Management and mechanisms of severe eosinophilic asthma

Rahul Shrimanker
St Edmund Hall
University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy

Michaelmas 2020

Contents

List of tables

List of figures

Abstract

Severe asthma is a complex problem in which eosinophilic asthma is an over-represented phenotype compared to mild and moderate asthma. Asthma attacks, for which persistent eosinophilic inflammation is a major risk factor, are the most clinically concerning aspect of this disease as they result in high healthcare use and spending, treatment related morbidity due to oral corticosteroids, and reduced quality of life for patients.

I studied the response to monitored inhaled corticosteroids (ICS) in patients with severe eosinophilic asthma. Patients who suppressed FeNO, a marker of eosinophilic inflammation, had marked reduction in mediators of eosinophilic inflammation in the airways. This was in contrast to those who did not suppress FeNO, suggesting ICS-resistant pathways. Prostaglandin D_2 (PGD2) was particularly non-responsive in this group with ICS-resistant eosinophilic inflammation.

I next studied the effect of timapiprant, an antagonist of the PGD_2 receptor CRTH2, in a randomised, placebo-controlled trial in severe eosinophilic asthma. This study found a trend towards a clinically significant lowering of sputum eosinophils compared to placebo, but no changes in lung function or symptom scores. These findings are in keeping with trials of other targeted anti-inflammatory treatments in asthma.

Finally, the effects of mepolizumab, an anti-IL-5 antibody treatment, on exacerbations of severe eosinophilic asthma were investigated for the first time. In novel *post hoc* analyses I found that exacerbations that occur on mepolizumab are less severe in terms of changes in lung function and symptoms, are associated with less eosinophilic airway inflammation, and may be less steroid-responsive than those on placebo.

In combination, these findings suggest a role for CRTH2 antagonists in the management of severe, treatment-refractory eosinophilic asthma. Further, larger studies required to explore this. The effects of anti-inflammatory treatments on asthma exacerbations needs to be studied in more detail as my analyses have shown them to be different in nature to those on placebo. The underlying drivers of exacerbations occurring on type-2 targeted treatments need to be further understood. These exacerbations may require a different treatment approach tailored towards the underlying pathology.

Acknowledgements

The work contained in this thesis would not have been possible without the contribution of a number of people to whom I am very grateful.

Foremost, I would like to thank my supervisor and mentor, Professor Ian Pavord, for his unending support, guidance, enthusiasm, and patience throughout this process.

Thank you to the specialist nurses and good friends, Clare Connolly and Katie Borg, who looked after our patients with such care and professionalism and made them feel valued at every stage.

Thank you to Professor Luzheng Xue for sharing his and expertise with flow cytometry, to Samantha Thulborn for her hard work with the huge number of sputum samples generated and the trial and laboratory management, and to Dr Tim Powell for his teaching and help with the laboratory work.

Thank you to my parents, Ila and Kishore, for their enduring love and support which they have shown me at every stage. Thank you to my partner Emily being there for me through the highs and lows of this work.

Finally, thank you to our patients who gave up their time and were enthusiastic and engaged throughout.

Statement of work personally performed

I designed the observational study of severe asthma, discussed in section 4, with Professor Pavord, wrote the protocol, and obtained regulatory and local approvals. I recruited all the patients and performed approximately 30% of the study visits. I performed the data analysis.

The randomised controlled trial of timapiprant, described in section 5, was designed by Professor Pavord. I co-wrote the protocol and subsequent amendments and obtained necessary regulatory and local approvals. I recruited all the study participants, performed all the screening visits and approximately 30% of the follow-up visits including sputum induction. I collected, processed, and performed approximately 10% of all the flow cytometry measurements. I performed the data analysis.

I performed all the measurements and analysis of sputum, blood and urine mediators presented in this thesis.

For the analysis of severe asthma exacerbations in section 6, I formulated the research questions and obtained the study data. When individual patient data was available, I performed the analysis, otherwise I requested specific additional summary data or analysis from the data holders.

Publications arising from this thesis

Original papers

Shrimanker, R., Keene, O., Hynes, G., Wenzel, S., Yancey, S., & Pavord, I. D. (2019). Prognostic and Predictive Value of Blood Eosinophil Count, Fractional Exhaled Nitric Oxide, and Their Combination in Severe Asthma: A Post Hoc Analysis. Am J Respir Crit Care Med, 200(10), 1308-1312. doi:10.1164/rccm.201903-0599LE

Shrimanker, R., Pavord, I. D., Yancey, S., Heaney, L. G., Green, R. H., Bradding, P., . . . Haldar, P. (2018). Exacerbations of severe asthma in patients treated with mepolizumab. Eur Respir J, 52(6). doi:10.1183/13993003.01127-2018

Luo, J., Wu, Y., Shrimanker, R., Go, S., Ye, Y., Hardman, C., . . . Xue, L. (2019). Resistance to apoptosis underpins the corticosteroid insensitivity of group 2 innate lymphoid cells. J Allergy Clin Immunol, 144(6), 1722-1726 e1710. doi:10.1016/j.jaci.2019.08.008

Hilvering, B., Hinks, T., Stöger, L., Marchi, E., Salimi, M., Shrimanker, R., . . . Go, S. (2018). Synergistic activation of pro-inflammatory type-2 CD8+ T lymphocytes by lipid mediators in severe eosinophilic asthma. Mucosal immunology.

Reviews and editorials

Shrimanker, R., & Pavord, I. D. (2018). Will precision medicine become an effective tool for airway disease? *Per Med, 15*(4), 243-245. doi:10.2217/pme-2018-0027

Shrimanker, R., Beasley, R., & Kearns, C. (2018). Letting the right one in: evaluating the generalisability of clinical trials. *Eur Respir J, 52*(6). doi:10.1183/13993003.02218-2018

Shrimanker, R., & Pavord, I. D. (2017). Protein Therapeutics in Respiratory Medicine. In *Protein Therapeutics* (pp. 587-610): Wiley-VCH Verlag GmbH & Co. KGaA.

Shrimanker, R., & Pavord, I. D. (2017). Interleukin-5 Inhibitors for Severe Asthma: Rationale and Future Outlook. *BioDrugs, 31*(2), 93-103. doi:10.1007/s40259-017-0215-8

Shrimanker, R., Choo, X. N., & Pavord, I. D. (2017). A new approach to the classification and management of airways diseases: identification of treatable traits. *Clin Sci (Lond), 131*(10), 1027-1043. doi:10.1042/CS20160028

Pavord, I. D., Hilvering, B., & Shrimanker, R. (2016). Emerging biologics in severe asthma. *Immunology and Allergy Clinics, 36*(3), 609-623.

Abstracts

Shrimanker, R., Borg, K., Connolly, C., Thulborn, S., Cane, J., Xue, L., . . . Pavord, I. D. (2019). Late Breaking Abstract - Effect of timapiprant, a DP2 antagonist, on airway inflammation in severe eosinophilic asthma. *European Respiratory Journal, 54*(suppl 63), RCT3784. doi:10.1183/13993003.congress-2019.RCT3784

Ye, Y., Shrimanker, R., Batty, P., Borg, K., Connolly, C., Thulborn, S., . . . Pavord, I. D. (2019). Late Breaking Abstract - An assessment of potential predictive biomarkers for the treatment of severe eosinophilic asthma with CRTH2 antagonists. *European Respiratory Journal, 54*(suppl 63), PA3708. doi:10.1183/13993003.congress-2019.PA3708

Shrimanker, R., Pavord, I., Price, R., Bradford, E., Keene, O., & Yancey, S. (2018). Fractional Exhaled Nitric Oxide and the Peripheral Blood Eosinophil Count as Biomarkers of the Response to Mepolizumab in Patients with Severe Eosinophilic Asthma. In *C101. ASTHMA CLINICAL AND MECHANISTIC STUDIES* (pp. A5964-A5964): American Thoracic Society.

Borg, C., Shrimanker, R., Connolly, C., Gittins, A., Thulborn, S., Cane, J., . . . Pavord, I. (2018). P135 Recognition of eosinophilic asthma: are routine clinical measures useful? In: BMJ Publishing Group Ltd.

Shrimanker, R., Hargaden, B., Bradding, P., Wardlaw, A., Brightling, C., Green, R., . . . Haldar, P. (2017). Mepolizumab and the response to oral prednisolone in patients with severe eosinophilic asthma. *European Respiratory Journal, 50*(suppl 61). doi:10.1183/1393003.congress-2017.PA3967

Shrimanker, R., Hargaden, B., Bradding, P., Wardlaw, A., Brightling, C., Green, R., . . . Heaney, L. (2017). Characterisation of exacerbations of severe eosinophilic asthma (SEA) on mepolizumab compared to placebo. *European Respiratory Journal, 50*(suppl 61). doi:10.1183/1393003.congress-2017.PA3971

1 Introduction

Asthma is a chronic, inflammatory condition of the lower airways and is the most common respiratory condition in the Western world. Despite effective treatments, asthma is still responsible for substantial morbidity, mortality, and healthcare utilisation. These patient, financial and societal costs are seen most in severe asthma, which persists despite the application of guideline-based treatments. Eosinophils are inflammatory white blood cells which are essential in the pathogenesis of asthma in around 30% of cases but are disproportionately highly represented in severe asthma, a complex and substantial clinical problem.

This chapter will review the current understanding of asthma biology, classification, treatment and outline the current clinical problems in managing severe eosinophilic asthma.

1.1 Asthma

Asthma is a common respiratory condition characterised by episodes of shortness of breath, wheeze, and cough due to airflow limitation as a result of an increased tendency of the airway to narrow (airway hyperresponsiveness), airway mucosal inflammation and increased airway mucus production.

1.1.1 History of asthma treatments

Symptoms of asthma have been recognised and described at least since the time of the Ancient Egyptians, from around 3000 BC, who used inhaled smokes of various plants as a treatment[1]. The first recorded use of the term asthma, from the Greek *aazein* for panting, was by Hippocrates (360-470 BC) and was used to include any form of breathlessness. The use of the label asthma has been added to and refined over time. The 19th Century physician Dr Henry Hyde Salter, who suffered from asthma from childhood onwards, wrote vivid descriptions of the effects of asthma and produced detailed diagrams of the airways including smooth muscle and the effects of smooth muscle constriction on bronchial diameter of constriction. Salter described asthma as "paroxysmal dyspnoea of a peculiar character with intervals of healthy respiration between attacks" [2]. This description, and many like it, emphasise the episodic nature of asthma with a remission of symptoms in between episodes of dyspnoea.

The symptoms of asthma were thought to be primarily due to bronchoconstriction due to airway smooth muscle hyper-reactivity and/or hypertrophy which was seen as the

key pathological process in asthma. The idea of asthma as a disease of airway smooth muscle abnormality and resultant bronchospasm led to the identification, development and application of bronchodilators as the mainstay of asthma treatment [3]. These treatments started with unselective β2-receptor antagonists such as the subcutaneous use of the newly identified hormone adrenaline in the early 1900's and isoprenaline in the 1940's. More selective β2-receptor agonists such as fenoterol and salbutamol were discovered in the 1960's and were delivered orally or by nebulisation with cumbersome equipment. It was noted that inhaled therapy had better clinical effect with fewer adverse events [4] and the invention of pressurised inhaler devices and improved drug preparation techniques allowed more selective β2-receptor agonists to be more easily administered.

Bronchodilator treatments were well tolerated and effective in the immediate relief of asthma symptoms and so were widely used as the mainstay of treatment for asthma. Despite seemingly effective treatments, spikes in asthma deaths were noted between the 1960s and 1980s and were thought to be related to over-use of bronchodilator treatments. Examination of these excess deaths helped lead to rethink of asthma mechanisms and treatments. Developing insights into the effects of allergens on mast cells, airway inflammatory mediators and airway inflammation on asthma symptoms led to the view that asthma was, as well as a condition of smooth muscle dysfunction, a condition of airway inflammation. This fundamental change in thinking led to the development of inhaled corticosteroids as an anti-inflammatory treatment for asthma. This change markedly improved asthma symptoms, quality of life, exacerbation

frequency and asthma related mortality [5]. The mechanisms involved in airway inflammation are discussed in detail in section 1.1.2.

It is now clear that asthma is a complex, heterogeneous condition and the term has, over time, evolved from a symptom-based descriptor to a more specific label encompassing symptoms, abnormality of airway function and airway inflammation.

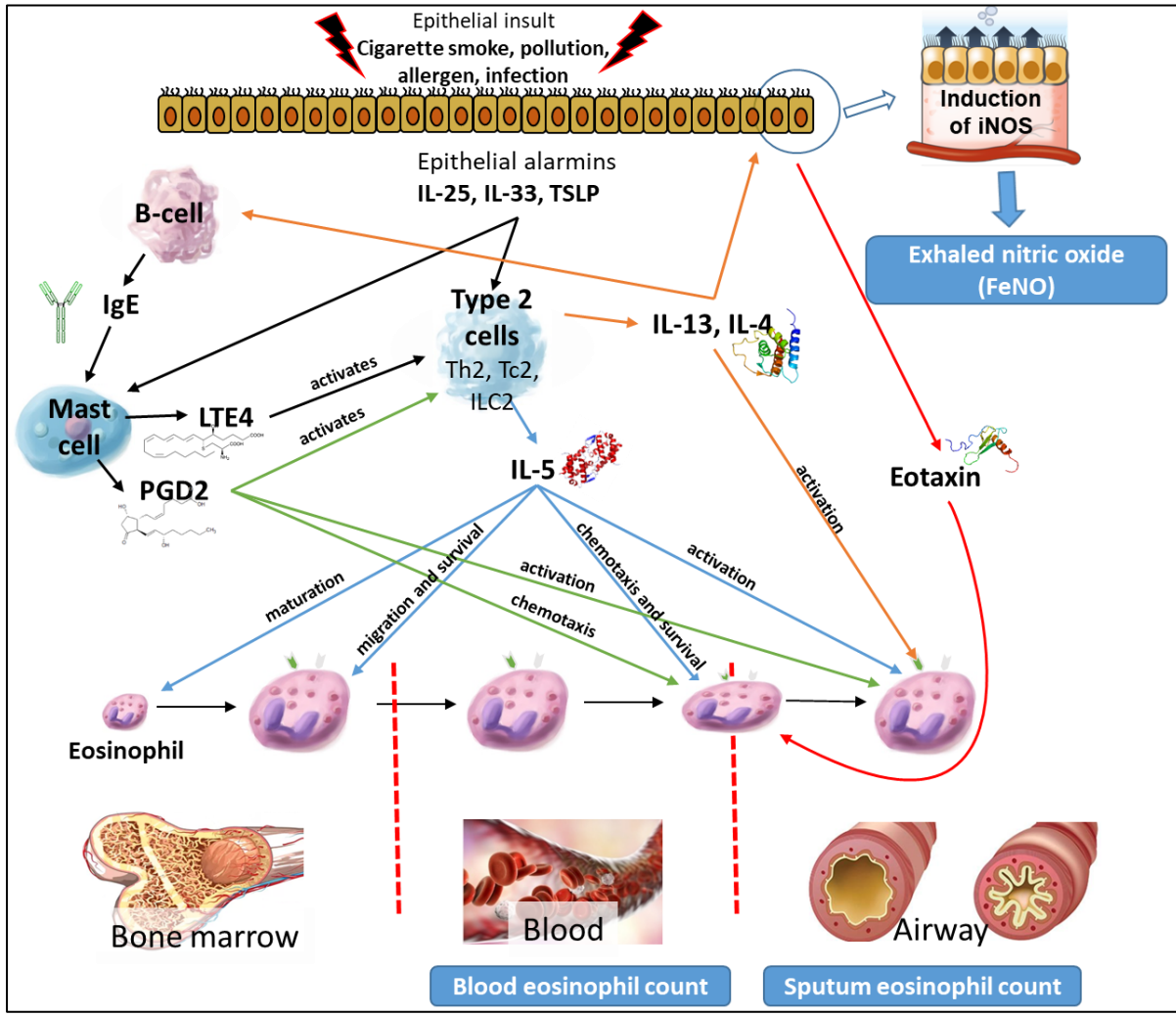
1.1.2 Pathology

Inflammation in the airways is driven by two main pathophysiological themes, namely type-2 driven mechanisms resulting in eosinophilic airway inflammation, and non-type-2 mechanisms such as the T-helper (Th)1 and Th17 pathways often resulting in neutrophilic airways inflammation. Interactions between the bronchial epithelium and external factors are thought to be the driving force in producing airways inflammation. Eosinophilic airway inflammation is thought to be triggered by type-2 cytokines produced by T cells and eosinophils, amongst other cells, as a result of airway epithelial insult by allergens, parasites or irritants that come into contact and pass through the epithelial barrier. Dendritic cells process antigens and trigger a response after binding to T-helper cells at regional lymphoid sites. CD4+ lymphocytes are polarised in to Th2 cells and produce the cytokines IL-4, IL-5, IL-9 and IL-13. IL-4 produced by Th2 cells causes a further drive for naïve Th cells to differentiate into Th2 cells and causes an immunoglobulin class switch, resulting in IgE production by B-cells. IgE subsequently binds to mast cells and eosinophils residing in the tissue, enabling them to release their

toxic granules upon antigen binding. Mast cells release a number of substances including the locally active histamine and the lipid mediators leukotriene E_4 and prostaglandin D_2 which further orchestrate eosinophilic inflammation.

IL-5 is essential for the production, maturation and activation of eosinophils. Locally it acts as a chemo-attractant and causes migration of eosinophils to sites of damage. IL-5 functions also in combination with IL-9 to recruit mast cells and eosinophils to an affected tissue site. IL-13 acts with IL-4 in inducing IgE production by B-cells, induces mucus production by goblet cells, causes goblet cell metaplasia and increases airway hyperresponsiveness by a direct effect on airway smooth muscle. The key known and hypothesised underlying mechanisms resulting in type-2 inflammation and their clinical implications are summarised in Figure 1.1.

Figure 1.1 Key pathways in the production of type-2 inflammation



Type-2 targeted treatments		
Target	**Drugs**	**Progress**
Ig-E	Anti Ig-E (omalizumab)	Available for atopic asthma
IL-5	Anti IL-5, Anti-IL5R (Mepolizumab, benralizumab, reslizumab)	Available for eosinophilic asthma
IL-13	Anti IL-13 and IL-4 (dupilumab)	Awaiting NICE approval for severe asthma
PGD2	Oral CRTH2 antagonists	Late phase clinical trials
TSLP	Anti-TSLP (tezepelumab)	Early phase clinical trials
IL-33	Anti-IL-33	Early phase clinical trials

Effect of treatments on biomarkers				
Treatment	**Blood eosinophils**	**Sputum eosinophils**	**FeNO**	**IgE**
Anti IgE (omalizumab)	↔	↔	↓	↔
Anti- IL-5 (Mepolizumab benralizumab, reslizumab)	↓↓	↓	↔	↔
Anti IL-13 and IL-4 (dupilumab)	↑	↓	↓↓	↔
CRTH2 antagonist	↔	↓	?	?
TSLP	↓↓	↓↓	↓↓	↓↓
IL-33	?	?	?	?

1.1.3 Severe asthma

Most patients with asthma can be well controlled on inhaled therapy consisting of beta-2 adrenergic receptor agonists to control bronchospasm and inhaled corticosteroids (ICS) to control the underlying inflammation. Various treatment guidelines for asthma suggest a stepwise increase in asthma medications and dose to achieve control [6-9]. These range from Step 1 where short-acting beta-2 agonists (SABA) are used as needed and the introduction of a low dose ICS can be considered, to Step 5 where patients require high dose ICS, inhaled long-acting beta-2 agonist (LABA) and additional systemic treatment such as oral corticosteroids or biological treatments [6]. Despite the effective treatments for asthma, around 10% of patients with asthma have severe asthma with ongoing symptoms and/or recurrent asthma attacks despite high dose asthma therapy at GINA steps 4 or 5 [7].The most recent, internationally agreed definition of severe asthma is shown in Table 1.1.

Severe asthma is a complex clinical problem with high level of morbidity and associated healthcare utilisation and spending. In addition to the morbidity and mortality burden due to asthma itself, there is substantial morbidity related to asthma treatments, particularly oral corticosteroids. Long term oral steroid treatment for asthma is associated with cardiovascular, psychiatric, gastrointestinal, bone and metabolic disturbance [10].

Table 1.1 ATS/ERS Definition of severe asthma for patients aged ≥6 years [7]

Asthma which requires treatment with guidelines suggested medications for GINA steps 4–5 asthma (high dose ICS# and LABA or leukotriene modifier/theophylline) for the previous year or systemic CS for ≥50% of the previous year to prevent it from becoming "uncontrolled" or which remains "uncontrolled" despite this therapy Uncontrolled asthma defined as at least one of the following: 1) Poor symptom control: ACQ consistently ≥1.5, ACT <20 (or "not well controlled" by NAEPP/GINA guidelines) 2) Frequent severe exacerbations: two or more bursts of systemic CS (≥3 days each) in the previous year 3) Serious exacerbations: at least one hospitalisation, ICU stay or mechanical ventilation in the previous year 4) Airflow limitation: after appropriate bronchodilator withhold FEV_1 <80% predicted (in the face of reduced FEV_1/FVC defined as less than the lower limit of normal) *Or* Controlled asthma that worsens on tapering of these high doses of ICS or systemic CS (or additional biologics)

GINA: Global Initiative for Asthma; LABA: long-acting β2-agonists; CS: corticosteroids; ACQ: Asthma Control Questionnaire; ACT: Asthma Control Test; NAEPP National Asthma Education and Prevention Program.

The assessment and management of severe asthma to achieve asthma optimal treatment and asthma control is complex. This is often because the the diagnosis of severe asthma is incorrect, or that asthma is uncontrolled because of a failure to master the basics of asthma management including treatment adherence, inhaler technique and smoking cessation. Another consideration is that asthma-like symptoms may also persist because of the presence of a comorbid condition such as obesity, rhinitis and psychosocial issues. Because of the complexity of the condition, it is recommended that patients with severe asthma are managed by a specialist in a centre with access to detailed airway tests and a multidisciplinary team that includes specialist nurses, physiotherapists and pharmacists [7, 11, 12].

1.1.4 Clinical problem – asthma attacks

Patients with asthma are at risk of asthma exacerbations (or asthma attacks) where symptoms and airflow limitation worsen and become less responsive to usual rescue treatment such as short-acting beta--agonists. These attacks are the most clinically important aspect of the disease as they can result in severe symptoms requiring unscheduled medical help including urgent review in primary or secondary care or hospital admission. Patients with asthma who have asthma attacks also experience a poorer quality of life compared to those who do not [13].

Asthma attacks still result in over 1200 deaths every year in the UK and, in the most recent figures available, over 1400 deaths were attributable to asthma in 2017. The National Review of Asthma Deaths (NRAD), a multi-agency review of all suspected asthma mortality in the UK [14], looked in detail 195 asthma deaths in 2012-2013 and concluded that the majority were the result of basic errors in asthma management and were thus readily preventable. Hospital admission rates and deaths from asthma in most developed countries halved in the 10-15 years after the publication of the first asthma treatment guidelines advocating earlier and more aggressive use of inhaled corticosteroids in 1990 [15, 16]. It is of concern, however, that these outcomes have not improved much over the last 10 years despite increased spending on asthma treatment [17].

As well as a high healthcare utilisation burden, asthma has a substantial financial cost. The cost of asthma to the UK is over £2.3 billion per year [18]. Patients with a recent history of an asthma exacerbation have particularly high annual healthcare costs, estimated to be three times those of a patient with asthma and no history of a previous exacerbation[19]. In severe asthma, there is an increased risk of asthma exacerbations and this small subgroup accounts for a disproportionately high amount of the healthcare spending, morbidity and mortality due to asthma [20, 21].

There has been an increasing focus on asthma exacerbations as their clinical importance and implications have been recognised [22, 23]. Treatments targeted at reducing asthma exacerbations are therefore an active area of interest and has led to trials designed at looking at treatment effects on asthma exacerbations as well as other measures associated with asthma such as symptom scores, quality of life indicators and lung function measurements. Although this focus on exacerbations is welcome, there are some problems when using exacerbations as a trial end point. GINA [6] define acute exacerbations as *"episodes of progressive increase in shortness of breath, cough, wheezing, or chest tightness, or some combination of these symptoms, accompanied by decreases in expiratory airflow that can be quantified by measurement of lung function".* GINA guidelines also define exacerbations as an acute loss of asthma control that requires urgent treatment. These treatments can range from increasing short-acting beta-2-agonist or increasing inhaled corticosteroid use to the use of oral steroids, which are the mainstay of treatment for asthma exacerbations. Treatment of exacerbations of

airways disease, encompassing asthma and COPD exacerbations is the most common indication for prescriptions of oral corticosteroids in the United Kingdom [24].

These definitions of exacerbations rely on subjective reports of symptoms from patients and the application of patient or physician directed treatments and so are open to bias, with either over or under-reporting and treatment of exacerbations possible. Trials of treatments aimed at reducing exacerbations have therefore mainly focused on severe exacerbations which are defined as the requirement for oral corticosteroid treatment. This treatment-defined definition is pragmatic and clinically relevant as exacerbation events requiring oral corticosteroid rescue treatment are more severe clinically and most of the treatment related morbidity in asthma is related to oral corticosteroids.

Ideally, a more biomarker and/or physiology directed definition of exacerbations is needed, but this requires a deeper understanding than we currently have of the interplay of airway calibre, mucus hypersecretion, airway inflammation and infection and their relationship to symptoms and quality of life.

1.1.5 Recognising asthma heterogeneity

"….. once we have cast diseases into these vast receptacles, these aetiological dustbins, they are satisfactorily accounted for. I believe that those brave enough to lift the lids off these bins and poke about among the rubbish there may find clinical salvage of inestimable value." – Dr Richard Asher, 1954 [25]

Asthma is a diagnosis used to encapsulate a constellation of symptoms including episodic shortness of breath, wheeze and cough and evidence of variable airflow obstruction. This umbrella term does not, however, convey any information about the underlying mechanisms resulting in these findings. A developing knowledge of the underlying mechanisms, such as those described in section 1.1.2 has shown asthma to be a heterogeneous disease involving several inflammatory pathways influenced by genetic, environmental, and infectious factors.

An early system to classify asthma according to its clinical features (or phenotype) was introduced by Dr Rackemann in 1947 [26]. This system classified asthma as either allergic ('extrinsic') or non-allergic ('intrinsic') based on the age of onset and the presence of allergies. Extrinsic asthma classically starts in the early school years and is typically associated with other allergic diseases such as rhinitis and eczema, often with a family history of allergic disease. Patients commonly notice wheeze and other symptoms after exposure to allergens such as house dust mite, animal dander and grass pollen. In contrast, intrinsic asthma classically starts in adulthood and is more common in females. There is no clear allergic trigger identified. Intrinsic asthma tends to present as more severe asthma with a higher rate of severe exacerbations and is less responsive to usual asthma treatments [27]. These clinical phenotypes are still used to classify asthma however it is now evident that there is no pathology that is unique to 'extrinsic' or 'intrinsic' asthma and that these labels are not helpful in determining treatments. An example of this is the use of omalizumab, an anti-IgE monoclonal antibody licenced for use in persistent, allergic asthma which has shown clinical efficacy in reducing

exacerbations [28-30]. Restrictions on its use based on patient weight, a requirement for allergy and a limited range of serum IgE levels have limited use of this agent and its impact has not been as great as that of biological agents in other disease areas. Two cohort studies have shown that omalizumab is as effective in patients with a label of non-allergic asthma as it is in those with allergic asthma. De Llano *et al.* studied 29 patients with non-allergic asthma and found the same improvement in lung function, symptoms and exacerbation frequency as seen in 226 patients with allergic asthma that were also treated with omalizumab [31]. Grimaldi-Bensouda *et al.* studied a cohort of 767 patients with uncontrolled asthma and found that omalizumab was effective in reducing exacerbations when given as an add-on treatment regardless of the label of allergic or non-allergic asthma.

Labels that are not related to an underlying mechanism (or endotype) or a response to treatment are problematic. Treatments can be withheld from patients that would benefit, as discussed above, or given to patients in whom there is no evidence of efficacy such as azithromycin given to current smokers with chronic obstructive pulmonary disease [32, 33]. Diagnostic labels can also hinder drug discovery. An example of this is the case of the anti-IL-5 monoclonal antibody mepolizumab. Two of the early trials of mepolizumab showed that it dramatically reduced the blood and sputum eosinophil count in patients with asthma but did not improve airway responsiveness, lung function or quality of life [34, 35]. The disappointing clinical effect of treatment led many to question whether eosinophilic inflammation played as important a role in asthma as previously thought. However, other potential explanations for the observed lack of

clinical efficacy was that the drug was being used in patients who did not have active eosinophilic inflammation, and that the trials were looking at the wrong outcomes. Patients with active eosinophilic disease are at an increased risk of exacerbations but it has been shown that symptoms and exacerbation risk are relatively independent each other and so reducing eosinophilic inflammation may not improve symptoms as much as reducing exacerbation frequency [36, 37]. This change in thinking led to the design and development of appropriate trials, in the right subjects, looking at the most responsive clinical outcomes. These have shown dramatic improvements in exacerbation rates in subjects with eosinophilic asthma who are treated with mepolizumab [38, 39].

It has been acknowledged that diagnostic labels and treatment guidelines can oversimplify a complex disease population and that a more personalised approach is preferable [40-42]. Agusti *et al.* suggest a 'treatable traits' approach - by deconstructing the umbrella term 'asthma' into its component parts and identifying the differing underlying mechanisms, treatments can be targeted to the processes that are active and relevant to the individual patient [43]. This requires recognising traits that are a) identifiable and b) treatable. Identification of traits requires the recognition and/or development of clinically accessible biomarkers; these are discussed further in section 1.1.7. Clinically useful treatable traits applicable to asthma, and potential treatments targeted at them, are summarised in Table 1.2.

Table 1.2 Treatable traits in asthma

Trait	How to measure	Definition	Treatment	Clinical effect	Strength of effect
Airflow limitation	Spirometry (primary care, respiratory clinic or lung function lab)	FEV1/FVC ratio of < 0.7	1. Bronchodilators (LABA, LAMA or LABA/LAMA)[44] 2. ICS[45] 3. Bronchial thermoplasty[46]	Improved daily symptoms, reduction in exacerbations, improved lung function	1. +++ 2. +++ 3. +
Airway hyperresponsiveness	Methacholine challenge Bronchodilator reversibility	PC20 < 8 mg/ml Improvement in FEV1 of 15% and/or 200ml	As above		
Eosinophilic airway inflammation	A. Induced sputum B. Blood C. FeNO	A. Eosinophil count ≥ 3% in sputum B. Eosinophil count ≥ 2% in blood C. FeNO > 25 ppb	1. Corticosteroids (inhaled or oral)[47, 48] 2. Biologics (anti-IL5, anti-IgE)[39, 49, 50]	Reduced exacerbations and improved daily symptoms	1. +++ 2. +++
Chronic airway infection	Sputum microscopy, culture and sensitivity	Colonisation of the airways by bacteria	Long-term low dose macrolide antibiotics[51]	Reduced exacerbations	++
Bronchiectasis	CT scan	Abnormal dilation of the small airways predisposing to excess sputum production and infections	1. Airway clearance techniques (e.g. physiotherapy)[52] 2. Mucolytics 3. Macrolides[53] 4. Surgery in single lobe bronchiectasis	Reduction in daily sputum production and number of exacerbations	1. + 2. + 3. ++ 4. +++ (in selected patients)
Cough reflex hypersensitivity	A. Cough questionnaire B. Cough counts C. Capsaicin challenge	A&B. > 100 coughs/day C. Research tool, no defined 'normal' value	1. Speech and language therapy[54] 2. Gabapentin[55]	Reduction in cough frequency +/- patient awareness of cough	1. ++ 2. +
Obesity	Weight in kilograms over height in metres squared	BMI > 30 kg/m2	Weight loss by[56-59] 1. Diet 2. Exercise 3. Bariatric surgery	Improved daily symptoms and lung function, reduction in severe exacerbations	++
Gastro-oesophageal reflux	A. Patient account	A. Symptoms	1. Proton pump inhibitors / H2 antagonists	Improved daily symptoms	1. + 2. +

	B. Oesophageal pH monitoring	B. Significant drops in oesophageal pH	2. Surgery		
Upper airways disease (e.g. vocal cord dysfunction)	A. Laryngoscopy B. Flow-volume loop	A. Paradoxical adduction of vocal cords during inspiration, expiration or both B. Flattened inspiratory curve	1. Speech and language therapy[60] 2. Psychotherapy	Improved daily symptoms and fewer exacerbations	1. ++ 2. +
Deconditioning	Cardiopulmonary exercise testing	Reduced exercise capacity with no pulmonary or cardiac limitation	Exercise and education	Improved daily symptoms Unknown effect on exacerbation	+
Poor treatment adherence	A. Prescription monitoring B. Chipped inhaler devices C. FeNO suppression testing	A/B. < 80% adherence = poor adherence < 50% adherence = very poor adherence C. FeNO value change over a week when monitored inhaler is used	1. Education[61] 2. FeNO suppression test as educational tool[62] 3. IM corticosteroids in those with eosinophilic disease and poor adherence	Improved daily symptoms, reduction in exacerbations, improved lung function	1. + 2. ++ 3. +++ (in selected patients)
Aspirin sensitivity / aspirin induced asthma	A. Aspirin challenge B. Prior history	Acute asthma symptoms on taking aspirin / NSAID drugs	1. Aspirin desensitisation[63] 2. Leukotriene receptor antagonists[64]		1. ++ 2. ++

+ Some effect of treatment or limited evidence of effect, ++ Moderate effect of treatment, +++ Good effect of treatment. FEV1 Forced expiratory volume in one second (litres), FVC Forced vital capacity (litres), LABA Long acting beta agonist, LAMA Long acting muscarinic antagonist, ICS inhaled corticosteroid, PC20 Provocative concentration, IL-5 Interleukin-5, IgE Immunoglobulin E, FeNO Fractional exhaled nitric oxide, ppb Parts per billion, NSAID non-steroidal anti-inflammatory drug

1.1.6 Severe eosinophilic asthma as a clinical phenotype

The clinical importance of recognising eosinophilic airway inflammation was described by Dr Morrow Brown in a study in 1958 [65]. Oral corticosteroids are a lifesaving treatment for asthma and represent one of the mainstays of treatment today. However, a Medical Research Council study in 1956 of oral corticosteroids in asthma [66] showed no benefit over rescue bronchodilator treatment. This was a surprise to experienced chest physicians, who had seen the beneficial effects of oral corticosteroids in their treatment of patients with asthma. Dr Morrow Brown conducted a further trial of oral corticosteroids in asthma, this time also evaluating the sputum of subjects prior to treatment using his medical student microscope. He found the presence of eosinophils in sputum was associated with the response to oral corticosteroids and that treatment was clearly effective in the subgroup with eosinophilic sputum[65]. These findings were, however, largely overlooked for many years with asthma treatments being dispensed in a non-targeted fashion.

The recognition of severe eosinophilic asthma as a pathologically distinct phenotype of asthma has led to a recent increase in targeted asthma research leading to new, effective asthma medications. Around 50% of severe asthma is associated with eosinophilic airway inflammation [67, 68]. There is much evidence to support the role of eosinophils in asthma with several studies showing increased eosinophils in the blood and in the airway in asthma compared to health. A cross-sectional study by Bousquet *et al.* [69] showed that the 43 participants with asthma had significantly higher levels of eosinophils and eosinophil cationic protein in bronchoalveolar lavage compared to 10 healthy controls. Participants with asthma also had significantly higher levels of

peripheral blood eosinophils compared to healthy controls. Levels of both blood and airway eosinophils were correlated with asthma severity and lung function suggesting a key role in asthma pathogenesis.

The presence of eosinophilic inflammation is associated with an increased risk of future exacerbations and a positive response to corticosteroid-based anti-inflammatory treatment [70]. A cohort study by Price *et al.* [71] of over 130, 000 primary care participants with asthma showed that patients with a blood eosinophil count of ≥ 400 cells/μL with a were at a higher risk of asthma exacerbation and poor asthma control than participants with a blood eosinophil count < 400 cells/μL. Around 15% of the primary care cohort had a blood eosinophil count ≥ 400 cells/μL. Another large asthma cohort, the Severe Asthma Research Program-3 (SARP-3), study has also shown that the blood eosinophil count is an independent risk factor for asthma exacerbations [72].

Targeting eosinophilic airways inflammation, rather than symptoms, leads to less frequent exacerbations, and a lower overall steroid burden whilst having no or minimal impact on symptoms or lung function. These observations provide evidence that symptoms and lung function are mechanistically disconnected from exacerbations. Green *et al.* did a randomised controlled trial of titrating asthma treatment based on the induced sputum eosinophils versus standard care as per the British Thoracic Society guidelines [48]. This study of 74 patients with moderate to severe asthma showed that inflammation guided treatment resulted in less airway inflammation and significantly fewer asthma exacerbations and hospital admissions without any overall increase in steroid use. Similar findings were reported by Jayaram *et al.* [73] in a population with less severe asthma and by Siva *et al.* [74] in patients with COPD.

1.1.7 Identifying eosinophilic airway inflammation

Of the measurements currently available to assess and characterise airway inflammation, differential sputum cell count is the most specific, discriminative, and well-validated and is regarded as the 'gold standard' for identifying airway inflammation (Figure 1.2) [70, 75-79]. Normal values have been well-documented [80] with a sputum eosinophil count of ≤ 3% of the total cells being normal [81]. A sputum eosinophil above this range is considered pathological airway inflammation and is predictive of a positive response to oral and inhaled steroids [9, 47]. However, there are practical and technical limitations to the use of induced sputum to assess airway inflammation. These include the technical difficulty of sputum processing immediately after production by an experienced technician, a 10-20% failure rate in induced sputum production, and the lack of an immediately available result to guide treatment [82]. Some patients are unable to tolerate the process of sputum induction, or find the procedure unpleasant, as it can cause bronchospasm and cough. FEV1 is monitored during sputum induction and the procedure is stopped if there is a significant fall from baseline, or if the patient finds the procedure intolerable. These limitations have mean that induced sputum evaluation is largely a research tool and not available in routine clinical practice. Clinicians and researchers have therefore been interested in simpler, more clinically accessible methods for identifying patients with eosinophilic airway inflammation.

A number of studies have shown that the peripheral blood eosinophil count is strongly correlated with sputum eosinophils and that increasing blood eosinophil counts are

highly predictive of a sputum eosinophilia [83, 84]. Bafadhel *et al.* demonstrated that patients with a peripheral blood eosinophil count of <2% of the total white cell count (equivalent to a total eosinophil count of around 0.15 x 10^9/L) at the time of an exacerbation of COPD were very unlikely to have a raised sputum eosinophil count [85] and went on to show that this cut point effectively stratified patients' response to oral prednisolone given to treat the exacerbation, with all the benefit occurring in patients with a blood eosinophil count > 2% [86]. This cut point has since been shown to be a reliable marker of response to mepolizumab in severe asthma [39] and exacerbation reduction with inhaled corticosteroids in patients with COPD [87-89] with no evidence of clinical efficacy in the 30-40% of patients with blood eosinophil counts <2% of the total white cell count. There appears to be increasing efficacy of inhaled corticosteroids in exacerbation reduction in COPD with increasing baseline eosinophil counts.

Other biomarkers available for assessment of eosinophilic airway inflammation, include blood indices such as the total and allergen-specific IgE, serum periostin and the exhaled breath test, fractional exhaled nitric oxide (FeNO) [78, 90]. Of these biomarkers, FeNO has the strongest positive correlation with eosinophilic airway inflammation and is most predictive of a response to inhaled corticosteroids [83, 91, 92].

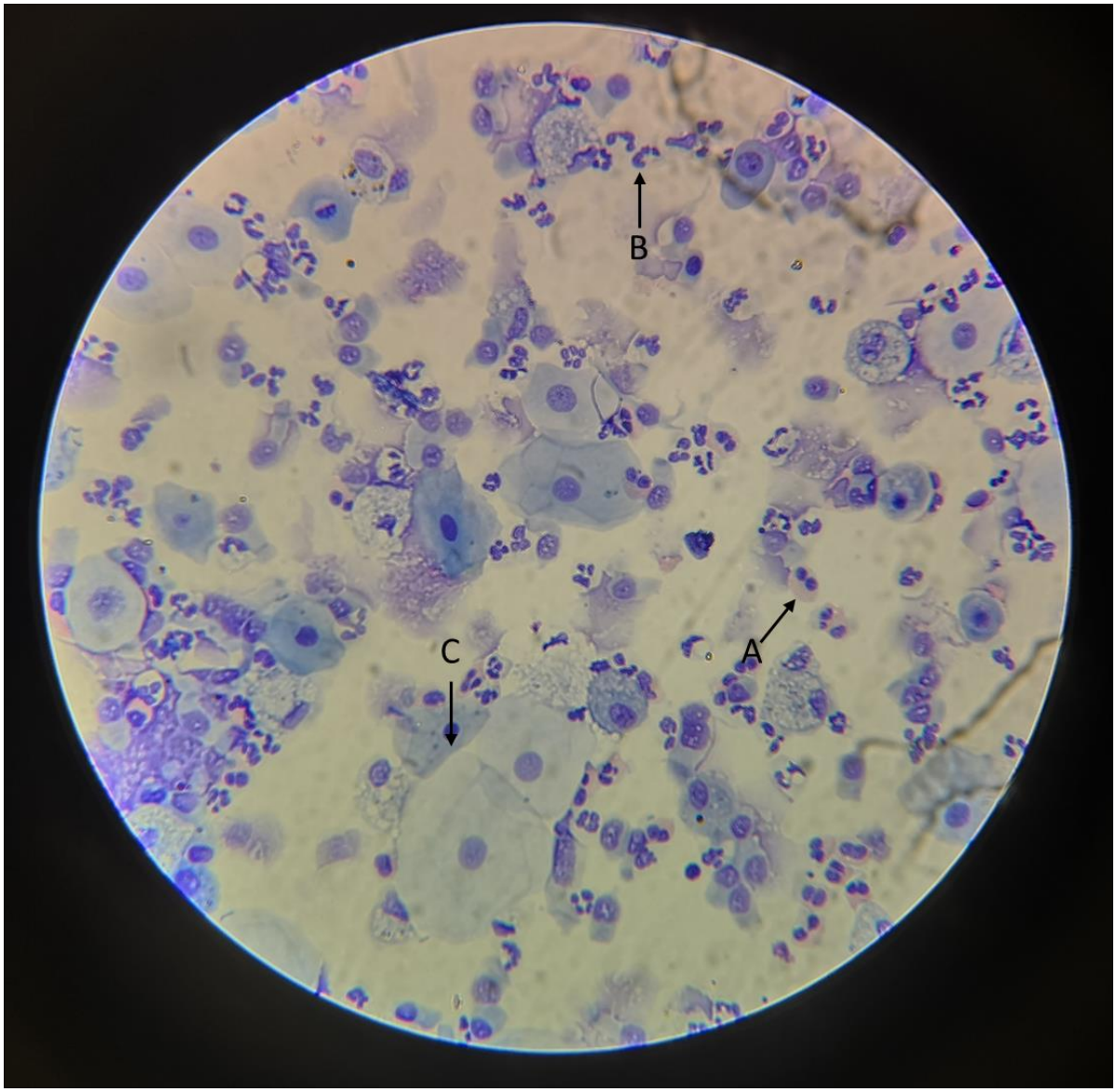
Nitric oxide (NO) is a molecule released by the airway epithelium in association with type-2, eosinophilic inflammation. The production of NO is regulated by the IL-4/IL-13 dependant inducible nitric oxide pathway [93] (Figure 1.1). NO was first detected in exhaled breath by chemiluminescence and mass spectrometry in 1991 [94]. Exhaled NO can be measured using a non-invasive breath test. The testing of FeNO requires little training and can be easily performed at the bedside in day-to-day clinical practice in

primary care and hospital outpatient settings. The measurement devise uses an electrochemical sensor and give a result of the fractional exhaled nitric oxide (FeNO) with the result reported as the parts per billion (ppb). FeNO interpretation in smokers merit comment as smoking exposure, whether active or passive, via conventional or electronic cigarettes, tends to reduce nitric oxide readings, irrespective of duration of the exposure, limiting the value of this test in current smokers [95, 96] . Different FeNO values can be used to identify eosinophilic airway inflammation with varying specificity and sensitivity depending on the value used. A FeNO of < 25 ppb is considered normal and suggests eosinophilic airway inflammation is unlikely, FeNO of 25-49 ppb reflects an intermediate probability of eosinophilic airway disease and FeNO of ≥ 50 ppb is widely accepted as being highly suggestive of eosinophilic inflammation [97-99]. In patients with inhaled corticosteroid responsive airway inflammation, FeNO falls rapidly after the application of inhaled steroids with changes seen 1 day after the first dose and a maximal effect seen from day 4 onwards [100]. Due to the limited systemic effect of inhaled corticosteroids, this effect is likely due to suppression of type-2 mediators within the airways. The reduction of FeNO in response to inhaled corticosteroids is dose-related when comparing low to medium/high doses of inhaled corticosteroids [101]. FeNO predicts response to inhaled corticosteroids improving asthma control as measured by the Asthma Control Questionnaire (ACQ). A randomised controlled study by Price *et al.* evaluated 294 participants with symptoms of cough, wheeze or dyspnoea but no confirmed diagnosis of asthma. 148 participants were randomised to inhaled corticosteroid treatment, and 146 to matching placebo. The investigators showed that ACQ was improved with inhaled corticosteroids compared to placebo in participants

with raised FeNO. The improvement in ACQ was correlated with the baseline FeNO, with the highest values showing the largest ACQ improvement [92]. A cohort study by Malinovschi *et al.* studied 153 inhaled corticosteroid naïve participants with asthma. Participants were given 500 μg inhaled beclomethasone daily. Study subgroups were defined as high FeNO (≥ 50 ppb), intermediate FeNO (≤ 25-50 ppb) or FeNO low (<25 ppb). The authors found that ACQ was significantly improved in a larger proportion of FeNO high or intermediate compared to FeNO low at 3 months (31%, 37% and 4% respectively) [91].

As well as being used to identify type-2 high airway inflammation and guide initial treatment, FeNO can be used to differentiate poor treatment adherence, a major cause of uncontrolled asthma and persistent airway inflammation, from treatment refractory disease. This can be done by attempting to control airway inflammation in an observed fashion by delivering high dose inhaled steroids and measuring the exhaled nitric oxide response – known as a FeNO suppression test [62]. Studies using FeNO to personalise asthma management have produced mixed findings due to differing study populations, the chosen FeNO treatment values and varying management protocols. Despite these limitations, there is consistent evidence that FeNO guided management results in fewer asthma exacerbations [102, 103] with potentially less overall treatment [104]. A well designed study of FeNO guided treatment in pregnant women with asthma by Powell and colleagues showed striking benefits including a 60% reduction in asthma exacerbations during pregnancy, improved perinatal outcomes and lower overall use of inhaled corticosteroids [105].

Figure 1.2 Induced sputum sample from a patient with asthma



Light microscopy of an induced sputum sample showing multiple cell types including A) eosinophil B) neutrophil and C) upper airways squamous cell.

1.2 The eosinophil

Eosinophils are potent granulocytic white blood cells which have a natural role in the innate immune response. They were first identified in 1879 by the German scientist Paul Ehrlich who developed a method for staining blood films with dyes to obtain differential blood counts [106]. Ehrlich invented the term 'eosinophil' to describe cells with granules which took up a bright red dye called Eosin and speculated that these granules contained secretory products which were important in disease.

1.2.1 Eosinophil function in health and disease

Eosinophils perform a number of functions in health and disease. Historically, it was thought that the primary role of eosinophils was in fighting parasitic helminth disease via the release of cytokines by cell degranulation and by phagocytosis. It is now clear that eosinophils have a much wider role which extends beyond their immune function in response to helminth infection.

In health, the majority of eosinophils are found in lymphoid tissues around the body including lymph nodes, thymus, spleen and in lymphoid tissue in the small intestine known as Peyer's patches. The role of eosinophils in the lymphoid tissue is thought to include the maturation and chemotaxis of other immune cells in response to infection. Eosinophils are also a major source of the activation and proliferation-induced ligand (APRIL), a survival factor for plasma cells, a key cell in the adaptive immune response, in

tissues [107]. Eosinophils are also able to modulate the immune response by acting as antigen presenting cells [108, 109]. In contrast to the cytotoxic action of the cytokines released during eosinophil degranulation in response to infection, eosinophils have also been shown to release several tissue growth factors and may play a role in tissue repair [110].

In health eosinophils are also found, in smaller numbers, in non-lymphoid tissue including adipose tissue. It is thought that in adipose tissue, eosinophils play a role in the prevention of insulin resistance and the development of type-2 diabetes mellitus. It has been shown that eosinophils in the adipose tissue are the main source of IL-4 which is used to promote and sustain alternatively activated macrophages (AAMs) which are essential for glucose homeostasis. A mouse model of type-2 diabetes mellitus showed that obesity was increased, and glucose tolerance decreased in eosinophil-deplete mice due to an attenuation of AAMs [111].

Eosinophils are associated with a number of non-infectious diseases. Eosinophilia can occur in haematological malignancies, including the myeloproliferative leukaemias and lymphoid malignancies such as lymphoma, due to hyperproliferation of eosinophils in the bone marrow. Eosinophilia may also occur due to immune-mediated disorders such as atopic dermatitis and drug hypersensitivity reactions. Both of these conditions are associated with a skin rash due to eosinophil cytokine release. Drug-related reactions can also result in a rare, more serious, eosinophil reaction known as drug-reaction with eosinophilia and systemic symptoms (DRESS). DRESS reactions often occur a number of weeks after starting the offending drug and can lead to multi-organ failure or death in some cases.

As seen in the airways in eosinophilic asthma, eosinophils can accumulate at mucosal membranes and cause local inflammation. This local mucosal inflammation can also be seen in the gastrointestinal tract and manifests as eosinophilic oesophagitis or eosinophilic gastroenteritis or colitis. The aetiology of these conditions is unclear but, like eosinophilic asthma, they respond to treatment with systemic steroids.

Eosinophils are the key effector cells in eosinophilic granulomatosis with polyangiitis (EGPA), an antineutrophil cytoplasmic antibodies (ANCA) associated systemic vasculitis which, by disease definition, coexists with asthma [112]. The cause of EGPA is unknown. The mainstay of treatment is with systemic corticosteroids and subsequent immunosuppression. Treatment with the anti-IL-5 monoclonal antibody, mepolizumab, used for the treatment of severe eosinophilic asthma, has shown efficacy in treating EGPA [113].

1.2.2 Eosinophil poiesis

Eosinophils are terminally differentiated multipotent hematopoietic stem cells that originate from the bone marrow. The differentiation process requires the commitment of multipotent CD34+ stem cells to an eosinophil lineage-committed progenitor lineage. For this to occur, a number of transcription factors including C/EBPε, GATA-1, PU.1, Helios, Aiolos, and XBP1 need to be present [114]. This drives differentiation into a granulocyte lineage with shared properties of basophils and eosinophils. Further differentiation and maturation occurs under the influence of cytokines including IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [115]. Terminal

differentiation into an eosinophil is dependent on the appearance of the receptor for the cytokine IL-5, the IL-5 receptor. Surface expression of the IL-5-receptor-alpha is one of the earliest eosinophil-specific lineage commitment events that occurs within the bone marrow [116].

1.2.3 Eosinophil life cycle and distribution

Eosinophils migrate in to circulating blood from the bone marrow, where they have matured, under the control of IL-5. Eosinophils make up a small fraction of the total blood white cell count, accounting for between 1-4% of the total count [117]. Eosinophils remain in the circulation for only a number of hours after before migrating into tissue.

Once established in tissue, eosinophil survival is dependent on several factors. Eosinophils usually survive for 2-5 days in the tissue compartment, although this may be extended to a number or weeks depending on the tissue and the surrounding cytokine environment [118]. The cytokines IL-3, IL-5 and GM-CSF [119] have been shown to be associated with increased eosinophil survival, whereas an absence of IL-5 promotes more rapid eosinophil apoptosis [120]. IL-3, IL-5 and GM-CSF can be produced by T-cells, macrophages, and epithelial cells as part of the inflammatory response. Eosinophils themselves can also make these survival factors when activated at the site of tissue inflammation [121]. This autocrine process may be another important factor in determining eosinophil tissue survival.

There is evidence to show that eosinophil apoptosis is delayed in asthma compared to health, and that the apoptosis can be affected by steroid treatment. Kankaanranta *et*

al. studied eosinophil apoptosis, measured by flow cytometry, in eosinophils isolated and cultured from the peripheral blood [122]. They studied 34 patients with asthma and 16 healthy controls. Eosinophils survived longer if taken from non-steroid taking asthma patients compared to healthy volunteers. In contrast, apoptosis occurred more rapidly in eosinophils isolated from patients with asthma who were taking steroid treatment. Interestingly, the use of β2-receptor antagonists was associated with decreased eosinophil apoptosis. They authors went on to neutralise IL-5, IL-3 and GM-CSF in their isolated eosinophil samples to investigate whether endogenous cytokines may explain the delayed apoptosis. Neutralising GM-CSF, but not IL-5 or IL-3, resulted in a small increase in eosinophil apoptosis suggesting possible a role for autocrine production of GM-CSF in eosinophil survival. Vignola *et al.* studied eosinophils in bronchial submucosa in bronchial biopsy samples [123]. 30 patients with asthma, 26 with chronic bronchitis and 15 healthy controls were evaluated. The authors found that there was a lower proportion of apoptotic cells found in the asthma subjects compared to health and chronic bronchitis. The number of non-apoptotic cells was correlated with GM-CSF levels in the bronchial sample as detected by immunohistochemistry.

1.2.4 Eosinophil shape change and chemotaxis

Eosinophils are predominantly found in tissue rather than in the circulation with around 99% of the total body eosinophils being tissue dwelling [124]. In health, eosinophils are found mainly in the intestinal tract with a small proportion found in the liver and spleen.

In eosinophilic disorders, eosinophils are present in higher than normal concentrations at the site of disease and cause or contribute to localised inflammation.

In both health and disease, eosinophils need to migrate from the circulation and then accumulate in tissue. This migration from blood to tissue is a step-wise process which is mediated by a number of chemokines and cellular interactions [125]. Firstly, mature eosinophils are released into the circulation under the control of IL-5. Secondly, eosinophils are concentrated and tethered to the vascular endothelium adjacent to the tissue. This step involves integrins which facilitate extra-cellular matrix adhesion such as VLA-4 (very late antigen 4) and upregulation of adhesion molecules including vascular cell adhesion molecule 1 (VCAM-1). In addition, cell adhesion molecules are expressed on the endothelial surface. One of the most potent is P-selectin, which is up-regulated at the site of inflammation, which binds to the high affinity glycoprotein P-selecting glycoprotein ligand 1 (PGSL-1) which is found on eosinophils and other white blood cells. Finally, transmigration across the vascular endothelium to exit the vascular compartment and further chemotaxis of the eosinophils to the site of inflammation. This requires the eosinophil to change shape to allow migration through vascular and tissue barriers and to migrate through the tissue interstitium. This final stage of eosinophil migration occurs mainly under the influence of chemokines. Eotaxins 1, 2 and 3, monocyte chemotactic proteins (MCPs) and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) are the chemokines most associated with eosinophil chemotaxis. These chemokines bind to the CC chemokine receptor (CCR) family. CCR3 binds eotaxins and is highly expressed on eosinophils and is also found on some T-cells including Th1 and Th2 cells. The type-2 cytokines IL-4 and IL-13 can indirectly influence

eosinophil chemotaxis as their presence can up-regulate the expression CC binding chemokines.

The ability to block eosinophil migration, and thus reduce or stop tissue eosinophilic inflammation is therefore an attractive treatment target. A study of the small molecule GW766994, an antagonist of the CCR3 receptor, was conducted by Neighbour *et al.* [126]. They studied 60 patients with eosinophilic asthma as defined by the sputum eosinophil count. In this short, 10-day, double-blind parallel group study of 300mg GW766994 vs matching placebo, the investigators found no difference in blood or sputum eosinophils between treatment groups. This was despite an ex-vivo effect on eosinophil chemotaxis by GW766994. The authors question the role of CCR3 in airway eosinophilia in asthma.

More recently, prostaglandin-D_2 has been identified as a potent chemoattractant for eosinophils and is discussed further in section 1.3.1.

1.2.5 Interleukin-5

Interleukin-5 (IL-5) is the key cytokine in the development of eosinophilic inflammation. It is encoded by a gene on the long arm of chromosome 5q in humans. This location on chromosome 5 is close to a cluster of genes responsible for encoding the type-2 cytokines IL-3, IL-4 and GM-CSF [127].

IL-5 is involved in all stages of the eosinophil life cycle, from haematopoiesis in the bone marrow [128, 129] to promoting recruitment of eosinophils in to the blood stream and chemotaxis in to tissue [130], increasing eosinophil survival in tissues [119] and

activation and control of eosinophil effector functions [131, 132]. IL-5 works via the IL-5 receptor (IL-5R) which is composed of 2 subunits - the IL-5Rα and the IL-5Rβ. The IL-5Rα is found exclusively on eosinophils and basophils and is responsible for binding IL-5. The IL-5Rβ subunit does not directly bind IL-5 but is responsible for signal transduction. IL-5Rβ is found on most white blood cells [133].

Il-5 is produced by a number of cells including CD4+ type-2 cells (Th2), CD8+ type-2 cells (Tc2), eosinophils, basophils, mast cells and innate lymphoid cells type-2 (ILC2) [134-137]. T-cells are the predominant source of IL-5 resulting in eosinophilic inflammation. Garsili *et al.* tested this in an antigen challenge mouse model in allergic mice. They showed that T-cell depleted mice were unable to mount an allergic, eosinophilic reaction in the lung compared to T-cell replete mice [138].

There is evidence to confirm that type-2 cytokines, and IL-5 in particular, are present and important in eosinophilic asthma. Broide *et al.* showed that IL-5 mRNA is upregulated and related to an increase in airway eosinophilic inflammation in an allergen challenge model in 5 atopic asthma patients [121]. Hamid *et al.* demonstrated that IL-5 mRNA was present in bronchial biopsies from individuals with eosinophilic asthma, but not in healthy controls. The investigators also showed that the level of IL-5 was correlated with asthma severity and the degree of eosinophilic inflammation [139]. The most compelling evidence of the role of IL-5 in eosinophilic inflammation comes from the studies of anti-il-5 monoclonal antibodies which selectively neutralise IL-5. An early study of the anti-IL-5 agent mepolizumab showed a marked decrease in blood and airway eosinophils [35].

Given the very selective and biologically relevant nature of cells that IL-5 works directly on, neutralising the action of IL-5, either by targeting IL-5 itself or the IL-5 receptor was an attractive target for the treatment of eosinophilic asthma. There are currently 3 anti-IL-5 monoclonal antibody therapies in clinical use and are discussed in detail in section 1.2.5.

1.2.6 Targeting interleukin-5 to treat eosinophilic asthma

Biologic treatments with monoclonal antibodies have been developed for a wide range of conditions across the fields of medicine with the first licenced use in 1986 of muromonab for the treatment of organ rejection following transplant. A further seven monoclonal antibodies were licenced during the 1990's. However, the first monoclonal antibody for respiratory disease was not in use until 2003 when omalizumab, a humanised immunoglobulin G (IgG) monoclonal antibody that binds to free IgE in the blood, was licenced for the use in persistent, allergic asthma. High cost and restrictions on use based on patient weight, a requirement for allergy and a limited range of serum immunoglobulin-E levels, limited use of this agent and its impact has not been as pronounced as that of biological agents in other disease areas. A rethink of the role of inflammation in airway disease has led to progress, with many targeted biological therapies now in use and under development. By far the most fruitful area to date has been in the treatment of severe, eosinophilic asthma, with several treatments recently licenced or in late stage clinical trials.

Given the very selective and biologically relevant nature of cells that IL-5 works directly on, neutralising the action of IL-5, either by targeting IL-5 itself or the IL-5 receptor, is

an attractive target for the treatment of eosinophilic asthma. There are currently 3 monoclonal antibody therapies in clinical use. Mepolizumab and reslizumab target the IL-5 receptor alpha binding domain of IL-5 whereas benralizumab targets the IL-5 receptor itself [140].

Mepolizumab

Mepolizumab, the first anti-IL5 treatment trialled in asthma, is a recombinant, humanised, IgG monoclonal antibody against IL-5. The first randomised controlled trial (RCT) of anti-IL-5 treatment in asthma investigated the effects of mepolizumab on the response to allergen challenge in patients with mild, allergic asthma. This trial showed dramatically reduced blood and sputum eosinophil counts in the 16 patients receiving mepolizumab compared to the 8 patients that received placebo, but no improvements in airway hyperresponsiveness or the airway response to inhaled allergen[34]. A subsequent, larger RCT conducted to evaluate the safety and efficacy of mepolizumab in 362 patients with moderate asthma selected on the basis of persistent symptoms and impaired lung function showed again that blood eosinophil counts were significantly lowered by the drug. The clinical end points of lung function and quality of life were not significantly improved [35]. As described in section 1.1.5, a fundamental change in thinking led to the design of trials to investigate whether treatment with IL-5 targeted therapy reduced the frequency of severe asthma attacks in patients with severe eosinophilic asthma.

A RCT conducted to evaluate the effect of monthly intravenous dosing of 750 mg of mepolizumab or placebo on exacerbation rates over one year and studied participants who had proven severe, eosinophilic asthma defined as a sputum eosinophil count of >

3% in the year leading up to recruitment. This trial studied 61 patients who took their regular medications in addition to the trial medications. Mepolizumab showed a 43% relative risk reduction of severe exacerbations and a modest improvement in asthma specific quality of life score [38]. Treatment with mepolizumab was associated with a marked reduction in the sputum and blood eosinophil counts, as seen in previous trials.

A further RCT of mepolizumab, also reported 2009, compared monthly intravenous mepolizumab to placebo over 6 months in 20 participants with severe asthma and persistent sputum eosinophils who required oral steroid treatment. Participants had a phased withdrawal of oral corticosteroids during the trial period. This trial showed that mepolizumab reduced blood and sputum eosinophils and allowed oral corticosteroids to be reduced significantly compared to placebo. Improvements were also seen in forced expiratory volume in one second (FEV1). The patients taking mepolizumab treatment were able to reduce their oral steroid dose by a mean of 83.8% compared to 47.7% for those taking placebo [141]. This magnitude of steroid reduction was much larger than seen in the only other monoclonal antibody available for asthma at the time, omalizumab. The Steroid Reduction with Mepolizumab Study (SIRIUS) trial looked specifically at reduction in need for oral steroid treatment [142]. It found that patients treated with mepolizumab were able to reduce their oral steroid dose by a median of 50%, compared to no reduction for those taking placebo. The mepolizumab treated group in this trial, despite lowering their steroids, had a lower rate of exacerbations and an improved symptom score compared to the placebo group.

A much larger, multi-centre RCT, the Dose Ranging Efficacy And safety with Mepolizumab in severe asthma (DREAM) trial was designed to define the dose of

mepolizumab and identify the optimum biomarker for predicting treatment response. It evaluated 3 doses of mepolizumab (75, 250 and 750 mg) delivered by monthly intravenous injection. 621 participants with severe eosinophilic asthma with a history of exacerbations were studied. Eligible participants for the trial needed to demonstrate evidence of eosinophilic airway inflammation as reflected by one or more of the following: sputum eosinophils > 3%, blood eosinophils > $0{\cdot}3 \times 10^9$/L, FENO > 50 ppb, and/or a prompt deterioration in asthma following a 25% reduction in corticosteroid treatment. This large study showed a large reduction in the number of severe exacerbations, with around a 50% reduction at all doses compared to placebo [39].

Significant reductions in the blood and sputum eosinophil count were seen and, whilst a dose-response relationship was seen for the later, no such effect was seen for clinical outcomes and blood eosinophil reductions. Moreover, the study showed that the blood eosinophil count was more predictive of a response to mepolizumab treatment than any other measure, suggesting that the active treatment target is the circulating eosinophil. The identification of a blood eosinophil level as a predictor of response to mepolizumab treatment is important as it is easily and reliably measured all over the world, unlike induced sputum differential cell counts which require specialist expertise in obtaining and evaluating the sample.

The finding that lower doses of mepolizumab were effective meant that subcutaneous administration was feasible as lower volumes of drug were required. The Mepolizumab as Adjunctive Therapy in Patients with Severe Asthma (MENSA) trial [143] used the key characteristic of the blood eosinophil count to identify eligible patients. Subjects needed to have a blood eosinophil count of > $0{\cdot}15 \times 10^9$/L at study entry, or of > $0{\cdot}3 \times 10^9$/L in

the past 12 months. This study used the lower dose of 75 mg intravenously compared to a subcutaneous dose of 100 mg and placebo. MENSA confirmed the results of previous trials, with a marked reduction in severe exacerbation rates and a clear relationship between clinical benefit and baseline blood eosinophil count. Unlike in other studies, there was clear evidence of improvement in lung function and symptom scores, potentially reflecting better identification of a treatment responsive population. Subgroup analysis showed a very large beneficial effect in the 30% of patients with a blood eosinophil levels of > 0·5 × 10^9/L [143]. No difference was seen between subcutaneous and intravenous dosing [143]. The efficacy of subcutaneous dosing meant that administering the drug is much simpler as it does not require the placement of an intravenous needle but is given as a small injection under the skin.

Mepolizumab received approval from the FDA (US Food and Drug Administration) and the European Medicines Agency in 2015 and the UKs NICE (National Institute for Health and Care Excellence) in 2017 and is now available for clinical use. With the increasing use and safety data since the approval for clinical use, mepolizumab can now be given at home by the patient via a pre-filled single dose injector.

Reslizumab

Reslizumab is another humanised IgG antibody against IL-5. It is delivered via intravenous infusion. An early trial of reslizumab in 106 subjects with severe eosinophilic asthma, as defined by sputum eosinophils of ≥ 3%, showed a reduction in sputum eosinophils and a small improvement in FEV1 with treatment compared to placebo [144]. In 2 parallel phase III clinical trial in a total of 953 participants with poorly controlled, moderate-severe eosinophilic asthma reslizumab at a dose of 3 mg/kg

showed a similar effect to mepolizumab with a marked reduction in exacerbations of 50-59% [50], although patients did need to have a higher blood eosinophil count of ≥ 0.4 x 10^9/L to enter the trail when compared to the mepolizumab trials. In contrast, a trial of reslizumab in 492 participants with uncontrolled asthma, but no selection criteria based on the presence of eosinophilic inflammation, showed no significant improvements in symptoms or lung function. Sub-group analysis showed improvements in the clinical outcome measures of asthma symptom control (Asthma Control Questionnaire, ACQ), FEV1 and rescue short-acting beta-2 agonist use in participants with blood eosinophils ≥ 0.4 x 10^9/L [145]. A 16 week trial of reslizumab in uncontrolled moderate and severe asthma patients with a blood eosinophil count of ≥ 0.4 x 10^9/L showed that a dose of 3 mg/kg showed improvements in FEV1 and symptom scores when compared to the lower dose of 0.3 mg/kg and placebo [146].

Benralizumab

Benralizumab is a humanised IgG antibody against the IL-5Rα subunit of the IL-5R and inhibits proliferation of IL-5 dependant cell lines [147]. It is given as a subcutaneous injection. Benralizumab has shown efficacy in reducing exacerbations in participants with blood eosinophils ≥ 0.3 x 10^9/L in a phase IIb dose ranging study trial evaluating monthly subcutaneous dosing treatment for one year in 606 participants [49]. The largest studies of benralizumab to date reported in October and November 2016. The SIROCCO trial randomised 1205 participants with severe asthma to receive placebo or 30mg Benralizumab 4 weekly or 8 weekly [148]. This study stratified subjects by their baseline eosinophil count or either above or below 0.3 x 10^9/L. The investigators found a 45-51% reduction in exacerbation rate in the eosinophilic group with both dosing

regimens, compared to a 17-30% reduction in the non-eosinophilic group. Lung function was also improved in the eosinophilic group for those receiving 8 weekly injections. The duplicate CALIMA trial with 1306 participants showed a 28-36% reduction in exacerbations for eosinophilic participants and an improvement in lung function with both dosing regimens [149]. A 28 week RCT of the oral steroid sparing effects of benralizumab evaluated showed a 50% reduction in oral steroid dose compared to placebo [150]. Benrealizumab is, like mepolizumab, approved for clinic use by NICE and can also be given by at home by the patient via a pre-filled single dose injector.

Benralizumab has also shown increased eosinophil cell death via antibody dependant, cell-mediated toxicity *in vitro* [151]. These mechanisms result in a rapid eosinopenia (low eosinophil count) following dosing with the drug and this was sustained for 8-12 weeks [152]. A RCT was conducted to evaluate whether a single dose of benralizumab given, alongside usual treatment, at the time of an acute asthma exacerbation reduced the occurrence of further exacerbations. The trial of 110 participants showed that in the 74 subjects receiving benralizumab, a single dose of benralizumab at 0.3 mg/kg or 1 mg/kg reduced the rate of exacerbations over the next 3 months by 49% [153]. This finding raises the possibility of using benralizumab as an add-on or alternative to usual treatment with oral steroids for an asthma attack. Using benralizumab in place of oral steroids may be logical, as it is likely that the main beneficial effect of oral steroids in treating an exacerbation is reducing circulating eosinophils. Further exacerbation trials are needed to answer this question. A summary of the key anti-IL5 trials are shown in Table 1.3.

Table 1.3 Summary of key anti-IL-5 drugs in asthma

Reference	Drug	Study population	No. of subjects	Dose	Blood eos* (x 10^9 / L)	Study period	Outcomes verses placebo
Leckie et al, 2000[34]	Mepolizumab	Mild allergic asthma	24	Single dose IV	0.38	16 weeks	• Lowered blood and sputum eosinophils • **No effect on late airway response to allergy or histamine**
Flood-Page et al, 2007[35]	Mepolizumab	Moderate ICS treated asthma	362	250 mg IV or 750 mg IV, 4 weekly	0.37	20 weeks	• Lowered blood and sputum eosinophils • **No significant differences in lung function, symptoms or SABA use** • Possible trend towards lower exacerbation rate in treatment groups
Haldar et al, 2009[154]	Mepolizumab	Refractory, eosinophilic (sputum≥3% eos) asthma with at least 2 courses of OCS in the preceding 12 months	61	750 mg IV 4 weekly	0.34 $	12 months	• Lowered blood and sputum eosinophils • **43% relative risk reduction for severe exacerbations** • Improved asthma symptom scores • No significant differences in lung function
Pavord et al, 2012[39]	Mepolizumab	Refractory, eosinophilic (sputum ≥ 3% eos or blood eos ≥ 0.3 x 10^9 / L or FENO ≥ 50 ppb) asthma with at least 2 courses of OCS in the preceding 12 months	621	75 mg, 250 mg or 750 mg IV 4 weekly	0.25 $	12 months	• **48-52% reduction in severe exacerbations** • **Rate of reduction in exacerbations varied according to the baseline blood eosinophil count** • Lowered blood and sputum eosinophils • No significant differences in lung function or symptom scores
Ortega et al, 2014[143]	Mepolizumab	Severe asthma with blood eos ≥ 0.15 x 10^9 / L at screening, or > 0.3 x 10^9 / L in the preceding 12 months	576	100 mg SC or 75 mg IV 4 weekly	0.30 $	40 weeks	• **Similar efficacy of subcutaneous and intravenous dosing** • Lowered blood eosinophils • Small improvement in FEV1 and improvement in QOL scores
Bel et al, 2014[142]	Mepolizumab	Severe asthma with blood eos ≥ 0.15 x 10^9 / L at screening, or > 0.3 x 10^9 / L in the preceding year AND OCS use for at least 6 months	135	100 mg SC 4 weekly	0.24 $	32 weeks	• **Median reduction of 50% OCS dose, compared to no reduction in placebo arm** • Reduced annualised exacerbation rate – 1.4 vs 2.1 • Lowered blood eosinophils • No significant change in lung function
Castro et al, 2011[144]	Reslizumab	Severe eosinophilic (sputum eos ≥ 3%) asthma	106	3 mg/kg IV 4 weekly	0.5 $$	15 weeks	• **Reduction in sputum eosinophils** • Small improvement in FEV1 • Possible trend towards improved symptom scores, more marked in subjects with nasal polyps
Castro et al, 2015[50]	Reslizumab	Moderate-severe, uncontrolled asthma (ACQ)	953	3 mg/kg IV 4 weekly	0.65	15 months	• **50-59% reduction in asthma exacerbations** • Small improvements in FEV1 and asthma symptom and control scores • Reduction in blood eosinophils

		with blood eos ≥ 0.4 x 10^9 / L in screening period					
Corren et al, 2016[145]	Reslizumab	Moderate-severe, uncontrolled (ACQ) asthma	496	3 mg/kg IV 4 weekly	0.28	28 weeks	• **No significant difference in FEV1 or ACQ** • Sub-group analysis showed improvements in FEV1, ACQ and rescue SABA use in subjects with blood eos ≥ 0.4 x 10^9 / L
Castro et al, 2014[49]	Benralizumab	Moderate-severe uncontrolled (ACQ) asthma with 2-6 courses of OCS in the preceding 12 months	606	2 mg SC, 20 mg SC or 100 mg SC 4 weekly	0.56 - eosinophilic subgroup 0.18 – non-eosinophilic subgroup	12 months	• **41% reduction in asthma exacerbations in the eosinophilic subgroup receiving the 100mg dose** • 20mg and 100mg doses reduced exacerbations in individuals with blood eos ≥ 0.3 x 10^9 / L. • Small improvement in FEV1 in eosinophilic subgroup
Nowak et al, 2015[153]	Benralizumab	Acute asthma exacerbation requiring ED assessment with one previous exacerbation in the preceding 12 months	110	0.3 mg/kg or 1 mg/kg SC once	0.25	24 weeks	• **Reduced asthma exacerbation rates by 49% and exacerbations resulting in hospitalization by 60%** • Reduced blood eosinophils • No difference in FEV1 or ACQ
Fitzgerald et al, 2016[149]	Benralizumab	Moderate-severe asthma with at least 2 courses of OCS in the last 12 months	1306	30 mg SC 4 weekly or 8 weekly	0.36	56 weeks	• **28-36% reduction in exacerbation rate with either dosing regime in subjects with blood eos≥ 0.3 x 109 / L** • Small improvements in FEV1 with both doses • Improvement in asthma symptom scores with both doses
Bleecker et al, 2016[148]	Benralizumab	Moderate-severe asthma with at least 2 courses of OCS in the last 12 months	1205	30 mg SC 4 weekly or 8 weekly	0.37	48 weeks	• **45-51% reduction in exacerbation rate with either dosing regime in subjects with blood eos≥ 0.3 x 109 / L** • Small improvements in FEV1 with both doses • Improvement in asthma symptom scores with 8 weekly dosing

All the above trials were randomised, double blind, placebo-controlled trials in subjects with asthma. Selected outcomes shown in bold.
*IL – interleukin, SABA – short acting beta-agonist. OCS – oral corticosteroids. Eos – eosinophils. FENO – fractional exhaled nitric oxide. FEV1 – forced expiratory volume in 1 second. QOL – quality of life. ACQ – asthma control questionnaire. SABA – short-acting beta-2 agonist. ED – emergency department. * x 10^9 cells / L $ geometric mean $$ median*

1.2.6.1 Summary of anti-IL5 treatments in asthma

In combination, these trials have shown that anti-IL5 treatment is effective at reducing the risk of severe asthma exacerbations when used in patients with active eosinophilic asthma as shown by sputum or blood eosinophils. Anti-IL5 treatments are well tolerated with an acceptable safety profile. There is a small increased risk of injection site reaction compared to placebo. Rarely, more serious hypersensitivity reactions including anaphylaxis have been reported. Rates of side effects such as nausea, headache and joint pains are similar to those seen in the placebo groups in these studies. The longer-term effects of anti-IL-5 treatment and its subsequent depletion of eosinophils is subject to ongoing study. Eosinophils may have a role in cancer rejection including the activation of macrophages the affecting the tumour vascularisation [155].

Currently, asthma guidelines do not recommend routinely measuring markers of eosinophilic inflammation [6], however the emergence of inflammation targeted therapies, understanding and defining the inflammatory phenotype is key in deciding which treatments to offer in severe asthma. Induced sputum testing gives a direct reflection of the inflammatory processes in the airway but is time consuming and requires technical expertise both in obtaining and processing the sample [97]. The blood eosinophil count and FENO are very accessible, minimally invasive biomarkers that predict eosinophilic airways inflammation to a good degree and could be measured routinely. The blood eosinophil count appears to be the best predictor of response to anti-IL5 treatment [156].

Whilst the reduction in future severe asthma exacerbations is a very clinically important endpoint, anti-IL-5 treatment may have other specific uses in asthma. Mepolizumab and benralizumab have been shown to allow a reduction in oral steroid dose, a cause of significant treatment related morbidity, with no worsening of asthma control [142]. There may be a role for the use of benralizumab in the treatment of acute eosinophilic asthma attacks [153], either as an adjunct to oral corticosteroids, which are currently the mainstay of treatment of severe asthma attacks, or in place of them as the main benefit from oral steroids seems to be the circulating eosinophil depleting effect, which is achieved rapidly with benralizumab. The future uses of anti-IL-5 treatment may be personalised depending on the patient history and clinical situation i.e. for the treatment of an acute exacerbation or for the prevention of future exacerbations.

Questions, however, remain about the use of anti-IL-5 treatments in asthma. It is not known what happens to the pattern of inflammation, or on asthma control when treatments are withdrawn – do they go back to baseline, or is there a lasting effect? Data from the post-treatment follow-up period of a mepolizumab study suggests that blood eosinophils and exacerbation frequency increase back to baseline over 3-6 months from stopping treatment [157]. Further studies are needed to explore this post-treatment period and to ascertain whether persistent or intermittent treatment is the optimum approach.

Another area of uncertainty is around the underlying pathophysiology and clinical management of asthma attacks for subjects on anti-IL-5 therapy. Blood eosinophils are quickly and fully depleted by anti-IL-5 treatment, raising the question as to whether asthma attacks on anti-IL-5 treatment are different in nature to those before therapy,

and whether they still be treated with circulating eosinophil depleting, oral steroid therapy. Close examination of these asthma attacks, with appropriate inflammatory phenotyping, on treatment with anti-IL-5 will help answer this question.

1.3 Prostaglandin D_2 (PGD_2) and chemoattractant homologous molecule expressed on Th_2 cells (CRTH2)

1.3.1 PGD_2 in asthma

PGD_2 is a major prostanoid lipid mediator. Prostaglandins are produced via the metabolism of arachidonic acid, which is liberated from phospholipids in cellular membranes by activities of phospholipases. Arachidonic acid is metabolised in to PGH_2 by cyclooxygenase (COX) enzymes COX-1 and COX-2 [158]. PGH_2 is a precursor of a number of PGs including PGD_2. PGH_2 is converted to PGD_2 by the action of two prostaglandin D synthases (PGDS) hematopoietic PGDS (H-PGDS), primarily expressed in mast cells, macrophages, dendritic cells and Th_2 cells, and lipocalin-type PGDS (L-PGDS), primarily expressed in the central nervous system [159]. PGD_2 is rapidly metabolised, with a half-life of around 30 minutes in the circulation [160].

PGD_2 mediates a number of the physiological changes seen in asthma through its direct local effects, and effects on the production of type-2 cytokines by type-2 cells. These effects include bronchoconstriction, vasodilation and subsequent increases in capillary permeability and airway oedema, and mucous production [161, 162]. The main source of PGD_2 in the airways are mast cells, although there is evidence that other cells such as eosinophils [163] and dendritic cells [164] also contribute to its presence. PGD_2 is found in increased concentrations in the airways of patients with stable asthma compared to healthy individuals. Crea *et al.* studied 11 patients with mild asthma and 11 healthy controls and showed that bronchoalveolar lavage (BAL) fluid taken at bronchoscopy had

twice the concentration for PGD_2 in asthma compared to health (0.6 ng/ml vs 0.3 ng/ml, p = 0.004) [165]. Interestingly, the authors found that leukotriene E4, the other major mast cell-derived lipid mediator in asthma, was not elevated (1.7 ng/ml vs 1.8 ng/ml, p > 0.05). Another study evaluating BAL PGD_2 found a larger difference between PGD_2 in asthma compared to health. The authors of this study evaluated 15 patients with allergic asthma, 11 patients with allergic rhinitis and 12 healthy controls. PGD_2 levels in BAL fluid were 12-22 fold higher in asthma compared to health ($p < 0.01$) and nearly 10 fold higher than in allergic rhinitis ($p < 0.01$) [166].

PGD_2 in the airways is markedly increased following IgE-mediated mast cell activation in atopic individuals. Wenzel *et al.* studied 17 participants and found that allergen challenge increased airway PGD_2 found in BAL fluid by 2.5 fold in atopic individuals without asthma (n = 3) and by 10 fold in patients who had both asthma and were atopic (n = 8). There was no change in non-atopic healthy controls (n = 6). A study by Murray *et al.* found a more dramatic increase, with a 150 fold increase in PGD_2 detected in BAL fluid following antigen challenge of individuals with asthma [167].

PGD_2 concentration in the airways in asthma is correlated with disease severity and disease control, with higher levels being associated with more severe disease, poorer asthma control, and increased exacerbation frequency [168]. The SARP asthma cohort evaluated the BAL fluid of 112 participants with asthma and found that levels of PGD_2 were 50% higher in severe asthma compared to mild or moderate asthma [169].

Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)

The biological actions of PGD_2 are exerted via three G-protein coupled receptors; D prostanoid receptor 1 (DP_1), chemoattractant homologous molecule expressed on Th2 cells (CRTH2, also known as D prostanoid receptor 2 (DP_2)) and thromboxane A_2 receptor. DP_1 is expressed by haematological and non-haematological cells including epithelial cells, fibroblasts, dendritic cells, T cells, basophils, eosinophils, and mast cells. Activation of the DP_1 receptor by PGD_2 has both inflammatory and anti-inflammatory effects. DP_1 activation results in increased intracellular cyclic-AMP (cAMP) which can inhibit effector cell function [170] and signalling for migration and activation of immune cells [171]. DP_1 activation with selective agonists result in airway smooth muscle relaxation and vascular dilatation [172]. The DP_1 receptor has not been shown to have any effect on increasing cell activation or migration and so is thought to be primarily responsible for the local effects of PGD_2. Activation of thromboxane A_2 increases the metabolism of PGD_2 [173].

CRTH2 is highly expressed on all type-2 cytokine (interleukins 4, 5, 9 and 13) producing cells including Th2 cells, type 2 innate lymphoid cells (ILC2), $CD8^+$ T cells (Tc2 cells), eosinophils and basophils [174, 175]. CRTH2 is recognised as the most reliable surface marker for identifying Th2 and Tc2 cells [175]. CRTH2 medicated signals are predominantly pro-inflammatory. CRTH2 effects are mediated by a reduction in intracellular cAMP and an increase in intracellular calcium which actives further

signalling pathways. Activation of CRTH2 by PGD_2 causes call activation, chemotaxis, production of type-2 cytokines, and reduced cell apoptosis [176-178].

PGD_2 is a potent chemoattractant of CRTH2 expressing cells. Monneret *et al.* investigated the effects of PGD_2 on *ex vivo* human white blood cells [179]. They found that PGD_2 was a potent chemoattractant for the eosinophils, which express CRTH2, but had no effect on neutrophils, which do not express CRTH2. They further investigated whether other PGD_2 receptors, such as the previously discussed DP_1 receptor, may be responsible for this effect. The effect of PGD_2 on DP_1 was blocked using the specific antagonist BWA868C. This had no effect on eosinophil chemotaxis but did increase eosinophil activation suggesting DP_1 has an inhibitory effect on eosinophils, and that the balance of DP_1 and CRTH2 (or DP_2) receptors on a particular cell may affect the response to PGD_2. As well as being a chemotaxin itself, PGD_2 can enhance the effects of other chemotaxins such as eotaxin [180]. PGD_2 induces chemotaxis, cell activation and type-2 cytokine production on a number of type-2 cells via CRTH2 including basophils, Th2 cells [181], Tc2 cells [182] and ILC2 cells [178].

These observations have shown that PGD_2 is a key mediator in the trafficking and activation of type-2 cells and their production of type-2 cytokines via CRTH2. Blocking CRTH2 is an attractive targeting type-2 inflammation. Xue *et al.* showed that the marked *ex vivo* PGD_2-CRTH2 mediated chemotaxis, cell activation and type-2 cytokine production in ILC2 cells can be blocked by the use of the selective CRTH2 antagonist TM30089 [178]. The chemotaxis and cytokine production by Th2 cells due to PGD_2 stimulation is also markedly attenuated by CRTH2 antagonism. This is seen in a mouse model studying the effects of the CRTH2 antagonist fevipiprant (QAW039). Fevipiprant

also reduced eosinophil shape change and chemotaxis in the mouse model [183]. A study of the CRTH2 antagonist AZD1981 in patients with chronic spontaneous urticaria showed that AZD1981 significantly reduced eosinophil shape change and chemotaxis in *ex vivo* blood samples from treated patients [184].

Due to their effects on type-2 inflammation, a key mechanistic pathway in the pathogenesis of asthma, CRTH2 antagonists have been studied in asthma and are discussed in section 1.3.3.

The role of CRTH2 antagonists in other diseases is also being explored. The CRTH2 expressing cells involved in the pathogenesis of atopic dermatitis made this an attractive disease to target with CRTH2 antagonists. Early results of trials of the CRTH2 antagonists timapiprant and fevipiprant in atopic dermatitis have shown no benefit over placebo. The full study reports have not yet been published. CRTH2 expressing cells have also been implicated in gastrointestinal disorders including inflammatory bowel disease and eosinophilic oesophagitis. A study by Radnia *et al*. showed elevated levels of CRTH2 positive cells in mice models of inflammatory bowel disease and in human samples in Crohn's disease compared to healthy controls [185]. They authors suggest that CRTH2 antagonists may have a role in treating inflammatory bowel disease. In a proof-of-concept study of 26 patients with eosinophilic oesophagitis, Straumann *et al.* showed that the CRTH2 antagonist timapiprant caused a 44% reduction in oesophageal biopsy eosinophils in 14 patients treated with timapiprant compared to no change in 12 patients treated with placebo [186]. Further studies to investigate CRTH2 antagonists in eosinophilic oesophagitis are underway.

1.3.2 CRTH2 expression in asthma

The source of type-2 cytokines that drive eosinophilic inflammation in asthma are all CRTH2 expressing cells as discussed above. There is evidence that CRTH2 expressing cells are enriched and up-regulated in the blood and airways of patients with eosinophilic asthma. A study evaluating CRTH2 expression in severe eosinophilic asthma compared to health evaluated 8 participants with asthma compared to 5 healthy controls. They found that overall CRTH2 expression was enhanced in asthma. The majority of this increased expression was due to increased eosinophils, however there were also increased levels of the type-2 cytokine producing Tc2 and Th2 cells in the severe asthma cohort [187]. Another study examining T cell subsets in asthma evaluated the blood of 46 patients with asthma, of which 11 were of the eosinophilic phenotype, and 11 healthy controls. The authors found that Tc2 cells were present in higher levels in eosinophilic asthma compared to non-eosinophilic asthma and compared to health. Regulatory T cells were also enriched in eosinophilic asthma. This study found that the level of Th2 cells were not different across phenotypes of asthma [188]. Hilvering *et al.* studied type-2 cytokine producing cells in the peripheral blood and in the airways in 2 cohorts of patients with severe asthma compared to healthy controls. 96 patients with asthma were studied (36 eosinophilic, 56 non-eosinophilic) with 38 healthy controls. In this cohort it was again seen that Th2 cell numbers were not raised in eosinophilic asthma compared to non-eosinophilic asthma [182]. They did, however, find that Tc2 cells were enriched in the airways as measured by sputum, bronchoalveolar lavage, bronchial biopsy, and peripheral blood in patients with severe eosinophilic asthma compared to health and severe non-eosinophilic asthma. Concentrations of the lipid

mediators PGD_2 and LTE_4 in the airways were also elevated in severe eosinophilic asthma. The investigators investigated the effect of these lipid mediators on *ex vivo* Tc2 cells and found that they both had a role in cell recruitment and activation. Of PGD_2 and LTE_4, PGD_2 was the post potent however they also worked synergistically with an additive effect on both recruitment and activation. The effects of PGD_2 in the *ex vivo* cell samples were largely via its action on CRTH2. This was demonstrated by blocking CRTH2 with the antagonist TM30089 which markedly attenuated the effects pro-inflammatory of PGD_2.

There has been much interest recently in type-2 innate lymphoid cells (ILC2) in the pathogenesis of asthma and particularly eosinophilic asthma. ILC2 are lineage negative, CRTH2 positive cells which are present in much lower numbers than Th2 cells but are capable of producing type-2 cytokines in a much higher concentration per cell than Th2 cells [189, 190]. Kim *et al.* studied 51 patients newly diagnosed with asthma prior to starting any asthma treatment and 18 healthy controls [191]. They found that ILC1, ILC2, and ILC3 numbers were all increased in induced sputum asthma compared to health. ILC2 counts were significantly increased in patients with eosinophilic asthma compared with those in patients with non-eosinophilic asthma, whereas ILC1 and ILC3 numbers were unchanged. Another study evaluating peripheral blood ILC2 numbers in 150 patients with asthma showed that ILC2 numbers were increased in the eosinophilic population compared to the non-eosinophilic population. They also found that ILC2 counts, as well as the FeNO and blood eosinophil count, had a positive correlation with the sputum eosinophil count [192]. Like Tc2 cells, ILC2 cells are activated by PGD_2 via CRTH2. This was demonstrated in a study by Xue *et al.* in a study of *ex* vivo ILC2s from

skin tissue. This study also showed that this effect is blocked by the CRTH2 antagonist TM30089.

In combination, these studies show that both Tc2 and ILC2 type-2 cytokine producing CRTH2 positive cells are present, enriched and active in asthma. Their migration and cytokine producing effects are mediated by PGD_2 via CRTH2, and this effect can be attenuated by CRTH2 antagonists. These observations suggest possible mechanistic pathways that rationalise the role for investigating CRTH2 antagonists in asthma.

1.3.3 Effects of CRTH2 antagonists in asthma

There are several highly specific CRTH2 antagonists which are currently being evaluated for asthma in phase 2 and 3 studies. The most studied antagonists, AZD1981, BI671800, timapiprant (OC000459) and fevipiprant (QAW039), have been shown to be safe and well tolerated with a side effect profile similar to placebo. They have shown mixed results when evaluating their clinical and biological effects. There are no CRTH2 antagonists which are currently approved for clinical use in asthma.

Timapiprant

A study by Barnes *et al.* evaluated timapiprant 200 mg twice daily compared to placebo in patients with mild persistent asthma who were inhaled corticosteroid (ICS) and oral corticosteroid naïve, a positive skin prick test and FEV1 ≥ 60 and ≤ 80% predicted [193]. The primary endpoint was comparison of FEV1 after 4 weeks of treatment with timapiprant compared to placebo. Induced sputum was also evaluated at baseline and after 4 weeks of treatment. 132 participants were randomised (65 timapiprant, 67

placebo). There was no significant improvement in FEV1. A per-protocol analysis showed a 9.2% improvement in FEV1 compared to 1.8% on placebo (p = 0.037). There were small improvements in the Asthma Quality of Life Questionnaire (AQLQ) and symptom scores however these did not meet the minimally clinically important difference. The geometric mean sputum eosinophil count reduced from 2.1% to 0.7% (p = 0.03) on timapiprant, however this was not significant when compared with the change on placebo (p = 0.37). A mechanistic study by Singh *et al.* evaluated the effect of 200 mg timapiprant on the asthmatic response to bronchial allergen challenge [194]. They conducted a randomised, double-blind, placebo-controlled, two-way crossover study of 16 days treatment with timapiprant in ICS and OCS naïve patients with asthma. 16 participants were recruited and underwent allergen challenge on placebo and timapiprant. Induced sputum, methacholine challenge and FEV1 were evaluated. The fall in FEV1 in response to allergen challenge was significantly reduced by treatment with timapiprant compared to placebo when assessed by area under the curve. Sputum eosinophil counts post-allergen challenge were over 3-fold lower after timapiprant treatment. There was no significant effect on bronchial response to methacholine. Pettipher *et al.* conducted a larger study evaluating 3 doses of timapiprant (either 25, 100 or 200 mg daily) in patients with persistent asthma who were ICS and OCS naïve with FEV1 ≥ 65 and ≤ 80% predicted [195]. This 12 week study evaluated FEV1 as the primary endpoint. The authors found that treatment with timapiprant improved FEV1 by 95 ml compared to placebo (p = 0.005). *Post hoc* analysis showed that this effect was more marked in the atopic, eosinophilic subgroup with an improvement in FEV1 of 220 ml. There were also improvements in AQLQ and ACQ.

AZD1981

Kuna *et al*. studied AZD1981 in two 4 week trials ('Study 1' and 'Study 2') which reported together in 2016 [196]. Study 1 recruited patients with stable asthma, on a low-medium dose ICS and a history of atopy as shown by positive skin prick test. Inhaled steroids were withdrawn during the run-in period and participants were randomised to 1000 mg AZD1981 twice daily or placebo. Study 2 recruited patients with persistent moderate-severe asthma, on high dose ICS but not on regular OCS with an FEV1 ≥ 40 and ≤ 85% predicted. Participants were randomised to 50, 400 or 1000 mg AZD1981 twice daily or placebo. The primary endpoint for both studies was the change in morning peak expiratory flow (PEF) from baseline after 4 weeks on treatment relative to placebo. Study 1 randomised 113 participants (57 AZD1981, 56 placebo) and Study 2 randomised 368 participants (277 to one of three doses of AZD1981, 91 placebo). Both studies found no improvement in the primary endpoint. There were small improvements in asthma symptoms as measures by the ACQ, but in both studies these were below the minimally clinically important difference. A larger, 12 week study by Bateman *et al.* studied 6 doses of AZD1981 (80 mg daily, 200 mg daily and 10 mg, 40 mg, 100 mg, or 400 mg twice daily) compared to placebo [197]. Participants had persistent moderate asthma despite treatment with a combination ICS and long-acting β2-agonist (LABA) inhaler. The primary end point was the mean change from baseline in pre-dose, pre-bronchodilator FEV1 over weeks 2, 4, 8, and 12 in the AZD1981-treatment group compared to the placebo group. 1144 participants were randomised (981 to one of 6 doses of AZD1981, 163 placebo). This large, dose ranging study did not show any improvements in FEV1 or

ACQ after 12 weeks of treatment. Biomarkers of eosinophilic inflammation were not measured in this large study and so subgroup analysis was not possible.

Fevipiprant

Two studies of fevipiprant were published in 2016. The first by Erpenbeck *et al.* studied fevipiprant 500mg daily compared to placebo for 28 days [198]. They recruited participants with mild-moderate allergic asthma, FEV1 ≥ 60% and ≤ 85%, and persistent symptoms. Participants on ICS, LABA or both entered a treatment washout period to gradually stop these treatments. The primary endpoint was trough FEV1 after 28 days treatment. 170 participants were randomised (82 fevipiprant, 88 placebo). There were no significant differences in trough FEV1 or ACQ. *Post hoc* subgroup analyses found patients with FEV1 < 70% predicted at baseline had a significant improvement with fevipiprant compared (207 mL; 90% confidence interval [CI]: 96, 319) and ACQ7 (0.41; 90%CI: -0.69, -0.13). Gonem *et al.* further investigated the anti-inflammatory effects of CRTH2 antagonists in the airways in a study of fevipiprant 225 mg twice daily for 12 weeks in moderate and severe asthma [199]. Participants needed to have a sputum eosinophil count of ≥ 2% at screening despite optimal asthma treatment. 61 participants were randomised (30 fevipiprant, 31 placebo). 23% of participants were on OCS. Treatment with fevipiprant significantly reduced sputum eosinophils compared to placebo with 4.5 and 1.3 fold reductions respectively (p = 0.0014). There were small improvements in FEV1, ACQ and AQLQ. There was no effect on the blood eosinophil count. A subgroup of participants underwent bronchoscopy. These revealed that treatment with fevipiprant decreased epithelial eosinophils and inflammation compared to placebo. The largest study of fevipiprant that has reported to date was

conducted by Bateman *et al.* who investigated 13 doses of fevipiprant in a dose ranging and clinical efficacy study in persistent mild-moderate allergic asthma on low dose ICS [200]. All participants had their treatment changed to low-medium dose inhaled budesonide during the run-in period. Any concomitant LABA/controllers were stopped. 1058 participants were randomised to one of 13 doses of fevipiprant (n = 782), placebo (n = 137), or active control with montelukast 10mg daily (n = 139) for 12 weeks. Fevipiprant showed an improvement in FEV1 of 112 ml which was significantly improved compared to placebo but not compared to active control. There was no dose-related effect of fevipiprant. There was no pre-specified subgroup improvement when stratified by prior lung function, blood eosinophils or symptoms.

1.3.3.1 Summary of CRTH2 antagonists in asthma

These trials have shown mixed results in their primary outcomes compared to placebo. CRTH2 antagonists, by their mechanism of action, target the pathways leading to eosinophilic airway inflammation. The majority of studies discussed above did not have a requirement for participants to demonstrate evidence of eosinophilic airway inflammation for study entry. Pettipher *et al.* showed that the maximum effect of CRTH2 antagonism was seen in an eosinophilic subgroup, however this was a *post hoc* analysis. Gonem *et al.* studied participants who had demonstrated eosinophilic airway inflammation at screening. In this population, CRTH2 antagonists improved airway inflammation, lung function and symptoms. As discussed in section 1.2.5 regarding anti-IL-5 treatments, it is essential that the correct target population is studied to properly evaluate the effects of the drug. Another factor in the interpretation of these studies is that they are all short studies, and mostly evaluating FEV1 as the primary outcome. As

we have seen in other treatments targeting eosinophils, the main effect is on exacerbation rates rather than lung function or symptoms. Further trials are required in populations of patients with eosinophilic asthma to understand the physiological and biological effects of these drugs. Longer duration studies are also required, and exacerbation frequency may be a more sensitive and clinically relevant outcome variable for these studies.

A summary of trials of CRTH2 antagonists in asthma is shown in Table 1.4.

Table 1.4 Summary of trails of CRTH2 antagonists in asthma

Reference	Drug	Study population	No. of subjects	Dose	Mean blood eos (x 10^9 / L)	Treatment period	Outcomes verses placebo
Barnes et al, 2012 [193]	Timapiprant (OC000459)	ICS and OCS naïve persistent asthma FEV1 60-80% predicted	132	200 mg BD	NS	28 days	• **No significant effect on FEV1 in the FAS**, some improvement seen in the PP analysis • Small improvement in AQLQ, below MCID
Singh et al, 2013 [194]	Timapiprant (OC000459)	Mild asthma ICS and OCS naïve	21	200 mg BD	NS	16 days**	• **Attenuation of the late asthma response to allergen challenge** • Lower falls in FEV1 and lower sputum eosinophils
Pettipher et al, 2014 [195]	Timapiprant (OC000459)	ICS and OCS naïve persistent asthma FEV1 65-80% predicted	519	25, 100 or 200 mg BD	NS	12 weeks	• **Improvement in FEV1, most marked in *post hoc* analysis of atopic/eosinophilic group** • Small improvements in ACQ and AQLQ
Erpenbeck et al, 2016 [198]	Fevipiprant (QAW039)	Mild-mod persistent asthma Positive skin prick ICS washout period	170	500 mg OD	NS	28 days	• **No improvement in FEV1** • *Post hoc* analysis showed some FEV1 improvement in participants with FEV1 <70%
Gonem et al, 2016 [201]	Fevipiprant (QAW039)	Mod-severe asthma Sputum eos ≥ 2% at screening	61	225 mg BD	0.28	12 weeks	• **Significant reduction in sputum eosinophils** • Improvements in FEV1, AQLQ
Bateman et al, 2017 [200]	Fevipiprant (QAW039)	Mild-mod allergic asthma	1058	13 doses from 1 mg OD to 150 mg BD [#]	NS	12 weeks	• **Improvement in FEV1** • No subgroup improvement when stratified by prior lung function, blood eosinophils or symptoms • No dose-related effect
Hall et al, 2015 [202]	BI 671800	ICS and OCS naïve persistent asthma FEV1 60-85%	389	50, 200 or 400 mg BD[##]	NS	6 weeks	• **Small improvement in FEV1 compared to placebo but smaller than active control[##]** • No change in ACQ in contrast to small improvement in ACQ with active control
Hall et al, 2015[202]	BI 671800	Persistent asthma On low dose ICS treatment	243	400 mg BD[#]	NS	6 weeks	• **Small improvements in FEV1 compared to placebo but not compared to active control[#]**

Kuna et al, 2016 [196]	AZD1981 'Study 1'	Stable asthma FEV1 65-110% ICS washout period	209	1000 mg BD	NS	4 weeks	• **No significant changes in lung function** • Small improvement in ACQ, below MCID
Kuna et al, 2016 [196]	AZD1981 'Study 2'	Persistent mod-severe asthma No OCS	510	50, 400 or 1000 mg BD	NS	4 weeks	• **No significant changes in FEV1** • Small improvement in ACQ, below MCID
Bateman et al, 2018 [197]	AZD1981	Persistent asthma on ICS No OCS	1144	10, 40, 100 or 400 mg BD OR 80 or 200 mg OD	0.26-0.32	12 weeks	• **No improvement in FEV1 or ACQ**

*All the above trials were randomised, double blind, placebo-controlled trials in subjects with asthma. *2-way crossover trial. Selected outcomes shown in bold. # trial had a placebo arm and an active control arm of montelukast 10mg daily. ## trial had a placebo arm and an active control arm of inhaled fluticasone propionate 220µg.*
OD – once daily. BD – twice daily. OCS – oral corticosteroids. Eos – eosinophils. FEV1 – forced expiratory volume in 1 second. QOL – quality of life. ACQ – asthma control questionnaire. SABA – short-acting beta-2 agonist. NS – not stated. FAS – full analysis set. PP – per protocol. MCID – minimally clinically important difference.

2 Hypotheses

I hypothesise that

1. Prostaglandin D_2 is an important and active mediator in inhaled corticosteroid-refractory, severe eosinophilic asthma.

To investigate the role of prostaglandin D_2, I will conduct a study to evaluate the effects of monitored inhaled corticosteroid treatment in severe eosinophilic asthma. Induced sputum will be collected before and after treatment and analysed for type-2 mediators including prostaglandin D_2. Between-group comparisons will be made between treatment-responsive and treatment-refractory airway inflammation

2. The CRTH2 antagonist timapiprant will reduce eosinophilic airway inflammation in patients with severe asthma and this effect will be due to effects on type-2 cytokine producing, CRTH2 positive cells.

To address this hypothesis, I will conduct a randomised, placebo-controlled trial of timapiprant in severe eosinophilic asthma. Induced sputum cell counts will be analysed to assess airway inflammation. Blood will be analysed by flow cytometry to evaluate CRTH2 cell numbers. Between-group comparisons will be made between timapiprant and placebo groups.

3. Treatment of severe eosinophilic asthma with type-2 targeted drugs affects the clinical phenotype of subsequent exacerbations due to the depletion of eosinophils.

To investigate this hypothesis, I will conduct *post hoc* analysis of exacerbation data of trials of mepolizumab. Mepolizumab was chosen as the type-2 targeted drug as it is the only type-2 treatment which has undergone a clinical trial which has reviewed participants and collected detailed biological and physiological data at the time of exacerbation. To evaluate the nature of exacerbations occurring on mepolizumab, biological, physiological and patient-reported symptom scores will be compared between mepolizumab and placebo groups.

3 Methods

3.1 Clinical methods

3.1.1 Physiological measurements

3.1.1.1 Vital signs and electrocardiogram

Vital signs were measured after 5 minutes sitting at screening, baseline and follow up visits. These are systolic and diastolic blood pressure, heart rate, oxygen saturations and oral body temperature. Devices approved for clinical use and regularly calibrated were used. Electrocardiograms were recorded after 5 minutes resting using a 12-lead ECG module with Spirotrac™ software (Vitalograph®, Bucks, UK).

3.1.1.2 Blood tests

Peripheral venous blood was be taken using standard techniques with the Vacutainer® holder and vacuum bottle system. A 4ml Vacutainer® EDTA and 7ml SST tube were taken for safety analysis. An additional 4 9ml Vacutainer® heparinised tubes and 7ml SST tubes were taken for exploratory lab work, as described in section 3.2. An additional blood sample was taken at the week 4 visit for pharmacokinetic studies, performed by Covance Inc. (New Jersey, USA). Safety analysis blood tests were processed in the Oxford University Hospitals Trust laboratory as per their usual practice. Blood tests performed were: Peripheral blood count, differential white cell count, platelet count, haematocrit, erythrocytes count, urea and electrolytes, glucose, serum proteins, calcium, phosphate, creatinine, total protein, albumin, bilirubin, liver function tests (AST, ALT, ALP, y-GT).

3.1.1.3 Urine tests

Urine samples were obtained at every visit. All samples were tested for protein, blood, ketones, glucose, bilirubin, specific gravity, and urobilinogen by dipstick. A urinary drugs of abuse screen was performed at the screening visit. Female participants of child-bearing age also had a urine pregnancy test performed. Urine samples were frozen in a plain universal and stored at -80°C for further analysis as described in section 3.2.3

3.1.1.4 Fractional exhaled nitric oxide (FeNO)

FeNO was measured using a HypAir FeNO+™ device (Medisoft®, Sorinees, Belgium). The measurements were conducted prior to spirometry. Readings were taken at a flow fate of 50 ml/second. The average (mean) of 3 adequate readings was taken.

3.1.1.5 Spirometry

Spirometry is a lung function test that requires inspiration to the inspiratory reserve volume, followed by a forced exhalation to the residual volume. This manoeuvre allows measurement of the forced expiratory volume expired in one second (FEV1), the total volume of air expired, or the forced vital capacity (FVC). These measurements also allow calculation of the FEV1 / FVC ratio, a marker of airflow obstruction. A Pneumotrac™ spirometer (Vitalograph®, Bucks, UK) was used with Spirotrac™ (Vitalograph®, Bucks, UK) software. The spirometer was calibrated each day according to the manufacturer's instructions. Spirometry was performed according to the joint European Respiratory Society (ERS) and American Thoracic Society (ATS) guidance [203] before and 20 minutes after 2.5 mg nebulised salbutamol. FEV1 and FVC were recorded as the best of 3 successive readings within 100 ml of each other.

3.1.1.6 Induced sputum testing

Induced sputum testing is a safe and non-invasive method of assessing lower airway inflammation. It has a higher yield than spontaneous sputum samples or broncho-alveolar lavage [82]. It involved the participant inhaling increasing concentrations of sterile hypertonic saline. Hypertonic saline can cause bronchoconstriction and so serial FEV1 measurement is performed to ensure participant safety. Sputum induction was performed at least 20 minutes after inhalation of 2.5 mg nebulised salbutamol. Participants inhaled serial concentrations of hypertonic saline (3%, 4% and 5%) for 5 minutes each via a nebuliser with a delivery rate of ~ 1 ml/minute. FEV1 was checked after each concentration and sputum induction was stopped if there was a fall of ≥ 20% of the post-bronchodilator value. Participants were encouraged to cough during and after each concentration. Any sputum produced was collected and stored at 4°C for processing as described in section 3.2.2. Study investigators were blinded to the sputum results until the trail database was locked.

3.1.2 Patient reported measurements

3.1.2.1 Participant questionnaires

Questionnaires were completed by the participants. They were given to the participants to complete before the other study measurements to avoid the influence of any clinical tests or investigator questioning. Asthma control questionnaire 5 (ACQ-5). The ACQ-5 is a five-item questionnaire, which has been developed as a measure of participant's asthma control that can be quickly and easily completed [204]. The questions are self-

completed by the participant. The five questions enquire about the frequency and/or severity of symptoms (nocturnal awakening on waking in the morning, activity limitation, and shortness of breath, wheeze). The response options for all these questions consist of a zero (no impairment/limitation) to six (total impairment/limitation) scale. This was completed at every scheduled visit after screening. Standardised Asthma Quality of Life Questionnaire (AQLQ(S)). The AQLQ(S) is a standardised version of the AQLQ. Five generic activities (strenuous exercise, moderate exercise, work-related activities, social activities, and sleep) in the AQLQ(S) replace the five patient-specific activities in the AQLQ [205]. Patients are asked to think about how they have been during the previous two weeks and to respond to each of the 32 questions on a 7-point scale (7 = not impaired at all - 1 = severely impaired). The overall AQLQ(S) score is the mean of all 32 responses and the individual domain score are the means of the items in those domains. This was completed at every scheduled visit after screening.

3.1.2.2 Daily diary and asthma management plan

Participants completed a daily diary throughout the study, recording daytime and night-time symptoms, twice daily peak expiratory flow (PEF), medication use including rescue β2-agonist and OCS, days off work and emergency care visits. PEF was recorded as the best of three successive readings using a standardised peak flow meter. Symptom scores from 0 to 3 (for day time symptoms: 0 = none 1 = occasional symptoms, 2 = symptoms most of the day, 3 = asthma very bad, unable to do normal activities at all; for night-time symptoms 0 = none, 1 = awoke once due to asthma, 2 = awoke 2-3 times due to

asthma, 3 = awake most of the night due to asthma) were recorded. All subjects were issued with an asthma management plan.

3.1.2.3 Recording of adverse events and concomitant medications

Adverse events and concomitant medications were recorded in source data throughout the study. Adverse events were characterised by severity, relationship to study medication, outcome, action taken, onset and resolution date and seriousness. The indication for any concomitant medications were recorded along with the dose and the duration of therapy.

3.1.3 Fractional exhaled nitric oxide suppression test

The goal of this test is to help stratify patients with high type-2 airways biology as demonstrated by an exhaled FeNO of 45 ppb or greater despite high dose inhaled corticosteroids. This test, which utilises directly observed inhaled corticosteroid treatments, can help identify patients who may be refractory to treatment with high dose inhaled corticosteroids or whose persistent type-2 biology may be due to poor treatment adherence. Following the above baseline measurements, patients were given a Flixotide® Evohaler® (GlaxoSmithKline, London, UK), containing 500µg of fluticasone propionate per actuation, with advice and demonstrations on inhaler technique and directions to take 2 puffs in the morning in addition to their usual inhaled asthma therapy. We attached an INCA™ (Vitalograph®, Bucks, UK) to each Flixotide® inhaler. This device contains a microphone which monitors the number and time of each actuation and whether an inhalation was efficient or not. Patients were also given a NIOX VERO® FeNO monitor and directed to take a morning and evening measurement daily. Patients were reviewed after 7 days of monitored inhaled corticosteroid therapy

for repeat clinical and physiological measurements and data was downloaded from the FeNO monitor and INCA™ device. A positive FeNO suppression test (i.e. FeNO suppressed suggesting poor treatment adherence rather than treatment refractory disease) was defined as previously defined by McNicholl *et al.* [62] who found that a 1.7 fold reduction in FeNO was a reliable cut-off for identifying treatment non-adherence.

3.2 Laboratory methods

3.2.1 Flow cytometry

Flow cytometry is a technique which uses light to measure the physical and biological characteristics of individual cells. A flow cytometer is a machine which allows cells, suspended in liquid, to pass single file through a high laser which then shines on to detectors. This single-cell process happens very quickly, allowing for the analysis of thousands of cells per second. The detector is connected to a computer which interprets the signals and shows the individual cell detection results in real-time. The pattern of the light scatter made by the cell interrupting the laser beam allows the computer analysis to give information on the physical characteristics of the cell. These measurements are the forward-scattered light (FSC) and the side-scattered light (SSC). The forward-scattered light is a measurement of the amount of the laser beam that passes around the cell and reflects the relative size of the cell. The side-scattered light is a measurement of light that is scattered by particles within the call and reflects the granularity of the cell. These measurements allow for identification of populations of cells within a sample and comparing relative numbers within each population. For example, when looking at whole blood, red cells can be seen as low FSC (small), low SCC (non-granular) cells, lymphocytes as higher FSC (larger), low SSC (non-granular) and the granulocytes as higher FSC (larger) and high SSC (granular). In addition to the physical properties of a cell, flow cytometry can be used to measure the biological characteristics of a cell using fluorescent labelled antibodies (or fluorochromes) and the effect of light fluorescence. When a light energy is absorbed by a molecule, electrons are raised from their resting state to an excited state. Electrons then return to the resting state by losing

energy including the emission of light. This light emitting effect is termed fluorescence. The light that is emitted by the excited electrons will always be of a lower energy (and therefore wavelength) than the exciting energy due to other sources of energy loss such as heat. These differences in wavelengths can be detected and measured. A number of lasers of differing wavelengths with a seperate detector for each laser can be used in combination. This is known as multi-colour, or polychromatic, flow cytometry. By adding a range of wavelength specific fluorochromes to the sample, multiple antibody binding sites can be identified on a single cell simultaneously. These methods allow for immunophenotyping of individual cells within a population based on their surface receptors.

3.2.1.1 Whole blood flow cytometry assay

Surface expression of CRTH2 expression was determined in whole blood samples by flow cytometry. The whole blood samples were processed as follows: FACS tubes labelled with the patient ID, "WB", and assigned as number 1 (stained) and another as number 2 (FMO). The whole blood (WB) antibody cocktail was prepared as (appendix, table 8.1). 50 µL of whole blood was added into tube 1 and tube 2 and then 50 µL of WB antibody mix added to tube 1 and tube 2. 2.5 µL of CRTH2-PE (Miltenyi Biotech, Bergish Galdbach, Germany) was added to tube 1 only. The tubes were Vortexed and left for 30 minutes in the dark at 4°C. 50 ml BD FACS Lysing solution (BD Biosciences, San Jose, USA) was prepared in Falcon Tube by adding 5 mL of 10X BD FACS Lysing Solution to 45 mL distilled water, mixed well and stored at room temperature. 1 mL of 1X BD FACS Lysing Solution was added to each tube and mixed, before adding another 1 mL of 1X

BD FACS Lysing Solution (total 2 mL). This was then incubated for 15 minutes at room temperature in the dark then centrifuged at 500 x g for 5 minutes. Supernatant was carefully decanted, and the cell pellet re-suspended in 1 mL of PBS. A further 1 mL of PBS was added and then Vortexed. Sample down at 500 x g for 5 minutes and then re-suspend in 250 µL of phosphate buffered saline. Samples were then run on a BD LSRFortessa™ (BD Biosciences, San Jose, USA) flow cytometer (appendix, section 8.1.1).

3.2.1.2 Peripheral blood mononuclear cells preparation and innate lymphoid type-2 assay

CRTH2 expression and innate lymphoid type-2 cells were measured in peripheral blood mononuclear cell fractions. Peripheral blood mononuclear cells (PBMC) are nucleated blood cells that have round nuclei. PBMC mainly consist of lymphocytes with a small number of monocytes and a few dendritic cells. PBMC can be isolated from the peripheral blood by centrifuging with a density gradient. The denser cells such as granulocytes and red blood cells will separate to below the density gradient layer, whereas the PBMC will stay above it with a plasma layer on top, allowing separation from the rest of the blood sample.

The PBMC samples were isolated and processed as follows: ILC2 antibody cocktail was made (appendix, table 8.2). Two 50 mL Falcon tubes were prepared and labelled with patient ID. Tube 1 is for diluting the blood, tube 2 for separation of PBMC layer with Lymphoprep™ (STEMCELL Technologies Inc.™. Vancouver, Canada). Up to 2 x 9 mL of whole blood in heparinised tubes were processed in one Falcon tube. Up to 18 ml of whole blood was diluted to 35 mL using PBS without Ca2+ and Mg2+ stored at room

temperature in tube 1 and mixed well. The heparinised blood tubes were rinsed out using PBS to acquire the maximum number of cells. 15 mL of Lymphoprep™ was carefully added to tube 2. 35 mL of the diluted blood was transferred to tube 2 by layering it above the Lymphoprep™ layer. Gentle pipetting was used to avoid mixing of the blood and Lymphoprep™. Tube 2 was centrifuged at 500 g for 25 minutes, acceleration 1 and deceleration 0. The formed PBMC layer was aseptically harvested to a new Falcon tube using a Pasteur's pipette and made up to a volume of 50 mL with PBS. Tube 2 was then centrifuged 300 g for 10 minutes at 4°C and the supernatant aspirated and discarded. Tube 2 was made up to a volume of 30 mL with PBS. A 20 µL aliquot was taken for counting by mixing with 80 µL 0.4% trypan blue (1:5 dilution), centrifuged at 200 g for 10 minutes at 4°C before calculating the re-suspension volume to get $10x10^6$ cells in 50 µL (2×10^8 cells/mL). Flow cytometry staining were prepared in 2 tubes, tube 1 = PBMC Stained and tube 2 = PBMC FMO. 50 µL of PBMCs were added to each tube with 50 µL of ILC2 antibody cocktail to each tube. 2.5 µL of CRTH2-PE (Miltenyi Biotech, Bergish Galdbach, Germany)) was added to tube 1 only. The tubes were Vortexed and left for 30 minutes in the dark at 4°C. The pellet was re-suspended in 1 mL of PBS, then a further 1 mL of PBS was added and Vortexed. Centrifuge at 500 g for 5 minutes. The supernatant was carefully decanted and discarded and the tube then vortexed to re-suspend the pellet. 200 µL of IC fixation buffer (eBioscience, Massachusetts, USA) was added and gently mixed by tapping the tube (not Vortex). Suspension was then fixed overnight at 4°C or 20 minutes at room temperature. Samples were then run on a BD LSRFortessa™ (BD Biosciences, San Jose, USA) flow cytometer (appendix, section 8.1.1).

3.2.1.3 Flow cytometry analysis

The flow cytometer data is relayed to a computer with analysis software that shows the individual cell results, or acquisitions, in real time as the cells pass through the array of lasers and detectors. All of the data from a cell undergoing polychromatic flow cytometry analysis cannot be displayed in one, 2-dimensional plot. Instead, 2 lasers, or channels, can be chosen and the cells appear as dots plotted on these axes. Alternatively, a histogram showing the intensity and number of acquisitions within each channel can be shown. These axes can be changed, both during acquisition or after all the cells are run. Cell populations can be selected, or gated, based on size, granularity and fluorochrome signals to determine cell subtypes.

Tc2 cells were gated as $CD3^{+}CD4^{-}CD8^{+}CRTH2^{+}$ cells and Th2 cells were gated as $CD3^{+}CD4^{+}CD8^{-}CRTH2^{+}$ cells in the lymphocyte population. ILC2 cells were defined as cells in the PBMC fraction that were, $CD45^{+}Lin^{-}IL\text{-}7RA^{+}CRTH2^{+}CD161^{+}$ (lineage markers CD3, CD4, CD8, CD14, CD16, CD19, CD56, CD123, CD11c, CD11b, and FcεR1).

3.2.2 Induced sputum processing

Sputum samples were induced and collected as detailed in section 3.1.1. Samples were processed within 4 hours. All steps until weighing sample after filtration were done in a Class II biological cabinet. Sputum samples were processed by an experienced technician.

3.2.2.1 Sample processing and storage

An empty petri dish was weighted and labelled with the patient identification. The whole sputum sample was transferred to the petri dish and weighed. Sputum plugs were selected and transferred to petri dish lid and move in small circular movements to reduce saliva presence and selected sputum was weighed. For consistency, aim for 0.25 g of sputum if possible. The selected sputum plugs were incubated in 8 x the selected sputum weight of Dulbecco's phosphate buffered Saline (DPBS). The sample was agitated with a Pasteur pipette until sample looked to be broken up and incubated for a minimum of 5 minutes then centrifuged at 200 g for 5 minutes at 4°C. 4 x the selected sputum weight of the supernatant was removed into 15ml tube and vortexed and split into aliquots of 100-500 µl of phosphate buffered saline (PBS) supernatant into 5 x labelled cryovials. PBS supernatant was then stored at -80°C. 4 x the selected sputum plug weight of 0.2% sterile dithiothreitol (DTT) was added to the remaining centrifuged sample (with pellet) and Vortexed for 15 seconds and placed on ice and left rocking for 15 minutes. PBS, equal to volume of DTT added, was added and Vortexed thoroughly. Debris was removed by filtering into a second Falcon tube through sterilised gauze placed in a sterile funnel. Sample was weighed after filtration. A live/dead and squamous cell count was performed by adding 10 µl of sample to 10 µl of Trypan Blue and mixing. Haemocytometer was flooded with 10 µl of cell solution. A minimum of 4 haemocytometer fields, or 100 cells were counted. Calculate total cell count/g of sputum and the percentage viability (appendix, section 8.2). Remaining DTT treated sample was centrifuged at 200 g for 5 minutes at 4°C. Supernatant was then removed into 15 ml tube, vortexed and split into aliquots of 100-500 µl of DTT supernatant. DTT

supernatant was stored -80°C. The pellet was re-suspended with re-suspension value (appendix, section 8.2) of DPBS and Vortexed.

3.2.2.2 Sputum slide creation and staining for cell counting

To make the slides for cell counting a Cytospin™ (Thermo Fisher Scientific, Massachusetts, USA) was used as follows. 2 x Cytospin™ slides were set up. 75 µl of the re-suspended sample was added to each funnel and spun at 450 rpm for 6 minutes. Slides were air dried for 15 minutes at room temperature. Slides were checked under the microscope. If there was cell crowding, sample was diluted with PBS and re-spun. If there were too few cells, a larger volume of sample was used and re-spun. Slides were incubated in methanol for 5 minutes, allow to air dry and then stored at room temperature. Cells slides were stained for counting the using Rapi-Diff II™ kit (Atom Scientific, Hyde, UK). 2 drops of Solution B were placed on area of cells, left for 5 minutes then rinsed with distilled water and allow to dry. 2 drops of Solution C were placed on area of cells and left for 10 minutes, rinsed with distilled water and allow to dry. One drop of DPX mountant reagent was placed on the slide, covered with a cover-slip and allow to dry.

3.2.2.3 Sputum slide counting

The prepared slides were counted by experienced, credited operators – either Mrs Samantha Thulborn or Dr Jennifer Cane. Cells were identified as neutrophils, macrophages, eosinophils, lymphocytes or epithelial cells (Figure 1.2). The total number of cells and the % of the total cell population were recorded.

3.2.3 Eicosanoid measurements by enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a method which uses antibody binding reactions to measure substances such as proteins, antigens and antibodies in biological samples. The assays used for these eicosanoid measurements use principle of competitive ELISA. This uses a primary, unlabelled, antibody being and the biological sample which contains the antigen of interest being added to an ELISA plate which is pre-coated with the same antigen. Any antigen which is not bound in an antigen-antibody complex is then removed by washing the plate. A secondary antibody that is specific to the primary antibody and incubated. The secondary antibody is labelled with an enzyme. A substrate is then added which causes the enzyme reaction to elicit a colour change reaction. This change in colour can be read by an optical plate reader. In the case of a competitive ELISA, the larger the colour change, the less antigen of interest in the biological sample.

Prostoglandin-D_2 (PGD_2) was measured in stored phosphate buffered saline (PBS) sputum supernatant samples (prepared as described in section 3.2.2.) and leukotriene-E_4 (LTE_4) in PBS sputum supernatant and stored urine samples (as described in section 3.1.1). PGD_2 was not measured in urine as it is rapidly metabolised and not reliably present in urine [206].

Commercially available ELISA kits were used for the PGD_2 and LTE_4 assays (Cayman Chemical, Michigan, USA). ELISA was run as per the manufacturer's instructions. Briefly, standards were made up using serial dilutions of the supplied assay-specific reagents

(either PGD_2 or LTE_4) to allow formulation of a standard curve. 50 µL of sample was added to each measurement well with 100 µL of ELISA buffer. 50 µL of assay tracer and 50 µL of monoclonal antibody were then added to each well. The ELISA plates were incubated overnight at 4°C, emptied and rinsed 5 times with ELISA buffer and then developed with Ellman's reagent. Standards and samples were run in duplicate. Plates were read on an optical plate reader at a wavelength of 405 nm at 30 minute intervals until the positive control wells had an optical density (OD) of between 0.3-1.5 units with the background was subtracted. Standard curves and sample concentrations were calculated from the OD. Serial dilutions of sputum supernatant and urine were tested and checked against the standard curve to obtain the optimum dilutions. Sputum was supernatant was tested neat, urine was tested at a dilution of 1:8 with phosphate buffered saline.

3.2.4 Measurement of cytokines in sputum and blood by multiplex array

Multiplex assays allow for the measurement of several cytokines within a single sample. We used the MSD U-Plex™ (Meso Scale Discovery, Maryland, USA) platform to measure type-2 cytokines. The U-Plex™ assay uses electrochemical luminescence which measures light which is emitted when electrochemical stimulation is applied to labelled antibodies to the analyte of interest. The assay can measure up to 10 analytes per sample. Eotaxin (CCL11), interleukin (IL) 4, IL-5, IL-9, IL-13, IL-17E/IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) were measured in cytokines in undiluted PBS sputum supernatant (as described in section 3.2.2) and stored blood serum (as described in section 3.2.1).

The assays were run as per the manufacturer's instructions. Briefly, the U-Plex plate was prepared with U-Plex linkers which had been mixed with the capture antibodies, incubated at room temperature for 60 minutes and then washed. Calibrator standards were reconstituted. 25 µL of either calibrator or sample were added to each well and incubated on a shaker at room temperature for 60 minutes. Plates were then washed 3 times before adding 50 µL of detection antibody solution to each well and incubating for 60 minutes at room temperature. Plates were then washed 3 times before adding 150 µL of read buffer and reading on an MSD instrument. Results were analysed using Discovery Workbench™ software (Meso Scale Discovery, Maryland, USA). The upper and lower limits of detection for the analytes, determined by the manufacturer documentation and the derived standard curves from experiment data, are shown in Table 3.1

Table 3.1 Upper and lower limits of detection of MSD assays

Analyte	LLD	ULD
Eotaxin	3.2	4800
IL-4	0.08	2100
IL-5	0.24	4000
IL-9	0.14	1500
IL-13	3.1	1900
IL-17E/IL-25	0.58	9200
IL-33	0.59	10300
TSLP	0.2	10100

Concentrations are shown in picograms per millilitre (pg/ml). LLD – lower limit of detection, ULD – upper limit of detection. IL – interleukin, TSLP – thymic stromal lymphopoietin

3.3 Observational study of severe asthma

I undertook a cohort study of severe asthma patients. Patients were recruited to an observational cohort study, the 'Oxford Airways Study'. I wrote the protocol for this

study and obtained local and national ethical and regulatory approvals. Participants were invited to participate in this study if they were being seen in the Oxford Airways Clinic for their routine clinical care. The aim of this study was the clinical and biological characterisation of participants with known or suspected airways disease at stable state and before and after key clinical interventions.

For patients who agreed to be in the study, written consent was provided by them to have the data from their routine clinical measurements, such as FEV1 and FeNO, entered into a trial database as well as undertaking additional tests such as symptom scores or further lung function tests. Participants also agreed to have samples such as blood tests and supernatant from induced sputum stored in a biobank and analysed for research purposes.

Patients were enrolled at the time of steady state, before key clinical interventions and at the time of exacerbation. Patients were then invited back for follow-up visits depending on their clinical course.

3.3.1 Baseline and follow-up measurements

Baseline measurements were carried out. Demographics, medication use and clinical characteristics were recorded. Pre and post-bronchodilator spirometry was conducted as described in section 3.1.1.5, blood tests including a full blood count were performed as described in section 3.1.1.2, asthma control questionnaire was measured as described in 3.1.2 and induced sputum testing was performed as described in section 3.1.1.6. Fractional exhaled nitric oxide was measured using a NIOX VERO® (NIOX,

Oxfordshire, UK) at an exhaled breath rate of 50 ml/s. The same measurements were performed at any subsequent follow-up visits.

Sputum supernatant was stored as described in section 3.2.2 and tested by ELISA for PGD_2 and LTE_4 and for cytokines my MSD multiplex as described in section 3.2.3.

4 Investigating the effects of inhaled steroids on markers of type-2 inflammation in the airways

4.1 Introduction

Patients with type-2 high asthma often have raised FeNO, indicating ongoing airway inflammation, despite treatment with inhaled corticosteroids. This may be due to treatment-refractory processes, or due to failure to deliver treatment either due to poor treatment adherence or poor inhaler technique. FeNO suppression using monitored inhaled steroids, as described in section 3.1.3, can be used to differentiate between treatment-refractory inflammation and poor treatment adherence. We investigated the effects of FeNO suppression on clinical measurements and the key cytokine and lipid mediators of type-2 inflammation (section 3.2.3) within the airways.

4.2 Methods

Patients with a confirmed diagnosis of asthma attending the Airways Clinic at Oxford University Hospitals NHS Trust were approached to be enrolled in the Oxford Airways Study as described in section 3.3. Written informed consent was obtained for patients who agreed to join the study. Patients performed routine FeNO measurements at every clinic visit. If, despite treatment with medium or high dose inhaled steroids [6], the FeNO was ≥ 45 ppb, the patients were offered FeNO suppression. For patients who consented, asthma control questionnaires, blood eosinophil counts, lung function and induced sputum samples were taken prior to FeNO suppression (day 0) and at the end of FeNO

suppression (day 7) in addition to daily FeNO measurements which were taken by the patient at home.

Sputum was processed as described in 2.2.2. Cell counts were performed and sample supernatants were analysed for the inflammatory cytokines IL-5, IL-13, eotaxin and thymic stromal lymphopoietin (TSLP) as described in and for the lipid mediators LTE_4 and PGD_2 as described in section 3.2.3.

4.3 Results

25 patients were recruited and had FeNO, blood, questionnaire and lung function measurements taken. 15 of these were able to produce sufficient sputum at both day 0 and day 7 for longitudinal mediator analysis.

The overall cohort was 64% female (n = 16) with a mean age of 48 years. The subgroup for sputum analysis was 53% female (n = 8) with a mean age of 53 years. The median ICS dose was 2000 µg beclometasone dipropionate equivalent. Baseline demographics of the cohort and sputum subgroup were similar and are shown in Table 4.1.

Table 4.1 FeNO suppression cohort demographics

	Total cohort (n = 25)	Sputum analysis subgroup (n = 15)
Age (years)	48 (16)	53 (16)
ICS dose (BDP equivalent, µg)*	1600 (1200)	1600 (1200)
Serum IgE (IU/L)	461 (617)	255 (336)
ACQ5	2.7 (1.2)	2.9 (0.9)
Mean VAS (mm)	35 (27)	40 (23)
Post bronchodilator FEV1 (L)	2.6 (0.9)	2.6 (1.1)
FEV1 (% predicted)	82 (18)	77 (18)
FeNO (ppb)	118 (74)	135 (86)
Blood eosinophils (*10^9/L)	0.54 (0.26)	0.48 (.20)

*Data shown as mean (SD) unless otherwise specified. VAS – visual analogue scale for cough, sputum, shortness of breath from 0 – 100mm, ICS – inhaled corticosteroid. BDP – beclometasone dipropionate, FeNO – fractional exhaled nitric oxide. * median (IQR)*

4.3.1 Effects of FeNO suppression test on clinical measurements

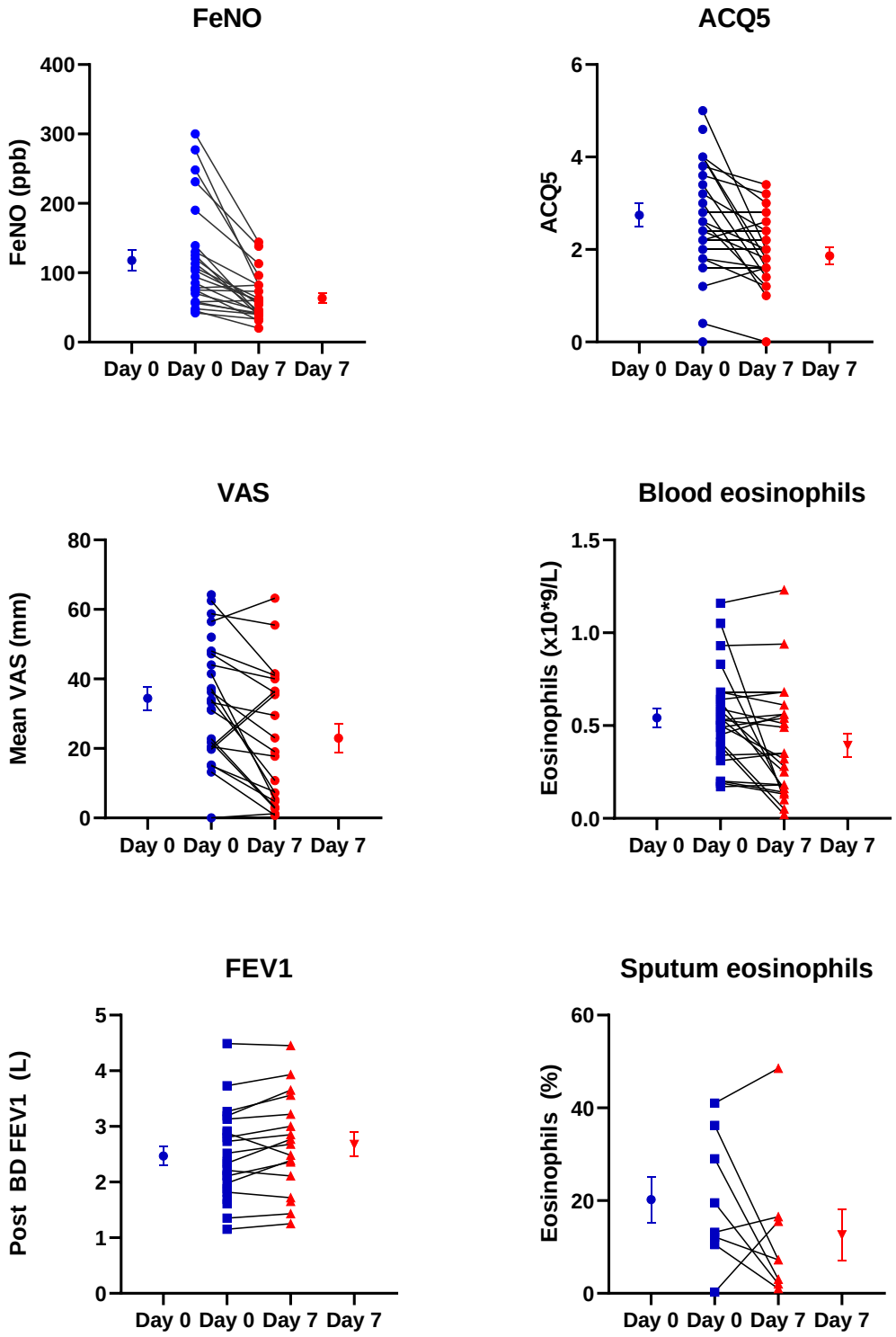
After 7 days of monitored ICS as per the FeNO suppression protocol 11 of the 25 participants had a positive FeNO suppression test as described in section 3.1.3. Asthma symptoms as measured by ACQ5 was improved by 0.75 [95% CI 0.33-1.1, $p < 0.001$) and symptoms measured by the mean VAS improved by 9 mm [3-15 mm, $p = 0.004$]. Post bronchodilator FEV1 improved by 134 ml [15-253 ml, $p = 0.29$]. FeNO was reduced by an average of 56 ppb [34-78 ppb, $p < 0.001$]. Interestingly, peripheral blood eosinophils were also reduced by 0.15*10^9/L [0.04-0.26, $p = 0.01$], Figure4.1. Similar results were seen when the sputum analysis subgroup, in which 6 of the 15 participants had a positive FeNO suppression test, were analysed. Of the 15 participants who were able to produce sputum samples at Day 0 and Day 7, 8 had paired, reliably countable and viable

cell counts for differential cell count at day 0 and 7. In this group, sputum eosinophils reduced by 7.6% from 20.2% to 12.6% [-20.9-5.7, p = 0.2].

I next investigated whether a positive FeNO suppression affected the positive clinical changes seen at day 7. For FeNO suppressors ACQ5 improved by 1.2 compared to 0.5 in the non-suppressed group (mean difference [95% CI] 0.7 [-0.2-1.5]). There was no difference in change in VAS. FEV1 improved by 187 ml in the FeNO suppressors compared to 118 ml in the non-suppressors (mean difference 69 ml [-183-321 ml]. Blood eosinophils decreased by 0.2 x 10^9/L in suppressors compared to 0.11 x 10^9/L in non-suppressors (mean difference 0.09 [-0.3-0.5 *10^9/L]).

In combination, these findings show clinically significant improvements in lung function, FeNO and symptom scores and a small effect on peripheral blood eosinophils with monitored inhaled corticosteroids. There is a trend towards a larger effect of inhaled corticosteroids on symptoms and lung function in participants with a positive FeNO suppression test.

Figure 4.1 Changes in clinical measurements between Day 0 and Day 7 of FeNO suppression test



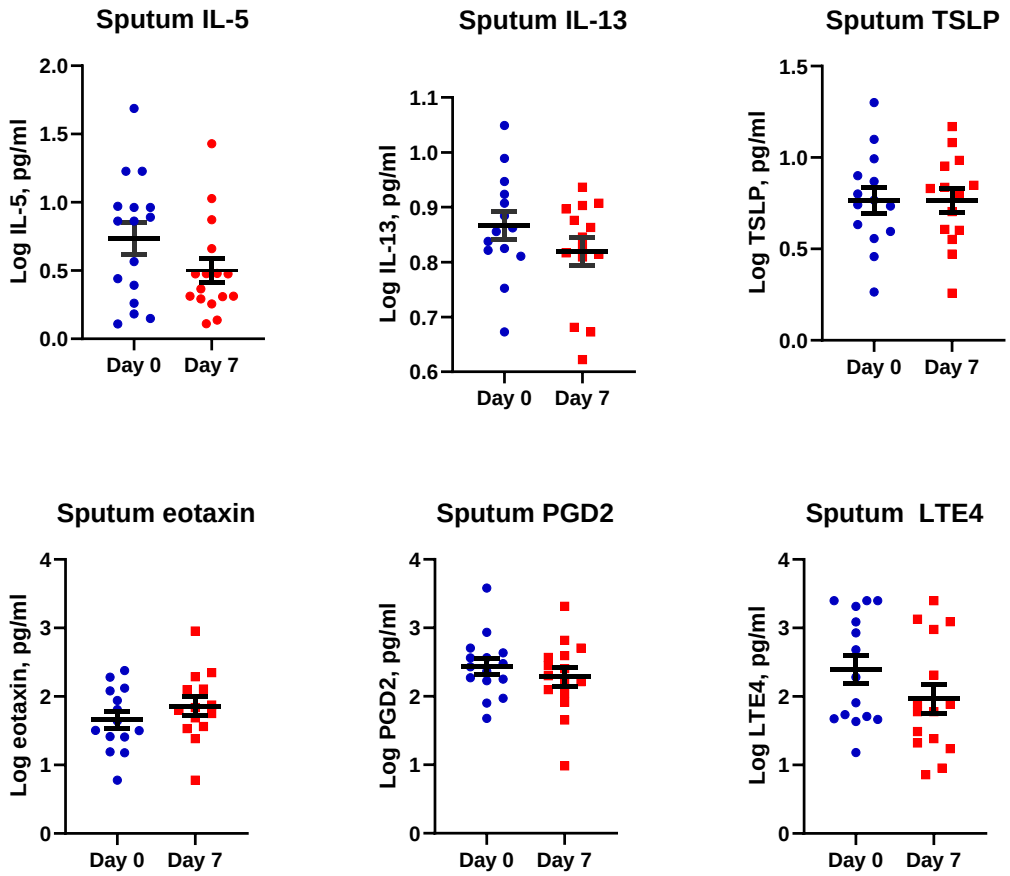
FeNO – fractional exhaled nitric oxide, ACQ5 – asthma control questionnaire 5, VAS – visual analogue scale, FEV1 – forced expiratory volume in 1 second, BD – bronchodilator. Error bars show mean and standard error of the mean

4.3.2 Effects of FeNO suppression test on type-2 mediators

Sputum IL-5 was significantly reduced from a geometric mean of 5.43 to 3.16 pg/ml, fold reduction [log SD] of 1.7 [0.41], p = 0.04 on Day 7 compared to Day 0. IL-13 was also significantly reduced from geometric mean 7.4 pg/ml to 6.6 pg/ml, fold change 1.1 [0.07], p = 0.027. TSLP was unchanged at Day 7 compared to Day 0 with concentrations of 5.83 and 5.81 pg/ml respectively, p = 0.99. There was a non-significant increase in eotaxin between Day 0 and Day 7 from 45.6 pg/ml to 72.1 pg/ml, p = 0.17. LTE_4 reduced significantly on Day 7 falling from 251.2 to 92.7 pg/ml, fold change 2.2 [0.57], p = 0.01. PGD_2 was numerically lower but not significantly reduced with values of 273.5 pg/ml and 193.2 pg/ml at Days 0 and 7 respectively, p = 0.14 (Figure 4.2). IL-25 and IL-33 were largely below the lower limit of detection of the assay (section 3.2.4) and did not change with FeNO suppression in participants in whom it was measurable.

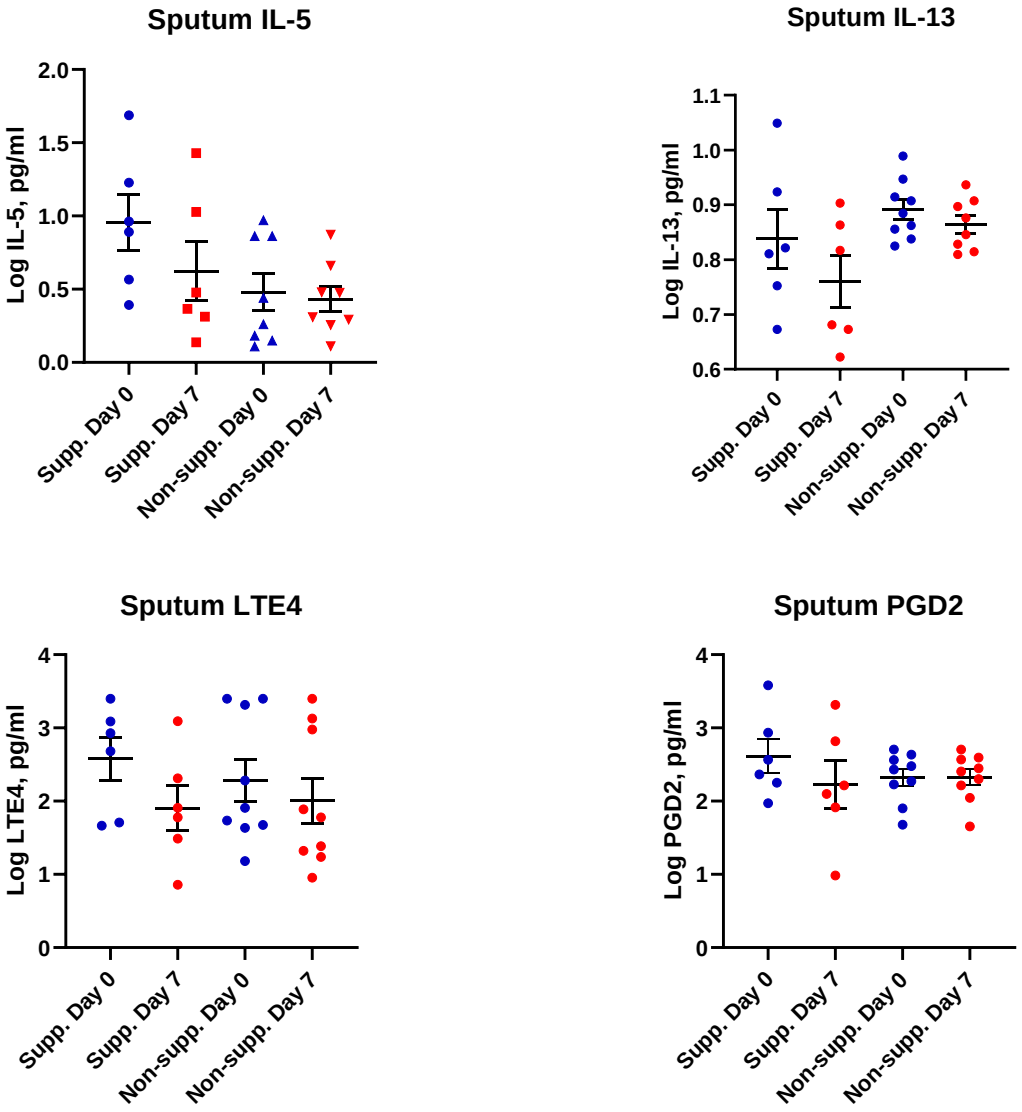
There were numerically larger falls in IL-5 (2.1 fold vs 1.1 fold, p = 0.2), IL-13 (1.2 fold vs 1.1 fold, p = 0.17) and LTE (4.7 fold vs 1.9 fold, p = 0.19) in FeNO suppressors (n = 6) than in FeNO non-suppressors (n = 9) respectively. PGD_2 fell significantly in FeNO suppressors but was unchanged in FeNO non-suppressors (2.4 fold reduction vs 1.0 fold change, p = 0.04) (Figure 4.3).

Figure 4.2 Changes in airway type-2 mediators with FeNO suppression



IL- interleukin, TSLP - thymic stromal lymphopoietin, PGD_2 – prostaglandin D2, LTE_4 – leukotriene E4, FeNO – fractional exhaled nitric oxide. Log refers to log_{10}, error bars show mean +/- standard error of the mean

Figure 4.3 Differences in airway type-2 mediators between FeNO suppressors and FeNO non-suppressors



Supp. – FeNO suppressors, Non-supp. – FeNO non-suppressors. Log refers to log_{10}, error bars show mean +/- standard error of the mean

4.4 Discussion

Despite baseline treatment with high doses of inhaled steroid, treatment with 7 days of monitored inhaled corticosteroid on top of usual asthma medications resulted in clinically and statistically significant improvements in clinical measures of airflow inflammation, asthma symptoms and airway inflammation. These improvements are likely due poor adherence to baseline medications rather than an effect of increased steroids as inhaled steroids have a dose plateau effect [207, 208] with minimal gains beyond a certain dose.

Monitoring of FeNO daily along with daily inhaler usage allowed evaluation of the clinical effects with a known adherence to medication. The clinical improvements most marked in patients who had a fall in FeNO, with smaller gains in patients who did not have a FeNO response. This suggests that the clinical improvements are being driven by a reduction in type-2 airway inflammation, the primary treatment target of inhaled steroids. This improvement in clinical measurements is mirrored by a reduction of the inflammatory mediators IL-5, IL-13 and LTE_4 in the airways. This finding shows the key mechanistic pathways, including IL-5 leading to the recruitment of eosinophils (IL-5) and the production of FeNO via the IL-13 dependent inducible nitric oxide synthase pathway, are attenuated by inhaled steroids. Again, it was seen that these reductions were most marked in FeNO suppressors. The smaller falls in FeNO non-suppressors suggests possible steroid resistant pathways leading to their production. There was no change in TSLP – an upstream inflammatory mediator produced by interactions at the epithelial barrier suggesting inhaled steroids do not affect this pathway. PGD_2 was markedly reduced in FeNO suppressors but unchanged in non-suppressors despite no significant

difference in baseline levels. As previously discussed in section 1.3, PGD_2 is a potent chemoattractant and activator of all CRTH2 expressing cells including eosinophils, Th2, Tc2 and ILC2 cells, which have all shown to play a role in the pathogenesis of severe eosinophilic asthma. These findings suggest that there may be an inhaled steroid resistant pathway contributing to PGD_2 in the airways. Targeting PGD_2 directly may therefore be an attractive treatment target in patients with inhaled steroid resistant disease.

5 The effect of timapiprant (OC000459) on eosinophilic airway inflammation and asthma control in subjects with refractory eosinophilic asthma: a randomised, double blind, placebo-controlled trial

Having shown that PGD2 is not supressed in patients with ICS resistant type-2 airway inflammationand previously shown that Tc2 cells are present in increased numbers and are hyper-responsive to PGD2 in this patient population, I investigated the effect of antagonism of CRTH2 with timapiprant in patients with severe eosinophilic asthma.

I conducted a randomised, double blind, placebo-controlled trial of 50 mg of timapiprant or placebo in 40 participants with severe eosinophilic asthma. The study was be conducted in accordance with the Good Clinical Practice (GCP) Guidelines as issued by the International Conference on Harmonisation [209], the Declaration of Helsinki [210] and the UK Statutory Instrument which incorporates the European Clinical Trial Directives (Directives 2001/20/EC and 2005/28/EC) and subsequent amendments. The trial was registered on the European Clinical Trials Database (EudraCT), EudraCT number: 2015-01833-26.

5.1 Objectives and endpoints

The objective of this study was to determine the effect of timapiprant on induced sputum eosinophil counts at 12 weeks of treatment compared to placebo. The primary endpoint was fold change in induced sputum eosinophil count from baseline to Week 12 compared to placebo. The secondary endpoints were change from baseline in the

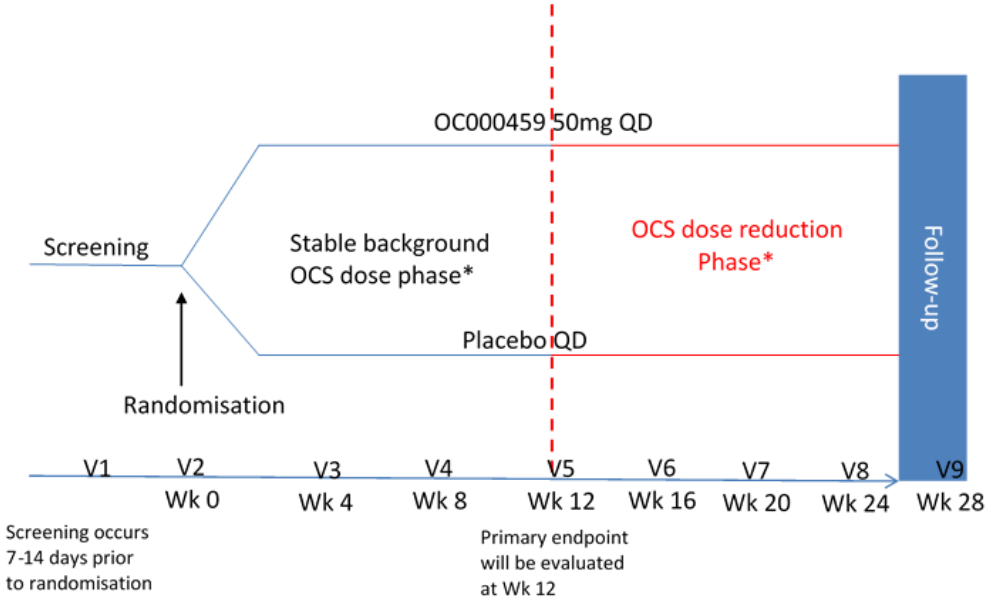
following: i) Induced sputum eosinophil count at week 12, ii) ACQ-5 at weeks 4, 8 and 12, iii) AQLQ(S) at weeks 4, 8 and 12, iv) FeNO at weeks 4, 8, and 12, v) FEV1 and FVC before and 20 minutes after inhaled salbutamol at weeks 4, 8, and 12, and v) induced sputum eosinophil count at weeks 4 and 8. Exploratory endpoints were i) to determine the effect of timapiprant on asthma control, ii) oral steroid usage and eosinophil airway inflammation during the OCS dose reduction phase of the study in subjects who were treated with OCS at baseline, iii) to evaluate the blood eosinophil count as a potential predictor of response to timapiprant treatment, iv) to evaluate the number of CRTH2-expressing cells in blood as a potential predictor of response to timapiprant treatment, and v) to evaluate urinary and sputum eicosanoid (PGD_2 and LTE_4) levels as potential predictors of response to timapiprant treatment. Safety endpoints were; to evaluate adverse events, clinically significant changes in clinical pathology (haematology, clinical chemistry and urinalysis), physical examination, ECG and vital signs at all time points.

5.2 Study design

I conducted a randomised, double blind, placebo controlled, parallel group comparison of the effects of oral timapiprant 50 mg once daily and matched placebo once daily on airways inflammation in subjects with severe eosinophilic asthma. Subjects who were not treated with oral corticosteroids at Baseline received 12 weeks treatment with either timapiprant or placebo. Subjects who were receiving oral corticosteroids at Baseline received either timapiprant or placebo for 24 weeks; the second 12 weeks of the study was set up to evaluate the effects of oral corticosteroid dose reduction on symptoms and measures of disease activity.

The study design in summarised in Figure 5.1. The oral corticosteroid reduction schedule from weeks 12-24 is shown in Table 8.3 (appendix).

Figure 5.1 Timapiprant (OC000459) study schedule



*Subjects not receiving background OCS at Baseline will undergo all on-therapy assessments at Visit 5 (week 12), but study medication will then no longer be administered. Subjects will return 4 weeks later for Visit 9 (Follow-up) assessments.
All other subjects will continue into the OCS dose reduction phase.

5.3 Study participants

Adult participants were recruited from a single centre from the Oxford Specialist Airways Clinic. Eligible participants had to have evidence of historical airflow obstruction, be on high dose inhaled corticosteroid treatment and meet the ERS/ATS criteria for severe asthma [7] and have evidence of eosinophilic airways inflammation as evidenced by a FeNO ≥ 50 ppb OR a blood eosinophil count ≥ 300 cells/µL OR a sputum eosinophil count ≥ 3% in the 24 months prior to the screening visit. Patients taking regular oral corticosteroids needed to be receiving 20 mg prednisolone or less and have had a stable dose for at least 4 weeks prior to screening and had to be a

minimum of 4 weeks from a course of rescue prednisolone. Participants had to have an induced sputum eosinophil count of ≥ 3% when measured at the screening visit.

Exclusion criteria included the current or recent use of type-2 targeted biological drugs, clinically significant abnormal serum biochemistry, haematology and urine examination values, hospital admission within the past 3 months, pregnancy or breastfeeding and the presence of another clinically important lung condition.

Full inclusion and exclusion criteria are listed in appendix section 8.3.1.

5.4 Study procedures

Written, informed consent was obtained prior to any study measurement or procedures being carried out. At each study visit, participants had vital signs measures, adverse events recorded, concomitant medications recorded, blood samples, urinalysis, FeNO measurement, pre and post-bronchodilator spirometry, ACQ5 and AQLQ questionnaires and diary card review. In addition, at prescribed visits, participants had a physical examination and electrocardiogram. For subjects taking oral corticosteroids, additional telephone follow-ups were conducted between weeks 12 and 24. The full schedule of study procedures is shown in appendix section 8.3.4.

5.5 Statistical analysis

Analyses were conducted using IBM SPSS® version 25 (IBM, New York, USA). Logged values refer to log to base 10.

5.5.1 Sample size and power calculation

The study was powered to detect a 3-fold reduction in the induced sputum eosinophil count. A 3-fold reduction was chosen as this reduction in sputum eosinophils has been shown to be clinically meaningful. Greene *et al*. showed that a 3-fold reduction in sputum eosinophils resulted in 67% fewer severe asthma exacerbations [48]. A 3-fold reduction in sputum eosinophils is also the same magnitude of reduction seen with the CRTH2 antagonist fevipiprant [199]. Using a two-sided test with a 5% significance level, in order to detect an assumed 3-fold reduction in induced sputum eosinophil counts between timapiprant and placebo (i.e. a difference in treatment group means of 0.477 on the logged scale) with at least 80% power and assuming a within-group standard deviation of 0.495 [141, 211] of change from baseline data on the logged scale, 18 evaluable subjects were required per group. Assuming approximately 90% of randomised subjects are evaluable, at least 20 subjects were required to be randomised to each treatment group (i.e. 40 subjects in total).

5.5.2 Randomisation

Randomisation was performed according to the method of minimisation, stratified by use of OCS at Screening (yes/no) and three categories of screening sputum eosinophil count (≥ 3% and < 10%, ≥ 10% and <35%, ≥35%). Randomisation was carried out using the electronic RRAMP system (OCTRU, Oxford, UK). The allocation ratio to timapiprant to placebo was 1:1.

5.5.3 Analysis sets

The primary analysis set is the Full Analysis Set (FAS) - all subjects who satisfied the randomisation criteria and received at least one dose of study medication and have at

least one post-baseline primary efficacy assessment. The full analysis set was considered the primary analysis population for efficacy endpoints. Secondary efficacy analysis wereconducted on the per-protocol set (PPS) – all subjects from the full analysis who have complied with the dosing regimen defined as at least 80% compliance with medication as confirmed by returned pill count), and the safety analysis set (SAS) – any subject who was randomised and received at least one dose of study medication. The primary endpoint was also evaluated in the following, pre-specified, subgroups; participants with ACQ-5 average score ≥ 1.5 at baseline; participants with at least 2 asthma exacerbations during the year prior to study start and; subjects with blood eosinophils ≥ 250 cells/µL at screening.

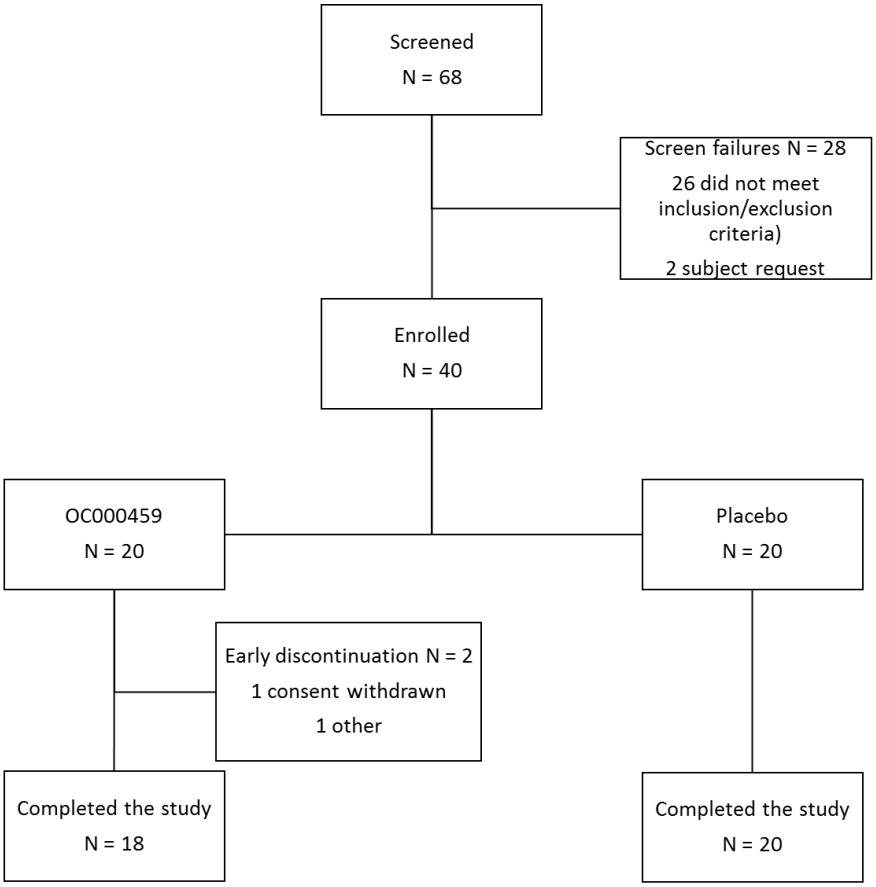
5.5.4 Analysis of endpoints

Statistical analyses were performed using appropriate two-sided hypothesis tests at the 5% significance level. Continuous variables were summarised using number of observations, mean (and/or geometric mean, where applicable), median, standard deviation, lower quartile, upper quartile, minimum and maximum values. Categorical variables were summarised presenting proportions (counts and percentages). The primary endpoint of timapiprant and placebo differences in the mean change from baseline at week 12 of the logged induced sputum eosinophil count data was analysed using analysis of covariance (ANCOVA) with use of OCS at Screening (yes/no) as factors and baseline sputum eosinophil count (Log_{10} value) as covariate. Further details of the statistical analyses are detailed in appendix section 8.3.3.

5.6 Results

A total of 68 participants were screened. The majority of participants who failed screening did so due to sputum eosinophil counts < 3%. 40 participants were enrolled and randomised to receive either 50 mg of timapiprant (n = 20) or placebo (n = 20) (Figure 5.2).

Figure 5.2 Timapiprant (OC000459) study CONSORT diagram



Participant demographic characteristics were similar in the timapiprant and placebo groups. Most subjects were male (15 [75.0%] subjects in the timapiprant group and 13 [65.0%] subjects in the placebo group. Mean (SD) age was 58 (15) years in the timapiprant group and 59 (11.0) years in the placebo groups. 5 (25%) of the participants

in the timapiprant group and 7 (35%) in the placebo group were taking maintenance oral corticosteroids at study screening.

At the screening visit, the average sputum eosinophil percentage was 17.49% (17.86% in the timapiprant group, 17.11% in the placebo group) which reduced to 10.82% (10.98% in the timapiprant group, 10.66% in the placebo group) at the randomisation visit. A summary of participant demographics is shown in Table 5.1.

Participant demographic characteristics were similar for the safety analysis set, the full analysis set and the per-protocol set. The numbers of participants in each analysis set are shown in Table 5.2.

Table 5.1 Timapiprant study baseline demographics and disease characteristics

	Timapiprant (n = 20)	**Placebo (n = 20)**	**Total (n = 40)**
Sex, male, n (%)	15 (75.0)	13 (65.0)	28 (70.0)
Age at screening, years	58 (15)	59 (11)	59 (13)
Race, n (%)			
White	18 (90.0)	18 (90.0)	36 (90.0)
Body mass index, kg/m²	27.6 (5.54)	29.3 (5.09)	28.5 (5.31)
Time since asthma diagnosis, years	30.0 (19.72)	26.2 (19.66)	28.1 (19.53)
Asthma exacerbations in the previous 12 months	3.2 (2.5)	3.9 (2.9)	3.6 (2.7)
0, n (%)	1 (5.0)	2 (10.0)	3 (7.5)
1, n (%)	6 (30.0)	4 (20.0)	10 (25.0)
2–4, n (%)	9 (45.0)	6 (30.0)	15 (37.5)
≥5, n (%)	4 (20.0)	8 (40.0)	12 (30.0)
Pre-bronchodilator FEV_1 % predicted	69.4 (26.9)	72.0 (22.9)	70.7 (24.7)
ICS dose, µg, BDP equivalent*	1200 (1000-2000)	1200 (1000-2000)	1200 (1000-2000)
On OCS, n (%)	5 (25)	7 (35)	12 (30)
Induced sputum eosinophil count, %			
At screening**	17.9 (1.6)	17.1 (1.4)	17.4 (1.5)
At randomization**	11 (1.4)	10.7 (1.4)	10.82 (1.4)
Blood eosinophil count (10^9/L)*	0.61 (0.38)	0.43 (0.53)	0.53 (0.45)
FeNO (ppb)	68.8 (56.4)	68.3 (53.0)	68.5 (54.0)

*Data shown as mean (SD) unless otherwise specified. *Median (range) **geometric mean (log SD). OCS – oral corticosteroids, ICS – inhaled corticosteroids, FeNO – fractional exhaled nitric oxide, FEV1 – forced expiratory volume in 1 second, ppb – parts per billion*

Table 5.2 Timapiprant study participant numbers by analysis group

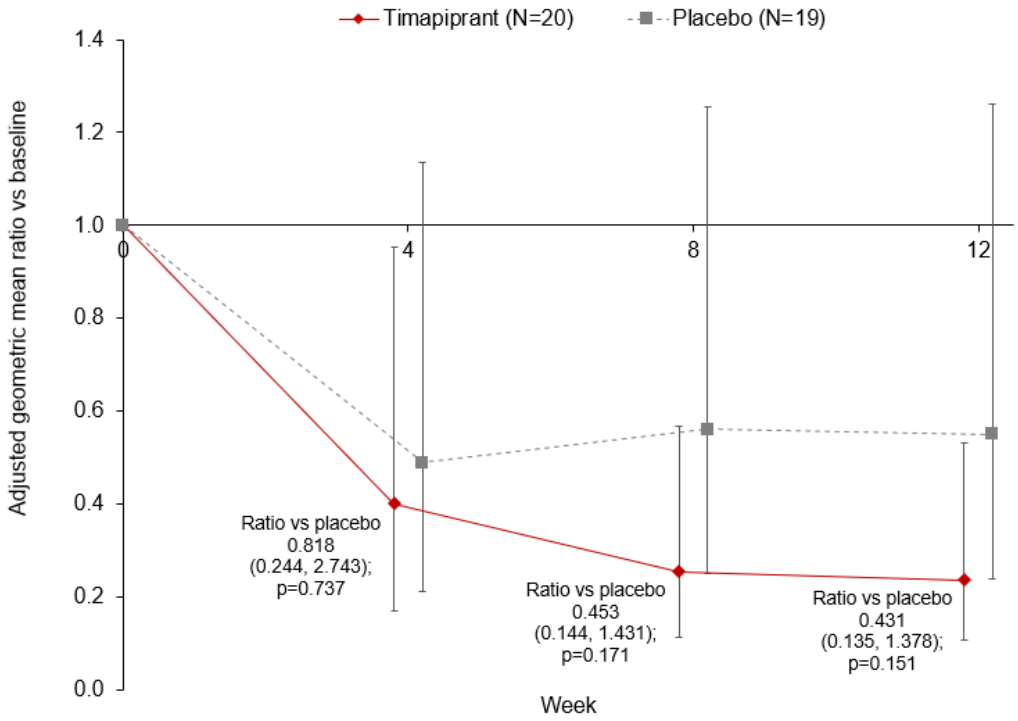
	Timapiprant **n = 20**	**Placebo** **n = 20**	**Total** **n = 40**
Safety analysis set	20 (100%)	20 (100%)	40 (100%)
Full analysis set	20 (100%)	19 (95%)	39 (97.5%)
Per protocol analysis set	18 (90%)	18 (90%)	36 (90%)

Data shown as n (%)

5.6.1 Sputum eosinophils

The primary endpoint was the fold change in sputum eosinophils from baseline to week 12 compared to placebo. Both groups were associated with a reduction from baseline (a 4.2-fold reduction with timapiprant, and a 1.8-fold reduction with placebo). The reduction was greater with timapiprant than placebo, with a ratio [95% CI] of 0.431 [0.135 – 1.378] representing a 2.3-fold reduction compared to placebo. The difference compared to placebo was not statistically significant (p = 0.151; Figure 5.3). Results were similar in the per protocol set and the safety analysis set. As seen at week 12, although there was a numerically greater reduction from baseline in sputum eosinophil count with timapiprant than placebo at weeks 4 and 8, the differences were not statistically significant between placebo and timapiprant groups (Figure 5.3). There was a suggestion of increasing efficacy over time. There was a numerical, but not statistically significant, decrease sputum eosinophil count from baseline to week 4, week 8 and week 12 (sputum eosinophil geometric mean 10.98%, 4.69%, 2.64% and 2.46% respectively).

Figure 5.3 Timapiprant study sputum eosinophil count adjusted geometric mean ratio vs baseline over the study duration (FAS)



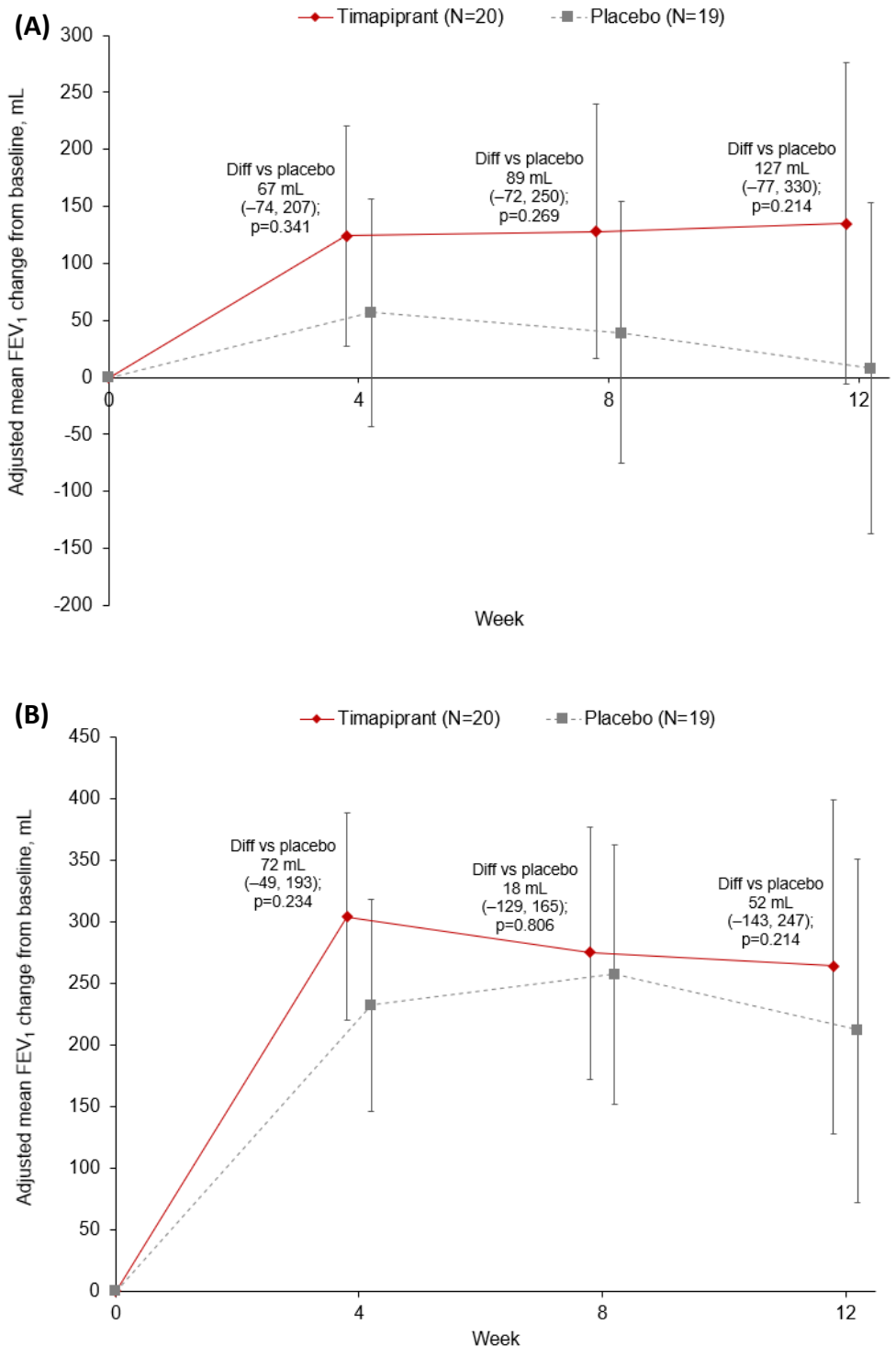
Data plotted are adjusted geometric mean ratio vs baseline and 95% confidence intervals

In pre-specified subgroup analyses, there was a significant reduction in sputum eosinophils at week 12 with timapiprant (n = 12) compared to placebo (n = 10) in participants with uncontrolled asthma, as evidenced by ACQ5 ≥ 1.5, with a ratio [95% CI] of 0.225 [0.077, 0.658] representing a 4.4 fold reduction compared to placebo, p = 0.009. In the other pre-specified subgroup analyses of participants with ≥ 2 exacerbations in the previous year and participants with blood eosinophils ≥ 250 cells/µL at screening, sputum reductions were larger than seen in the FAS with timapiprant compared to placebo, however these did not reach statistical significance.

5.6.2 Lung function

Pre- and post-bronchodilator lung function endpoint outcomes of FEV1 measurement showed a similar pattern to sputum eosinophil measurements. There was a greater improvement from baseline with timapiprant than placebo at all weeks 4, 8 and 12, especially for the pre-bronchodilator measurements. However, none of the differences were statistically significant when compared to placebo (Figure 5.4 a and b). The maximum change compared to placebo was seen at week 12 when timapiprant showed a 127 ml [95% CI -77, 330] improvement in pre-bronchodilator FEV1 compared to placebo. Of note, the baseline value in both evaluations was pre-bronchodilator FEV_1, and consequently the changes from baseline were greater in the post- than pre-bronchodilator evaluations.

Figure 5.4 Timapiprant study FEV_1 adjusted mean change from baseline over the study duration assessed (A) pre-bronchodilator, and (B) post-bronchodilator (FAS)



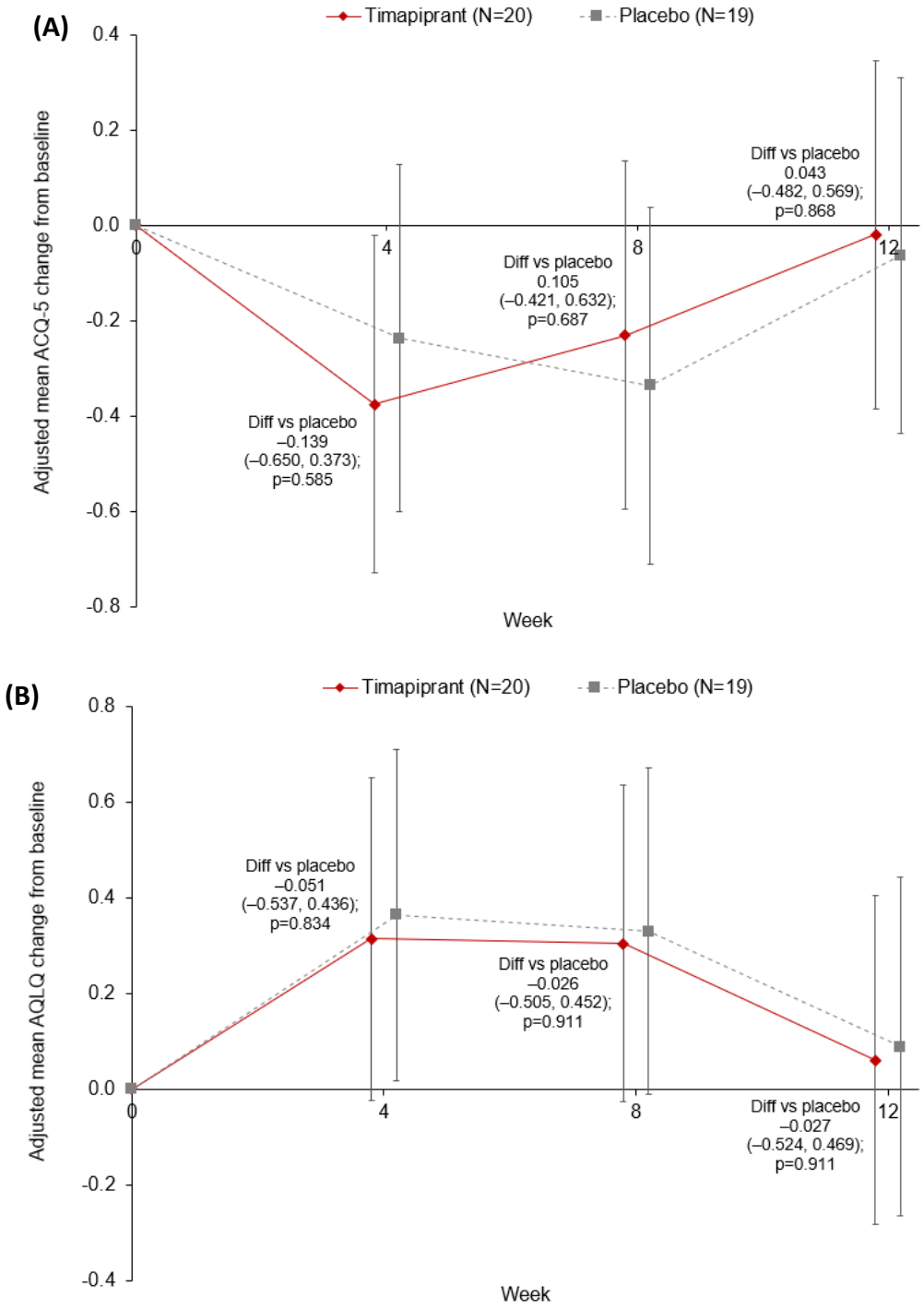
Data plotted are adjusted mean changes from baseline and 95% confidence intervals. FEV_1, forced expiratory volume in 1 second; FAS, full analysis set

5.6.3 Symptoms and quality of life

Asthma symptoms, as measured by the ACQ-5, did not show any clinically significant changes from baseline in the timapiprant or placebo groups (Figure 5.5a) Asthma quality of life, measured by the Asthma Quality of Life Questionnaire (AQLQ), also did not have any clinically significant changes from baseline in either treatment group (Figure 5.5b).

The proportion of ACQ-5 responders that achieved an improvement of the minimally important clinical difference (MCID) of 0.5 or greater in their score did not differ between groups at any visit (35.0 vs 26.3% at Week 4, for timapiprant and placebo, respectively, odds ratio 1.76 [95% CI 0.27, 11.59]; 25.0 vs 36.8% at Week 8, 0.43 [0.08, 2.41]; 30.0 vs 42.1% at Week 12, 0.33 [0.06, 1.88]). Similarly, the proportion of AQLQ responders achieving the AQLQ MCID of 0.5 did not differ between groups at any visit (30.0 vs 36.8% at Week 4, for timapiprant and placebo, respectively, odds ratio 0.73 [95% CI 0.09, 5.92]; 30.0 vs 31.6% at Week 8, 1.15 [0.24, 5.61]; 30.0 vs 26.3% at Week 12, 1.45 [0.28, 7.43]).

Figure 5.5 Timapiprant study (A) ACQ-5 and (B) AQLQ adjusted mean changes from baseline over the study duration (FAS).

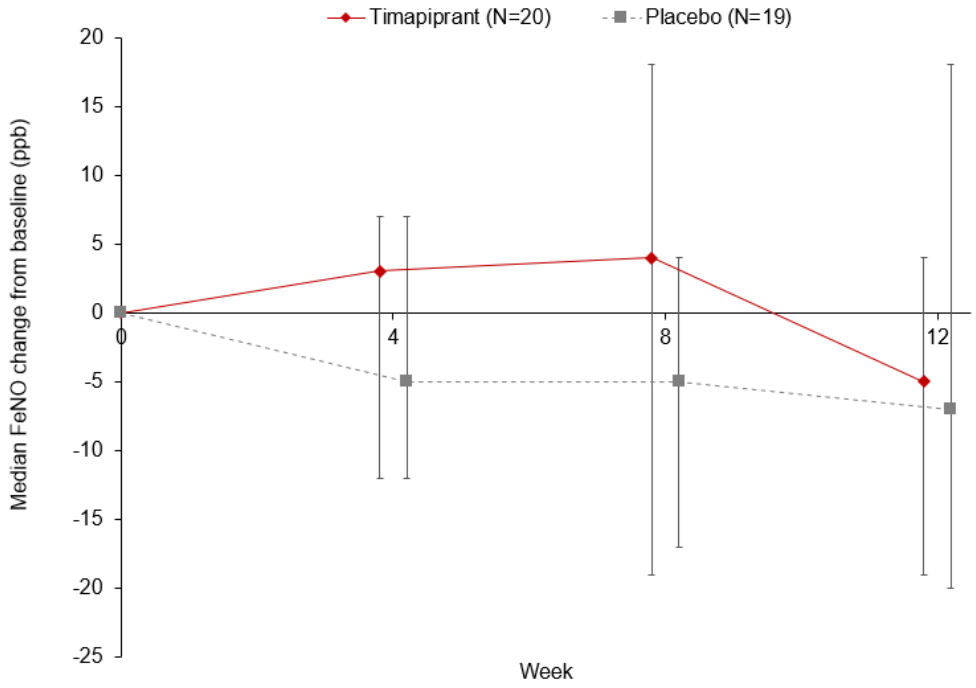


Data plotted are mean changes from baseline and 95% confidence intervals. ACQ5 - Asthma Control Questionnaire 5; AQLQ, Asthma Quality of Life Questionnaire; FAS, full analysis set.

5.6.4 FeNO

Overall changes from baseline in FeNO were small, with no clinically relevant or statistical differences between the two groups at any time point. There was high inter-patient variability in these measurements between visits (Figure 5.6).

Figure 5.6 Timapiprant study FeNO median changes from baseline over the study duration (FAS).



Data plotted are median changes from baseline and interquartile ranges. FeNO - forced exhaled nitric oxide; FAS, full analysis set.

5.6.5 Oral corticosteroid reduction

Participants in the timamiprant and placebo groups who were on maintenance oral corticosteroids at week 12 were invited to participate in an oral steroid withdrawal as previously described. 4 participants in the timampirant group and 7 in the placebo group completed the protocol to week 22. The 4 participants in the timampirant group were

able to reduce their oral corticosteroids to ≤ 5mg prednisolone / day compared to 6 out of 7 (87%) participants in the placebo arm. All of the timampiprant group were able to reduce their oral steroid dose by ≥ 50% compared to 87% of the placebo group. Given the small numbers in each group no statistical comparisons have been made for this exploratory endpoint.

5.6.6 Safety analysis

Adverse events (AEs) were experienced by 90% of patients in each group. The majority were mild or moderate in severity and were not considered related to treatment. Only two events were severe (one in each group), both of which were also serious, but neither considered related to treatment. One patient experienced AEs resulting in study drug discontinuation in the timapiprant group – three events: pruritus, back pain and flatulence. All events were moderate, unrelated to study treatment, and had resolved by the end of the study. However, the patient stopped taking study medication due to the events and so the reason was recorded as 'withdrew consent'. There were no clinically relevant findings for any of the other safety evaluations, in terms of haematology, clinical chemistry or urinalysis. There were no changes from baseline in overall vital signs (diastolic and systolic blood pressure, heart and respiratory rate, temperature, oxygen saturation and weight), with no clinically relevant findings in ECG analyses.

5.7 Biological effects and biomarkers of response to timapiprant

5.7.1 Effects of timapiprant on sputum mediators

There was no significant change in the concentrations of cytokines IL-5, IL-13, eotaxin or TSLP in the airways after 12 weeks of treatment with either timapiprant of placebo. There was no significant change in the concentrations of the lipid mediators LTE_4 and PGD_2 in either treatment group.

5.7.2 Effects of timapiprant on blood cytokine levels

Blood cytokines (in serum or plasma) were mostly below the lower limit of detection in this highly sensitive multiplex assay (section 3.2.4). For participants with detectable blood cytokine levels, there was no change with treatment and no correlation between blood cytokine levels and sputum eosinophil counts.

5.7.3 Effects of timapiprant on circulating CRTH2 cells

Treatment with 12 weeks of timapiprant did not significantly change the level of total circulating CRTH2 cells. Specifically, there was no significant difference in circulating basophils, eosinophils, Th2 cells, Tc2 cells or ILC2 after 12 weeks treatment either when measured as total cell numbers, or as a percentage of the white cell population.

5.7.4 Biomarkers of response

I next investigated whether any baseline measurements may be predictive of a response to timapiprant. There was no correlation between reduction in sputum eosinophils after 12 weeks of timapiprant and baseline IL-5, IL13, eotaxin, TSLP, PGD_2 or LTE_4. Baseline sputum eosinophil count was strongly correlated with reduction in sputum eosinophils after 12 weeks of timapiprant treatment, $r = 0.76$, $p = 0.001$. Blood eosinophils were

also correlated with reduction in sputum eosinophils, r = 0.58, p = 0.01. The total number of CRTH2 cells was positively correlated with reduction in sputum eosinophils, r = 0.67, p = 0.001. A similar result was seen if the percentage of CRTH2 positive cells was used rather than the total number. Th2 and ILC2 cells, either total numbers or percentages, were not correlated with the reduction in sputum eosinophils. The number, and percentage, or Tc2 cells were correlated with the reduction in sputum eosinophils on timapiprant, r = 0.51, p = 0.03.

5.8 Discussion

This single centre, proof of concept, randomised placebo-controlled trial investigated the effect of timapiprant on eosinophilic airway inflammation in severe eosinophilic asthma. Although the reduction in sputum eosinophil count was greater with timapiprant than placebo at Week 12, the difference between the changes in the timapiprant and placebo groups was not statistically significant, and so the primary objective was not met. Despite this, the study provided several interesting findings. Importantly, treatment with timapiprant was associated with a significant reduction from baseline in sputum eosinophil count at all three time points (weeks 4, 8 and 12), with a gradual increase in effect over the study duration. In contrast, the reduction from baseline in the placebo group was not statistically significant at any time point, with wide confidence intervals and no change in placebo effect over time. The magnitude of effect on sputum eosinophils seen with timapiprant was very similar to that seen with the CRTH2 antagonist fevipiprant in the only other study to measure the effects of CRTH2 antagonism on airway inflammation [199] with 4.2 and 4.3 fold reduction from baseline respectively. This observation suggests a consistent class effect of CRTH2 antagonism in the reduction of airway inflammation. The observed variance in the standard deviation of the sputum eosinophil results were greater than those expected and used for the power calculation, hence the study was likely underpowered, increasing the chances of a type 2 error.

There was a significant reduction in mean sputum eosinophil count between the screening and baseline visits (7–14 days run-in period during which medication was unchanged). This effect was equal in both treatment groups and likely represents an

overall improvement in adherence to background medication and regression to the mean. This reduction, in both groups, during the run-in period suggests a robust treatment-related effect is being measured. There was, however, a further fall in sputum eosinophils from Week 0 to Week 4 in both groups, after which the sputum eosinophil percentages remained stable in the plaebo group but continued to fall in the timapiprant group. This fall to Week 4 may reflect a further reflection of increased adherence due to study entry and regression to the mean. A longer run-in period of 6 weeks, rather than 1-2 weeks, may show any treatment-related effect more clearly.

Pre-dose FEV1 was numerically greater throughout the study with timapiprant compared to placebo. This observed effect was seen starting from Week 4 and maintained through to Week 12. The magnitude of FEV1 improvement effect is consistent with the previously observed changes in eosinophilic asthma in previous studies of timapiprant [195] and fevipiprant [199] . The post-bronchodilator data are more challenging to interpret, given the baseline value was assessed as the pre-bronchodilator value of the same study visit, so the results may primarily demonstrate the effect of salbutamol. However, timapiprant had a numerically greater effect than placebo at all three visits.

Timapiprant had no effect on the ACQ-5 and AQLQ evaluations, either overall or in the responder analyses (with fewer than half of the patients experiencing a clinically relevant improvement in either measure). These results are consistent with other studies of anti-inflammatory treatment such as mepolizumab, in which the effect of mepolizumab on ACQ-6 and AQLQ did not differ from that of placebo [39] and fevipiprant [199].

The analysis of the primary endpoint in the FAS was consistent with the results in the three sensitivity analyses – a not unexpected finding, given that the majority of patients were included in all four analyses. A more unexpected finding was from the pre-specified, *post-hoc* analyses, in which the greatest effect of timapiprant on sputum eosinophil count was in the subgroup with ACQ-5 ≥ 1.5 at baseline, with a significant 4.4-fold reduction vs placebo. This may be because uncontrolled, symptomatic asthma as reflected by the ACQ-5, is a marker of poor adherence to inhaled treatment and the addition of timapiprant caused a greater reduction in this group that was relatively under-treated at baseline. An alternative explanation may be that uncontrolled, symptomatic asthma is more likely to be present in patients with timapiprant-responsive airway inflammation. Detailed physiological and biological phenotyping of these patients, compared to well controlled patients, may reveal other potential biomarkers of respone. The use of timapiprant in the uncontrolled, symptomatic asthma population in a larger study warrants further investigation. The effect of timapiprant on sputum eosinophils was also greater in the other two subgroups than in the FAS, but the reductions vs placebo did not reach statistical significance. This demonstrates the need to identifying a specific phenotype of patients who may gain particular benefit from therapy.

This study showedthat baseline sputum and blood eosinophil counts were correlated with the reduction in sputum eosinophils with treatment with timapiprant. The total number, or percentage, or circulating CRTH2 positive cells are also correlated with response to treatment. This finding is unsurprising, as the majority of circulating CRTH2 positive are eosinophils. However, this analysis also found that Tc2 cells were correlated

with response to treatment, whereas Th2 and ILC2 cells were not. These findings support the previous findings by Hilvering *et al.* that Tc2, but not Th2, cells are enriched in the airways and blood of patients with severe eosinophilic asthma [182].

Although sputum eosinophils were significantly reduced compared to baseline with timapiprant treatment, there was no effect on FeNO, a biomarker of type-2 inflammation. These findings are in keeping with a study of fevipiprant in eosinophilic asthma [199] and also the lack of effect on FeNO of anti-IL-5 treatments in severe asthma [39]. There was also no change in mediators of type-2 inflammation, including IL-5, IL-13 and PGD_2 in the airways with treatment with timapiprant.

As seen in previous studies, timapiprant was well tolerated, with a similar adverse event profile to placebo and no relevant findings in the other safety parameters. The study population was predominantly male, Caucasian and had an average age of 59 years. This may have implications as to the generalisability of the data to differing populations.

In conclusion, this proof-of-concept trial provided additional data about timapiprant efficacy in severe eosinophilic asthma. Although the trial did not meet its primary efficacy endpoint, there was a suggestion of efficacy in this population that warrants further investigation. Although there were statistically and clinically meaningful reductions in sputum eosinophils in the timapiprant group compared to baseline, the greater than expected observed variance in sputum eosinophils would suggest a larger sample size is required to detect a statistically significant response. As with other anti-inflammatory treatments in eosinophilic asthma, it is likely that the key impact of CRTH2 antagonists will be in exacerbation reduction. The results support the ongoing development in a selected patient population.

6 Mepolizumab and exacerbations of severe eosinophilic asthma

6.1 Prognostic and predictive value of blood eosinophil count, fractional exhaled nitric oxide, and their combination in severe asthma

6.1.1 Introduction

Patients with severe eosinophilic asthma have a high risk of exacerbations requiring rescue oral corticosteroid treatment. Monoclonal antibody treatments inhibiting IL-5 directly or via the IL-5Rα or IL13/IL4 via the IL-4Rα reduce exacerbations of severe, eosinophilic asthma with evidence to type-2 inflammation as shown by a raised peripheral blood eosinophil count or fractional exhaled nitric oxide (FeNO) [39] [212]. Both of these biomarkers have been associated with an increased risk of exacerbations [213].

The key cytokine for the development of eosinophils is IL-5 whereas FeNO is regulated by the IL-13 dependant inducible nitric oxide pathway [93]. These markers of differing pathways active in producing type-2 inflammation suggests that their combination might provide additive prognostic and predictive information. I tested this hypothesis in a post-hoc analysis of a placebo-controlled trial of anti-IL-5 (mepolizumab) in patients with severe asthma.

6.1.2 Methods

I undertook a *post-hoc* analysis of a phase 2b study of mepolizumab in patients with severe eosinophilic asthma (DREAM) [39]. This study was selected as it was the only randolised controlled trial of mepolizumab to assess both FeNO and blood eosinophils at baseline. DREAM evaluated placebo and 3 doses of mepolizumab (75, 250 and, 750

mg IV 4 weekly) for 52 weeks. Participants had a history of 2 or more exacerbations requiring oral corticosteroids in the previous year and evidence of eosinophilic inflammation as reflected by one of more of the following: a peripheral blood eosinophil count ≥ 300 cells/μL; a sputum eosinophil count ≥ 3%; FeNO ≥ 50 ppb; prompt deterioration of asthma control after a 25% or less reduction in regular maintenance inhaled or oral corticosteroids. As the DREAM study did not show a dose-related effect of active treatment or evidence of an interaction between dose and predictive value of biomarkers, our analysis is based on the combined effect of the different doses.

Participants were divided into subgroups depending on their baseline peripheral blood eosinophil count (PBE) and FeNO. PBE were defined as high (≥ 150 cells/μL) or low (<150 cells/μL) and FeNO as high (≥ 25 ppb) or low (< 25 ppb). These cut points were chosen because of pre-existing evidence linking them to eosinophilic airway inflammation and response to corticosteroids [214]. Baseline demographics, clinical characteristics and annualised exacerbation rates were calculated based on 4 biomarker subgroups; PBE high-FeNO high, PBE high-FENO low, PBE low-FENO high, PBE low-FeNO low. An additional analysis was carried out using a PBE cut point of 300 cells/μL.

The DREAM study was a multi-centre, randomised, double blind, placebo-controlled trial. The primary interest of this analysis was severe exacerbation rate, defined as the requirement for rescue oral corticosteroids, as the main benefit of mepolizumab treatment is to reduce the rate of exacerbations and exacerbation rate was the primary outcome measure of the trial. Change in pre-bronchodilator FEV1 after 52 weeks of treatment was also investigated.

6.1.3 Results

606 DREAM participants had baseline blood eosinophil and FeNO measurements. The study population had a mean of 3.6 exacerbations per patient per year in the year prior to study enrolment. Lung function was reduced, with a mean FEV1 of 60% predicted and there was a high symptom burden with a mean ACQ6 score of 2.3 (< 1.5 indicating good control). The baseline demographics and clinical characteristics of the study patients across biomarker subgroups and treatment are shown in table 6.1. Placebo and mepolizumab groups were well matched overall, as werethe biomarker subgroups.

The risk of exacerbations was highest in placebo treated patients with high baseline PBE and FeNO. The efficacy of active treatment was most marked in this group with mepolizumab showing 61% exacerbation rate reduction, compared to 33% exacerbation rate reductions in the PBE high-FeNO group. Mepolizumab did not have a significant effect on exacerbation rate in the PBE low subgroups regardless of FeNO. Pre-bronchodilator FEV1 tended to increase with mepolizumab compared to placebo in PBE high groups regardless of FeNO, whereas there was no improvement in FEV1 in the PBE groups (Figure 6.1). To investigate whether the level of blood eosinophils affected this, patients were stratified by a PBE of 300 cells/μL and a similar pattern was seen (Figure 8.1, section 8.4, appendix).

Table 6.1 Baseline demographics and disease characteristics of DREAM patients divided by baseline PBE and FeNO subgroup and randomised treatment.

	PBE <150 cells/µL, FeNO <25 ppb		PBE <150 cells/µL, FeNO ≥25 ppb		PBE ≥150 cells/µL, FeNO <25 ppb		PBE ≥150 cells/µL, FeNO ≥25 ppb		Total	
	Placebo (n = 18)	**Mepolizumab (n = 57)**	**Placebo (n = 14)**	**Mepolizumab (n = 57)**	**Placebo (n = 35)**	**Mepolizumab (n = 127)**	**Placebo (n = 84)**	**Mepolizumab (n = 214)**	**Placebo (n = 151)**	**Mepolizumab (n = 455)**
Age, years	44.3 (12.07)	52.7 (11.79)	45.5 (10.55)	50.6 (8.27)	45.9 (11.05)	48.0 (10.99)	46.9 (11.54)	49.2 (11.49)	46.2 (11.33)	49.5 (11.10)
Female, n (%)	11 (61)	38 (67)	7 (50)	34 (60)	29 (83)	83 (65)	49 (58)	132 (62)	96 (64)	287 (63)
ICS Dose (µg/day)[1]	1053 (356)	1108 (434)	1033 (115)	1077 (567)	1175 (464)	1150 (523)	1169 (608)	1094 (447)	1145 (523)	1109 (483)
Maintenance OCS use, n (%)	2 (11)	18 (32)	5 (36)	22 (39)	8 (23)	25 (20)	27 (32)	75 (35)	42 (28)	140 (31)
Pre-bronchodilator FEV1, mL,	2253 (812.2)	1747 (631.1)	2105 (811.7)	1888 (647.0)	1611 (506.3)	1845 (636.2)	1919 (605.8)	1911 (678.8)	1905 (656.9)	1869 (657.4)
Pre-bronchodilator %predicted FEV1	66.3 (17.05)	59.2 (14.99)	58.2 (12.90)	60.6 (15.22)	53.9 (12.77)	59.3 (16.57)	60.5 (15.54)	60.3 (16.24)	59.4 (15.21)	59.9 (16.01)
Post-bronchodilator FEV1, mL	2688 (821.8)	2008 (706.1)	2648 (948.3)	2178 (725.5)	1927 (652.8)	2154 (680.0)	2311 (725.0)	2297 (764.4)	2298 (777.1)	2206 (733.8)
ACQ-6 Score	2.6 (1.20)	2.1 (1.02)	2.5 (0.82)	2.5 (0.98)	2.4 (1.11)	2.1 (1.13)	2.5 (1.08)	2.4 (1.11)	2.5 (1.07)	2.3 (1.10)
PBE count, cells/µL*	80 (0.57)	50 (0.95)	50 (0.89)	80 (0.72)	350 (0.66)	350 (0.54)	450 (0.61)	410 (0.63)	280 (1.01)	240 (1.03)
FeNO count, ppb*	14.6 (0.39)	12.3 (0.45)	42.3 (0.49)	54.0 (0.47)	14.4 (0.34)	15.2 (0.42)	55.5 (0.55)	51.2 (0.50)	33.7 (0.79)	30.7 (0.79)
Exacerbations in year prior to study, mean (SD)	2.7 (1.87)	3.3 (2.50)	3.5 (1.99)	3.9 (3.06)	2.9 (1.09)	3.0 (2.03)	4.4 (4.87)	3.8 (3.16)	3.8 (3.83)	3.5 (2.81)
Requiring hospitalization, n (%)	4 (22)	20 (35)	3 (21)	17 (30)	7 (20)	28 (22)	25 (30)	44 (21)	39 (26)	109(24)

*1. ICS doses are presented as ex-valve/metered doses and are based on conversions to an FP equivalent dose. Data shown as mean (SD) unless otherwise specified. *geometric mean (log SD). ACQ, asthma control questionnaire; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 second; ICS, inhaled corticosteroids; OCS, oral corticosteroids; PBE, peripheral blood eosinophil count; ppb, parts per billion; SD, standard deviation*

6.1.4 Discussion

In patients with severe asthma treated with placebo who had high blood eosinophil counts and FeNO, the rate of severe exacerbations requiring oral corticosteroid treatment was up to twice that seen in placebo treated patients with low or discordant biomarker results. The biomarker subgroups all had similar baseline lung function, symptom scores and exacerbation risk indicating that the increased risk of exacerbation events is independent of these other markers of asthma control and risk. This indicates that biomarker profiling of patients with severe asthma adds predictive value to a traditional risk assessment.

To evaluate the relationship between biomarker profile and treatment efficacy, our findings were compared with published results of the phase 3 trial of monoclonal antibody dupilumab (QUEST), a monoclonal antibody that blocks IL-13 and IL-4 by binding to the IL-4 receptor-α [212]. This study evaluated 2 doses of dupilumab (200 mg or 300 mg SC 2 weekly) for 52 weeks in 1902 patients with moderate-severe persistent, uncontrolled asthma as per the GINA guidelines [6]. Both doses had equivalent efficacy and, as combined data is not available, data is presented from the 934 patients randomised to dupilumab 200 mg every two weeks or matched placebo. Information on change in pre-bronchodilator FEV1 by biomarker profile was not available for QUEST (Figure 6.1). The efficacy of dupilumab on exacerbations was most marked in the PBE high-FENO high group with a 68% reduction. There was also an exacerbation rate reduction of 33% in the PBE high-FeNO low group which was similar to the effect seen in this subgroup with mepolizumab treatment. In the PBE low-FeNO low group neither biologic had a significant effect on exacerbations. In the PBE low-FeNO high group a 39% exacerbation rate reduction was seen with dupilumab. Although not statistically

significant, this finding contrasts to the absence of effect seen with mepolizumab in this subgroup.

The study populations differed significantly with the DREAM population having a higher exacerbation risk and a higher proportion of patients using high dose inhaled or regular oral corticosteroids. However, the higher risk of exacerbation in patients with higher blood eosinophils and FeNO was seen in both populations suggesting a true effect seen across a range of asthma severity. This is in keeping with earlier studies showing that a composite profile of biomarkers of type-2 airway inflammation provides prognostic information over and above a assessment of risk of exacerbation based on traditional asthma measures although this earlier study used a composite score of blood eosinophils, serum periostin and FeNO biomarkers [215]. This analysis extends these earlier findings by showing that patients with both high blood eosinophil counts and FeNO also had the greatest response to biological treatment with mepolizumab and dupilumab, indicating that biomarker profiles have predictive as well as prognostic value.

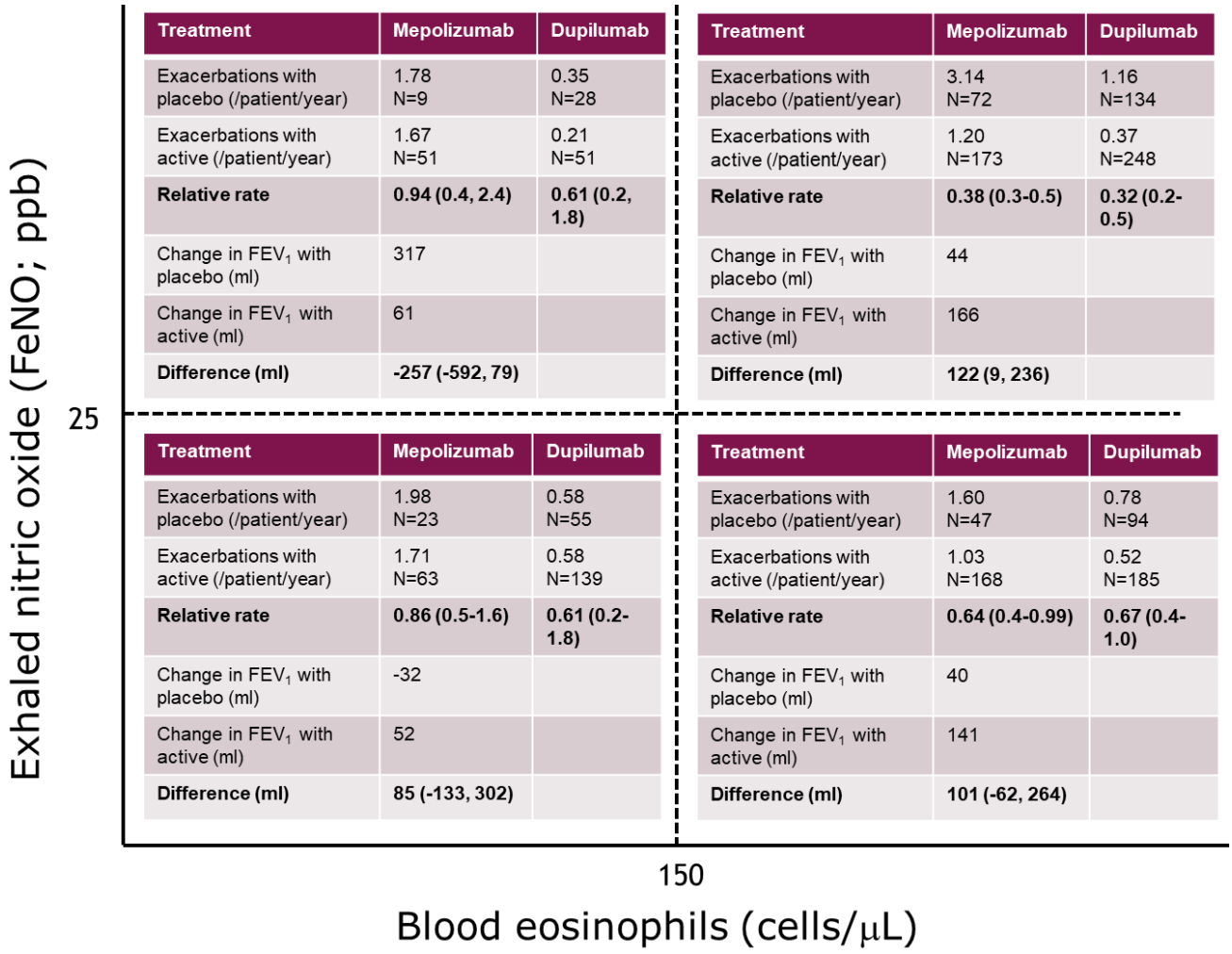
The greater prognostic value of the combined biomarker profile makes biological sense given the biomarkers relate to different aspects of type-2 immune responses in the airway. The peripheral blood eosinophil count reflects airway and systemic IL-5 production and is reduced markedly by anti-IL-5 but not dupilumab. In contrast, FeNO reflects airway IL-13 activity as it is reduced markedly by anti-IL-13 and dupilumab but not anti-IL-5. Thus, the two measures provide a more complete assessment of type-2 immune responses in the airway. It is also likely that the combination of a systemic and local airway measure adds precision to an assessment in only one of these compartments. We did not find an improvement for mepolizumab in patients with high FeNO but low PBE as might be expected as FeNO is a

marker of IL-13 activity in the airway. In contrast, there was a trend for benefit of dupilumab in this group.

Caution is required in interpreting this post-hoc subgroup analysis since the number of patients in some subgroups is small and the populations studied in DREAM and QUEST were different. It is acknowledged that the relative efficacy of mepolizumab and dupilumab treatment in subgroups may reflect the play of chance or differences in patient populations as well as the different cytokine associations of the biomarkers. However, it is striking that the relative exacerbation rate reductions in three of the four subgroups were very similar.

The findings of this analysis suggest that biomarker profiles might have value in identifying patients suitable for different biological agents. Formal head-to-head studies in similar patient populations are needed to assess this possibility prospectively. Future studies should also model the relationship between biomarker values and exacerbations more completely, allowing more accurate inferences to be drawn on individual patient responses.

Figure 6.1 Mepolizumab annualised exacerbation rates compared to placebo by biomarker subgroups



Treatment	Mepolizumab	Dupilumab
Exacerbations with placebo (/patient/year)	1.78 N=9	0.35 N=28
Exacerbations with active (/patient/year)	1.67 N=51	0.21 N=51
Relative rate	**0.94 (0.4, 2.4)**	**0.61 (0.2, 1.8)**
Change in FEV_1 with placebo (ml)	317	
Change in FEV_1 with active (ml)	61	
Difference (ml)	**-257 (-592, 79)**	

Treatment	Mepolizumab	Dupilumab
Exacerbations with placebo (/patient/year)	3.14 N=72	1.16 N=134
Exacerbations with active (/patient/year)	1.20 N=173	0.37 N=248
Relative rate	**0.38 (0.3-0.5)**	**0.32 (0.2-0.5)**
Change in FEV_1 with placebo (ml)	44	
Change in FEV_1 with active (ml)	166	
Difference (ml)	**122 (9, 236)**	

Treatment	Mepolizumab	Dupilumab
Exacerbations with placebo (/patient/year)	1.98 N=23	0.58 N=55
Exacerbations with active (/patient/year)	1.71 N=63	0.58 N=139
Relative rate	**0.86 (0.5-1.6)**	**0.61 (0.2-1.8)**
Change in FEV_1 with placebo (ml)	-32	
Change in FEV_1 with active (ml)	52	
Difference (ml)	**85 (-133, 302)**	

Treatment	Mepolizumab	Dupilumab
Exacerbations with placebo (/patient/year)	1.60 N=47	0.78 N=94
Exacerbations with active (/patient/year)	1.03 N=168	0.52 N=185
Relative rate	**0.64 (0.4-0.99)**	**0.67 (0.4-1.0)**
Change in FEV_1 with placebo (ml)	40	
Change in FEV_1 with active (ml)	141	
Difference (ml)	**101 (-62, 264)**	

FEV_1 – forced expiratory volume in 1 second, ppb – parts per billion

6.2 Cross-sectional examination of exacerbations of severe asthma in patients treated with mepolizumab compared to placebo

6.2.1 Introduction

Mepolizumab, a humanised monoclonal antibody that neutralises IL-5, reduces exacerbations of severe eosinophilic asthma and COPD [216, 217]. The beneficial effect of treatment is most obvious in patients with a raised peripheral blood eosinophil count, a group who are at high risk of exacerbation off treatment [217, 218]. Even in this population, exacerbation rates whilst receiving mepolizumab are around 1/patient/year. The nature of these remaining exacerbations has not been described. I carried out a post-hoc comparison of exacerbations occurring during treatment with mepolizumab or placebo in a previously reported, double-blind, placebo-controlled trial of mepolizumab in severe eosinophilic asthma [216]. I tested the hypothesis that exacerbations in each group differ with respect to change in symptom scores, FEV_1 and inflammatory profile.

6.2.2 Methods

Retrospective analyses of exacerbations occurring during a placebo controlled double blind trial of mepolizumab 750 mg IV given every four weeks for 52 weeks [216] were conducted. The study involved 61 patients with severe asthma, who had an induced sputum eosinophil count of ≥ 3% at screening or at some point over the previous 12 months, and two or more exacerbations of asthma treated with systemic corticosteroids in the last year. 29 patients were treated with mepolizumab and 32 with placebo. Patients were asked to contact the study team for assessment at the time of a clinical deterioration suggestive of an

exacerbation, ideally prior to starting rescue treatment according to their self-management plan. Patients were seen prior to starting rescue prednisolone treatment or as soon as possible after. Cough, breathlessness and wheeze visual analogue scale (VAS) [48], spirometry, Asthma Control Questionnaire (ACQ5), and induced sputum inflammatory cell counts were measured. Individual patient and exacerbation source data were used for analysis. Exacerbations were analysed as individual events. Further analysis was conducted at the patient level to investigate whether there was a significant effect of clustering due to some patients having multiple exacerbations.

6.2.3 Results

Exacerbation data were available for 159 episodes in 43 patients treated with oral prednisolone. Baseline characteristics of patients who had an exacerbation and were included in our analysis were similar to the overall trial population. All patients were on high dose inhaled corticosteroids and 50% of patients in each group were on maintenance oral corticosteroids. There was no significant difference between the characteristics of the placebo and mepolizumab treated groups (Table 6.2).

105 exacerbation events occurred in 25 patients in the placebo arm and 54 events in 18 patients in the mepolizumab arm. In 83 exacerbations (28 mepolizumab, 55 placebo) patients were reviewed prior to prednisolone treatment and in 76 (26 mepolizumab, 50 placebo) patients were seen after starting prednisolone for a mean (SD) of 5.4 (8.8) days in the placebo arm and 5 (7.5) days in the mepolizumab arm. The median number of exacerbations evaluated per patient was 3 (range 1-9).

Table 6.2 Characteristics of participants with exacerbations in the mepolizumab and placebo treated groups

	Placebo (n = 25)	**Mepolizumab (n = 18)**
Age, years	49 (11)	46 (10)
BMI, kg/m^2	29 (6.4)	29 (7.7)
Exacerbations in the previous year	5.7 (2.8)	5.9 (2.4)
ICS dose*	1000 (800-1600)	1000 (800-1600)
On ICS, n (%)	13 (52%)	9 (50%)
IgE	423 (678)	344 (380)
Post BD FEV1, % predicted	77 (26)	74 (19)
Blood eosinophils, cells/mm^3**	0.31 (0.3)	0.30 (0.35)
Sputum eosinophils, %**	5.7 (0.69)	5.8 (0.76)
VAS symptom score. mm	41 (26)	36 (22)
AQLQ	4.6 (1.2)	4.8 (1.0)
ACQ 5	2.4 (1.4)	2.0 (1.1)

*Data shown as mean (SD) unless otherwise specified. * Median (IQR), ** geometric mean (log SD). BMI – body mass index, ICS – inhaled corticosteroids, IgE – immunoglobulin E, BD – bronchodilator, FEV1 – forced expiratory volume in 1 second, VAS – visual analogue scale, AQLQ – asthma quality of life questionnaire, ACQ – asthma control questionnaire.*

Overall, exacerbations on placebo were associated with a higher sputum eosinophil percentage than those on mepolizumab (geometric mean 5.4% vs 2.6%, p = 0.03). There was no difference in total VAS, ACQ5, FEV1, or change in these measures from the baseline study visit or immediately pre-exacerbation visits between placebo and mepolizumab groups.

However, closer examination of the data showed that there were marked differences in the patients seen before and those seen during rescue prednisolone treatment. In patients seen prior to rescue prednisolone treatment, change from baseline in symptom VAS was greater (40 vs 16 mm; mean difference 24 mm, 95% CI 15-34 mm; p < 0.001) and geometric mean sputum eosinophil count was higher in the placebo arm than the mepolizumab arm (6.7 vs

2.6%; p = 0.046). In contrast, in patients seen when taking rescue prednisolone treatment, patients on placebo tended to have less mean change in VAS than patients on mepolizumab (14 vs 28 mm; mean difference -14 mm; 95% CI -3 – 31mm; p=0.1;) and a less obvious difference in geometric mean sputum eosinophil count (2.7 vs 1.1%; p = 0.1; Table 6.3). All elements of the total VAS (cough, wheeze, breathlessness) were equally affected. There were no differences in the sputum total cell count or mean sputum neutrophil differential count (Table 6.3). There was no significant difference in the change from baseline ACQ5 or FEV_1 in the placebo versus mepolizumab arms either in the pre-treatment or treated exacerbation groups (Table 6.3). Similar results were seen if the last stable visit measurements were used in place of the study baseline values.

To investigate whether these observed differences were driven by repeat exacerbation events within the same patient, sensitivity analysis was conducted using either the mean of each patient's exacerbation data or using the data from their first exacerbation. Similar results were seen in these sensitivity analyses compared to the primary analysis suggesting that our findings were not driven by individual patients with frequent events.

Table 6.3 Changes seen during exacerbation in the pre-treatment and during-treatment groups on mepolizumab and placebo treatment

	Exacerbation – seen pre-treatment			**Exacerbation – seen during treatment**		
	Placebo	**Mepolizumab**	***p***	**Placebo**	**Mepolizumab**	***p***
ΔMean VAS (mm)	40 (3)	16 (4)	*<0.001*	14 (6)	28 (8)	*0.1*
ΔACQ5	1.0 (0.1)	0.6 (0.2)	*0.1*	0.3 (0.2)	0.8 (0.3)	*0.2*
ΔFEV1 (L)	-0.11 (0.1)	-0.16 (0.1)	*0.65*	-0.12 (0.1)	-0.20 (0.1)	*0.55*
Sputum total cell count (cells x10^6/ml)	5.7 (1.2)	4.7 (1.9)	*0.66*	6.0 (2.7)	4.9 (1.8)	*0.74*
Sputum eosinophils (%) *	6.7% (0.1)	2.6% (0.2)	*0.046*	2.7% (0.1)	1.1% (0.1)	*0.1*
Sputum neutrophils (%)	57.5% (4.6)	57.2% (6.1)	*0.96*	54.8 (5.8)	59 (5.8)	*0.62*

*Data shown as mean (standard error). Δ - Change from baseline. * Geometric mean (log standard error). FEV_1 – forced expiratory volume in 1 second, ACQ – asthma control questionnaire, VAS – visual analogue scale*

6.2.4 Discussion

The most striking finding of this retrospective analysis of exacerbations of severe eosinophilic asthma occurring whilst on mepolizumab and placebo is the marked difference in change in VAS in patients assessed before and after rescue prednisolone was started. In patients on placebo the change in VAS was much greater in patients assessed before, compared to after, rescue treatment whereas this was not evident in patients receiving mepolizumab. The most plausible explanation for these differences is that exacerbation events occurring on mepolizumab are less severe in terms of symptoms, but also less responsive to prednisolone.

There is evidence to support this interpretation. Exacerbations occurring on mepolizumab were associated with a lower induced sputum eosinophil count compared to those occurring on placebo, particularly in the group who had not started rescue prednisone. Moreover, studies in asthma [73] and COPD [219] have shown that non-eosinophilic exacerbations are

less severe, are associated with a longer duration and a poorer response to corticosteroids than eosinophilic episodes [219-221].

Similar trends were seen when symptoms were assessed by ACQ5, although this measure proved to be less responsive than total VAS, probably because it assesses symptoms over 1 week. There were no differences in change in FEV_1 at exacerbation, suggesting that changes in this measure are disassociated from changes in symptom scores.

These findings are preliminary and are based on a post-hoc retrospective analysis, so some caution is required. However, they are potentially important as they suggest that different mechanisms might drive exacerbations occurring whilst on mepolizumab treatment. A possible explanation for this difference between exacerbations is that patients on mepolizumab are better controlled at steady state and so are more sensitive to deteriorations in symptoms and seek intervention for symptoms sooner. This explanation is unlikely as mepolizumab treatment has very little effect on symptom or quality of life scores suggesting the overall symptom burden is similar.

When stable (i.e. not during exacerbation), phenotypes of airway inflammation in asthma tend to be predominantly eosinophilic or neutrophilic, with mixed inflammatory phenotypes being seen infrequently [36, 222]. Airway inflammation in asthma during exacerbation is poorly studied, however cluster analysis in COPD suggests discrete exacerbation phenotypes can be identified based on patterns of airway inflammation [85]. It appears that these can be differentiated using biomarkers, such as the blood eosinophil count, at the time of exacerbation [86]. As neutrophilic and eosinophilic tend to occur as mutually exclusive patterns of inflammation, it is likely that there is interplay between the underlying inflammatory pathways. For example, cigarette smoke causes a rise in neutrophilic airway

inflammation and a reduction in eosinophilic airway inflammation [223]. A similar pattern is seen with inhaled corticosteroids [224]. One hypothesis is that eosinophilic inflammation is protective against airway infection, which would lead to neutrophilic inflammation, and suppressing eosinophilic inflammation makes airway infection more likely. This raises the possibility that different, potentially non-corticosteroid based treatment approaches, may be sufficient for those exacerbations which are not eosinophilic in nature as they may be driven by infection or another non-steroid responsive pathology. Future studies evaluating the symptom, physiologic, airway inflammatory and microbiological characteristics of exacerbation events occurring on mepolizumab are required to investigate this possibility.

6.3 Longitudinal examination of exacerbations of severe asthma in patients treated with mepolizumab compared to placebo

6.3.1 Introduction

Mepolizumab, a humanised monoclonal antibody targeting IL-5, has been shown to deplete circulating eosinophils and reduce exacerbations of severe eosinophilic asthma [39]. The exacerbation reduction seen with treatment is most marked in patients with higher peripheral blood eosinophil counts [156]. This is a group who are at high risk of exacerbations despite treatment with high dose inhaled corticosteroids (ICS) and other controller medication. However, while most patients experience no exacerbations with mepolizumab treatment, roughly one exacerbation/year is reported at the population level.

The preliminary analysis, detailed in section 6.2, showed, in an analysis of exacerbations occurring in 60 patients receiving mepolizumab or placebo, that untreated exacerbations occurring on mepolizumab are associated with lower sputum eosinophil counts and lower decrements in symptom scores measured by the visual analogue scale (VAS) compared to placebo [225]. In contrast, in patients assessed whilst taking rescue prednisolone, the fall in symptom VAS tended to be greater in the mepolizumab compared to placebo treated group. This analysis suggested that this might reflect a different, less corticosteroid responsive mechanism of exacerbation in patients treated with mepolizumab. To further characterise the changes in symptom and lung function during prednisolone treated exacerbations occurring on mepolizumab, I carried out a post-hoc comparison of diary card data from exacerbations occurring during treatment with mepolizumab or placebo in three previously reported placebo-controlled trials [39, 143, 226]. This study investigates whether

exacerbations differ with respect to change from baseline in peak expiratory flow (PEF) and symptom scores, and in the rate of recovery following oral prednisolone treatment.

6.3.2 Methods

Data from diary cards was reviewed from 3 studies involving 1743 patients with severe eosinophilic asthma: DREAM (1), a 52-week study of 3 doses of mepolizumab (75, 250 or 750 mg IV 4 weekly) versus placebo; MENSA (2), an 32-week study of 2 doses of mepolizumab (75 mg IV or 100 mg SC 4 weekly) versus placebo; and MUSCA (3), a 24-week study of mepolizumab 100 mg SC 4 weekly versus placebo. All studies recruited patients with a history of 2 or more exacerbations in the previous year and evidence of eosinophilic airway inflammation. As similar efficacy on exacerbation rate reduction has been shown across all doses of mepolizumab, dose groups were combined for analysis. Patients completed a daily diary card during treatment including a 6 point symptom score assessing asthma symptoms in the previous 24 hours (0 – no symptoms, 5 – worst symptoms; Table 6.4) and a best-of-three morning peak expiratory flow (PEF) recorded in L/min. Patient medication usage was also recorded.

Exacerbations were defined as worsening symptoms and/or rescue inhaler use that required rescue oral corticosteroids (OCS) for 3 or more days. Events with at least 20 days of diary data in the period from 14 days prior to starting OCS (day -14) to 14 days after starting OCS (day 14) were included in the analysis. For each endpoint, the baseline value was taken to be that recorded at Day -14, or the most recent measurement prior to this point if a day -14 value was unavailable. Change from day -14 in PEF and symptom score was estimated for each event at each day of the 29-day exacerbation period. In addition, rate of change in endpoints

was estimated in the week after OCS initiation using linear regression. For each endpoint, within subject means were derived for individuals with multiple events prior to analysis. Unpaired t-tests were used to compare mean changes at Day 0 and mean recovery rates between treatment groups.

Table 6.4 Symptom scoring used for diary card recording

Symptom score	**Definition**
0	No symptoms during the previous 24-hours
1	Symptoms for one short period during the previous 24-hours
2	Symptoms for two or more short periods during the previous 24-hours
3	Symptoms for most of the previous 24-hours which did not affect my normal daily activities
4	Symptoms for most of the previous 24-hours which did affect my normal daily activities
5	Symptoms so severe that I could not go to work/school or perform normal daily activities

6.3.3 Results

One or more exacerbation occurred on treatment in 322 out of 623 patients treated with placebo and 419 out of 1120 patients treated with mepolizumab. Sufficient diary card data was available to compute the change in PEF from day-14 (baseline) to the exacerbation event (day 0) up until day 14 post exacerbation for 1021 exacerbations, 473 occurring in 247 subjects on placebo and 548 in 331 subjects on mepolizumab. For change in symptom scores sufficient diary card data was available from 1026 exacerbations, 476 occurring in 248 patients on placebo and 550 in 332 patients on mepolizumab.

The baseline demographics of the study participants who experienced one or more exacerbation during the study are shown in Table 6.5 for all studies combined. Baseline participant age, sex, BMI, maintenance OCS use or maintenance OCS dose and lung function measures were similar between placebo and mepolizumab treated groups.

Table 6.5 Baseline demographics of patients who had one or more exacerbation during the study

	Mepolizumab	Placebo
Number of patients with ≥1 exacerbation	419	322
Male	37%	34%
Age	50 (12)	50 (13)
BMI	28.9 (6.1)	28.4 (6.5)
Exacerbations in previous year	3.9 (3.1)	3.9 (3.4)
Prebronchodilator FEV_1 (% predicted)	57.3 (17.2)	59.3 (16.6)
Blood eosinophil count (cells/mm^3)*	0.23 (1.1)	0.34 (1.0)

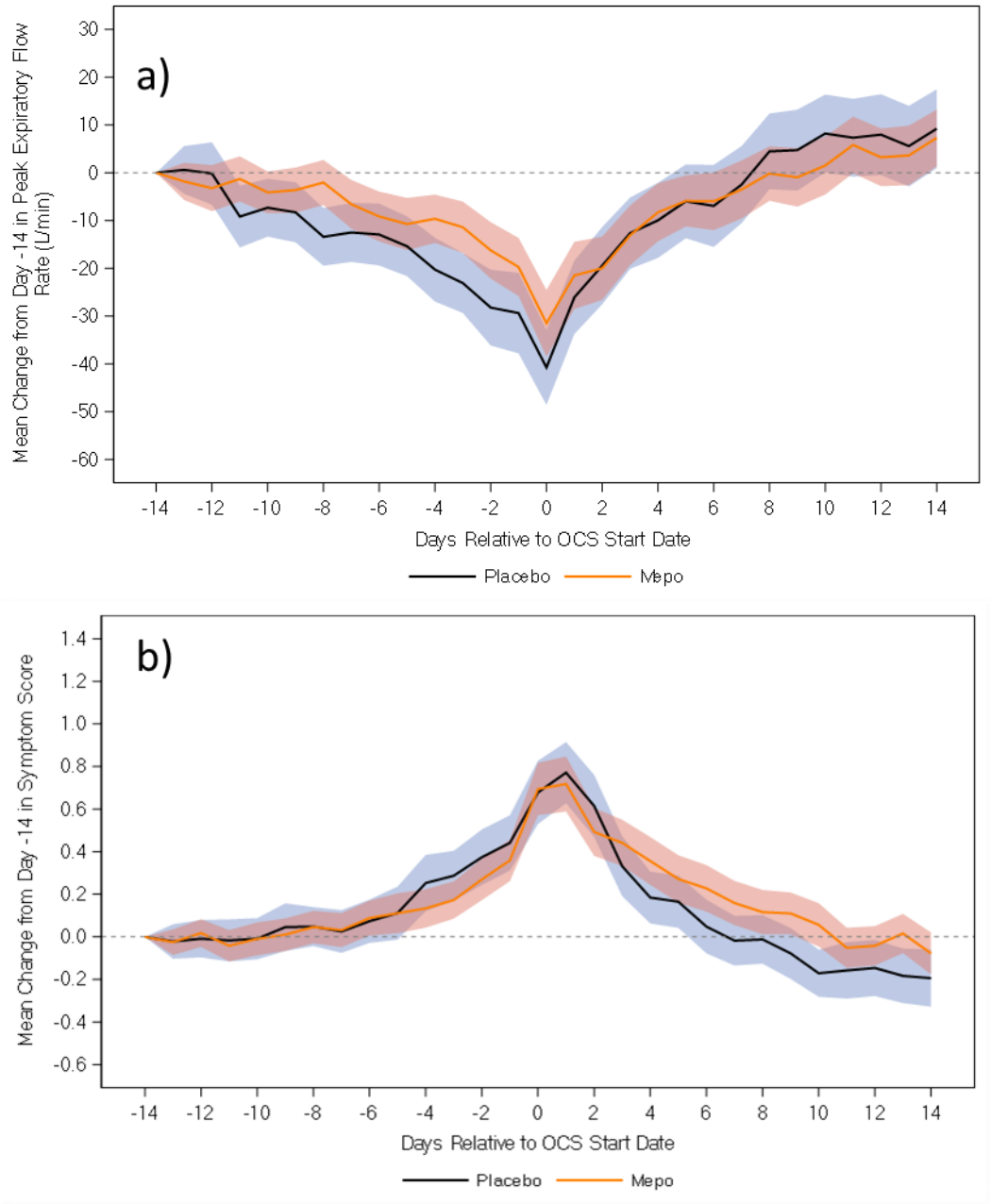
Baseline PEF	252 (98)	264 (109)
Baseline symptom score	1.9 (1.2)	1.8 (1.2)
Day -14 PEF**	245 (108)	262 (107)
Day -14 symptom score**	1.4 (1.4)	1.6 (1.3)

Data shown as mean (SD) unless otherwise stated Geometric mean, SD of log-transformed eosinophil count. FEV_1 – forced expiratory volume in 1 second, BMI – body mass index, PEF – peak expiratory flow*

Exacerbations on placebo were associated with a larger mean drop in PEF compared to mepolizumab at the time of starting OCS (day 0) vs day -14 (-39.0 vs -29.5 L/min; mean difference 9.5; 95% CI 0.6, 19.6 Figure 6.2a), representing a 15 and 12% decrease from day -14 respectively. PEF from day 0 to 7 recovered at 4.8 and 3.6 L/min per day (mean difference 1.2 L/min; 95% CI -0.2, 2.6 L/min), representing a percentage of the total fall in PEF recovered per day of 12% for both treatments.

The mean increase in daily symptom score at day 0 compared to day -14 was 0.64 and 0.68 points for placebo and mepolizumab (mean difference 0.03; 95% CI -0.15, 0.22; Figure 6.2b), representing an increase of 44 and 45% respectively. The rate of recovery of symptom scores from day 0 to 7 was 0.49 points/week vs 0.40 points/week (mean difference 0.09; 95% CI -0.01, 0.19), representing 19 and 13% of the total drop recovered/day for placebo and mepolizumab groups (Figure 6.2b). The profile of symptoms and PEF from day -14 to 14 did not differ by mepolizumab dose (appendix, Figure 8.2).

Figure 6.2 Changes in PEF and symptom scores during exacerbation on mepolizumab compared to placebo



Panel a) shows changes in PEF, panel b) shows changes in symptom scores. PEF – peak expiratory flow, OCS – oral corticosteroids.

6.3.4 Discussion

This analysis, for the first time, describes the change in PEF and symptom scores for exacerbations occurring in patients treated with placebo and mepolizumab. This analysis found that in patients treated with mepolizumab, exacerbations are less severe in terms of falls in PEF but result in similar increases in symptom scores. In contrast, the rate of recovery following treatment with prednisolone was similar for PEF but showed a strong trend to be slower for symptoms scores. These findings support the suggestion that exacerbation events occurring in patient treated with mepolizumab may be different and are in keeping with the hypothesis that they are associated with a diminished response to prednisolone.

The previous cross-sectional analysis (section 6.2) had shown that exacerbations occurring on mepolizumab are associated with a lower induced sputum eosinophil count compared to those occurring on placebo [154, 225]. In patients with chronic obstructive pulmonary disease non-eosinophilic exacerbations have been shown to be associated with a less marked fall in lung function and a slower symptom recovery after treatment with prednisolone when compared to eosinophilic events [227]. The findings of a similar profile of exacerbations in patients with severe eosinophilic asthma treated with mepolizumab suggests that similar heterogeneity of exacerbations is seen in patients with severe asthma and raises the possibility of a distinct phenotype of exacerbation not responsive to mepolizumab treatment.

The presence of a non-mepolizumab responsive exacerbation type is supported by the finding in two large placebo controlled studies of patients with eosinophilic COPD that exacerbation events treated with antibiotics alone are not prevented by mepolizumab treatment whereas oral corticosteroid treated events are prevented in a blood eosinophil dependent manner [228]. Further studies are required to determine whether mepolizumab exacerbation events

differ with respect to inflammatory mechanisms and triggers. Our findings could reflect the removal of one of many inflammatory and environmental events such as viral infection or response to airborne pollutants, contributing to an exacerbation [229] and studies are also required to investigate this possibility. Finally, prednisolone has been shown to be less effective than placebo as a treatment for non-eosinophilic exacerbations in patients with COPD[227] and studies are required to establish whether this is also the case for exacerbations occurring in a patient treated with mepolizumab.

There are limitations with this post-hoc analysis that require further discussion. Firstly, the comparison of mepolizumab and placebo presented here is observational in nature, rather than a randomised comparison. In addition, approximately 20% of exacerbations had insufficient diary card data to be included for analysis. However, these patients had similar baseline characteristics to patients who were included, so this is unlikely to bias results. Secondly, I elected to include all doses of mepolizumab in this analysis as they had equivalent efficacy in reducing exacerbations. No relationship between mepolizumab dose and the profile of exacerbations was observed, so it is unlikely that higher doses, which reduce sputum eosinophils more completely [39], have a greater attenuating effect on the response to prednisolone but recognise that the power to show such an effect may be limited. Thirdly, some patients had multiple exacerbations. However, the approach used was to base the analysis by first estimating within-subject means in patients with more than one exacerbation event. This methodology reduces the possibility of frequently exacerbating patients skewing the analysis and gives a more conservative estimation of change compared to one that treats each event individually. Fourthly, the cross-sectional examination of exacerbations on mepolizumab (section 6.2) identified a difference in symptom scores but not FEV_1 between on mepolizumab and on placebo exacerbations whereas the current study has shown a

reduction in PEF fall but no change in symptom scores. The use of different measures of symptoms and lung function complicates this comparison but both studies show similar trends and are collectively in keeping with less severe events occurring in patients treated with mepolizumab. Finally, an exacerbation was defined as the decision to start prednisolone, which was made by the patient or their usual physician according to their asthma care plan. To help account for potential bias and over-treatment with prednisolone, all events in the DREAM and MENSA clinical trials were corroborated by diary card review showing either a decrement in PEF, a 50% increase in rescue medication use or increased asthma symptom scores for at least 2 consecutive days. This approach found that a very low percentage of events were rejected as true exacerbations and so this was not applied to the MUSCA study which examined all prednisolone treated exacerbations. Nevertheless, this validation step could have reduced the true between-treatment differences in exacerbations severity.

In conclusion, the findings of this analysis suggest that exacerbations occurring in patients treated with mepolizumab are associated with a smaller fall in PEF and a trend to a slower recovery in symptom scores following treatment with prednisolone. In these respects they resemble the corticosteroid unresponsive, non-eosinophilic exacerbations described in patients with COPD[227]. To fully understand the impact of IL-5 blockade on exacerbations, prospective studies are required to characterise symptom, physiologic, airway inflammatory, microbiological and treatment response characteristics of exacerbation events occurring on mepolizumab. These findings represent a strong basis for these studies.

7 Discussion

Severe asthma is a complex problem in which eosinophilic asthma is over-represented compared to mild and moderate asthma. Severe exacerbations, defined as those requiring rescue treatment with oral corticosteroids, are the most important aspect of this disease in terms of patient perception, treatment-related morbidity, and healthcare utilisation.

A proportion of severe eosinophilic asthma which remains uncontrolled is due to problems with treatment adherence or treatment delivery, such as poor inhaler technique. However, there are patients who have truly treatment-refractory disease and have persistent type-2 inflammation despite optimal application of inhaled steroid treatment. I have shown that these patients fail to suppress mediators of type-2 inflammation compared to patients whose inflammation is controlled by inhaled steroids. PGD_2 is an important mediator in type-2 inflammation and is predominantly produced by mast cells. It has local effects on the airway and a key role in the recruitment and activation of type-2 effector cells. I have shown, for the first time, that PGD_2 is particularly non-responsive to inhaled steroids in patients with inhaled steroid refractory type-2 inflammation. Previous *in vivo*, *ex vivo* and *in vitro* studies have shown that steroids can inhibit the release of inflammatory mediators from mast cells [230-232]. The mechanisms causing resistance of these inhibitory mechanisms remain unclear and require further investigation. Better understanding of the causes of inhaled steroid resistance may reveal new therapeutic avenues for treatment-refractory asthma.

The key receptor for the biological effects of PGD_2 on type-2 inflammation is the CRTH2 receptor. Attenuating the effects of PGD_2 by blocking the CRTH2 receptor is therefore an attractive treatment target. I conducted a randomised, placebo-controlled trial of the CRTH2

antagonist timapiprant in severe eosinophilic asthma. This was the first trial of timapiprant to evaluate airway inflammation and investigate whether it has any oral steroid-sparing effect.

Prior to starting the study, there were a number of organisational and logistical challenges that I needed to overcome. Firstly, the original funding for the study from the manufacturer fell through during trial set up. This required a change of trial management from a commercial clinical research organisation to using local research infrastructure. This involved designing and setting up a trial database, organising the randomisation process, re-writing sections of the protocol and patient information sheets and designing data collection sheets prior to resubmitting the trial protocols for new ethical and regulatory approvals. Secondly, the original sponsor for the study, the drug manufacturer Atopix Therapeutics, was acquired by the pharmaceutical company Chiesi mid-way through the study. This required further changes to the protocol and setting up a new data management system to comply with the new company's 'in house' procedures, before having to update all of the approvals. These factors made setting up and starting the study more difficult and delayed the planned start date by over 12 months. One of the biggest challenges was recruiting the participants as well as undertaking the laboratory-based experiments in a shorter time frame. These challenges, however, did give me a very good understanding of all the processes involved in setting up, running and recruiting to a clinical trial.

My study of timapiprant showed that in patients with severe, treatment refractory, eosinophilic asthma, there was a trend for timapiprant to significantly reduce airway eosinophils compared to placebo. This finding is in keeping with the findings with fevipiprant in severe eosinophilic asthma and suggests a real class effect. This reduction of eosinophils in the airway was not associated with a change in peripheral blood eosinophils, FeNO or

circulating type-2 cells. This localised response suggests that the effects of timapiprant are due either to direct effects on the eosinophils, by reducing chemotaxis into the airway in response to PGD_2 or increasing local eosinophil apoptosis. Another possible mechanism of this eosinophil-reducing effect is reduction or attenuation of type-2 cells within the airways. Further investigation of direct effects on CRTH2 antagonists on airway eosinophils and the number and activity of type-2 cells may yield further insights into the therapeutic potential of this class of medications. The effect of timapiprant on reducing airway eosinophils was correlated with the baseline numbers of circulating Tc2 cells, a class of type-2 cells which has shown to be enriched in the blood and airway of patients with eosinophilic asthma. The number of Tc2 cells in the airways may be another biomarker of response to CRTH2 antagonists. As seen with other studies of anti-inflammatory treatments for asthma, the reduction in eosinophils was not associated with an improvement in symptoms. The most marked change in this study of timapiprant was the reduction in eosinophilic airway inflammation as measured by induced sputum, a measure that is strongly correlated with exacerbations risk. It is therefore likely that the key clinical benefit of CRTH2 antagonists, as seen in studies of biologic treatments targeting IL-5, is likely to be a reduction in exacerbation risk. This study had some limitations. Following the analysis at the end of the study, it was apparent that the study was underpowered to detect the assumed 3-fold reduction in sputum eosinophils. This was due to a higher than assumed variance in sputum eosinophils. It may also be that the most treatment-responsive population was not picked. Subgroup analysis showed that timapiprant was most effective in participants with uncontrolled symptoms as measured by the ACQ-5. Larger studies of timapiprant in eosinophilic asthma, with a focus on uncontrolled patients, may reveal a more marked treatment effect.

The risk of exacerbations in asthma has been shown to be related to the amount of airway eosinophilic inflammation as measured by sputum eosinophils, and by the more clinically accessible biomarkers of the peripheral blood eosinophil count and FeNO. In the *post-hoc* analysis of the DREAM study evaluating mepolizumab, I found that these biomarkers offer insights in to further phenotyping type-2 asthma. The blood eosinophil count is related to IL-5, whereas FeNO is driven by IL-13 dependant pathways. I found that these biomarkers are additive in assessing exacerbation risk, and in predicting response to mepolizumab. This relationship between FeNO and response to mepolizumab had not previously been described. These findings were consistent in another study population evaluating the antil-IL-4-receptor alpha drug dupilumab, which blocks the action of IL-13 and IL-4. The analysis suggested that FeNO was a better predictor of response to dupilumab whereas the blood eosinophil count was more closely associated with the response to mepolizumab. These findings suggest that measuring both biomarkers together can give a more detailed evaluation of risk, response to treatment and potentially guide treatment choices. Evaluation of these biomarkers at the time of exacerbation, and their change in response to treatment may offer further predictive information and should be studied prospectively.

Treating type-2 inflammation with targeted anti-inflammatory treatment is effective in reducing airway inflammation, as shown with CRTH2 antagonists, and in reducing exacerbation frequency, as seen with anti-IL-5 treatment. Anti-IL-5 treatments for severe asthma reduce exacerbation risk by depleting or reducing circulating eosinophils. The effects of type-2 targeted treatments on the underlying phenotype of asthma is unclear. The effects of anti-IL-5 treatment on exacerbations of severe eosinophilic asthma have not been previously described. I investigated the effects of the anti-IL-5 monoclonal antibody mepolizumab on exacerbations and found, in two separate, novel analyses, that

exacerbations on mepolizumab treated with oral steroids were associated with less eosinophilic airway inflammation, lower symptom scores and smaller changes in peak flow compared to placebo. There was also a trend towards a longer recovery compared to exacerbations. These findings suggest that exacerbations on mepolizumab are different in nature to those occurring on placebo, and that a different treatment strategy may be required for them. A limitation of these studies in to mepolizumab-treated exacerbations is that they are retrospective, *post hoc* analyses. Prospective examination of exacerbations on type-2 targeted treatments are required as exacerbations on eosinophil-depleting treatments may have a different underlying pathophysiology. A clinically relevant question that requires further study is whether exacerbations on type-2 targeted are driven by infection rather than type-2 airway inflammation, and whether biomarkers of type-2 inflammation, measured at the time of exacerbation can be used to phenotype exacerbations.These studies may reveal underlying pathways and biomarkers that can be used design studies to investigate whether targeting the exacerbation phenotypes with a phenotype-specific treatment is superior to the current approach of treating all exacerbations with oral steroids.

8 Appendix

8.1 Flow cytometry

Table 8.1 Whole blood antibody cocktail composition

Epitope	Fluorchrome	Dilution	For 2.5 sample
CD193	APC	1:100	2.5
CD8	Pacific Blue	1:50	5
CD19	PE-Cy7	1:50	5
CD3	PerCP	1:50	5
CD14	PE-Texas Red	1:1000	2.5 (of 1:10)
CD4	AlexaFluor700	1:200	2.5
CD16	FITC	1:200	1.25
TOTAL Ab (µL)			25
PBS (µL)			101.25
2.5 µL of CRTH2-PE to be added to tube 1			

Table 8.2 ILC antibody cocktail composition

Epitope	Fluorchrome	Dilution	For 2.5 sample
FcεRI	PerCP	1:200	1.25
CD16	PerCP	1:200	1.25
CD117	APC	1:100	2.5
CD161	PE-Dazzle[2]s	1:100	2.5
ST2L	FITC	1:100	2.5
CD127/IL-7RA	eFluor450[3]	1:100	2.5
CD11b	PerCP	1:100	2.5
CD19	PerCP-Cy5.5	1:100	2.5
CD56	PerCP	1:100	2.5
CD8	PerCP	1:100	2.5
CD45	PE-Cy7	1:100	2.5
CD4	PerCP	1:100	2.5
CD3	PerCP	1:50	5
CD123	PerCP	1:50	5
CD14	PerCP	1:50	5
L/D	Zombie Aqua[1]	1:500	5 (of 1:10)
CD11c	PerCP	1:25	10
TOTAL AB (µL)			57.5
PBS (µL)			67.5
2.5 µL of CRTH2-PE to be added to tube 1			

[1] Zombie Aqua = BV510 channel on Fortessa
[2] PE-eFluor610 = PE-Texas Red channel on Fortessa
[3] eFluor450 = BV421 channel on Fortessa

8.1.1 Flow-cytometer set-up, calibration and data acquisition

The goal of this procedure is to ensure consistent median fluorescence intensity (MFI) readings over time. This is achieved by calibrating the machine and running new compensation beads each day a patient sample is being measured.

Flow cytometers are highly sensitive machines which can detect small differences in cell size and fluorescence. These readings can are affected by small changes in the alignment of the lasers, laser voltages, change in the optical filters and the sensitivity and resolution of the optical detector [233]. To standardise measurements to account for small variations in these variables, beads of known size and shape can be run through the machine and analysed to calibrate the flow cytometer. All fluorochromes have an emission light spectrum for a given excitation spectrum. The excitation spectrum is the light wavelengths that add energy to a fluorochrome, causing it to emit light in another range of wavelength when it loses that energy. These emission spectra cover a range of wavelengths and so can overlap. When this occurs, fluorescence signals from a fluorochrome may be detected by more than one detector. To correct for this, a process of compensation is used. This ensures that the fluorescence detected in a detector comes from the fluorochrome that is being measured. Compensation beads can be made up with the fluorochromes being studied and software compensation can be applied.

The BD LSRFortessa™ machine (BD Biosciences, San Jose, USA) was allowed to warm up for a minimum of 30 minutes. Rainbow Compensation Beads (BD Biosciences, San Jose, USA) were run on the cytometer. Laser voltages were adjusted, if needed, to ensure 90-95% of the events were in the pre-specified gates. The same gating was used for each sample run.

Compensation beads were made up with mouse and rat antibody coated beads (Table 8.3), The compensation beads were then run in their own light channel. Software compensation was then applied to the patient sample to be analysed.

Table 8.3 Flow cytometer compensation bead set-up

Fluorochrome	**Antigen/Fluorochrome**
Mouse beads	
FITC	CD16-FITC
PerCP	CD3-PerCP
APC	CD193-APC
AlexaFluor700	CD4-AF700
APC-Cy7	CD45-APC-Cy7
Pacific Blue	CD8-Pacific Blue
eFluor450	CD127-eFluor450
BV510	CD193-BV510[1]
BV711	CD68-BV711
PE-Texas Red	CD14-PE-Texas Red
PE-eFluor610	CD161-PE-eFluor610
PE-Cy7	CD19-PE-Cy7
PE-Cy7	CD45-PE-Cy7
Rat beads	
PE	CRTH2-PE

8.2 Sputum analysis

Cell viability formula:

*Total Number of Cells ($x10^6$) = (Live + Dead Cells/ No. of squares) x 2 x Filtrate Weight (ml) / 100

* x 1000 = Re-suspension Value (µl) – Gives 1 x 10^6 cells/ml

Total cell count (10^6 cells/g sputum)= * / Selected Sputum Weight (g) = ____

% Viability = (Live / Live + Dead) x 100 = _____

8.3 Timapiprant study

8.3.1 Timapiprant study population

Inclusion criteria:

Subjects must:

- Provide written informed consent to participate in the study.
- Be age 18 years and over.
- Evidence of reversible airflow obstruction documented within the previous 24 months or prior to Randomisation by one of the following measures:
    - airway reversibility (>12% increase in FEV_1 and 200ml following 2.5mg nebulised salbutamol),
    - airway hyper-responsiveness (methacholine provocative concentration (PC20) causing a 20% fall in FEV1 of ≤8 mg/mL),
    - airflow variability in clinic FEV1 ≥15% between two consecutive clinic visits, *or* airflow variability as indicated by >20% diurnal variability in PEF observed on 3 or more days.
- Meet the ERS/ATS criteria for severe asthma at Screening:
    - A high dose inhaled corticosteroid and long acting β_2-agonist or leukotriene modifier/theophylline used for the previous year
    - OCS used for ≥50% of the previous year.
- Have documented evidence of eosinophilic airway inflammation during the 24 months prior to Screening:
    - an induced sputum eosinophil count ≥3%; **or**

- a peripheral blood eosinophil count ≥0.25 x 10^9/l that was related to asthma; **or**
- an exhaled nitric oxide concentration (FeNO) >50 ppb.

- Have evidence of eosinophilic airway inflammation at Screening as reflected by an induced sputum eosinophil count of ≥3%.Subjects who are taking OCS must be receiving a 20 mg prednisolone equivalent dose or less daily for at least four weeks before the first dose of study drug1. The dose of OCS should be unchanged for at least 14 days prior to the Baseline visit.
- Able to comply with the study protocol.
- Women of child bearing potential must have a negative pregnancy test at Screening and on Day 1 and be prepared to use two forms of reliable contraception (hormonal, either oral or implant; intrauterine device; intravaginal barrier device; condom) during the study and for three months thereafter.
- Male subjects with female partners of child bearing potential must agree to take adequate contraceptive precautions for the entire duration of study participation.

Exclusion criteria:

- Treatment with Xolair™ or anti-Th2 biologicals within 6 months prior to screening.
- Subjects with clinically significant abnormal serum biochemistry, haematology and urine examination values (not associated with the study indication) within 21 days of the first dose.
- Subjects who have been hospitalised in the last 3 months.
- History of more than 2 episodes of confirmed bacterial lower respiratory tract infection or current lower respiratory tract infection.
- Subjects who are current smokers or have a smoking history of >15 pack years.
- Significant comorbidity that in the Investigator's opinion is likely to impact the subject's participation in a 26 week study.
- Presence of a clinically important lung condition other than asthma, e.g. malignancy or previous history of cancer in remission for less than 12 months, clinically important liver or kidney disease, uncontrolled clinically significant cardiovascular or metabolic disease, hypereosinophilic syndromes such as Churg-Strauss Syndrome or eosinophilic oesophagitis.
- Receipt of any of the following medications – more than 1g of paracetamol per day, prednisolone at a dose of more than 20mg per day, regular non-steroidal anti-

inflammatory drugs (NSAIDs) for more than 3 days in the two weeks prior to randomisation.

- A history (or suspected history) of alcohol misuse or substance abuse within 2 years prior to screening, or positive drugs of abuse result at screening.
- Participation in another clinical study (observational or interventional) within 3 months of the first dose of study drug or at any time during this study. Studies that require consent only to store data and that do not require follow up are not considered to be observational or interventional and are exempt from this exclusion.
- A history of hypersensitivity and/or idiosyncrasy to any of the test compounds or excipients employed in this study (see Section 6).
- Subjects who are pregnant or breastfeeding. Patients should not be enrolled if they plan to become pregnant during the time of study participation.
- Subjects who have known evidence of lack of adherence to controller medications and/or ability to follow physician's recommendations.
- Subjects with ALT or AST at screening ≥2xULN.
- Subjects with active hepatitis, chronic active hepatitis or evidence of chronic liver disease (e.g. alcoholic liver disease, fatty liver disease, autoimmune hepatitis, etc.)

Withdrawal criteria:

- Subject experiences a serious or severe adverse event that prevents him/her from continuing as determined by the Principal Investigator.
- Subject incurs a significant protocol violation as determined by the Principal Investigator or Medical Monitor.
- Subject requires uptitration or initiation of OCS, theophylline or leukotriene modifier between Baseline and Week 12.
- Subject requests early discontinuation.
- Reasonable and justified request of the Sponsor.
- Investigator's request (e.g. if the Investigator considers that the subject's health is compromised by remaining in the study or the subject is not sufficiently co-operative).
- Subject is lost to follow-up. A subject should only be designated as lost to follow-up only if the site is unable to establish contact with the subject. The site must attempt to contact the subject on multiple occasions and only determine the subject to be 'lost to follow-up' after there have been at least 3 documented attempts, via at least 2 different methods (phone, text, e-mail, certified letter, etc), to contact the subject.
- Subject experiences an increase in ALT as follows:
    - ALT ≥8xULN

- ALT ≥5xULN for more than 2 weeks
- ALT ≥3xULN and either TBL ≥2xULN or INR >1.5 if not receiving anticoagulants
- ALT ≥3xULN with the appearance of fatigue, nausea, vomiting, right upper quadrant pain or tenderness, fever, rash, and/or new eosinophilia (>5%)

Reasons for all withdrawals must be captured in the Case Report Form (CRF) and can include: an adverse event, lost to follow-up, protocol violation, lack of efficacy, sponsor terminated study, noncompliance, pregnancy, abnormal laboratory results including clinically significant abnormality identified on the ECG.

Screening Failures

Subjects will be assigned a study number at the time of signing the consent. Subjects who do not progress to the Baseline visit (Visit 2) will be deemed a screening failure. Information to be collected for screen failure subjects will be detailed in the CRF completion guidelines. Re-screening of subjects will be allowed only upon approval by the medical monitor.

8.3.2 Oral corticosteroid withdrawal schedule

Table 8.4 Timapiprant oral corticosteroid withdrawal schedule

Study visit	**Prednisolone Equivalent Dose/mg/Day**				
OCS dose at Baseline	20	15	10	7.5	5.0
Visit 5: Week 12-14	10	10	5	5.0	2.5
After Telephone consultation 1 (T1) Week 14-16	5	5	2.5	2.5	2.5 every other day
After Visit 6: Week 16-18	2.5	2.5	2.5 every other day	2.5 every other day	2.5 every other day
After Telephone consultation 2 (T2) Week 18-20	2.5 every other day	2.5 every other day	2.5 every other day	2.5 every other day	0
After Visit 7: Week 20-22	2.5 every other day	2.5 every other day	0	0	0
After Telephone consultation 3 (T3) Week 22-24	0	0	0	0	0

OCS – oral corticosteroids. Criteria to be considered for not reducing OCS Criteria to be considered for NOT reducing OCS dose: mean morning PEF over 7 days prior to clinic visit or telephone consultation is <80% of mean morning PEFs from the previous visit; asthma-related night time awakenings over 7 days prior to visit >50% increase over the previous clinic visit or telephone consultation; rescue medication use requiring 12 puffs or more of rescue inhaler on any one day in the prior week; change in ACQ-5 ≥ +0.5 from the prior visit; acute exacerbation requiring hospital treatment in previous 4 weeks; symptoms of adrenal insufficiency.

8.3.3 Timapiprant study statistical analysis

Analyses were conducted using IBM SPSS® version 25 (IBM, Ney York, USA). Logged values refer to log to base 10.

Sample size and power calculation

The sample size assessment was based on an analysis of the treatment and placebo group differences in the mean change from baseline to week 12 of the logged induced sputum eosinophil count using the full analysis set.

Using a two-sided test with a 5% significance level, in order to detect an assumed 3-fold reduction in induced sputum eosinophil counts between OC000459 and placebo (i.e. a difference in treatment group means of 0.477 on the logged scale) with at least 80% power and assuming a within-group standard deviation of 0.495 of change from baseline data on the logged scale, 18 evaluable subjects are required per group. The standard deviation is derived from an estimate derived from the literature [141, 211].

Assuming approximately 90% of randomised subjects are evaluable, at least 20 subjects are required to be randomised to each treatment group (i.e. 40 subjects in total).

Randomisation

All subjects who remained eligible for the study following screening were randomised to receive OC000459 50 mg or matching placebo. Randomisation was performed according to the method of minimisation, stratified by use of OCS at Screening (yes/no) and three categories of screening sputum eosinophil count (≥ 3% and < 10%, ≥ 10% and <35%, ≥35%). Randomisation was carried out using the electronic RRAMP system (OCTRU, Oxford, UK). The allocation ratio to OC000459 and placebo was 1:1.

Analysis sets

Full analysis set - all subjects who satisfied the randomisation criteria and received at least one dose of study medication, and have at least one post-baseline primary efficacy assessment. The full analysis set will be considered the primary analysis population for efficacy endpoints.

Per-protocol set - all subjects from the full analysis who have complied with the dosing regimen defined as at least 80% compliance with medication as confirmed by returned pill count). They must also have valid sputum eosinophil counts at baseline (or screening if baseline is not available) and week 12 (or week 8 if week 12 is not available). Efficacy analyses will be performed on the per-protocol analysis set as a secondary analysis population.

Analysis of endpoints

Statistical analyses will be performed using appropriate two-sided hypothesis tests at the 5% significance level. Continuous variables will be summarised using number of observations, mean (and/or geometric mean, where applicable), median, standard deviation, lower quartile, upper quartile, minimum and maximum values. Categorical variables will be summarised presenting proportions (counts and percentages). Summary tables will be presented by treatment group (OC000459 and placebo) and for all subjects.

Primary endpoint - the OC000459 and placebo differences in the mean change from baseline at week 12 of the logged induced sputum eosinophil count data. The data will be analysed using analysis of covariance (ANCOVA) with use of OCS at Screening (yes/no) as factors and baseline sputum eosinophil count (Log_{10} value) as covariate:

For each subject, the primary variable being analysed is calculated as follows:

log10 (sputum eosinophil (%) at Week 12) – log10(sputum eosinophil (%) at Baseline)

= log10 ([sputum eosinophil (%) at Week 12]/[sputum eosinophil (%) at Baseline]).

Secondary - FEV1 was compared by treatment group. The differences in FEV1, FVC and FEV1/FVC between before and after nebulised salbutamol were summarised. Absolute and change from baseline ACQ-5, assessments at 4, 8, and 12 weeks were summarised and statistically compared by treatment group. In addition, the proportion of subjects with changes in excess of the minimally important difference were analysed. Absolute and change from baseline quality of life as assessed by AQLQ(S) at 4, 8, and 12 weeks was summarised by treatment group. In addition, the proportion of subjects with changes in excess of the minimally important difference was analysed. Absolute and change from baseline FeNO at 4, 8, and 12 weeks was summarised by treatment group. Absolute, fold and absolute change from baseline sputum eosinophil count at 4 and 8 weeks will be summarised by treatment group.

Missing and censored data

For the statistical analysis of quantitative efficacy endpoints at Week 12 a Last Observation Carried Forward (LOCF) approach will be applied in the case of a missing Week 12 assessment. Only post-baseline data will be considered for this approach (i.e., baseline value will not be carried forward to replace missing post-baseline assessments (i.e. Week 4 will not have LOCF applied as the preceding visit is baseline Week 0)). Where subjects have missing data at Week 12, then the Week 8 assessment will be used, if both Week 12 and Week 8 are missing then the Week 4 assessment will be used. Sensitivity analysis will be conducted to assess the LOCF imputation.

For the primary endpoint, data will be considered missing if the study visit is within 2 weeks of taking rescue oral corticosteroids due to the effect of corticosteroids on the outcome variable.

8.3.4 Timapiprant study schedule

Table 8.5 Timapiprant study schedule – not taking oral corticosteroids at baseline

	Informed consent	**Visit 1 (Screening Day ≥-7 to -14))**	**Visit 2 (Randomisation Week 0)**	**Visit 3 (Week 4) ±4 Days**	**Visit 4 (Week 8) ±4 Days**	**Visit 5 (Week 12) ±4 Days**	**Follow up (Week 16 or early withdrawal) ±4 Days**
Informed consent	X						
Medical History		X					
Physical Exam		X					X
Demographics		X					
Weight & Height		X					Weight only
ECG		X				X	X
Adverse events		X	X	X	X	X	X
Concomitant medications		X	X	X	X	X	X
Vital Signs		X	X	X	X	X	X
Blood samples		X	X	X	X	X	X
Drugs of abuse screen		X					
Urinalysis		X	X	X	X	X	X
Urine pregnancy test in women of childbearing potential		X	X	X	X	X	X
FeNO			X	X	X	X	X
Pre and post bronchodilator spirometry		X*	X	X	X	X	X
AQLQ(S)			X	X	X	X	X
ACQ-5			X	X	X	X	X
Induced sputum eosinophil count		X	X	X	X	X	X
Bronchoscopy (optional – separate consent required)			X			X	
Dispense IMP			X	X	X		
Daily diary			X	X	X	X	
Trough blood sample for pharmacokinetics				X			

*Note: Screening Visit will occur at least 7 days and no more than 14 days before the Visit 2 (Randomisation) occurs * Spirometry before and after inhaled salbutamol can be evaluated at Screening if historical data are not available in source documents.*

Table 8.6 Timapiprant study schedule – taking oral corticosteroids at baseline

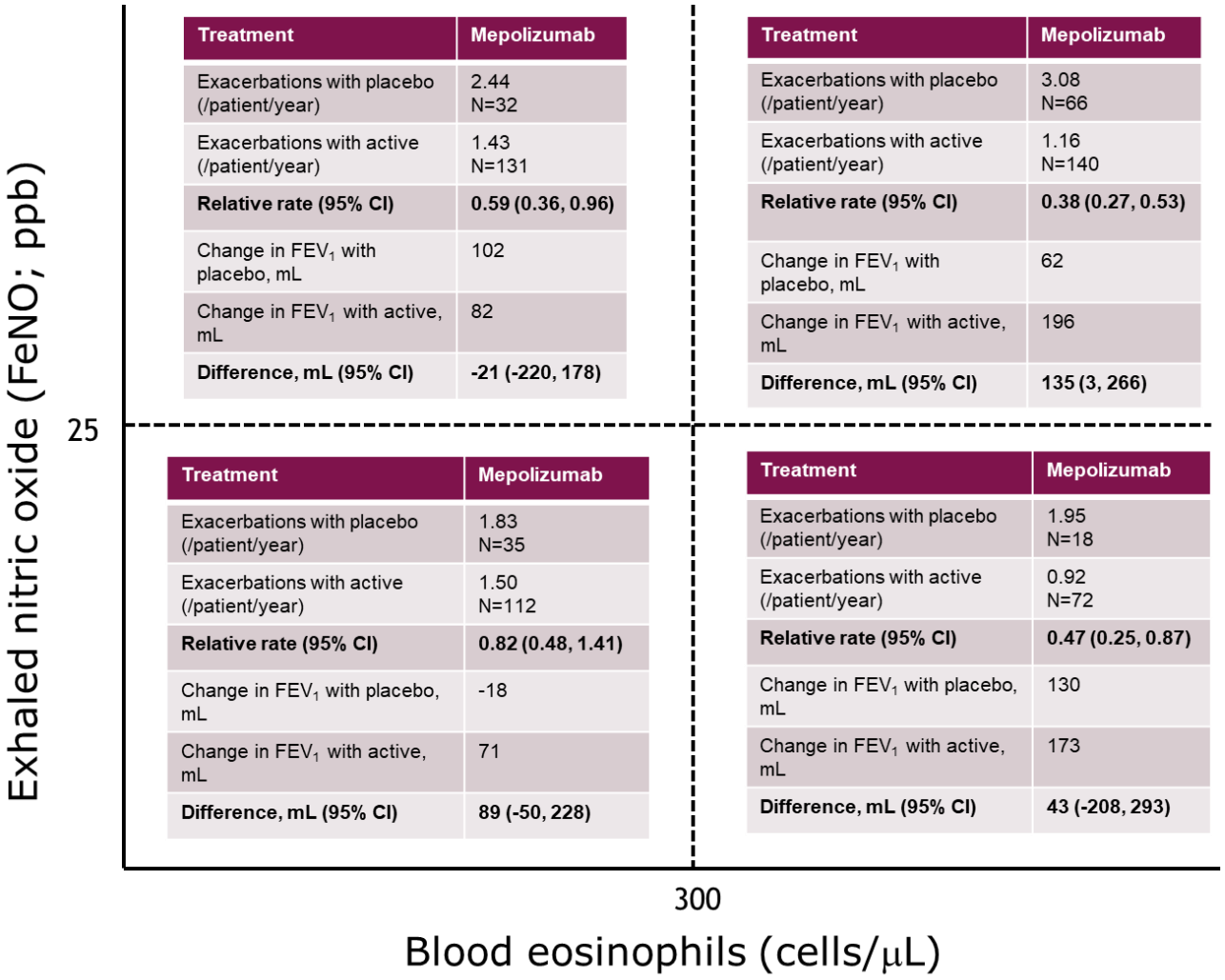
	Informed consent	**Visit 1 (Screening Day ≥-7 to -14)**	**Visit 2 (Randomisation week 0)**	**Visit 3 (week 4) ±4 Days**	**Visit 4 (week 8) ±4 Days**	**Visit 5 (week 12) ±4 Days**	**T1 (Week 14) ± 4 Days**	**Visit 6 (week 16) ±4 Days**	**T2 (Week 18) ± 4 Days**	**Visit 7 (week 20) ±4 Days**	**T3 (Week 22) ± 4 Days**	**Visit 8 (week 24) ±4 Days**	**Follow up (week 28 or early withdrawal) ±4 Days**
Informed consent	X												
Medical History		X											
Physical Exam		X											X
Demographics		X											
Weight & Height		X											Weight only
ECG		X				X						X	X
Adverse events		X	X	X	X	X	X	X	X	X	X	X	X
Concomitant medications		X	X	X	X	X	X	X	X	X	X	X	X
Vital Signs		X	X	X	X	X		X		X		X	X
Blood samples		X	X	X	X	X		X		X		X	X
Drugs of abuse screen		X											
Urinalysis		X	X	X	X	X		X		X		X	X
Urine pregnancy test in women of child bearing potential		X	X	X	X	X		X		X		X	X
FeNO			X	X	X	X		X		X		X	X
Pre and post bronchodilator spirometry		X[1]	X	X	X	X		X		X		X	X
AQLQ(S)			X	X	X	X		X		X		X	X
ACQ-5			X	X	X	X	X	X	X	X	X	X	X
Induced sputum eosinophil count		X	X	X	X	X		X		X		X	X

	Informed consent	**Visit 1 (Screening Day ≥-7 to -14)**	**Visit 2 (Randomisation week 0)**	**Visit 3 (week 4) ±4 Days**	**Visit 4 (week 8) ±4 Days**	**Visit 5 (week 12) ±4 Days**	**T1 (Week 14) ± 4 Days**	**Visit 6 (week 16) ±4 Days**	**T2 (Week 18) ± 4 Days**	**Visit 7 (week 20) ±4 Days**	**T3 (Week 22) ± 4 Days**	**Visit 8 (week 24) ±4 Days**	**Follow up (week 28 or early withdrawal) ±4 Days**
Bronchoscopy (optional – separate consent required)			X			X						X	
Dispense IMP			X	X	X	X		X		X			
Assess clinical status and prescribe OCS[2]						X	X	X	X	X	X		
Daily Diary			X	X	X	X	X	X	X	X	X	X	
Trough blood sample for pharmacokinetics				X									

OCS – oral corticosteroids, IMP – investigational medicinal produce, FeNO – fractional exhaled ntric oxide, ACQ – asthma control restionnaire, AQLQ – asthma quality of life questionnaire, ECG – electrocardiogram. 1. Spirometry before and after inhaled salbutamol can be evaluated at Screening if historical data are not available in source documents. 2. Subjects should be clinically assessed before each step of the OCS dose reduction is prescribed. Telephone consultation (T1, T2, T3) to assess subject's clinical status before OCS dose reduction occur

8.4 Biomarker analysis

Figure 8.1 Mepolizumab annualised exacerbation rates compared to placebo by biomarker subgroups using higher blood eosinophil cut-point



Treatment	Mepolizumab
Exacerbations with placebo (/patient/year)	2.44 N=32
Exacerbations with active (/patient/year)	1.43 N=131
Relative rate (95% CI)	**0.59 (0.36, 0.96)**
Change in FEV_1 with placebo, mL	102
Change in FEV_1 with active, mL	82
Difference, mL (95% CI)	**-21 (-220, 178)**

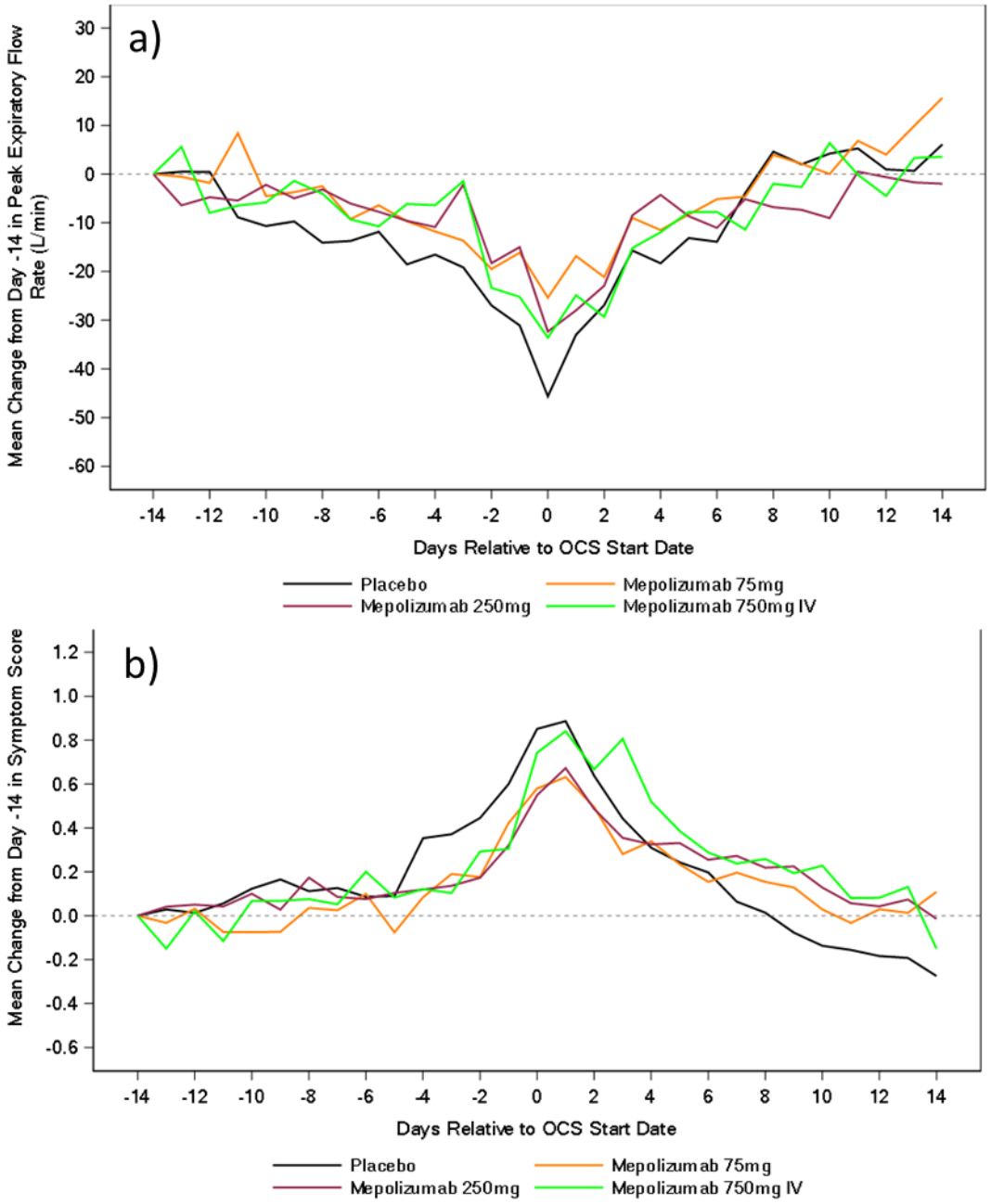
Treatment	Mepolizumab
Exacerbations with placebo (/patient/year)	3.08 N=66
Exacerbations with active (/patient/year)	1.16 N=140
Relative rate (95% CI)	**0.38 (0.27, 0.53)**
Change in FEV_1 with placebo, mL	62
Change in FEV_1 with active, mL	196
Difference, mL (95% CI)	**135 (3, 266)**

Treatment	Mepolizumab
Exacerbations with placebo (/patient/year)	1.83 N=35
Exacerbations with active (/patient/year)	1.50 N=112
Relative rate (95% CI)	**0.82 (0.48, 1.41)**
Change in FEV_1 with placebo, mL	-18
Change in FEV_1 with active, mL	71
Difference, mL (95% CI)	**89 (-50, 228)**

Treatment	Mepolizumab
Exacerbations with placebo (/patient/year)	1.95 N=18
Exacerbations with active (/patient/year)	0.92 N=72
Relative rate (95% CI)	**0.47 (0.25, 0.87)**
Change in FEV_1 with placebo, mL	130
Change in FEV_1 with active, mL	173
Difference, mL (95% CI)	**43 (-208, 293)**

8.5 Mepolizumab exacerbation study

Figure 8.2 Changes in PEF and symptom scores during exacerbation on mepolizumab compared to placebo by mepolizumab dose



Panel a) shows changes in PEF, panel b) shows changes in symptom scores. PEF – peak expiratory flow, OCS – oral corticosteroids.

9 References

1. Cohen, S.G. *Asthma in antiquity: the Ebers Papyrus*. in *Allergy Proc*. 1992.
2. Salter, H.H., *On asthma: its pathology and treatment*. 1864, Philadelphia,: Blanchard and Lea. viii, 17 -260 p.
3. Chu, E.K. and J.M. Drazen, *Asthma: one hundred years of treatment and onward.* Am J Respir Crit Care Med, 2005. **171**(11): p. 1202-8.
4. Tattersfield, A.E., *Current issues with beta2-adrenoceptor agonists: historical background.* Clin Rev Allergy Immunol, 2006. **31**(2-3): p. 107-18.
5. Barnes, P.J., *Efficacy of inhaled corticosteroids in asthma.* J Allergy Clin Immunol, 1998. **102**(4 Pt 1): p. 531-8.
6. Global Initiative for Asthma. *Global Strategy for Asthma Management and Prevention.* www.ginasthma.org. 2016 [cited 2016 11th May].
7. Chung, K.F., et al., *International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma.* Eur Respir J, 2014. **43**(2): p. 343-73.
8. British Thoracic, S. and N. Scottish Intercollegiate Guidelines, *British guideline on the management of asthma.* Thorax, 2014. **69 Suppl 1**: p. 1-192.
9. Jatakanon, A., et al., *An inhaled steroid improves markers of airway inflammation in patients with mild asthma.* Eur Respir J, 1998. **12**(5): p. 1084-8.
10. Lefebvre, P., et al., *Acute and chronic systemic corticosteroid-related complications in patients with severe asthma.* J Allergy Clin Immunol, 2015. **136**(6): p. 1488-1495.
11. Israel, E. and H.K. Reddel, *Severe and Difficult-to-Treat Asthma in Adults.* N Engl J Med, 2017. **377**(10): p. 965-976.
12. Lodrup Carlsen, K.C., et al., *Assessment of problematic severe asthma in children.* Eur Respir J, 2011. **37**(2): p. 432-40.
13. Andersson, F., S. Borg, and E. Stahl, *The impact of exacerbations on the asthmatic patient's preference scores.* J Asthma, 2003. **40**(6): p. 615-23.
14. Levy, M.L., *National Review of Asthma Deaths (NRAD).* Br J Gen Pract, 2014. **64**(628): p. 564.
15. *Guidelines for management of asthma in adults: II--Acute severe asthma. Statement by the British Thoracic Society, Research Unit of the Royal College of Physicians of London, King's Fund Centre, National Asthma Campaign.* BMJ, 1990. **301**(6755): p. 797-800.
16. *Guidelines for management of asthma in adults: I--Chronic persistent asthma. Statement by the British Thoracic Society, Research Unit of the Royal College of Physicians of London, King's Fund Centre, National Asthma Campaign.* BMJ, 1990. **301**(6753): p. 651-3.
17. Martinez, F.D. and D. Vercelli, *Asthma.* Lancet, 2013. **382**(9901): p. 1360-72.
18. Shaw, D.E., R.H. Green, and P. Bradding, *Asthma exacerbations: prevention is better than cure.* Ther Clin Risk Manag, 2005. **1**(4): p. 273-7.
19. Hoskins, G., et al., *Risk factors and costs associated with an asthma attack.* Thorax, 2000. **55**(1): p. 19-24.
20. Kang, H.R., et al., *Risk factors of asthma exacerbation based on asthma severity: a nationwide population-based observational study in South Korea.* BMJ Open, 2018. **8**(3): p. e020825.

21. Eisner, M.D., et al., *Risk factors for hospitalization among adults with asthma: the influence of sociodemographic factors and asthma severity.* Respir Res, 2001. **2**(1): p. 53-60.
22. Tattersfield, A.E., et al., *Exacerbations of asthma: a descriptive study of 425 severe exacerbations. The FACET International Study Group.* Am J Respir Crit Care Med, 1999. **160**(2): p. 594-9.
23. FitzGerald, J.M. and P.G. Gibson, *Asthma exacerbations . 4: Prevention.* Thorax, 2006. **61**(11): p. 992-9.
24. van Staa, T.P., et al., *Use of oral corticosteroids in the United Kingdom.* QJM, 2000. **93**(2): p. 105-11.
25. Asher, R., *Straight and crooked thinking in medicine.* Br Med J, 1954. **2**(4885): p. 460-2.
26. Rackemann, F.M., *A working classification of asthma.* Am J Med, 1947. **3**(5): p. 601-6.
27. Romanet-Manent, S., et al., *Allergic vs nonallergic asthma: what makes the difference?* Allergy, 2002. **57**(7): p. 607-13.
28. Busse, W., et al., *Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma.* J Allergy Clin Immunol, 2001. **108**(2): p. 184-90.
29. Busse, W.W., *Anti-immunoglobulin E (omalizumab) therapy in allergic asthma.* Am J Respir Crit Care Med, 2001. **164**(8 Pt 2): p. S12-7.
30. Hanania, N.A., et al., *Exploring the Effects of Omalizumab in Allergic Asthma.* American Journal of Respiratory and Critical Care Medicine, 2013. **187**(8): p. 804-811.
31. de Llano, L.P., et al., *Effects of omalizumab in non-atopic asthma: results from a Spanish multicenter registry.* J Asthma, 2013. **50**(3): p. 296-301.
32. Vermeersch, K., et al., *Azithromycin during Acute COPD Exacerbations Requiring Hospitalization (BACE): a Multicentre, Randomized, Double-blind, Placebo-controlled Trial.* Am J Respir Crit Care Med, 2019.
33. Han, M.L.K., et al., *Predictors of Chronic Obstructive Pulmonary Disease Exacerbation Reduction in Response to Daily Azithromycin Therapy.* American Journal of Respiratory and Critical Care Medicine, 2014. **189**(12): p. 1503-1508.
34. Leckie, M.J., et al., *Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response.* Lancet, 2000. **356**(9248): p. 2144-8.
35. Flood-Page, P., et al., *A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma.* Am J Respir Crit Care Med, 2007. **176**(11): p. 1062-71.
36. Haldar, P., et al., *Cluster analysis and clinical asthma phenotypes.* Am J Respir Crit Care Med, 2008. **178**(3): p. 218-24.
37. Jatakanon, A., S. Lim, and P.J. Barnes, *Changes in sputum eosinophils predict loss of asthma control.* Am J Respir Crit Care Med, 2000. **161**(1): p. 64-72.
38. Haldar, P., et al., *Mepolizumab and exacerbations of refractory eosinophilic asthma.* N Engl J Med, 2009. **360**(10): p. 973-84.

39. Pavord, I.D., et al., *Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial.* Lancet, 2012. **380**(9842): p. 651-9.
40. Beasley, R., et al., *The further paradoxes of asthma management: time for a new approach across the spectrum of asthma severity.* Eur Respir J, 2018. **52**(5).
41. O'Byrne, P.M., C. Jenkins, and E.D. Bateman, *The paradoxes of asthma management: time for a new approach?* Eur Respir J, 2017. **50**(3).
42. Chung, K.F., *Personalised medicine in asthma: time for action: Number 1 in the Series "Personalised medicine in respiratory diseases" Edited by Renaud Louis and Nicolas Roche.* Eur Respir Rev, 2017. **26**(145).
43. Agusti, A., et al., *Precision medicine in airway diseases: moving to clinical practice.* Eur Respir J, 2017. **50**(4).
44. Tashkin, D., et al., *A 4-year trial of tiotropium in chronic obstructive pulmonary disease (UPLIFT trial).* Rev Port Pneumol, 2009. **15**(1): p. 137-40.
45. Calverley, P.M., et al., *Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease.* N Engl J Med, 2007. **356**(8): p. 775-89.
46. Castro, M., et al., *Effectiveness and safety of bronchial thermoplasty in the treatment of severe asthma: a multicenter, randomized, double-blind, sham-controlled clinical trial.* Am J Respir Crit Care Med, 2010. **181**(2): p. 116-24.
47. Brightling, C.E., et al., *Sputum eosinophilia and short-term response to prednisolone in chronic obstructive pulmonary disease: a randomised controlled trial.* Lancet, 2000. **356**(9240): p. 1480-5.
48. Green, R.H., et al., *Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial.* Lancet, 2002. **360**(9347): p. 1715-21.
49. Castro, M., et al., *Benralizumab, an anti-interleukin 5 receptor alpha monoclonal antibody, versus placebo for uncontrolled eosinophilic asthma: a phase 2b randomised dose-ranging study.* Lancet Respir Med, 2014. **2**(11): p. 879-90.
50. Castro, M., et al., *Reslizumab for inadequately controlled asthma with elevated blood eosinophil counts: results from two multicentre, parallel, double-blind, randomised, placebo-controlled, phase 3 trials.* Lancet Respir Med, 2015. **3**(5): p. 355-66.
51. Albert, R.K., et al., *Azithromycin for prevention of exacerbations of COPD.* N Engl J Med, 2011. **365**(8): p. 689-98.
52. Murray, M.P., J.L. Pentland, and A.T. Hill, *A randomised crossover trial of chest physiotherapy in non-cystic fibrosis bronchiectasis.* Eur Respir J, 2009. **34**(5): p. 1086-92.
53. Fjaellegaard, K., et al., *Antibiotic therapy for stable non-CF bronchiectasis in adults - A systematic review.* Chron Respir Dis, 2016.
54. Chamberlain Mitchell, S.A., et al., *Physiotherapy, and speech and language therapy intervention for patients with refractory chronic cough: a multicentre randomised control trial.* Thorax, 2017. **72**(2): p. 129-136.
55. Ryan, N.M., S.S. Birring, and P.G. Gibson, *Gabapentin for refractory chronic cough: a randomised, double-blind, placebo-controlled trial.* The Lancet, 2012. **380**(9853): p. 1583-1589.
56. Maniscalco, M., et al., *Weight loss and asthma control in severely obese asthmatic females.* Respir Med, 2008. **102**(1): p. 102-8.

57. Eneli, I.U., T. Skybo, and C.A. Camargo, Jr., *Weight loss and asthma: a systematic review.* Thorax, 2008. **63**(8): p. 671-6.
58. Spivak, H., et al., *Weight loss and improvement of obesity-related illness in 500 U.S. patients following laparoscopic adjustable gastric banding procedure.* Am J Surg, 2005. **189**(1): p. 27-32.
59. Juel, C.T., et al., *Asthma and obesity: does weight loss improve asthma control? a systematic review.* J Asthma Allergy, 2012. **5**: p. 21-6.
60. Bahrainwala, A.H. and M.R. Simon, *Wheezing and vocal cord dysfunction mimicking asthma.* Curr Opin Pulm Med, 2001. **7**(1): p. 8-13.
61. Lindsay, J.T. and L.G. Heaney, *Nonadherence in difficult asthma - facts, myths, and a time to act.* Patient Prefer Adherence, 2013. **7**: p. 329-36.
62. McNicholl, D.M., et al., *The utility of fractional exhaled nitric oxide suppression in the identification of nonadherence in difficult asthma.* Am J Respir Crit Care Med, 2012. **186**(11): p. 1102-8.
63. Xu, J.J., L. Sowerby, and B.W. Rotenberg, *Aspirin desensitization for aspirin-exacerbated respiratory disease (Samter's Triad): a systematic review of the literature.* Int Forum Allergy Rhinol, 2013. **3**(11): p. 915-20.
64. Dahlen, S.E., et al., *Improvement of aspirin-intolerant asthma by montelukast, a leukotriene antagonist: a randomized, double-blind, placebo-controlled trial.* Am J Respir Crit Care Med, 2002. **165**(1): p. 9-14.
65. Brown, H.M., *Treatment of chronic asthma with prednisolone; significance of eosinophils in the sputum.* Lancet, 1958. **2**(7059): p. 1245-7.
66. Medical Research Council, *Controlled trial of effects of cortisone acetate in chronic asthma; report to the Medical Research Council by the subcommittee on clinical trials in asthma.* Lancet, 1956. **271**(6947): p. 798-803.
67. Schleich, F.N., et al., *Importance of concomitant local and systemic eosinophilia in uncontrolled asthma.* Eur Respir J, 2014. **44**(1): p. 97-108.
68. Wenzel, S.E., et al., *Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics.* Am J Respir Crit Care Med, 1999. **160**(3): p. 1001-8.
69. Bousquet, J., et al., *Eosinophilic inflammation in asthma.* N Engl J Med, 1990. **323**(15): p. 1033-9.
70. Nair, P. and F.E. Hargreave, *Measuring bronchitis in airway diseases: clinical implementation and application: Airway hyperresponsiveness in asthma: its measurement and clinical significance.* Chest, 2010. **138**(2 Suppl): p. 38S-43S.
71. Price, D.B., et al., *Blood eosinophil count and prospective annual asthma disease burden: a UK cohort study.* Lancet Respir Med, 2015. **3**(11): p. 849-58.
72. Denlinger, L.C., et al., *Inflammatory and Comorbid Features of Patients with Severe Asthma and Frequent Exacerbations.* Am J Respir Crit Care Med, 2017. **195**(3): p. 302-313.
73. Jayaram, L., et al., *Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations.* Eur.Respir.J, 2006. **27**(3): p. 483-494.
74. Siva, R., et al., *Eosinophilic airway inflammation and exacerbations of COPD: a randomised controlled trial.* Eur Respir J, 2007. **29**(5): p. 906-913.
75. Hargreave, F.E. and P. Nair, *REbuttal from drs hargreave and nair.* Chest, 2011. **139**(6): p. 1275-1277.

76. Hargreave, F.E. and P. Nair, *Point: Is measuring sputum eosinophils useful in the management of severe asthma? Yes.* Chest, 2011. **139**(6): p. 1270-3.
77. Peters, S.P., *Counterpoint: Is measuring sputum eosinophils useful in the management of severe asthma? No, not for the vast majority of patients.* Chest, 2011. **139**(6): p. 1273-5; discussion 1275-8.
78. Nair, P., *Update on clinical inflammometry for the management of airway diseases.* Can Respir J, 2013. **20**(2): p. 117-20.
79. Pavord, I.D., *Sputum induction to assess airway inflammation: is it an inflammatory stimulus?* Thorax, 1998. **53**(2): p. 79-80.
80. Belda, J., et al., *Induced sputum cell counts in healthy adults.* Am J Respir Crit Care Med, 2000. **161**(2 Pt 1): p. 475-8.
81. Spanevello, A., et al., *Induced sputum cellularity. Reference values and distribution in normal volunteers.* Am J Respir Crit Care Med, 2000. **162**(3 Pt 1): p. 1172-4.
82. Pavord, I.D., et al., *The use of induced sputum to investigate airway inflammation.* Thorax, 1997. **52**(6): p. 498-501.
83. Westerhof, G.A., et al., *Biomarkers to identify sputum eosinophilia in different adult asthma phenotypes.* Eur Respir J, 2015. **46**(3): p. 688-96.
84. Pignatti, P., et al., *Correlation between sputum and blood eosinophils in asthmatic and COPD patients with comorbidities.* European Respiratory Journal, 2018. **52**(suppl 62): p. PA4418.
85. Bafadhel, M., et al., *Acute exacerbations of chronic obstructive pulmonary disease: identification of biologic clusters and their biomarkers.* Am J Respir Crit Care Med, 2011. **184**(6): p. 662-71.
86. Bafadhel, M., et al., *Blood eosinophils to direct corticosteroid treatment of exacerbations of chronic obstructive pulmonary disease: a randomized placebo-controlled trial.* Am J Respir Crit Care Med, 2012. **186**(1): p. 48-55.
87. Pascoe, S., et al., *Blood eosinophil counts, exacerbations, and response to the addition of inhaled fluticasone furoate to vilanterol in patients with chronic obstructive pulmonary disease: a secondary analysis of data from two parallel randomised controlled trials.* Lancet Respir Med, 2015. **3**(6): p. 435-42.
88. Siddiqui, S.H., et al., *Blood Eosinophils: A Biomarker of Response to Extrafine Beclomethasone/Formoterol in Chronic Obstructive Pulmonary Disease.* Am J Respir Crit Care Med, 2015. **192**(4): p. 523-5.
89. Pavord, I.D., et al., *Blood eosinophils and inhaled corticosteroid/long-acting beta-2 agonist efficacy in COPD.* Thorax, 2016. **71**(2): p. 118-25.
90. Dasgupta, A. and P. Nair, *When are biomarkers useful in the management of airway diseases?* Pol Arch Med Wewn, 2013. **123**(4): p. 183-8.
91. Malinovschi, A., et al., *FeNO as a predictor of asthma control improvement after starting inhaled steroid treatment.* Nitric Oxide, 2014. **40**: p. 110-6.
92. Price, D., et al., *Using fractional exhaled nitric oxide (FeNO) to diagnose steroid-responsive disease and guide asthma management in routine care.* Clin Transl Allergy, 2013. **3**(1): p. 37.
93. Arron, J.R., et al., *Noninvasive biomarkers that predict treatment benefit from biologic therapies in asthma.* Ann Am Thorac Soc, 2013. **10 Suppl**: p. S206-13.

94. Gustafsson, L.E., et al., *Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans.* Biochem Biophys Res Commun, 1991. **181**(2): p. 852-7.
95. Malinovschi, A., et al., *The value of exhaled nitric oxide to identify asthma in smoking patients with asthma-like symptoms.* Respir Med, 2012. **106**(6): p. 794-801.
96. Dressel, H., et al., *Exhaled nitric oxide: independent effects of atopy, smoking, respiratory tract infection, gender and height.* Respir Med, 2008. **102**(7): p. 962-9.
97. Schleich, F., S. Demarche, and R. Louis, *Biomarkers in the Management of Difficult Asthma.* Curr Top Med Chem, 2016. **16**(14): p. 1561-73.
98. Schleich, F.N., et al., *Exhaled nitric oxide thresholds associated with a sputum eosinophil count >/=3% in a cohort of unselected patients with asthma.* Thorax, 2010. **65**(12): p. 1039-44.
99. Pavord, I.D., *Asthma control, airway responsiveness and airway inflammation.* Clin Exp Allergy, 2009. **39**(12): p. 1780-2.
100. Heaney, L.G., et al., *Remotely Monitored Therapy and Nitric Oxide Suppression Identifies Nonadherence in Severe Asthma.* Am J Respir Crit Care Med, 2019. **199**(4): p. 454-464.
101. Anderson, W.J., et al., *Inhaled corticosteroid dose response using domiciliary exhaled nitric oxide in persistent asthma: the FENOtype trial.* Chest, 2012. **142**(6): p. 1553-1561.
102. Essat, M., et al., *Fractional exhaled nitric oxide for the management of asthma in adults: a systematic review.* European Respiratory Journal, 2016.
103. Petsky, H.L., et al., *A systematic review and meta-analysis: tailoring asthma treatment on eosinophilic markers (exhaled nitric oxide or sputum eosinophils).* Thorax, 2012. **67**(3): p. 199-208.
104. Smith, A.D., et al., *Use of exhaled nitric oxide measurements to guide treatment in chronic asthma.* N Engl J Med, 2005. **352**(21): p. 2163-2173.
105. Powell, H., et al., *Management of asthma in pregnancy guided by measurement of fraction of exhaled nitric oxide: a double-blind, randomised controlled trial.* Lancet, 2011. **378**(9795): p. 983-990.
106. Ehrlich, P., *Beitrage zur Kenntnis der granulierten. Bindegewebszellen und der eosinophilen Leukozyten.* Arch Anat Physiol, 1879. **3**: p. 166-169.
107. Berek, C., *Eosinophils: important players in humoral immunity.* Clin Exp Immunol, 2016. **183**(1): p. 57-64.
108. Marichal, T., C. Mesnil, and F. Bureau, *Homeostatic Eosinophils: Characteristics and Functions.* Front Med (Lausanne), 2017. **4**: p. 101.
109. Shi, H.Z., et al., *Lymph node trafficking and antigen presentation by endobronchial eosinophils.* J Clin Invest, 2000. **105**(7): p. 945-53.
110. Lee, J.J., et al., *Eosinophils in health and disease: the LIAR hypothesis.* Clin Exp Allergy, 2010. **40**(4): p. 563-75.
111. Wu, D., et al., *Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis.* Science, 2011. **332**(6026): p. 243-7.
112. Ntatsaki, E., et al., *BSR and BHPR guideline for the management of adults with ANCA-associated vasculitis.* Rheumatology (Oxford), 2014. **53**(12): p. 2306-9.

113. Wechsler, M.E., et al., *Mepolizumab or Placebo for Eosinophilic Granulomatosis with Polyangiitis.* N Engl J Med, 2017. **376**(20): p. 1921-1932.
114. Fulkerson, P.C., *Transcription Factors in Eosinophil Development and As Therapeutic Targets.* Front Med (Lausanne), 2017. **4**: p. 115.
115. Takamoto, M. and K. Sugane, *Synergism of IL-3, IL-5, and GM-CSF on eosinophil differentiation and its application for an assay of murine IL-5 as an eosinophil differentiation factor.* Immunol Lett, 1995. **45**(1-2): p. 43-6.
116. Bochner, B.S., *The eosinophil: For better or worse, in sickness and in health.* Ann Allergy Asthma Immunol, 2018. **121**(2): p. 150-155.
117. Shamri, R., J.J. Xenakis, and L.A. Spencer, *Eosinophils in innate immunity: an evolving story.* Cell Tissue Res, 2011. **343**(1): p. 57-83.
118. Park, Y.M. and B.S. Bochner, *Eosinophil survival and apoptosis in health and disease.* Allergy Asthma Immunol Res, 2010. **2**(2): p. 87-101.
119. Simon, H.U., et al., *Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia.* J Immunol, 1997. **158**(8): p. 3902-8.
120. Kankaanranta, H., E. Moilanen, and X. Zhang, *Pharmacological regulation of human eosinophil apoptosis.* Curr Drug Targets Inflamm Allergy, 2005. **4**(4): p. 433-45.
121. Broide, D.H., M.M. Paine, and G.S. Firestein, *Eosinophils Express Interleukin-5 and Granulocyte Macrophage-Colony-Stimulating Factor Messenger-Rna at Sites of Allergic Inflammation in Asthmatics.* Journal of Clinical Investigation, 1992. **90**(4): p. 1414-1424.
122. Kankaanranta, H., et al., *Delayed eosinophil apoptosis in asthma.* J Allergy Clin Immunol, 2000. **106**(1 Pt 1): p. 77-83.
123. Vignola, A.M., et al., *Evaluation of apoptosis of eosinophils, macrophages, and T lymphocytes in mucosal biopsy specimens of patients with asthma and chronic bronchitis.* J Allergy Clin Immunol, 1999. **103**(4): p. 563-73.
124. Rosenberg, H.F., S. Phipps, and P.S. Foster, *Eosinophil trafficking in allergy and asthma.* J Allergy Clin Immunol, 2007. **119**(6): p. 1303-10; quiz 1311-2.
125. Wardlaw, A.J., *Eosinophil trafficking in asthma.* Clin Med (Lond), 2001. **1**(3): p. 214-8.
126. Neighbour, H., et al., *Safety and efficacy of an oral CCR3 antagonist in patients with asthma and eosinophilic bronchitis: a randomized, placebo-controlled clinical trial.* Clin Exp Allergy, 2014. **44**(4): p. 508-16.
127. van Leeuwen, B.H., et al., *Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5.* Blood, 1989. **73**(5): p. 1142-8.
128. Clutterbuck, E.J., E.M. Hirst, and C.J. Sanderson, *Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GMCSF.* Blood, 1989. **73**(6): p. 1504-12.
129. Lopez, A.F., et al., *Regulation of human eosinophil production and function by interleukin-5.* Immunol Ser, 1992. **57**: p. 549-71.
130. Warringa, R.A., et al., *Modulation of eosinophil chemotaxis by interleukin-5.* Am J Respir Cell Mol Biol, 1992. **7**(6): p. 631-6.

131. Carlson, M., C. Peterson, and P. Venge, *The influence of IL-3, IL-5, and GM-CSF on normal human eosinophil and neutrophil C3b-induced degranulation.* Allergy, 1993. **48**(6): p. 437-42.
132. Kita, H., et al., *Release of granule proteins from eosinophils cultured with IL-5.* J Immunol, 1992. **149**(2): p. 629-35.
133. Takatsu, K., *Interleukin-5 and IL-5 receptor in health and diseases.* Proc Jpn Acad Ser B Phys Biol Sci, 2011. **87**(8): p. 463-85.
134. Rosenberg, H.F., K.D. Dyer, and P.S. Foster, *Eosinophils: changing perspectives in health and disease.* Nat Rev Immunol, 2013. **13**(1): p. 9-22.
135. Sawicka, E., et al., *Tc2 cells respond to soluble antigen in the respiratory tract and induce lung eosinophilia and bronchial hyperresponsiveness.* Eur J Immunol, 2004. **34**(9): p. 2599-608.
136. Van Gool, F., et al., *Interleukin-5-producing group 2 innate lymphoid cells control eosinophilia induced by interleukin-2 therapy.* Blood, 2014. **124**(24): p. 3572-6.
137. Hogan, S.P., et al., *Eosinophils: biological properties and role in health and disease.* Clin Exp Allergy, 2008. **38**(5): p. 709-50.
138. Garlisi, C.G., et al., *T cells are necessary for Th2 cytokine production and eosinophil accumulation in airways of antigen-challenged allergic mice.* Clin Immunol Immunopathol, 1995. **75**(1): p. 75-83.
139. Hamid, Q., et al., *Interleukin-5 mRNA in mucosal bronchial biopsies from asthmatic subjects.* Int Arch Allergy Appl Immunol, 1991. **94**(1-4): p. 169-70.
140. Amini-Vaughan, Z.J., M. Martinez-Moczygemba, and D.P. Huston, *Therapeutic strategies for harnessing human eosinophils in allergic inflammation, hypereosinophilic disorders, and cancer.* Curr Allergy Asthma Rep, 2012. **12**(5): p. 402-12.
141. Nair, P., et al., *Mepolizumab for prednisone-dependent asthma with sputum eosinophilia.* N Engl J Med, 2009. **360**(10): p. 985-93.
142. Bel, E.H., et al., *Oral glucocorticoid-sparing effect of mepolizumab in eosinophilic asthma.* N Engl J Med, 2014. **371**(13): p. 1189-97.
143. Ortega, H.G., et al., *Mepolizumab treatment in patients with severe eosinophilic asthma.* N Engl J Med, 2014. **371**(13): p. 1198-207.
144. Castro, M., et al., *Reslizumab for poorly controlled, eosinophilic asthma: a randomized, placebo-controlled study.* Am J Respir Crit Care Med, 2011. **184**(10): p. 1125-32.
145. Corren, J., et al., *Phase 3 Study of Reslizumab in Patients With Poorly Controlled Asthma: Effects Across a Broad Range of Eosinophil Counts.* Chest, 2016. **150**(4): p. 799-810.
146. Bjermer, L., et al., *Reslizumab for Inadequately Controlled Asthma With Elevated Blood Eosinophil Levels: A Randomized Phase 3 Study.* Chest, 2016. **150**(4): p. 789-798.
147. Ghazi, A., A. Trikha, and W.J. Calhoun, *Benralizumab--a humanized mAb to IL-5Ralpha with enhanced antibody-dependent cell-mediated cytotoxicity--a novel approach for the treatment of asthma.* Expert Opin Biol Ther, 2012. **12**(1): p. 113-8.
148. Bleecker, E.R., et al., *Efficacy and safety of benralizumab for patients with severe asthma uncontrolled with high-dosage inhaled corticosteroids and long-acting*

beta2-agonists (SIROCCO): a randomised, multicentre, placebo-controlled phase 3 trial. Lancet, 2016.
149. FitzGerald, J.M., et al., *Benralizumab, an anti-interleukin-5 receptor alpha monoclonal antibody, as add-on treatment for patients with severe, uncontrolled, eosinophilic asthma (CALIMA): a randomised, double-blind, placebo-controlled phase 3 trial.* Lancet, 2016.
150. Nair, P., et al., *Oral Glucocorticoid-Sparing Effect of Benralizumab in Severe Asthma.* N Engl J Med, 2017. **376**(25): p. 2448-2458.
151. Kolbeck, R., et al., *MEDI-563, a humanized anti-IL-5 receptor alpha mAb with enhanced antibody-dependent cell-mediated cytotoxicity function.* J Allergy Clin Immunol, 2010. **125**(6): p. 1344-1353 e2.
152. Busse, W.W., et al., *Safety profile, pharmacokinetics, and biologic activity of MEDI-563, an anti-IL-5 receptor alpha antibody, in a phase I study of subjects with mild asthma.* J Allergy Clin Immunol, 2010. **125**(6): p. 1237-1244 e2.
153. Nowak, R.M., et al., *A randomized trial of benralizumab, an antiinterleukin 5 receptor alpha monoclonal antibody, after acute asthma.* Am J Emerg Med, 2015. **33**(1): p. 14-20.
154. Haldar, P., et al., *Mepolizumab and exacerbations of refractory eosinophilic asthma.* New England Journal of Medicine, 2009. **360**(10): p. 973-984.
155. Carretero, R., et al., *Eosinophils orchestrate cancer rejection by normalizing tumor vessels and enhancing infiltration of CD8(+) T cells.* Nat Immunol, 2015. **16**(6): p. 609-17.
156. Ortega, H.G., et al., *Severe eosinophilic asthma treated with mepolizumab stratified by baseline eosinophil thresholds: a secondary analysis of the DREAM and MENSA studies.* Lancet Respir Med, 2016.
157. Haldar, P., et al., *Outcomes after cessation of mepolizumab therapy in severe eosinophilic asthma: a 12-month follow-up analysis.* J Allergy Clin Immunol, 2014. **133**(3): p. 921-3.
158. Oguma, T., K. Asano, and A. Ishizaka, *Role of prostaglandin D(2) and its receptors in the pathophysiology of asthma.* Allergol Int, 2008. **57**(4): p. 307-12.
159. Arima, M. and T. Fukuda, *Prostaglandin D(2) and T(H)2 inflammation in the pathogenesis of bronchial asthma.* Korean J Intern Med, 2011. **26**(1): p. 8-18.
160. Schuligoi, R., et al., *PGD2 metabolism in plasma: kinetics and relationship with bioactivity on DP1 and CRTH2 receptors.* Biochem Pharmacol, 2007. **74**(1): p. 107-17.
161. Black, J.L., et al., *A comparison of the contractile activity of PGD2 and PGF2 alpha on human isolated bronchus.* Prostaglandins, 1986. **32**(1): p. 25-31.
162. Marom, Z., J.H. Shelhamer, and M. Kaliner, *Effects of arachidonic acid, monohydroxyeicosatetraenoic acid and prostaglandins on the release of mucous glycoproteins from human airways in vitro.* J Clin Invest, 1981. **67**(6): p. 1695-702.
163. Luna-Gomes, T., et al., *Eosinophils as a novel cell source of prostaglandin D2: autocrine role in allergic inflammation.* J Immunol, 2011. **187**(12): p. 6518-26.
164. Shimura, C., et al., *Dendritic cells express hematopoietic prostaglandin D synthase and function as a source of prostaglandin D2 in the skin.* Am J Pathol, 2010. **176**(1): p. 227-37.

165. Crea, A.E., J.A. Nakhosteen, and T.H. Lee, *Mediator concentrations in bronchoalveolar lavage fluid of patients with mild asymptomatic bronchial asthma.* Eur Respir J, 1992. **5**(2): p. 190-5.
166. Liu, M.C., et al., *Evidence for elevated levels of histamine, prostaglandin D2, and other bronchoconstricting prostaglandins in the airways of subjects with mild asthma.* Am Rev Respir Dis, 1990. **142**(1): p. 126-32.
167. Murray, J.J., et al., *Release of prostaglandin D2 into human airways during acute antigen challenge.* N Engl J Med, 1986. **315**(13): p. 800-4.
168. Balzar, S., et al., *Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program.* Am J Respir Crit Care Med, 2011. **183**(3): p. 299-309.
169. Fajt, M.L., et al., *Prostaglandin D(2) pathway upregulation: relation to asthma severity, control, and TH2 inflammation.* J Allergy Clin Immunol, 2013. **131**(6): p. 1504-12.
170. Tilley, S.L., T.M. Coffman, and B.H. Koller, *Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes.* J Clin Invest, 2001. **108**(1): p. 15-23.
171. Yoshimura-Uchiyama, C., et al., *Differential modulation of human basophil functions through prostaglandin D2 receptors DP and chemoattractant receptor-homologous molecule expressed on Th2 cells/DP2.* Clin Exp Allergy, 2004. **34**(8): p. 1283-90.
172. Giles, H., et al., *The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist.* Br J Pharmacol, 1989. **96**(2): p. 291-300.
173. Pettipher, R. and T.T. Hansel, *Antagonists of the prostaglandin D2 receptor CRTH2.* Drug News Perspect, 2008. **21**(6): p. 317-22.
174. Cosmi, L., et al., *CRTH2: marker for the detection of human Th2 and Tc2 cells.* Adv Exp Med Biol, 2001. **495**: p. 25-9.
175. Cosmi, L., et al., *CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease.* Eur J Immunol, 2000. **30**(10): p. 2972-9.
176. Xue, L., A. Barrow, and R. Pettipher, *Interaction between prostaglandin D and chemoattractant receptor-homologous molecule expressed on Th2 cells mediates cytokine production by Th2 lymphocytes in response to activated mast cells.* Clin Exp Immunol, 2009. **156**(1): p. 126-33.
177. Xue, L., et al., *Prostaglandin D2 causes preferential induction of proinflammatory Th2 cytokine production through an action on chemoattractant receptor-like molecule expressed on Th2 cells.* J Immunol, 2005. **175**(10): p. 6531-6.
178. Xue, L., et al., *Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells.* J Allergy Clin Immunol, 2014. **133**(4): p. 1184-94.
179. Monneret, G., et al., *Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor.* Blood, 2001. **98**(6): p. 1942-8.
180. Schratl, P., et al., *Hierarchy of eosinophil chemoattractants: role of p38 mitogen-activated protein kinase.* Eur J Immunol, 2006. **36**(9): p. 2401-9.

181. Hirai, H., et al., *Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2.* J Exp Med, 2001. **193**(2): p. 255-61.
182. Hilvering, B., et al., *Synergistic activation of pro-inflammatory type-2 CD8+ T lymphocytes by lipid mediators in severe eosinophilic asthma.* Mucosal immunology, 2018.
183. Sykes, D.A., et al., *Fevipiprant (QAW039), a Slowly Dissociating CRTh2 Antagonist with the Potential for Improved Clinical Efficacy.* Mol Pharmacol, 2016. **89**(5): p. 593-605.
184. Oliver, E.T., et al., *Effects of an Oral CRTh2 Antagonist (AZD1981) on Eosinophil Activity and Symptoms in Chronic Spontaneous Urticaria.* Int Arch Allergy Immunol, 2019. **179**(1): p. 21-30.
185. Radnai, B., et al., *Eosinophils Contribute to Intestinal Inflammation via Chemoattractant Receptor-homologous Molecule Expressed on Th2 Cells, CRTH2, in Experimental Crohn's Disease.* J Crohns Colitis, 2016. **10**(9): p. 1087-95.
186. Straumann, A., et al., *Anti-eosinophil activity and clinical efficacy of the CRTH2 antagonist OC000459 in eosinophilic esophagitis.* Allergy, 2013. **68**(3): p. 375-85.
187. Hilvering, B., et al., *Severe asthma is characterised by high levels of CRTH2 expressing cells in peripheral blood.* European Respiratory Journal, 2015. **46**(suppl 59).
188. Chantveerawong, T., et al., *CRTH2 expression on T cell subsets in asthma.* Journal of Allergy and Clinical Immunology, 2018. **141**(2).
189. Chen, Z., et al., *Peripheral pathological ILC2: An inflammatory indicator for the assessment of asthma control in clinics.* European Respiratory Journal, 2016. **48**(suppl 60): p. PA561.
190. Spits, H. and T. Cupedo, *Innate lymphoid cells: emerging insights in development, lineage relationships, and function.* Annu Rev Immunol, 2012. **30**: p. 647-75.
191. Kim, J., et al., *Innate immune crosstalk in asthmatic airways: Innate lymphoid cells coordinate polarization of lung macrophages.* J Allergy Clin Immunol, 2019. **143**(5): p. 1769-1782 e11.
192. Liu, T., et al., *Type 2 innate lymphoid cells: A novel biomarker of eosinophilic airway inflammation in patients with mild to moderate asthma.* Respir Med, 2015. **109**(11): p. 1391-6.
193. Barnes, N., et al., *A randomized, double-blind, placebo-controlled study of the CRTH2 antagonist OC000459 in moderate persistent asthma.* Clin Exp Allergy, 2012. **42**(1): p. 38-48.
194. Singh, D., et al., *Inhibition of the asthmatic allergen challenge response by the CRTH2 antagonist OC000459.* Eur Respir J, 2013. **41**(1): p. 46-52.
195. Pettipher, R., et al., *Heightened response of eosinophilic asthmatic patients to the CRTH2 antagonist OC000459.* Allergy, 2014. **69**(9): p. 1223-32.
196. Kuna, P., L. Bjermer, and G. Tornling, *Two Phase II randomized trials on the CRTh2 antagonist AZD1981 in adults with asthma.* Drug Des Devel Ther, 2016. **10**: p. 2759-70.

197. Bateman, E.D., et al., *Efficacy and safety of the CRTh2 antagonist AZD1981 as add-on therapy to inhaled corticosteroids and long-acting beta2-agonists in patients with atopic asthma.* Drug Des Devel Ther, 2018. **12**: p. 1093-1106.
198. Erpenbeck, V.J., et al., *The oral CRTh2 antagonist QAW039 (fevipiprant): A phase II study in uncontrolled allergic asthma.* Pulm Pharmacol Ther, 2016. **39**: p. 54-63.
199. Gonem, S., et al., *Fevipiprant, a prostaglandin D2 receptor 2 antagonist, in patients with persistent eosinophilic asthma: a single-centre, randomised, double-blind, parallel-group, placebo-controlled trial.* Lancet Respir Med, 2016. **4**(9): p. 699-707.
200. Bateman, E.D., et al., *Fevipiprant, an oral prostaglandin DP2 receptor (CRTh2) antagonist, in allergic asthma uncontrolled on low-dose inhaled corticosteroids.* Eur Respir J, 2017. **50**(2).
201. Gonem, S., et al., *Fevipiprant, a prostaglandin D2 receptor 2 antagonist, in patients with persistent eosinophilic asthma: a single-centre, randomised, double-blind, parallel-group, placebo-controlled trial.* Lancet Respir Med, 2016. **4**(9): p. 699-707.
202. Hall, I.P., et al., *Efficacy of BI 671800, an oral CRTH2 antagonist, in poorly controlled asthma as sole controller and in the presence of inhaled corticosteroid treatment.* Pulmonary Pharmacology & Therapeutics, 2015. **32**: p. 37-44.
203. Miller, M.R., et al., *Standardisation of spirometry.* Eur Respir J, 2005. **26**(2): p. 319-38.
204. Juniper, E.F., et al., *Measurement properties and interpretation of three shortened versions of the asthma control questionnaire.* Respir Med, 2005. **99**(5): p. 553-8.
205. Juniper, E.F., et al., *Validation of a standardized version of the Asthma Quality of Life Questionnaire.* Chest, 1999. **115**(5): p. 1265-70.
206. Awad, J.A., J.D. Morrow, and L.J. Roberts, 2nd, *Detection of the major urinary metabolite of prostaglandin D2 in the circulation: demonstration of elevated levels in patients with disorders of systemic mast cell activation.* J Allergy Clin Immunol, 1994. **93**(5): p. 817-24.
207. Masoli, M., et al., *The dose-response relationship of inhaled corticosteroids in asthma.* Curr Allergy Asthma Rep, 2004. **4**(2): p. 144-8.
208. Holt, S., et al., *Dose-response relation of inhaled fluticasone propionate in adolescents and adults with asthma: meta-analysis.* BMJ, 2001. **323**(7307): p. 253-6.
209. Branch, S.K., *Guidelines from the International Conference on Harmonisation (ICH).* J Pharm Biomed Anal, 2005. **38**(5): p. 798-805.
210. World Medical, A., *World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects.* JAMA, 2013. **310**(20): p. 2191-4.
211. Dasgupta, A., et al., *Sample sizes for clinical trials using sputum eosinophils as a primary outcome.* Eur Respir J, 2013. **42**(4): p. 1003-11.
212. Castro, M., et al., *Dupilumab Efficacy and Safety in Moderate-to-Severe Uncontrolled Asthma.* N Engl J Med, 2018. **378**(26): p. 2486-2496.

213. Malinovschi, A., et al., *Exhaled nitric oxide levels and blood eosinophil counts independently associate with wheeze and asthma events in National Health and Nutrition Examination Survey subjects.* J Allergy Clin Immunol, 2013. **132**(4): p. 821-7.e1-5.
214. Pavord, I.D., et al., *The current and future role of biomarkers in type 2 cytokine-mediated asthma management.* Clin Exp Allergy, 2017. **47**(2): p. 148-160.
215. Heaney, L.G., et al., *Research in progress: Medical Research Council United Kingdom Refractory Asthma Stratification Programme (RASP-UK).* Thorax, 2016. **71**(2): p. 187-189.
216. Haldar, P., et al., *Mepolizumab and exacerbations of refractory eosinophilic asthma.* New England Journal of Medicine. , 2009. **360**(10): p. 973-984.
217. Pavord, I.D., et al., *Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial.* The Lancet, 2012. **380**(9842): p. 651-9.
218. Ortega, H.G., et al., *Severe eosinophilic asthma treated with mepolizumab stratified by baseline eosinophil thresholds: a secondary analysis of the DREAM and MENSA studies.* Lancet Respiratory Medicine, 2016. **4**(7): p. 549-556.
219. Bafadhel, M., et al., *Blood eosinophils to direct corticosteroid treatment of exacerbations of chronic obstructive pulmonary disease: a randomized placebo-controlled trial.* American journal of respiratory and critical care medicine, 2012. **186**(1): p. 48-55.
220. Green, R.H., et al., *Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial.* The Lancet, 2002. **360**(9347): p. 1715-1721.
221. Green, R.H., et al., *Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids.* Thorax, 2002. **57**(10): p. 875-879.
222. Moore, W.C., et al., *Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program.* Am J Respir Crit Care Med, 2010. **181**(4): p. 315-23.
223. Nishimura, T., et al., *Suppressive effect of environmental tobacco smoke on murine Th2 cell-mediated nasal eosinophilic inflammation.* Asia Pac Allergy, 2020. **10**(2): p. e18.
224. Fahy, J.V., *Eosinophilic and neutrophilic inflammation in asthma: insights from clinical studies.* Proc Am Thorac Soc, 2009. **6**(3): p. 256-9.
225. Shrimanker, R., et al., *Exacerbations of severe asthma in patients treated with mepolizumab.* Eur Respir J, 2018. **52**(6).
226. Chupp, G.L., et al., *Efficacy of mepolizumab add-on therapy on health-related quality of life and markers of asthma control in severe eosinophilic asthma (MUSCA): a randomised, double-blind, placebo-controlled, parallel-group, multicentre, phase 3b trial.* Lancet Respir Med, 2017. **5**(5): p. 390-400.
227. Bafadhel, M., et al., *Blood eosinophils to direct corticosteroid treatment of exacerbations of chronic obstructive pulmonary disease: a randomized placebo-controlled trial.* Am J Respir Crit Care Med, 2012. **186**.
228. Pavord, I.D., et al., *Mepolizumab for Eosinophilic Chronic Obstructive Pulmonary Disease.* N Engl J Med, 2017. **377**(17): p. 1613-1629.

229. Pavord, I.D., et al., *Multiple inflammatory hits and the pathogenesis of severe airway disease.* Eur Respir J, 2006. **27**(5): p. 884-888.
230. Belvisi, M.G., *Regulation of inflammatory cell function by corticosteroids.* Proc Am Thorac Soc, 2004. **1**(3): p. 207-14.
231. Wershil, B.K., et al., *Dexamethasone and cyclosporin A suppress mast cell-leukocyte cytokine cascades by multiple mechanisms.* Int Arch Allergy Immunol, 1995. **107**(1-3): p. 323-4.
232. Cole, Z.A., G.F. Clough, and M.K. Church, *Inhibition by glucocorticoids of the mast cell-dependent weal and flare response in human skin in vivo.* Br J Pharmacol, 2001. **132**(1): p. 286-92.
233. Perfetto, S.P., et al., *Quality assurance for polychromatic flow cytometry using a suite of calibration beads.* Nature Protocols, 2012. **7**: p. 2067.