3 Overview of heterogeneity in asthma

3.3.1 Heterogeneity of disease expression

Heterogeneity in asthma is complex and multifactorial and summarised in [Fig 4]. As discussed, the absence of a disease specific marker compromises diagnostic rigour. In clinical practice, asthma is more accurately viewed as a syndrome of symptomatic variable airflow obstruction. The British guidelines for asthma recommend a number of different approaches to securing a diagnosis of asthma [Fig 5]. However, diagnostic congruity between different approaches assumes the information obtained with each is equivalent; an assumption that remains to be validated. It is therefore likely that the clinical diagnosis of asthma includes a number of different if related disease entities. As [Fig 4] illustrates, diagnostic criteria applied to airways disorders poorly inform underlying pathological mechanisms. Markers for each step of the disease pathway exhibit heterogeneity of expression within the asthma population. Taken together, countless permutations of marker expression across the various steps of the disease process are possible. Heterogeneity is complicated further by time (natural variation in disease activity) and therapy. Glucocorticoid therapy is associated with changes in the airway inflammatory cell profile and airway physiology; evidence of structural change is demonstrable with chronic treatment [section 2.4.2]. The effect of these additional dimensions is likely under-estimated in cross-sectional studies. Whether the additional heterogeneity arising from treatment should be considered an integral part of the disease or a confounder for which adjustment is needed remains a matter of debate. A number of co-morbidities are associated with chronic asthma [Table 1]. These may represent both overlapping and unrelated pathologies that co-exist to either mimic or exacerbate clinical asthma symptoms. In common with other chronic disease, the clinical expression of asthma is associated with a significant and variable psychosocial component (190). This is frequently overlooked and may be difficult to quantify.

3.3.2 Heterogeneity of treatment response

Heterogeneity in the response to asthma pharmacotherapy is increasingly considered an important component of the failure to achieve control in refractory asthma.

Multiple factors have been identified to date. In general terms, symptoms associated with co-morbidities pathologically unrelated to asthma and psychosocial morbidity will be refractory to asthma pharmacotherapy. Obesity is associated with a high symptom burden (191) and a poor response to glucocorticoid therapy (192). Mechanisms for this are poorly understood but small studies have demonstrated improvement in asthma control with weight loss (193). Current smoking is also associated with a high symptom burden and impaired glucocorticoid responsiveness (194). One study has demonstrated a significant reduction in asthma symptoms and improvement in the cutaneous response to corticosteroid following smoking cessation for 6 weeks (195).

The profile of airway inflammation is an important predictor of the short term response to glucocorticoid therapy. Both eosinophilic airway inflammation and elevated exhaled nitric oxide are associated with a good response in steroid naïve patients with asthma. The former is also predictive of response in prednisolone dependent, severe asthma. Smoking is associated with predominantly non-eosinophilic pattern of airway inflammation in asthma (196). This observation may in part explain the observed lack of response to inhaled steroid in this group.

At a molecular level, *in vitro* studies suggest a potential role for vitamin D3 in promoting the anti-inflammatory effects of dexamethasone in glucocorticoid unresponsive asthma (197). Targeted gene studies have identified polymorphisms of the beta2 adrenoceptor to be associated with heterogeneity in the response to short acting beta agonists but not long acting beta agonists (198) and polymorphisms of the 5-lipoxygenase biosynthetic and receptor pathway are associated with a differential response to monteleukast (199).

3.4 Problems arising from asthma heterogeneity

It is recognised that heterogeneity is arguably the major intellectual limitation to progress in our understanding of asthma (200). The Ciba guest symposium of 1959 (185) was one of the first forums to attempt characterisation of heterogeneity in airways disease. An excerpt from the conclusion encapsulates the problems of

heterogeneity. While the comment is made with reference to emphysema, it is equally applicable to asthma:

> *…(emphysema) is used to indicate various morbid states of the lung differing widely in their pathology, symptomatology and prognosis. This results in confusion and misunderstanding between investigators working in different centers and in different branches…*

From a clinical perspective, a one-size-fits-all approach fails to be effective in all patients. Perhaps one of the greatest barriers to the characterisation of heterogeneity has been the success of glucocorticoid therapy in the management of asthma. Glucocorticoids are broad spectrum anti-inflammatory therapies that are effective in a large proportion of patients, irrespective of the underlying pathological processes. However, heterogeneity in the response to asthma therapy is a highly relevant clinical issue for the subgroup of patients with difficult asthma, requiring management in secondary care. In this group, heterogeneity is striking and the aetiology of poor clinical control is broad and multifactorial. A systematic approach to the evaluation of these patients, together with a multi-disciplinary and individualised management plan is recommended for optimising care (19).

From a scientific perspective, genetic and molecular association studies necessarily require the study of populations that are homogeneous in respect of their underlying disease pathways. Heterogeneity in such study samples increases the likelihood of type II errors. This is especially pertinent for gene association studies where functional single nucleotide polymorphisms have a very low independent attributable risk (less than 5%) (200). A number of novel and primarily engineered molecular therapies have been developed over the past decade and trials with these drugs are invaluable not only for informing clinical efficacy but for also providing a novel strategy to understand elements of asthma immunobiology *in vivo*. Meaningful interpretation of outcomes from such studies requires careful molecular characterisation of the participating cohort. Molecular association studies therefore form the basis of future biomarker and drug development.

3.5 Principles of characterising asthma heterogeneity - Lessons from biology

The purpose of characterising heterogeneity in asthma is to identify and understand homogeneous subpopulations within the whole, for aspects of disease that are clinically and biologically meaningful. The assumption made is that such subgroups exist within the asthma population.

The accepted paradigm for characterising heterogeneity is found in the biological taxonomy (the science of classification) of organisms; the principles developed on this platform are applicable to any biological system in which heterogeneity exists. In brief, biological taxonomy is founded on the principle that the greater the number of shared characteristics between two organisms, the greater the probability of a biological relationship existing between them. Detailed physical characterisation or 'phenotyping' is therefore fundamental. Classification or taxonomy refers to the construction of models for placing phenotypes in a manner that informs underlying relationships. Characterising heterogeneity is therefore a 2-step process of *phenotyping* and *classification*.

Both phenotypes and classification models may change on the basis of the information gathered or available, new techniques and the goals that are defined. Such changes are influenced by advances in scientific understanding and the taxonomy of any biological system should therefore be considered a dynamic process. The history of biological taxonomy illustrates this well. Developed primarily as a method for systematic nomenclature in the 18th century, the focus of taxonomy shifted to a model representative of evolutionary relationships. The change in emphasis did not alter the methodological principle of grouping by observable characteristics. Indeed, the scope of such methodology was broadened with the development of numerical taxonomy in the mid-twentieth century (201). This field utilised computer based mathematical algorithms (cluster analysis) to measure the 'evolutionary distance' between organisms on the basis of considerably larger numbers of recorded characteristics. The technique was popular for its objectivity and capacity for processing information. More recently, the availability of genetic information from advances in DNA

sequencing techniques has led to the replacement of phenotype based numerical taxonomy with phylogenetics, which compares genetic data from organisms past and present to construct evolutionary trees using predictive mathematical models. In this context, it is worth noting that scientists have been aware for almost a century that evolution is a function of heritable traits and that an unreliable and often poor relationship exists between phenotype and genotype. Yet, as a scientific discipline applicable to biological taxonomy, phylogenetics only became plausible with the ability to sequence DNA efficiently and rapidly. Thus, the 'evolution' of taxonomy is dependent upon developments in many other fields. The terms 'systems biology' and 'systems medicine' have been created to highlight the need for a co-ordinated and multi-disciplinary approach to the ongoing refinement of taxonomy for different biological systems.

3.6 Hurdles to characterising heterogeneity in asthma

A number of hurdles exist to characterising heterogeneity in asthma and airways diseases more generally are summarised:

1. **Absence of a reliable nomenclature**: as discussed, there is poor specificity on the basis of clinical symptoms and physiological criteria for definitions of the different airways diseases. As the history of biological taxonomy illustrates, reliable nomenclature provides a platform for extending the role of taxonomy. In this context, it may be preferable to begin with the taxonomy of airways disease in order to develop a systematic nomenclature.
2. **Complexity of heterogeneity:** this includes the multi-dimensional nature of the disease, the unpredictable effects of treatment and psychosocial wellbeing on observable characteristics and the natural fluidity of characteristics over time. While some aspects of heterogeneity are beyond control, an important step in characterising heterogeneity is to identify techniques to deconstruct the multi-dimensionality of disease expression in a systematic way.
3. **Limited repertoire of measurement tools:** observable characteristics include all characteristics that can be seen or are unseen but measureable. However, what

is ‘measurable’ depends not only on the availability of tools but their appropriateness for use in large and representative population samples. One example is the measurement of airway inflammation. Although measurable at bronchoscopy, the inclusion of airway inflammation as a measurable characteristic has only gained recognition since the development of sputum induction. The characterisation of heterogeneity will benefit from the development of more such non-invasive tools to measure other ‘hidden’ components of disease.

4. **Historical selection criteria:** there is a tendency, particularly in pharmacotherapy studies, to include participants that fulfil ‘disease specific’ criteria. This is usually in an effort to gather a purer disease phenotype. Poor representation of real world disease within study samples of asthma and COPD have been reported (202). Studies that seek to characterise heterogeneity must take an alternative approach and include populations with fewer disease specific criteria.
5. **Limited therapeutic options:** the importance of disease phenotypes are frequently judged by their perceived clinical relevance. This is one reason for the wide acceptance of inflammatory phenotypes of asthma. However, the clinical relevance of phenotypes is limited by the paucity of therapeutic options that are presently available. Furthermore, there is a risk that potentially meaningful phenotypes that may inform the direction of future research are lost due to a lack of immediate clinical relevance.

3.7 Goals of characterising heterogeneity in asthma

The goals of asthma characterisation are a product of the limitations that may be overcome by removing heterogeneity. The primary goals of clinical relevance differ from those of scientific relevance at face value. Furthermore, there are differences in the methodology needed to identify phenotypes in the two groups. Thus complex, sophisticated, labour-intensive and expensive techniques may be utilised to generate phenotypes and classification models for scientific purpose. In contrast, an important aspect of ‘clinically relevant’ characterisation is the ability to identify phenotypes using simple bedside tests. Thus parallel models for clinical and scientific purpose may

converge after multiple iterations to yield a single model that is fit to meet the purposes of both [Fig 6]. An important aspect of the schema shown is the identification of novel biomarkers of biological significance that are developed for commercial use and fed into the clinical model to yield new clinical phenotypes that form the basis of further scientific investigation.

3.7.1 Biomarkers and endotypes

A biomarker is broadly defined as any measurable characteristic that may be used as an indicator of the risk, presence or severity of a disease state. There is considerable interest and investment in the search for novel biomarkers that may inform asthma phenotypes. In this role, biomarkers of potential utility must be detectable in the presence of disease and be independent of disease activity and therefore effects of therapy. The term 'endophenotype' or 'endotype' has been coined in psychiatry to describe such stable phenotypes, with the inference that they are defined by unique and specific genetic or molecular characteristics (203). This concept has recently been proposed for application in asthma (200) as phenotypes of asthma to date have been defined by biomarkers of disease activity, leading to the problem of phenotypic uncertainty with time and therapy.

3.8 Overview of recognised asthma phenotypes

The history of asthma phenotypes dates back to the 17th century when William Harvey suggested a distinction between asthma of bronchial or cardiac origin. Since that time, there have been several important shifts in the ideology governing asthma diagnosis and classification. In the 19th century, the recognition of 'allergic excitation' together with the development of skin testing techniques encouraged a very narrow perspective of asthma as a disease that was necessarily associated with and precipitated by allergy, verifiable with skin testing. Through diligent collection of case records for patients attending his clinic, Rackemann clearly illustrated the absence of an identifiable allergic trigger in a large proportion of cases with clinical asthma (204). He suggested a number of other associations that were significant for this group and

proposed a model for asthma classification on this basis. In his paper describing intrinsic asthma Rackemann (205) summarises this change to a broader perspective:

> *'In the beginning all was allergy that wheezed, and if the methods peculiar to allergy could not reveal the cause, these methods were deemed faulty. It was recognised, however, that the simple allergic process could be aggravated and continued by secondary infections. Still later, primary infections came to be regarded as the cause of asthma …'*

A natural consequence of a more inclusive approach to diagnosis has been heterogeneity that clinicians have sought to classify in a variety of different ways. A number of systems for asthma classification have been formally proposed over the past eighty years; a sample of the phenotypes described is presented in table [Table 7], together with a summary of the utility of each model for fulfilling the goals of classification discussed above.

In light of their clinical origins, the phenotypes are predictably better at meeting clinical goals of classification. Most phenotypes are labile with time and therapy. Exceptions to this are those characterised on the basis of triggers and asthma related outcomes; extrinsic asthma and aspirin sensitive asthma may represent endotypes.

There is some debate about whether severe asthma constitutes a phenotype of asthma. In many ways this is analogous to whether old age is a phenotype of man. Old age is of no relevance in the setting of phenotypes constructed to examine phylogenetic relationships. However, old age has prognostic importance and may help inform factors associated with the mechanisms of ageing. In the same way, severe asthma is prognostically significant and therefore a phenotype of clinical relevance. It is also proposed that there may be specific genetic factors that predict 'severity' which may only be identified by characterising severe asthma as a phenotype (200). The alternative view is that severity is most often a descriptive term applied to qualify phenotypic expression and has no independent phenotypic significance.

Phenotyping on the basis of asthma outcomes is also an approach that has been used. Definitions of outcomes are more explicit and therefore less heterogeneous than the

disease itself. They may also be readily applied to construct models that inform risk associations.

Although models differ in the characteristics used to construct them, they share a number of common principles. Excepting inflammatory phenotypes, all are based on clinical observation and all classification has been performed using subjective stratification criteria. It is also apparent that each model is defined by a single aspect of disease. Given the variable relationships that exist between the different asthma domains, it is difficult to integrate the information presented between models.

3.9 Moving forwards with characterising asthma heterogeneity

3.9.1 Multi-dimensional phenotyping

Characterising heterogeneity on the basis of multiple aspects of disease is biologically appealing. Firstly, it incorporates a broader spectrum of observable characteristics, increasing the likelihood of identifying meaningful (biological) relationships. Secondly, it defines each phenotype on the basis of the *relationship* expressed between different domains of disease rather than the absolute expression of a single aspect of disease. In biological terms, examining relationships between domains is likely to better inform underlying processes and pathways. Additionally, this will be associated with greater phenotypic stability. As an example, non-eosinophilic asthma may indicate either a true phenotype or well controlled eosinophilic asthma. The two are indistinguishable on the basis of inflammatory characteristics alone. The inclusion of clinical symptom expression as a characteristic may reveal concordance of expression in eosinophilic asthma and discordance in the true non-eosinophilic phenotype. Furthermore, such a system would correctly classify poorly controlled eosinophilic asthma (high levels of eosinophilic inflammation + high symptoms) with well controlled eosinophilic asthma (non-eosinophilic + few symptoms).

Two important questions arise:

i. What are the different domains of the asthma phenotype and how might they be identified?
ii. How can this information be processed to identify phenotypes of asthma?

3.9.2 Multivariate mathematical techniques for characterising asthma heterogeneity

The rapid escalation of computer processing power over the past fifty years has made feasible the analysis of progressively larger and more complex datasets. This has necessitated the design of mathematical algorithms to accompany specific aspects of data management. Such algorithms are advantageous for having the capacity to

process large volumes of information quickly, reliably and objectively. Furthermore, different algorithms are suited to addressing particular questions and may be modified to address a specific scientific problem. Multivariate techniques may therefore provide a solution to the questions posed and help overcome the limitations of historical phenotypes. In the next section I describe the two groups of algorithms considered in this thesis.

3.10 Multivariate techniques and characterising heterogeneity

3.10.1 Introduction

It is said that statistical analysis is the process of making scientific inferences from data that contain variability (206). Novel challenges have arisen with the statistical analysis of high volume, complex data. Methodologically, classical analysis techniques are alone insufficient and not well suited to address the scientific questions posed by such data. In particular, such techniques are not designed to explore underlying pattern structure and between-group comparisons are undermined by the uncertainty of how to define statistical significance after multiple comparisons. These challenges have encouraged the emergence of new statistical paradigms, most notably in the field of multivariate statistical methodology (207).

Multivariate techniques are now an established part of research associated with large datasets. Originally, such algorithms were applied to the analysis of large population datasets of the social and political sciences. Algorithms developed for the primary purpose of biological taxonomy led to the birth of a new discipline – numerical taxonomy; variations of these techniques were subsequently applied to the classification of medical disorders, most notably in psychiatry (208). More recently, the development of technologies enabling high throughput, high efficiency output of molecular and genetic data have greatly extended the role of multivariate statistical analyses to the examination of biological data. Bioinformatics is an empirical science concerned with the application of computer science to the field of molecular biology, with the primary goal of characterising vast and complex datasets to better understand underlying biological processes. At a macroscopic level, parallels with the challenges in asthma are easy to see and bioinformatics solutions to analysis problems are therefore a useful guide.

Factor analysis and cluster analysis are two related multivariate modelling techniques that are designed primarily for performing systematic classification. Although conceptually similar, the two groups of techniques differ in the algorithms they employ, with implications for their respective suitability to different classification

tasks. In broad terms, both techniques seek to identify patterns within data. These patterns inform the likely underlying structure of the data that is the basis for classification. Each group is comprised of a number of algorithms that differ in the mathematical rules governing pattern recognition and classification processes. This can lead to considerable variability in the interpretation of data structure. An understanding of the underlying principles of these techniques is therefore a necessary prerequisite for their appropriate use in research practice.

In this section, I describe the principles and limitations of factor and cluster analysis techniques and discuss how each may be utilised for the characterisation of asthma heterogeneity.

3.10.2 Factor analysis

Overview

Factor analysis (209) includes a group of algorithms that are primarily used to identify patterns of variation for variables within a dataset. In brief, variables that group together are represented by a factor which is mathematically the vectorial sum of contributions from each component variable. The factor therefore represents a weighted sum of data from all the grouping variables, and may be used in place of the individual variables in further analysis, without a significant loss of information.

Methodology

The vectorial representation of factor analysis is informative [Fig 7].The sum of each variable within a dataset is plotted in space as a vector. Variables that exhibit similar patterns of expression therefore plot close together (NB the cosine of the angular relationship between individual vectors is synonymous with the Pearson coefficient of correlation between the two variables).

Factor analysis techniques use a 2-step algorithm to classify patterns of expression. The first step is primarily a data reduction step. In this step, a factor 'axis' is constructed that maximises representation of the common variability within the data (in effect, linear regression). A second factor is then constructed to account specifically for variability that is *not* included by the first. This factor is therefore mathematically independent of the first and may be represented geometrically by an axis that is perpendicular to the first factor axis. Iterations continue until all of the variability within the dataset is accounted for by independent factors.

The second step of the algorithm is an optimisation step. The factor axes are rotated to maximise their representation of variability for groups of variables rather than the whole dataset. With rotation, relationships between each factor axis and individual variables will change; however the proportion of variance of the dataset accounted for by the factor remains constant. Rotation is important for changing the emphasis of factors from a data reduction model representative of the whole dataset to a

structural model in which individual factors define clustering variables. The rotated factor solution is believed to yield 'invariant factors' i.e. the factor model is less sensitive to the removal or addition of one or a few dataset items.

Factor analysis techniques differ in their use of algorithms to achieve the steps described. The two techniques used commonly in medical research are principal factor analysis (PFA) and principal components analysis (PCA). PFA uses only the common variability of each item while PCA uses all of the variability for each item, both common and unique. PFA may be better for studies that are aimed at characterising data structure while PCA is considered more appropriate where the primary goal is data reduction. In practice, differences in outputs between the two techniques are frequently minor as most variability for individual items is common when a large number of variables are included. Factor rotation algorithms may be 'orthogonal' (independence between factor axes is retained) or oblique (unconstrained rotation of individual axes). The former retains statistical independence of the factors, which is desirable for further analysis. Oblique rotation attempts to maximise the association between groups of variables and each factor axis. However, the violation of orthogonal constraints makes interpretation of the factor structure more difficult.

Interpreting factor analysis outputs

The outputs obtained with factor analysis are complex and use terminology that is incomprehensible to readers without prior knowledge of the subject. This presents a significant problem for conveying important scientific information in studies where the technique has been applied, particularly as the use of factor analysis and other multivariate techniques is increasingly commonplace. An example of a factor output that summarises the points discussed here is given [Fig 8].

A critical part of the assessment of factor analysis models is evaluation of their validity. For a factor analysis model to be representative of the data from which it is derived, it should account for the majority of the variance of the dataset (this information is generally provided as part of the result summary). Similarly the relevance of each factor selected should be made on the basis of the proportion of total variance that is accounted for by the factor. The '***eigenvalue***' is used to quantify this and is calculated

as the sum of variances accounted for by all the contributing variables to the factor. An eigenvalue of less than 1 implies that the factor accounts for less variance than a single variable in the dataset and is often used as a cut off for determining the number of factors that are included in the model. Finally, the proportion of the total variance of each item accounted for by the model is expressed as the ***'communality'***. Variables with low communalities are therefore not adequately represented in the factor model and inferences about these items from the model may not be accurate.

The rotated factor matrix presents a summary of the factor model. Each factor may be defined according to the pattern of highly loading variables. A common inference made is that variables correlating closely with a given factor also correlate closely with each other. This is not necessarily true and should be cross-checked with the correlation matrix that is also presented as part of the output.

Uses of factor analysis

The main uses of factor analysis are data reduction and the identification of groups of variables sharing related patterns of expression. From a mathematical perspective, data reduction is invaluable to help overcome the problems associated with the application of classical statistical tests to complex datasets. A small number of factors derived from a large number of data items will reduce the effects of multiple comparisons and the orthogonal relationship between factors enables their further use as independent variables for analyses such as multiple regression. Data reduction is achieved using either the factor score for each factor (weighted sum of the contribution of all variables to the factor) or a single representative variable (with a high loading coefficient to the factor, implying a majority contribution to the factor score) for further analysis. The former requires a more complete dataset as the factor scores use data from each of the contributing variables. In contrast, the use of a single representative variable may overcome the problem of missing data.

As a technique that identifies relationships between groups of variables, factor analysis makes statistical inferences that may contribute significantly to identifying and understanding underlying mechanisms and processes. From a biomedical perspective it is important to remember that the relationships defined by factor

analysis are mathematical and not biological. Identified patterns are therefore *hypothesis generating* and can help direct further study; factor analysis should not be used in isolation to draw biological conclusions. However, in his text Rummel, a political scientist and keen advocate of factor analysis takes a different view and makes a rather philosophical argument:

> *'To explain an event is to be able to predict it … To explain that the Roman Empire fell because of disunity and moral decay, is to say that, given the presence of these two elements in an empire with the characteristics of the Roman Empire, the empire will break up or be conquered… Prediction itself is based on the identification of causal relations, i.e. regularity. Therefore, if a factor can be called a cause, it can be called an explanation.'*

Applying factor analysis to asthma

The properties of factor analysis make it a powerful tool for characterising heterogeneity. A large number of measurable characteristics are routinely recorded as part of clinical assessment in asthma. Yet little is known about the relationships that exist between these variables and whether the information that is gathered may be organised in a structured manner. Factor analysis lends itself to tackling these questions. Rosi and colleagues (142) performed a factor analysis of eight measured characteristics recorded in 99 consecutive patients with asthma. The authors identified 3 factors associated with the eight measurements and based on the loading patterns, these factors could be identified as being representative of lung function (FEV_1, FVC, IVC); airway dysfunction (bronchodilator reversibility, airway hyperresponsiveness); and eosinophilic airway inflammation (sputum eosinophils, ECP) respectively. The factor model presents a view of the independent components or domains that together constitute the clinical phenotype of asthma. A similar exploratory factor analysis by Lapperre and colleagues (210) was performed in 114 patients with COPD using data from ten measured characteristics. In this study 4 factors were identified. 3 of the factors described domains of disease that were identical to Rosi's study in asthma. A single item (exhaled nitric oxide) loaded on the fourth factor and therefore added little to the model. The identification of similar patterns of expression of variables measured across different categories of airways disease supports the idea

that there is considerable overlap between disease groups. Furthermore, the result indicates that the domains identified are invariant and may be used to characterise phenotypes of airways disease, more generally.

3.10.3 Cluster Analysis

Overview

Cluster analysis is a generic term for a broad range of numerical methods that are designed primarily to identify groups or clusters of homogeneous observations within heterogeneous multivariate datasets (211). Cluster analysis is conceptually similar in its properties to factor analysis as a technique for data reduction and identifying relationships. Whereas factor analysis classifies patterns of expression of *variables*, cluster analysis performs classification of the *population* based on patterns of expression for specified variables. In mathematical terms, a cluster refers to a collection of items that exhibit 'internal cohesion' (within-group homogeneity) and 'external isolation' (between-group separation). Cluster analysis algorithms are designed to fulfil both conditions.

Methodology and nomenclature

Cluster analysis techniques are broadly of two types: i) hierarchical and ii) non-hierarchical or optimisation clustering techniques. Broadly speaking, all clustering techniques follow a 2-step algorithm. The first step involves quantifying similarity between cases. This is a geometrical distance in space. The second step involves placing cases into groups on the basis of measured similarity. Group allocation is an iterative process.

Measuring similarity

Similarity may be defined on the basis of one (monothetic) or several (polythetic) variables and is generally measured as the geometric distance between two cases plotted in space. Several geometric measures of distance are available with differing influences on outcome [Table 9]. For polythetic clustering, the number of variables used determines the dimensionality of the distance calculation; this calculation assumes that each dimension is perpendicular to every other (i.e. statistically independent) in space. The inclusion of highly correlated variables violates this assumption leading to inaccurate measures of distance and an unreliable cluster

model. Increasing dimensionality requires larger sample sizes to identify cluster structure. Therefore, a limitation exists on the maximum number of variables that may be chosen to perform polythetic cluster analysis reliably, for a given sample size. Formann (212) suggests a minimum sample size of 2^k where k= number of variables used for clustering.

Grouping techniques

Hierarchical techniques are constrained by the assumption that all cases within the dataset arise from or converge to a single group. Agglomerative hierarchical analysis refers to 'building up' from cases to progressively larger groups. In divisive hierarchical analysis, the sequence occurs in the opposite direction. Outputs from a hierarchical analysis can be presented graphically in the form of a dendrogram [Fig 9]. The distance axis is a quantitative presentation of similarity. Each node at which branching occurs represents a cluster. The position on the distance axis at which the node is found refers to the level of similarity between objects within the clusters at that level. Phylogenetic trees are constructed using hierarchical clustering techniques; the distance axis here is considered to represent 'evolutionary distance'.

Non-hierarchical cluster analysis begins with a pre-specified number of clusters to which cases are allocated [Fig 10]. Although not as popular as hierarchical clustering, non-hierarchical techniques are favoured by some for greater mathematical compliance. The clusters formed are not constrained by a hierarchical pathway, theoretically allowing better optimisation of cluster structure. Furthermore, in hierarchical analysis the branching structure is irreversible, with few exceptions. Thus, if a partition is incorrectly or inappropriately positioned during the iterative process, it cannot usually be repaired. In contrast, the iterative processes used in non-hierarchical algorithms assume a fluid cluster structure that is not defined until the cluster centres are optimised. A propensity for self-repair therefore exists.

Despite these theoretical advantages in methodology, non-hierarchical algorithms are limited by the contentious issue of how to objectively pre-specify the number of clusters. Several approaches to this problem have been proposed (213), without consensus. Techniques are likely to differ in their appropriateness, according to

circumstance. A subjective approach to the problem is satisfactory, particularly when an informed estimate of the underlying data structure is feasible (211). In this case, the cluster structure using one more and one fewer clusters than the presumed optimum number is interrogated. Objective methodology is perhaps more desirable for exploratory data mining. One technique considered to perform well is the silhouette method, which is an iterative algorithm evaluating the difference between within-cluster variance of a cluster before partition with the sum of within-cluster variances after optimal division of the cluster. Division of the cluster is accepted if the difference in variances exceeds a threshold (214). In some circumstances, the 'elbow-method' applied in factor analysis to yield a scree plot may also be used to determine the number of clusters. In v-fold cross validation, an algorithm is applied that partitions the available data into training and test sets. Successive iterations attempt to identify the number of clusters that minimises classification error between training and test sets. Another approach is to explore the degree of separation that is achieved with successive cluster divisions. This approach is analogous to that taken for the evaluation of dendrograms, where 'big' (a subjective quantity) changes between successive nodes along the distance axis support consideration of further subgroups. In this context, though less conspicuous, it is noteworthy that the problem of subjectivity also exists for hierarchical techniques.

Within the families of hierarchical and non-hierarchical cluster analysis, various algorithms exist that differ according to the mathematical functions used for measuring similarity and grouping cases into clusters [Table 9]. For a given dataset, these differences can lead to considerable variability in cluster structure. This can be compounded by variability with repetition of the same algorithm; something that is more likely for datasets without a clear underlying cluster-structure. The iterative pathway will form clusters that are dependent upon the position of the first partition. Repetition of a cluster analysis at multiple random starting points is therefore recommended to validate stability of a model (215).

Missing data

The absence of data that is required to quantify similarity of a case with other members of the population excludes it from the analysis. Missing information is commonplace in population datasets, even when these are developed as part of well organised research studies. Polythetic clustering is particularly vulnerable to this problem. Techniques exist that adjust for missing data in a manner that maximises the likelihood of appropriate case allocation. However, greater reliability with these methods is achieved when there is a low volume of missing data that is missing at random (216).

Uses of cluster analysis

As discussed, the biological application of cluster analysis is most often as a technique for identifying homogeneous subgroups within a heterogeneous population. This informs taxonomic structure that can be used to direct mechanistic study. In psychiatry, cluster analysis has played a useful role for both informing and validating aspects of disease nomenclature (217;218). The technique has also been applied to risk stratify subgroups within a heterogeneous population of attempted suicide cases (219). Conceptually, the identification of risk groups rather than risk factors in this way may be a better approach for studying complex and multifactorial outcomes.

In polythetic clustering, groups are representative of distinct patterns of expression for the variables used to perform the analysis. Applying this principle to asthma, the approach may identify distinct patterns of expression between the multiple dimensions of the asthma phenotype that are of mechanistic importance (220).

3.11 Conclusions

Multivariate techniques have a growing application in the applied sciences. However, the idea that these numerical approaches yield results that are objective and free of *apriori* bias should not be considered dogma. Applying multivariate techniques to biological disciplines relies appropriately on subject specific expertise for deciding the factors included in analysis and the interpretation of the results obtained. Both these aspects have a significant subjective weighting. In an exploratory context, multivariate techniques are perhaps better considered as quantitative approaches to a qualitative solution. From a methodological perspective, it is clear that neither technique uses an accepted standardised approach. Far from being 'black-box' techniques, there is considerable opportunity for customising the analysis to meet study and subject specific aims. However, different methods can lead to significantly different outputs. Until a validated and standardised methodology is available, conclusions derived from the outcomes of such studies require cautious interpretation. Variability is also compounded by differences in the composition of populations sampled. Results from a single study site therefore require replication at other centres for validation.

Despite these limitations, factor and cluster analysis offer a novel approach for tackling the complexity of asthma heterogeneity. The two groups of methods provide complementary information and may be used in tandem: factor analysis can be used to define the distinct domains or dimensions that make up the asthma phenotype and cluster analysis may be used to study distinct patterns of domain co-expression in the asthma population.

3.12 Figures and Tables

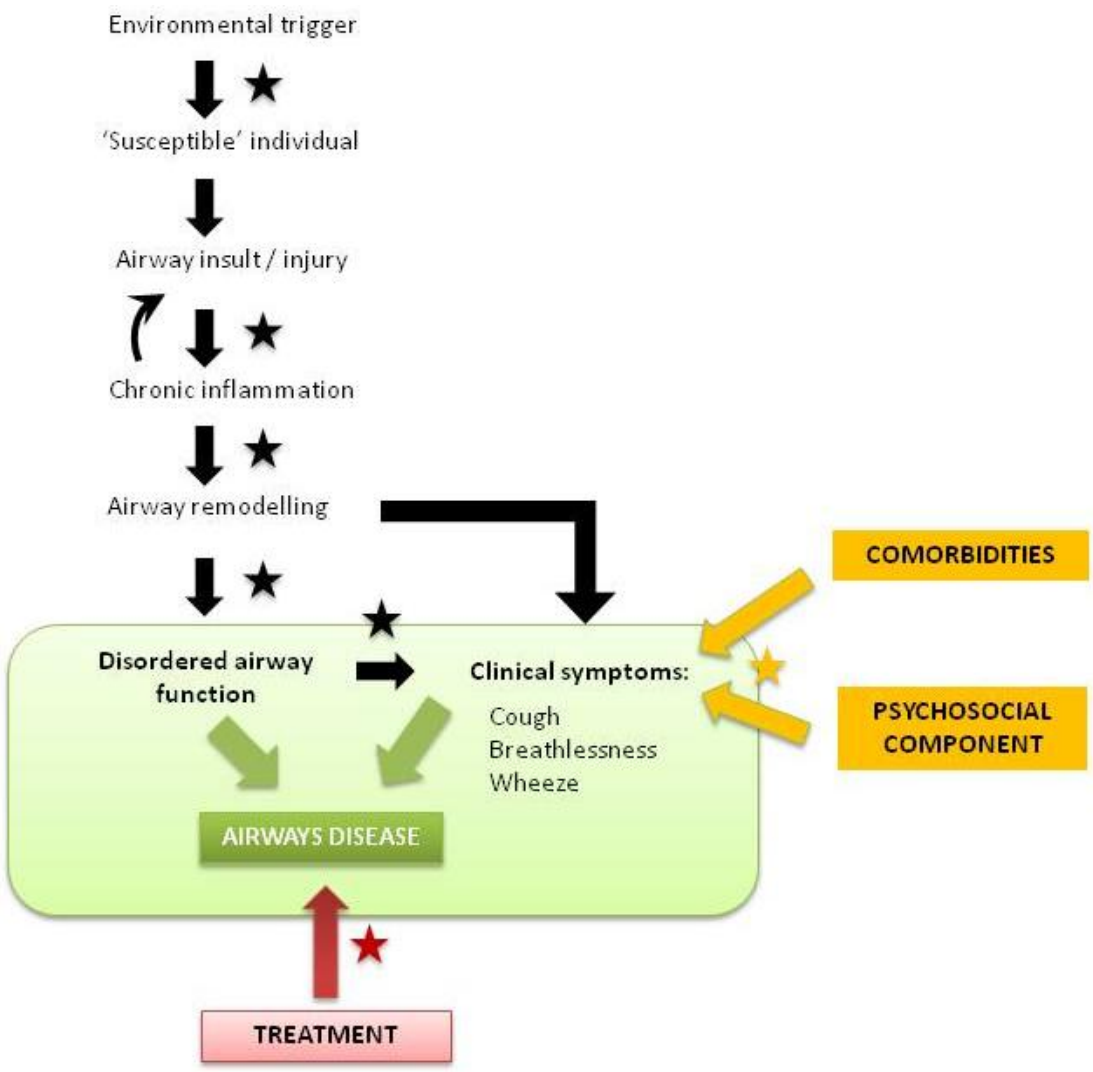


Figure 4: A common pathway to chronic airways disease and sources of heterogeneity

A shared sequence of events governs the manifestation of all chronic airways diseases. Differences between the disorders are likely to originate from the differing effects of environmental triggers in susceptible individuals.

Pathological heterogeneity (★) is a function of the spectrum of possible expression at each proposed step of disease pathogenesis. Clinical heterogeneity originates from pathological heterogeneity and is modified by a spectrum of responses to treatment, (★) together with the confounding effect of co morbidities and psychosocial factors. (★)

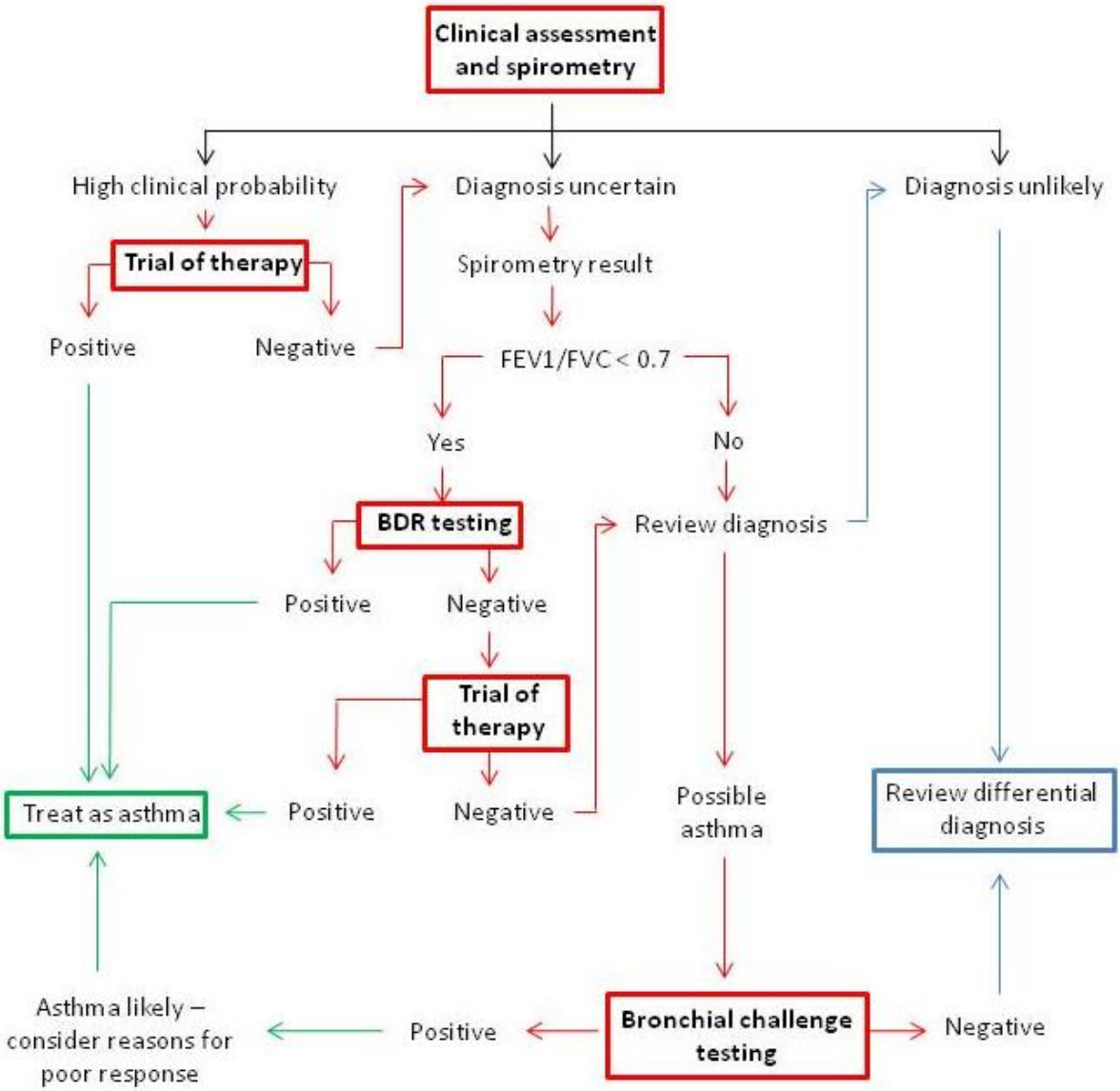


Figure 5: Algorithm of asthma diagnosis

Adapted from the BTS/SIGN guidelines 2008 (6).

The schema illustrates a stepwise approach to the diagnosis of asthma. A number of different criteria may be applied for establishing a diagnosis of asthma. However it is not known whether the different criteria are equivalent.

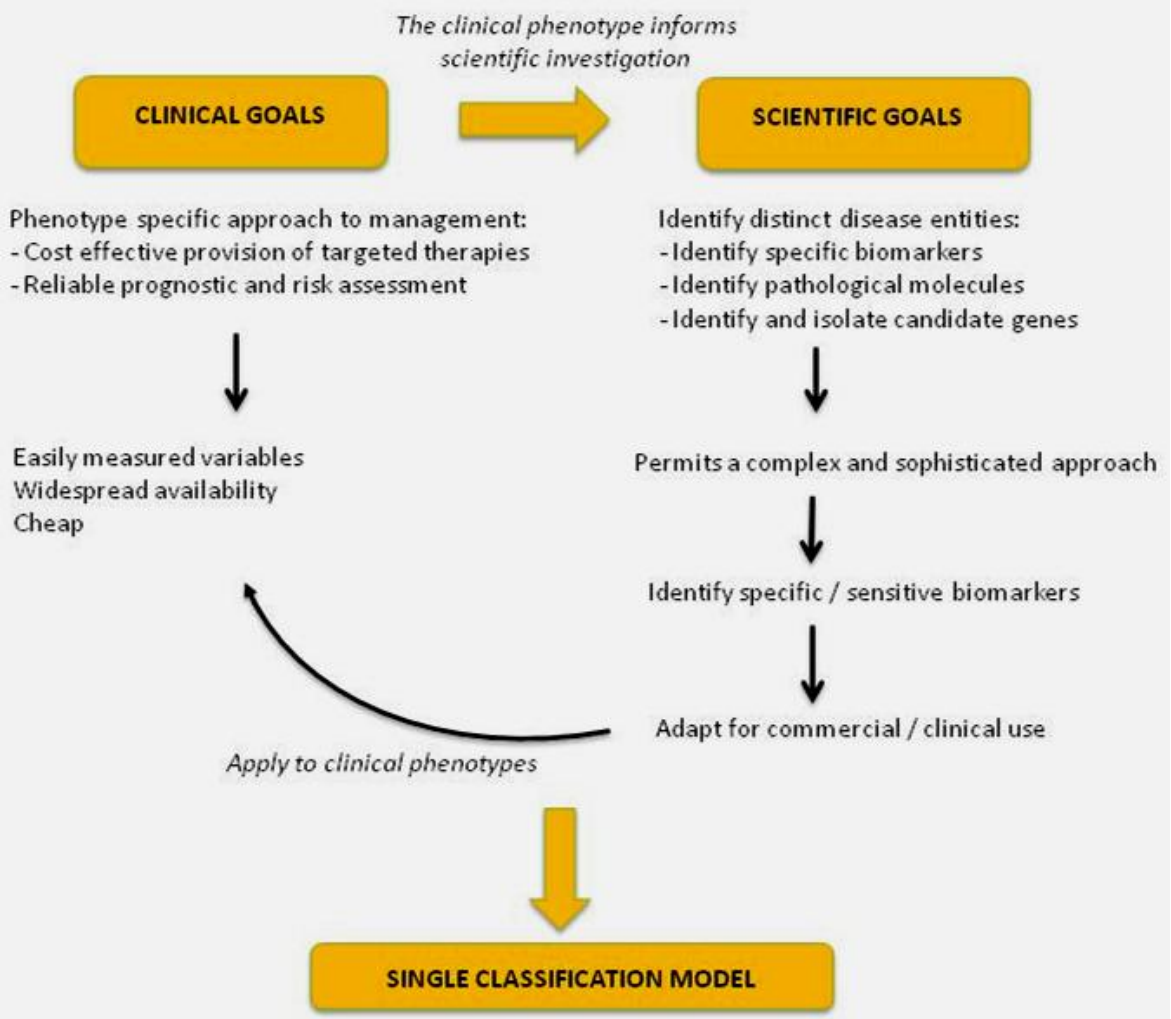


Figure 6: Developing a unified model of classification

Classification is a dynamic process. The schema illustrates differences in priorities and requirements for the development of classification models of clinical and scientific interest. Such models may develop in parallel to inform the other. Over several iterations, convergence to a single model may be achieved.

Table 7: Reported classification models of asthma

Characteristic measured	Author	Asthma phenotypes	Clinical utility			Scientific utility			Stable with time and therapy
			Informs therapy	*Informs risk and prognosis*	*Informs specific management needs*	*Informs pathology*	*Yields biomarkers for clinical application*	*Associated with specific genetic polymorphisms*	
Trigger based	Rackemann F 1927 (1)	Extrinsic / intrinsic asthma	++	+	++	+	+	UK	+
	Burge PS 1992 (2)	Occupational asthma	±	+	++	+	+	UK	±*
	Samter M 1968 (3)	Aspirin sensitive	+	+	++	+	-	UK	+
Variable airflow obstruction (Peak flow)	Ayres J 1998 (4)	Type 1 & 2 brittle asthma	+	+	+	-	+	UK	-
	Turner-Warwick M 1977 (5)	Brittle / morning dipper / irreversible / drifter	+	+	-	-	+	UK	-
Airway pathology	Pavord I 1999 (6)	Eosinophilic / non-eosinophilic	++	+	+	++	++	UK	-
Treatment requirement	Global Initiative for Asthma 1995, American Thoracic Society 2000 (7)	Refractory (severe asthma / difficult asthma	NA	++	+	±	-	UK	±
Asthma outcome	Wenzel S 2006 (8)	Exacerbation prone	-	++	+	-	-	UK	-
	Boulet L 1995 (9)	Fixed airflow obstruction	-	+	-	+	-	+	+

Legend Table 7

UK= unknown

* Occupational asthma is a stable phenotype in that a defined trigger predictably causes and aggravates asthma in a susceptible individual; however, early avoidance of the occupational agent may prevent chronic asthma from developing at all.

1 Rackemann FM. J Lab Clin Med 1927; 12:1185-1197.
2 Burge PS. Br Med Bull 1992; 48(1):221-230.
3 Samter M, Beers RF, Jr. Ann Intern Med 1968; 68(5):975-983.
4 Ayres JG, Miles JF, Barnes PJ. Thorax 1998; 53(4):315-321.
5 Turner-Warwick M. Br J Dis Chest 1977; 71(2):73-86.
6 Pavord ID, Brightling CE, Woltmann G, Wardlaw AJ. Lancet 1999; 353(9171):2213-2214.
7 ATS Refractory Asthma Workshop Committee. Am J Respir Crit Care Med 2000; 162:2341-2351.
8 Wenzel SE. Lancet 2006; 368(9537):804-813.
9 Boulet L, Belanger M, Carrier G. Am J Respir Crit Care Med 1995; 152(3):865-871.

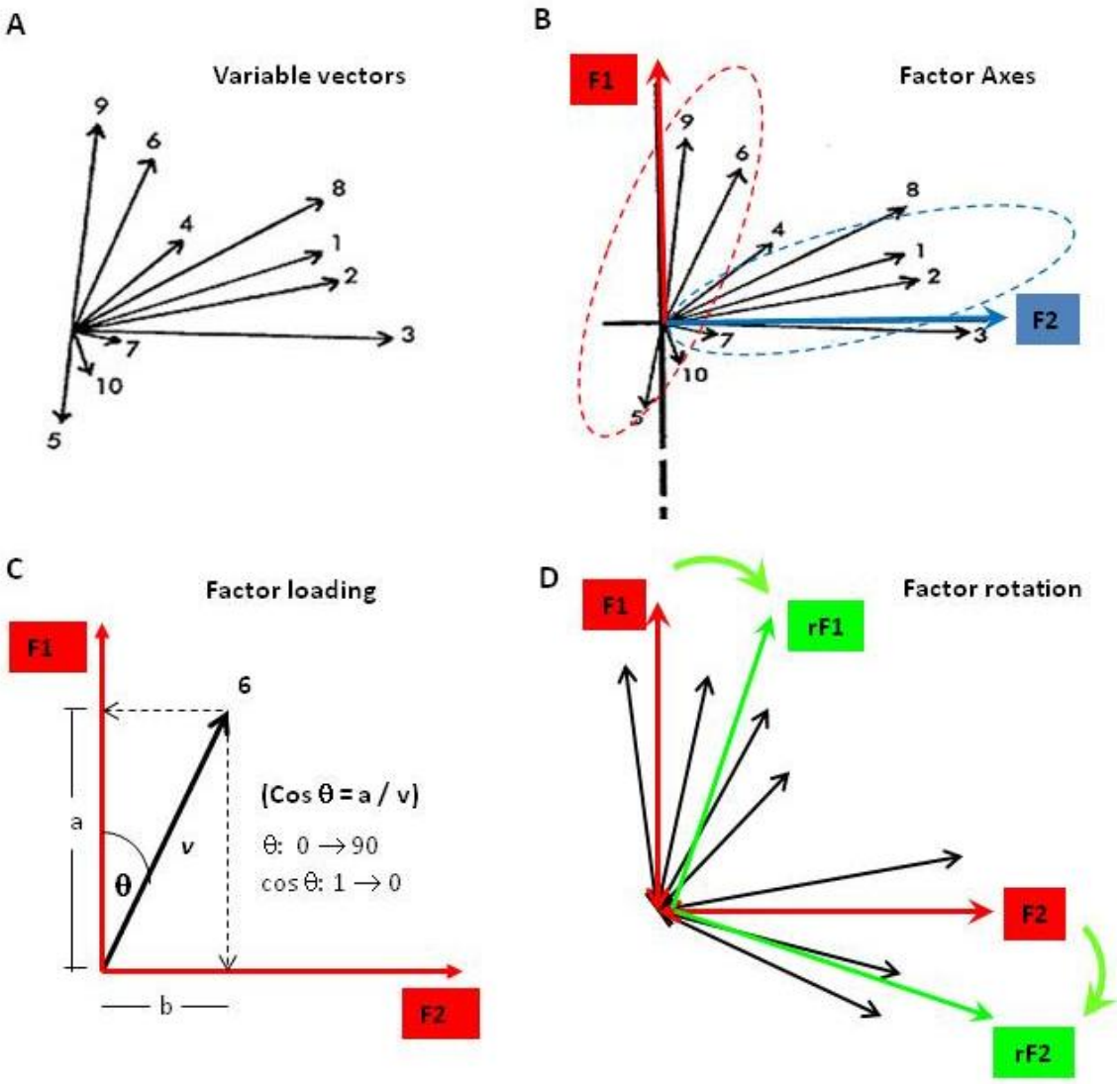


Figure 7: Aspects of factor analysis

A: Measured variables within a dataset are represented in space as vectors from a common origin. The direction taken by each vector depends on the relationship of that variable with all other variables included for analysis.

B: Factor axes (F1 and F2) are constructed sequentially to maximise capture of residual variance for plotted variables. As each factor accounts for variance not captured by earlier factors, it follows that the axes are mathematically independent from one another – in vectorial terms, they are perpendicular.

C: The loading of each variable vector onto a factor axis is expressed as the loading coefficient. This is mathematically the cosine of the angle between the vector and axis and is equivalent to the Pearson coefficient of correlation.

D: Rotation of plotted factor axes (rF1 and rF2) maximises the loading of clusters of variables. For orthogonal rotation, the perpendicular relationship between factor axes is retained.

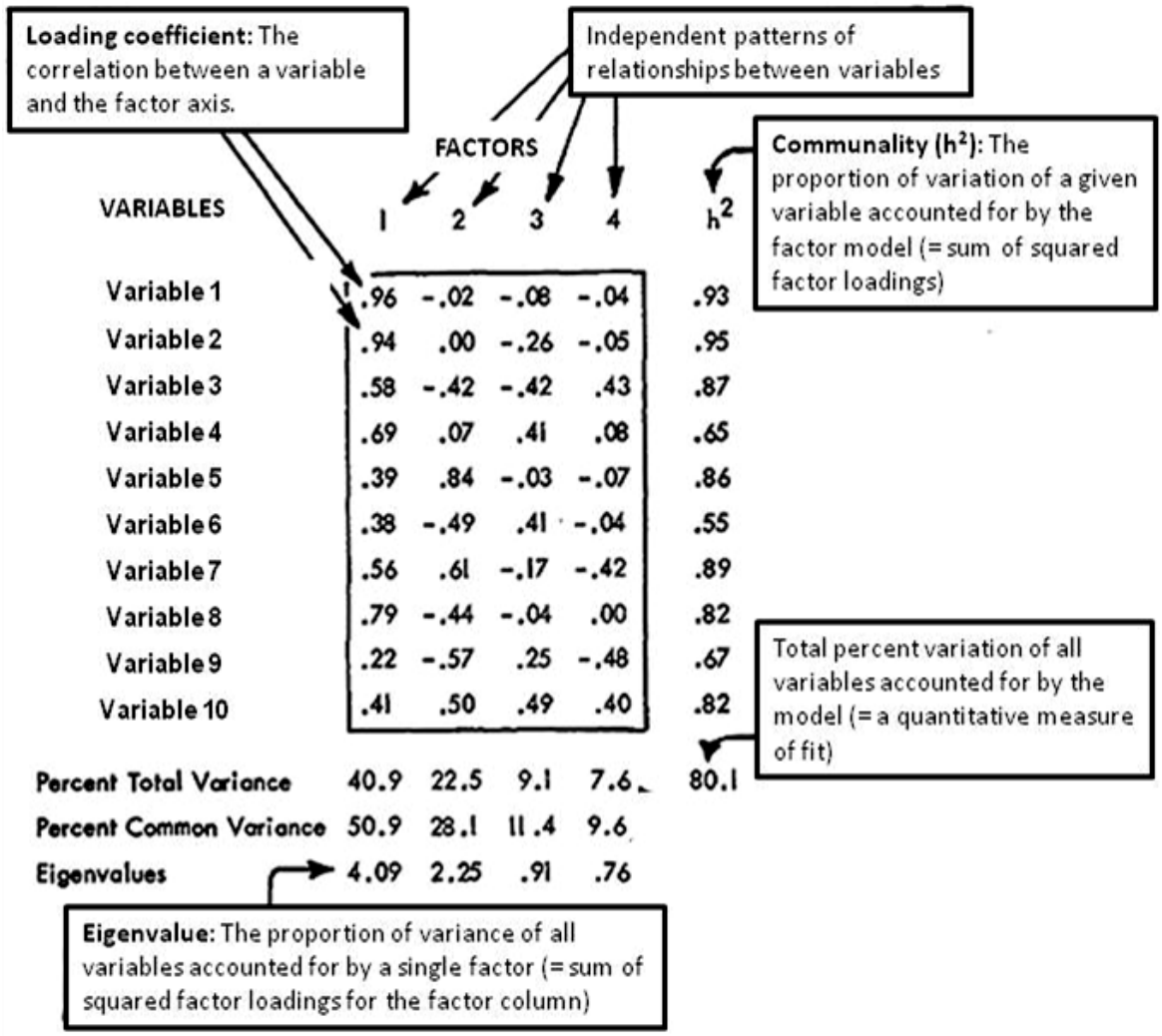
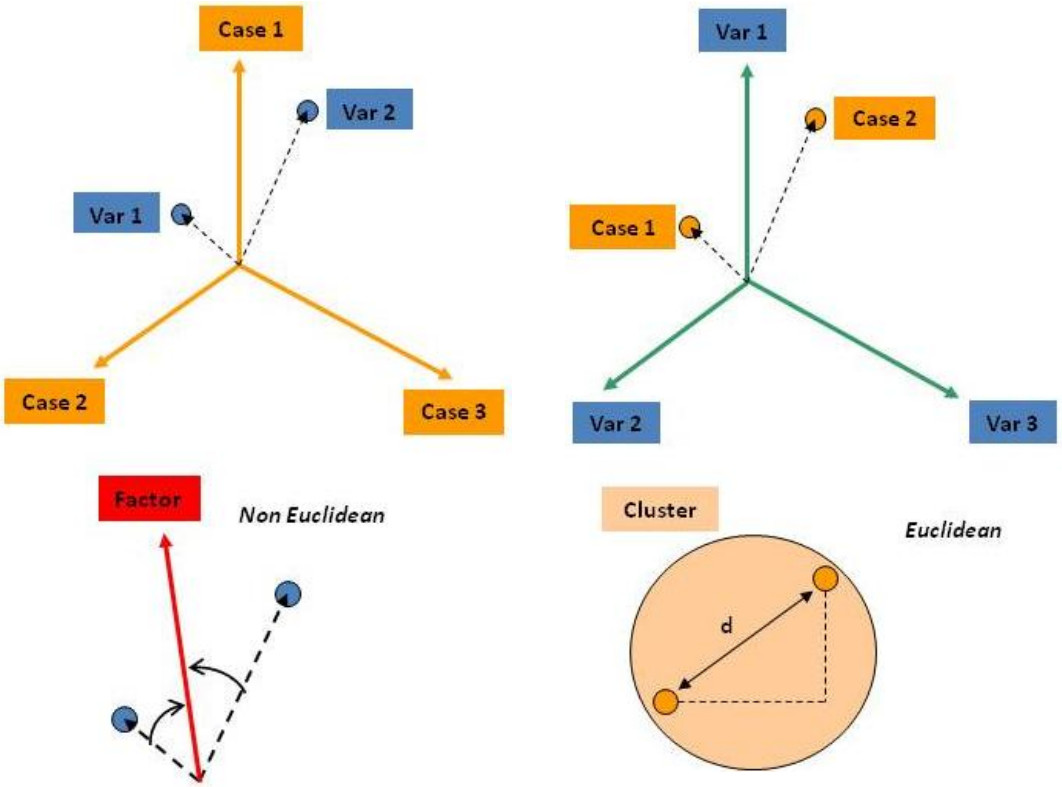


Figure 8: Elements of a factor analysis output

Adapted from Rummel R.J. 2002 (221)

Table 8: Comparison of factor and cluster analysis

	Factor Analysis	Cluster analysis
Plotting data for reduction	The population sum for each variable is calculated and plotted as a vector in space	The geometrical position of each datapoint is computed from the vectorial sum of the clustering variables
Measuring similarity	Angular relationship between constructed vectors	Geometrical distance in space between plotted datapoints
Grouping	Defined according to the angular relationship between vectors and constructed factor axes	*Hierarchical methods:* Either group together or divide preformed groups according to threshold distances between pairs of datapoints [Fig 9] *Non-hierarchical methods:* Construct a prespecified number of cluster centres. Clusters are defined according to the geometrical distance between datapoints and cluster centres [Fig 10]
Outcome	Useful for characterising relationships between variables within a dataset	Useful for grouping cases within a dataset on the basis of shared similarity for chosen variables



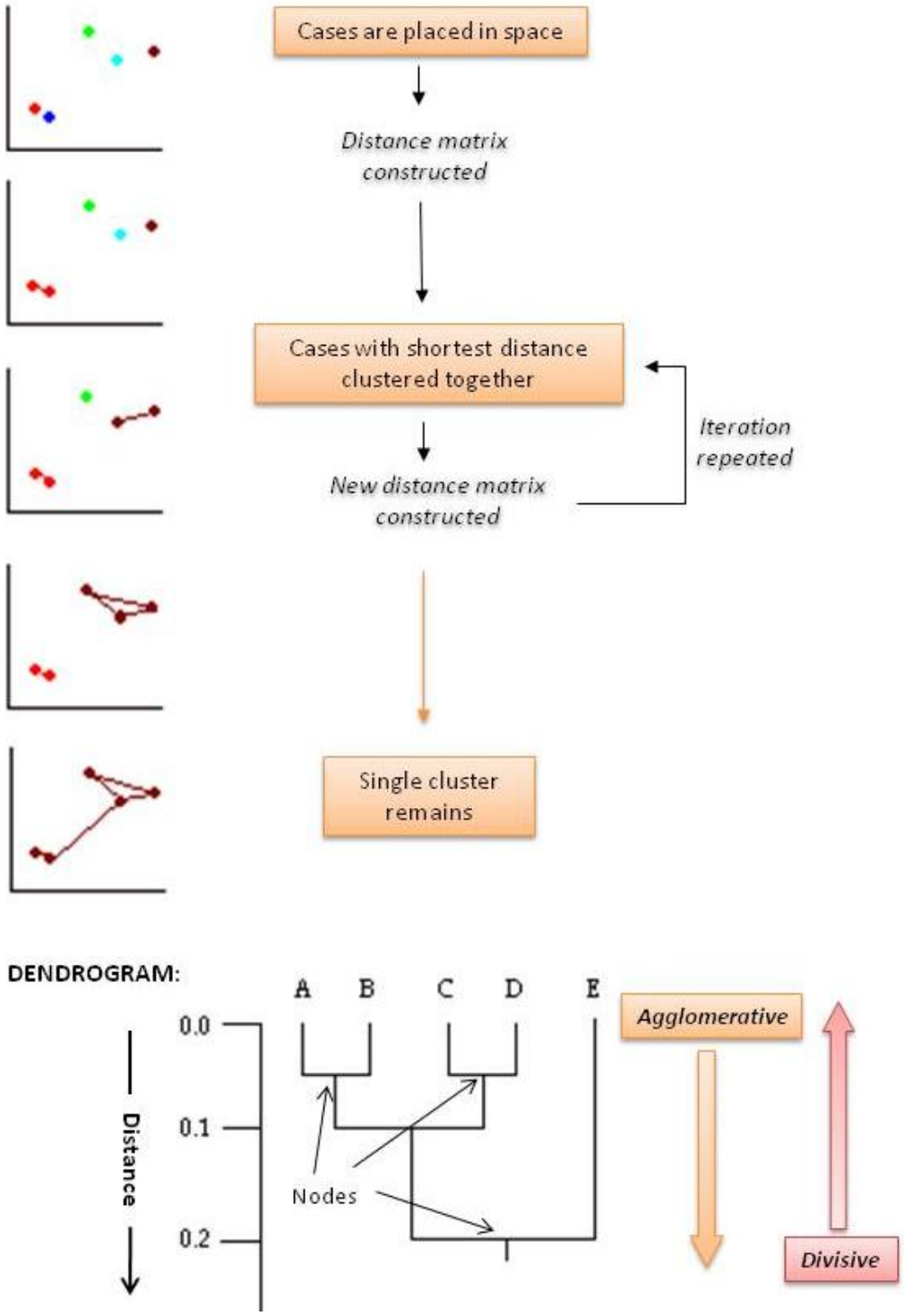


Figure 9: Hierarchical clustering methodology

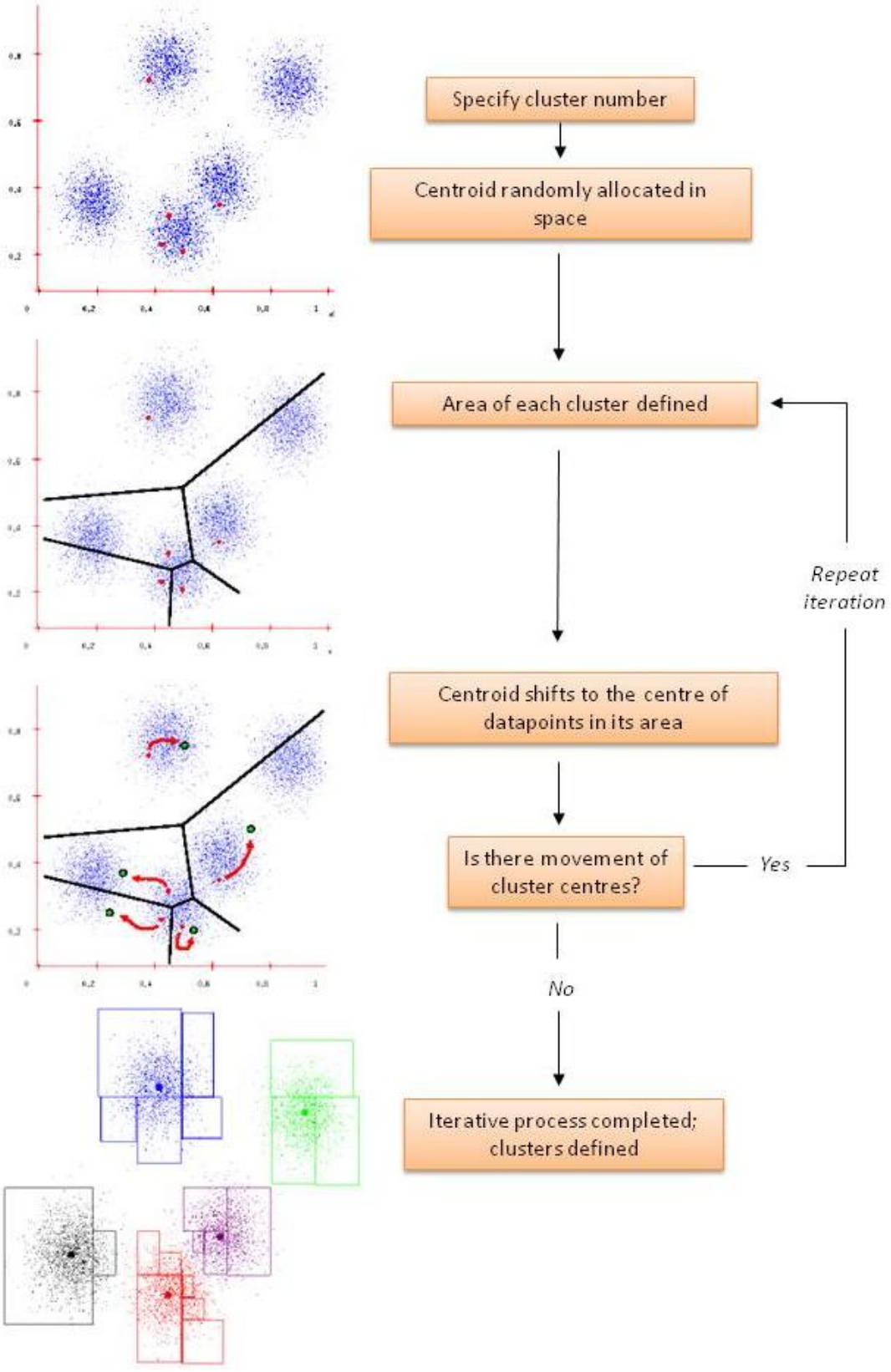


Figure 10: Non-hierarchical clustering methodology

Table 9: Overview of different mathematical algorithms commonly applied in cluster analysis

Measuring distance (x,y)		
Method	**Mathematical expression**	**Comments**
Euclidean distance	$\{\Sigma_i(x_i-y_i)^2\}^{1/2}$	Distance between two objects is not affected by addition of new objects Scale dependent
Squared Euclidean	$\Sigma_i(x_i-y_i)^2$	As above Increasing weight given as distance between objects increases
City-block (Manhattan) distance	$\Sigma_i\|x_i-y_i\|$	Sum of average distance across dimensions The distance measure is broadly similar to Euclidean distance, with dampening of the effect of outliers (distances not squared)
Chebychev distance	$\text{Max}\|x_i-y_i\|$	Describes the relationship between two objects on the basis of the largest distance between them for any one dimension. Objects grouping together must necessarily be similar across all specified dimensions.

Grouping algorithms - distance criteria for determining linkage			
Method	**Alternative name**	**Description**	**Comments**
Single linkage	Nearest neighbour	Minimum distance between two objects from neighbouring clusters.	Unbalanced and chain like clusters
Complete linkage	Furthest neighbour	Distance between the two furthest objects of neighbouring clusters.	Forms compact clusters that tend to be of similar size. Not appropriate if underlying data structure is less uniform.
Average linkage	PGMA	Average distance between all object pairs between neighbouring clusters	Produces compact and regular clusters but performs better when the underlying data is not uniform. A weighted form of the algorithm may be applied that corrects for differences in cluster size
Centroid linkage	UPGMC	Distance between the centre of neighbouring clusters	Assumes points can be measured in Euclidean space.
Median linkage	WPGMC	Weighted form of centroid linkage, taking into account differences in cluster size	Position of new cluster after linkage is placed in a position intermediate between the forming clusters, dependent upon their respective sizes.
Wards method	Minimum sum of squares	An analysis of variance approach, with the aim of minimizing the sum of sqaures after fusion of two clusters.	This approach is regarded to be very efficient but is susceptible to outliers and creates small spherical clusters that may not be appropriate

Adapted from Everitt B *et al* 2001 (211)
Abbreviations: PGM = Pair group method; C = Centroid; A = Arithmetic average; W = Weighted; U = Unweighted
Example: PGMA = Pair group method (using) arithmetic average

4. HYPOTHESES

- I hypothesise that the multivariate statistical techniques of principal components analysis and cluster analysis can be used characterise asthma heterogeneity and lead to the formulation of clinically meaningful phenotypes of asthma.

- I hypothesise that the application of the multivariate techniques independently to populations of refractory and mild to moderate asthma will reveal important differences in phenotypic characteristics between the groups that will inform further the basis for refractory asthma.

- I hypothesise that asthma phenotypes characterised by discordance between the expression of symptoms and eosinophilic airway inflammation will benefit from a management algorithm titrating glucocorticoid therapy to maintain a normal sputum eosinophil count.

- I hypothesise that eosinophils are important effector cells in the pathogenesis of severe asthma exacerbations and treatment with mepolizumab, a specific inhibitor of eosinophilic inflammation will improve asthma control in patients with eosinophilic asthma and a history of recurrent severe exacerbations by lowering the frequency of severe exacerbations.

5. METHODS

5.1 Multivariate statistical methods

5.1.1 Data extraction and utilisation

The data used for analysis in the factor and cluster analyses were derived from 4 separate patient datasets:

1. GPIAG and Leicester Asthma and Dysfunctional breathing (GLAD) study (N=70) [NCT00515840]
2. Intensive Asthma Study (N=114) [ISRCTN 08067387] (222)
3. Clinical database of patients attending the Glenfield Hospital Difficult Asthma Clinic (N=187)
4. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial (N=68) (23)

Datasets 1 and 2 comprised the asthma population managed in Primary Care.

Dataset 3 included only subjects with refractory asthma, defined in accordance with the criteria of the American Thoracic Society [Table 2](14).

Dataset 4 included subjects with both refractory and less severe asthma and comprised the population for assessment of longitudinal outcome in separate clusters.

All the datasets had a longitudinal design for serial data collection; only data obtained at the baseline visit was used. Cases with missing data that was needed to perform multivariate analysis were excluded.

5.1.2 Factor analysis

Principal components analysis with orthogonal varimax rotation was performed using 22 commonly measured clinical variables. A description of the measurement of relevant variables is described in the clinical methods [section 5.2]. An eigenvalue > 1 was chosen apriori as the criterion to determine the number of factors included in the

rotated solution. Variables with a low communality (<60%) were excluded as they did not contribute significantly to the factor model. As the primary purpose of this analysis was to identify variables that characterised statistically independent domains for further utilisation in cluster modelling (see below), variables that significantly cross-loaded between factors (loading coefficient >0.4) were also excluded from the model. 16 variables remained after exclusions and their patterns of loading in the final rotated solution are presented in table [Table 10]. Based on these patterns, we identified the factors as being representative of:

1. Symptoms
2. Atopy / Allergy
3. Eosinophilic inflammation
4. Psychological status
5. Variable airflow obstruction

Together, these factors define the structure of the clinical asthma phenotype. Additional variables not incorporated by this model but considered to be significant determinants of the asthma phenotype were included for cluster analysis. This is discussed further below.

Table 10: Orthogonal varimax rotation of 16 commonly measured clinical parameters for asthma assessment

Variable	Factors				
	1	2	3	4	5
PEF Variability					0.771
SPT Cat fur		0.923			
SPT Dog dander		0.889			
SPT D. Pteronyssinus		0.704			
SPT Grass Pollen		0.875			
Nocturnal Symptoms	0.821				
Daytime Symptoms	0.898				
Activity Symptoms	0.779				
Dyspnoea	0.871				
Wheeze	0.862				
Anxiety Score				0.914	
Depression Score	0.374			0.840	
Exhaled NO 50ml/sec			0.785		
Sputum Eosinophils			0.774		
Blood Eosinophils			0.778		
FEV 1 response to BD					0.751

Loading coefficients ≤0.3 have been omitted for clarity
The KMO measure of sampling adequacy was 0.776
The Bartlett test of sphericity had a significance of <0.0001
>75% of the total variance was explained by the factors

5.1.3 Cluster Analysis

Overview

Uniform cluster analysis methodology was applied to each population using a two step approach. 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 was used to define likely cluster boundaries (223). This estimate was pre-specified in a k-means cluster analysis that was used as the principal clustering technique (224). The k-means cluster analysis was repeated with one more or less pre-specified clusters and the most representative model was selected in each population. To ensure repeatability and stability within each model, the k-means algorithm was repeated several times in each dataset at random starting points using an alternative statistical software package (STATA) and also repeated within subpopulations of each dataset.

Selecting variables for cluster modelling

Using principal components analysis, authors have previously identified a consistent factor structure for clinical variables commonly measured in clinical practice to characterise airways diseases (142;210) . When considering variable selection for the cluster analysis, our aims were:

1. To choose variables that were measured in clinical practice and contributed to the clinical evaluation of asthma
2. To select only variables that were considered important in defining the disease phenotype rather than being a product of the disease process
3. To avoid choosing a number of different variables that were representative of the same aspect of the disease as this would introduce further bias when the cluster analysis was performed

As described, the results of principal components analysis for our datasets identified 5 independent domains from which to select variables for input to the cluster model. To avoid weighting the analysis, we selected only one parameter that was representative

of each factor. These were: atopic status (allergy domain); peak expiratory flow variability measured as amplitude percent mean of the lowest and highest readings over 2 weeks (variable airflow obstruction domain); induced sputum eosinophil count (airway inflammation domain); Modified JACQ score (symptoms domain). Psychological status was considered a consequence of the disease and therefore not included as an input parameter. However, performing the cluster analysis with the inclusion of the anxiety score (the parameter with the higher loading coefficient in the principal components analysis) did not alter the structure of the clusters (results not shown). Additionally, variables not loading significantly on the factor model but considered significant determinants of the asthma phenotype were also included. These were: gender (225), age of asthma onset (226) and body mass index (227). Methacholine PC20 was included for the primary care population only. This measure was only available in 10% of our refractory asthma patients, representing cases in which there was diagnostic doubt. The inclusion of only those patients with methacholine PC20 data would therefore likely have introduced selection bias. Post bronchodilator FEV1 and the frequency of asthma exacerbations were considered a consequence of the disease and therefore omitted as *input* characteristics.

5.2 Clinical Methods

5.2.1 Allergen Skin Testing

Atopic status was defined according to results of skin prick testing with 4 common UK aerollergens: cat fur; dog dander; grass pollen; and *Dermatophagoides pteronyssinus.* A positive response, defined as a weal ≥ 2mm larger than negative control (saline) for one or more allergen constituted atopy. All subjects were instructed to withhold therapy with anti-histamines for at least 3 days prior to testing.

5.2.2 Fractional exhaled nitric oxide (FeNO)

FeNO was measured online at a flow rate of 50 ml/sec (Niox chemiluminescence analyser, Aerocrine, Sweden) as the best of two readings as previously described (228). In accordance with European Respiratory Society recommendations, the test was performed prior to any other and before administration of any inhaled medication on the day. Subjects were instructed to refrain from taking long acting beta agonist or antihistamine medication for 48 hours prior to the test.

5.2.3 Spirometry

Spirometry was performed using a rolling seal spirometer (Vitalograph, Buckinghamshire, UK) as the best of three blows within 100 mls. Reversibility was assessed twenty minutes after inhalation of 200µg albuterol.

5.2.4 Methacholine challenge testing

Airway hyperresponsiveness was measured as the concentration of methacholine required to cause a 20% fall in FEV_1 (PC_{20}MCh), using the tidal breathing method, as previously described (229). For safety reasons, testing was reserved for subjects that were stable and with FEV_1 > 1 litre. Subjects inhaled saline followed by doubling doses of methacholine 0.03 -16 mg/ml via a Wright's nebuliser with an output of 0.13 ml/min. Subjects were instructed to continue breathing with a tidal pattern during inhalation of the solution for 2 minutes. A nose clip was applied to ensure respiration

was entirely via the oral route. FEV_1 was measured at 30 seconds and 60 seconds after each dose of methacholine. Spirometry was repeated at 3 minutes if the FEV_1 at 90 seconds was lower than the reading at 30 seconds. The lowest FEV_1 after each dose of methacholine was used to calculate the % drop from baseline. The procedure was terminated if the drop exceeded 20% or the highest dose of methacholine was achieved. PC_{20}MCh was calculated by linear interpolation of the log-dose response curve. If FEV_1 dropped >20% after inhalation of saline, PC_{20}MCh was assigned a value of 0.015.

5.2.5 Sputum Induction

Sputum induction was performed as previously described (107). Subjects were pre-treated with inhaled albuterol 200 mcg, 10-30 minutes before the procedure to minimise bronchoconstriction and post bronchodilator FEV_1 was measured prior to starting the induction. Subjects inhaled increasing concentrations of saline (3, 4 and 5%) in sequence for 5 minutes via an ultrasonic nebuliser (Medix, Harlow, UK; output 0.9 ml/min). Tidal breathing was employed with application of a nose clip. After each period of inhalation, the subject blew their nose and rinsed their mouth to avoid nasal and oral contamination respectively prior to expectoration into a sterile pot. FEV_1 was measured after each inhalation. The procedure was stopped if the FEV_1 fell by > 20%; if the fall in FEV_1 was 10-20%, induction was repeated at the same concentration of saline.

5.2.6 Quantitative asthma symptom scores

Asthma symptoms are a measure of clinical asthma control. Symptoms were quantified using two methods: 1. The Juniper Asthma Control Questionnaire (JACQ) and 2. A visual analogue scale (VAS) for symptoms of cough, breathlessness and wheeze. These are briefly described.

JACQ

This is a well known and validated 7-point questionnaire that includes 6 questions about different aspects of symptom control; the seventh field measures airflow

obstruction, expressed as % predicted FEV_1 (230). Each question provides a severity scale of 0-6 points for the subject to describe their control for the preceding 2 weeks. Scores for the seven fields are added and the total divided by seven to give the overall score. A score of ≥1.57 indicates suboptimal control of asthma symptoms; a change in the JACQ score of 0.5 points between visits is clinically significant (230).

A modified form of the JACQ was used in both the studies of this thesis. This included responses to the 5 questions about symptoms and excluded the fields examining frequency of short acting beta agonist use and lung function. The modified JACQ score was obtained by dividing the total score by 5. This is a validated form of the JACQ (231) that enables quantification of symptoms alone and excludes the confounding effects of behaviour (frequency of short acting beta agonist use) and airflow obstruction.

Visual Analogue Scale

The visual analogue score has been developed and validated to examine the perception of dyspnoea by patients with airways disease on a linear scale of 100 mm that may be presented either horizontally or vertically (232). There is near perfect correlation between the two designs (233). A horizontal scale of 100 mm was presented to subjects for each of the symptoms of cough, breathlessness and wheeze (23). Subjects were asked to plot their perceived control for each symptom over the preceding 2-weeks on the scale, with 0 mm representing no symptoms and 100 mm representing worst ever symptoms. Analyses were performed for each symptom independently and for a composite score that was the arithmetic mean of scores for all three symptoms.

5.2.7 Asthma Control Score

Based on the validated horizontal scales described above, a horizontal 100 mm scale was constructed for subjects to respond to the question *'How do you feel your asthma has been during the study, compared to how you remember it to have been before entering the study?'*

The scale was bipolar and is presented below [Fig 11]:

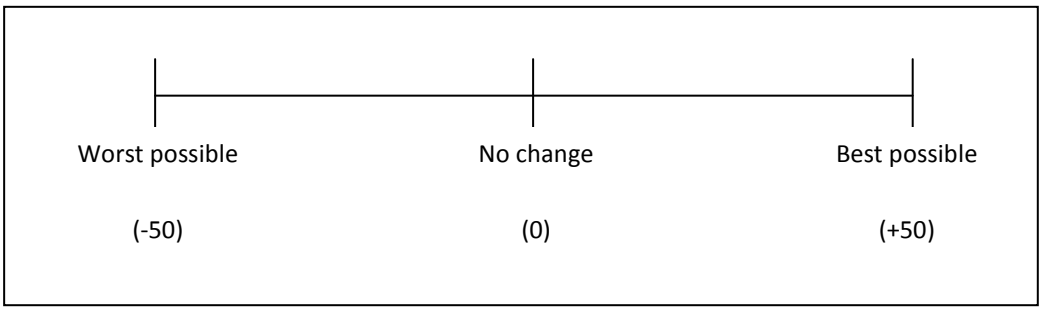


Figure 11: Asthma control score

The asthma control score was completed by subjects at the final study visit in the clinical trial with mepolizumab.

5.2.8 Visual analogue scale for Nasal Polyps

This is a validated (234) 100 mm horizontal visual scale that measures symptoms associated with nasal polyps across 5 domains: sense of smell, nasal secretion, pressure over sinuses, nasal obstruction, and headache. Subjects with a history of nasal polyps, defined as a history of previous polypectomy or a positive diagnosis at nasendoscopy, were asked to score the severity of their polyp associated symptoms for each of the 5 domains. Composite scores were calculated as the arithmetic mean of scores for all domains.

5.2.9 Juniper Asthma Quality of Life Questionnaire (AQLQ)

The Juniper asthma quality of life questionnaire is a validated scoring system for quantifying asthma related quality of life (235). It comprises 32 items that are each scored between 1 and 7. Higher scores indicate better quality of life. Scores for the 32 items reduce to being representative of 4 domains: symptoms (12 items); activities (11 items); emotion (5 items); and environment (4 items). A score is calculated for each domain as the arithmetic mean of item scores for that domain and a composite score is calculated from the arithmetic mean of the domain scores. We used a standardised version of the AQLQ that pre-specifies the 5 activities upon which subjects respond (236).

5.3 Bronchoscopic methods

All bronchoscopies were performed by blinded senior clinicians, in accordance with published guidelines (237). Subjects were pre-treated with nebulised salbutamol 2.5 mg. During bronchoscopy, subjects had a bronchial wash with 20 ml isotonic saline; six endobronchial biopsies were collected from middle and lower lobe carinae and if tolerated, bronchoalveolar lavage was performed using warmed isotonic saline, administered as three sequential 60 ml boluses into the right middle lobe. The bronchial wash and lavage fluid were processed as previously described (130). Briefly, fluid was filtered through 48μm gauze and diluted to a concentration of $0.5x10^6$ cells ml^{-1} for cytospin preparation. Cytospins stained with Romanowski stain were counted by a blinded individual and cell counts were expressed as a percentage of at least 400 inflammatory cells. Biopsy specimens were fixed in acetone containing the protease inhibitor phenylmethansulfanyl fluoride (PMSF) and embedded in glycol methacrylate, after storage for 24 hours at -20°C (238). Immunostaining was performed for major basic protein (MBP) and measurements were made by two blinded individuals independently for the number of MBP^+ cells/mm^2 in submucosa and thickness of the subepithelial layer, recorded as the mean of fifty measurements over a distance of at least 1mm (239).

5.4 Radiological methods

Helical thin section computed tomography (CT) scan has been used to assess airway remodelling in patients with asthma (240). Subjects were administered a dose of long acting β_2-agonist within 3 hours of the CT being undertaken. The scan was performed at full inspiration and limited from the aortic arch to the carina, to capture the right upper lobe apical segmental bronchus (RB1). All scans were obtained using the Siemens Sensation 16 mutislice scanner at 0.75mm collimation, 120kV, 50mAs, pitch 1.1, scan length 53 mm and scan time of 2.85 s. Images were reconstructed at 0.75mm slice thickness using a 512x512 matrix and a very sharp reconstruction algorithm (B70-f). RB1 bronchus on the CT images from all subjects was identified and the airway wall cross sectional geometry was measured with a semi-automated program (Emphylyx-J

V 1.00.01; British Columbia University, Vancouver) using the full width half maximum (FWHM) technique. Wall area (WA), lumen area (LA), maximum airway diameter (Dmax) and minimum airway diameter (Dmin) were measured. WA and LA were corrected for size dependant error and oblique orientation as described below. The total area (TA) and percentage wall area (%WA) were derived from the LA and WA (TA = LA + WA; %WA = WA/TA x 100). All airway dimensions were corrected for body surface area.

We designed an airway phantom modelling the right upper lobe ASB (RB1) down to the 12^{th} generation airways to assess the accuracy and repeatability of manual and automated measures of cross-sectional airway geometry and to derive ways of predicting and minimising observer error. We derived correction equations by looking at the best parabolic planar 3 dimensional fit of the phantom tube measured wall area/luminal area, the maximum/minimum diameter of the airway luminal ratio (a marker of oblique orientation) and the true wall area/luminal area measured by stereomicroscopy to the nearest micron. For each tube 7 values of maximum/minimum ratio and corresponding geometry (wall area and luminal area) measured using the full width half maximum (FWHM) method were derived based upon reconstructing each phantom tube at 10º increments from 0º (perpendicular to the long axis of the tube) to 60º corresponding to a ratio of largest to smallest diameter of 1.0 to 2.0. The final correction equations were derived using all 63 measurements of the 9 phantom tubes. Correction equations were generated using a custom program (LeoStatistic, Version 14.5, www.leokrut.com). The correction equations derived from multivariate analysis using parabolic approximation were:

$$\text{True LA} = 20 - 0.014(\text{Measured LA} - 20)^2 + 3.7(\text{Dmax/Dmin} - 2.1)^2 \quad \mathbf{[r^2 = 0.85]}$$

$$\text{True WA} = 50 - 0.0073(\text{Measured WA} - 92)^2 + 7.5(\text{Dmax/Dmin} - 2.3)^2 \quad \mathbf{[r^2 = 0.80]}$$

6. STUDIES

6.1 STUDY 1: CLUSTER ANALYSIS AND CLINICAL ASTHMA PHENOTYPES

6.1.1 At a glance commentary

Scientific knowledge on the subject

Although several models of asthma classification have been proposed, a system defining the phenotypes of clinical asthma that incorporate the different aspects of the disease has not been developed.

What this study adds to the field

Cluster analysis techniques may be used to classify asthmatic patients into phenotypic groups that exhibit clinically relevant differences in outcome with a management strategy that utilises a measure of eosinophilic inflammation for titrating corticosteroid therapy.

6.1.2 Abstract

Rationale

Heterogeneity in asthma expression is multidimensional, including variability in clinical, physiological and pathological parameters. Classification requires consideration of these disparate domains in a unified model.

Objectives

To explore the application of a multivariate mathematical technique, k-means cluster analysis, for identifying distinct phenotypic groups.

Methods

We performed k-means cluster analysis in three independent asthma populations. Clusters of a population managed in primary care (n=184) with predominantly mild to

moderate disease, were compared with a refractory asthma population managed in secondary care (n=187). We then compared differences in asthma outcomes (exacerbation frequency and change in corticosteroid dose at 12 months) between clusters in a third population of 68 subjects with predominantly refractory asthma, clustered at entry into a randomised trial comparing a strategy of minimising eosinophilic inflammation (inflammation guided strategy) with standard care.

Results

Two clusters (early onset atopic and obese, non-eosinophilic) were common to both asthma populations. Two clusters characterised by marked discordance between symptom expression and eosinophilic airway inflammation (early onset symptom predominant and late onset inflammation predominant) were specific to refractory asthma. Inflammation guided management was superior for both discordant subgroups leading to a reduction in exacerbation frequency in the inflammation predominant cluster [3.53 (SD 1.18) vs 0.38 (SD 0.13) exacerbation/patient/year, p=0.002] and a dose reduction of inhaled corticosteroid in the symptom predominant cluster (mean difference 1829µg beclomethasone equivalent/ day (95% CI 307 – 3349 µg) p=0.02).

Conclusions

Cluster analysis offers a novel multidimensional approach for identifying asthma phenotypes that exhibit differences in clinical response to treatment algorithms.

6.1.3 Introduction

Asthma impacts significantly on the rising burden of chronic disease in developed countries. 5-10% of sufferers have refractory asthma that remains poorly controlled despite maximal inhaled therapy (241). Effective clinical care is complicated by heterogeneity in the physiological, pathological and molecular abnormalities associated with refractory asthma (124). Current descriptions of asthma phenotypes are limited by subjectivity and poor coherence. A robust system of classification that incorporates the multidimensionality of asthma is needed to identify subgroups with consistent patterns of disease (220;242). This may provide a framework for identifying distinct phenotypes, with specific pathophysiological abnormalities that predict response to particular therapies (19) and help focus current genetic and molecular studies.

The taxonomy of organisms remains the paradigm for biological models of classification. It is based empirically upon the principle that similarity measured across a number of different characteristics, predicts relationships of biological significance with greater probability. Cluster analysis refers to a group of multivariate mathematical algorithms that broadly perform two distinct functions: 1. Quantification of similarity between individuals within a population on the basis of the (multiple) specified variables; 2. Grouping of individuals into clusters such that similarity between members of the same clusters is strong and between different clusters is weak (211;243). The principal advantage of performing classification numerically is objectivity and methodology for including multiple variables that assume equal weighting, which helps minimise *a priori* bias. Numerical taxonomy or taximetrics is the branch of taxonomy that has developed to utilise mathematical algorithms such as cluster analysis for this purpose (201) and the principle has been extended for use in other areas of biomedical science, notably bioinformatics and psychiatry (208). In the latter, cluster analysis techniques have been used to identify patterns of symptom expression that have been used to define diagnostic categories (208).

We postulated that cluster analysis could be applied for classifying clinical phenotypes of asthma. We examined this hypothesis using the k-means clustering algorithm to

classify two distinct asthma populations: a group recruited from primary care with asthma of predominantly mild to moderate severity and a group from secondary care that met pre-specified criteria for refractory asthma (14). The clinical relevance of these clusters was evaluated further by investigating differences in asthma outcomes between clusters identified in a separate cohort of patients with predominantly refractory asthma that participated in a recently completed randomised study at our centre comparing a management strategy aimed at titrating steroid therapy to maintain a normal sputum eosinophil count, with a conventional clinical protocol (23). Some of the results of this study have been previously reported in the form of an abstract (244).

6.1.4 Methods

Subjects

We studied three discrete populations with asthma. All patients had a physician diagnosis of asthma and sufficient symptoms to warrant at least one prescription for asthma therapy in the previous 12 months. All patients were current non-smokers and ex-smokers had a less than 10 pack year smoking history. The 2 larger datasets comprised cross-sectional data for performing cluster analysis to identify the major disease patterns existing respectively within primary care and refractory asthma populations. Our first dataset was comprised of baseline data from patients with asthma (N=184) recruited from primary care practices for 2 prospective clinical studies at our centre: the GLAD Study (N=70) (ISRCTN 47153522) and the recently completed Intensive Asthma Study (N=114) (223). The studies shared common subject selection criteria and recruitment techniques.

Our second dataset (N=187) was comprised of data from patients with a diagnosis of refractory asthma, made in accordance with ATS criteria (14) by a respiratory physician with a specialist interest in this field. All the patients attended our specialist Glenfield Hospital refractory asthma clinic for assessment and management of their asthma. The analysis was performed on consecutive patients attending the clinic between 2004 and 2006 with a full complement of data collected as part of their routine baseline

assessment during their first visit to our centre. The systematic recording and validation of data for some aetiological factors such as nasal polyps, aspirin sensitivity and ethnicity is not routinely performed at our Centre. This data was therefore not available as part of the analysis. However, to be representative of the secondary care asthma population, we chose to include all patients meeting ATS criteria for refractory asthma. Thus, patients in whom non-adherence with therapy is likely to have been a major determinant were not excluded. This is in contrast to our third population (described below) who were recruited to a clinical trial in which suspected or documented therapy non-adherence was an exclusion criterion of the study.

The third dataset comprised baseline and longitudinal data collected from a prospective clinical study (23). The study compared severe exacerbation frequency over 12 months in 74 patients with predominantly refractory asthma managed according to regular monitoring of airway inflammation using induced sputum (sputum arm) with the aim of titrating steroid therapy to maintain normal eosinophil counts or standard clinical care (clinical arm). Sufficient baseline data was available in 68 of the 74 study participants to perform cluster analysis. 59 out of the 68 patients (86.7%) met ATS criteria for refractory asthma.

Cluster analysis methodology

Uniform cluster analysis methodology was applied to each population and described in the methods [section 5.1.3]. Discriminant function analysis was performed using both forward and backward stepwise algorithms on each cluster model to evaluate the input variables that were significant determinants of model structure. This is discussed in greater detail in the on line supplement.

Statistical methods

The between cluster comparison of baseline parameters that were not input parameters was performed using one way analysis of variance for parametric variables, the chi-squared test for proportions and Kruksal Wallis for non parametric variables. For the analysis of outcome data in the prospective study, our clustering algorithm was applied to the baseline study data and outcomes were compared

between study arms for each cluster using the independent t-test. Univariate analysis of variance with the cluster model as a covariate was performed to verify the significance of this as an independent factor for any observed differences in outcome (see on line supplement). The measured outcomes were pre-specified and included the frequency of severe exacerbations, measured as the number of rescue courses of oral corticosteroid and the change in corticosteroid dose at 12 months. All statistical analyses were performed using SPSS v.14. Additionally, STATA was used to perform repetitions of cluster models with the k-means algorithm for demonstrating repeatability.

Approval from the Local Research Ethics Committee was obtained for data analysis and publication following informed consent for the respective clinical studies and as part of a clinical database for patients attending the Glenfield Hospital Difficult Asthma Clinic.

6.1.5 Results

Compared with our secondary care, refractory asthma population the primary care population had milder disease with significantly fewer symptoms, less airway dysfunction and lower levels of eosinophilic airway inflammation, while taking a significantly lower mean dose of inhaled corticosteroids [Table 11].

The cluster structure described for each population was reproducible when repeating the algorithm using STATA and within randomly selected subsets of each population (data not shown). Statistical validity for the results was supported by identifying similar clusters of refractory asthma within the independent study cohort of Green et al.

A 3-cluster model best fit the primary care population dataset [Table 12], [Fig 12]. Cluster 1 described a subgroup with early onset, atopic asthma. This cluster had evidence of airway dysfunction, symptoms and eosinophilic airway inflammation. Clinically, this cohort was associated with a significantly greater number of previous hospital attendances and asthma exacerbations requiring oral corticosteroids when compared with the other primary care subgroups. Cluster 2 described an obese

subgroup with a female preponderance, evidence of asthma symptoms and an absence of eosinophilic airway inflammation. The third cluster was labelled benign asthma as cases within this subgroup had little evidence of active disease. Asthma symptoms, airway inflammation and measures of airway dysfunction were frequently within normal limits and 58% of this cohort did not have evidence of significant airway hyperresponsiveness at the time of assessment. Consistent with a milder disease profile, patients from this cluster had very low rates of hospital attendance for asthma and severe exacerbation frequency in the previous 12 months [Table 12].

We identified four clusters in the secondary care, refractory asthma population [Table 13], [Fig 12]. Clusters 1 and 2 had a profile that closely resembled the respective clusters in primary care. Thus, early onset atopic asthma and obese, non eosinophilic asthma were common to asthma populations across the spectrum of severity. The principal distinction between the clusters in each population was the difference in absolute values of different objective measures of disease severity. In comparison with primary care, early onset atopic asthma in secondary care exhibited greater airway dysfunction, symptoms and eosinophilic airway inflammation on a higher dose of corticosteroid therapy. However, the *pattern* of expression of these variables, demographic data and measures of asthma control were consistent between clusters of the two populations. The subpopulation of this phenotype with refractory asthma had a significantly higher rate of failed attendance of appointments in the 12 months after referral to the clinic compared with the other phenotypes of refractory asthma [Table 13].

Clusters 3 and 4 were specific to the refractory asthma population and both exhibited marked dissociation between eosinophilic inflammation and asthma symptoms. Cluster 3 described an early onset, symptom predominant group with minimal eosinophilic disease. Cluster 4 described an eosinophilic inflammation predominant group with few symptoms, late onset disease and a greater proportion of males.

Discriminant function modelling identified the majority of input parameters used in the cluster analysis of both populations to be significant determinants of cluster membership. The discriminant function model of primary care and refractory asthma

clusters required 7 of 8 input parameters (excluding atopic status) and 5 of 7 parameters (excluding atopic status and gender) respectively. The accuracy of the discriminant function models for predicting cluster membership were 94.6% (primary care) and 96.8% (refractory asthma).

Cluster analysis was performed from baseline data in 68 patients of the prospective study dataset. 3 clusters were identified; all were comparable with clusters observed in the larger refractory asthma population. The original study demonstrated a significant reduction in severe exacerbation frequency in the sputum arm with no significant difference in corticosteroid usage between the groups. The present cluster specific analysis revealed that all of the benefit for preventing exacerbations occurred in the inflammation predominant cohort [3.53 (SD 1.18) vs 0.38 (SD 0.13) exacerbation/patient/year, p=0.002] [Table 14]. Additionally, sputum guided therapy allowed successful down titration of corticosteroid therapy in early, symptom predominant asthma [mean difference 1829μg beclomethasone equivalent per day (95% CI 307 – 3349 μg); p=0.02], without compromising asthma control. A univariate analysis of variance with the cluster model as a covariate identified both treatment grouping and the cluster model as significant determinants for observed differences in exacerbation frequency (p=0.002 study groups; p=0.03 cluster model) but only the cluster model was a significant determinant for differences in inhaled corticosteroid dose (p=0.07 for treatment groups and p=0.005 for cluster model).

6.1.6 Discussion

The need for classifying asthma heterogeneity has gained urgency with the parallel development of better tools for measuring disease characteristics that highlight disparity in clinical, physiological and pathological markers, together with novel and specific molecular therapies that are only likely to be efficacious in particular subgroups of asthma. This study is the first to apply principles of cluster analysis for the identification of clinical asthma phenotypes. We have further shown that phenotypes constructed in this way exhibit clinically relevant differences in outcome with management strategies that utilise a measure of eosinophilic inflammation for titrating corticosteroid therapy.

Asthma classification is complicated by the multidimensional nature of the disease. This prompted our consideration of cluster analysis techniques for this purpose. We selected the k-means clustering algorithm as it maximises separation between clusters, thereby offering the greatest scope for identifying distinct groups within the population. Both familiar and previously uncharacterised asthma subgroups were identified that are more representative of multidimensionality. The identification of early onset atopic asthma, an established asthma phenotype, validates the method for identifying the other subgroups against an accepted reference (204). Discriminant function analysis demonstrated the majority of the clustering parameters to be significant for cluster modelling, supporting multidimensionality. Atopic status was not identified as a significant discriminator influencing cluster membership in either primary care or secondary care. However, the prevalence of atopy did differ significantly between clusters and its inclusion to describe the phenotypes is therefore appropriate.

We chose to consider the 2 asthma population datasets independently when performing cluster analysis. This enabled clearer identification of factors that are specifically associated with refractory asthma, a condition that is sufficiently disparate to be considered a distinct disease entity by several authors (245).

The early onset, atopic asthma phenotype was common to both asthma populations, differing only in the severity of disease expression. We identified significantly higher rates of non-attendance for clinic appointments in the refractory subgroup, which has been associated with poorer therapeutic compliance (246). Our finding of uncontrolled eosinophilic airway inflammation was in keeping with this. Our failure to identify the same phenotype in the recruited prospective study cohort may be because poor compliance was an exclusion criterion for the study. Although equivalent measures of compliance were not obtained in our primary care population, it may be an important factor distinguishing this phenotype between the two populations. Strategies for improving compliance may therefore have a greater role in the management of this subgroup of refractory asthma. The obese, non-eosinophilic phenotype common to both populations was characterised by symptoms that were not associated with eosinophilic airway inflammation. Given the recognised association between

eosinophilic airway inflammation and steroid responsiveness in airways disease (169), the reported steroid resistance of asthma in obese patients (192) may in part be explained by the general pattern of airway inflammation seen with this phenotype.

The traditional paradigm of a direct relationship between eosinophilic inflammation and symptoms underpins present therapeutic guidelines that recommend symptom led titration of corticosteroid therapy (6). Our analysis suggests a symptom led approach would be effective for mild to moderate asthma in primary care for patients with early onset atopic asthma and benign asthma, where concordance was observed between inflammation and symptoms. However, discordance between these domains is a prevalent characteristic of refractory asthma and is also a feature of the obese, symptom predominant, non-eosinophilic phenotype seen in primary care. This may be a significant factor predisposing to failure with a conventional protocol and supports a role for measuring eosinophilic airway inflammation in these subgroups. For symptom predominant phenotypes, the aetiology of symptoms is multifactorial and not closely related to underlying eosinophilic airway inflammation. Overtreatment with corticosteroids may therefore occur. In keeping with this, a recent study using exhaled nitric oxide (FeNO) as a measure of eosinophilic airway inflammation in asthma showed that FeNO guided management resulted in lower inhaled corticosteroid use without compromising asthma control (22). In contrast, the inflammation predominant phenotype will be undertreated, leading to uncontrolled eosinophilic inflammation that is associated with a greater risk of future severe asthma exacerbations (112). Our hypothesis is supported by the results of the longitudinal cluster specific analysis that demonstrated a 10 fold reduction in exacerbation frequency for this phenotype with a management strategy that measures eosinophilic airway inflammation to titrate therapy.

This study has several limitations. Our primary care cohort is unlikely to have been completely representative of this asthma population. Our data was gathered from subjects that had enrolled on two other studies and were therefore more likely to include subgroups that were agreeable and / or able to participate in a study. A selection bias is likely to have existed as 9% of people invited to participate were enrolled to the studies. In keeping with this, our sample had a higher than expected

mean age and subject proportion with late onset disease (Table 6.1.7). The generalisability of this group to the asthma population as a whole requires caution. However, our primary interest was to identify different phenotypes. In this context, an atypical sample was more likely to reveal less common phenotypes that exist in primary care. In contrast, our difficult asthma population was selected at random from our local clinic and is almost certainly a representative population of local disease phenotypes. Although we have used the k-means clustering algorithm, it is well recognised that populations of both disease and health have a continuous spectrum of expression. The use of an algorithm that separates the population into discrete clusters may not be realistic. Alternative clustering techniques that use a probabilistic approach for cluster structure and membership within a dataset may provide additional information and should be explored (213). Nevertheless, our analysis supports the hypothesis that subgroups of clinical relevance exist within asthma populations and can be revealed using cluster analysis. Despite our efforts to be objective, there were several areas of subjectivity including our selection of variables for clustering and deciding on the number of clusters for each population. Although our choice of clustering parameters was broad, we cannot exclude the possibility that other variables may be of greater significance in developing meaningful phenotypes. Additionally, the possible association between specific cluster profiles and well recognised aetiological factors such as nasal polyps and aspirin sensitivity could not be explored. An advantage of multivariate techniques is that no single variable should be critical for determining the model. One of the drawbacks of using a non-hierarchical clustering technique is the need to pre-specify the number of expected clusters. There are no well validated techniques for predicting the number of clusters within a given population. We estimated this from dendrogram plots obtained using the hierarchical Ward's method. The study also does not address the question of stability in cluster membership over time and with changes in treatment. Within each population, there was no significant difference in treatment regimens and doses between clusters. Thus differences observed between clusters may be considered a product of differences in the underlying disease profile together with differences in the response to therapy. These two factors are likely to be closely related. An additional confounder is the variability in treatment adherence. Poor adherence is an important driver of difficult

asthma and adherence levels at the time that measurements were performed should be factored into any consideration of responses to anti-inflammatory therapy that are likely to influence the phenotype. We identified poorer clinic attendance rates for members of the early onset atopic cluster of our refractory asthma population (Table 13). This is a useful surrogate of treatment adherence, although more direct and objective measures of adherence such as the proportion of collected prescriptions have been developed (247). Inclusion of such a variable in the cluster model may be informative. Although longitudinal change in cluster membership has not been explored, our analysis indicates cluster profiling at baseline is predictive of response to a management strategy prospectively for at least 12 months. It is also notable that four of the parameters we used for clustering (age of onset, gender, atopic status and BMI) are relatively invariant and not generally affected by time and therapy.

In summary, this study supports a role for, the utilisation of multivariate techniques in the classification of asthma populations. Clinically important prognostic differences identified between the phenotypes within this model may provide a reliable framework for exploratory molecular and genetic studies, presently undermined by population heterogeneity.

6.1.7 Figures and Tables

Table 11: Comparison of baseline characteristics in the 3 asthma populations

Variable	Primary Care N=184	Secondary Care N=187	Longitudinal Cohort N=68	P-value‡
Gender (% female)	54.4	65.8	47.1	0.082
Age (years) [S.D.]	49.2 [13.9]	43.4 [15.9]	52.4 [14.6]	<0.001
Age of Onset (years) [S.D.]	24.7 [19]	20.3 [18.4]	31.1 [23.7]	<0.001
Atopic Status (% positive)	72.8	73.8	57.4	0.365
Body Mass Index (kg/m^2) [S.D]	27.5 [5.4]	28.5 [6.5]	28.0 [5.9]	0.55
†PC_{20} Methacholine (mg/ml)	1.04 [1.13]	-†	0.67 [0.68]	0.19
Peak flow variability (amp % mean)	17 [0.38]	32.2 [0.48]	13.8 [0.29]	<0.001
% FEV_1 change with bronchodilator	1.63 [1.16]	12.8 [0.41]	3.2 [1.04]	<0.001
Post bronchodilator FEV_1 (% predicted)	91.4 [21]	82.1 [21.1]	80.2 [20.6]	0.013
Sputum eosinophil count (%)	1.32 [0.62]	2.9 [0.99]	2.4 [0.81]	0.08
FeNO* (ppb)	31.6 [0.33]	43 [0.32]	4.32 [0.64] *	<0.001
Sputum neutrophil count (%)	55.09 [0.31]	46.7 [0.32]	41.1 [0.35]	0.04
Modified JACS ^ [S.D.]	1.36 [0.74]	2.02 [1.16]	1.42 [1.26]	<0.001
Dose of Inhaled Corticosteroid (BDP equivalent / mcg) [S.D.]	632 [579]	1018 [539]	1821 [1239]	<0.001
Long Acting Bronchodilator use (%)	40.2	93	86.7	<0.001

‡ Significance figures are derived using one way analysis of variance between the three populations for continuous variables or chi squared test for proportions.

† Bronchial challenge testing is not routinely performed in secondary care for refractory asthma. The comparison given is between the primary care asthma population and the longitudinal study cohort.

***** FeNO was measured using the Niox analyser at 50ml/sec in the primary care population and secondary care population. The Logan analyser was used at a flow rate of 250 mls/sec in the longitudinal study cohort. A strong linear correlation of 0.97 exists between the two measurement protocols. The statistical comparison is between FeNO levels in primary and secondary care using Niox.

Table 12: Clusters in Primary Care

Variable	Primary Care N=184	Cluster 1 Early onset atopic asthma N=61	Cluster 2 Obese Non-eosinophilic N=27	Cluster 3 Benign Asthma N=96	Significance (p-value)‡
†Gender (% female)	54.4	45.9	81.5	52.1	0.006
Age (years) [S.D.]	49.2 [13.9]	44.5 [14.3]	53.9 [14]	50.8 [13]	0.003
†Age of Onset (years) [S.D.]	24.7 [19]	14.6 [15.4]	35.3 [19.6]	28.2 [18.3]	<0.001
†Atopic Status (% positive)	72.8	95.1	51.9	64.6	<0.001
†Body Mass Index (kg/m^2) [S.D]	27.5 [5.4]	26.1 [3.8]	36.2 [5.5]	26 [3.6]	<0.001
†PC_{20}* Methacholine (mg/ml) PC_{20} > 8 mg/ml (N) {%}	1.04 [1.13] 64 {34.7}	0.12 [0.86] 2 {3.3}	1.60 [0.93] 6 {22.2}	6.39 [0.75] 56 {58.3}	<0.001
†Peak flow variability * (amp % mean)	17 [0.38]	20 [0.47]	21.9 [0.32]	14.8 [0.32]	0.039 0.00
% FEV_1 change with bronchodilator *	1.63 [1.16]	4.5 [0.91]	1.82 [1.16]	0.83 [1.22]	<0.001
Post bronchodilator FEV_1 (% predicted)	91.4 [21]	86.9 [20.7]	91.5 [21.4]	94.2 [20.7]	0.107
†Sputum eosinophil count * (%)	1.32 [0.62]	3.75 [0.64]	1.55 [0.51]	0.65 [0.44]	<0.001
FeNO*^ (ppb)	31.6 [0.33]	57.5 [0.27]	25.8 [0.29]	22.8 [0.27]	<0.001
Sputum neutrophil count (%) *	55.09 [0.31]	45.87 [0.24]	72.71 [0.13]	57.56 [0.36]	0.038
†Modified JACS [S.D.]	1.36 [0.74]	1.54 [0.58]	2.06 [0.73]	1.04 [0.66]	<0.001
Dose of Inhaled Corticosteroid (BDP equivalent / mcg) [S.D.]	632 [579]	548 [559]	746 [611]	653 [581]	0.202
Long Acting Bronchodilator use (%)	40.2	34.4	48.2	41.7	0.442
Previous hospital admission or emergency attendance (number per patient)	0.60 [1.57]	1.04	0.26	0.20	0.037
Previous outpatient attendance (% attended)	15%	22%	19%	6%	0.121
Severe asthma exacerbations (requiring oral corticosteroids) in past 12 months (number per patient)	1.25 [1.94]	1.86 [0.32]	1.07 [0.32]	0.39 [0.18]	0.002

† Variables included in the cluster analysis.

* Geometric mean [$\log_{10}$ standard deviation]

The shaded column represents a cluster not observed in the secondary care asthma population.

‡ Comparison between clusters using ANOVA for continuous variables and Chi squared test for proportions. Significance values for variables included in the cluster analysis are a product of the cluster algorithm and are provided for illustrative purposes only.

Table 13: Clusters in secondary care

Variable	Secondary Care N=187	Cluster 1 Early onset, Atopic N=74	Cluster 2 Obese, non-eosinophilic N=23	Cluster 3 Early symptom predominant N=22	Cluster 4 Inflammation predominant N=68	Significance (p-value) ‡
†Gender (% female)	65.8	75.7	87	68.2	47.1	<0.001
Age (years) [S.D.]	43.4 [15.9]	39.4 [15.7]	42.7 [11.1]	35.5 [15.5]	50.6 [15.1]	<0.001
†Age of Onset (years) [S.D.]	20.3 [18.4]	12.7 [12.9]	15.4 [15.2]	12.6 [15]	32.6 [19.1]	<0.001
†Atopic Status (% positive)	73.8	83.8	65.2	81.8	63.2	0.024
†Body Mass Index (kg/m^2) [S.D.]	28.5 [6.5]	27.6 [4.5]	40.9 [6.5]	23.6 [3.1]	27 [3.9]	<0.001
†Peak flow variability * (amp % mean)	32.2 [0.48]	46.1 [0.35]	21.2 [0.76]	24.2 [0.65]	27.6 [0.36]	0.002
% FEV_1 change with bronchodilator *	12.8 [0.41]	24.5 [0.31]	9.3 [0.35]	4.5 [0.33]	9.8 [0.34]	<0.001
Post bronchodilator FEV_1 (% predicted) [S.D.]	82.1 [21.1]	79.0 [21.9]	79.0 [18.5]	79.5 [26.1]	87.2 [18.5]	0.093
†Sputum eosinophil count * (%)	2.9 [0.99]	4.2 [0.76]	1.3 [1.01]	0.1 [0.9]	8.4 [0.64]	<0.001
FeNO*^ (ppb)	43 [0.32]	51.2 [0.36]	24.2 [0.27]	22.6 [0.30]	53.1 [0.32]	<0.001
Sputum neutrophil count (%) *	46.7 [0.32]	45.4 [0.39]	49.3 [0.22]	51.3 [0.23]	45.9 [0.29]	0.892
†Modified JACS [S.D.]	2.02 [1.16]	2.63 [0.93]	2.37 [1.09]	2.11 [1.11]	1.21 [0.95]	<0.001
Dose of Inhaled Corticosteroid (BDP equivalent / mcg) [S.D.]	1018 [539]	1168 [578]	1045 [590]	809 [396]	914 [479]	0.008
Long Acting Bronchodilator use (%)	93.0	91.9	95.4	90.9	94.1	0.999
Maintenance oral corticosteroid use (%)	31.7	32.4	22.7	22.7	36.8	0.604
Median Nijmegen Score [IQR] (% with score> 23) §	16 [7-26.5]	20.5 [12-30.25] (44.6)	23 [12-33] (52.2)	16.5 [4.5-28] (31.8)	9 [1-17] (19.1)	0.004
Median Anxiety Score [IQR] (% with score≥ 11) §	7 [4-10]	7.5 [4.8-10.3] (24.3)	8 [3-14] (34.8)	6 [3.8-8.3] (13.6)	6 [3-9] (19.1)	0.34
Median Depression Score [IQR] (% with score≥ 11) §	4 [2-7]	4.5 [2-8] (13.5)	5 [2-7] (4.3)	4 [2-7] (4.5)	3 [1-6] (7.4)	0.104
Courses of oral corticosteroids for asthma exacerbations (number / case / yr)	4.05 [2.33]	4.62 [0.27]	3.90 [0.38]	3.57 [0.49]	3.43 [0.27]	0.02
Hospital admissions for asthma (number / case / yr)	1.54	1.64	1.61	1.54	1.23	0.703
Failed Clinic Appointments (% DAC appointments/ yr)	20.0	26.2	15.7	19.0	14.8	0.027

Table Legend:

† Variables included in the cluster analysis
* Geometric mean [log_{10} standard deviation]
‡ Comparison between clusters using ANOVA for continuous variables and Chi squared test for proportions. As for the other tables, significance values for variables included in the cluster analysis are a product of the cluster algorithm and should not be further interpreted.
ˆ Measured with NIOX at a flow rate of 50 mls/sec
Shaded columns represent clusters not identified in the primary care asthma population.
A comparison of pre-specified asthma outcomes between the two management protocols analysed according to cluster allocation of subjects at study entry.

* Expressed as equivalent dose of beclomethasone

Table 14: Cluster specific outcomes of longitudinal study

Outcomes	Study Group		Sig
1. Obese Female			
	Clinical N=10	**Sputum N=8**	
Δ Inhaled corticosteroid dose* /µg per day (SEM)	-400 (328)	-462 (271)	0.89
Severe exacerbations over 12 months (SEM)	1.40 (0.78)	1.50 (0.80)	0.93
Number commenced on oral corticosteroids	2	1	0.59
2. Inflammation predominant			
	Clinical N=15	**Sputum N=24**	
Δ Inhaled corticosteroid dose* /µg per day (SEM)	+753 (334)	+241 (233)	0.22
Severe exacerbations over 12 months (SEM)	3.53 (1.18)	0.38 (0.13)	0.002
Number commenced on oral corticosteroids	2	9	0.17
3. Early Symptom predominant			
	Clinical N=7	**Sputum N=4**	
Δ Inhaled corticosteroid dose* /µg per day (SEM)	+1429 (429)	-400 (469)	0.022
Severe exacerbations over 12 months (SEM)	5.43 (1.90)	2.50 (0.87)	0.198
Number commenced on oral corticosteroids	6	0	-

A comparison of pre-specified asthma outcomes between the two management protocols analysed according to cluster allocation of subjects at study entry.

* Expressed as equivalent dose of beclomethasone

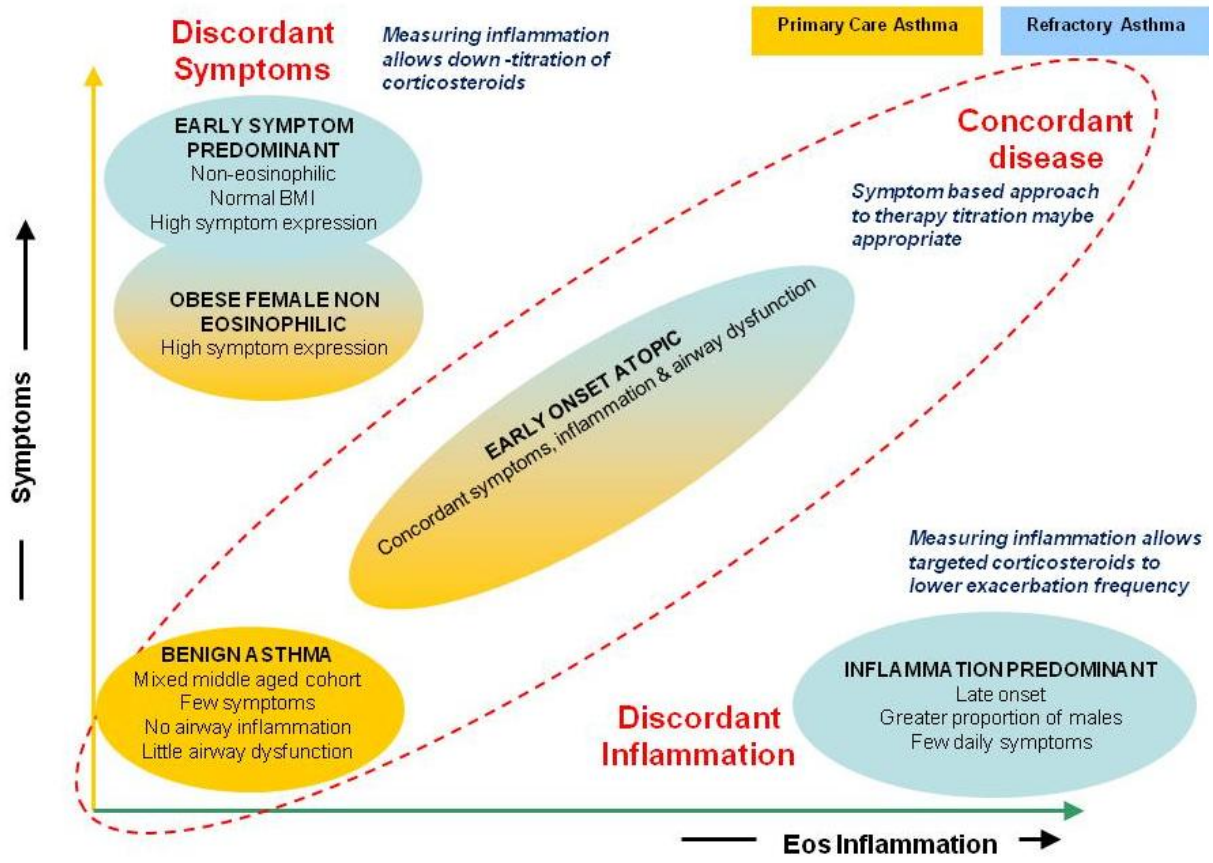


Figure 12: Clinical phenotypes of asthma identified with cluster analysis

A summary of phenotypes identified using cluster analysis in primary and secondary care asthma populations. The clusters are plotted according to their relative expression of symptoms and inflammation as these are the two clinically pertinent and modifiable dimensions of the disease. The plot highlights greater discordance to be a feature of secondary care asthma. Although reasons for this dissociation are unclear, the utilisation of measures of airway inflammation in these subgroups is clinically informative.

6.2 STUDY 2: MEPOLIZUMAB (ANTI-IL 5) AND EXACERBATIONS OF REFRACTORY EOSINOPHILIC ASTHMA

6.2.1 Abstract

Background

Asthma exacerbations are associated with significant morbidity, mortality and healthcare utilisation. Preventing exacerbations remains an important goal of therapy. Evidence suggests eosinophilic airway inflammation is associated with exacerbation risk.

Methods

We conducted a randomised, double blind, placebo-controlled parallel group study of mepolizumab, a monoclonal antibody targeting interleukin-5, in sixty-one subjects with refractory eosinophilic asthma and a history of recurrent severe exacerbations. Subjects received twelve infusions of either mepolizumab (n=29) or placebo (n=32) at monthly intervals. The primary outcome measure was the number of severe exacerbations over the 50 week treatment phase, defined as episodes of acute asthma requiring treatment with oral corticosteroids. Important secondary outcomes were change in asthma symptoms, the Asthma Quality of Life Questionnaire (AQLQ), lung function, airway hyperresponsiveness and eosinophil counts in the blood and sputum.

Results

Mepolizumab therapy was associated with a significant reduction in the number of severe exacerbations over 50 weeks (2.0 vs 3.4; relative risk 0.57, 95% confidence interval [CI] 0.32, 0.92; p=0.02) and an improvement in AQLQ (0.55 vs 0.19; mean difference 0.35, 95% CI 0.08, 0.62; p=0.02). Mepolizumab significantly lowered blood (p<0.001) and sputum (p=0.002) eosinophil counts. There was no difference between groups for symptoms, post bronchodilator forced expiratory volume in one second (FEV_1) or airway hyperresponsiveness. The only serious adverse effects reported were

hospitalisations with acute severe asthma. These occurred in 3 subjects randomised to mepolizumab and 11 randomised to placebo (p=0.07).

Conclusions

Mepolizumab therapy reduces exacerbations and improves AQLQ in subjects with refractory eosinophilic asthma. Our study supports a role for eosinophils as important effecter cells in the pathogenesis of severe asthma exacerbations in this patient population. (ISRCTN trial registration number: 75169762)

6.2.2 Introduction

Asthma is a complex chronic inflammatory disorder of the bronchial tree that presents clinically with variable symptoms of cough, breathlessness and wheeze, punctuated by periods of more severe and sustained deterioration in control requiring emergency treatment, termed exacerbations. Exacerbations are associated with significant morbidity, mortality and health care costs (248).

Exacerbations differ from day-to-day symptoms in that they respond poorly to usual inhaled therapy and are more closely linked to increased airway inflammation (249). Eosinophilic airway inflammation may be particularly important as infiltration of the airway mucosa with activated eosinophils is seen in post-mortem studies of patients who have died of acute severe asthma (83) and markers of eosinophilic airway inflammation increase well before the onset of exacerbations induced by withdrawal of corticosteroid treatment (111;112). Moreover, management strategies that control eosinophilic airway inflammation as well as the clinical manifestations of asthma are associated with a reduction in exacerbation frequency (23;25).

Mepolizumab, a humanised monoclonal antibody against interleukin (IL)-5, offers the prospect of clarifying the role of eosinophils in exacerbations, as it is a selective and effective inhibitor of eosinophilic inflammation (64;81;183;173). Clinical trial experience with this agent in asthma has been disappointing (64;183) although studies have focused on outcome measures that are not closely associated with eosinophilic airway inflammation and have included populations selected on the basis

of clinical and physiological characteristics rather than the presence of eosinophilic airway inflammation(250).

We have tested the hypothesis that eosinophils are important in the pathogenesis of asthma exacerbations in a double blind, randomised placebo controlled parallel group study investigating the effect of 12-months treatment with mepolizumab on exacerbation frequency in subjects with refractory asthma and evidence of eosinophilic airway inflammation despite maximum tolerated corticosteroid treatment. Secondary aims included assessing the effects of treatment on airway inflammation, asthma symptoms, asthma related quality of life, FEV_1 and airway structure assessed using Computerised Tomography (CT).

6.2.3 Methods

Subjects

All subjects were older than 18 years and had a clinical diagnosis of asthma supported by one or more of the following: maximum diurnal peak expiratory flow (PEF) variability >20% over 14 days; improvement in FEV_1 of >15% after 200 μg inhaled albuterol; and/or a provocative concentration of inhaled methacholine required to cause a 20% fall in FEV_1 (PC_{20}) of < 8 mg/ml. Subjects were recruited among patients attending a Refractory Asthma Clinic, providing secondary asthma care for a mixed urban and rural population of 1 million and tertiary care for 4 million. Patients attending this clinic have a standardised assessment, which includes non-invasive assessment of airway inflammation using induced sputum every 2-4 months. Inclusion criteria were: a diagnosis of refractory asthma according to American Thoracic Society criteria (14); a sputum eosinophil count >3% on at least one occasion in the previous two years despite maximum tolerated corticosteroid treatment; and at least 2 exacerbations requiring rescue prednisolone treatment in the previous twelve months. Subjects had stable treatment requirements and were exacerbation free for > 6 weeks prior to study enrolment. Exclusion criteria were: current smoking; serological evidence of parasitic infection; significant co-morbidity; possibility of conception; and poor treatment adherence. All subjects provided written informed consent. The study

protocol was approved by the local research ethics committee and the National Medicines and Healthcare products Regulatory Agency (MHRA).

Study Design

The study was a single centre randomised double blind placebo controlled clinical trial conducted between April 2006 and August 2008. The study measurements are described in [section 5.2] and the protocol is summarised in [Fig 13].

Routine study visits

All subjects had demographic details collected and pre- and post- bronchodilator spirometry performed at a baseline visit. Regular treatment was kept constant from this point until study completion. After a two week run-in, baseline methacholine PC_{20} was measured followed a day later by FE_{NO}, symptom scores and AQLQ. Subjects were treated with a 2-week course of high dose prednisolone (0.5 mg/kg/day to a maximum of 40 mg/day) after the baseline visit and again after completion of the final treatment visit. The purpose of this was twofold: 1. To assess the responsiveness of symptoms, FeNO and FEV1 to a corticosteroid trial and compare between and within group and within subject effects before and after 12 doses of treatment with mepolizumab and 2. To minimise the confounding effect of reversible airway inflammation on measurements of airway wall geometry with CT scanning. The subgroup of participants consenting to bronchoscopy had the procedure performed before prednisolone at both timepoints of the study. At visit 3, after completing the 2-week course of prednisolone and before receiving the first study treatment, subjects had a further assessment of symptom scores, FE_{NO}, spirometry before and after bronchodilator and, in those consenting, a CT.

Subjects were randomised using minimisation (251) to receive twelve infusions of either intravenous mepolizumab 750mg or matched placebo (150mls 0.9% NaCl) at monthly intervals between visits 3 and 14. The criteria used for minimisation were frequency of exacerbations in the previous 12 months, the baseline sputum eosinophil count and whether subjects were receiving maintenance oral prednisolone therapy. FE_{NO}, spirometry before and after bronchodilator and symptom scores were recorded

at each visit; AQLQ was measured at visits 5, 8, 11 and 14; and methacholine PC_{20} was measured the day before visits 8 and 14. The treatment phase finished 2 weeks after visit 14, 50 weeks after treatment was started. At this time, consenting subjects had a bronchoscopy and all subjects were issued with a further 2-week course of oral prednisolone as described above, followed by measurement of FE_{NO}, symptom scores, spirometry and CT scans.

At the final study visit, both subjects and blinded members of the study team were asked to complete a study evaluation form. Both parties were asked for their opinion on study conduct and comment on the possible treatment group to which the subject was randomised. Subjects were additionally asked to comment on how they viewed their global asthma control over the period of the study, both in free text and by marking an asthma control horizontal visual analogue scale [Fig 11]. This scale was designed to be 100 mm in length, ranging from -50 mm (worst possible control) to +50 mm (best possible control) with 0 (no change in control) in the middle [section 5.2.7].

Exacerbation visits

Exacerbation events during the treatment phase of the study were managed in accordance with standard clinical guidelines (6). Subjects initiating treatment themselves in the community did so with guidance from their personalised management plan. In all cases, subjects were instructed to seek medical advice either prior to, or as soon as possible after starting therapy. Oral prednisolone therapy was prescribed at a dose of 0.5 mg/kg/day to a maximum of 40 mg/ day. In subjects presenting to the investigating team prior to or within 48 hours of starting therapy, a decision to commence or continue treatment with oral prednisolone and therefore record the event as a severe exacerbation was based on clinical assessment. Events that were not treated with oral prednisolone but did result in subjects seeking medical advice were also recorded but not included in analysis of the primary outcome. Decisions on the need for adjunctive therapy such as antibiotics and the need for hospitalisation were physician led. Subjects reviewed within 72 hours of an exacerbation had symptom scores, FE_{NO}, PEF and spirometry before and after

bronchodilator performed. In addition, sputum samples were obtained for cell counts and microbial analysis.

Because of the expected anti-eosinophil effects of mepolizumab (41;64;173), results of FE_{NO}, sputum and blood leucocyte differential counts obtained during scheduled and unscheduled visits were not disclosed to investigators. Exacerbation events requiring hospitalisation were managed by the admitting clinical team, whose members were unaware of treatment assignments.

Safety Assessment

Safety was assessed clinically and on the basis of laboratory tests and measurement of vital signs before and after infusion and adverse event reports. All adverse events during the study were recorded in accordance with local research guidelines. A copy of serious adverse events was also submitted to GSK as part of their ongoing collection of data.

Statistical Analysis

The primary outcome measure of the study was the number of severe asthma exacerbations per subject. Severe exacerbations were defined as periods of deterioration in asthma control requiring treatment with high dose oral prednisolone for at least five days (6). Exacerbation events occurring in the 50 weeks between completion of the first treatment visit and two weeks after the final treatment visit were included in the analysis. A recurrence in asthma symptoms shortly after completing a course of prednisolone was recorded as a separate exacerbation event if the subject had a prior return to baseline control for a period of at least five days. Secondary outcome measures were: change in the differential blood and percent sputum eosinophil counts; FE_{NO}; post bronchodilator FEV_1 % predicted; methacholine PC_{20}; AQLQ; symptom scores; CT measured airway wall geometry; and bronchoscopic measures of eosinophilic airway inflammation.

All subjects that completed at least one treatment visit were included in an intention-to-treat analysis of the primary outcome. For subjects that dropped out, the adjusted exacerbation frequency was calculated as: recorded exacerbations + (visits

remaining/total visits x group mean exacerbation frequency). Exacerbation frequency was compared between the study groups using a negative binomial model and verified with the Mann Whitney U-test, as previously described (252). In a study of a similar cohort (23) the mean (SD) exacerbation number was 3.2 (2.1) per subject per year . Based on a mean exacerbation number of 2 per subject per year, the study required sixty subjects to detect a 50% reduction in exacerbation frequency with 80% power. Secondary outcome parameters were log transformed where appropriate. Between and within-group comparisons were made for mean change between baseline and the mean or geometric mean of the post-treatment values using the unpaired and paired t-tests respectively for parametric distributions and the Mann Whitney U-test for non-parametric distributions. Proportions were compared with Fishers exact test. Statistical software packages used during various analyses included SPSS version 13, STATA version 7 and Graph Pad Prism version 4.

6.2.4 Results

Enrolment and subject characteristics

The enrolment pathway of eligible patients is summarised in [Fig 14]. Sixty one of the sixty three subjects screened started treatment and constituted the intention to treat population. Thirty two subjects were randomised to receive placebo. Overall, 94.9% of treatment visits were completed. Subjects that withdrew completed a mean of 4.6 treatment visits (38.3%). Subjects were well matched with respect to baseline characteristics [Table 15].

As figure 14 shows, 149 of 449 patients (33%) attending our refractory asthma clinic would have met the eligibility criteria for the study.

Efficacy

Severe exacerbation frequency

The median treatment period was 348 days for mepolizumab and 340 days for placebo (p=0.3). During this period a total of 57 severe exacerbations occurred in the

mepolizumab treated group and 109 severe exacerbations in the group assigned to receive placebo [Fig 15]. The mean number of severe exacerbations per subject was 2.0 in the mepolizumab treated group and 3.4 in the placebo group (relative risk 0.57; 95% confidence interval [CI] 0.32, 0.92; p=0.02). The difference in exacerbation number remained significant with non-parametric analysis (p=0.04). A further 27 episodes were recorded, in which subjects attended for assessment of deteriorating control but did not receive oral corticosteroid therapy. 17 of these episodes occurred in subjects assigned to mepolizumab and 10 visits occurred in the placebo group. There was a significant difference between the groups in the proportion of exacerbation visits that did not result in treatment with oral corticosteroids (23% for mepolizumab group vs 8.4% for placebo group; p=0.004). From a clinical perspective, exacerbations not requiring oral corticosteroids had significantly lower symptom scores, using both the JACQ (p<0.001) and visual analogue scale (p<0.001); significantly better peak expiratory flow (p<0.001) and post bronchodilator FEV_1 (p=0.001); and significantly less reversibility following treatment with short acting bronchodilator (p=0.005). Assessment of airway inflammation identified significantly lower sputum eosinophil counts (p=0.02) but raised total sputum neutrophils (p=0.01) [Table 16].

Thirty-one percent of mepolizumab treated subjects had no severe exacerbations during the study period, compared with 16% in the placebo group (p=0.23). The mean duration of prednisolone therapy per exacerbation was similar between the groups (mepolizumab 10.9 days vs. placebo 11.7 days; p=0.31). There were 3 hospital admissions in 2 subjects for exacerbations of asthma in the mepolizumab group compared with 11 admissions in 5 subjects for the placebo group (p=0.07). The cumulative days in hospital was significantly lower in subjects randomised to mepolizumab treatment than placebo (12 days vs. 48 days; p<0.001). There was no within-group or between-group difference in the proportion of exacerbations occurring by season or month (p=0.74 and p=0.98 respectively for between-group comparisons).

Treatment for an exacerbation was initiated by the subject in 20%, by the primary care physician or another hospital in 25 % and by the study team in 55% of occasions. In the 77% of exacerbations assessed at or within 72 hours of initiating prednisolone therapy,

there was no difference between the groups in PEF, pre- and post-bronchodilator FEV_1, symptom scores and rescue bronchodilator use. Sputum was obtained in 62% of exacerbations. The sputum eosinophil count at exacerbation differed significantly between the groups [GM (log SEM) 4.4% (0.1) placebo vs 1.5% (0.1) mepolizumab; p=0.005]. The proportion of subject exacerbations associated with a clinically significant sputum eosinophilia (greater than 3%) was 59.3% in the placebo group, compared with 35.7% in the mepolizumab group (p=0.04). In 19% of placebo exacerbations with sputum eosinophilia, the baseline sputum eosinophil count was normal, indicating a significant rise at the time of exacerbation. In contrast, all eosinophilic exacerbations in the mepolizumab group occurred in subjects with a high sputum eosinophil count at baseline. The sputum neutrophil count (absolute count per milligram of selected sputum) did not differ between the study groups (p= 0.87).

Exacerbations treated with antibiotics were characterised by higher symptoms scores with the Juniper Asthma Control Questionnaire (p=0.02); higher sputum neutrophil counts (p=0.02) and lower FeNO (p=0.02). Comparing clinical characteristics of severe exacerbations with eosinophilic and non-eosinophilic sputum identified no difference between the groups for symptoms. However, eosinophilic exacerbations were associated with significantly greater bronchodilator reversibility (p=0.005) and FeNO (p<0.001).

Inflammatory markers

Mepolizumab therapy was associated with a significant between and within-group reduction of both blood and sputum eosinophil counts [Fig 17], [Table 18]. The geometric mean of blood eosinophil counts during the treatment phase, as compared with the baseline value was reduced by a factor of 6.6 in the mepolizumab group and by a factor of 1.1 in the placebo group, with the changes from baseline differing between the groups by a factor of 6.1 (95% CI 4.1 to 8.9; p<0.001). Sputum induction resulted in an assessable cytospin preparation on 90 % of visits. The geometric mean sputum eosinophil count was reduced by a factor of 7.1 with mepolizumab and by a factor of 1.9 in the placebo group, with the changes from baseline differing by a factor of 3.7 (95% CI 1.6 to 8.4; p=0.002). There were no significant between-group

differences in the change from baseline of FE_{NO} (p=0.29) or sputum total neutrophil count (p=0.68) [F and F].

Paired bronchial biopsy specimens (specimens obtained before and after the treatment phase) were available in 14 subjects (9 mepolizumab), paired bronchoalveolar lavage (BAL) specimens in 11 subjects (8 mepolizumab) and bronchial wash specimens in 10 subjects (7 mepolizumab). The change from baseline in eosinophil counts following mepolizumab therapy, compared with placebo, were reduced by a factor of 2.1 (95% CI 0.6 to 68.1; p=0.68) for bronchial biopsies; by a factor of 8.2 (95% CI 0.9 to 75.4; p=0.06) for BAL specimens; and by a factor of 16 (95% CI 1.8 to 140; p=0.02) for bronchial wash samples [F and F].

Inflammation and exacerbation frequency

A linear model with univariate analysis of variance, using tertiles of area under the curve sputum eosinophil counts as a covariate, identified this to be a significant independent factor associated with the frequency of exacerbations in subjects assigned to both placebo (R^2 = 0.16, p=0.03) and mepolizumab (R^2 = 0.16, p<0.05) [Fig 16]. 16% of subjects assigned to mepolizumab therapy had a mean study sputum eosinophil count in the highest tertile. This subgroup accounted for 27% of severe exacerbations with mepolizumab. A similar association between the level of control for sputum eosinophil counts and other secondary outcome measures was not observed.

All subjects assigned to receive mepolizumab were given the same dose of drug at each visit (750 mg). There was no association between control of eosinophilic inflammation in blood or sputum, measured as area under the curve during the treatment phase, and mepolizumab dose corrected for weight (data not shown).

Other outcomes

There were no significant differences between the groups in the change from baseline in symptom scores, whether assessed using visual analogue scales or the JACQ [F and F]. The mean improvement in AQLQ from baseline in the mepolizumab group was 0.55 compared to 0.19 with placebo (mean difference 0.35; 95% CI 0.08 to 0.63; p=0.02)

[Table 18]. There were no significant between-group differences in changes from baseline for post-bronchodilator FEV_1 or methacholine PC_{20} [Table 18].

CT scans were obtained before and after the treatment phase of the study in 26 subjects from each group. There was a significant between-group difference in the change from baseline for airway wall area (mean difference 1.1 mm^2/ m^2 body area; 95% CI 0.2 to 2.1; p=0.02) and total area (mean difference 1.5 mm^2/ m^2 body area; 95% CI 0.2 to 2.8; p=0.03) [Table 18].

Outcomes from corticosteroid trial

The clinical response to prednisolone therapy was measured as the change in symptom scores and post bronchodilator FEV1 respectively. The response was compared within groups at baseline and at the end of the treatment phase. Between-group differences were examined as the mean difference of within-group change in prednisolone response of symptoms and post bronchodilator FEV 1 at the two time points. A wide spectrum of response was observed for both symptom scores and FEV1 and there was no significant association between the magnitude of response and the baseline sputum eosinophil count, grouped in tertiles [Table 20]. There was a poor correlation, expressed as the coefficient of agreement in tertiles of response, for symptoms and FEV1 to prednisolone (κ=0.17, p=0.07). There were no significant between-group differences in the change in symptom scores after prednisolone treatment, before and after the treatment phase of the study with mepolizumab or placebo [Table 20]. Nine subjects randomised to mepolizumab had a greater than 0.5 point decrease in their JACQ score after the 2-week course of prednisolone before commencing treatment with mepolizumab. These subjects had a similar within-group fall in JACQ scores following the prednisolone course given after completing the study (mean reduction 1.2 points before mepolizumab and 0.9 points afterward; mean difference -0.3; 95% CI -1.0 to 0.4; p=0.32).

In contrast, mepolizumab therapy was associated with a significant within-group and between-group attenuation of improvement in post bronchodilator FEV1 after prednisolone therapy at the end of the study for the tertile of subjects with the greatest response to prednisolone at baseline [within group difference (pre-treatment

prednisolone response – post-treatment prednisolone response) (95% CI) placebo group 29 mls (-235 to 294), p=0.81; mepolizumab group 500 mls (66 to 934), p=0.03; between group difference (95% CI) 471 mls (21 to 961), p=0.04] [Table 20].

Post-hoc analysis identified FEV1 response to prednisolone, expressed in tertiles, as the only significant baseline predictor of mepolizumab efficacy during the study. Subjects with an improvement in FEV1 had a significant reduction compared with placebo in severe exacerbations with mepolizumab therapy during the study [tertile 2 (FEV1 response -50 ml to 200 ml) relative risk (RR) with mepolizumab 0.4, p=0.01; tertile 3 (FEV1 response 220 ml to 2350 ml), RR with mepolizumab 0.3, p=0.03]. In contrast, tertiles of FEV1 response to salbutamol at baseline was inversely related to the efficacy of mepolizumab for preventing exacerbations [Table 17].

End of study evaluation

At study completion subjects were asked to document their presumed treatment allocation. 45% of subjects were 'unsure', 36% selected the correct group and 19% were incorrect. There was no difference in the proportions of each response between the study groups (p=0.42).

Compared with placebo, subjects assigned to mepolizumab had a significantly higher asthma control score measured on the visual analogue scale [mean (SEM) 20.5 (4.3) for mepolizumab and 8.0 (4.1) for placebo; mean difference between groups 12.5 (95% CI 0.6 to 24.3); p=0.04] [Table 18]. Within the study population, we found a significant difference between tertiles of the asthma control score for mean JACQ score, % predicted post bronchodilator FEV_1, asthma quality of life, change in asthma quality of life and frequency of severe exacerbations during the treatment phase [Table 19].

Safety

Intravenous mepolizumab was well tolerated over 12 months. The only serious adverse effects reported were hospitalisations with acute severe asthma. No local effects of infusion were observed. One subject was withdrawn from the study after

developing a transient maculopapular rash 24 hours after receiving the first infusion of mepolizumab.

6.2.5 Discussion

We found that mepolizumab treatment significantly reduced the number of asthma exacerbations requiring corticosteroid therapy and increased asthma-related quality of life in subjects with refractory eosinophilic asthma and a history of recurrent exacerbations. There was no significant improvement in symptoms or FEV_1, parameters that are commonly used for quantifying asthma control. Treatment effectively lowered blood and sputum eosinophil counts and was well tolerated over 12 months.

Previous studies of mepolizumab in less severe asthma have been too short to evaluate the effect of treatment on exacerbation frequency although the largest study to date did show a similar 50% reduction in severe exacerbations, which approached statistical significance (183). The absence of effect of mepolizumab on symptoms, FEV_1 and airway responsiveness in our study is also in keeping with the experience of others in less severe asthma (64;81;173;183). Treatment had a larger effect on blood and sputum than biopsy eosinophil numbers, consistent with earlier work (173) although a proportion of patients had a persistent sputum eosinophilia at exacerbation despite mepolizumab therapy. Further studies are required to investigate the mechanisms underlying heterogeneity in the biological response to mepolizumab and the relative resistance of tissue dwelling eosinophils to anti-IL-5.

We have previously shown that the main effect of a management strategy that suppresses eosinophilic airway inflammation is a reduction in exacerbation frequency and have suggested a causal link between eosinophilic airway inflammation and exacerbations (23). This view is strongly supported by the current study as mepolizumab is a selective inhibitor of eosinophilic airway inflammation (176;177). Most severe asthma exacerbations are thought to be causally linked to viral infections, which typically cause non-eosinophilic inflammation (253). How then does mepolizumab lead to a significant reduction in exacerbation frequency? One explanation is that viral infections only cause severe exacerbations when there is

uncontrolled eosinophilic airway inflammation (254). A second possibility is that viral infections are less important and fluctuating eosinophilic airway inflammation more important in the aetiology of severe exacerbations in this group of patients. However, it is important to be mindful that eosinophils are not the sole determinant of severe exacerbations in this group. We found that 16% of the variance in the exacerbation rate was attributable to the mean eosinophil count, expressed in tertiles over the period of observation, in both treatment groups (figure 16). This implies that non-eosinophilic mechanisms remain important for this population and specific anti-eosinophilic treatment will not ameliorate severe exacerbation risk completely. This is supported by the observation that severe exacerbations were reported in subjects that had well controlled eosinophilic airway inflammation for the duration of the study. Nevertheless, the contribution of eosinophilic airway inflammation is sufficient in this population to identify a significant reduction in severe exacerbation frequency with treatment. As a corollary, it is apparent that in statistical terms, a threshold is likely to exist for the variance of severe exacerbations attributable to sputum eosinophils that will determine whether or not specific anti-eosinophil therapies such as mepolizumab will be effective and therefore the phenotypes of asthma that would be appropriate for clinical trials of efficacy.

Mepolizumab treatment had no significant overall effect on asthma symptoms and lung function during the study. However responsiveness of these clinical parameters to a short course of oral prednisolone was observed in some subjects. Interestingly, the response of symptoms to prednisolone remained intact for responders after mepolizumab treatment, when eosinophilic airway inflammation was suppressed. In contrast, mepolizumab was associated with attenuation of the FEV1 response to prednisolone in responders at baseline. These findings help delineate biological mechanisms that are associated with individual components of clinical disease expression. Eosinophilic inflammation is responsible for mediating a greater degree of FEV1 responsiveness to glucocorticoid, compared with symptoms for which a signal was not found. This supports the idea that symptoms are multifactorial and not necessarily related to disease activity. The importance of eosinophilic inflammation for both FEV1 responsiveness to glucocorticoid therapy and exacerbations is emphasised

by the association between these two clinical endpoints such that efficacy of mepolizumab for preventing severe exacerbations may be predicted by baseline FEV response to prednisolone. Together these observations suggest that mepolizumab may prevent exacerbations by blocking a pathway of eosinophil mediated decline in lung function associated with increasing risk of precipitating a severe exacerbation event; this is distinct from the effects on lung function of airway smooth muscle as FEV1 response to salbutamol did not correlate with the FEV1 response to prednisolone and was not an independent predictor of mepolizumab efficacy. The dissociation between exacerbation risk and eosinophilic airway inflammation on one hand and day-to-day clinical manifestations of asthma on the other has important implications for the way asthma is managed and assessed in this group of patients.

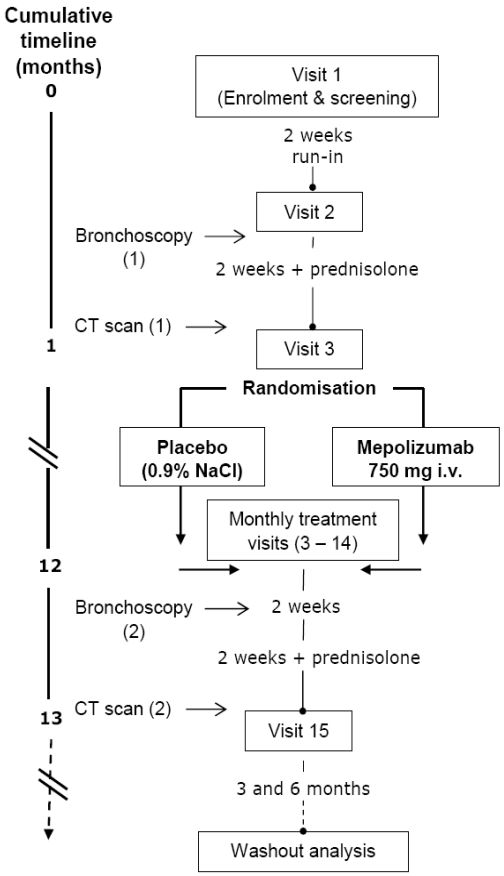
There was a small but statistically significant improvement in asthma quality of life with mepolizumab therapy, perhaps reflecting the value of exacerbation prevention to patients. Interestingly, a non-specific linear scale of asthma control that was designed to measure patient perception of asthma correlated significantly with lung function, symptoms and exacerbation control during the study and was significantly higher (indicating better control) in subjects receiving mepolizumab, compared with placebo. This suggests that mepolizumab therapy is associated with asthma wellbeing that is not reliably measured with the tools used routinely. More sensitive objective tools for measuring asthma associated quality of life are needed.

We found that CT measured airway wall thickness and total wall area was reduced in those subjects treated with mepolizumab compared with placebo. The CT scans were undertaken after a 2-week course of prednisolone and after administration of bronchodilators, so the findings are unlikely to be confounded by bronchomotor tone and acute airway inflammation. Whether the changes in airway wall dimensions translate to important long-term clinical effects requires further investigation.

The profile of therapeutic effect seen with mepolizumab treatment illustrates well how we can learn more about the pathogenesis of different airway responses with selective inhibitors of inflammation. The patients studied had refractory eosinophilic asthma despite maximum tolerated therapy, which in many cases included regular oral

corticosteroids. Although they resemble the exacerbation prone phenotype of severe asthma described by Moore et al (146) and the eosinophilic inflammation predominant phenotype described by us (255) , heterogeneity in the response to mepolizumab and oral prednisolone suggest that even this carefully selected population may not be homogeneous. Patients with eosinophilic airway inflammation and a history of frequent severe exacerbations are relatively prevalent in a population with refractory asthma and there is an important unmet need for better treatment. A well-tolerated agent that reduces the frequency of exacerbations, time spent in hospital because of asthma exacerbations and the need for rescue oral corticosteroid treatment would be an attractive treatment option. Our results should not be extrapolated beyond the highly selected group of patients recruited here. However, further clinical trials should be done to establish more clearly the risk and benefits of treatment in a wider population. Many patients with fluctuating respiratory symptoms and eosinophilic airway inflammation do not meet current criteria for a diagnosis of asthma (144;256;257) and we have previously argued that new ways of classifying airway disease are needed to allow proper evaluation of new therapeutic entities (220). Investigators planning future trials should be mindful of disease characteristics that suggest a response to therapy and select patients with airways disease and eosinophilic airway inflammation rather than those that meet arbitrary physiological criteria.

6.2.6 Figures and tables



	Visit Number	Symptom scores	Asthma quality of life	Bronchoscopy	CT Scan	Spirometry	Exhaled nitric oxide	Sputum induction	Blood leucocyte count	Bronchial provocation test
		Summary of longitudinal measurements								
Treatment Visits	1	X				X		X	X	
	2		X	X			X			X
	2-weeks prednisolone				X					
	3	X				X	X			
	4	X				X	X			
	5	X	X			X	X	X	X	
	6	X				X	X			
	7	X				X	X			
	8	X	X			X	X	X	X	X
	9	X				X	X			
	10	X				X	X			
	11	X	X			X	X	X	X	
	12	X				X	X			
	13	X				X	X			
	14	X	X	X		X	X	X	X	X
	2-weeks prednisolone				X					
	15	X				X	X			

Figure 13: Overview of study protocol

Bronchial provocation testing was performed a day before visits 2, 8 and 14.

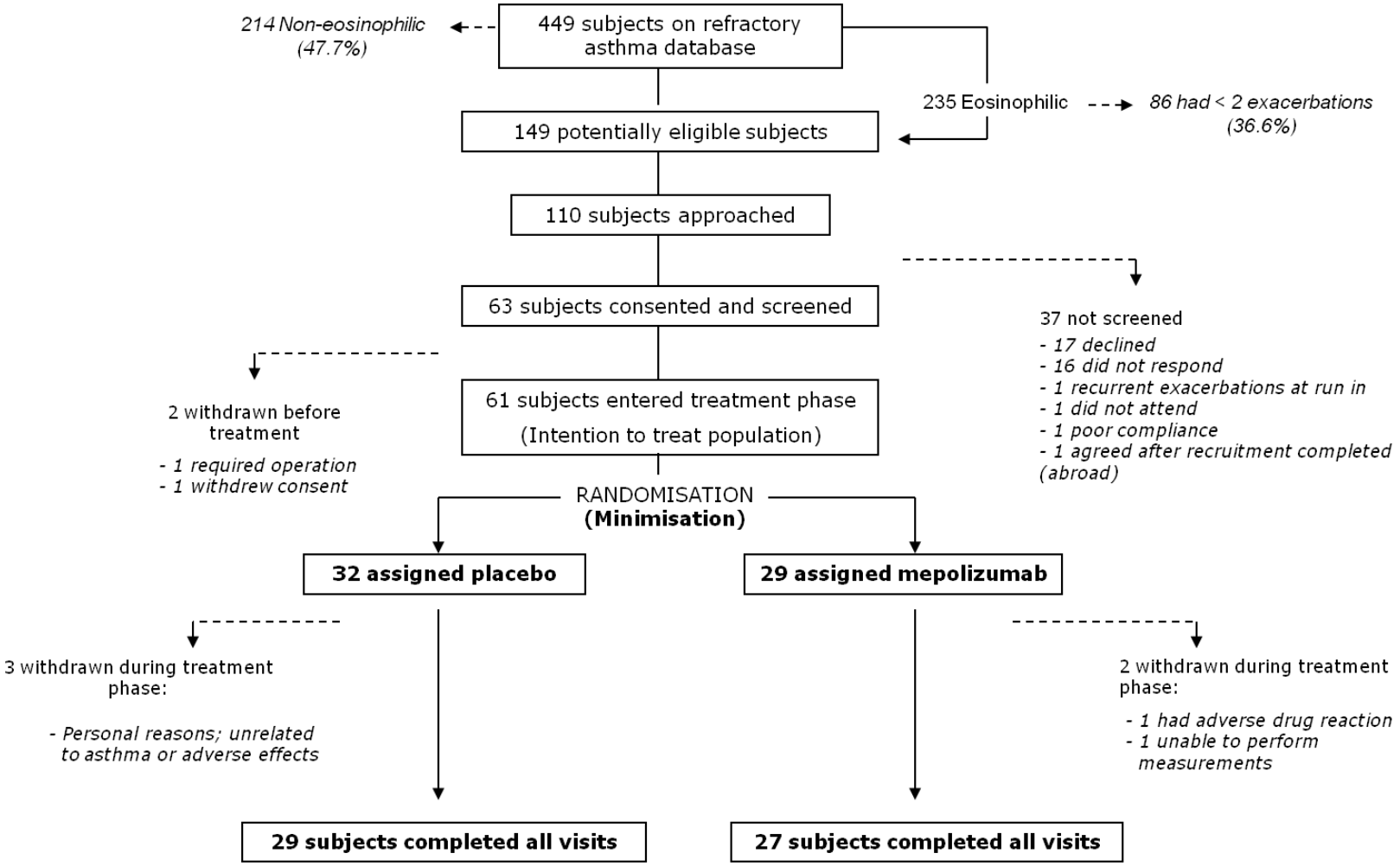


Figure 14: CONSORT - Eligibility, recruitment and randomisation

Table 15: Baseline characteristics of intention to treat population

	Mepolizumab (N=29)	Placebo (N=32)	Significance
Gender - male/female	14/15	18/14	0.80
Age - yrs (Range)	48 (21-63)	50 (24-72)	0.34
Age of onset - yrs (Range)	26 (2-53)	26 (2-57)	0.99
Body Mass Index - kg m-2	29.4 ± 7.3	29.2 ± 5.9	0.92
Atopic Status - %positive ^	67.9	68.8	0.78
Total IgE - kU l^{-1} *	177.8 ± 0.47	195 ± 0.64	0.75
Nasal polyps - % positive	34.4	31.2	0.59
‡Severe exacerbations in previous year	5.5	5	0.71
Previous ITU admission for asthma - %	27.5	31.25	0.78
Methacholine PC_{20} - mg ml^{-1} *	0.6 ± 1.24 (N=16)	1.1 ± 1.1 (N=18)	0.38
Post bronchodilator FEV1 - % predicted †	78.1 ± 20.9	77.6 ± 24.1	0.93
FEV1/FVC ratio - % †	72.2 ± 9.6	67.7 ± 13.5	0.15
FEV1 Bronchodilator responsiveness - %	9.1 ± 14.2	7.0 ± 13.1)	0.57
‡Sputum eosinophil count - % *	6.84 ± 0.64	5.46 ± 0.75	0.60
Blood eosinophil count - $x10^9$ l^{-1} *	0.32 ± 0.38	0.35 ± 0.30	0.57
FE_{NO} - ppb *†	44.4 ± 0.40	35.5 ± 0.40	0.31
Juniper asthma control score	1.98 ± 1.07	2.38 ± 1.35	0.28
Asthma Quality of Life Score	4.72 ± 1.26	4.84 ± 1.13	0.71
BDP equivalent Inhaled corticosteroid dose - µg per day † (Range)	2038 (1000-4000)	1711 (1000-4000)	0.03
Long acting beta agonist use - % using	92.9	90.6	0.99
‡Regular oral prednisolone - % using Mean (range) daily dose of maintenance prednisolone - mg	57.1 9 (5-20)	53.1 10 (2-40)	0.80 0.72
Monteleukast use - % using	21.4	25	0.76
Methotrexate use for asthma	0	2	0.49

$^{\Delta}$ Plus-minus figures are means ± standard deviation unless otherwise stated

Significance figures are p-values obtained by performing a two-sided independent t-test for variables with a parametric distribution, the Fisher exact test for comparison of proportions and a Mann Whitney U-test for comparison of non-parametric variables

‡ Parameters used for stratifying randomisation with minimisation. Minimisation was performed by an independent clinician (CEB)

^ Positive atopic status defined on the basis of a positive skin test to any of four specified aeroallergens (see on-line supplement)

* Figures presented are the geometric mean ± $\log_{10}$ (standard error)

† Abbreviations used: BDP = beclomethasone dipropionate; FE_{NO} = Fraction exhaled nitric oxide. This was measured at a flow of 50ml/sec (see on-line supplement); FEV_1 = Forced expiratory volume in 1 second; FVC = Forced vital capacity

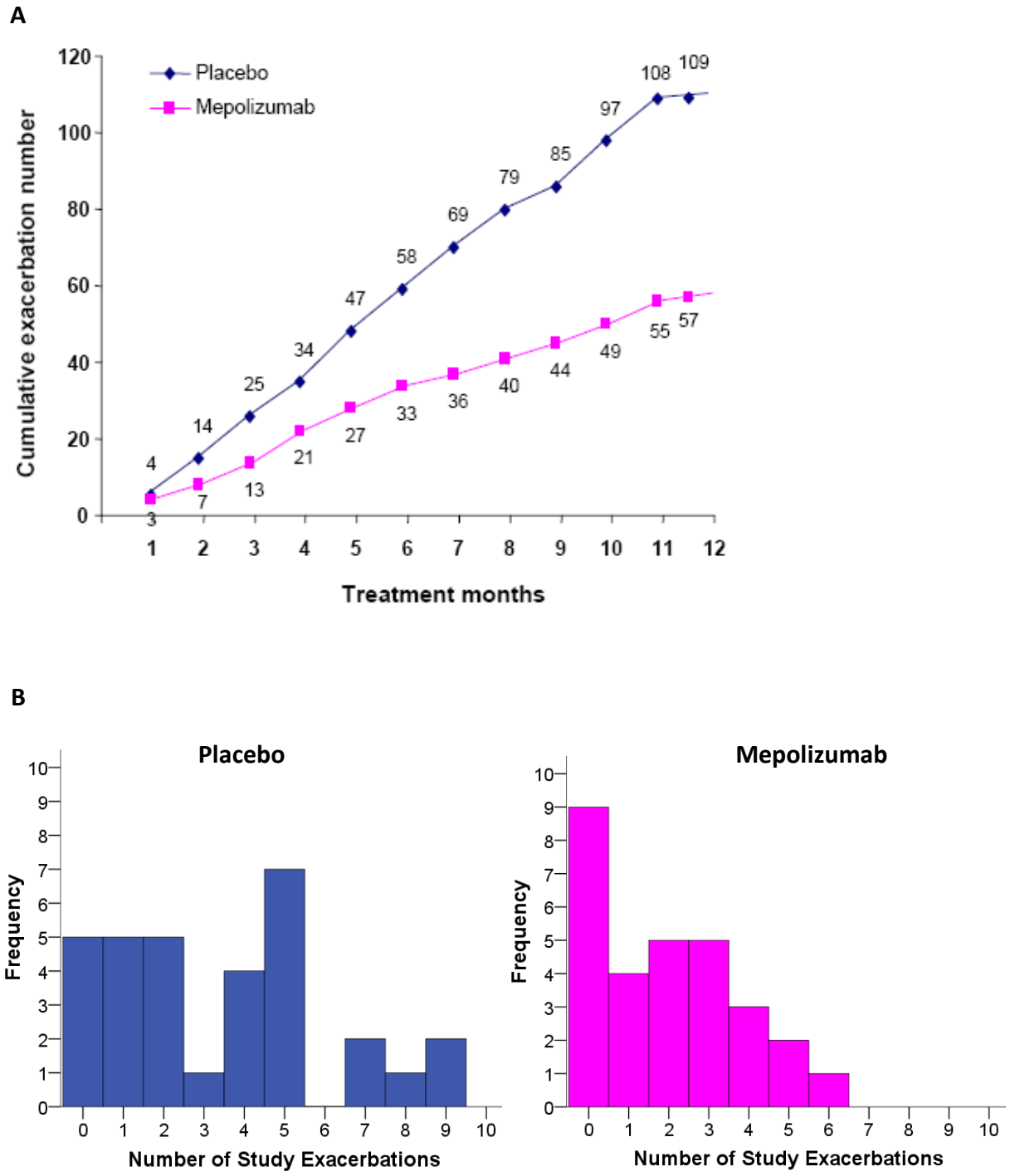


Figure 15: Profile of severe exacerbations during treatment phase of study

A: Cumulative number of severe exacerbations in each treatment group
B: Frequency distribution for exacerbation number per subject in each treatment group
The mean exacerbation number over the 50 week treatment period was 2 and 3.4 with mepolizumab and placebo treatment respectively (relative risk 0.57; 95% CI 0.32, 0.92; p=0.02 with a negative regression model.)

Table 16: Comparison of exacerbation visits treated and not treated with prednisolone

	Moderate Exacerbation (N=27)		Severe exacerbation (N=158)		
	N	Mean Std. Error Mean	N	Mean Std. Error Mean	Sig
Sputum obtained	18/27 (67%)		89/158 (56%)		0.40
Sput Neuts / mg of selected sputum	16	24137 ± 18529	92	4024 ± 778	0.01
% Sput Eos ^	16	1.0 ± 0.2	98	2.9 ± 0.1	0.02
FeNO $_{50}$ ^	18	37 ± 0.1	76	53 ± 0.04	0.1
Post BD FEV_1 (% best)	20	100.7 ± 2.8	100	88.7 ± 1.8	0.001
Pre BD FEV_1 (% best)	21	96.0 ± 2.8	106	79.5 ± 2.0	0.001
% FEV_1 BD Response	20	4.3 ± 2.0	97	11.7 ± 1.5	0.005
JACQ Score	22	2.4 ± 0.2	124	3.4 ± 0.1	<0.001
Mean VAS / mm	24	38 ± 4.4	129	60.2 ± 2.0	<0.001
PEF (% best)	25	73.4 ± 2.6	145	55.7 ± 1.3	<0.001
Antibiotics given	10/27 (37%)		65/159 (41%)		0.83
Receiving mepolizumab (% of group)	17 (63%)		53 (34%)		

Overview of the clinical characteristics for periods of deterioration in asthma control that prompted subjects to seek medical advice.

^ Geometric mean with log_{10} standard error of the mean

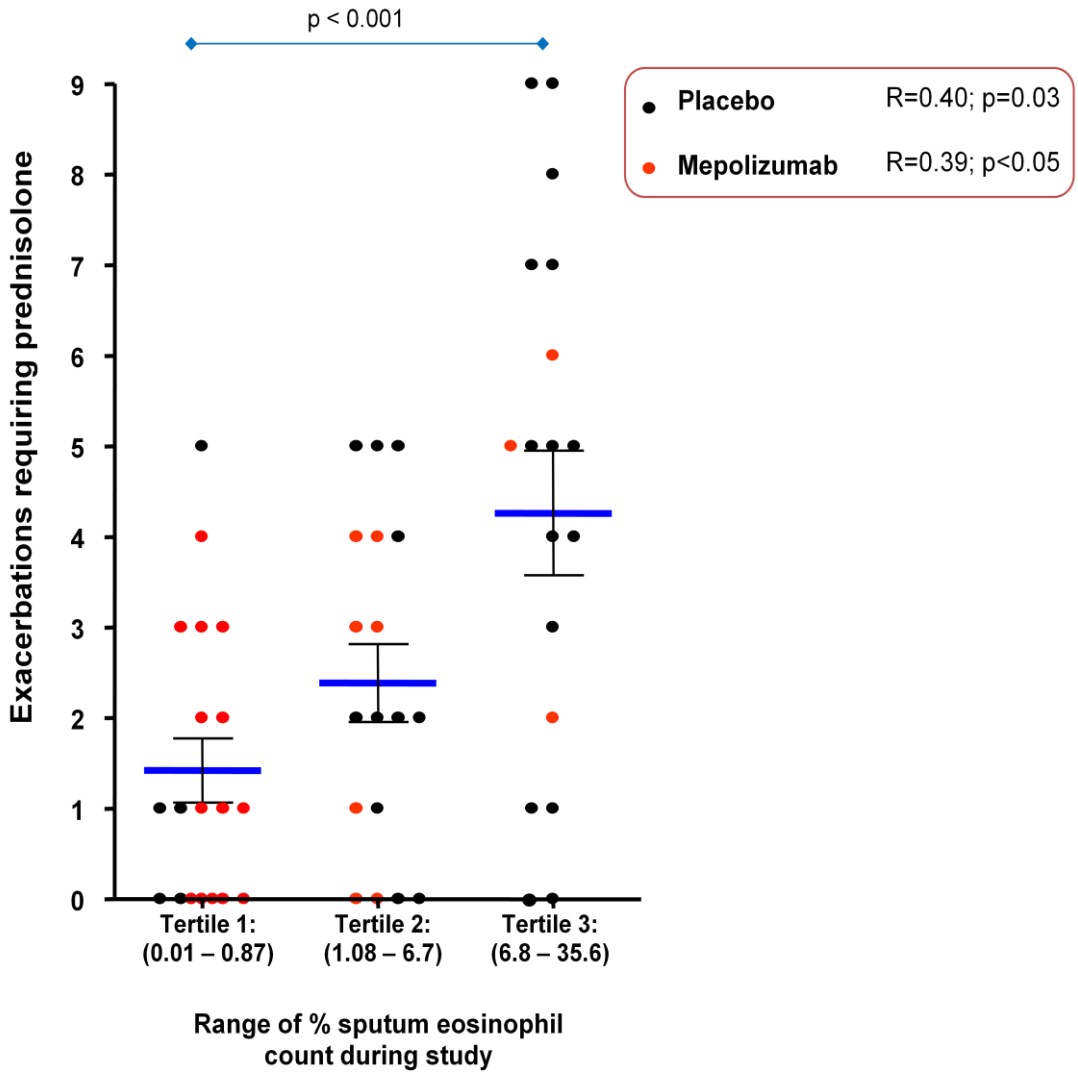


Figure 16: Exacerbation frequency and mean control of sputum eosinophil count during treatment phase of study

The figure demonstrates actual numbers of severe exacerbations occurring in subjects, stratified by tertiles of mean sputum eosinophil count during the study. There was a significant difference in exacerbation rates across tertiles for all subjects ($p<0.001$). Differences remained significant in a univariate model for each treatment group. The variance in exacerbations due to the sputum eosinophil count was identical for both groups in the model, supporting the idea that the primary effect of mepolizumab in the study was to reduce exacerbations by controlling the sputum eosinophil count.

Table 17: Response to meploizumab in baseline tertiles of response to prednisolone and Salbutamol

	Exacerbation no/patient/50 weeks		
	Mepolizumab	Placebo	p Value
Change in FEV_1 after prednisolone (ml)			
<−50	2.4	2.0	0.63
−50 to 220	2.1	5.0	0.02
>220	0.8	2.9	0.02
Change in FEV_1 after salbutamol (ml)			
<50	1.3	3.8	0.02
50 to 150	1.7	3.6	0.11
>150	2.6	2.4	0.85

The response to prednisolone represents the change in post-bronchodilator FEV 1 measured at the same time of day before and 1-2 hours after the final dose (day 14) of prednisolone. FEV 1 was measured before and 20 minutes after inhaled salbutamol. Between treatment group comparisons of the rate of severe exacerbation for each tertile of FEV1 response has been performed using the Mann-Whitney U test.

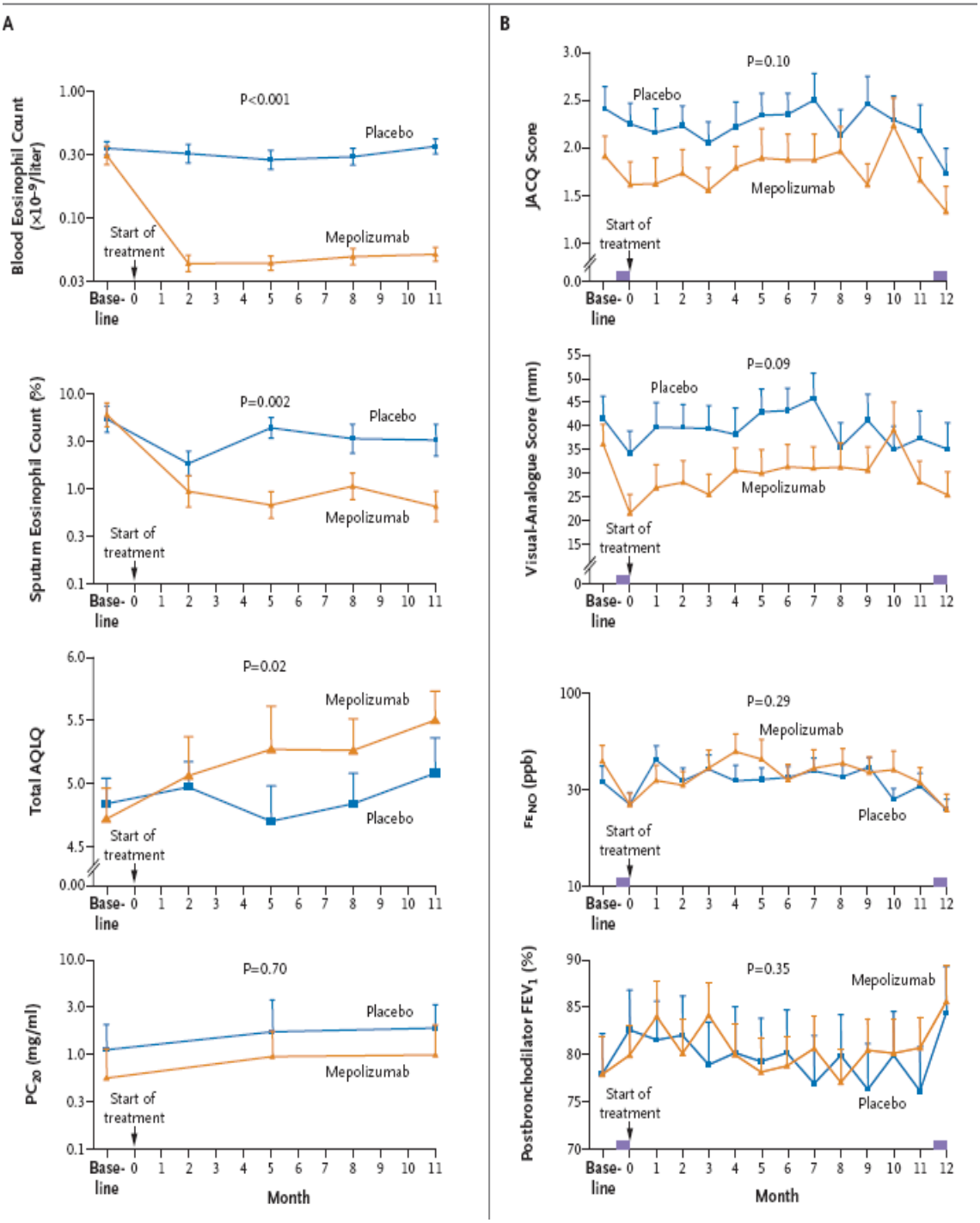


Figure 17: Secondary outcomes during treatment phase of study

Parameters were evaluated before and after prednisolone 0.5 mg/kg/day to a maximum of 40 mg/ day for 14 days, given at the beginning and end of the treatment phase. The shaded purple bars in panel B epresent the 2-week prednisolone course (see main text).

P-values represent the significance of the mean difference between the groups for the change from baseline to the mean or geometric mean of the post-treatment values. This data is presented in tabular form [Table 18].

Abbreviations: B=Baseline visit (study visit 1); PS=Post steroid visit (study visit 15)

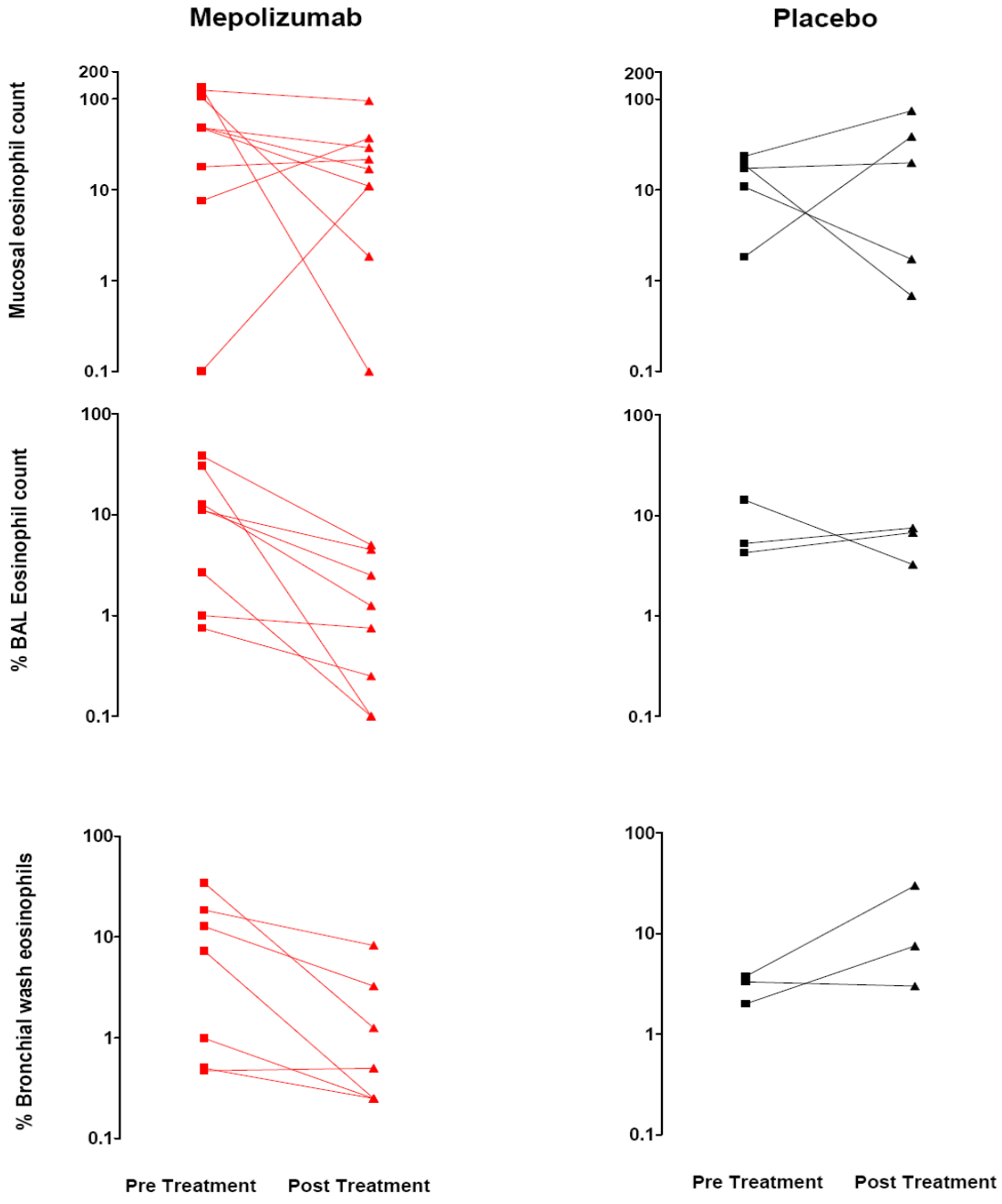


Figure 18: Eosinophil counts in different lung compartments at bronchoscopy

Individual bronchial biopsy, bronchoalveolar lavage (BAL) and bronchial wash eosinophil counts before and after mepolizumab and placebo treatment.

Table 18: Comparison of changes in secondary outcomes between treatment groups

	Mepolizumab		Placebo		Between group difference in change† (95% CI)	Significance ‡
	Baseline	Change from baseline §	Baseline	Change from baseline §		
Fraction exhaled nitric oxide (ppb) *	44.4 ± 0.4	0.85 (0.67 to 1.04)	35.5 ± 0.4	0.99 (0.80 to 1.19)	1.2 (0.9 to 1.6)	0.29
Total sputum neutrophil count (cells per mg selected sputum)	2534 ± 4890	-1291 (-3363 to 779)	1062 ± 1210	370 (-417 to 1157)	1662 (-1085 to 4410)	0.22
Modified Juniper Asthma Control Score	1.98 ± 1.07	-0.17 (-0.47 to 0.13)	2.38 ± 1.35	-0.21 (-0.52 to 0.11)	-0.04 (-0.46 to 0.38)	0.65
Visual analogue scale symptom score	36.2 ± 22.0	-7.7 (-15.2 to -0.3)	40.6 ± 26.2	-3.2 (-9.0 to 2.7)	4.6 (-4.7 to 13.9)	0.36
Asthma quality of life score	4.61 ± 1.21	0.55 (0.14 to 0.97)	4.77 ± 0.99	0.19 (-0.06 to 0.44)	-0.35 (-0.63 to 0.08)	0.02
Asthma control score (analogue scale) #	20.4 ± 22.4		8.0 ± 22.6		12.4 (0.60 to 24.2)	0.04
Post bronchodilator FEV1 (litres)	2.31 ± 0.82	0.06 (-0.09 to 0.21)	2.39 ± 0.85	0.12 (-0.03 to 0.26)	0.05 (-0.15 to 0.26)	0.61
^Methacholine PC_{20} (mg/ml^{-1}) *	0.6 ± 1.2	0.9 (-1.5 to 2.1)	1.1 ± 1.1	0.4 (-0.6 to 1.5)	-0.5 (-3.1 to 2.1)	0.70
Blood eosinophil count ($x10^9\ l^{-1}$) *	0.32 ± 0.38	0.15 (0.11 to 0.20)	0.35 ± 0.30	0.9 (0.7 to 1.17)	6.1 (4.1 to 8.9)	<0.001
Sputum eosinophil count (%) *	6.8 ± 0.6	0.14 (0.07 to 0.25)	5.46 ± 0.75	0.51 (0.28 to 0.91)	3.7 (1.6 to 8.4)	0.002
Bronchial wash eosinophil count (%) *	3.1 ± 0.8	0.19 (0.04 to 0.81)	3.1 ± 0.1	3.0 (0.2 to 45.7)	16.0 (1.8 to 140)	0.02
Bronchoalveolar lavage eosinophil count (%) *	5.5 ± 0.7	0.1 (0.02 to 0.50)	5.6 ± 0.3	0.8 (0.05 to 12)	8.2 (0.9 to 75.4)	0.06
Bronchial subepithelial eosinophil count (number per unit area) *	47.6 ± 0.4	0.41 (0.03 to 5.3)	10.9 ± 0.5	0.85 (0.04 to 19.1)	2.1 (0.06 to 68.1)	0.68
CT % Wall Area	66.3 ± 4.5	-1.2 (-2.5 to 0.1)	65.0 ± 5.3	-0.4 (-2.1 to 1.4)	-0.8 (-2.9 to 1.3)	0.43
Wall area/ BSA (mm^2m^{-2})	12.1 ± 3.9	-0.6 (-1.3 to 0.1)	11.6 ± 3.9	0.5 (-0.1 to 1.2)	-1.1 (-2.1 to -0.2)	0.02
Luminal area/ BSA (mm^2m^{-2})	6.4 ± 2.8	0.08 (-0.2 to 0.4)	6.5 ± 2.7	0.4 (-0.1 to 0.9)	-0.3 (-0.9 – 0.3)	0.26
Total area/ BSA (mm^2m^{-2})	18.4 ± 6.5	-0.5 (-1.5 to 0.4)	18.0 ± 6.4	0.9 (-0.04 to 1.9)	-1.5 (-2.8 to -0.2)	0.03

Mean (SD) pre-treatment values and post-treatment change within and between groups with 95% confidence intervals (CI).

* Geometric mean (log SD) with mean fold change and 95% CI. ^ For methacholine $PC_{20,}$ the change from baseline is expressed as doubling doses.

§ Change was calculated as a difference between the mean or geometric mean of the post treatment values and the baseline values. For parameters expressed as geometric mean, the change is expressed as a fold change.

‡ Significance refers to the between group difference in change.

An increase in asthma quality of life indicates improvement. An increase in symptom scores indicates worsening asthma symptoms.

17 subjects from each group had bronchial provocation testing performed. In the mepolizumab group, 9, 8 and 7 subjects had adequate samples for measurement of biopsy samples, bronchoalveolar lavage and bronchial wash at both time-points of the study. The corresponding figures in the placebo group were 5,3 and 3 subjects.

† The between group difference was calculated as the difference in change from baseline with placebo and mepolizumab.

The asthma control score was a non-specific measure of asthma well-being performed on a 100 mm horizontal analogue scale at the end of the treatment phase of the study only.

Abbreviations used: FEV_1 = Forced expiratory volume in 1 second; PC_{20} = Provocative concentration of methacholine required to induce a fall in the FEV_1 of 20% from baseline; CT = Computerised tomography; WA= Wall area; BSA= Body surface area; LA= Luminal area; TA= Total area

Table 19: Relationship between asthma control score and validated measures of asthma

Variables *	Asthma control score tertiles ^			Significance ~
	1 (-40 to 0)	2 (1 to 23)	3 (26 to 50)	
Juniper asthma control score	2.45 ± 0.79	2.10 ± 0.87	1.09 ± 0.82	<0.001
% FEV1	63.9 ± 14.9	71.4 ± 19.7	81.3 ± 15.9	0.01
Asthma quality of life score	4.3 ± 0.9	4.9 ± 1.2	5.9 ± 0.9	<0.001
Δ asthma quality of life score	-0.12 ± 0.62	0.34 ± 0.90	0.85 ± 0.92	0.002
Severe exacerbations	3.4 ± 2.3	4.0 ± 2.4	0.9 ± 1.4	<0.001§

* Values presented for each variable are the mean ± standard deviation during the treatment phase of the study

~ Between-tertile significance testing performed with one way analysis of variance for all variables except severe exacerbations (§ Kruksal Wallis).

^ Range of scores for each tertile given in brackets

Table 20: Clinical response to 2-weeks oral prednisolone in study groups, before and after completing treatment phase of study

A

	Pre treatment		Post treatment		Mean difference in change (95% CI) ^	Sig‡
	Pre-steroid measurement	Mean change (95% CI) with prednisolone†	Pre-steroid measurement	Mean change (95% CI) with prednisolone†		
Placebo Group						
Modified JACQ	2.38 ± 1.25	-0.19 (-0.49 to 0.1)	2.10 ± 1.43	-0.07 (-0.33 to 0.20)	-0.13 (-0.73 to 0.3)	0.40
Post BD FEV1 (litres)	2.39 ± 0.85	0.17 (0.01 to 0.35)	2.56 ± 0.87	0.20 (0.05 to 0.34)	0.04 (-13.2 to 14.0)	0.84
FE_{NO} (ppb) *	35.6 ± 0.40	0.8 (0.62 to 0.97)	39.8 ± 0.23	0.7 (0.49 to 1.06)	1.3 (0.8 to 2.0)	0.26
Mepolizumab Group						
Modified JACQ	1.91 ± 1.09	-0.3 (-0.60 to 0.00)	1.62 ± 1.19	-0.15 (-0.50 to 0.21)	-0.12 (-0.4 to 0.15)	0.37
Post BD FEV1 (litres)	2.3 ± 0.82	0.07 (-0.07 to 0.22)	2.38 ± 0.76	0.10 (-0.03 to 0.23	-0.03 (-21.3 to 20.7)	0.90
FE_{NO} (ppb) *	44.4 ± 0.40	0.6 (0.50 to 0.76)	34.5 ± 0.33	0.7 (0.48 to 0.89)	1.1 (0.6 to 2.1)	0.74

B

Tertiles of FEV_1 response	Placebo					Mepolizumab					Between group comparison	
	N	Pre-study (SEM)	Post-study (SEM)	Mean difference (95% CI)	Sig	N	Pre-study SEM	Post-study SEM	Mean difference (95% CI)	Sig	Mean difference (95% CI)	Sig
1 (-700 mls to -80 mls)	7	-307 (63)	160 (114)	-467 (-733 to -201)	0.01	11	-216 (54)	234 (98)	-450 (-769 to -131)	0.01	17 (-364 to 398)	0.93
2 (-50 mls to 200 mls)	8	94 (39)	118 (85)	-24 (-254 to 206)	0.81	9	33 (29)	21 (57)	12 (-126 to 151)	0.84	36 (-213 to 285)	0.76
3 (220 mls to 2350 mls)	12	350 (25)	321 (135)	29 (-235 to 294)	0.81	6	483 (69)	-17 (173)	500 (66 to 934)	0.03	471 (21 to 961)	0.04

Panel A: Overall within-group response in clinical parameters for each study group. Mean (SD) values for measurements prior to receiving prednisolone. * Geometric mean (log SD) for corresponding FE_{NO}.
† Change was calculated as a difference between the mean or geometric mean of the post prednisolone values and the baseline values. For $FE_{NO,}$ the change is expressed as a fold change. **^**The mean difference in change between post treatment and pre treatment changes with prednisolone in each group. For $FE_{NO,}$ the difference is expressed as a fold change.
‡ Significance refers to the difference in change between the response to prednisolone pre and post treatment.
The geometric mean sputum eosinophil counts before prednisolone pre and post treatment were: 5.8% and 3.2% in the placebo group and 6.5% and 0.6% in the mepolizumab group.

Panel B: Within and between group comparison of change in FEV_1 with prednisolone before and after treatment, stratified by tertiles of response for the cohort at baseline. High responders (tertile 3) at baseline in mepolizumab treated subjects failed to show any response after the treatment phase. A similar change was not seen for JACQ (data not shown).

6.3 CLINICAL AND PATHOLOGICAL OUTCOMES AFTER MEPOLIZUMAB THERAPY: A 12 MONTH WASHOUT ANALYSIS

6.3.1 Introduction

Mepolizumab is an efficacious and specific inhibitor of eosinophilic inflammation. We have previously shown a significant reduction in the frequency of severe exacerbations occurring in subjects with refractory eosinophilic asthma receiving mepolizumab therapy for 12 months (258). This benefit correlated with biological efficacy of the drug for lowering blood and sputum eosinophil counts during the study and provided further evidence in support of an effector role for eosinophils in the pathogenesis of severe asthma exacerbations.

Previous studies have reported differences in the pharmacological and biological half life of mepolizumab following cessation of therapy (63). In cynomolgus monkeys, the pharmacological half life reported after 2-doses of mepolizumab was 13 ± 2 days. More recently, a 3-dose human study performed in subjects with moderate asthma reported a terminal half life for the drug of 21 days (183). The biological half life has been generally expressed as the period for which the blood eosinophil count remains suppressed. Studies indicate this is a variable time period that is dependent upon the dose and duration of mepolizumab therapy and the underlying disorder being treated (173;174;177;183). In mild asthma, three 750 mg infusions of mepolizumab led to suppression of the blood eosinophil count for 9 weeks after the final dose (173).

The clinical and biological effects, measured in blood and sputum, of stopping mepolizumab in subjects with refractory asthma are not known. Furthermore, there is no data in either humans or animal models of asthma after 12 months of high dose mepolizumab therapy. A theoretical risk of 'rebound' has been suggested previously (180), based on *in vitro* observations that anti-IL5 therapy is associated with upregulation of IL-5 synthesis by Th2 cells; upregulation of IL-5R expression by eosinophils; and persistence of preformed IL-5 in complex with the drug for a variable period of time after cessation of therapy (177). This complex may act as a depot for IL-5 if dissociation occurs prior to clearance.

A significant increase in the frequency of severe exacerbations that is associated with a rise in the sputum eosinophil count following cessation of mepolizumab would further strengthen the evidence in favour of a role for eosinophilic airway inflammation in the pathogenesis of severe exacerbations.

As part of a washout analysis, subjects completing our 12-month study of mepolizumab in refractory asthma were followed up at 3-monthly intervals for a further 12 months. The aims of this period of observation were:

1. To examine the kinetics of recovery in blood and sputum eosinophil counts.
2. To examine the change in clinical markers of asthma control and whether any evidence could be found for rebound following cessation of therapy in asthma symptom scores, spirometric measures of lung function and the frequency of severe exacerbations.
3. To examine whether a temporal correlation was apparent between the change in biological and clinical control of disease.

6.3.2 Methods

Study Design

This was an unblinded, prospective, observational study of subjects that completed a 12-month randomised, double-blind, placebo controlled parallel group study of mepolizumab therapy in refractory eosinophilic asthma (259). Subjects were followed up at 3-monthly intervals in our Refractory Asthma Clinic for a period of 12 months as part of routine clinical care.

The final visit for treatment with either mepolizumab or placebo was assigned as the baseline visit for the washout analysis. This was the final visit during the study at which blood and sputum samples were collected. The 12 month period of follow up was calculated from this time point. All subjects received a 2-week course of oral prednisolone 2-weeks after this visit as part of the trial protocol.

At each follow-up visit, subjects were assessed clinically by a senior respiratory physician. The number of severe exacerbations requiring oral prednisolone during the

previous 3 months and exacerbations requiring hospitalisation were recorded. Adjustments to regular treatment were permitted as clinically indicated. Subjects completed a Juniper Asthma Control Questionnaire (JACQ). FeNO was measured at a flow rate of 50ml/sec with mini NIOX and was performed as the first test at each visit. All subjects were asked to withhold regular long acting bronchodilators for at least 24 hours prior to their clinic visit. Spirometry was performed before and 20 minutes after inhalation of 400 mcg of Salbutamol. Sputum induction was then performed using a standardised protocol [section 5.2.5] and blood samples were taken to measure the differential leucocyte count.

Analysis

Between and within treatment-group comparisons were performed for blood and % sputum eosinophil counts, JACQ scores, post bronchodilator FEV1 and the number of severe exacerbations. Variables were log transformed to approximate a normal distribution where appropriate. Between-group comparisons across the 12 months were calculated as the difference in the mean change from baseline for each group. Statistical comparison was performed using the independent t-test and Mann Whitney U-test for parametric and non-parametric distributions respectively. Within-group change in variables, both across all visits and between consecutive visits, was analysed using repeated measures analysis of variance in a general linear model. For results that reached statistical significance, the change between the visits was validated using the paired t-test or Wilcoxon sign ranked test for parameteric and non-parametric variables respectively. For repeated measures analysis, missing data was handled by replacing with the subject's mean when a single datapoint was not available. If more than one datapoint was missing, the subject was excluded from that analysis. Between and within-group comparisons of exacerbations over the 12 months were generally evaluated with non-parametric tests, both across and between visits. Severe exacerbation rates were normally distributed in the mepolizumab group during the observation period. It was therefore possible to apply a repeated measures model to examine between-visit changes in severe exacerbations within the mepolizumab group. Statistical analyses were performed using the following software: SPSS v.16 (SPSS, Inc); Prism v.5 (GraphPad software, Inc); and Stata® v.7 (StataCorp, LP).

6.3.3 Results

Subjects and follow up

56 subjects (27 assigned to mepolizumab) completed the original study. Characteristics of these subjects at the baseline visit for follow up are summarised in table [Table 21]. Of this cohort, one subject that received mepolizumab dropped out from further follow up due to a move away from the area and was not included in any follow up analyses.

Overall, 41 of 55 subjects (75%) attended for all 4 follow up visits; the remainder attended 3 visits. There was no difference in the proportion attending all visits between treatment groups (p=0.54). There was no difference in the mean period of follow up between the treatment groups [mean ± SEM placebo group 364 ± 11.4 days, mepolizumab group 384 ± 8.3 days; mean difference 20 days (95% CI -8.3 to 48.2), p=0.16].

Changes to drug therapy

There were no between-group differences of changes to any regularly prescribed asthma medication for the 12 months. The mean dose of inhaled corticosteroid increased by 95± 103 mcg BDP equivalent in the placebo group and fell by 89 ± 151 mcg BDP equivalent in the mepolizumab group (mean difference 185 mcg [95% CI -184 to 554]; p=0.31). There were no between-group differences in the proportion of subjects either starting or stopping add-on therapies prescribed at steps 4 and 5 of the BTS algorithm. These included methotrexate, monteleukast, oral theophylline and prednisolone [Table 22]. For subjects on maintenance prednisolone prior to the study, it was stopped at follow up in 5 of 15 subjects that received mepolizumab (33%) and in 3 of 15 subjects (20%) that received placebo (p=0.68 for between group comparison). Prednisolone was started in 6 of 14 subjects (43%) that received placebo and 2 of 11 subjects (18%) that received mepolizumab (p=0.23). For subjects that continued with a maintenance dose of prednisolone, there was no between or within-group difference in the change from baseline of the dose of prednisone taken regularly [mean difference in change between groups = 0.2 mg (95% CI -5.1 to 5.5), p=0.94].

Changes in eosinophilic inflammation

Significant between-group differences in change from baseline of the blood and eosinophil counts occurred over the 12 months of observation [Table 23]. Cessation of mepolizumab therapy was associated with a 6.1 fold increase in the blood eosinophil count (95% CI 3.9 to 9.7; p<0.001) and 1.8 fold increase in the sputum eosinophil count (95% CI 1.1 to 3.0; p=0.03) compared with the placebo group. A significant within-group rise across visits for subjects formerly treated with mepolizumab occurred for the blood eosinophil count (4.6 ±1.2 fold; p<0.001) but not for the sputum eosinophil count (3.1 ± 1.2 fold; p=0.24). Significant between visit changes in the blood eosinophil count were observed at 0-3 months (p<0.001) and 3-6 months (p=0.003) [Fig 19], [Table 23]. For the sputum eosinophil count, a significant rise occurred at 0-3 months (p=0.03); thereafter, the sputum eosinophil count did not change significantly [Fig 19], [Table 23]. No significant within-group change for either blood or sputum eosinophils was observed across or between visits in subjects that received placebo.

Severe Exacerbations

The frequency of severe exacerbations in the 12 months after the final treatment visit of the study was 3.1 per subject in the placebo group and 3.9 per subject in the mepolizumab group [rate ratio 1.25 (95% CI 0.71 to 1.91); p=0.54]. There was no significant within group change for subjects that received placebo between the treatment and observation periods (p=0.39). In contrast, there was a significant within-group increase in the frequency of severe exacerbations between the two time periods for subjects that were treated with mepolizumab (p=0.009). In a repeated measures model, a significant increase in the exacerbation rate occurred for the interval 3-6 months after the final treatment visit (p=0.01), preceded by the rise in sputum eosinophils [Fig 20].

Changes in other clinical measures of asthma

A significant between-group difference was observed in the mean change from baseline of both JACQ symptom scores and post-bronchodilator FEV1 [Table 23]. For

the latter, the significant difference was due to an increase in the FEV1over the 12 months in subjects that had received placebo, rather than any significant drop occurring following cessation of mepolizumab therapy. However, within-group changes were not statistically significant in either group.

Asthma symptoms rose by a mean of 0.54 points over the 12 months after the final treatment visit for subjects that received mepolizumab. This was both statistically significant and of a clinically significant magnitude [Table 23]. In contrast, symptoms remained unchanged in subjects that received placebo. However, most of the between-group difference could be attributable to a significant fall in asthma symptoms (p=0.049) occurring in the placebo group for the interval 9-12 months after the final treatment visit of the study [Fig 21].

6.3.4 Discussion

We found that eosinophilic inflammation in blood and sputum increased to baseline levels 6 months after stopping treatment with mepolizumab in patients with refractory eosinophilic asthma. This was accompanied clinically by an increase in the frequency of severe exacerbations and asthma symptoms but no change in pulmonary function.

Previous studies using mepolizumab have been performed in subjects with milder asthma and treatment was given for a considerably shorter duration. However in clinical practice, mepolizumab therapy is likely to be reserved for patients with refractory asthma that have a stronger eosinophilic drive and be given for longer than 3 months. Biological and clinical outcomes reported in these studies are therefore of little practical relevance.

Our study is the first to report changes that occur after stopping mepolizumab therapy in an appropriate study population. Biologically, the effect on blood eosinophils was most significant and in keeping with the inverse response observed when therapy was given. This supports the idea that the biological influence of mepolizumab is primarily seen in the circulatory compartment. The rise in the blood eosinophil count began soon after stopping therapy (in the first 3 months) and continued to rise significantly to baseline over 6 months. At 3 months, the blood eosinophil count remained

significantly lower in mepolizumab treated subjects. This is longer than the 9 weeks of suppression reported with 3 months of mepolizumab in mild asthma by Flood-Page and colleagues (173) and may indicate the cumulative effect of 12 months of therapy in our study. However, in the study by Leckie and colleagues, a single dose of mepolizumab in mild asthma was associated with suppression of the blood eosinophil count for 4 months (64).

Previous studies have not examined the effect of stopping mepolizumab on eosinophilic airway inflammation. We found the sputum eosinophil count rose significantly to baseline levels within 3 months of stopping mepolizumab. This suggests that although the blood eosinophil count remained significantly suppressed at 3 months, the rise in this interval was sufficient to overcome the effects of mepolizumab on eosinophilic inflammation in the airway. From a biological perspective, this illustrates well the powerful chemotactic drive that is present in refractory eosinophilic asthma – a lower circulating eosinophil load is sufficient to increase the eosinophil count in sputum. However, it is also possible that an uncertain contribution is made by migration into the airway of resident tissue eosinophils. We and others have reported evidence of persistent tissue eosinophilia after mepolizumab therapy (173;256). Clinically, sputum eosinophils are closely associated with severe exacerbation risk. Our results therefore suggest that the blood eosinophil count may not be appropriate for measuring the biological half-life of mepolizumab in asthma.

The frequency of severe exacerbations increased significantly after stopping mepolizumab. Twelve months after stopping mepolizumab, exacerbation frequency was not significantly different between subjects of the two study groups. A significant rise in exacerbations occurred in the interval 3-6 months after stopping mepolizumab and was preceded by the significant rise in sputum eosinophils [Fig 20]. These findings are in keeping with earlier studies of corticosteroid withdrawal in asthma that have consistently reported a high and rising sputum eosinophil count to be predictive of a subsequent loss of asthma control or severe exacerbation event (111-113;260). In this study, the rise in sputum eosinophils occurred after stopping a highly specific anti-eosinophil therapy. This further supports the idea that eosinophils have a direct role in the pathogenesis of severe exacerbations.

The finding of increased asthma symptoms following cessation of mepolizumab is both interesting and unexpected. Increased symptoms were not accompanied by evidence of lung function decline. In our clinical trial, mepolizumab therapy was not associated with a significant improvement in symptoms. Despite this, it is notable that although not statistically significant, mean symptom scores for subjects receiving mepolizumab were considerably lower than for subjects in the placebo group at the end of the treatment phase of the study [Table 23]. The rise in symptoms after stopping therapy may therefore in part represent regression to the mean. An increasing frequency of severe exacerbations will have also contributed to higher symptom scores at each routine visit.

The study has a number of limitations. It is primarily a descriptive account of a period of unblinded observation. As part of the original study design, all subjects received a 2-week course of oral prednisolone at the beginning of the observation period. Together with changes to regular medication that were permissible throughout the period, it is probable that dynamic changes in blood and sputum eosinophilia will have been subject to the confounding effects of these anti-inflammatory therapies. However, the mean change in maintenance prednisolone and inhaled corticosteroid therapy was very small in both groups, implying that the effect of these changes is unlikely to have affected the results.

From a clinical perspective, a significant strength of this study design is that it demonstrates well how clinical control and its effects on clinical care are affected by stopping mepolizumab therapy. Our data does not provide any evidence for a rebound effect and deterioration in clinical control beyond that achieved prior to starting therapy.

The changes in clinical and biological control observed during the 12 month period after stopping mepolizumab in patients with refractory eosinophilic asthma are a valuable adjunct to the findings made on therapy. The reversal of changes in blood and sputum eosinophilic inflammation, coupled with an increase in severe exacerbation frequency support further a role for this cell in the pathogenesis of these events. Our study indicates that beneficial effects of mepolizumab last for

approximately 3 months after treatment is stopped. The absence of evidence for clinical or biological rebound is reassuring and favours a role for mepolizumab use in clinical practice.

6.3.5 Figures and Tables

Table 21: Study group characteristics of subjects participating in 12 month washout analysis

	Placebo (N=29)	Mepolizumab (N=27)	Significance[§]
Gender / % female	48.3	55.6	0.61
Age / years (SEM)	49 (2.0)	47 (1.8)	0.44
Onset / years (SEM)	24 (3.4)	26 (3.1)	0.75
Body Mass Index / kgm^{-2} (SEM)	29.4 (1.1)	29 (1.4)	0.81
Atopic Status / % positive	72.4	66.7	0.77
***Total Ig E / kU l^{-1}**	234 (1.2)	191 (1.2)	0.52
JACQ Score	2.17 (0.3)	1.66 (0.2)	0.16
Post bronchodilator FEV1 / % predicted	76.7 (4.7)	80.7 (3.2)	0.49
***FeNO / ppb**	34 (1.2)	35 (1.2)	0.93
***Blood Eosinophils / $x10^9$ l^{-1}**	0.90 (1.1)	0.05 (1.1)	<0.001
***% Sputum Eosinophils**	4.6 (1.4)	2.1 (1.4)	0.14
Total Sputum Neutrophils / mg selected sputum	2700 (980)	1200 (490)	0.19
^Inhaled corticoisteroid dose / mcg BDP equivalent (IQR)	1800 (1000 to 2000)	2000 (2000 to 2000)	0.009
Theophylline use / %	41.4	29.6	0.41
Leukotriene receptor antagonist use / %	27.6	22.2	0.76
Methotrexate use / %	7 (N=2)	0	0.49
Maintenance prednisolone / %	55.2	59.3	0.79

* Values are geometric mean (standard error of the mean expressed as fold difference)
^ Median with interquartile range
§ Significance calculated using Fisher's exact test for comparison of proportions, independent t-test for comparison of parametric variables and Mann Whitney U-test for comparison of non-parametric variables

Table 22: Overview of changes to add-on therapies during washout

Therapy	Outcome after treatment phase	Study Groups	
		Placebo	Mepolizumab
Methotrexate	Not started	22	27
	Started	4	0
	Stopped	1	0
	Continued	1	0
Leukotriene Receptor Antagonist	Not started	17	21
	Started	3	0
	Stopped	1	1
	Continued	7	5
Theophylline	Not started	15	17
	Started	1	2
	Stopped	3	3
	Continued	9	5
Prednisolone	Not started	8	10
	Started	***6***	***2***
	Stopped	3	5
	Continued	12	10

Table 23: Between and within-group changes in measured variables

Mean Change from end of study	Within group change across visits				Between group change across visits	
	Placebo	F-Value [Sig]	Mepo	F-Value [Sig]	Ratio fold change (Mepo/placebo)	Sig
Blood Eosinophils/ Fold change (SEM)	0.74 (1.07)	1.6 [0.17]	4.6 (1.2)	19.9 [<0.001]	6.1 (3.9 to 9.7)	<0.001
Sputum Eosinophils/ Fold change (SEM)	1.7 (1.1)	1.1 [0.35]	3.1 (1.2)	1.5 [0.24]	1.8 (1.1 to 3.0)	0.03
FeNO/ Fold change (SEM)	0.95 (1.1)	0.7 [0.85]	0.96 (1.1)	0.7 [0.78]	1.0 (0.8 to 1.3)	0.92
	Placebo	F-Value [Sig]	Mepo	F-Value [Sig]	Mean difference in change (Mepo – placebo)	Sig
Post BD FEV_1 / % change from end of study	7.7 (0.5)	1.6 [0.17]	-1.1 (1.0)	0.92 [0.45]	-8.8 (-6.1 to -11.5)	0.002
Post BD FEV_1 / mls	93 (15)		-48 (31)		142 (58 to 226)	0.006
Modified JACQ	0.05 (0.14)	1.6 [0.17]	0.59 (0.16	4.9 [0.001]	0.54 (0.01 to 1.06)	0.047

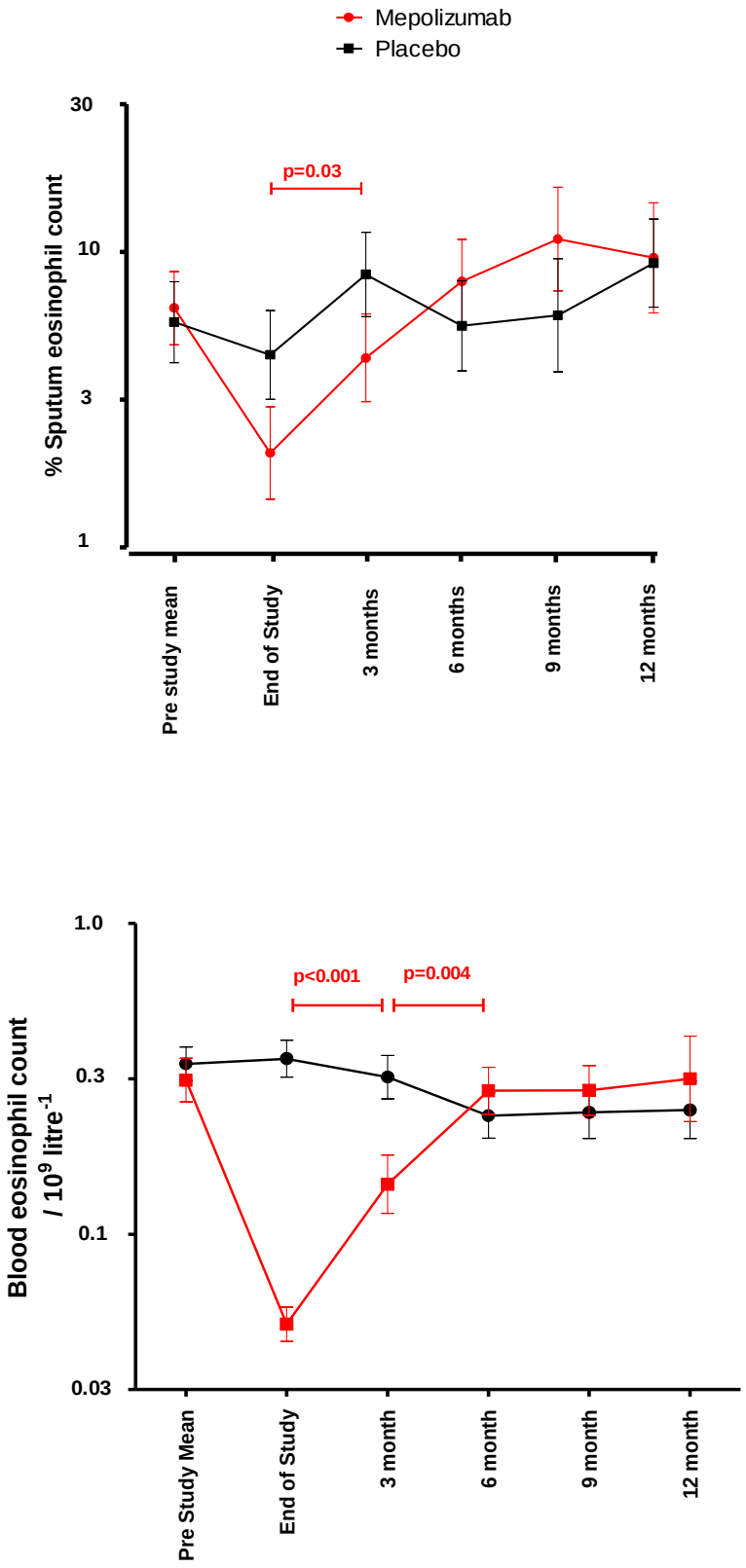


Figure 19: Change in eosinophilic inflammation during washout

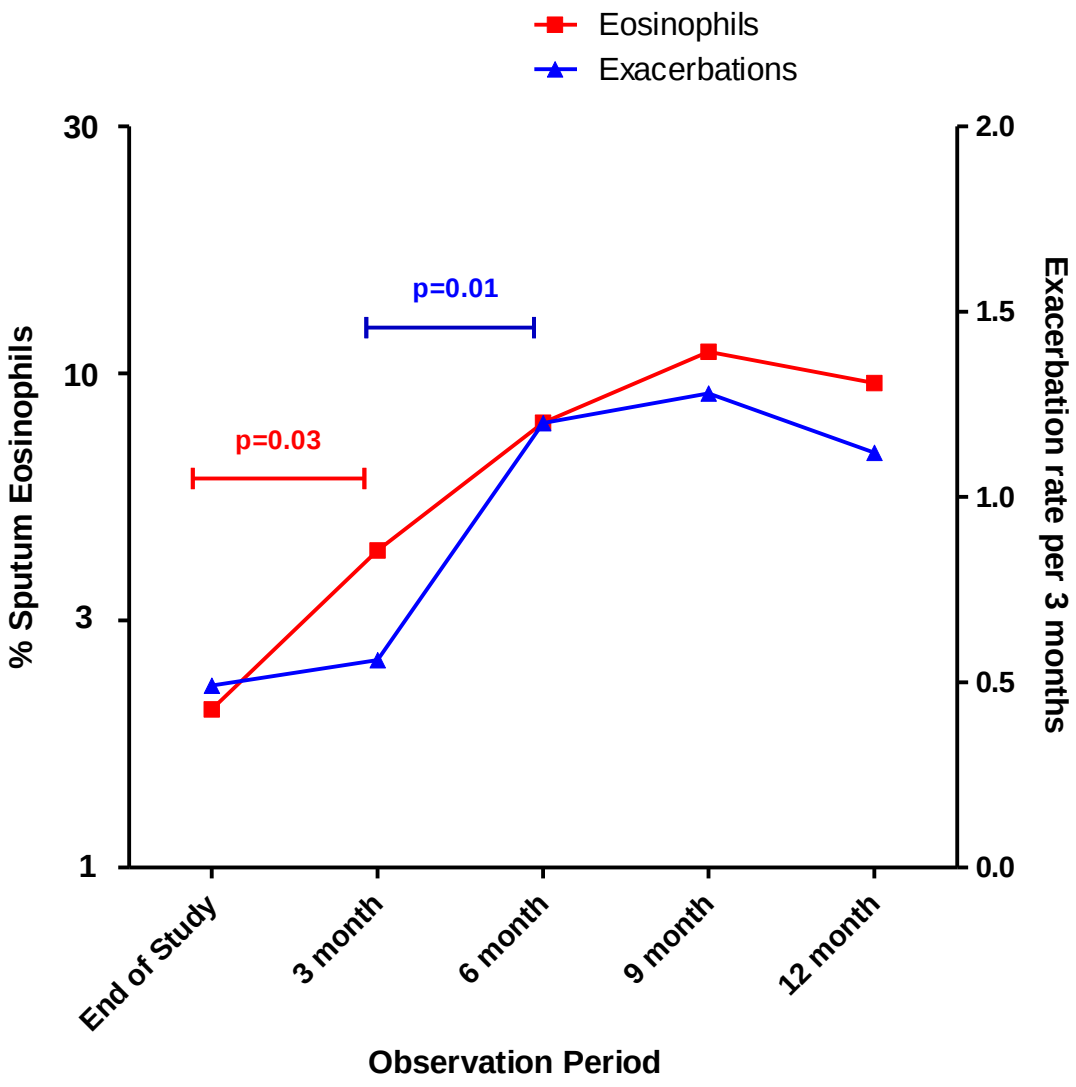


Figure 20: Association of sputum eosinophils with severe exacerbation frequency during washout for subjects that received mepolizumab

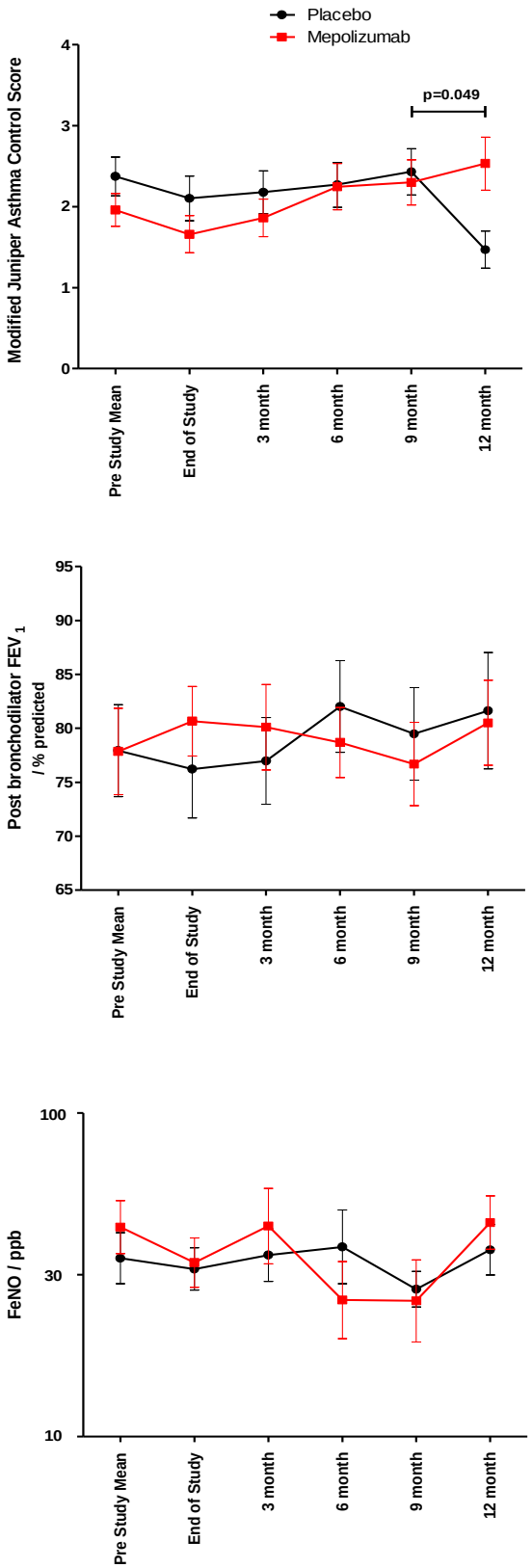


Figure 21: Change in other measures of clinical asthma control during washout

7. CONCLUSIONS AND FURTHER WORK

7.1 Introduction

This thesis has been concerned primarily with understanding further the role of eosinophils in the clinical expression of asthma. An answer to this apparently simple question remains elusive. An important reason for this is the complexity of heterogeneity that exists within the asthma population. It is well recognised that asthma is comprised of several measurable components that encompass aspects of airway pathology and physiology. How these relate to each other and with clinical disease presentation is poorly understood. Moreover, it is clear that clinical disease expression exhibits redundancy such that both eosinophilic and non-eosinophilic patterns of airway inflammation may present in similar ways. The development of specific and efficacious anti-eosinophilic therapies over the past decade was expected to provide the answer. However, the absence of any clinical benefit with these therapies has raised serious doubts concerning any important role for eosinophils in clinical asthma.

In this thesis, the problems described have been approached in two ways. Firstly, we explored the use of multivariate statistical techniques to characterise and categorise clinical heterogeneity in the asthma population. In the second part of this thesis we performed a clinical trial using mepolizumab in a well defined target population with refractory eosinophilic asthma and a history of frequent severe exacerbations to explore the hypothesis that eosinophils are important in the pathogenesis of severe exacerbations.

In this concluding chapter, I have summarised our results and outlined possible directions for future research.

7.2 Multivariate statistics and asthma phenotypes

In light of the multi-dimensional complexity of asthma, we explored the use of multivariate techniques for phenotyping. These techniques are naturally attractive in this setting for being able to process information from multiple domains together and provide a solution that is composite.

Using cluster analysis we identified four major clusters comprising the local difficult asthma population. Qualitatively, the clusters differed in many ways; however of clinical relevance was the clear difference identified between clusters for their respective associations between asthma symptoms and underlying eosinophilic airway inflammation. The level of concordance or discordance between these domains was associated with differences in outcome between inflammation guided and conventional clinical management algorithms. For clusters with considerable discordance between expression of symptoms and eosinophilic airway inflammation, inflammation guided therapy was associated with successful down-titration of maintenance glucocorticoid therapy in symptom predominant asthma and a significant reduction in severe exacerbations for eosinophilic, inflammation predominant asthma. Of interest was the finding of two clinically distinct eosinophilic subgroups raising the possibility that even within this pathologically defined population, clinical heterogeneity exists, adding to the complexity of defining the role of eosinophils.

The encouraging results obtained with cluster analysis support further evaluation and application of these techniques. A number of mathematically distinct but related algorithms exist and new algorithms are being developed that are designed to be fit for purpose. Developing clustering techniques for phenotyping asthma will require a multi-disciplinary approach. Inherently, these techniques are dependent upon the underlying population to be clustered and the variables used to perform clustering. Analyses performed at different centres may yield broadly comparable results but differences will exist. Identifying differences that are of clinical or scientific importance and distinguishing these from ‘natural variations’ due to differences in population characteristics will be a challenge. Cluster analysis is cross sectional. To be of scientific merit, the validity of any model constructed with these techniques requires hypothesis

driven, prospective longitudinal follow up. Important questions to explore are whether phenotypes of asthma identified at stable state with cluster analysis are of prognostic value, particularly with respect to their association with phenotypes at exacerbation and the clinical response to specific therapies. From a biological standpoint, cluster analysis may be helpful for identifying differences in the cellular, biochemical and genetic expression of different asthma phenotypes, particularly as vast quantities of molecular and genetic information can now be routinely gathered with 'biomic' platforms. In this context, multivariate techniques offer the prospect of moving beyond the search for single molecules and characterising associations between clinical phenotypes and biological 'signatures'.

7.3 Mepolizumab in refractory eosinophilic asthma

Our clinical trial with mepolizumab in refractory eosinophilic asthma was the first study in asthma to show clinical benefit with a specific anti-eosinophilic therapy. In so doing, the study illustrated the important principle of using a targeted approach with specific therapies. We hypothesised that eosinophils have an important effector role in the pathogenesis of severe exacerbations. However, exacerbations are heterogeneous and are likely to be eosinophil driven events in subjects with a history of eosinophilic airway inflammation. This formed the basis of the inclusion criteria for the study. The study was single-centre and performed in a relatively small cohort of patients. Larger studies are therefore needed for validation. A role for eosinophils in exacerbation pathogenesis was strengthened by the observation that the frequency of exacerbations increased in the 3 month interval after eosinophilic airway inflammation rose significantly in the post-study washout period.

Dissociation between symptoms, lung function and eosinophilic inflammation has been suggested in earlier studies. The dissociated clinical response to mepolizumab in the study was in keeping with these earlier observations. An important confounder of our results is the failure of mepolizumab to effectively ameliorate tissue inflammation. In addition to trialling mepolizumab in larger populations of eosinophilic asthma, similar studies are needed with alternative, specific anti-eosinophilic therapies that

have greater tissue activity. In this setting, a strategy using a dual approach, targeting both eosinophilopoiesis and chemotaxis, may be particularly effective and requires further investigation. One approach that has been reported is the use of antisense oligonucleotide therapy to target CCR 3 and the common beta-subunit receptor for IL 5, IL 3 and GM-CSF. In this study, attenuation of the inflammatory and airway responses following allergen challenge was achieved in mild asthmatics (261).

Despite the selective inclusion criteria used at study entry, there was considerable heterogeneity in the response to mepolizumab. This is not unexpected and would be in keeping with our finding of distinct phenotypes of eosinophilic asthma.

The basis for observed differences in clinical expression between the clusters of eosinophilic asthma is unclear. We suggest that the clusters may differ in respect of the anatomical site of tissue eosinophil accumulation and / or the level of eosinophil activation. Accumulation more distally in a clinically silent portion of the lung or tissue accumulation with 'latent' eosinophils may give rise to the inflammation predominant cluster of eosinophilic asthma. These possibilities require further study and may have implications for differences in the pathogenesis of severe exacerbations between the groups. This is discussed further below.

7.4 Characterising severe exacerbations

An important limitation of clinical trials is the absence of an objective definition for severe exacerbations. The accepted definition is subjective and based on the clinically determined need for a course of high dose oral glucocorticoid therapy. In recurrent exacerbators, frequent courses of prednisolone are an important source of morbidity. Yet exacerbations, like stable asthma are heterogeneous and the efficacy of prednisolone for different types of exacerbation is likely to vary.

7.4.1 Non-eosinophilic exacerbations

In their studies, Jayaram (25) and Nair (262) safely withheld oral glucocorticoid therapy for treat non-eosinophilic exacerbations. In our study, severe exacerbations in subjects receiving mepolizumab were eosinophilic on only one third of occasions. This would

suggest that if eosinophilic airway inflammation is an important determinant of glucocorticoid response at the time of exacerbation then the number of exacerbation episodes requiring treatment with prednisolone could be reduced still further in patients given mepolizumab. Studies to determine responsiveness to oral glucocorticoids at the time of severe exacerbation are of considerable clinical importance. Biomarkers are needed that are measurable at the time of exacerbation and inform prognosis, both in respect of severity of the presenting event and the likely response to glucocorticoids.

7.4.2 Exacerbations in eosinophilic patients

In a post-hoc analysis of the mepolizumab study, we found FEV1 response to oral glucocorticoid at baseline was a significant predictor of response to mepolizumab over 12 months. Moreover, for subjects receiving mepolizumab, the FEV1 response to glucocorticoid therapy was attenuated at the end of the study. Together, these observations suggest a role for eosinophils in both lung function decline and exacerbations. In subjects where the two are related, a linear chain of events is suggested with a direct role for accumulating eosinophilic airway inflammation in declining lung function that precipitates a clinical exacerbation [Fig 22]. In this setting, it follows that poorly-controlled asthma progresses to a severe exacerbation that is glucocorticoid responsive. Of the two eosinophil-predominant phenotypes we described in difficult asthma, this linear one-step model would best fit with subjects that have concordant inflammation and symptoms.

In contrast, for subjects with eosinophil inflammation predominant asthma, eosinophils may have a less direct role in the pathogenesis of severe exacerbations. I hypothesise that a second unpredictable triggering event catalyses the rapid onset of a severe exacerbation in which activated tissue eosinophils that are already resident have an important effector role [Fig 22]. One implication of this proposed multi-step or multi-hit model is that severe exacerbations are less frequent for this phenotype, but when they occur, may be of greater severity. In keeping with this, we found that compared with the concordant cluster, subjects of the inflammation predominant cluster had fewer severe exacerbations per year but the number of past hospital and

intensive care unit admissions for exacerbations were similar between the groups. If this model is accurate then the efficacy of anti-eosinophilic therapies for preventing severe exacerbations may not be readily measurable in subjects with inflammation predominant asthma.

7.4.3 Summary

From our data, two important questions arise:

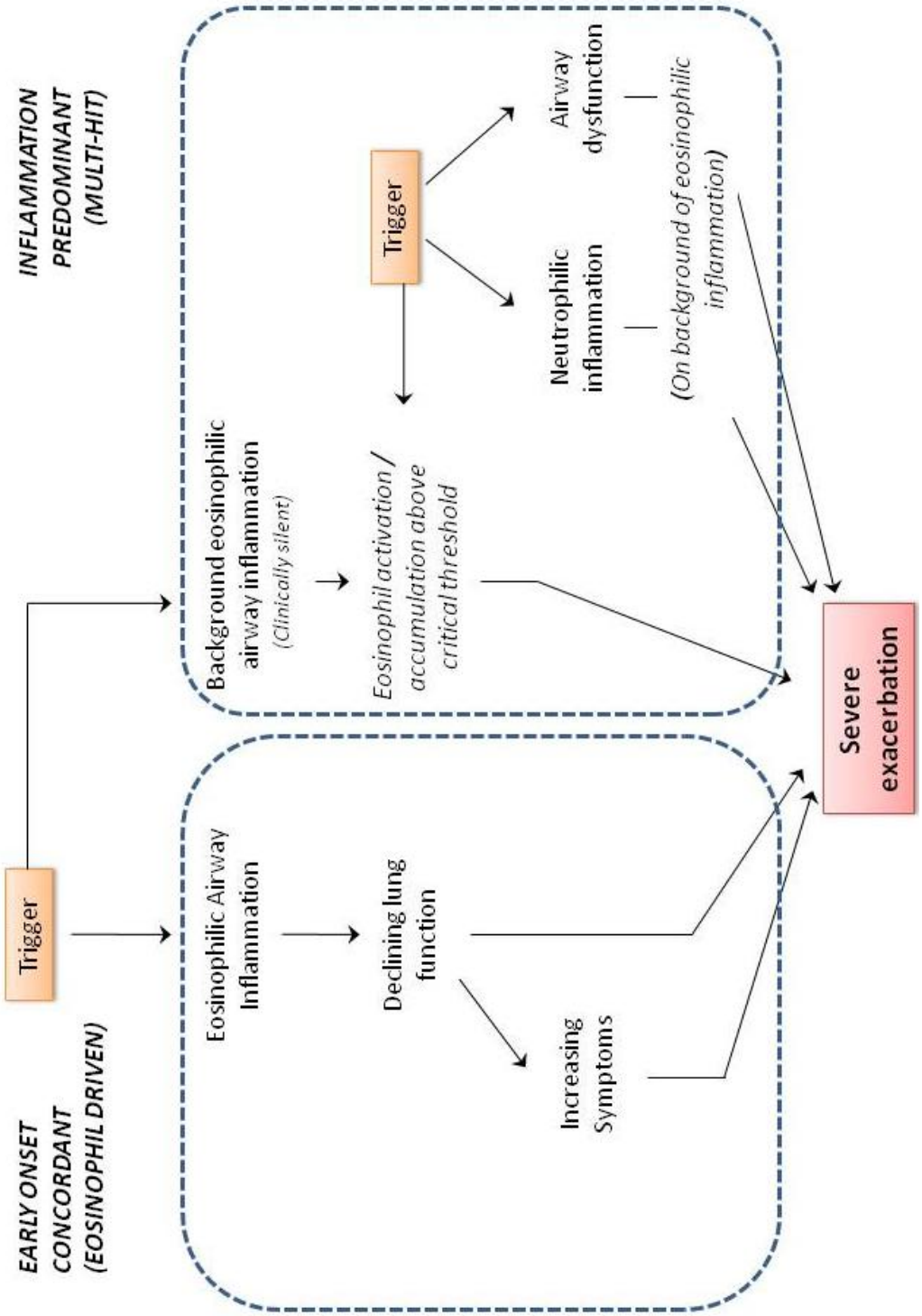
1. Does FEV_1 response with oral glucocorticoid therapy in refractory asthma at stable state, predict glucocorticoid responsiveness at the time of exacerbation?

2. Can we use cluster information obtained at stable state to predict the likely pattern and profile of subsequent exacerbations?

The holy grail of asthma management is to develop patient-specific personalised prescriptions. Correctly predicting the exacerbation type to which a patient is susceptible raises the possibility of providing an enhanced self management plan and may avoid the need to measure biomarkers at the time of exacerbation.

It is hoped that the outcomes presented in this thesis positively inform the direction of future research to answer the important questions that will benefit the future care of patients with asthma.

Figure 22: Proposed models of exacerbation pathogenesis in eosinophilic asthma phenotypes



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