IDENTIFICATION OF CLINICAL, IMMUNOLOGICAL AND GENETIC MARKERS OF ASTHMA SUBGROUPS

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Abstract

Asthma is a heterogeneous disease thought to be driven by multiple overlapping clinical and immunological features present to different extents in different patients. Genome wide association studies (GWAS) have identified genetic signals associated with the risk of developing asthma, with many of these signals being in loci/genes involved in the type 2 inflammatory pathway. New asthma biological drugs that target specific components of the type 2 inflammation pathway have been brought into clinic over the last decade e.g. anti-IL5 (Mepolizumab). However, identifying patients who may or may not respond is still difficult. This thesis used three approaches; clinical, blood inflammatory and genetic profiling to explore asthma heterogeneity, with implications on treatment and wider asthma management. 4,205 asthma patients aged 16-60 were selected from the Genetics of Asthma Severity and Phenotypes (GASP) database; a Luminex assay was used for quantification of the serum biomarkers in 708 asthma patients, and 3,037 patients were genotyped for 25 genetic variants associated with moderate-severe asthma. The clinical data showed patients with childhood onset of asthma are more atopic compared to the adult-onset groups (IgE: <16 150 KU/L, 16-34 85.5 KU/L & 35-60 89.5 KU/L; $p= 7.8x10^{-8}$). Serum data demonstrated multiple Th2 cytokines are present in the blood with highly correlated expression including IL-4 and IL-5 ($r^2= 0.51$; p= <0.001). Some patients had multiple drivers of inflammation as 24.4% of patients overlapped for Th1, Th2 and Th17 cytokines using the top quartiles of biomarker levels. The genetic risk factors for moderate-severe asthma are implicated in the clinical features of asthma. rs61816764 (*FLG*) and rs9303277 (*IKFZ3*), showed the strongest effect size with childhood onset ($p= 2.67x10^{-4}$ and $2.20x10^{-7}$; respectively). These data provide greater insight on the ability of stratified medicine, using genetics and inflammatory profile, to help identify the type of inflammation in asthma, guide treatment for biological therapy and even identify patients that may be suitable for combination biologic therapy.

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Abstracts

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Abbreviations

ACOS0- Asthma COPD Overlap Syndrome

ACT- Asthma Control Test

ACQ- Asthma Control Questionnaire

AERD- Aspirin-Exacerbated Respiratory Disease

ALI- Air Liquid Interface

AUGOSA- Asthma UK Genetics of Severe Asthma

BALF- Bronchoalveolar Lavage Fluid

BMI- Body Mass Index

BTS- British Thoracic Society

CD4- Cluster of Differentiation 4

CF- Cystic Fibrosis

CI- Confidence Interval

COPD- Chronic Obstructive Pulmonary Disease

COX- Cyclooxygenase

CRF- Case Report Forms

CRISPR-Cas9- Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9

CXCL8- C-X-C Motif Chemokine Ligand 8

CysLT1- Cysteinyl Leukotrienes 1

DCs- Dendritic Cells

ECM- Extra Cellular Matrix

EDTA- Ethylenediamine Tetraacetic Acid

EGFR- Epidermal Growth Factor Receptor

ELISA- Enzyme-Linked Immunoassay

eQTL- Expression Quantitative Trait Loci

Fc- Fragment crystallisable

FcεRI- High-Affinity IgE Receptor

FeNO- Fractional exhaled nitric oxide

FEV_1- Forced Expiratory Volume in 1 second

FRET- Fluorescence Resonant Energy Transfer

FVC- Forced Vital Capacity

GASP- Genetics of Asthma Severity and Phenotypes

GATA3- Guanine Adenine Thymine Adenine sequence-binding protein 3

GERD- Gastroesophageal Reflux Disease

GINA- Global Initiative for Asthma

GWAS- Genome Wide Association Study

HBECs- Human Bronchial Epithelial cells

HWE- Hardy Weinberg Equilibrium

ICS- Inhaled Corticosteroids

ICU- Intensive Care Unit

IFN-γ- Interferon-γ

IgE- Immunoglobulin E

IL- Interleukin

IL-4α- Interleukin 4 Receptor Alpha Chain

IL-5α- Interleukin 5 Receptor Alpha Chain

ILC2- 2 Innate Lymphoid Cells- Type 2

KASP- Kompetitive Allele-Specific PCR

LABA- Long-Acting β2-Agonists

LAMA- Long-Acting Muscarinic Antagonists

LD- Linkage Disequilibrium

LTRA- Leukotriene Receptor Antagonists

MAF- Minor Allele Frequency

MUC5AC- Mucin 5AC

NICE- National Institute of Clinical Excellence

NK- Natural Killer

NSAID- Non-Steroidal Anti-Inflammatory Drugs

NTC- No Template Control

OCS- Oral Corticosteroids

OR- Odds Ratio

PC- Principle Component

PE- Phycoerythrin

PEF- Peak Expiratory Flow

PCR- Polymerase Chain Reaction

PPB- Parts per Billion

QC- Quality Control

RAF- Risk Allele Frequency

RCF- Relative Centrifugal Force

RPM- Rotations per Minute

SABA- Short-Acting β2-Agonists

SARP- The Severe Asthma Research Program

SD- Standard Deviation

SNP- Single Nucleotide Polymorphism

SST- Separating Serum Tubes

STAT6- Signal Transducer and Activation of Transcription 6

ST2- Suppression of Tumorigenicity 2

TENOR- The Epidemiology and Natural History of Asthma: Outcomes and Treatment Regimens

TGF-β- Transforming Growth Factor-β

Tfh- Follicular Helper Cells

Th0- Naïve T-Helper Cells

Th1- T-Helper 1 Cells

Th2- T-Helper 2 Cells

Th17- T-Helper 17 Cells

TLR2- Toll-Like Receptor-2

TNF- Tumour Necrosis Factor

TSLP- Thymic Stromal Lymphopoietin

TSLPR- Thymic Stromal Lymphopoietin Recptor

uBIOPRED- (Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes)

Ultra-LABA- Ultra-Long-Acting β2-Agonists

V2G- Variant-Gene

Chapter 1- General Introduction

Asthma is a heterogeneous disease thought to be driven by multiple overlapping clinical and immunological features that are present to different extents in different patients. Accumulating evidence suggests that asthma *per se*, as well as specific sub-types of disease, are driven by a combination of genetic and environmental factors. Currently, monoclonal antibody drugs are being developed to target specific areas of the Type 2 inflammatory pathway but there is still lack of research to see which patients may benefit from these drugs. Asthma genome wide association studies (GWAS) have identified genetic signals associated with the risk of developing the disease. Many of these signals are in loci that suggest genes/pathways that are involved in the innate (first line of defence against pathogens) and adaptive (immunity occurring after exposure to pathogen or antigen) immune responses and make potential therapeutic targets. A recent moderate-severe asthma GWAS identified previously reported signals that have been associated with asthma and a novel signal in the MUC5AC locus, specific to moderate to severe asthma (Shrine et al., 2019).

This thesis is set out to look at whether genetic risk factors for asthma found in Type 2 pathway associated genes drive the expression of the Th2 cytokines, receptors and signalling proteins as well as activity of the pathway leading to asthma relevant clinical phenotypes. This is important as it may help identify what type of inflammation is driving a patient's asthma by using their clinical, blood and genetic profile, which may then allow their asthma therapy to be individually tailored. Secondly, this thesis will use serum biomarker data to identify patients who may be more amenable to the new and upcoming asthma biological therapies that target the Type 2 pathway, as well as see how they overlap and clinically compare. Finally, this thesis will take the 25 genetic signals identified in the moderate-severe asthma GWAS by Shrine et al (Shrine et al., 2019) to see if carrying the relevant risk allele for each of these signals drives identified serum biomarkers and/or clinical features associated with asthma.

1.1 Asthma Background

Asthma is now recognised as a heterogeneous chronic inflammatory disease and is thought to be caused mostly by an individuals' genetic and epigenetic variability, in combination with environment exposures such as viruses, allergens and pollution (Carr and Bleecker, 2016, Loxham and Davies, 2017). Asthma effects around 235 million people worldwide (World Health Organization, 2019 figures) with 5.4 million people in the UK receiving treatment for asthma and around 200,000 people in the UK having severe asthma (Asthma UK, 2021). In 2017 1,484 people died from an asthma attack in the UK (Asthma UK, 2021) and asthma deaths in England and Wales have increased by 33% in the last decade (2008-2018) (Iacobucci, 2019). Another study looked at mortality rates in England and Wales between 2001 and 2017 and found a 50% increase in asthma deaths from asthma patients over the age of 75 years (Shaw et al., 2019). Common asthma symptoms include breathlessness, wheezing, coughing and chest tightness. Wheezing is a common clinical feature which is caused by bronchoconstriction of the bronchi and bronchioles via contraction of the airway smooth muscle (Ha and Rogers, 2016). Common triggers of asthma are allergens such as house dust mite and pollen and non-allergens including weather, air pollution, viruses, smoking and stress/emotion. Overall, asthma is characterised by distinct inflammation processes by CD4 T-helper 2 (Th2) cells as will be further described in section 1.2 figure 1.2.1 (Mitchell and O'Byrne, 2017) (Barnig et al., 2018).

1.1.1 Development of Asthma

There are multiple risk factors that contribute to the development of asthma. One common risk factor for asthma is atopy which is defined as producing immunoglobulin E (IgE) antibodies in response to an exposure of a common allergen (Comberiati et al., 2017). Children are more likely to develop asthma if they suffer from other atopic diseases such as eczema, this is known as "atopic march" (discussed further in chapter 3.1.1) (Hill and Spergel, 2018). Another potential explanation for the development of asthma is the hygiene hypothesis. The hygiene hypothesis proposes that children are less likely to develop asthma and eczema when exposed infectious agents and unhygienic practices. For example, children that have grown up in rural or farm

environments are more likely to be protected from developing immune-mediated and inflammatory diseases, such as asthma, hay fever and eczema. Whereas children not growing up in an agricultural setting are more susceptible to asthma or atopic diseases (Von Ehrenstein et al., 2000) (Haspeslagh et al., 2018). Multiple studies have been able to support this, one study showed that muramic acid (found in bacteria cell walls) concentration was significantly higher in from dust samples from farm children's mattresses than non-farms (157 vs 131 ng/mg respectively). Their data showed that environmental exposure to bacteria is inversely correlated with the prevalence of asthma symptoms such as wheeze supporting the hygiene hypothesis (Braun-Fahrländer et al., 2002). However, the hygiene hypothesis cannot be applied to the development of all asthma types. Other risk factors of asthma include respiratory viral infections specifically rhinovirus (the common cold), occupational exposures and smoking (Jartti et al., 2020) (Dao and Bernstein, 2018) (Jaakkola et al., 2019). In addition to environmental factors, there is strong evidence that genetics also contribute to the predisposition of asthma; this is known as heritability (discussed further in section 1.6 and chapter 6). Therefore both heritability and the environment are likely to contribute to the development of asthma (discussed further in section 1.6.1) (Hernandez-Pacheco et al., 2019).

1.1.2 Diagnosis and Current Management of Asthma

There is currently no gold standard diagnosis for asthma and so diagnosis is based on current symptoms and airway function tests. Symptom patterns can give an indication in the diagnosis of asthma for example symptoms that worsen at night or in the morning including a lower peak flow in the morning, during exercise, exposure to allergens and/or cold air (Wong et al., 2016). A family history of atopy and asthma is also an indicator of asthma (Thomsen, 2015b). Measuring airflow limitation with spirometry which is a pulmonary function test that assesses the severity and variability of airflow obstruction, which can help confirm the diagnosis of asthma. Airflow obstruction is calculated by the forced expiratory volume in 1 second (FEV_1)/ forced vital capacity (FVC) ratio. The reference range is 0.75-0.80 and a FEV_1/FVC ratio of <0.70 indicates obstruction indicating the presence of an obstructive

pulmonary disorder such as asthma or chronic obstructive pulmonary disease (COPD). However, a negative result does not rule out the diagnosis of asthma (Horak et al., 2016). A significant increase in FEV_1 of 200 mL or more and a 12% improvement from baseline after the administration of a bronchodilator, is also an indication of potential asthma (Ye et al., 2018). As well as symptoms and spirometry there are other tests that may be carried out to help diagnose asthma such as fractional exhaled nitric oxide (FeNO) and sputum or blood eosinophil count. FeNO is used to assess airway inflammation and if a result of 40 parts per billion (ppb) or higher is recorded, then it is considered supportive of an asthma diagnosis (Turner et al., 2019) (see chapter 4.1.1.1 for further discussion on nitric oxide). Even though sputum and blood eosinophil counts are commonly used in asthma, according to the National Institute of Clinical Excellence (NICE) 2021 guidelines, they are not used as diagnostic tests for asthma. Instead they are mainly used to diagnose patients who are thought to have severe asthma and also a tool for guiding and tailoring asthma treatment in order to reduce exacerbations (Walsh et al., 2016) (Saglani and Menzie-Gow, 2019, Pavord et al., 2018) (see chapter 4.1.1.3-4 for further discussion on blood and sputum eosinophils).

The most widely used asthma guidelines worldwide are those issued by the Global Initiative for Asthma (GINA). In the UK NICE and British Thoracic Society (BTS) have also published guidelines for diagnosing asthma (see Table 1.1.2a for an overview of the diagnosis tests in asthma). Overall, asthma can be difficult to diagnose and even misdiagnosed due to its heterogeneous presentation from patient to patient and the lack in a gold standard test for asthma.

Test	Diagnosis for asthma	Reference
Peak flow variability	More than 20% variability is a positive test.	(NICE, 2021) (BTS, 2019) (GINA, 2019)
Spirometry	An FEV_1/FVC of <0.7 is considered positive for obstructive airway disease.	(NICE, 2021) (BTS, 2019) (GINA, 2019)
Bronchodilator reversibility	An improvement of FEV_1 of >12% after using a bronchodilator is considered positive.	(NICE, 2021) (BTS, 2019) (GINA, 2019)
Airway hyperreactivity	A drop of >20% in FEV_1 in a direct bronchial challenge test with histamine or methacholine is considered positive.	(NICE, 2021) (BTS, 2019) (GINA, 2019)
Fractional exhaled nitric oxide	FeNO level of >40 ppb (or >50 ppb*) is considered positive.	(NICE, 2021) (BTS, 2019) (GINA, 2019)*

Table 1.1.2 Current tests used to diagnose asthma according to NICE, BTS and GINA

The severity of asthma is defined by the GINA guidelines that assess how controlled asthma is based on symptoms, lung function, need for reliever and exacerbations. These guidelines have been developed in order to provide consistency of asthma treatment around the world (see figure 1.1.2a). These guidelines take a step-by-step approach to treatments, with the overall aim being to achieve daily asthma control and prevent future exacerbations using the lowest possible level of medication type and dosage. Severe asthma patients have uncontrolled asthma despite the suggested use of medium-high dose of inhaled corticosteroids (ICS), long-acting beta-adrenoceptor agonists (LABA) in the GINA guidelines steps 4 or 5 (Global Initiative for Asthma, 2019) (Papi et al.).

The British Thoracic Society guidelines have a step up and step-down approach to asthma i.e., if short acting beta-2 antagonists (SABA) are used more than 3 times a week, the patient is stepped up to the next line of treatment; low dose ICS along with SABA. Again, if asthma symptoms are still uncontrolled and lung function is still poor then you step up again to the next line of treatment and add on an additional therapy such as inhaled LABA to the current therapy the patient is on. This approach is continued until the patient's asthma is controlled and then you start to step down in the treatment line to find and maintain the lowest therapy and dose (see figure 1.1.2b) (British Thoracic Society, 2019). However, there is a need for new medicines

to treat severe refractory asthma patients as proportion of these patients still have uncontrolled asthma despite taking high dose corticosteroids. Additionally, many asthma patients on high dose steroid treatment can suffer with life limiting side effects such as osteoporosis and depression etc.

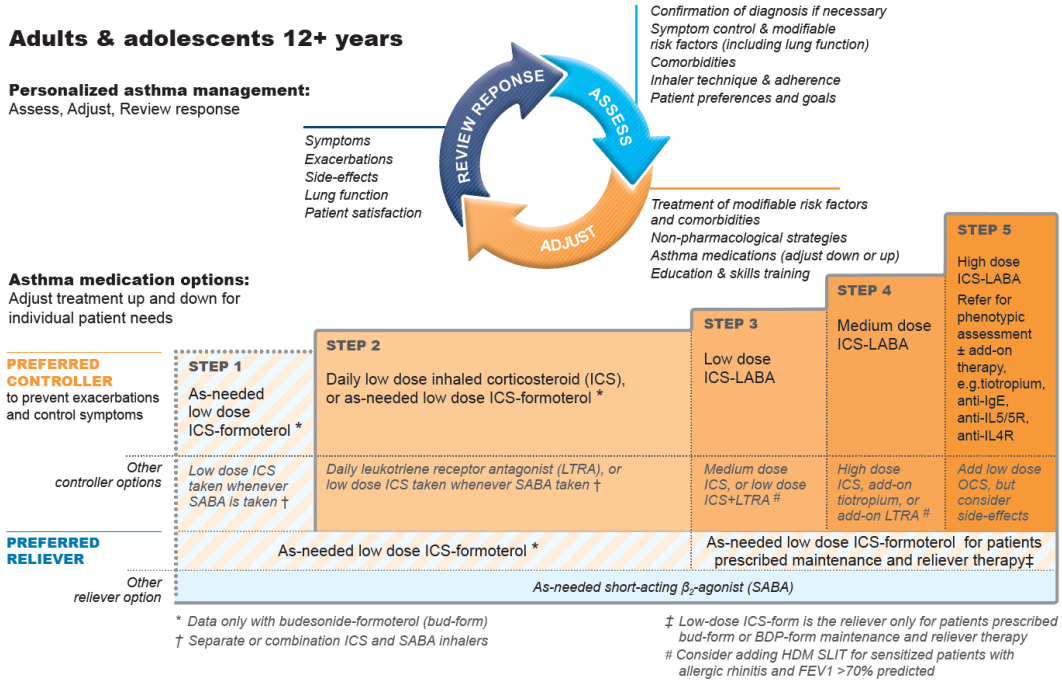


Figure 1.1.2a The GINA asthma treatment strategy in adults and adolescents (+12 years): the step-by-step strategy developed to achieve daily control of asthma symptoms and prevent future exacerbations. GINA recommends starting on a low dose ICS and review asthma symptoms every 2-3 month's, in addition to lung function, side effects and patient satisfaction, in addition to assessing inhaler techniques, adherence, other comorbidities and symptom control. This will then allow clinicians and healthcare specialists to decide whether treatment will need modifying such as increasing or decreasing ICS dose, treating other comorbidities or even educating and training skills including inhaler technique. (© 2020, Global Initiative for Asthma, available from www.ginasthma.org, published in Fontana, WI, USA).

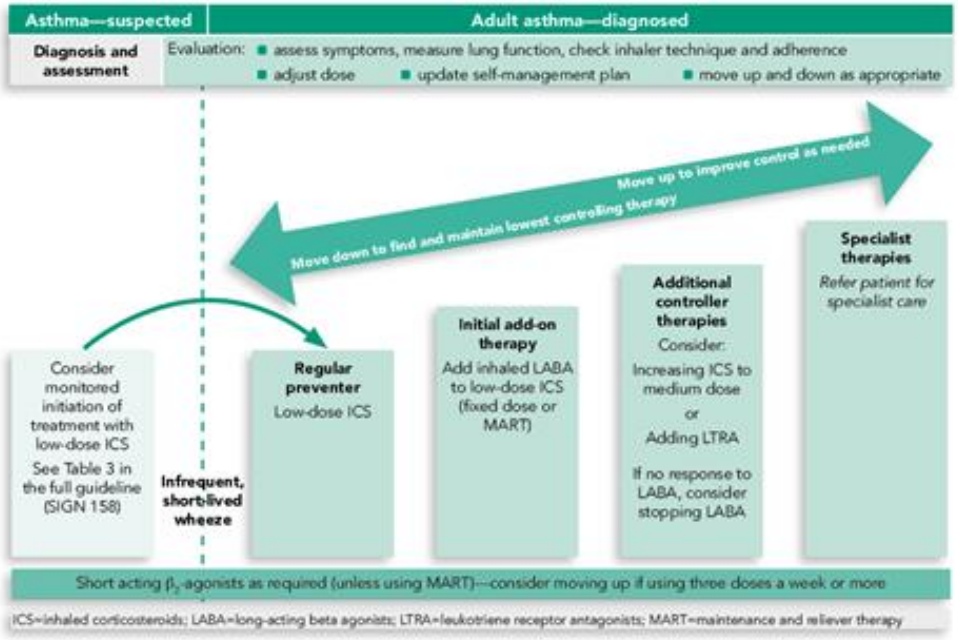


Figure 1.1.2b Summary of Asthma Management in Adults: Current 2019 step up, step down approach to asthma in order to control asthma symptoms and improve lung function as well as maintaining the lowest controlling therapy (British guideline on the management of asthma, 2019).

1.1.3 Severe Asthma

Approximately about 5-10% of the asthma population present with severe asthma (Chen et al., 2016). Severe asthma accounts for more than 50% of the costs attributed to the disease and is a significant cause of mortality (Canonica et al., 2020). It accounts for ~65,000 UK hospital admissions each year and ~1400 death a year in the UK (Carlsson and Bayes, 2020). Severe asthma is defined as asthma that is difficult to control despite standard current treatment. Guidelines from GINA define asthma severity based on the level of asthma treatment used to maintain sufficient control, with individuals maintained on step 5 therapy (oral steroids with or without biological therapy), classified as severe asthma patients (See 1.1.2a for GINA 2020 treatment strategy guide). Severe patients will usually have poor lung function, frequent severe exacerbations, and presence of comorbidities (Soenjoyo et al., 2019). A variety of triggers can cause these patients to exacerbate the most common being viral respiratory tract infections (Gern, 2015).

In cases of severe exacerbations, the patient may need urgent action to prevent a fatal outcome, whereas in a moderate asthma exacerbation the patient may need a change in treatment (Pavord et al., 2017). An exacerbation

is identified by an increase in asthma symptoms and a fall in lung function, patients with a less 50% peak expiratory flow (PEF) of baseline or predicted should be referred to hospital. The treatment aims are to rapidly relieve bronchoconstriction and stop inflammation of the airways (Carlsson and Bayes, 2020). Severe asthma patients are usually on treatments with high-dose ICS, a second controller and/or systemic corticosteroids. In the last decade therapy has advanced and there has been a new human monoclonal antibody drugs developed and approved for severe asthma patients that target the Type 2 pathway. These drugs have been included in Step 5 in the latest GINA recommendations along with the other medications (GINA 2020). However, there is not a universal response to the new therapies. Therefore, a couple of key questions that is yet to be answered is which patients will benefit the most from these new drugs and identifying biomarkers to help clinicians decide which drug will be suitable for which patient. These monoclonal antibody drugs will be discussed further in section 1.5.

1.2 Airway Inflammation in Asthma

Inflammation is the body's immune response to a stimulus such as a virus or allergen and acts by trying to remove the stimuli and initiate the healing process. Inflammation is an important defence mechanism that is vital to health. Lung inflammatory diseases are involved with complex interactions with structural and immune cells. Excessive acute inflammation following lung injury can impair gas exchange. Chronic inflammation and unresolved lung injury is frequently seen in many respiratory diseases such as asthma, chronic obstructive pulmonary disorder (COPD) and cystic fibrosis (CF) (Chen et al., 2017). Asthma causes airway hyper responsiveness, smooth muscle contraction, mucus hypersecretion and structural changes in the airways such as thickening of the basement membrane and goblet cell enlargement also defined as airway remodelling. All these changes in the airways eventually leads to limited airflow and even obstruction of the airways (Hough et al., 2020). Allergens, cytokines and infectious agents are the main stimuli that activate the inflammation signalling pathways in epithelial cells in asthma. Additionally, in asthma, mast cells, eosinophils and $CD4^+$ T lymphocytes are the predominant immune cell types involved in the inflammatory process, particularly in type 2 inflammation. These mediators, cell types and stimuli drive the inflammation process in asthma resulting in breathing difficulties and increase of an exacerbation in a asthma patient (Barnig et al., 2018) (Aghasafari et al., 2019).

Asthma endotypes describe the distinct pathophysiological mechanism at cellular and molecular level. The most well-established asthma endotypes are Type 2-high and Type 2-low. Type 2-high asthma also known as eosinophilic asthma, tends to have airway eosinophilia and are associated with predominantly Th2 cytokines (IL-4, IL-5 and IL-13) meaning that patients will have elevated Th2 cytokines in the blood and/or sputum. Type 2-low asthma is essentially non-Type 2 driven asthma will have an absence of airway eosinophilia and will include inflammation from either type 1 (IFN-γ and IL-6) or type 17 (IL-17A) pathways meaning they will have elevated non-Th2 cytokines in the blood/sputum. Type 2-high patients are usually childhood onset atopic asthma whereas Type 2-low patients typically have adult onset of

asthma, females, smokers and non-atopic (Kuruvilla et al., 2019). Even though patients may experience similar clinical symptoms their response to therapeutics are different therefore, personalised medicine as a way forward to target patient endotypes. Type 2-high patients tend to be more responsive to ICS and biological inhibitors of Type 2 inflammation whereas Type 2-low patients do not respond as well to ICS and biological Th2 inhibitors and are more likely to be steroid resistant and have severe neutrophilic asthma (Fahy, 2015) (Pazdrak et al., 2016) (Athari, 2019). Type 2, Type 1 and Type 17 inflammation will be further discussed in detail below.

1.2.1 Type 2 Inflammation

Figure 1.2.1 demonstrates the Type 2 inflammation pathway in the airways. Type 2 immune responses in asthma are initiated by master regulatory cytokines called alarmins which include IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). These alarmins are released by bronchial epithelial cells in response to an environmental stimulus such as a virus i.e. Rhinovirus, allergen or air pollutant. IL-33 and TSLP activate airway dendritic cells (DC) which then induces the differentiation of naïve T cells (Th0) into Th2 cells. IL-25 and IL-33 act together on mast cells, basophils and group 2 innate lymphoid cells (ILC2) that drive a cascade of downstream events to induce Th2 adaptive immune response. ILC family is comprised of five subsets (natural killer cells, lymphoid tissue inducer cells and three helper ILCs. ILC2 cells are important in directing a strong type 2 response and partake in inflammatory responses and tissue repair and are the predominant ILC population in the lung in comparison to other mucosal surfaces (Helfrich et al., 2019). Th2 cells then migrate to the airway epithelium and the sub epithelial mucosa where they over secrete type 2 cytokines IL-4, IL-5 and IL-13, ILC2 also secrete high amounts of these Th2 cytokines. These cytokines then result in pathological changes in the airways such as smooth muscle contraction and airway remodelling. IL-4 is important in the differentiation of naïve T-helper (Th0) cells to Th2 cells, which sequentially Th2 cells facilitate the production of IgE from B-cells . Whereas, IL-5 are involved in the activation and recruitment of eosinophils and mast cells and IL-13 induces goblet cells hyperplasia, mucus hypersecretion and airway hyperresponsiveness (Caminati et al., 2018).

These pathological changes results in an increased risk of an asthma exacerbation, which could lead to hospitalisation and even life-threatening fatality.

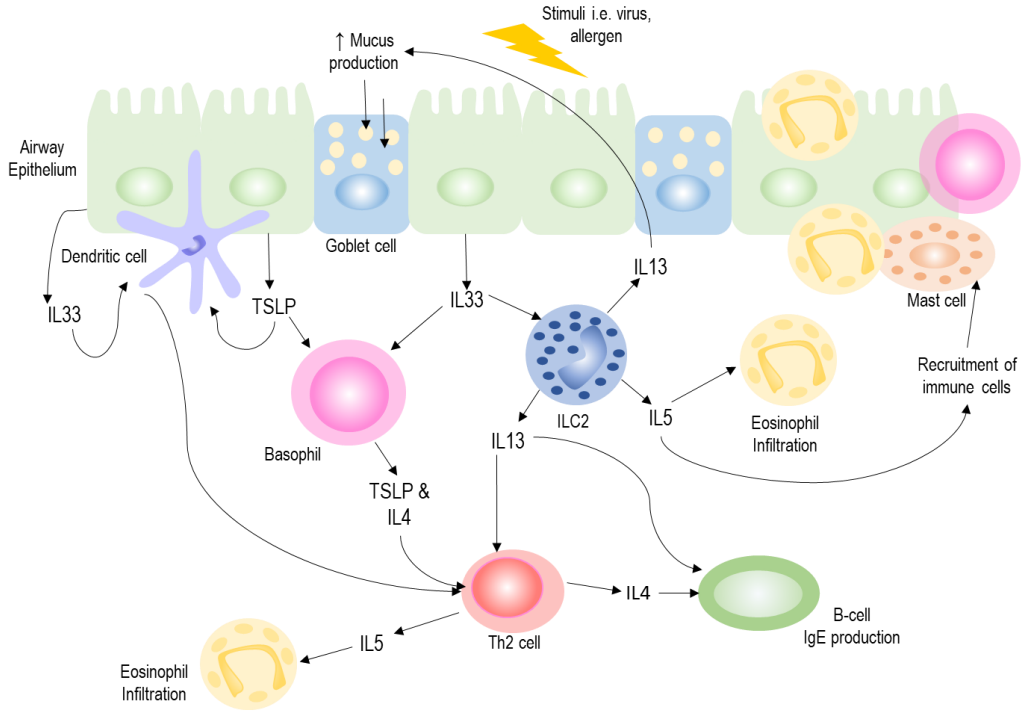


Figure 1.2.1 Type 2 inflammation pathway: an environmental stimulus i.e. a virus causes the release of IL-33 and TSLP from epithelial cells. IL-33 and TSLP activate dendritic cells, basophils and ILC2 cells which induce Type 2 adaptive immune response, this causes Th2 cytokines IL-4, IL-5 and IL-13 to be produced. This response results pathological changes in the airways such as airway remodelling, increased mucus production, smooth muscle contraction and accumulation of inflammatory cells thus increasing the risk of an asthma exacerbation.

1.2.1.1 Alarmins (IL-33 and TSLP)

The innate immune pathway starts at the airway epithelium, where alarmins (IL-33 and TSLP), epithelial derived mediators, initiate the type 2 signalling pathway. IL-33 is a member of the IL-1 family of cytokines that has been featured in type 2 immunity due to the association of genetic variants of both IL-33 and its receptor, ST2, with atopic asthma (Kuruvilla et al., 2019). IL-33-ST2 seems to be the key amplifier of Type 2 asthma when the airways come into contact with a stimulus i.e. allergen (Moffatt et al., 2010). IL-33 has also been associated airway remodelling in asthma with one study showing IL-33 to be correlated with basement membrane thickness with (r^2= 0.831, p= <0.05) and also relative protein levels of collagen increasing after 24 h with IL-33 stimulation (p= <0.05) (Guo et al., 2014). Additionally, serum IL-33 levels have

also been shown to be significantly elevated (p= 0.0001) in asthma patients (322.6 pg/mL; n= 89) compared to healthy controls (139.7 pg/mL; n= 57) (Momen et al., 2017).

TSLP acts on a broad range of immune cell types such as DCs, basophils and mast cells. TSLP is highly produced by epithelial cells lining the barrier surfaces of the lungs and even skin and gut. TSLP is a member of the IL2 cytokine family and shares homology with IL-7, its receptor is composed of the IL-7Rα chain paired with a TSLP-specific receptor component, TSLPR (West et al., 2012). TSLP plays a big role in the development of allergic lung inflammation, one mouse study showed that TSLPR knock out mice did not develop an inflammatory lung response to inhaled antigens (Al-Shami et al., 2005). Even though TSLP is involved in Type 2 inflammation, it is however key in controlling inflammatory responses against Th1 and Th17 inflammation. This demonstrates that TSLPs function in complex but also promotes and controls several inflammation pathways (Becerra-Díaz et al., 2017). Serum TSLP levels have also been shown to be elevated in stable asthma patients (203 pg/mL; n= 41) compared to healthy controls (44 pg/mL; n= 14) (p= 0.0012) (Skrgat et al., 2015). Finally, levels of IL-33 and TLSP in the bronchoalveolar lavage fluid (BALF) have been shown to be elevated in asthma patients (n= 70) verses controls (n= 30) (IL-33: p= 0.0002, TLSP: p= 0.009). They have also shown to be correlated inversely with FEV_1 (IL-33: r= -0.488, p= <0.0001; TSLP: r= -.0565, p= <0.0001) and even when treated without and with ICS or IC/OCS IL-33 and TSLP were still elevated (IL-33: p= 0.0342 and p= 0.0012 & TLSP: p= 0.0428 and p= <0.0001) (Li et al., 2018). This suggests that IL-33 and TSLP are involved in airway inflammation as well as impairing lung function even with intensive corticosteroid treatment. This also highlights their usefulness as an immunological tool in asthma diagnosis as well as desirable targets for asthma treatment since they are master regulators of Th2 inflammation therefore makes them ideal to target to block further downstream effects of type 2 inflammation. Clinical trials have already shown usefulness in an anti-TSLP and anti-IL-33 therapy in improving asthma symptoms which will be further discussed in section 1.5.2.

1.2.1.2 Th2 cytokines (IL-4, IL-5 and IL-13)

Cytokines IL-4, IL-5 and IL-13 are produced by Th2 cells as well as ILC2s via an allergic sensitisation and exposure to inhaled allergens in the airways. The production of Th2-cell-associated cytokines via $CD4^+$ T cells is under genetic control and epigenetic control (Lambrecht et al., 2019). IL-4 and IL-13 were the first Th2 related interleukins discovered in the 1980s and have been associated with atopy and allergic diseases. IL-4 is a major cytokine that drives IgE synthesis by B cells. IL-4 is mainly produced by T follicular helper cells (Tfh) in the lymph nodes. Tfh cells control IgE synthesis via secretion of IL-4 to allergen-specific B cells. IL-4 is also secreted and activates mast cells, basophils and eosinophils. IL-13 is made by canonical Th2 cells in the lung effector sites. IL-13 is known to induce goblet cell differentiation, mucus productions, bronchial hyperresponsiveness, IgE synthesis and recruit eosinophils and basophils to the airways. IL-4 and IL-13 are closely linked as they both activate the α-subunit of the IL-4 receptor (IL-4Rα) and initiates downstream phosphorylation of the transcriptional factor signal transducer and activator of transcription 6 (STAT6). STAT6 activates the Th2 lineage in Th0 cells as well as inducing the expression of GATA3 and involved in mucus production as STAT6 and GATA3 both activate the production of the Th2 cytokine i.e. IL-13 which is involved with stimulation of mucus production in goblet cells (Marone et al., 2019). The GATA3 is involved with the maintenance and function of mature ILC2s and is highly expressed in Th cells, it is also the master transcription factor for controlling Th2 cell cytokine production (Bagnasco et al., 2016) (Lambrecht et al., 2019).

IL-5 is an important biological factor for eosinophil differentiation, growth, activation, survival and induction for airway eosinophilia which is a key feature of asthma (Pelaia et al., 2019). Suggesting, IL-5 plays a crucial role in the pathogenesis of eosinophilic asthma. IL-5 is also more responsive to corticosteroids as it has the potential to induce eosinophilic apoptosis (Zhang et al., 2000). IL-5 is produced by ILC2 and Th2 cells, commonly found in the blood and BALF of asthma patients. In addition to these cells eosinophils, natural killer (NK) cells and mast cells also produce IL-5. The release of IL-5 from ILC2 cells is dependent on GATA3 activation induced by epithelial innate

cytokines (IL-33 and TSLP) (Hoyler et al., 2012). The biological effects of IL-5 start by IL-5 binding to the α subunit of IL-5 receptor (IL-5Rα) inducing its interaction with βc subunits and which then causes the activation of several complex intracellular signalling network. The activation of these signalling networks leads to eosinophil maturation and activation (Pelaia et al., 2019) (Nagase et al., 2020).

Many studies have also shown how these Th2 cytokines levels in the blood and airways are elevated compared to healthy non-asthma patients. Th2 cytokines levels in the BALF of asthma patients have been shown to be significantly elevated compared to healthy controls (IL-4: p= 0.0007, IL-5: p= 0.013 and IL-13: p= 0.0003), regardless of whether ICS or IC/OCS therapy was administered (IL-4: p= 0.0188 and p= 0.0389; IL-5: p= 0.0034 and p= 0.0132 & IL-13: p= 0.0196 for ICS only) (Li et al., 2018). Other studies have also shown an increase in Th2 cytokines in the sputum and blood across asthma severity. For example, IL-13 was elevated in the sputum of severe asthma patients (GINA 5, n= 10) compared to controls (n= 4) p= 0.027 (Saha et al., 2008). Also, levels of IL-4 in the peripheral blood of children with moderate-severe asthma were elevated compared to controls (179.59 pg/mL, n= 1158 and 162.53 pg/mL, n= 1075; respectively, p= <0.001) (Cui et al., 2017). Overall, these Th2 cytokines have shown to play a crucial role in airway inflammation and also contribute to the many hallmarks of asthma such as increasing mucus production, IgE synthesis, bronchial remodelling and airway hyperresponsiveness. All of these changes drive the activation and remodelling of the airway epithelium and subepithelial tissue, increasing the risk of exacerbation. There have been more recent developments in targeted therapies against these Th2 cytokines that have demonstrated efficacy in managing asthma in severe patients.

1.2.2 Th17 Inflammation

Th17 driven inflammation is known as one of the Th2-low endotypes in asthma, with cytokine IL-17 being the key role in Th17 response when released by T-helper 17 cells and causes inflammation (Becerra-Díaz et al., 2017). IL-17A and IL17F are the two main cytokines involved in Th17 response, the pathway is usually involved as an antimicrobial defence

mechanism against extracellular pathogens such as bacterial or fungal infections. When a pathogen is introduced, the uptake and processing of the antigens from the pathogen caused naïve Th0 cells in the lymph nodes to differentiate into Th17 cells. Multiple factors can influence Th17 differentiation, with one study showing how ongoing TGF-β and IL-6 signalling via STAT3 induces Th17 development and is also suggested to be responsible for Th17 maintenance (Harbour et al., 2020). Th17 cells then migrate to the subepithelial layer, where on pathogen recognition they release IL-17 cytokines such as IL-17A and IL-17F, which trigger antimicrobial responses in cells nearby. Some of these antimicrobial responses include epithelial cells releasing CXCL8 (IL-8) which promotes neutrophil production and an antimicrobial peptide i.e. β-defensins, S100 proteins and Lipocalin-2 that disrupts the stability of the microbial membrane (Hynes and Hinks, 2020).

Individuals with Th17 inflammation tend to be more resistant to steroid treatments and have severe neutrophilic asthma. Increased levels of IL-17A have been found in the bronchial walls of severe asthma patients and have been associated with neutrophilic infiltration and steroid resistance. IL-17A has been known to increase smooth muscle cell proliferation and drive collagen deposition. IL-17 activity results in neutrophilic inflammation and IL-8 drives neutrophil recruitment. Clinical characteristics that have been associated with Th2-low endotypes are obesity, smoking and late age of onset of asthma (Kuruvilla et al., 2019). Plasma IL-17A levels have also shown been shown to be elevated in severe asthma patients compared to controls (4.32 pg/mL, n= 373 and 2.27 pg/mL, n= 881; respectively, p= <0.0001) (Zhou et al., 2017). Furthermore, another study showed IL-17 serum levels to be increased in severe asthma patients (Mild: 14.21 pg/mL, moderate: 12.22 pg/mL and severe: 24.72 pg/mL; p-value not stated). The same study also stated that IL-17 values of > 20 pg/mL were encountered in 31% of severe asthma patients compared to 13% in the mild group and 8% in the moderate group (p= <0.001) (Agache et al., 2010). These studies suggest that levels of IL-17 may play a role in severe asthma compared to mild-moderate forms of the disease.

In addition, IL-17 has also been demonstrated to regulate Th2 immune responses; it has been shown that there has been a mix of Th2/Th17 T cell

population in some asthma patients *in vivo*. One study demonstrated that Th17 cells upregulated Th2 cell mediated eosinophilic airway inflammation as well as inducing Th17 cells mediated neutrophilic airway inflammation (Wakashin et al., 2008). There has also been much evidence in an overlap between Th2 and Th17 inflammation responses in some asthma patients. One study demonstrated that some asthma patients had a combined increased sputum eosinophils and neutrophils suggesting both Th2 and Th17 interactions (Hastie et al., 2010).

Overall, it remains unclear whether there is a direct association with IL-17 driving the disease pathology of asthma. Efforts have been made to target the IL-17 pathway in asthma by biologics being created to target IL-17 (see section 1.5.2 for asthma biologics). With around half of patients having Type 2-low asthma and being less responsive to therapies, its important new asthma drugs are being researched and developed to target these asthma patients and improve understanding of Th17 inflammation in asthma.

1.2.3 Th1 Inflammation

Th1 inflammation is a Th2-low endotype in asthma with interferon-ɣ (IFN-ɣ) being the hallmark cytokine in the pathway that is produced by Th1 cells (Lambrecht et al., 2019). Like patients with Th17 inflammation, Th1 inflammation is usually found in more severe asthma patients that are unresponsive to steroid treatments and also associated with bronchial hyperresponsiveness. A study has shown that there were higher IFN-ɣ mRNA expression in those with moderate to severe asthma than mild asthma ($p = <0.01$) as well higher non-allergic asthma than allergic asthma ($p = 0.04$) (Truyen et al., 2006). As well as IFN-ɣ Th1 cells also secret IL2 and lymphotoxin-α, these cytokines bring out the defence against intracellular pathogens such as bacteria and viruses. Both Th1 and Th17 cells are characterised by their expression of the transcription factor T-bet and by the production of IL-2, IFN-γ, and lymphotoxin-α (Annunziato et al., 2015).

It is thought that Th1 mediated immunity is initiated in asthma patients in response to a viral infection. When an intracellular pathogen is introduced, the Th0 cells become activated through antigen exposure from the pathogen

which afterwards caused the Th0 cells to differentiate into either a Th1 or Th2 phenotype. This differentiation of either Th1 or Th2 can depend on multiple stimulatory factors for example, the amount of antigen presented and the presence of local cytokines particularly IL-4 as it is key to Th2 cell differentiation (Robinson et al., 2017) (Annunziato et al., 2015). AdditionallyIL-12 produced by DCs has been shown to be responsible for Th1 cell differentiation in response to a pathogen that interacts with pathogen recognition receptors on DCs. There has also been additional cytokines noted to part of Th1 cell activity including IL-23 which is suggested to have activities on memory T-cells and IL-27 which is thought to be involved in early Th1 initiation (Brombacher et al., 2003).

Th1 and Th2 cells often act together in an immune response but after prolong immunisation, the response can become predominately Th1 or Th2 with Th1 cells supporting cell mediated responses and Th2 cells supporting allergic responses (Chaplin, 2010). It has also been shown that IFN-γ can supress allergic airway inflammation in animal models and that it can also override the production of IgE and eosinophilia (Durrant and Metzger, 2010). One study showed that IFN-γ inhibited Th2 induced effects that promotes pathological responses in asthma in a mouse model such as inhibiting mucus production and eosinophilia (Mitchell et al., 2011). IFN-γ has been shown to be elevated in exhaled breath condensate of asthma patients (2654 fg/mL) compared to the control patients (1872 fg/mL) (Naumov et al., 2019) however, another study showed IFN-γ levels in the BALF were not elevated or reduced in asthma versus control patients even when on ICS or ICS/OCS medication (Li et al., 2018). Moreover, IFN-γ sputum mRNA expression has been shown to be significantly elevated in patients with asthma exacerbations with confirmed viral infection compared to those patients that did not exacerbate (2.47, 1.73 gene expression log transformed respectively; p= 0.002) (Van Eerdewegh et al., 2002). This suggests that viral infections induce the expression of IFN-γ which may bring on asthma exacerbations.

Overall, the role of IFN-γ in asthma remains controversial, with studies finding not only that IFN-γ inhibits Th2 responses, but that high expression of IFN-γ is found in severe asthma patients who do not respond well to current therapies.

Further understanding the mechanisms and pathways of Th1 cell immunity and IFN-ɣ in asthma may provide targets for new asthma therapies.

1.2.4 Cytokine profiling used to characterise asthma subgroups

Due to the heterogonous nature of asthma, a few studies have been able to characterise its inflammatory profile using various analysis methods such as using mRNA expression. A study of 205 asthma patients were defined as having Th2 high or Th2 low based on the sputum mRNA expression levels of particular cytokines (i.e., IL-4, IL-5, IL-13, IL-17A etc.). Their data showed that 52.8% of the patients had a classic Th2-high sputum cytokine profile however, when unsupervised cluster analysis was applied, it did not support a priori classification of Type 2 versus non-type 2 molecular phenotypes (Seys et al., 2017).

One other study used principal component (PC) analysis to identify cytokine profiles and asthma outcomes in the serum of adult asthma patients. Two clusters were identified a “pro-inflammatory cytokine profile” which consisted of cytokines involved with eosinophilic asthma (IL-5, IL-7 and IL-13) or neutrophilic asthma (IL-8 and TNF-α), and an “anti-inflammatory cytokine profile” (IL-1Ra and IL-10). The anti-inflammatory cytokine profile was associated with lower risk of worsening of asthma control (adjusted OR: 0.67 vs 1.23), exacerbations (adjusted OR: 0.68 vs 1.09) and low IgE levels (128 vs 166 IU/mL; p= 0.04) in adult asthma patients compared to the pro-inflammatory cytokine profile (Akiki et al., 2017). These studies suggest that cytokine profiling may help identify the type of inflammation driving a patient’s asthma, those with subsequent risk of exacerbations or worsening of asthma and help inform the decision on a suitable biological treatment for each patient. However, there is still a need for more research to further define a patient cytokines profiles using sputum or serum as this may help identify subgroups of asthma patients which could benefit from the new biological treatment. Part of this project will look into cytokine profiling in the sera of asthma patients to see whether patients display Type 2 and/or non-Type 2 inflammation and which of these may be more amenable to the new biological therapies.

1.3 Airway changes in asthma

The lower respiratory airways is made up of the trachea, two bronchi (larger airways that supply air to each lung), bronchioles (smaller airways) and alveolar ducts where gas exchange occurs. The trachea consists of ciliated pseudostratified columnar cells supported with C shaped rings of hyaline cartilage. The major cell types in the large and small airways are the ciliated, undifferentiated columnar and basal cells. In the larger airways goblet cells (secretory cells) are also present but differentiate to club cells (another secretory cell type) in the small airways. The alveoli are the final portion of the airway with a single cell layer of pneumocytes in close proximity to the capillaries to allow gas exchange and also contain surfactant to reduce surface tension at the pulmonary air-liquid interface (Crystal et al., 2008) (van den Bosch et al., 2021).

Asthma can cause structural changes in the airways which is termed airway remodelling. The main changes include epithelial shedding, thickening of the basement membrane, goblet cell hyperplasia, smooth muscle cell hyperplasia and hypertrophy, angiogenesis and sub epithelial fibrosis (Figure 1.3) (Bonser and Erle, 2017) (Crystal et al., 2008). These changes are key processes in airway remodelling, and the consequences of these structural changes are alteration in barrier function, increase mucus secretion, narrowing of the airways and alterations in tissue and mechanical properties which in turn has an effect on lung function (Guida and Riccio, 2019). These structural changes are thought to be caused by infectious agents, allergens, inhaled particulates and the host's response to these stimuli that cause ongoing tissue injury (Erle and Sheppard, 2014). It is thought that epithelial cell injury from a stimulus may induce necrotic cell death, which when followed by stimulation of transforming growth factor-β (TGF-β) can initiate tissue regeneration. This constant cell damage and stimulation of growth factors can result in abnormal tissue repair and so causes airway remodelling such as thickening of the airway smooth muscle (Hough et al., 2020) (Stewart et al., 2012) (Kicic et al., 2006). Chronic airway inflammation is also thought to be a major driving force in the initiation of the remodelling process. This theory is only supported by the fact steroid treatment in asthma patients not only reduces airway inflammation

but has also shown to have beneficial effects on airway remodelling such as increasing vascular permeability, angiogenesis and restoring normal beta-2-adrenergic responsiveness (Chetta and Olivieri, 2012).

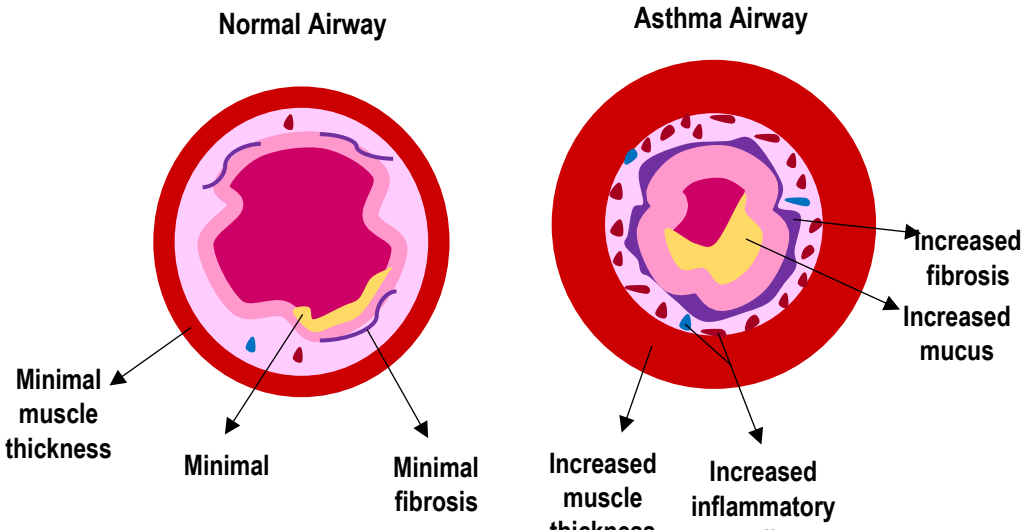


Figure 1.3 Normal verses a chronic asthma airway: Airway structural changes are seen in the chronic asthma airway such as increased mucus production, increased muscle thickness, narrower lumen and increased numbers of inflammatory cells in comparison to the mild asthma airway.

In an asthmatic airway, there is also an increase in extracellular matrix (ECM) proteins in the basement membrane region, lamina propria and submucosa, which contributes to airway thickening and obstructing airflow. The ECM is a group of proteins and glycoproteins such as collagen, elastin, fibronectin and proteoglycans. There is a high abundancy of collagen fibres found in the lung. Collagen has excellent tensile strength but low elasticity and contributes to the overall structure of the lung and so overproduction of collagen causes lung stiffness. Additionally, fibroblasts are a major creator of EMC (Hough et al., 2020). It has also been shown that airway epithelial cells in asthma patients stimulate naïve lung fibroblasts to produce a higher fold of collagen (COL1A1: 2.2-fold & COL3A1: 10.8-fold) and fibronectin than in on-asthma controls (Reeves et al., 2014).

Overall, airway remodelling causes change in composition, thickness, mass or volume of structural components observed in the airway wall of asthma patients compared to healthy individuals. These are distinct features of airway remodelling in asthma which in turn causes airflow limitation, increases the

severity of asthma and increases the risk of an exacerbation. There has been much investigation on how airway remodelling occurs with some suggestions including an individual's response to a stimuli or chronic inflammation. Over the years efforts have been made to try target airway remodelling with the hope to develop more effective treatments for patients who do not respond to steroid treatment.

1.3.1 Airway epithelial cell types

The upper airways are continuously lined with epithelial cells, forming a highly regulated physical barrier that lines the airway lumen and creating an interface between the host and environment which acts as the first line of defence against inhaled foreign particles such as allergic antigens. The airway epithelial cells have pattern recognition receptors that detect environmental stimuli which then triggers the epithelial cells to release soluble cytokines, chemokines, growth factors etc. (Ziegler and Artis, 2010). The major cell airway types are undifferentiated columnar, basal, ciliated and secretory cells. Basal cells are airway epithelial precursor cells, and the basal layer is located below the ciliated columnar epithelium. Basal cells have high epidermal growth factor receptor (EGFR) expression levels, which then decrease when basal cells differentiate into ciliated and goblet cells (Gon and Hashimoto, 2018). Ciliated cells facilitate the transport of inhaled foreign particles from getting deep into the lungs (mucociliary escalator). Goblet cells support this function by the ciliated cells by producing mucus as part of a biochemical barrier system. These two specialised cells make up and maintain the mucociliary apparatus. When a particle is inhaled it mixes with the mucus which then gets transported by the cilia cells via cilia beating, this process is called mucociliary clearance which is essential for keeping the airways clear (Ma et al., 2018) (Potaczek et al., 2020).

The airway epithelial cells play a crucial role in innate immune functions in the lungs (Gon and Hashimoto, 2018). In asthma patients the airway epithelium has some abnormalities as the bronchi appears injured. The airway epithelial cells appear hyperplastic, particularly the goblet cells and they also lose their cilia. As a result of all these epithelial changes, this causes a breakdown in the epithelial cell tight junction integrity and upregulation of mucus production

(Guida and Riccio, 2019). Environmental factors are thought to disrupt the tight junction barrier via protease activation, as this accelerates epithelial permeability which makes it easier for allergens to infiltrate the airway submucosa. It has been suggested that Th2 cytokines alter tight junction functions by disrupting the airway epithelial barrier (Gon and Hashimoto, 2018).

Goblet cell hyperplasia is a feature of asthma. Asthma patients also tend to secrete more mucin particularly mucin 5AC protein (MUC5AC) in comparison to patients without asthma (Ma et al., 2018). One study demonstrated that patients with mild asthma demonstrated goblet cell hyperplasia and increased stored mucin in the airway epithelium whereas patients with moderate asthma has increased levels of stored mucin but higher levels of secreted mucin. They also suggested that acute degranulation of hyperplastic goblet cells may be a mechanism for asthma exacerbation in mild and moderate asthma (OrdoÑEz et al., 2001). As mentioned, cilia cells are important in mucociliary clearance and in asthma there is a disruption of ciliated cell functions and murine asthma models have reported to show a reduction in the number of ciliated cells (Reader et al., 2003). IL-13 has also been shown to alter epithelial cell morphology and inhibit ciliated cell differentiation in vitro (Laoukili et al., 2001). Additionally in asthma patients where the mucociliary apparatus is modified, there are is an increase in mucin gene expression due to an increase number of goblet cells and a reduction in the number of ciliated cells (Curran and Cohn, 2010). It has also been reported that in severe asthma patients there is a decrease in ciliary beat frequency (Tilley et al., 2015). Altogether the airway epithelium is composed of a variety of highly differentiated and functionally specialised cells. In healthy patients the epithelial cells along with the mesenchymal cells and the innate and adaptive immune cells maintains the local integrity via various regulatory mechanisms such as the release of a variety of cytokines and chemokines, mucociliary clearance etc. Pathological influences may disrupt this system causing changes in these mechanisms resulting in the epithelial cells to alter during differentiation.

1.4 Asthma Heterogeneity

Asthma was regarded as a atopic disease involving allergen exposure, immunoglobulin E (IgE)-mediated sensitisation with Th2 cell inflammation and IL-5 mediated eosinophil airway inflammation, which resulted in reversible airflow obstruction and clinical symptoms in some patients (Pembrey et al., 2018). However, this has been challenged and disproved as sputum studies have demonstrated that less than 50% of asthma cases are caused by eosinophilic airway inflammation (Brooks et al., 2016). It has been well established since the 1950s that there are many phenotypes of asthma. Different asthma phenotypes have been proposed as a way of identifying different asthma groups. Phenotypes are features shared by most asthma patients, which include demographic features, clinical features, physiological and immunological data. The benefit of categorising patients into specific asthma phenotypes is that it can allow personalised treatment which has become important as there are multiple biological therapies being offered today that target different inflammation pathways (Kaur and Chupp, 2019) (Schoettler and Strek, 2020) (Hamilton and Lehman, 2019). Before phenotypes, categorisation was based on multiple of clinical factors such as age of onset, asthma severity, atopic/non-atopic, induced by viral infection, steroid reactivity, induced sputum etc. The earliest attempts to define asthma phenotypes were very simple and were classified based on age of onset or atopic/non-atopic. However, studies have now used a statistical approach such as cluster analysis which has introduced more representative categories (Hirose and Horiguchi, 2017) (Pembrey et al., 2018) (Kaur and Chupp, 2019).

Cluster analysis offers a multidimensional approach for identifying clinical phenotypes of asthma by grouping similar observations into a number of clusters based on the observed values for several variables for each individual asthma patient (Haldar et al., 2008). The first asthma study to perform a cluster analysis identified two common clusters that were common in two asthma populations (Haldar et al., 2008). They used three separate asthma populations (n= 184, n= 187 and n= 68; respectively) all with physician diagnosed asthma from either primary or secondary care settings. The results identified two clusters that were common to first and second asthma

populations (n= 184 and n= 187) which were early-onset atopic (primary care n= 61 and secondary care n= 74) and obese non-eosinophilic (primary care n= 27 and secondary care n= 23). Figure 1.4a displays the summary of phenotypes identified in both primary and secondary care asthma populations (Haldar et al., 2008). Another study called The Severe Asthma Research Program (SARP) conducted a cluster analysis using lung function tests (baseline FEV_1 and FEV_1 post bronchodilator) and age of onset and identified 5 clusters (see Figure 1.4b) (Moore et al., 2010).

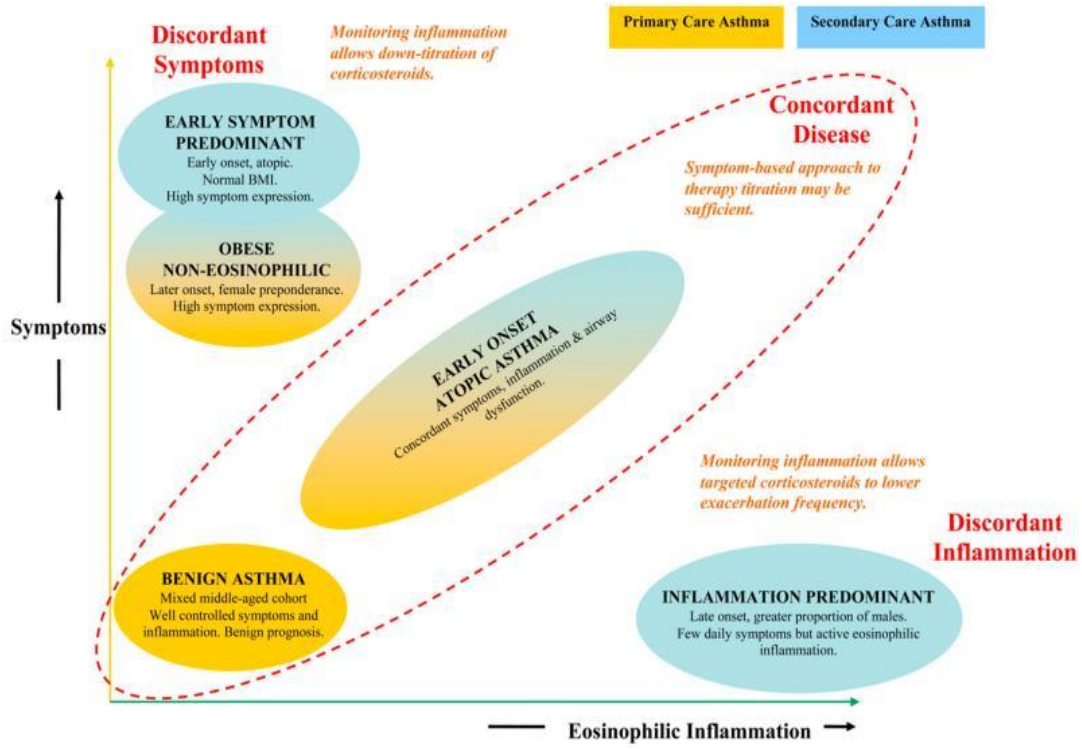


Figure 1.4a Cluster of clinical phenotypes of asthma identified from the Haldar et al, 2008 study: the clusters are plotted according to their relative expression of symptoms and eosinophilic inflammation. 5 clinical phenotypes were identified using both primary and secondary care populations. **Cluster 1 early age of onset atopic asthma:** greater airway dysfunction and consistent symptoms and inflammation, **cluster 2 obese non-eosinophilic:** late onset, high symptoms and a female predominance, **cluster 3 early symptom predominant:** with little to none inflammation with early age of onset and high symptom expression, **cluster 4 inflammation predominant:** with little to no symptoms, late age of onset and typically more males and **cluster 5 benign asthma:** well controlled symptoms with little inflammation (Haldar et al., 2008).

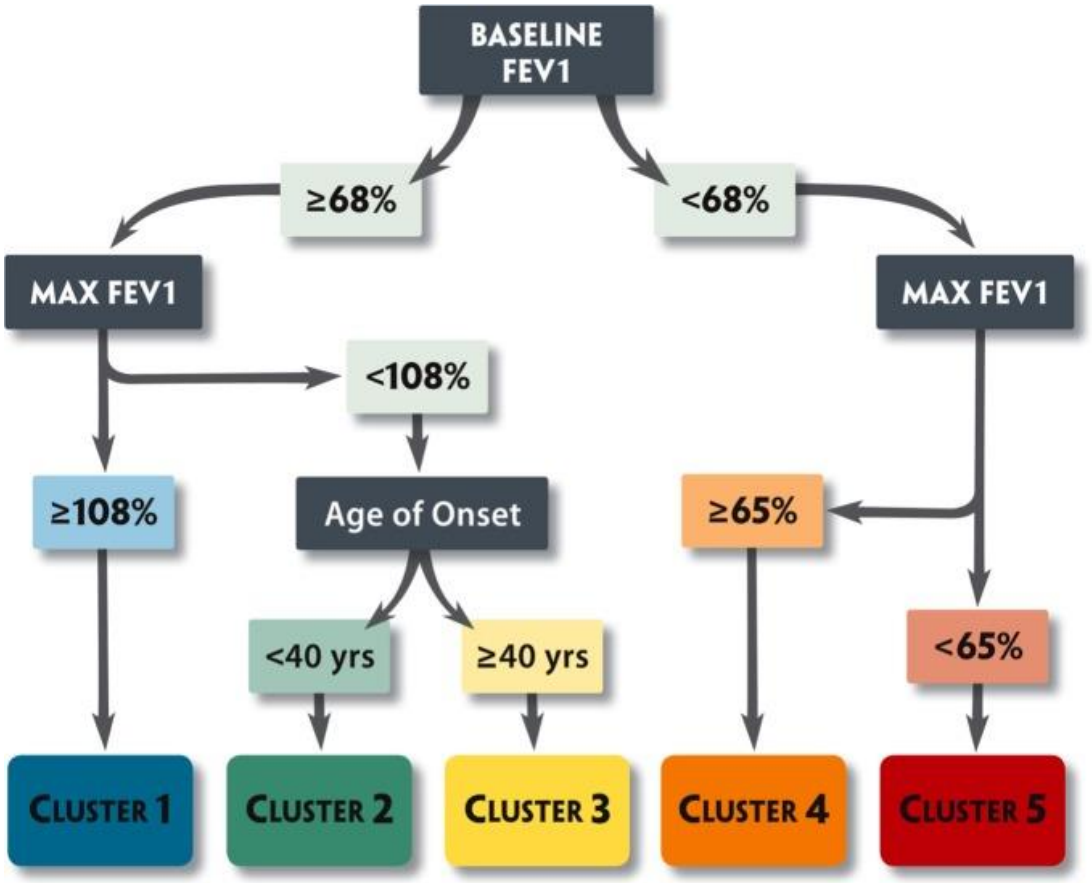


Figure 1.4b five clusters identified from SARP cluster analysis study using pre and post FEV_1 and age of onset: cluster 1: childhood onset mild atopic asthma, **cluster 2:** early age of onset mild to moderate atopic asthma, **cluster 3:** adult onset, non-atopic asthma with predominately females and high body mass index (BMI), **cluster 4:** childhood onset with low FEV_1 and severe atopic asthma and **cluster 5:** adult onset of asthma with severe atopic and fixed airflow (Moore et al., 2010).

These two cluster analysis studies found very similar asthma clinical phenotype cluster groups to each other as they both identified early age of onset and atopic asthma and obese non-atopic asthma. Moreover, asthma phenotypes sometimes overlap which makes it difficult to classify patients into a specific phenotype group (Desai and Oppenheimer, 2016). Discussed below are the most common and well described asthma phenotype groups to date.

1.4.1 Atopic asthma

Atopic or allergic asthma is probably the most common overall phenotypes of asthma which occurs in roughly 50% of adult asthma patients (Thomsen, 2015a) (Schreiber and Mailaender, 2017). Atopic and allergic asthma are often used interchangeably but there is a distinct difference. Atopy is defined as becoming sensitized and producing immunoglobulin E (IgE) antibodies in response to an exposure of a common allergen (Comberiati et al., 2017).

Allergy is defined as hypersensitivity reaction by an immunological mechanism via an allergen (IgE mediated or non-IgE mediated) (Tanno et al., 2016). Atopic asthma is typically presented in early childhood and is associated with a family history of asthma or/and atopy, allergic rhinitis, atopic dermatitis and early exposure to allergens (Pakkasela et al., 2020). Allergic asthma is thought to be predominately mediated by the Type 2 inflammatory pathway and is also associated and diagnosed with high serum IgE levels, airway eosinophilia and a positive skin prick test (Hamilton and Lehman, 2019). IgE plays a key role in the development of allergic asthma and a marker of atopy (Thomson and Chaudhuri, 2012). In atopic patients' exposure to an allergen initiates a series of complex events causing the production of allergen specific IgE. IgE then becomes attached to fragment crystallisable (Fc) receptors of inflammatory cells such as mast cells and basophils. This results in degranulation of these inflammatory cells which cause them to release pro-inflammatory mediators such as histamine, prostaglandins, chemokines and cytokines. This causes the airway smooth muscles to contract which in turn narrows and constricts the airways (Athari, 2019). Patients with atopic asthma have elevated IgE levels which increases airway inflammation and bronchial hyperresponsiveness due to ongoing chronic allergic activation of IgE thus increase asthma exacerbation in patients (Normansell et al., 2014). This has made IgE an attractive target for therapy as IgE has an important role in the pathogenesis in asthma exacerbations with Omalizumab being the current anti-IgE drug for severe atopic asthma patients which will be further discussed in section 1.5.2.

1.4.2 Neutrophilic asthma

Neutrophilic asthma is not as well defined as the other asthma phenotypes but occurs in roughly 30% of asthma patients (Gibson and Foster, 2019). These patients will have an absence of markers related to Th2 high endotypes such as eosinophilia but instead is generally characterised by neutrophilic inflammation especially in patients with severe asthma. Neutrophilic asthma is seen mostly in severe asthma patients who tend to be steroid-insensitive. It has been considered that the frequent use of high-dose steroids could be a contributor to neutrophilic inflammation, as steroids have shown to prevent

apoptosis in neutrophils (Ronchetti et al., 2018) (Kuruvilla et al., 2019). The Th17 inflammatory pathway has been thought to be one of the main drivers of neutrophilic asthma as these patients have been shown to have over expression of Th17-derived cytokines such as IL-17A. The exact cause of neutrophilic asthma is not entirely clear but other factors including allergens, viral infections, and environmental pollutions have been thought to initiate Th17 airway inflammation (Th17 described in chapter 1.6.2) (Lambrecht et al., 2019) (Hamilton and Lehman, 2019).

1.4.3 Aspirin-exacerbated respiratory disease

Aspirin-exacerbated respiratory disease (AERD) occurs in a subset of late onset of asthma patients typically with a Th2-high endotype. Roughly, around 7% of asthma patients present with AERD (Rajan et al., 2015). AERD is caused by a non-allergic hypersensitivity that can provoke severe respiratory symptoms including allergic rhinitis and asthma exacerbations from nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin. Aspirin inhibits cyclooxygenase (COX) which is a synthetic enzyme of prostaglandins. This hypersensitivity reaction in AERD is usually from NSAIDs that have a stronger COX-1 inhibitory activity. However, certain COX-2 inhibitors such as celecoxib can be used in patients with AERD (Taniguchi et al., 2019) (Kuruvilla et al., 2019). Once COX-2 is covalently modified by aspirin promotes synthesis of AT-RvD1 an aspirin triggered resolvin which is a specialized pro-resolving lipid mediator that has shown to decrease smooth muscle cells migration (Mottola et al., 2017). Leukotriene receptor antagonists (LTRAs) have been shown to be an effective treatment with patients with AERD due to the overproduction of cysteinyl leukotrienes (CysLT1) and so targeting the leukotriene pathway has shown to improve asthma symptoms and lung function (Rodríguez-Jiménez et al., 2018). Additionally, since AERD patients do not have clinical atopy, the biological monoclonal antibody Omalizumab has been shown to decrease urinary CysLT1 and prostaglandin D_2 metabolites, both are which involved in acute aspirin induced reactions (Laidlaw, 2019), suggesting Omalizumab may be beneficial for patients with AERD.

1.4.4 Obesity

Obesity is a major risk factor of asthma, there is likely to be a few phenotypes within obese asthma however, the most common is adult onset of the disease in mostly females (Haldar et al., 2008). These patients tend to have more severe asthma, lack of atopy, poor asthma control and do not respond well to medication such as ICS and LABAs (Tashiro and Shore, 2019). Around 48% of adult patients with severe asthma are obese in the UK (Gibeon et al., 2013). Cluster analysis have also shown that this group of patients have poor lung function compared to other type of asthma patients (Moore et al., 2010). Systemic inflammation has been shown to occur in adult obese asthma and is thought to play an important role in these patients, with data showing IL-6 levels being significantly elevated in obese asthma patients (n= 68) compared to non-obese asthma patients (n= 47) (p= <0.0001) (Scott et al., 2011). It has also been shown there is little airway sputum eosinophil inflammation but there is significant inflammation in adipose tissue in these patients. Studies have shown female asthma patients with a high BMI will usually have increased neutrophils levels in the blood and sputum compared to asthma patients with a low BMI. For example, one study showed neutrophilic asthma was present in a greater proportion of obese than of non-obese asthmatic females (42.9% versus 16.2% respectively; p= 0.017, n= 65) (Scott et al., 2011). Another study showed particularly female patients, who had a BMI over >30 had an increased blood neutrophils compared to those with a BMI of <25 (4.2 10^3/µL, n= 63; 3.4 10^3/ µL, n= 213 respectively; p= 0.0001) (Telenga et al., 2012). Overall, obesity causes significant changes to the lung physiology, increase accumulation of fat in the thoracic can lead to compression of the lung and reduction in lung volume. It has been shown that with weight loss their asthma symptoms such as lung function, asthma control which overall improves their quality of life. Obese asthma patients with neutrophilic airway inflammation has been shown that their asthma improves with weight loss (Peters et al., 2018) (Tashiro and Shore, 2019).

1.4.5 Age of onset

Asthma can be developed at any age however, there are some clinical differences between early and late age onset of asthma. Early onset of asthma

is usually associated with poor symptom control despite even taking high dose ICS and atopy which many cohort studies have shown. Whereas late onset of asthma is normally associated with females, smokers and greater airflow obstruction (Tan et al., 2015). There are many factors that can cause a person to develop late onset asthma, one cause is suggested to be from occupational exposures such as work related stress, around 5-25% of asthma cases are thought to be occupational (Eng et al., 2011) (Rabell-Santacana et al., 2015). Early sensitisation to multiple allergens has been associated with early onset of asthma. Studies have shown that children who have been raised on animal farms are more likely to be protected from developing childhood asthma and atopic sensitisation (Riedler et al., 2001). It is thought that the wide range of the environmental microorganisms could explain the protection, as the microbial exposure was inversely related to the risk of childhood asthma (Ege et al., 2011). More on age of onset is further discussed in chapter 3.1.1

1.4.6 Eosinophilia

Eosinophilia is a key clinical feature of asthma, it plays a role in airway remodelling as well as leading to airflow obstruction and increasing exacerbation risk (Bakakos et al., 2019). High eosinophil count has been associated with severe asthma with the prevalence of eosinophilic asthma in patients suggested to be 32-40% (de Groot et al., 2015) (Hasegawa et al., 2015). It has been known for a while that eosinophilic inflammation is a key feature of poorly controlled severe asthma and frequent exacerbations (Bousquet et al., 1990). More about eosinophilic asthma is discussed further in chapter 3.1.3.

1.5 Asthma Treatments

Once asthma has been diagnosed, it is essential to ensure that each patient receives appropriate treatment. A well-controlled asthma patient will be free from respiratory symptoms day and night i.e. wheezing and coughing, having little or no need for reliever medication, can lead a normal and active lives and continue to have a normal or their best possible lung function (Larsson et al., 2020). There has been a range of tools validated in order to assess asthma control including Asthma Control Questionnaire (ACQ) (Juniper et al., 2006) and Asthma Control Test (ACT) (Nathan et al., 2004), these ask about symptoms, frequency of reliever usage and lung function. Annual asthma reviews have been shown to help improve asthma control and reduce exacerbations. It allows practitioners to check patients' inhaler technique, discuss current maintenance therapy and educate on asthma and treatment. However, even though achieving well-controlled asthma reduces the risk of exacerbations, it does not completely eliminate the risk of an exacerbation even with mild asthma patients (Larsson et al., 2020). Bronchodilators have played a key role in the treatment of asthma for numerous of years and are still currently used to this day to manage asthma. Additionally, there are now new monoclonal antibody drugs that target asthma on a molecular level. The first one being Omalizumab (anti-IgE) approved back in 2003 and to date more have been released and have shown promising results so far (Tanaka, 2015).

1.5.1 Current Asthma Treatments

1.5.1.1 Beta-2 adrenergic receptor agonists

Beta-2 adrenergic receptor agonists have been used to treat asthma since the early 1900s and are the first line of treatment for asthma before introducing ICS. Beta-2 adrenergic receptor agonists work by relaxing airway smooth muscle. There are three main classes of beta-agonists, SABA, LABA and ultra-long-acting beta-2-agonists (ultra-LABA). SABAs have a short half-life and are used as quick relievers whereas LABAs and ultra-LABAs have a longer life of

action and so provide sustained symptomatic relief. SABAs e.g. Salbutamol provide almost instant symptomatic relief in battling bronchoconstriction and acute exacerbations. LABAs e.g. Salmeterol are the preferred add on therapy for patients with uncontrolled asthma despite taking ICS treatment as well (Cazzola et al., 2013)

1.5.1.2 Inhaled corticosteroids

ICS are the foundation of asthma management in adults and GINA guidelines recommend that patients who use their SABAs twice or more a month or awaken at night due to symptoms should be administered ICS (GINA, 2020). Data has shown that ICS decrease hospital admissions by 83% in children with moderate to severe asthma compared with those on the placebo (Sawanyawisuth et al., 2020). Another study in adult asthma patients showed a reduction in hospital admissions by 34% (95% CI: 24-42%) with regular use of ICS (Suissa et al., 2002). ICS work by supressing activated inflammatory genes and increasing transcription of anti-inflammatory genes, therefore reducing airway inflammation. This airway suppression causes ICS to reduce airway hyperresponsiveness and control asthma symptoms (Ye et al., 2017). ICS is the main treatment recommended for the long-term management of asthma that remains uncontrolled on conjunction with short-acting bronchodilators. Treatment with low-dosage ICS is effective in reducing asthma exacerbations. If symptoms persist ICS dosage is increased to a moderate dosage however, adding a LABA to low-dose ICS is thought to be more effective. Only high ICS dosage is recommended if control still cannot be achieved with medium dosage ICS and/or LABA (GINA, 2020). Overall, ICS are an effective treatment in most asthma patients in reducing symptoms and exacerbations.

1.5.1.3 Long-acting muscarinic antagonists

Long-acting muscarinic antagonists (LAMA) are a class of bronchodilators which have a different mechanism of action to LABAs. LAMAs reduce bronchoconstriction in the lungs by binding to muscarinic acetylcholine receptors, which then cause a reduction in smooth muscle contraction and mucus secretion (Buhl et al., 2018). Furthermore, this has been shown to reduce inflammation and asthma-related airway remodelling in asthma animal

models. According to GINA, LAMAs such as Tiotropium are given in step 4 as add on therapy when patients cannot control their asthma with high dose ICS/LABA therapies (Hamelmann, 2018).

1.5.1.4 Oral Corticosteroids

OCS are used in the maintenance of severe asthma and in managing acute exacerbations. Corticosteroids are effective at targeting components of the Type 2 pathway. Eosinophils particularly respond well to OCS as they apoptosis due to down regulation of IL-5. OCS also inhibit transcription at the genomic level of IL-4 and IL-5, which results in inhibiting this section of the Type 2 inflammatory cascade that is responsible for the maturation, chemotaxis and survival of eosinophils. It is thought that OCS decrease mucus secretion by having direct effects on the submucosal glands and reduction in the inflammatory milieu of the airway wall (Ramsahai and Wark, 2018). Long term use of high-dose ICS and OCS can cause adverse effects for some patients such as reduction in bone density, hypertension, cataracts and glaucoma (Ferrara et al., 2019) (Bleecker et al., 2020). Despite taking the conventional asthma medication and additional add on therapies many patients still suffer with their symptoms, particularly severe difficult to control asthma patients. In the past decade there has been development in new biologics that target the Type 2 pathway. A few of these biologics are currently available in clinics and some are in the last phases of clinical trials. Below I will discuss the current and upcoming biological add on therapies that are coming into clinical use.

1.5.2 New and Upcoming Asthma Biologics in Clinic and in Clinical Trials

Over the past decade biological therapies have been developed to target patients with severe uncontrolled asthma despite being on high dose ICS. These biological therapies are composed of specific antibodies that are directed against cytokines and small signalling proteins that are involved in asthma pathogenesis particularly the Type-2 pathway (Figure 1.5.2). Th2 cytokines IL-4, IL-5 and IL-13 and IgE have been major targets for therapeutics in the recent years as they play a role in immune response and allergy. The first biological therapy to enter into clinic was the anti-IgE drug Omalizumab approximately 15 years ago. Built on the success of showing how targeting

specific components of the Type-2 pathway can give therapeutic benefit in asthma, more biologics have been approved with additional ones in late clinical trial phases which have shown promising results so far. Unlike other asthma medication where they are administered orally, biologics are given either as subcutaneous injection or as an intravenous infusion (Busse, 2019) (Johnson et al., 2019) (Pavord et al., 2019) (Nakajima et al., 2020). Below discusses the current biologics available in clinics and a couple of biologics that are in the later stages of clinical trials.

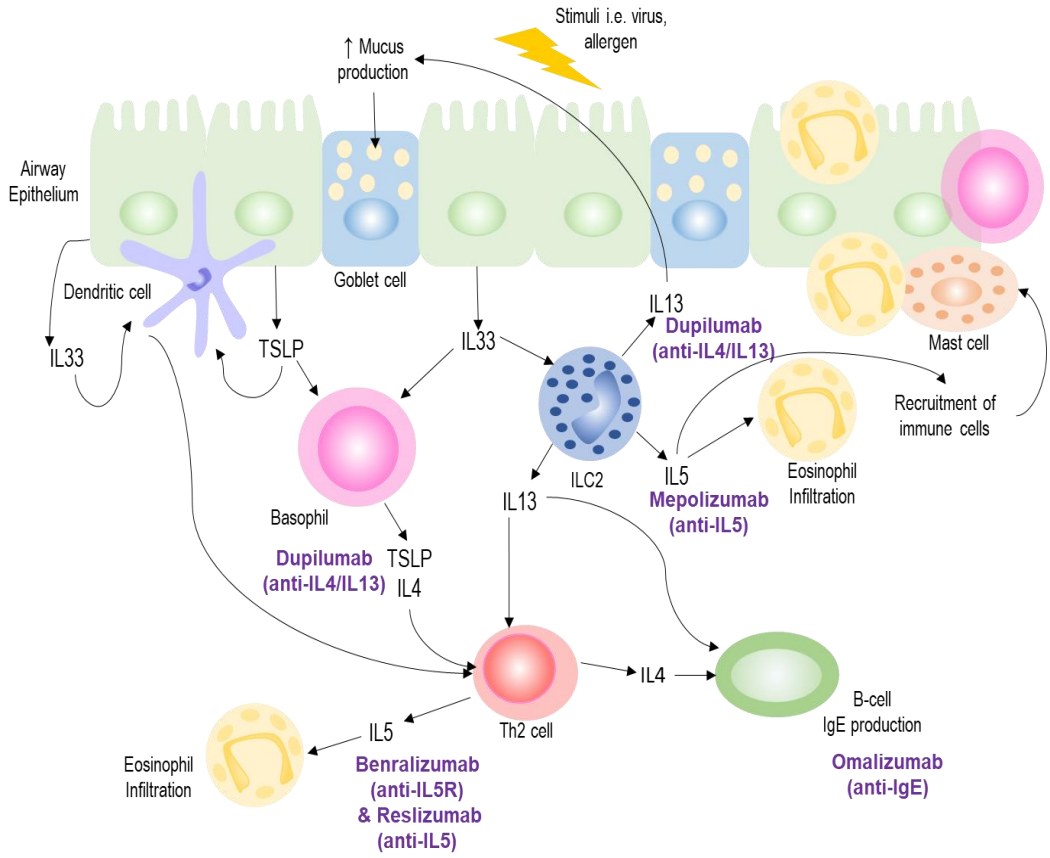


Figure 1.5.2 monoclonal antibody drugs (highlighted in purple) that are currently available in clinic and how they target the type 2 pathway: Dupilumab blocks IL-4/IL-13, Mepolizumab blocks IL-5, Dupilumab blocks anti-IL-4R/IL-13, Benralizumab blocks IL-5R, Reslizumab blocks IL-5 and Omalizumab blocks IgE.

1.5.2.1 Anti-IgE Therapy (Omalizumab)

Omalizumab has been the only biological therapy available for patients with uncontrolled moderate-severe allergic asthma, for these patients this therapy has been very effective for them (Menzella et al., 2017). Omalizumab is an anti-IgE monoclonal antibody treatment, which works by inhibiting free IgE by 96% by binding to a high-affinity IgE receptor (FcεRI) by attaching itself to the

same antigenic epitope on IgE. Omalizumab also gradually downregulates FcεRI expression on mast cells and basophils which makes these cells less sensitive to allergen stimulation thus, reducing eosinophilic influx and activation (MacGlashan et al., 1997) (Doroudchi et al., 2020) (Johnson et al., 2019). This drug has proven to be very effective in asthma patients with uncontrolled severe allergic IgE mediated asthma who are sensitised to at least one aeroallergen and also have IgE levels within the specified range (30-1500 IU/mL). Additional criteria to start Omalizumab for are positive skin prick test, total IgE at 30-1500 IU/mL asthma exacerbations requiring OCS despite daily high-dose ICS and frequent symptoms day or night for patients >6 years and a FEV_1 of <80% got patients >12 years (GINA, 2020) (Nygaard et al., 2017). Approximately more than 50% of people have allergic IgE mediated asthma and therefore could benefit from Omalizumab (Normansell et al., 2014) (Samitas et al., 2015). One study demonstrated that starting Omalizumab in children before starting school in autumn reduced the risk of exacerbations likely driven by viral infections (Teach et al., 2015). Another study noted that patients who took Omalizumab had an improved mean ACT score from 12.4 to 20.5, suggesting that the drug improves asthma symptoms (López Tiro et al., 2015). Finally, one other study showed that FEV_1 improved at 6 months (baseline FEV_1 (L): 1.9±0.7 & 6 months FEV_1 (L) 2.1±0.8) in patients that responded to Omalizumab (n= 164) and remained stable for two years (12 months FEV_1 (L): 2.0±0.8 & 24 months FEV_1 (L): 2.0±0.8), p= <0.0001 (Paganin et al., 2017).

1.5.2.2 Anti-IL-5 Therapy (Mepolizumab, Reslizumab and Benralizumab)

Mepolizumab, Reslizumab and Benralizumab are monoclonal antibodies that binds to IL-5 or IL-5Rα. IL-5 is a key chemokine for eosinophils and Mepolizumab and Reslizumab bind and deplete IL-5, which reduces peripheral eosinophils counts. Alternatively, Benralizumab binds to IL-5Rα, which in turn triggers natural killer cell cytotoxic killing of eosinophils and blocks IL-5 resulting in nearly depleting the body's eosinophils. These drugs target eosinophilic driven asthma and have proven to be effective in asthma patients with eosinophilia particularly in high baseline blood eosinophilia than sputum eosinophilia (Doroudchi et al., 2020). One recent study showed that patients

with severe eosinophilic asthma after 1 year of treatment with Mepolizumab resulted in a significant reduction of annual exacerbation (before treatment: 4.3 ± 2.3 and after 1 year: 1.3 ± 1.8; p= <0.0001) (Kallieri et al., 2020). In a phase II and III clinical trials for Benralizumab patients with eosinophilic asthma that received the drug showed a reduction in annual exacerbations in both trials. A 57% reduction in exacerbations when taking 20 mg of Benralizumab when compared to the placebo (p= 0.015) (Castro et al., 2014) and a 36% reduction in exacerbation in eosinophil high individuals (>300 cells/µL) receiving Benralizumab every 4 weeks when compared to the placebo group (p= 0.0018) (FitzGerald et al., 2016). One phase III Reslizumab study showed an improvement in FEV_1 (270 mL difference in change in FEV_1; p= 0.04) in the eosinophil high group. The study also demonstrated that a greater proportion of patients achieved a decrease in ACQ-7 score of ≥ 0.5 at week 16 with Reslizumab compared with the placebo (p= 0.01) (Corren et al., 2016).

1.5.2.3 Anti-IL-4 and anti-IL-13 Therapy (Dupilumab)

Dupilumab is a monoclonal antibody that binds IL-4Rα, which blocks both IL-4 and IL-13 receptors. The inhibition of IL-4 or IL-13 cytokines alone was proven to be ineffective in asthma, likely due to biological redundancy of their signalling pathways. IL-4 and IL-13 are major drivers of the Th2 pathway which is known to drive airway inflammation in asthma and additional effects including increase mucus production, goblet cell hyperplasia and smooth muscle hypotrophy. Dupilumab is given to patients with moderate-severe asthma with eosinophilic asthma or OCS dependency (Johnson et al., 2019) (Doroudchi et al., 2020). A phase II study Dupilumab demonstrated at 87% reduction in asthma exacerbation rates compared to the placebo group (p=<0.0001) (Wenzel et al., 2013). In a subsequent phase III trial severe asthma patients who received Dupilumab, had significantly lower rates of exacerbation (Annual exacerbation rate: 0.46, p=<0.0001), improved lung function (high dose group FEV_1 (L): 0.47 & placebo group FEV_1 (L): 0.22, p=<0.00001) and improved asthma control (ACQ-5 scores were lower with Dupilumab verses the placebo group) than those who received the placebo (Castro et al., 2018). Dupilumab has also been shown to be improve clinical

features such as lung function particularly in patients with elevated biomarkers of type 2 inflammation, such as blood eosinophils FeNO (Castro et al., 2020).

1.5.2.4 Anti-TSLP (Tezepelumab)

Tezepelumab is an anti-TSLP monoclonal antibody that binds to TSLP and prevents its interaction with the TSLP receptor complex. TSLP is thought as a master regulator of the Th2 immune response occurring at barrier surfaces such as the skin and lungs which the epithelial cells produce (Nakajima et al., 2020). Even though Tezepelumab has not been released into clinic yet, studies have shown promising results. A phase II clinical trial on Tezepelumab revealed that asthma patients who received Tezepelumab showed significantly lower rates of exacerbations (reduction by 61% in the low dose, 71% in the medium dose, and 66% in the high dose) than those who received the placebo (p=<0.0001). Additionally, the trial also showed an improvement in FEV_1 at 52 weeks in all Tezepelumab groups versus the placebo group (difference: 0.12 L with the low dose; p=0.01, 0.11 L with the medium dose; p=0.02, and 0.15 L with the high dose; p=0.002) (Corren et al., 2017). A previous study involving the effects of anti-TSLP on allergen-induced asthmatic responses demonstrated that Tezepelumab significantly decreased levels of blood (p=0.004) and sputum eosinophils (p=0.004) in asthma patients (Gauvreau et al., 2014). As of writing, there are currently two clinical phase III trials underway that will examine whether Tezepelumab can reduce annual asthma exacerbation rates (NAVIGATOR trial) and reduce the required daily dose of OCS without loss of symptom control (SOURCE trial) (Nakajima et al., 2020). Recent phase 3 clinical trial data have shown encouraging results in Tezepelumab treating severe asthma patients by reducing exacerbation rates and improving lung function. The data showed that annual rate of asthma exacerbations was 0.93 (95% CI: 0.80-1.07) with Tezepelumab and 2.10 (95% CI: 1.84-2.39) with placebo. At 52 weeks, an improvement in pre-bronchodilator FEV_1 was observed on treatment with Tezepelumab (0.23 L) versus placebo (0.09 L; p= <0.001) (Menzies-Gow et al., 2021).

1.5.2.5 Other biologics in early phase clinical trials

Lebrikizumab is an anti-IL-13 monoclonal antibody that binds specifically to IL-13 with high affinity and blocks its activity. IL-13 is central to Th2 inflammation

and contributes to many features of asthma such increase mucus production (Bujarski et al., 2016). Studies have shown inconsistant results from Lebrikizumab regarding improvement of lung function. A previous study showed that Lebrikizumab has the greatest improvement in change in FEV_1 in periostin high patients when compared to placebo (8.2 percentage point higher; p=0.03), whereas periostin low patients did not have a significantly improved FEV_1 compared to the placebo group (p=0.61). This suggested that periostin could be an important biomarker for stratified medicine as periostin high patients may respond to this drug better (Corren et al., 2011). However, one clinical trial study assessing Lebrikizumab in mild-moderate asthma patients on SABA alone found that Lebrikizumab did not significantly improve FEV_1 (p= 0.06). This suggests that IL-13 may not be a dominant driver in lung function in mild-moderate patients (Korenblat et al., 2018). Serious adverse effects, including increased concentrations of blood eosinophils and eosinophilic pneumonia have been reported for this drug (Hanania et al., 2016).

IL-33 has been a target of interest in asthma as it is produced by the airway epithelial cells and smooth muscle cells and is also expressed on immune cells such as dendritic cells, macrophages and mast cells (Zhu et al., 2018a). One phase II clinical trial for an IL-33 antibody REGN3500 showed an improvement in lung function and asthma control in patients with blood eosinophil levels ≥300 cells/μL (Sanofi, 2019). Further clinical trial data on other IL-33 antibodies has yet to be released, but there have been promising results seen in animal model studies too. One study showed that knocking out IL-33 in mice prevented them from developing lung eosinophilia after allergen induction (Kamijo et al., 2013). Another mouse model study demonstrated while IL-33 drove inflammation and remodelling in the airways, blocking IL-33 with an IL-33 neutralising antibody improved inflammation and remodelling of the lung epithelium. Additionally, IL-33 normalised the levels of key inflammatory mediators associated with airway inflammation and exacerbations, including eosinophilic infiltration, and was able to re-establish the presence of ciliated cells over goblet cells (Allinne et al., 2019).

Finally, one non-Th2 inflammation targeting biologic is Brodalumab, a monoclonal antibody that binds to IL17RA, thereby blocking activity of IL17A, IL17B, and IL25 (Zhu et al., 2018a). A clinical trial in moderate-severe asthma patients demonstrated that Brodalumab did not improve asthma clinical features such as FEV_1 ($p= 0.85$) and symptom score ($p= 0.37$) after 12 weeks of treatment compared to placebo. However, they did show significant difference in asthma control scores (estimated treatment difference of 0.53; $p= 0.02$) in a small subgroup with high bronchodilator reversibility FEV_1 (≥20%) at a dose of 210 mg (Busse et al., 2013).

1.5.2.6 Summary

Overall, these drugs that have made it into clinic have shown promising results so far for severe/difficult to treat asthma patients (Table 1.5.2). Omalizumab appears to effect a decline in severe exacerbations, daily asthma symptoms and improve lung function in patients with allergic asthma. Whereas Mepolizumab, Reslizumab, Benralizumab and Dupilumab have shown to reduce annual exacerbations, improve lung function and reduce asthma symptoms in patients with eosinophilic asthma. Overall, this improves the patients' quality of life and allows them to lead a normal and active life. Additionally, there more biologics that are in the early and late stages of clinical trials that have the potential to come into clinic in the near future. However, it is still unsure who will benefit the most from these new drugs and the cost of these drugs are high so it is important to identify biomarkers that will help clinicians decide who to give which drug too. Finally, there is also a need for more biologics that target patients with Th2-low asthma as currently the biologics available in clinic target Th2 inflammation and also Brodalumab failed clinical trials as a biologic.

Biologics in clinic	Target	Clinical features effective on	References
Omalizumab	IgE	Reduces exacerbations by ~25% Improves lung function Decrease ICS use Improves asthma symptoms	(López Tiro et al., 2015) (Paganin et al., 2017) (McGregor et al., 2019)
Mepolizumab	IL-5	Reduces exacerbations by ~50% Decrease OCS use to complete removal (~14%)	(Kallieri et al., 2020) (Doroudchi et al., 2020) (McGregor et al., 2019)
Reslizumab	IL-5	Reduces exacerbations by ~50-60% Improves lung function Improves asthma symptoms	(Corren et al., 2016) (Doroudchi et al., 2020) (McGregor et al., 2019)
Benralizumab	IL-5R	Reduces exacerbations by ~25-60% Improves lung function Decreases OCS use to complete removal (~50%) Improves asthma symptoms	(Castro et al., 2014) (FitzGerald et al., 2016) (McGregor et al., 2019)
Dupilumab	IL-4/IL-13R	Reduces exacerbations by ~50-70% Improves lung function Decreases OCS use to complete removal (~50%) Improves asthma symptoms	(McGregor et al., 2019) (Wenzel et al., 2013) (Castro et al., 2018) (Castro et al., 2018)

Table 1.5.2 Summary of current drugs available in clinic for uncontrolled moderate-severe asthma patients and their therapeutic benefits on asthma clinical features.

1.6 Genetics of Asthma

Studies have shown asthma runs in families, and children are at an increased risk by 50% of developing asthma if their parents already have asthma (Thomsen, 2015b). Asthma is not caused by a single mutation to one gene and so the transmission of asthma does not follow Mendelian inheritance like in monogenic diseases such as cystic fibrosis (autosomal recessive). Instead, asthma is a polygenic multifactorial disorder meaning many factors contribute to the development of asthma. These factors include both genetic and environmental, the combined action of multiple genes with one another and environmental factors causes asthma (Thomsen, 2015b) (Carr and Bleecker, 2016).

Twin studies have supported a genetic contribution to asthma development, with asthma being more likely to occur in an individual if they have a genetically close family member with asthma. One study reported that monozygotic twins have a higher risk (6-fold increase) of developing asthma then dizygotic twins (3-fold increase), if their twin was asthmatic (Thomsen et al., 2010). There are currently a number of genetic variants that are associated with asthma susceptibility with some specific genotypes increasing the likelihood to developing atopy (Marenholz et al., 2015) or responsiveness to bronchodilator therapy (Ortega et al., 2014) (Jovicic et al., 2018).

Early investigations into the of role genetic variation in the development of asthma were carried out using approaches such as candidate gene studies and positional cloning. Candidate gene association studies have been utilised to examine variation across specific genes with known or suggested functions in asthma pathogenesis. The most common approach is a case-control study (individuals with and without the specific disease) in which these earlier studies identified single nucleotide polymorphisms (SNPs) or insertion deletion (indel) polymorphism in genes associated with the risk of developing asthma (Ober and Yao, 2011) (Melén and Pershagen, 2012). Positional cloning examines the segregation of genetic variation with disease through generations of families. This approach identified several plausible genes associated with asthma, e.g., *ADAM33* involved in airway remodelling, *HLA-G* involved in

innate/adaptive immunity, *PLAUR* involved in airway remodelling, *PCDH1* involved in epithelial cell function (Lee et al., 2011) (Sayers, 2019).

Relative with asthma	Individual's risk of developing asthma (%)
No family history	5
Uncle/aunt/nephew/niece	10
Half sibling	10
Full sibling	25
One parent	25
Dizygotic twin	35
Both parents	50
Monozygotic twins	75

Table 1.2 the percentage risk of an individual developing asthma with genetically close family member having asthma already. However, this can vary from other genetic and environmental factors. Used from (Thomsen, 2015b).

1.6.1 Gene-environment interactions

Both genetic and environmental risk factors are suggested to play a role in the development of asthma (Lee et al., 2015). Environmental exposure in early life can play an important role in the development of asthma as environmental exposures can have differing impacts on asthma development dependent on the developmental window during specific genetic responses. The differential influence of genetic and environmental factors is hypothesised to lead to disease heterogeneity and severity observed in asthma (Castro-Giner et al., 2006). Many gene-environmental studies have looked at how genetic responses to environmental exposure influence the risk of asthma throughout life, particularly in childhood. A widely studied environmental exposure, smoking and environmental tobacco smoke has been known to cause asthma in children and adults. Previous studies have shown associations between 17q12-21 variants (*GSDML*) and early-onset asthma (Moffatt et al., 2007) (Moffatt et al., 2010). Data has shown that the 17q12 variant, rs2305480 also showed associated with increased childhood onset of asthma symptoms (OR: 1.17, CI: 1.11-1.24) but the effect was seen stronger in children who were exposed to smoke during and infancy (OR: 1.30, CI: 118-1.43) (van der Valk et al., 2012). It is thought that *GSDML* is involved in epithelial barrier function and mechanisms by which tobacco smoke affects airway inflammation are thought to result from a genetically compromised barrier function of the airway mucosa, allowing airborne allergens to penetrate the mucosa (Kabesch,

2006). Another study in asthma patients who were exposed to environmental tobacco smoke found that the *CD14* genotypes GG or GC were associated with lower baseline FEV_1 (family based: p=.00009 & cross-sectional cohort: p=0.03). CD14 is expressed on the surface on monocytes, macrophages and neutrophils and is thought to be a player in biological responses environmental microbial products. It suggests that genetic variation in the CD14 gene may modify the interaction between environmental tobacco smoke, and asthma severity effecting lung function (Choudhry et al., 2005).

Most gene-environment studies are focused on the childhood onset of asthma. These studies have revealed novel genes that have not involved previously with the pathogenesis of asthma. However, there are still inconsistencies between studies in regard to the direction of effect within certain genetics variants. This is thought to be due to the study designs lacking power, use of populations with different ethnic origins and differences in the characterisation of the exposure or/and disease phenotype. There has also been little replication in gene-environment interactions in previously reported genetic variants and environmental exposures i.e. tobacco smoke (Morales and Duffy, 2019). Finally, there are very few gene-environmental studies looking at other important risk factors of asthma including weather, medication and diet. Overall, gene-environment interactions have shown some importance in the development of asthma but there is a lack of understanding of their complex interaction between one another.

1.6.2 Asthma Genome Wide Association Studies

More recent advances in genotyping technologies and extensive mapping of variation in the human genome has provided new opportunities for genome-wide scanning to identify novel genetic drivers of asthma and asthma related phenotypes. Genome-wide association studies (GWAS) are observational studies that are driven by a hypothesis-free approach that compares the frequencies of genetic variants between cases-controls using large population-based cohorts (100,000+) for gene discovery.

The main principle of GWAS is that common diseases such as asthma are likely to be driven by many common variants of small effect such as SNPs.

Modern commercial DNA microarrays, such as Illumina and Affymetrix platforms, allow for extensive coverage of the human genome, genotyping between 0.5 - 2.5 million SNPs. Although historically lacking coverage of rare SNPs, with a low minor allele frequency (MAF) of <5%, current arrays such as the UKBioBank array have now included increasing numbers of these rare variants as well as indels covering further variation (Shrine et al., 2019). Rare SNPs are more likely to be functional or deleterious and are considered to have a greater impact on disease risk than common SNPs. Even though there have been some great successes in many GWASs there are however some limitations. Genotyping and association testing very large numbers of variants can introduce false positive findings and therefore, significance thresholds need to be corrected with multiple testing using, for example Bonferroni correction, which generates smaller p-value such as p= $<5x10^{-8}$ in both the discovery and replication stages. A second limitation is that, it has been commented that SNPs identified in GWAS only explain a small proportion of the heritability of complex traits like asthma and do not necessarily pinpoint the causal variants and genes (Tam et al., 2019).

GWAS has shown great success in identifying an increasing number of novel genetic signals associated with asthma. The first novel asthma locus identified by GWAS was *ORMDL3* and *GSDMB* genes located on chromosome 17q12-21.1 (Moffatt et al., 2007). In this study, there were 994 cases, and 1243 control of European descent and findings were replicated in 2320 cases and 3301 controls. This genetic region has also been associated with childhood asthma in subsequent studies that include ethnically diverse populations. From then on, there have been several asthma GWAS which have identified and importantly replicated signals related to asthma. Most notably the size of the studies has significantly increased, e.g., 28,399 cases (self-reported asthma) and 128,843 controls with 27 independent loci identified (Pickrell et al., 2016). These GWAS have highlight alterations in innate and adaptive immune responses genes e.g., *CD247, IL13, IL33, LRCC32, HLA, CLEC16A, GATA3, TSLP, CAMK4, TNFSF4*, airway hyper-responsiveness e.g., *GSDMB, ORMDL3* and epithelial homeostasis e.g., *IL33, TSLP*. Asthma GWAS have also looked at a combination of phenotypes such as blood eosinophil counts,

IgE levels, disease severity and hospitalisations (Gudbjartsson et al., 2009) (Moffatt et al., 2010) (Weidinger et al., 2008) (Shrine et al., 2019) (Wan et al., 2012) (Bønnelykke et al., 2014).

In the last decade GWAS studies have identified over 200 loci that have been associated with asthma and some with severe asthma (Schoettler and Strek, 2020). GWAS has helped understand complex diseases such as asthma in the last decade but have only so far explained a small proportion of the hereditability (Tam et al., 2019). GWAS has also provided a great amount of information about which genes may be involved in asthma, but it is limited regarding its translation of GWAS data to pathways and new mechanistic and clinical understanding. However, new approaches have been developed to translate genetics to increase mechanistic and clinical understanding on how these genes drive asthma development and progression. Gene modification approaches through molecular biology techniques such as genotyping and CRISPR/Cas9 have allowed a better understanding of determining phenotypic and mechanistic effects of the activity of these genes.

1.6.3 GWAS studies that have found genetic signals related to moderate-severe asthma

The first GWAS to identify genetic variants associated with severe asthma was carried out using The Epidemiology and Natural History of Asthma: Outcomes and Treatment Regimes (TENOR) cohort (Li et al., 2010). Associations with previously reported asthma loci were found e.g., in the *RAD50/IL13* and *HLA-DR/HLA-DQ* regions but these did not meet genome wide significance. Similarly, the Asthma UK Genetics of Severe Asthma (AUGOSA) study of moderate-to-severe asthma replicated the known 17q21 association at *ORMDL3*/*GSDMB*/*ZPB2* (Wan et al., 2012). However, these signals also did not meet genome-wide significance, potentially due to lack of statistical power as the consisted of 933 patients and 3346 controls in the first stage of the study with replication stage included 231 cases and 1345 controls without asthma.

The most recent and largest moderate-severe asthma GWAS was from a European population, two stage case-control design (Shrine et al., 2019). Cases were either doctor diagnosed or defined based on the appropriate

medication for moderate to severe asthma per GINA guidelines definitions. The controls were defined as not having asthma, atopy through lack of relevant medication or other chronic lung diseases such as chronic bronchitis or emphysema. 5135 cases and 25 675 controls were used for stage 1 and 5414 cases and 21 471 controls for stage 2. 24 genetic signals in association with moderate to severe asthma were identified that reached genome wide significance, with several of these signals being involved in genes associated with the Type 2 pathway (Figure 5.1.1). 21 of these signals have also been previously identified in other studies, which have contained mild asthma patients. This suggests that there may be some shared genetic factors between mild and moderate to severe asthma patients. Three of them 24 signals identified were novel to this study, *GATA3* (rs10905284), *MUC5AC* (rs11603634) and *KIAA1109* (rs560026225). *MUC5AC* was not significantly associated with asthma when mild asthma patients were included in the analyses, whereas *GATA3* and *KIAA1109* signals were associated with all asthma including mild patients. Although, the signal for *GATA3* in moderate-severe asthma was different than the one identified with mild asthma patients included.

eQTL analyses found that the rs11603634 proxy SNP rs11602802 (r^2= 0.46 European population) is an eQTL with *MUC5AC* with the bronchial brush epithelial cells. *MUC5AC* mRNA expression levels from bronchial epithelila cells were elevated in patients with severe asthma carrying the risk allele (A) of SNP rs11602802 (proxy for SNP rs11603634 A:G) in two independent analyses (p= 0.039 and p= 0.022). Similarly, the proxy SNP rs17454584 for *KIAA1109* (rs560026225) (r^2= 0·57) is an eQTL for *KIAA1109* in lung tissue, with the asthma risk allele (GATT) associated with decreased expression of *KIAA1109.* No significant eQTL association was seen for the *GATA3* signal (Shrine et al., 2019). The *KIAA1109* signal has previously been associated with allergic disease (Zhu et al., 2018b), type 1 diabetes (Plagnol et al., 2011) and ulcerative colitis (Anderson et al., 2011). The rs10905284 (*GATA3*) asthma risk allele C, has also been associated with rheumatoid arthritis (Okada et al., 2014) and increased concentrations of blood eosinophils (Astle et al., 2016).

This study reported for the first time that the *MUC5AC* locus was specifically associated with increased risk of developing moderate to severe asthma. *MUC5AC* has been shown to regulate mucin production and it is thought that alterations in the FOXA transcription activity causes this increased MUC5AC expression. MUC5AC protein concentrations have also been seen to increase in sputum of patients with asthma during exacerbations compared to patients with controlled asthma and controls (Lachowicz-Scroggins et al., 2016). Finally, MUC5AC has also been linked to airway hyper-responsiveness and mucus plugging during exacerbation (Bonser and Erle, 2017). These findings could potentially provide an understanding for this difficult to treat subgroup of asthma patients and so targeting mucins may have therapeutic value.

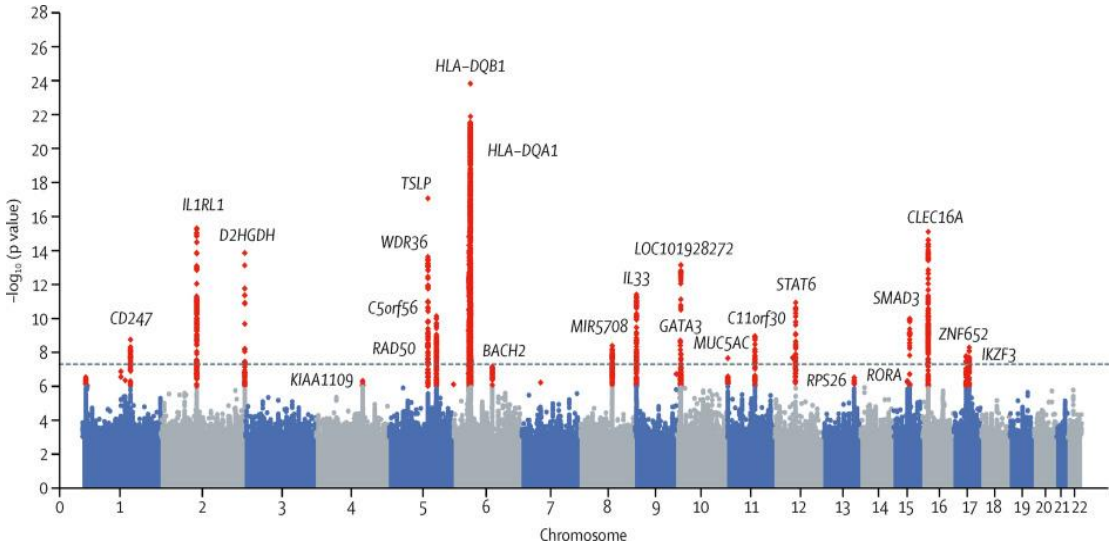


Figure 1.2.3 Moderate to severe asthma GWAS stage 1 analyses Manhattan plot: the x axis is the chromosome number; the y axis is the $\log_{10}$ p-value and the dotted line represents the genome wide significance p-value ($p=<5x10^{-8}$). The red points are the signals that met the criteria for the stage 2 follow-up, the loci are labelled with the nearest gene for signals reaching genome wide significance in meta-analysis. Figure taken from (Shrine et al., 2019).

1.7 Summary

To conclude, asthma is a complex disease, with multiple factors such as genetics and environmental that can contribute to the development of the disease. Studies have shown how heterogeneous asthma is, with age of onset ranging from childhood to very late adulthood and patients presenting little to high symptoms and/or inflammation. Depending on its severity, asthma can cause changes to the structure of the airways, causing problems such as impaired gas exchange and an increase in mucus production. This in turn causes worsening of asthma symptoms, increased risk of exacerbations and hospital admissions. With airway inflammation driving asthma, studies have demonstrated that asthma can divided into Type 2-high and Type 2- low. Type 2-high are patients that have mainly airway eosinophilia known as Th2. Whereas Type 2-low is patients that have an absence of airway eosinophilia, and their asthma is driven Th1 or Th17 immune cells.

5-10% of asthma patients have a severe form of the disease despite taking high dose ICS and OCS. New biologics that target these different pathways are being developed and many are currently in phase II or III clinical trials or already available to use in clinic, with most of the new biologics target components of the Th2 pathway. However, there are still severe asthma patients that still do not respond to these new biologics. Currently, biomarkers such as eosinophil counts and/or FeNO, along with clinical phenotypes, are used to assess who may benefit from these new biologics. With more newly developed drugs coming into clinic, it is important to identify new biomarkers that are easy to measure and are non-invasive, in order to administer the most appropriate drug to the patient. This will help avoid any drug wastage, as these are costly, minimise any side effects and improve the patients' quality of life.

1.8 Hypotheses and Aims

1.8.1 Hypotheses

1. A greater understanding of clinical and immunological features will identify specific sub-groups of asthma that provide insight into the drivers of i) adult vs. childhood disease, ii) exacerbation and iii) eosinophilic asthma.
2. Serum cytokines represent an accessible measure to further characterise asthma patients and this profile is related to i) age of onset ii) exacerbation and iii) eosinophilic asthma.
3. Genetic variants identified as risk factors for the development of moderate-severe asthma drive specific inflammation and clinical features of asthma.

1.8.2 Aims

- Clinically and immunologically characterise patients to understand the drivers of i) adult vs childhood asthma, ii) those patients that exacerbate, iii) eosinophilic vs non eosinophilic asthma
- Define the relationship between different Type 2 and non-Type 2 biomarkers and determine whether they are co-ordinately expressed in asthma patients.
- Identify the inflammatory signature associated with different asthma patient groups e.g. those prone to hospital admissions.
- Define the relationship between patients who are in the top 25% for the Type 2 biomarkers that are current drug targets.
- Identify whether the genetic variants identified in GWAS as asthma risk alleles drive expression of the Type 2 biomarkers and if they lead to clinical Type 2 phenotype.

Chapter 2- Using Clinical and Immunological Data to Further Define Asthma Subgroups

The Genetics of Asthma Severity and Phenotypes (GASP) is a UK non-related cohort of adults with asthma. GASP was utilised to identify differences in clinical and immunological features of the following subsets of asthma patients; i) early onset of asthma patients, ii) asthma patients more prone to exacerbations and iii) those which meet the eosinophil criteria for Mepolizumab. These three questions are relevant to asthma as age of onset is one of the key variables identified in asthma heterogeneity. Hospital admissions are also clinically relevant today as there are many patients that are prone to exacerbate despite taking high dose inhaled corticosteroids. Finally, Mepolizumab is a new drug in clinic that targets IL-5 and patients must have a blood eosinophil count of >300 cells/µL to go on the drug; therefore, looking at patients who meet the blood eosinophil count is clinically relevant to see how they differ to those who do not meet the criteria.

2.1 Introduction

2.1.1 Age of Onset

Although many patients develop asthma during childhood, asthma can occur at any time in life. Age of onset classification is most commonly split into childhood onset (early onset <16) and adult onset (late onset) (Hirano and Matsunaga, 2018). Studies have shown that late onset of asthma tends be associated with obesity, a female predominance, worse lung function and lack of atopy (Hirano and Matsunaga, 2018). The Severe Asthma Research Program (SARP) identified a cluster of asthma patients that consisted mostly of older women (71%, mean age of 50 years) with a high BMI of >30 (58%) as well as an FEV_1 of <80% predicted (71%) (Moore et al., 2010). It is also recognised that patients with late onset of asthma tend to have multiple comorbidities such as gastroesophageal reflux, COPD and pulmonary fibrosis (Trivedi and Denton, 2019), poor inhaler technique and mood disorders such as anxiety and depression (Labor et al., 2018). Other studies have demonstrated that many late onset of asthma patients may be more severe than the early onset of asthma patients as they are more likely to have

uncontrolled asthma and less responsive to steroid therapy (Wu et al., 2015) (Hirano and Matsunaga, 2018) (Trivedi and Denton, 2019). Patients who develop asthma as a young adult tend to be similar to childhood onset of asthma with atopy being a risk factor. Asthma development in middle age tends to be mostly women who are obese, current or ex-smokers, from a lower socio-economic status and non-atopic (Sood et al., 2013) (Tan et al., 2016). In older age of onset atopy does not appear to be a risk factor however, disease severity is worse in comparison to early age of onset and is usually more progressive. Lung function is also worse amongst late adult onset as they tend to have more airflow obstruction which is thought to be caused by the presence of other comorbid lung diseases such as COPD and old age (Trivedi and Denton, 2019).

Asthma in children is a chronic disease. However, there are periods where the disease can go into remission or resolve all together. Furthermore it is also known that individuals whom have had asthma as a child may also have it reoccur again in adult life (Hirano and Matsunaga, 2018). There have been several suggested risk factors in developing asthma as a child including genetics i.e. sex, medical and family history and environmental risk factors such as maternal smoking (Paaso et al., 2014) (Trivedi and Denton, 2019). Both genetic and environmental factors contribute to the risk of asthma, with genetic factors thought to account for 35–95% of the susceptibility to develop asthma (Ober and Yao, 2011) (Turner, 2017). Using age of onset as a variable in GWAS studies has identified many loci associated with childhood onset of asthma such as *ORMDL3, GSDMB* and *FLG* (Moffatt et al., 2007) (Pividori et al., 2018). Gene-environment interactions are known to be important in the development of asthma in children.

As previously mentioned, atopy is known to be strongly associated with early onset of asthma. The term “atopic march” has been used clinically to describe individuals with atopic disease, it starts off as atopic dermatitis (eczema) in infancy, progressing to IgE mediated food allergy, allergic rhinitis (hay fever) and then asthma later in childhood. All of these conditions carry a complex pathophysiology involving aspects of the immune system (Hill and Spergel, 2018) (Trivedi and Denton, 2019). Numerous cohort studies have shown atopy

to be very high risk in early onset of asthma (Tan et al., 2016) (Chaudhuri et al., 2016) with one study showing that children with atopy in early childhood had a much greater risk of developing asthma by the age of ten (odds ratio = 17.14) (Kusel et al., 2012). Defects in airway epithelial cells are thought to be the facilitator of allergic sensitisation. Exposure to harmful stimuli may impede epithelial barrier function resulting in an increased access of harmful stimuli to innate immune cells and blood vessels which in turn leads to sensitisation and type 2 inflammation (Hellings and Steelant, 2020). Barrier dysfunction is thought to be the key facilitator in atopic march which usually leads to the development of asthma in children. On the whole early onset of asthma tends to be easier to control using existing medication such as ICS and those that are difficult to treat or who are uncontrolled tend to have comorbidities which contribute to it (Ullmann et al., 2018).

The Tucson Children's Respiratory Study was the first longitudinal study where children were enrolled at birth and the natural history of asthma was assessed. The study demonstrated that asthma is heterogeneous and identified important risk factors associated with airway obstruction such as wheezing (Martinez, 2002). Wheezing patterns in childhood has been thought to have an association with asthma outcomes. One study that characterised childhood wheezing into 6 phenotypes from infancy to adolescence and observed their associations with asthma. They showed that early childhood wheezing before the age of 4 years and persisting throughout childhood into adolescence had strong associations with airway obstruction. It was also found that there was a higher prevalence of atopy in the wheezing phenotypes that was strongly associated with persistence of wheezing after onset and continuous wheezing from infancy than those with remitting phenotypes (Granell et al., 2016). It has also been suggested that an inappropriate response to respiratory viruses particularly rhinovirus and early allergic sensitisation are strong contributors to the start and persistence of childhood onset of asthma (Martinez, 2019). Studies have shown that children who had the most common rhinoviruses isolated from their upper airways during wheezing illnesses were more likely to develop childhood asthma compared to those who did not have rhinovirus related wheezing (Lemanske et al., 2005) (Kusel et al., 2007).

Overall childhood onset of asthma and adulthood share some clinical similarities and differences with childhood onset being more atopic and easier to treat and adult onset being associated with increased prevalence in women, obese patients and poorer lung function. Table 3.1.1 summaries the key features of adult and childhood onset of asthma.

Features	Childhood onset	Adult onset
Symptoms	Cough, wheezing, dyspnoea and chest tightness.	Wheeze, shortness of breath, cough, chest tightness, sputum production and aspirin sensitivity.
Treatment response	Easier to treat with ICS	More difficult to treat
Severity	Less severe	More severe
Clinical features associated	High blood IgE levels Positive skin prick tests	Worse FEV_1pp Nasal polyps Sinusitis Aspirin-exacerbated respiratory disease (AERD)
Risk factors associated	Atopy: allergic rhinitis, eczema Family history	Smoking Obesity Lower socio-economic background Female

Table 3.1.1 Overview of the Clinical Features between Childhood verses Adulthood Onset of Asthma (Tan et al., 2016) (Hirano and Matsunaga, 2018) (Trivedi and Denton, 2019) (Liu et al., 2020).

2.1.2 Exacerbations in Asthma

It is known that asthma exacerbations are frequent in patients with a previous exacerbation history and severe uncontrolled asthma. Cohort studies describe 8.4% (from 211,807 patients) and 12.5% (from 222,817 patients) patients experience 1 or more exacerbation during a 12 month follow up period (Suruki et al., 2017). An asthma exacerbation is an acute or chronic episode of increased cough, chest tightness and/or wheezing which is associated with a decrease in lung function (GINA 2020). Now with that the inflammatory nature

of asthma has been recognised and with the introduction of inhaled corticosteroids and new biologics coming into clinic, asthma control and quality of life of asthma patients has improved substantially. Nonetheless, there are still patients who exacerbate with varying severity which range from increased symptoms to life threatening episodes (Chung et al., 2014).

There have been many asthma cohort studies with the aim of identifying clinical features of asthma patients that exacerbate as well as identifying clinical factors that may predict future exacerbations. One study looking at two large databases from the United States (US) and UK which involved around >200,000 asthma patients demonstrated that patients that had ≥2 exacerbations tend to have a higher GINA score of 4-5. Additionally, they showed that patients who had exacerbations leading to emergency department/hospital re-admission had a 32-35% increased risk of a following exacerbation within the next 12 months especially in those patients with the most severe disease (Suruki et al., 2017). Another smaller scale study of 2780 patients demonstrated similar findings with exacerbation frequency higher in patients with a recent exacerbation history compared to those who did not exacerbate. The same study suggested that recent severe asthma exacerbations are a strong independent factor in predicting future exacerbations and so should be considered as part of the clinical assessment of patients who have severe or difficult to treat asthma (Miller et al., 2007).

It has also been shown that exacerbations have been directly linked to a decline in lung function. One study looking at asthma exacerbations and its association with lung function in patients with severe eosinophilic asthma observed a direct relationship between the numbers of exacerbations and FEV_1pp decline (Ortega et al., 2018). Additionally, another study looking at baseline lung function on future exacerbations in 1,865 moderate-severe asthma patients showed that a baseline FEV_1pp (pre-bronchodilator) of 50-79% or <50% was associated with a higher risk of severe exacerbations compared to FEV_1pp of ≥80% (IRR: 1.46 and 2.03 respectively). It was also suggested that baseline lung function may be an important predictor of future asthma exacerbations (Khan et al., 2018). In addition to a decline in lung function, poor symptom control has been identified as symptom of asthma

exacerbations. A recent study looking at the association of asthma control tests (ACT) and questionnaires with asthma exacerbation showed that they may provide useful information in predicting future asthma exacerbations. Their results showed that ACQ scores were higher and ACT were lower in the exacerbation group compared to the non-exacerbation group ($p= <0.05$) (Uematsu et al., 2018).

As well as lung function decline and poor symptom control, smoking history is also associated with an increase in exacerbations rates. A multicentre study looking at cigarette smoke in adults with asthma exacerbations in the emergency department found that a third of emergency department asthma exacerbations were current smokers and that this data has not changed from another multicentre study in the late 1990s (Silverman et al., 2017). Additionally, another study looked at the effects of second hand smoke in children with asthma. They showed children were nearly twice as likely to be hospitalised with an asthma exacerbation (odds ratio [OR] 1.85, 95% confidence interval [CI] 1.20-2.86, $p= 0.01$) and more likely to have lower FEV_1/FVC value (OR -3.34, 95% CI -5.35 to -1.33, $p= 0.001$) from second hand smoking (Wang et al., 2015).

Finally, viral infections have been known to cause asthma exacerbations with 60-70% caused by viral infections particularly rhinovirus. There are multiple mechanisms suggested that cause viral exacerbations including increased eosinophilic inflammation of the airway and lower airway neutrophilic inflammation (Adeli et al., 2019). One study showed that viruses were detected in 44% of exacerbations with a decrease in peak expiratory flow (PEF) rate of 50 mL/minute or more, and the most commonly identified virus being the rhino virus (Nicholson et al., 1993).

Even though there has been a considerable decline in asthma mortality rates over the last few decades, recent asthma exacerbation studies have shown us are that there are still a proportion of asthma patients that still have uncontrolled or difficult to treat asthma with multiple factors increasing the risk of an exacerbation (Figure 3.1.2). But with the development of precision medicine and targeted therapies it has become more hopeful that there will be

a further decline in mortality rates and improved management of exacerbations.

Figure 2.1.2 Overview of the risk factors associated with asthma exacerbations (Suruki et al., 2017) (Miller et al., 2007) (Silverman et al., 2017) (Ortega et al., 2018) (Adeli et al., 2019).

2.1.3 Blood Eosinophils as a Target for Therapy in Asthma

Eosinophils are cells of the innate immune system and in which play key roles in responses such as infection and allergic disease (Weller and Spencer, 2017). Eosinophils accumulate at sites of parasitic infections, allergic inflammation and also contribute to the development of asthma. In asthma eosinophils play a role in airway remodelling and airway hypersensitivity and it is now known that eosinophilia is one of the many characteristic features of asthma that leads to airflow obstruction and increase risk of exacerbations (Bakakos et al., 2019) (Nakagome and Nagata, 2018). Eosinophilia is part of the Th2 inflammatory pathway which is a feature in atopic asthma (Matucci et al., 2018). High blood eosinophil counts have been associated with atopic diseases such as atopic dermatitis and rhinitis (Froidure et al., 2016). Blood eosinophil counts have also been used to evaluate the responsiveness to

therapies such as ICS and biologics (Bakakos et al., 2019) (Matucci et al., 2018). There is a link between high blood eosinophils and high serum IgE levels (Froidure et al., 2016). Another other study looking at the associations between blood eosinophils and lung function in adults with and without asthma demonstrated that higher eosinophil levels were associated with lower FEV_1pp and FEV_1/FVC pre- and post- bronchodilator in participants with asthma. This suggests that blood eosinophils are associated with decline in lung function and airflow obstruction (Hancox et al., 2018). However, a study looking at the characterisation of severe asthma phenotype demonstrated that blood eosinophilia is a poor marker of asthma severity as there were no statistically significant differences between the groups (p=0.19) and the blood eosinophil counts (log), Mild: -0.72, Moderate: -0.63 & Severe: -0.75 (Moore et al., 2007).

Asthma GWAS have found multiple loci that are involved in the pathogenesis of asthma. One study with 21,510 European subjects reported SNPs that reached genome wise significance near the *IL1RL1, IKZF2, GATA2* and *SH2B3* genes (Gudbjartsson et al., 2009). *IL1RL1* has been reported as an asthma gene in many asthma GWAS (Moffatt et al., 2010) (Pickrell et al., 2016) (Shrine et al., 2019). *IL1RL1* is thought to play a part in severe and eosinophilic asthma phenotypes and drive Th2 inflammation. One study looking at the phenotypic and functional translation of the *IL1RL1* locus polymorphisms in asthma lung tissue and airway epithelium identified 3 independent signals that were associated with blood eosinophil counts, FEV_1 and severe asthma (Portelli et al., 2020).

IL-5 is the key cytokine that is involved in the maturation, proliferation, activation and survival of eosinophils and so biological treatments targeting IL-5 and IL-5R have been investigated and developed as a therapeutic target for patients with severe asthma. Biologicals targeting IL-5, Mepolizumab, Reslizumab and Benralizumab have been approved and included into guidelines as add-on therapy for severe eosinophilic asthma (Emma et al., 2018). Eosinophil counts have been used as a biomarker in identifying patients who have severe asthma and that may benefit from asthma biologics. Patients who are approved for Mepolizumab have to meet certain criteria with one of the criteria being a blood eosinophil count of over >300 cells/µL within the last

12 months (GINA, 2020). There has now been some data released showing the percentage of asthma patients who have met the criteria for Mepolizumab have responded. One study showed that Mepolizumab treatment effectively reduced symptoms of severe eosinophilic asthma in the majority of patients however, 53% still remained uncontrolled with an ACQ score of ≥1.5 (Harvey et al., 2019).

2.1.4 Introduction to the Genetics of Asthma Severity and Phenotypes Cohort

GASP (Shrine et al., 2019) is an UK non-related adult asthma cohort which has patients recruited from the UK over several years. This was the first time the cohort was used to answer clinical questions based on age of onset, hospital admissions and blood eosinophil levels to see if they identified any new or previously reported clinical subsets of patients. Age of onset was studied to see if the cohort presented any clinical features based on childhood verses adult onset. However, age of onset was divided into three groups <16 (childhood), 16-34 (young adult) and 35-60 (older adult) as it is known that these three groups vary clinically. Patients with hospital admission data were also studied. There is great interest in how these patients present clinically especially those prone to frequent exacerbations and also factors that could be used predict potential future exacerbations. Finally, since new biologics are coming into clinical for severe asthma patients' biomarkers such as eosinophils are being used to see whether these patients are eligible for the drugs. Hence, patients with eosinophil blood count data were split based on the criteria for the Mepolizumab drug. This was used to identify sub-groups including characterisation of these subgroups to provide insight into the clinical features of these patients. (Note: the blood eosinophil count is only one of several criteria's the patients have to meet to be eligible for the drug).

2.2 Aims

The aim of this chapter is to answer clinically relevant questions including:

1. Identify clinical and immunological features present in childhood versus adulthood onset of asthma.
2. Identify the clinical and immunological features present in asthma patients that are prone to exacerbate.
3. Identify the clinical and immunological features present in asthma patients that meet the blood eosinophil level criteria for the Mepolizumab drug.

2.3 Methods

2.3.1 Inclusion and exclusion criteria for the GASP cohort

Adult patients with asthma were recruited from 18 UK centres that were either from hospital clinics, lung function laboratories, in-patients under the care of the respiratory team or attending the site for other research projects. For patients to be recruited to the GASP study the following criteria had to be met (Figure 2.3.1): Patients had to have physician-diagnosed asthma of at least one year duration and had to be on prescribed medication for asthma. Asthma patients had to also be between the ages of 16 to 60 years old. Patients over the age of 60 were not recruited because older patients generally have a decline in lung function with age and also are at more risk of have other respiratory comorbidities which may confound diagnosis. Patients were excluded if they were unable to comprehend the study and provide consent e.g., insufficient command of English in the absence of someone to adequately interpret. Patients were also excluded if they had a known lung, chest wall, neuromuscular or cardiac disease or abnormality that would confound the Spirometry test, peak flow and the asthma control questionnaire (ACQ) score.

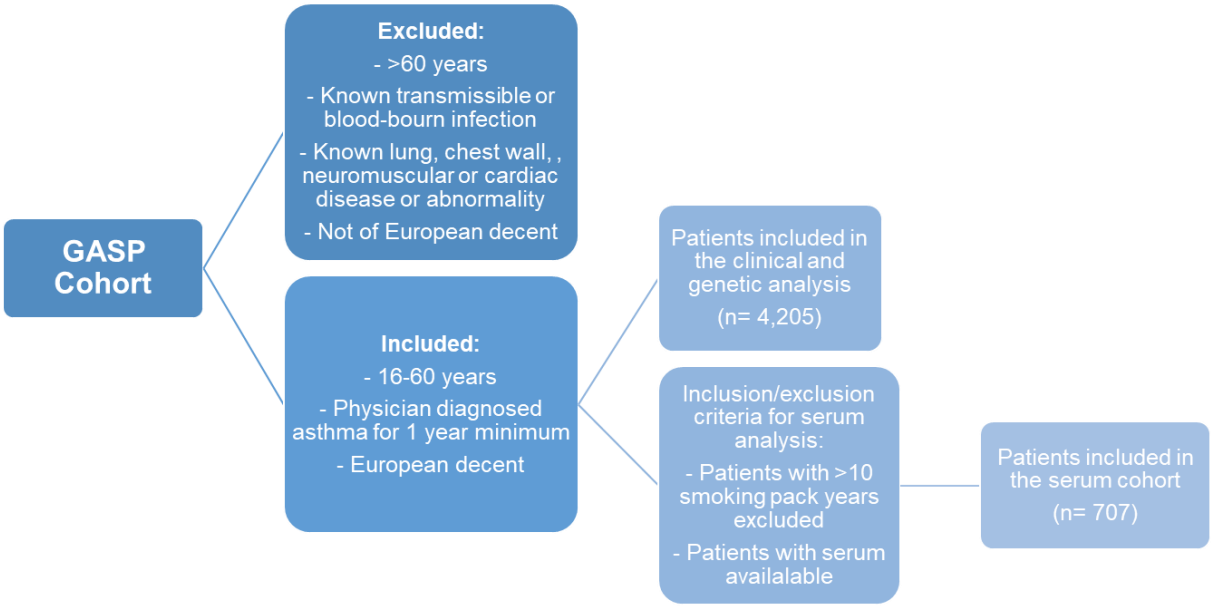


Figure 2.3.1 Consort of the inclusion and exclusion criteria of the GASP database and those selected from the GASP for the serum cohort

2.3.2 Quality control of the GASP cohort

Clinical and immunological data for each patient was captured across multiple centres through numerous ways e.g., spreadsheets and case report forms (CRF) and so there was a possibility of miss entering wrong values. Prior to starting the analysis, the data was quality control checked twice to ensure there were no abnormal values or missed typed values in the data set that would disrupt the analysis and give a false result. This quality control check was done by randomly checking 10% of the total number of patients in the cohort available at that time to see if the data on the database matched the data on the paper hard copy or original spreadsheet. The database was also checked for values that were non physiological and in doubt such as lung function values, weight, height etc. This was done by ordering each variable from high to low or low to high and checking the top and bottom values of each variable. Any values that looked irregularly high or low were checked against the original hard copy and were corrected or deleted. In addition to this, any irregular values were also consulted with clinical colleagues. Values that were

very low or high that matched the hard copy were deleted due to the fact they may have had other underlying health conditions that may affect the analysis.

2.3.3 Statistical analysis

All statistical analyses were carried out on IBM SPSS Statistics 24. Details of specific statistical methods are described.

To identify whether the continuous variables were either parametric or non-parametric, histograms were created with a distribution curve to see if they looked normally distributed. The mean was given for parametric data along with the standard deviation and the median with the range was given for non-parametric data. When two independent samples were being compared either a Student's t-test (parametric) or a Mann-Whitney U test (non-parametric) was used. When more than two independent samples were being compared either a one-way ANOVA (parametric) or a Kruskal-Walis test (non-parametric) was carried out. For some of the variables including height, weight and IgE a univariate model was used to take into account covariates such as age and gender. For testing the relationship between categorical variables, a Chi squared test was used. Correction for multiple testing will be used throughout the analysis using Bonferroni as this will give confidence in the results and get rid of any finding that are likely not to lead to anything.

2.4 Results

2.4.1 Demographic and immunological features of the GASP cohort

Table 2.4.1 shows the overall demographics for the cohort. There was a total of 4,205 asthma patients from 37 UK centres available for analysis. This cohort was mainly enriched with GINA 3-5 patients as recruitment centres were asked to primarily focus on recruiting these patients. Not all the data was available for all the 4,205 patients' particularly immunological data. Based on 3,534 patients with age seen data the mean age of the cohort was 42 years. The mean age of onset was 18 years (n= 2,137) and 65.9% (n= 2553) of the cohort were predominately females. The median BMI of the cohort was 27.89 kg/m^2 (n= 2739, 14.84-78.16), suggesting the cohort was obese (BMI= ≥25kg/m^2). 64.5% were non-smokers (n= 2116) and the other 35.5% were either ex-smokers (22.5%, n= 737) or current smokers (13%, n=426) with smoking pack years ranging from 0.3-161 (n=936). The mean lung function values were 82.03% (S.D: 22.21, n= 3,000) for FEV_1pp and 0.72 (S.D: 0.13, n= 2,682) for FEV_1/FVC. A smaller portion of the cohort had further lung function and immunological tests performed. The additional lung function features available were the percentage FEV_1 reversibility which is the percentage change in FEV_1 before and after bronchodilator treatment, to which the median value was 8.40% (range: -26.28-68.75%, n= 683) and the peak flow which the mean value was 390.2 L/min (S.D: 131.02, n= 1,324). The immunological features available for the cohort were blood total IgE levels and blood eosinophil levels. The median value for the blood total IgE levels were 130.0 KU/L (range: 0.60-4,900 KU/L, n= 1,879) and for the blood eosinophils were 0.21x10^9/L (0-5.42x10^6/L, n=811). 60.7% (n= 2,193) of the cohort had allergic rhinitis and 37.6% (n= 1,206) had atopic dermatitis. Additional clinical features for the cohort were hospital admissions within the last year for an asthma exacerbation of which 68.4% (n= 762) had never had a hospital admission and 31.6% (n= 352) have had one or more, of which 5.7% (n= 63) have been in the intensive care unit (ICU). The mean ACQ score for ACQ6 and ACQ7 are also available for a quarter of the cohort with the mean ACQ score for both 6 and 7 being 1.94 (n= 1,052 & n= 1,016; respectively) suggesting that most of the cohort did not have their asthma symptoms well managed (based on BTS

guidelines). An ACQ score of 1.5 or more is considered clinically significant as it suggests that a patients asthma is poorly controlled (Juniper et al., 2006). This was also in line with the initial aims of the project to recruit a cohort enriched for moderate-severe asthma patients. Finally, GINA data was also available for nearly half the cohort with the majority having moderate/severe asthma (GINA 3/4: 66.4% [n= 1,176] & GINA5: 6.4% [n= 213]) which what would be expected as the cohort was designed to have predominantly moderate to severe asthma patients. For the rest of the cohort 6.4% (n= 114) were mild asthma patients (GINA 1) and 15.2% (n= 269) were mild/moderate asthma patients (GINA 2).

Demographics, Clinical and Immunological Features of the GASP Cohort	
Subjects (n)	4205
Age seen (years: Mean & S.D)	42 (11.93) (n= 3534)
***Age of onset (years: Mean & S.D)**	18.6 (15.66) (n= 2137)
***Age of onset (n):**	2217
<16	1090 (51.0%)
16-34	619 (29%)
>35	428 (20%)
Gender (n):	3875
Female	2553 (65.9%)
Height (m: Mean & S.D)	1.67 (0.09) (n= 3231)
Weight (kg: Mean & S.D)	81.01 (20.64) (n= 2768)
BMI (Kg/m^2: Median & Min-Max)	27.89 (14.84-78.16) (n= 2739)
Smoking status (n):	3279
Never	2116 (64.5%)
Ex-Smoker	737 (22.5%)
Current	426 (13.0%)
Smoking Pack Years (Median & Min-Max)	6 (0.3-161) (n= 936)
FEV_1pp (Mean & S.D)	82.03 (22.21) (n= 3000)
FEV_1FVC (Mean & S.D)	0.72 (0.13) (n=2682)
Change in FEV_1% Reversibility (Median & Min-Max)	8.40 (-26.28-68.75) (n= 683)
Peak Flow (L/min: Mean & S.D)	390.2 (131.02) (n= 1324)
Blood total IgE (KU/L: Median & Min-Max)	130.0 (0.60-4900) (n= 1879)
Blood Eosinophils (x10^9/L: Median & Min-Max)	0.21 (0.0-5.42) (n= 811)
** **Allergic rhinitis (n):**	2193
Yes	1331 (60.7%)
****Atopic dermatitis (n):**	1206
Yes	453 (37.6%)
Hospital admissions (n):	1114
Never	762 (68.4%)
Once	185 (16.6%)
More than once	104 (9.3%)
High dependency/ICU	63 (5.7%)
Mean ACQ Score 7 (Mean & S.D)	1.94 (1.27) (n= 1016)
Mean ACQ Score 6 (Mean & S.D)	1.94 (1.36) (n=1052)
GINA (n):	1772
1 (Mild)	114 (6.4%)
2 (Mild/Moderate)	269 (15.2%)
3/4 (Moderate/ Severe)	1176 (66.4%)
5 (Severe)	213 (12%)

Table 2.4.1 Demographics, clinical and immunological features of the GASP cohort n=4205: Data is presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). FEV_1pp: forced expiratory volume in 1 second percentage predicted; FEV_1/FVC: forced expiratory volume in 1 second / forced vital capacity ratio; IgE: immunoglobulin E;

ACQ: asthma control questionnaire; GINA: global initiative for asthma. *Age of onset- some only known as childhood onset not the actual age so marked as <16 therefore numbers vary for both variables. **Allergic rhinitis and atopic dermatitis are doctor diagnosis and/or self-reported.

2.4.2 Childhood onset of asthma are more atopic than adult onset of asthma

Table 3.4.2 shows the demographics, clinical and immunological data stratified by three age of onset groups (<16, 16-34 and 35-60). There were 1,090 asthma patients in the childhood onset group (<16), 619 asthma patients in the young adult-onset group (16-34) and 428 asthma patients in the older adult-onset group (35-60).

Age seen was highly significant $p=1.66x10^{-95}$ this is likely to be a biased finding due to the older age of asthma onset groups will inevitably be recruited at an older age. Table 3.4.2 shows that there were more females in the 16-34 group (74%, n=458) than the <16 and 35-60 groups (67%, n=729 & 66.8%, n=286 respectively) however, this was not statically significant. Weight was higher in the 35-60 group (86.12 kg, n=377) compared to the other groups which were statistically significant p=0.001, figure 3.4.2.A shows the direction of effect was coming from the <16 and 35-60 group as the 35-60 group were significantly higher compared to the <16 group (p=0.001). This suggests that older age of onset asthma patients are more likely to be overweight than the childhood or earlier adult onset. In appendix 7.1 table 7.1.1, describes a control cohort of 823 patients, these patients were recruited from Nottingham for a historic study and have now been added to the GASP database as a control arm. In the control cohort mean weight shows to be 75.22 kg (n=823) suggesting that asthma patients may generally be more overweight, particularly older age of onset. There were more non-smokers in the <16 group (68.9%, n=745) but more smokers/ex-smokers in the 16-34 (36.8%, n=227) and 35-60 groups (40.3%, n=172), this was not statistically significantly. However, smoking pack years was statistically significant p=1.3x10-7 across the groups, being lower in the <16 group (5 pack years, 0.04-75, n=292) and higher in the 16-34 (9 pack years, 0.03-161, n=189) and 35-60 groups (10 pack years, 0.05-120, n=152), suggesting that patients who have childhood onset of asthma are less likely to start smoking compared to patients who have adult onset of asthma.

FEV_1pp is shown to be statistically lower (p=0.003) in the <16 (80.17%, S.D: 21.15, n=944) and 16-34 groups (79.11%, S.D: 23.06, n=534) compared to

the 35-60 group (83.92%, S.D: 21.4, n=371). Figure 3.4.2.B shows that the direction of effect was coming from the 35-60 group as FEV_1pp was lower in the <16 patients compared to the 35-60 (p=0.014) and also lower in the 16-34 patients compared to the 35-60 patients (p=0.003). Although, peak flow was higher in the >16 group (412.1 L/min, S.D: 130.68, n=421) compared to the 16-34 and 35-60 groups (383.06 L/min, S.D: 124.84, n=256 and 388.07 L/min, S.D: 136.07, n=164; respectively) this, however, did not meet statistical significance. Indicating that adult onset of asthma may have slightly improved lung function than those who have childhood or early adulthood onset of asthma.

Patients in the <16 group had a higher total blood IgE levels (150 KU/L, 0.6-4850, n=485) compared to the 16-34 and 35-60 groups which had lower blood IgE (85.5 KU/L, 1-4190, n=288 & 89.5 KU/L, 2-3528, n=214; respectively), which showed to be statistically significant, $p=7.8x10^{-8}$. Figure 3.4.2.C shows that the direction of effect is coming from the <16 patients as they showed to be significantly higher compared to the 16-34 patients (p=<0.0001) and the 35-60 patients (p=<0.0001). As well, <16 patients had high prevalence of allergic rhinitis (63.6%, n=567) and atopic dermatitis (32.6%, n=125) than the 16-34 and 35-60 patients (allergic rhinitis: 58.6%, n=268 & 51.9%, n=163 and atopic dermatitis: 21.9%, n=47 & 18.28%, n=26; respectively) which also showed to be statistically significant (allergic rhinitis: p=0.001 & atopic dermatitis: p=0.001). These data suggests that patients with childhood onset of asthma are more atopic than adulthood onset of asthma.

	<16	16-34	35-60	P value
Subjects (n)	1090	619	428	-
Age seen (years: Mean & S.D)	38.43 (12.07) (n=1082)	44.46 (9.83) (n=614)	51.28 (5.91) (n=428)	1.66E-95
Gender (n): Female	1088 729 (67%)	620 458 (74%)	428 286 (66.8%)	0.006
Height (m: Mean & S.D)	1.67 (0.47) (n=1019)	1.66 (0.09) (n=583)	1.67 (0.47) (n=410)	0.203
Weight (kg: Mean & S.D)	81.53 (20.89) (n=913)	83.31 (20.6) (n=507)	86.12 (22.62) (n=377)	**0.001**
BMI (Kg/m^2: Median & Min-Max)	28.04 (14.84-59.52) (n=904)	29.06 (16.90-78.16) (n=499)	29.63 (15.82-57.61) (n=369)	0.543
Smoking status (n): Never Ex-Smoker Current	1082 745 (68.9%) 215 (19.9%) 122 (11.3%)	616 389 (63.1%) 143 (23.2%) 84 (13.6%)	427 255 (59.7%) 114 (26.7%) 58 (13.6%)	0.008
Smoking Pack Years (Median & Min-Max)	5.0 (0.04-75) (n=292)	9 (0.03-161) (n=189)	10 (0.05-120) (n=152)	**$1.3x10^{-7}$**
FEV_1pp (Mean & S.D)	80.17 (21.15) (n=944)	79.11 (23.06) (n=534)	83.92 (21.4) (n=371)	**0.003**
FEV_1FVC (Mean & S.D)	0.72 (0.13) (n=910)	0.72 (0.13) (n=513)	0.73 (0.11) (n=370)	0.131
Change in FEV_1% Reversibility (Median & Min-Max)	10.24 (-20.12-68.75) (n=273)	9.29 (-18.62-61.48) (n=176)	6.86 (-26.28-61.21) (n=135)	0.261
Peak Flow (L/min: Mean & S.D)	412.1 (130.68) (n=421)	383.06 (124.84) (n=256)	388.07 (136.07) (n=164)	0.01
Blood total IgE (KU/L: Median & Min-Max)	150 (0.6-4850) (n=485)	85.5 (1-4190) (n=288)	89.5 (2-3528) (n=214)	**$7.8x10^{-8}$**
Blood Eosinophils (x10^9/L: Median & Min-Max)	0.23 (0.0-5.15) (n=155)	0.25 (0.01-5.42) (n=102)	0.2 (0.1-2.60) (n=77)	0.985
****Allergic rhinitis (n): Yes**	892 567 (63.6%)	457 268 (58.6%)	314 163 (51.9%)	**0.001**
****Atopic dermatitis (n): Yes**	383 125 (32.6%)	215 47 (21.9%)	143 26 (18.3%)	**0.001**
Hospital admissions (n): Never Once More than once High dependency/ICU	594 397 (66.8%) 97 (16.3%) 60 (10.1%) 40 (6.7%)	286 193 (67.5%) 52 (18.2%) 27 (9.4%) 14 (4.9%)	196 146 (74.5%) 28 (14.3%) 14 (7.1%) 8 (4.1%)	0.388
Mean ACQ Score 7 (Mean & S.D)	1.95 (1.26) (n=521)	1.99 (1.31) (n=253)	1.77 (1.22) (n=175)	0.165
Mean ACQ Score 6 (Mean & S.D)	1.94 (1.35) (n=531)	2.0 (1.38) (n=263)	1.76 (1.28) (n=185)	0.137
GINA (n): 1 2 3/4 5	807 35 (4.3%) 107 (13.3%) 557 (69.0%) 108 (13.4%)	424 18 (4.2%) 47 (11.1%) 293 (69.1%) 66 (15.6%)	280 11 (3.9%) 40 (14.3%) 194 (69.3%) 35 (12.5%)	0.778

Table 2.4.2 Demographics, clinical and immunological features stratified by age of onset: Data are presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). BMI: body mass index; FEV1pp: forced expiratory volume in 1 s percentage predicted; FVC: forced vital capacity; IgE: immunoglobulin E; ICU: intensive care unit; ACQ: asthma control questionnaire; GINA: global initiative for asthma. *Age of onset- some patients are only

known as childhood onset not the actual age so is classed as <16 therefore, numbers vary for both age of onset variables. **Allergic rhinitis and atopic dermatitis are doctor diagnosis and self-reported.

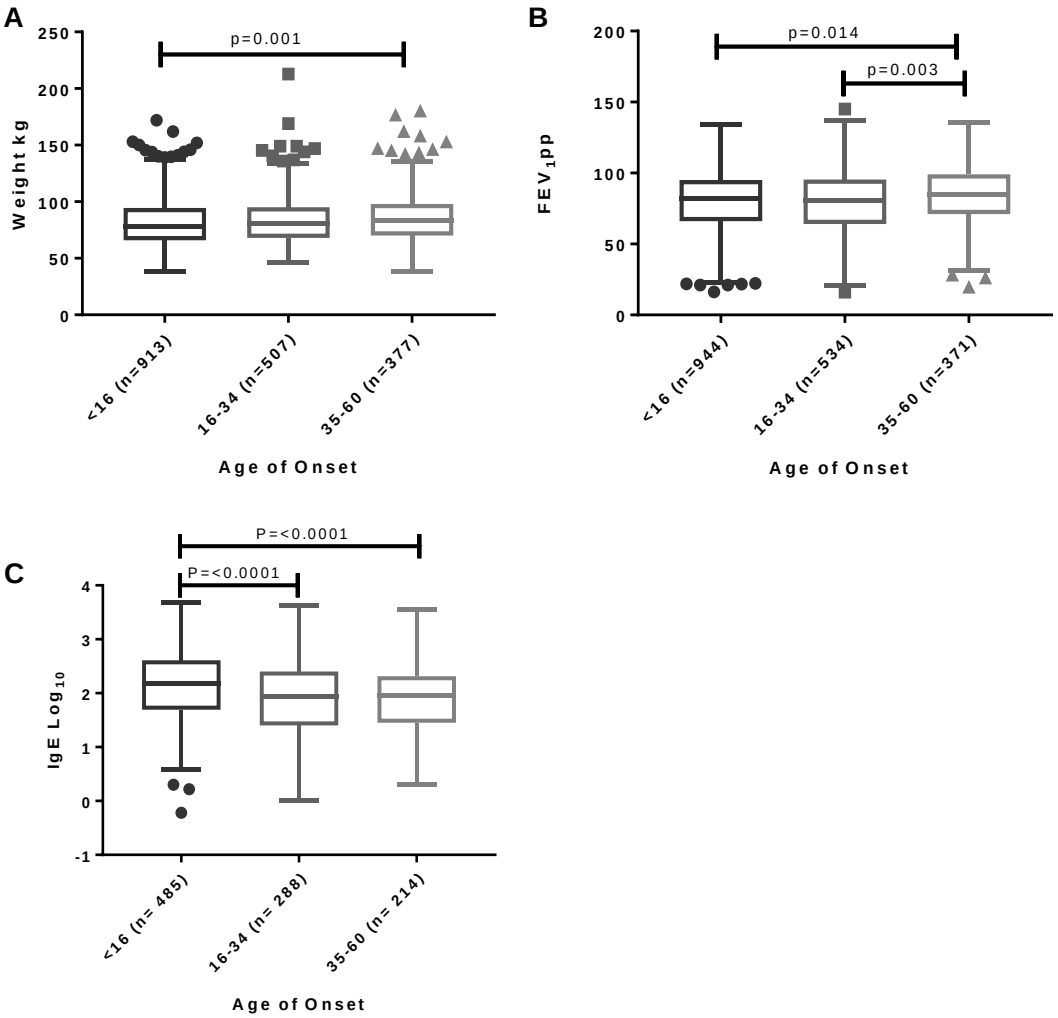


Figure 3.4.2 Clinical and immunological features that made Bonferroni (p=0.003) correction stratified by age of onset: A: weight split by asthma age of onset shows weight is higher in the 35-60 age of onset patients than the <16 patients' p=0.001. B: FEV_1pp split by asthma age of onset shows lung function is lower in the <16 patients compared to the 35-60 p=0.014 and lower in the 16-34 patients compared to the 35-60 patients p=0.003. C: Blood IgE levels split based on asthma age of onset shows IgE levels were higher in the <16 group compared the 16-34 group p=<0.0001 and 35-60 group.

2.4.3 Patients prone to exacerbation are more severe with worse lung function and poor asthma symptom control

Table 3.4.3 shows the demographics, clinical and immunological features stratified based on whether asthma patients have had an exacerbation within the last 12 months. The data shows that those who had an exacerbation are more likely to be younger (mean: 39.6 years, n=347) than those who have not (mean: 43.4 years, n=755), and this was shown to be statistically significant $p=6.5x10^{-7}$. Patients who had an exacerbation had an earlier age of onset of asthma (15.9 years, n=332) than those who have not had an exacerbation (18.6 years, n=711), they were also more likely to be female (75.8%, n=75.8%). Weight was also higher in patients who had exacerbated (86.44 kg, S.D: 22.57, n=309) than patients who had not exacerbated (83.02 kg, S.D: 20.14, n=714). However, these clinical differences between groups did not meet Bonferroni correction. Although, BMI was significantly higher ($p=4.6x10^{-5}$) in patients that have had an exacerbation (29.97 kg/m^2, range: 16.11-57.61, n=303) than those have not exacerbated (28.66 97 kg/m^2, range: 14.84-78.16, n=706) suggesting that patients with a higher BMI are more likely to exacerbate however, the effect size is small. Smoking pack years was slightly higher in patients who have exacerbated (5 pack years, range: 0.15-52.5, n=90) than patients who have not exacerbated (3.63 pack years, range: 0.03-18, n=166), this also did not meet statistical significance.

The data also demonstrated patients who had exacerbated had worse baseline lung function as FEV_1pp showed to be considerably lower in patients that have exacerbated (80.73%, S.D: 20.51, n=316) compared to the group that have not exacerbated (86.46%, S.D: 86.46, n=728), this was seen to be statistically significant $p=2.9x10^{-5}$. More patients had allergic rhinitis in the non-exacerbating group (70.7%, n=473) in comparison to the exacerbation group (62.4%, n=174) however, this was not statistically significant. Additionally, patients that had an exacerbation demonstrated poor asthma control as the data for the mean ACQ6 and ACQ7 score was higher (2.47, S.D: 1.44, n=269 and 2.44 S.D: 1.34, n=259; respectively) in comparison to the patients who have not exacerbated (1.73, S.D: 1.25, n=665 and 1.75, S.D: 1.18, n=648; respectively). Both ACQ scores were shown to be highly statistically significant

$p=3.80x10^{-14}$ (ACQ7) and $p=1.20\ x10^{-14}$ (ACQ6). It is suggested that a ACQ score of 1.5 or more is considered that a patients asthma is poorly controlled (Juniper et al., 2006). Patients who exacerbated were more likely to have severe asthma according to the GINA score than those who have not exacerbated. 26.5% (n=89) of asthma patients who exacerbated were in GINA 5 whereas only 11.9% (n=87) of patients who did not exacerbate were in GINA 5, this was shown to be highly statistically significant $p= 6.28x10^{-12}$. Overall, the data suggests that patients who exacerbate are more likely to have an elevated BMI, have severe asthma based on GINA with a reduced lung function and poor asthma control.

	Never	Ever	P Value
Subjects (n)	762	352	-
Age seen (years: Mean & S.D)	43.4 (11.55) (n=755)	39.6 (12.33) (n=347)	**$6.5x10^{-7}$**
***Age of onset (years: Mean & S.D)**	18.6 (16.02) (n=711)	15.9 (15.03) (n=332)	0.012
***Age of onset (n):** **<16** **16-34** **>35**	711 372 (52.3%) 193 (27.1%) 146 (20.5%)	332 189 (56.9%) 93 (28%) 50 (15.1%)	0.102
Gender (n): **Female**	760 527 (69.3%)	351 266 (75.8%)	0.027
Height (m: Mean & S.D)	1.67 (0.09) (n=712)	1.66 (0.09) (n=316)	0.569
Weight (kg: Mean & S.D)	83.02 (20.14) (n=714)	86.44 (22.57) (n=309)	0.005
BMI (Kg/m^2: Median & Min-Max)	28.66 (14.84-78.16) (n=706)	29.97 (16.11-57.61) (n=303)	**$4.6x10^{-5}$**
Smoking status (n): **Never** **Ex-Smoker** **Current**	760 586 (77.1%) 160 (21.1%) 14 (1.8%)	351 252 (71.8%) 86 (24.5%) 13 (3.7%)	0.060
Smoking Pack Years (Median & Min-Max)	3.63 (0.03-18) (n=166)	5 (0.15-52.5) (n=90)	0.004
FEV_1pp (Mean & S.D)	86.46 (20.14) (n=728)	80.73 (20.51) (n=316)	**$2.9x10^{-5}$**
FEV_1FVC (Mean & S.D)	0.75 (0.11) (n=669)	0.74 (0.1) (n=287)	0.082
Peak Flow (L/min: Mean & S.D)	428.75 (130.37) (n=263)	408.74 (144.76) (n=93)	0.218
Blood total IgE (KU/L: Median & Min-Max)	104.0 (2-3528) (n=238)	167.5 (2-4850) (n=112)	0.131
****Allergic rhinitis (n):** **Yes**	669 473 (70.7%)	279 174 (62.4%)	0.012
****Atopic dermatitis (n):** **Yes**	134 50 (37.3%)	65 27 (41.5%)	0.566
Mean ACQ Score 7 (Mean & S.D)	1.75 (1.18) (n=648)	2.44 (1.34) (n=259)	**$3.80x10^{-14}$**
Mean ACQ Score 6 (Mean & S.D)	1.73 (1.25) (n=665)	2.47 (1.44) (n=269)	**$1.20\ x10^{-14}$**
GINA (n): **1** **2** **3/4** **5**	733 53 (7.2%) 136 (18.6%) 456 (62.3%) 87 (11.9%)	336 4 (1.2%) 35 (10.4%) 208 (61.9%) 89 (26.5%)	**$6.28x10^{-12}$**

Table 3.4.3 Demographics, clinical and immunological features stratified by hospital admissions: Data are presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). BMI: body mass index; FEV1pp: forced expiratory volume in 1 s percentage predicted; FVC: forced vital capacity; IgE: immunoglobulin E; ICU: intensive care unit; ACQ: asthma control questionnaire; GINA: global initiative for asthma**.** * Age of onset- some patients are only known as childhood onset not the actual age so is marked as <16 therefore, numbers vary for both age of onset variables. **Allergic rhinitis and atopic dermatitis are doctor diagnosis and self-reported.

2.4.4 Do clinical variables associated with exacerbation per se show a relationship with the nature of exacerbation?

The clinical features that were statistically significant after Bonferroni correction in table 3.4.3 were then followed up in table 3.4.4 and the patients with exacerbation data were further split into hospital admission frequency for exacerbations and the severity of exacerbations i.e., ICU. Patients that had an exacerbation in the last year were then categorised as having either one exacerbation, more than one or being in the high dependency/ICU.

For age seen patients who had not had an exacerbation were older (43.2 years) than those who have had an exacerbation in all three groups (40, 39.4 and 39.0 respectively) this was still shown to be statistically significant after Bonferroni correction $p=1.5x10^{-5}$. Figure 3.4.4A shows that patients who have never had a hospital admission are significantly older than those who have had one admission (p=0.002), more than one hospital admission (p=0.006) or been in the high dependency/ICU (p=0.021). BMI was higher in the exacerbation groups (30 kg/m^2, 30.41 kg/m^2 and 30.20 kg/m^2 respectively) in comparison to the patients that have not exacerbated (28.66 kg/m^2) however, this was not statistically significant p=0.023.

FEV_1pp data across the four groups showed to decrease quite noticeably the more exacerbations the patient had. Patients who never had an exacerbation had a mean FEV_1pp of 86.46% (S.D:20.14, n=728) whereas, patients who were in the exacerbation groups such as the high dependency/ICU patients had a mean FEV_1pp of 76.14% (S.D:19.92, n=53) (once: 82.62%, S.D: 1.35, n=167 and more than once: 79.98%, S.D: 22.08, n=96) which showed to be significantly lower $p=7.3x10^{-5}$. Figure 3.4.4B shows that patients who have had more than one hospital admission or been in the high dependency/ICU have statistically significant lower lung function than those who have not had a hospital admission (more than once: p=0.017 & high dependency/ICU: p=0.002).

Both mean ACQ scores showed to increase the more exacerbations the patient had (Mean ACQ7: Never: 1.75 S.D: 1.18, n=648, Once: 2.23, S.D: 1.25, n=149, More than once: 2.56, D.D: 1.37 and High dependency/ICU: 2.99,

S.D: 1.57, n=40; Mean ACQ6: Never 1.73, S.D: 1.35, n=665, Once: 2.24, S.D: 1.35, n=154, More than once: 2.65, S.D: 1.46, n=73 and High dependency/ICU: 3.01, S.D: 1.57, n=42) and was also highly statistically significant for both scores $p=4.09x10^{-15}$ (ACQ7) and $p=9.63x10^{-16}$ (ACQ6). Figure 3.4.4C and D shows that patients who have had either one or more than one hospital admission, had a statistically significantly higher ACQ 6 and 7 score than those who have never had a hospital admission (ACQ6 & 7: p=<0.0001). Additionally, Figure 3.4.4C and D shows that patients who had been admitted to ICU had significantly higher ACQ6 & 7 score than those who had never had a hospital admission or who had one hospital admission (ACQ6 & 7: p=<0.0001 and p=0.003 respectively).

Lastly GINA severity was also seen to be highly significant $p=4.33x10^{-16}$ across the hospital admission groups. 49.2% (n=30) of patients in the High dependency/ICU group were GINA 5 patients whereas, patients who exacerbated once only 16.2% were (n=28) in GINA 5. This supports the data in table 3.4.3.1 and illustrates those patients who have frequent or are admitted into high dependency/ICU are more likely to be younger, have very severe asthma with lower lung baseline function and also poor symptom control in comparison to those who have not exacerbated.

	Never	Once	More than once	High dependency/ ICU	P-Value
Subjects (n)	762	185	104	63	-
Age seen (years: Mean & S.D)	43.4 (11.55) (n=755)	40.0 (12.38) (n=181)	39.4 (11.77) (n=103)	39.0 (13.12) (n=63)	**$1.5x10^{-5}$**
BMI (Kg/m^2: Median & Min-Max)	28.66 (14.84-78.16) (n=706)	30.0 (16.11-57.61) (n=162)	30.41 (19.38-55.17) (n=88)	30.20 (16.85-54.90) (n=53)	0.023
FEV_1pp (Mean & S.D)	86.46 (20.14) (n=728)	82.62 (1.35) (n=167)	79.98 (22.08) (n=96)	76.14 (19.92) (n=53)	**$7.3x10^{-5}$**
Mean ACQ Score 7 (Mean & S.D)	1.75 (1.18) (n=648)	2.23 (1.25) (n=149)	2.56 (1.37) (n=70)	2.99 (1.44) (n=40)	**$4.09x10^{-15}$**
Mean ACQ Score 6 (Mean & S.D)	1.73 (1.25) (n=665)	2.24 (1.35) (n=154)	2.65 (1.46) (n=73)	3.01 (1.57) (n=42)	**$9.63x10^{-16}$**
GINA (n):	733	173	102	61	
1	53 (7.2%)	4 (2.3%)	0	0	
2	136 (18.6%)	22 (12.7%)	9 (8.8%)	4 (6.6%)	**$4.33x10^{-16}$**
3/4	456 (62.3%)	119 (68.8%)	62 (60.8%)	27 (44.3%)	
5	87 (11.9%)	28 (16.2%)	31 (30.4%)	30 (49.2%)	

Table 3.4.4 Clinical features of asthma patients stratified by hospital admission frequency: Data are presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). BMI: body mass index; FEV1pp: forced expiratory volume in 1 s percentage predicted; ICU: intensive care unit; ACQ: asthma control questionnaire; GINA: global initiative for asthma**.** P= 0.008 with Bonferroni correction.

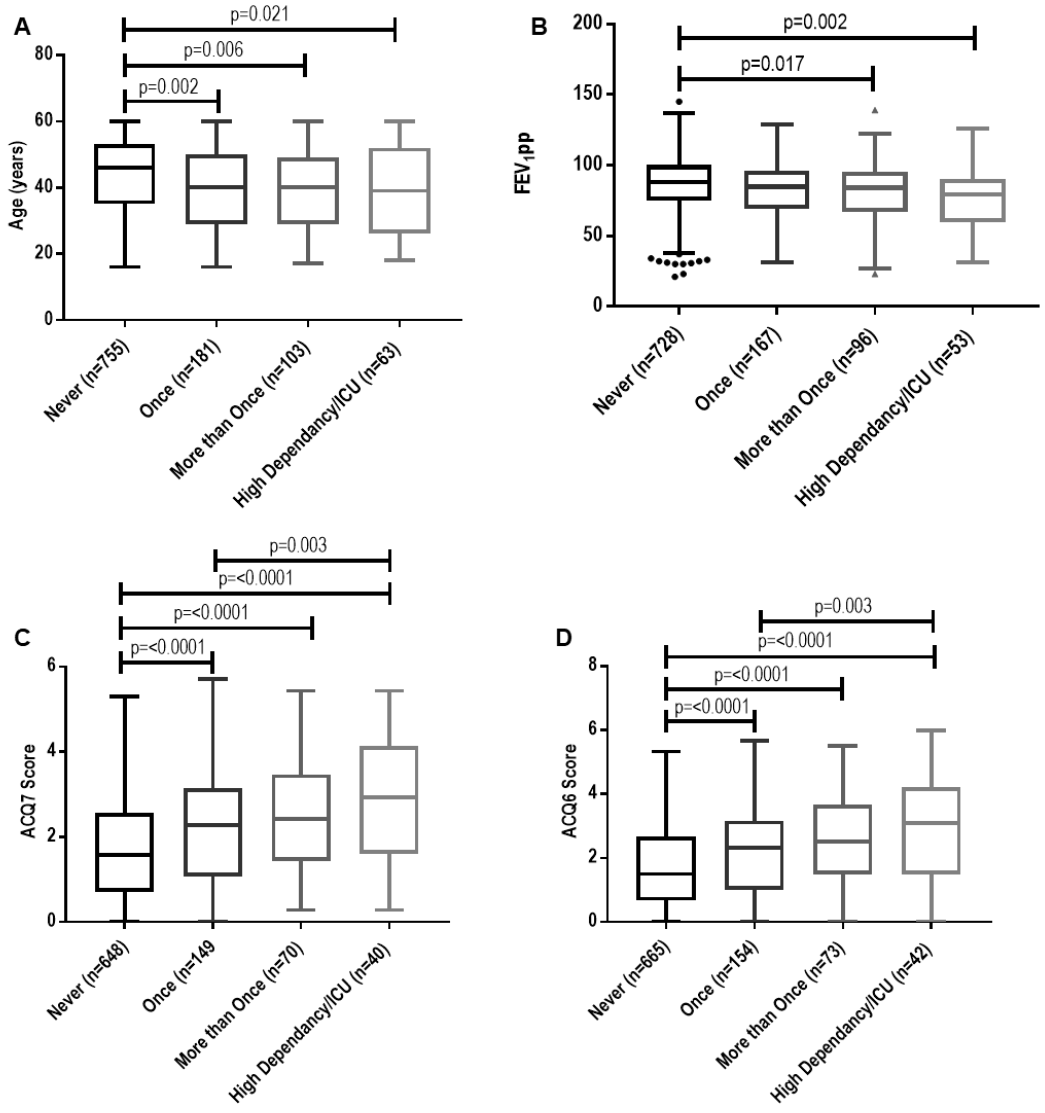


Figure 3.4.4 Clinical and immunological features that made Bonferroni (p=0.003) correction stratified by hospital admission frequency: A: age seen (years) split by hospital admissions shows patients who have never had a hospital admission tend to be older than those who had one or more hospital admissions (p=0.002, p=0.006 & p=0.021). B: FEV_1pp split by hospital admissions shows lung function declines as the frequency of hospital admissions increases (p=0.017 and p=0.002). C: ACQ7 score split based on hospital admissions shows ACQ7 score increases as hospital admission frequency increases (p=<0.0001 & p=0.003). D: ACQ6 score split based on hospital admissions shows ACQ7 score increases as hospital admission frequency increases (p=<0.0001 & p=0.003). E: GINA split based on hospital admissions shows patients who have had more hospital admissions or have been in high dependency/ICU are more likely to be severe asthma patients (GINA 5) $p=4.33x10^{-16}$).

2.4.5 Blood eosinophils do not identify distinct asthma patient subsets

Table 3.4.5 shows the demographics, clinical and immunological features of asthma patients stratified based on whether they meet the blood eosinophil count criteria for the monoclonal anti-IL-5 drug Mepolizumab. The current blood eosinophil count for the Mepolizumab drug according to the GINA report (GINA, 2020) is ≥300 cells/µL. 811 patients from the GASP cohort have blood eosinophil counts, of which 304 meet the blood eosinophil count for the drug. The asthma patients who meet the eosinophil count criteria have a lower smoking pack years (9.25 packs a year, range: 0.05-120, n=70) compared to the other group who do not meet the eosinophil count (20 packs a year, range: 0.04-108, n=110), this was shown to be statistically significant after Bonferroni correction p=0.0003 as shown in Figure 3.4.5A. Additionally, total blood IgE levels are higher in the >300 cells/µL group (250.0 KU/L, range: 1.66-4700, n=245) compared to the <300 cells/µL group (109.0 KU/L, range: 1-4900, n=390) which was also statistically significant, p= 7.30×10^{-7} as displayed in Figure 3.4.5B. Overall the data shows that blood eosinophil count does not identify an asthma sub-phenotype that is blood eosinophil high and clinically distinct.

	<300 cells/µL	>300 cells/µL	P Value
Subjects (n)	507	304	-
Age seen (years: Mean & S.D)	45.07 (10.87) (n=505)	44.38 (11.34) (n=303)	0.391
***Age of onset (years: Mean & S.D)**	21.30 (15.91) (n=196)	19.48 (16.40) (n=138)	0.309
***Age of onset (n):** **<16** **16-34** **>35**	196 90 (45.9%) 58 (29.6%) 48 (24.5%)	138 65 (47.1%) 44 (31.9%) 29 (21%)	0.745
Gender (n): **Female**	507 353 (69.6%)	304 197 (64.8%)	0.155
Height (m: Mean & S.D)	1.65 (0.09) (n=459)	1.66 (0.1) (n=267)	0.147
Weight (kg: Mean & S.D)	79.57 (20.47) (n=460)	80.5 (21.75) (n=266)	0.563
BMI (Kg/m^2: Median & Min-Max)	28.17 (15.41-51.53) (n=456)	27.52 (17.78-60.24) (n=264)	0.430
Smoking status (n): **Never** **Ex-Smoker** **Current**	503 276 (54.9%) 121 (24.1%) 106 (21.1%)	303 166 (54.8%) 85 (28.1%) 52 (17.2%)	0.265
Smoking Pack Years (Median & Min-Max)	20.0 (0.04-108) (n=110)	9.25 (0.05-120) (n=70)	**0.0003**
FEV_1pp (Mean & S.D)	75.42 (24.11) (n=424)	71.59 (22.57) (n=219)	0.052
FEV_1FVC (Mean & S.D)	0.67 (0.14) (n=469)	0.66 (0.13) (n=279)	0.273
Change in FEV_1% Reversibility (Median & Min-Max)	7.86 (-8.79-61.48) (n=155)	8.17 (-26.28-68.75) (n=122)	0.398
Peak Flow (L/min: Mean & S.D)	361.74 (126.41) (n=374)	362 (127.8) (n=176)	0.982
Blood total IgE (KU/L: Median & Min-Max)	109.0 (1-4900) (n=390)	250.0 (1.66-4700) (n=245)	**$7.30x10^{-7}$**
****Allergic rhinitis (n):** **Yes**	223 108 (48.4%)	162 89 (54.9%)	0.207
****Atopic dermatitis (n):** **Yes**	193 68 (35.2%)	93 30 (32.3%)	0.619

Table 3.4.5 Demographics, clinical and immunological features stratified on the eosinophil count criteria for the monoclonal anti-IL-5 antibody drug: Data are presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). BMI: body mass index; FEV1pp: forced expiratory volume in 1 s percentage predicted; FVC: forced vital capacity; IgE: immunoglobulin E. P= 0.003 with Bonferroni correction. * Age of onset- some patients are only known as childhood onset not the actual age so is marked as <16 therefore, numbers vary for both age of onset variables. **Allergic rhinitis and atopic dermatitis are doctor diagnosis and self-reported.

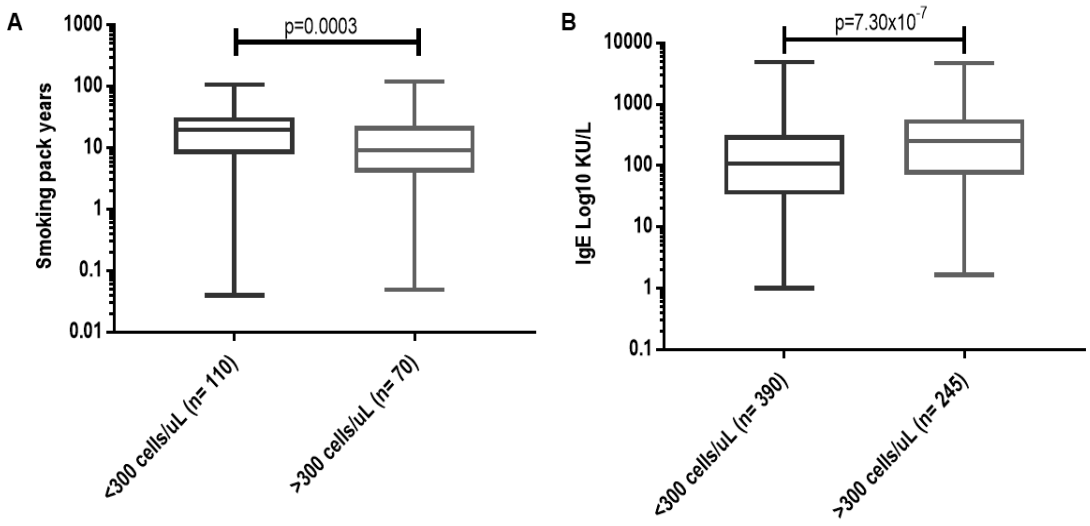


Figure 3.4.5 Clinical and immunological features that made Bonferroni (p=0.003) correction stratified by blood eosinophil count criteria for the monoclonal anti-IL-5 antibody drug: A: smoking pack years split by eosinophil count shows patients who have a higher eosinophil count have lower smoking pack years than patients who have a low eosinophil count p=0.0003. B: IgE levels split by eosinophil count shows IgE levels are higher in patients who have a higher eosinophil count than those with a lower eosinophil count $p=7.30x10^{-7}$.

2.4.6 Summary Table

Table 4.6.1 describes are the key findings from the three clinical questions asked around asthma age of onset, hospital admissions and patients who are eosinophilic high (>300 µl/cells).

Childhood Asthma	Exacerbation Prone	Eosinophilic High
Higher serum IgE levels	Reduce lung function in those with increased frequency of exacerbations	Higher serum IgE levels
Lower smoking pack years	Higher mean ACQ score and score increased with exacerbation frequency	Lower smoking pack years
High prevalence of atopy	Lower age seen	
Reduce baseline lung function	More severe GINA 5 patients	

Table 3.4.6 Summary table of clinical and immunological features that define groups based on the three clinical questions related to age of onset, hospital admissions and eosinophilia.

2.5 Discussion

The aims of this chapter were to answer clinically relevant questions and provide new insight into looking at the clinical presentation of i) adult-onset asthma verses childhood onset, ii) exacerbation prone patients and iii) patients who meet the blood eosinophil criteria for the Mepolizumab drug. The main findings were that childhood onset of asthma presented more atopy compared to the adult-onset groups. Patients that were prone to exacerbations where more severe according to GINA score, had a higher ACQ score and noticeably reduced lung function. Additionally, as the frequency of exacerbation history increases lung function gradually declines and ACQ score slowly increased. A specific sub-phenotype of patients was not identified in eosinophilic high patients, other than they had higher serum IgE levels and lower smoking pack years.

To date this is the largest UK asthma cohort study with 4,205 patients; 78.4% were moderate to severe asthma patients. The asthma patients in the GASP cohort overall shows to be predominantly females, have a high BMI and with some evidence of airflow obstruction. The majority of the GASP cohort had never smoked or were ex-smokers and showed some evidence of having poorly controlled asthma. Some of the demographic and clinical features found in GASP are also similar in other asthma cohorts such as the uBIOPRED and SARP. uBIOPRED had 610 adults (421 severe asthma, 88 mild/moderate and 101 controls) with the severe asthma patients being predominantly females (59%), had a high BMI of ~29 kg/m^2, majority were non-smokers (only 42 current smokers) and they also showed evidence of airflow obstruction with the mean FEV_1/FVC in the severe group being 0.64 (severe non-smoking group n=311) and 0.72 in the mild/moderate group (Shaw et al., 2015). The SARP I cohort is a severe asthma cohort with 438 adult asthma patients (204 severe, 70 moderate and 164 mild) however, to date the SARP III cohort has now 526 adult asthma patients (Teague et al., 2018). The SARP I has a predominance of females (72% mild, 56% moderate and 64% severe; p=0.05), majority were non-smokers (<5 pack years) and also showed a decline in FEV_1/FVC% across asthma severity (80% mild, 67% moderate and 65% severe; p=<0.0001) (Moore et al., 2007). The main similarities between the 3

cohorts are that they mostly have moderate-severe asthma patients and show some sort of airway obstruction. Both cohorts also had additional features such as skin prick tests and uBIOPRED had blood neutrophil counts, FENO levels and sputum eosinophil and neutrophil counts which GASP did not have. Overall GASP captured the majority of the same demographics and clinical features as the other two cohorts but in larger quantities.

It has been known that childhood asthma is different to adult-onset asthma since the 1960's e.g. childhood asthma patients are more atopic and adult onset. Methods such as cluster analyses, GWAS genetic studies have helped further define childhood and adult asthma more accurately. Additionally, heritability estimates are also higher for childhood onset of asthma as genetics seem to play a greater role in childhood onset, as asthma and allergic diseases in childhood are highly heritable and many these genetic variants are in the Type 2 pathway (Ullemar et al., 2016). Patients with childhood asthma onset are less severe compared to adult onset and the majority of childhood onset asthma manifests as an allergic phenotype and allergic comorbidities such as eczema and allergic rhinitis. Additionally, children with persistent wheeze throughout childhood have increased risk of developing childhood asthma (Dharmage et al., 2019). As for adult onset of asthma it is usually characterised as predominately obese females which may be non-atopic, have severe eosinophilic inflammation and/or airflow limitation (Amelink et al., 2013) (Teague et al., 2018). One potential biased finding which needs to be addressed is the age seen, this was shown to be highly statistically significant. This is most likely to be because the older age of onset coming into hospital or GP in later life for diagnosis and so will therefore be enrolled into the study at an older age.

The GASP cohort showed the early onset of asthma group being more atopic than the tow adult-onset groups. This confirms previous studies that have identified clusters of childhood onset patients that are more atopic and display atopic comorbidities such as allergic rhinitis (Fitzpatrick et al., 2011) (Fleming et al., 2015). Another clinical study showed atopy was also observed in individuals with early onset of asthma in comparison to late onset of asthma. The study also found that early onset of asthma patients had higher numbers

of hospital admissions in the past year compared to the late onset of asthma patients (Chaudhuri et al., 2016) however, this feature was not seen in the GASP cohort. This demonstrates that atopy in childhood onset adds further burden to the treatment of the disease and also suggests that “atopic march” plays a big part of the development of asthma in children.

As for the adult-onset groups, it was demonstrated that there was a higher predominance of females, increase in weight, along with a higher smoking pack years and lower lung function. These features of adult onset of asthma are in good agreements with multiple studies that have looked at the clinical characteristics between early and late age of onset of asthma. For example one study showed that the late onset individuals were also predominantly females and current smokers in comparison to the early age of onset group (Tan et al., 2016), in the GASP cohort the childhood onset group had lower smoking pack years suggesting that asthma patients are less likely to smoke as an adult if they have childhood onset of asthma. Another study identified that with advancing age patients have greater airflow limitation, less allergen sensitisation and are more obese (Teague et al., 2018). One other study identified three phenotypic clusters related to adult onset of asthma one of which demonstrated a persistent airflow limitation with high eosinophilic asthma and one other cluster was characterised with obese females (Amelink et al., 2013). It is thought that sex hormones play a role in asthma pathogenesis as adult onset of asthma has shown to be more predominant in females. Various factors such as changes in ovarian hormone concentration during the menstrual cycle, pregnancy and menopause in women may affect asthma symptoms and control. Mouse models have shown that ovarian hormones are important in inducing allergic airway inflammation (Riffo-Vasquez et al., 2007). Oestrogen has also been studied in mouse models and has shown that the oestrogen receptor-α is critical in regulating airway responsiveness (Carey et al., 2007). Neutrophils have been shown to be more highly activated in obesity in circulation and in adipose tissue vasculature, this indicates the low grade inflammation seen in obesity is likely from the result of chronic activation of the innate immune response which then may extend to the airways in some individuals (Shah et al., 2010). It has also been suggested

that leptin and other adipokines may be important mediators of airway disease in obese patients with asthma (Sideleva et al., 2012). Finally, it is thought that the relationship between obesity and asthma could be due to changes in energy relating hormones and comorbidities of obesity that could worsen asthma particularly gastroesophageal reflux disease (GERD) and Type 2 diabetes (Shore, 2008).

GASP showed patients in the 16-34 adult-onset group had the lowest FEV_1pp additionally, PEF was also lower in the adult onset groups this however, did not meet Bonferroni correction but suggests there may be more airflow obstruction due to older age, from smoking and even other undiagnosed lung comorbidities such as COPD. It is possible that many of the older onset of asthma patients would meet the criteria for COPD based on the spirometry results. Asthma and COPD can present with overlapping clinical features making distinguishing between the two challenging. A proportion of patients are sometimes diagnosed with asthma COPD overlap syndrome (ACOS) as they have overlapping phenotypes of both asthma and COPS. Therefore it is possible that some of these asthma patients may have ACOS (Corlateanu et al., 2017). Finally, a reduced airflow could be due ongoing airway inflammation and hyperresponsiveness (BRINKE et al., 2001). Overall, the clinical features identified in childhood and adult onset of asthma group in the GASP cohort showed to be on par with features found in other asthma cohort studies and demonstrate just how heterogeneous asthma is.

Asthma exacerbations is a critical clinical question as there is great interest in studying the group of asthma patients that exacerbate and whether there are any predicting factors for future exacerbations. One study identified clinical features in exacerbation-prone asthma patients in the context of race and found that as well as having common risk factors such as exacerbation history of 2 or more in the last year and a lower baseline lung function, black patients also had unique risk factors. Some of those risk factors include allergic rhinitis and chronic sinusitis (Grossman et al., 2019). Firstly, the significant finding of age seen between never and ever exacerbating is most probably biased. This is due to the fact patients that come into clinic or hospital from an exacerbation

are likely to be earlier in life as their asthma is not controlled and so end up in this study.

Studies have shown that patients who are prone to exacerbate are generally more severe, have a reduce in lung function and poor symptom control (Suruki et al., 2017), (Ortega et al., 2018) (Uematsu et al., 2018). This cohort demonstrated similar findings, between the never and ever exacerbating groups. The patients that had an exacerbation with the last year were more severe (GINA 3/4 or 5) with a lower FEV_1pp, a higher mean ACQ score, BMI and younger. Some additional findings such as smoking being a risk factor for an exacerbation has also been seen in other studies (Silverman et al., 2017). Obesity has been known to worsen asthma symptoms and also make them less responsive to ICSs and LABAs therefore increasing the risk of an exacerbation (Holguin et al., 2011) (Boulet and Franssen, 2007). When hospital admissions were broken down into frequency of hospital admissions, these significant clinical findings mentioned above showed a clear trend across the four hospital admission groups. Lung function declined rapidly as hospital admission frequency increased, mean ACQ score increased significantly across the groups and nearly 50% of the patients that were in high dependency/ICU were in GINA 5. This supports other studies suggest that severe asthma patients with frequent exacerbations along with poor lung function and symptom control could be predictive factors for future exacerbations in asthma patients. This suggests that these patients who are prone to exacerbation do not have their asthma very well controlled or may not be responsive to the current therapy they are on. This indicates the need for new asthma drugs that will target these group of patients prone to multiple exacerbations and/or severe exacerbations.

There are multiple cluster analysis studies that have identified groups of patients that have eosinophilic asthma meaning they have high eosinophil levels in the blood (Haldar et al., 2008) (Nadif et al., 2018). Patients in the higher eosinophil group were observed to see how they differ clinically to eosinophilic low patients. As previously mentioned, eosinophils play a big role in the inflammation of asthma and high eosinophil levels is usually associated with severe asthma and Th2 endotype asthma. Eosinophils are a commonly

used biomarker in measuring severe asthma as well as being a requirement for the new biological anti-IL-5 agent Mepolizumab (Matucci et al., 2018). The use of blood eosinophil biomarker for airway eosinophilia is based upon the relationship between blood and sputum eosinophil counts, however airway eosinophils are thought to be a better reflection on airway inflammation and so a high blood eosinophil levels may indicate airway eosinophilia (Pavord et al., 2012) (Bakakos et al., 2019). It has been shown that there is some correlation between sputum and blood eosinophils in asthma patients ($r=0.68$; $p=<0.0001$) and so supports the fact that a high blood eosinophil count reflects airway eosinophilia (Pignatti et al., 2019).

Patients that had blood eosinophils data were split into two groups based on the blood eosinophil count criteria for the Mepolizumab drug. The data showed that blood IgE levels were higher in higher eosinophil group compared to the lower eosinophil group. Blood eosinophil levels has shown to be associated with blood IgE levels, one study identified this association between blood and sputum eosinophil count and serum IgE with severe asthma ($p=0.002$) (Kumar et al., 2017). This association between IgE and eosinophils levels is likely due to Th2 cells being involved in controlling and producing IgE because Th2 cells produce IL-4 and IL-13, which then influences the functioning and production of eosinophils through the actions of IL-5 (Matucci et al., 2018). High eosinophil counts and IgE levels are usually associated with patients who have atopy particularly those with early onset of asthma so an allergy stimulus would therefore produce more IgE which in turn then produces eosinophils. However, no association was found between age of onset and atopy with eosinophil count in this cohort. Finally, the data from GASP showed that high smoking pack years was seen to be statistically significant with a lower blood eosinophil count). Studies has shown that cigarette smoke has effects on the immune inflammatory response. One study indicated that cigarette smoke regulates inflammatory mechanisms of eosinophil migration therefore reducing the number of eosinophils (Botelho et al., 2011). In COPD patients where the majority are smokers, eosinophil levels have been shown to be lower in the blood which suggests that smoking has an effect on eosinophil levels (Oliver et al., 2019). Another study looking at whether blood eosinophil and monocyte

counts are related to smoking also demonstrated the same effect and found that patients who smoked had lower blood eosinophil levels and when patients stopped smoking their blood eosinophil count increased (Jensen et al., 1998). This suggests that not only smoking has a huge impact on health and asthma, but it may also affect asthma drug treatment choices especially for the new biologicals coming into clinic where blood eosinophil count is a commonly used biomarker. Overall, the data shows that a high blood eosinophil count does not identify an asthma sub-phenotype that is clinically distinct.

A limitation to this part of the study was the majority of the cohort are moderate to severe asthma patients which have been recruited from a hospital setting and so it could be seen biased when looking at the effects of asthma severity with some of the clinical features described above. However, this is still the largest moderate to severe asthma cohort in the world currently with a large amount of clinical and immunological data. Additionally, the cohort shows similarities to other severe asthma cohorts regarding the clinical characteristics and so it shows the GASP cohort is a good representation of moderate to severe asthma patients.

In summary, GASP has identified that childhood onset of asthma is more likely to be atopic than the adult onset of asthma who are more likely to smoke, be heavier and have lower lung function. Patients who had a hospital admission within the last 12 months had a high GINA score, reduce lung function and high ACQ score compared to those who never had one. Finally, patients who were eosinophilic high did not identify as a clinically distinct sub-phenotype of patients.

Chapter 3- Can serum biomarkers help define asthma subgroups?

3.1 Introduction

Many approaches have been used to define asthma into subgroups including clinical, immunological and transcriptomic data. Studies have used clinical (e.g. demographic, lung function and atopy) and immunological data (e.g. total serum IgE and blood eosinophils) from asthma cohorts to preform cluster analysis (Haldar et al., 2008) (Moore et al., 2010). This has identified subgroups of asthma patients (see chapter 1.3 for more detail) such as childhood atopic asthma, high eosinophilic asthma, and obese non-eosinophilic asthma. Transcriptomics, which is the study of gene expression in blood, sputum, tissue etc. has also been used to identify subsets of asthma. U-BIOPRED used blood transcriptomics identified differences in gene expression between subjects with and without asthma (n= 610) and identified 1,693 differentially expressed genes in the severe asthma group vs the control group (Bigler et al., 2017). The study however was not able to match these transcriptomic changes with any specific clinical cluster stressing the complexity and incomplete understanding of asthma. Serum and blood biomarkers are now being used as approaches to further identify subgroups of asthma patients. Serum IgE is already used as a biomarker for atopy and treatment response and has correlated positively with asthma severity (Kuruvilla et al., 2019). The development of specific blood serum or plasma biomarkers are particularly attractive because they reflect systemic pathological changes and are relatively easy to access compared to lung samples. This study set out to look at cytokines and small signalling proteins of the Th1, Th2 and Th17 pathway as serum biomarkers of asthma patients.

3.1.1 The Importance of Using Biomarkers in Asthma for Stratified Medicine, Aiding Diagnosis and Guide Asthma Treatment

A biological biomarker (biomarker) is a measurable indicator of a biological state or condition and is mostly measured in biological fluids such as blood, sputum or urine (Califf, 2018). Biomarkers are important in the development of medical diagnostics, prognostics and therapeutics. However, there is still a

sensitivity issue that exists due to low abundance of biomarkers particularly cytokines and the sensitivity of existing technologies (Alam et al., 2017) (Akiki et al., 2017). Additionally, a potential problem for the use of cytokines as disease serum biomarkers is the lack of organ specificity since serum cytokine levels can change in response to injury and damage in all tissue types (Yi et al., 2018). Biomarkers have helped in the phenotyping and endotyping of asthma and only a few biomarkers that reflect the Type 2 airway inflammation are currently readily available for clinical use.

As treatments that target the Type 2 inflammation pathway become more available in clinic, it is important to know which patients have Type 2 inflammatory asthma so clinicians can identify who may benefit from these new therapies. Currently blood and sputum eosinophils are used as biomarkers for many of the new biologics such as Mepolizumab (see chapter 4.1.1 for other current Type 2 biomarkers and more on blood eosinophils as a biomarker). However, blood and sputum eosinophil levels have shown to be very variable and also non-specific as high levels can be caused by several types of inflammation not just asthma (see chapter 2.1.3 for more detail on eosinophil variability). However, there is still a great unmet need for biomarkers that reflect the non-Type 2 inflammation pathway (see chapter 1.2.1 for Th2 inflammation).

Stratified medicine is based on identifying patients with specific disease mechanisms and their response to treatments to ensure patients get the right treatment that is suited for them. Biomarkers in asthma may have diagnostic properties that help reflect underlying airway inflammation and clinical presentation. For example sputum neutrophilia is a good indication of airway neutrophilia and severe asthma which is unrelated to Type 2 inflammation whereas sputum eosinophilia indicates Type 2 inflammation (Wan and Woodruff, 2016). They may also help predict and/or identify patients who would respond to specific therapies and/ or prognostic properties that identify patients at risk of poor outcomes such as asthma exacerbation. Additionally, there are a few biomarkers that have pharmacodynamic properties which would be helpful in measuring the biological response to therapy (Narendra et al., 2019) (Califf, 2018). The identification of biomarkers can help with stratified

medicine as they potentially aid diagnosis, guide treatment decisions and allow clinicians to predict and monitor response to treatment.

3.1.2 Measuring Serum Cytokines and Small Signalling Proteins in asthma patients to guide treatment response

Measuring cytokines in the serum of asthma patients is not commonly used however, cytokines are key to recently developed asthma monoclonal antibody therapies. Some of these therapies may not be effective in all asthma patients, therefore measuring cytokine levels could provide useful information for understanding the pathologic process and monitoring of disease progression and inflammation. It is already well known that cytokines IL-4, IL-5 and IL-13 are the key mediators of Type 2 inflammation along with IL-33, TLSP, ST2, periostin and eotaxin also being involved either upstream or downstream of the Type 2 pathway. Whereas non-Type 2 inflammatory cytokines such as IL-6, IL-17A and IFN-γ are involved in neutrophilic, Th17 and Th1 inflammation pathways respectively as further discussed in chapter 1.2 (Fahy, 2015) (Athari, 2019). (See chapter 1.2 for more in-depth detail on Th1, Th2 and Th17 inflammation). Little is known about the association between serum cytokine levels and the effect of asthma symptom control such as lung function. Cytokine levels are often measured in induced sputum but measuring cytokines in serum could be an alternative as blood collection is less invasive and more practical in large studies or clinical practice. Measuring cytokines in serum also has its limitations as cytokine levels can increase after acute or chronic infections, tissue injury, autoimmune diseases and other disorders. Cytokines have also been known have short half-lives for example IL-13 and IL-4 (Perkins et al., 2006) (Hall et al., 2017) in peripheral blood as levels start to decrease when left at room temperature. Thus serum should be separated from blood cells quickly to prevent degradation or absorption (Zhou et al., 2010).

Additionally, cytokine levels can be affected by drugs particularly steroid treatments as it is known that they suppress cytokines. One study demonstrated the effects of corticosteroids on cytokine production from asthma lung lymphocytes and showed that treatment with Dexamethasone had reduced effects on IL-13 in moderate severe asthma patients compared

to the controls (p= 0.01) (Kaur et al., 2014). Other pre-analytical factors such as physical activity, blood collection, handling, processing and storage can have an effect on the blood cytokine levels (Zhou et al., 2010). Additionally, in general the levels of many cytokines circulating in the blood of either healthy or diseased patients are below the detection limit of many commercially available assay kits and the cytokines that can be measured in the blood can also show considerable variability (Aziz, 2015).

Studies have measured cytokines and small signalling proteins such as periostin, IL17, IL8, IL-13, and IL-5 using ELISA's and multiplex assays. One study of 110 patients (60 refractory asthma, 30 with non-refractory asthma and 20 healthy controls) used a multiplex assay to measure 244 biomolecules in BALF and serum and BALF immune cells. Although they managed to measure many of the cytokines they did however, find difficulty in measuring and detecting Th2 cytokines IL-13, IL-5 and IL-4. Their data found 13 of the biomolecules showed a significant positive correlation with the BALF neutrophils including IL-8 and IL-6 (R= 0.7 and R= 0.52; respectively p= <0.001) (Alam et al., 2017). However, another study has been able to measure cytokines such as TLSP and IL-13 using an ELISA assay (Lee et al., 2016).

Serum periostin is a matricellular protein secreted by airway epithelial cells in response to IL-13 and so it is thought that periostin could be a surrogate marker for IL-13 activity (Narendra et al., 2019). Serum periostin has been used as a predictive biomarker of treatment response for Lebrikizumab (anti-IL-13). One study showed that patients with high serum periostin levels had improved change in percentage FEV_1 with Lebrikizumab versus the placebo (37.5 mg: 6.8%, 125 mg: 10.7% and 250 mg: 10.1%) compared to those who had low periostin levels (37.5 mg: -1.9%, 125 mg: 2.7% and 250 mg: 7.2% (Figure 3.1.2) (Hanania et al., 2015). Another study that looked at biomarkers that predicted the response to Omalizumab demonstrated that patients with high periostin levels of ≥50 ng/mL had a more noticeable reduction exacerbation rate of in comparison to patients with low levels of periostin (Hanania et al., 2013). However, the limitations with using serum periostin is that levels may fluctuate with age and also may be elevated in other diseases such as atopic dermatitis, cancer, idiopathic pulmonary fibrosis which may

affect its ability to be an independent biomarker for asthma. Although periostin may have good prognostic and pharmacodynamic properties, it currently still remains an investigational biomarker (Narendra et al., 2019).

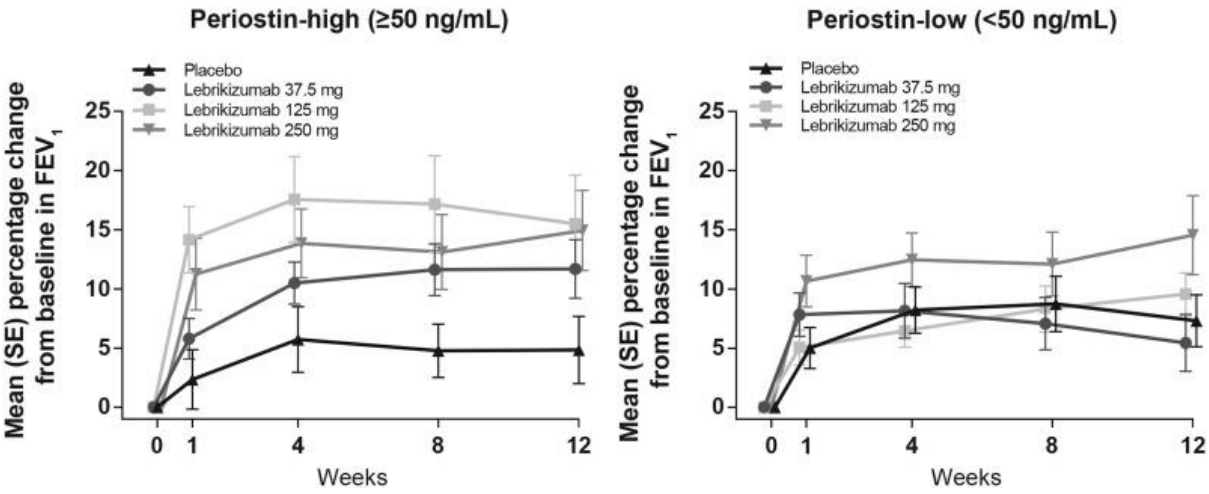


Figure 3.1.2 The mean relative change in percentage FEV_1 from baseline up to week 12 for the placebo and 3 different Lebrikizumab dosage groups in periostin-high and periostin-low patients. Taken from (Hanania et al., 2015).

3.1.3 Utilising a Subset of the GASP Cohort with Sera to Further Define Asthma Subgroups

As mentioned in chapter 2.1.4 the GASP cohort consists of UK non-related asthma patients aged 16-60 and a subset of these asthma patients have serum samples available (n=708). However, for this part of the study patients who are ex-smokers or current smokers who had smoking pack years of more than 10 were excluded (n=19). This is due to the high smoking pack years effecting lung function values and potentially cytokine levels in the blood. With many of the new biologics coming into clinic targeting the Type 2 inflammation pathway, it is still unknown who will benefit from these new therapeutic drugs. In this study IL-4, IL-5, IL-13, IL-33, TLSP, ST2, Periostin, Eotaxin, IL-6, IL17A and IFN-ɣ were measured in the sera from a custom made Luminex assay from R&D Systems. Looking at the different cytokines and small signalling proteins from different inflammation pathways may give insight to see whether patients express more Type 2 or non-Type 2 biomarkers and therefore may also suggest what type of inflammation they have. Additionally, these

biomarkers can see if they identify any clinical features of asthma such as hospital admissions, ACQ score and lung function. A discovery and replication cohort will also be created from the GASP cohort in order to provide confidence in findings.

3.2 Hypothesis

The hypothesis underlying this work is that cytokine and/or protein biomarkers in the serum of asthma patients can identify subsets of asthma patients with different inflammatory drivers and disease presentation.

3.2.1 Aims

1. Define the relationship between the Type 2 and non-Type 2 biomarkers and determine whether they are co-ordinately expressed in the asthma patients.
2. Identify the inflammatory signature associated with different clinical asthma patient groups:
    I. GINA asthma severity score
    II. Exacerbation prone
    III. Lung function
    IV. Asthma control questionnaire (ACQ)

3.3 Methods

Patients that had serum samples were selected from the GASP cohort of 4,205 (see chapter 2.3 for the inclusion criteria for the GASP cohort (Figure 2.3.1):). The study aimed to have two representative cohorts (discovery and replication) of a cross section of asthma patients. This section describes and explains the inclusion and exclusion criteria for the serum biomarker study and the Luminex assay principle and procedure that was used to measure the biomarkers in the sera.

3.3.1 Inclusion and exclusion criteria for the serum biomarker study

To be included in the study patients had to meet to the following criteria under ethics GM129901 which is described in Figure 3.3.1. Patients had to have physician-diagnosed asthma of at least one year and had to be on prescribed medication for asthma. Asthma patients had to also be between the ages of 16 to 60 years old. Patients over the age of 60 were not recruited because older patients generally have a decline in lung function with age and also are at more risk of have other respiratory comorbidities. All patients had to be non-related meaning only one patient from each family could be involved in the study. Patients recruited also had to be of European descent of at least two generations therefore both parents and grandparents have to be European. Patients from other ethnic groups were not included due to the fact it would lead to a relatively small numbers of these different groups. This was thought to be unethical as it exposes them to inconvenience with no prospect of comprising a sufficiently large, ancestrally similar, population for useful results to be obtained.

Patients were excluded from the study if they had a known highly transmissible or serious blood-borne infection that would render the whole blood and saliva samples a serious biohazard including Hepatitis B, Hepatitis C, and HIV. Patients were excluded if anyone was unable to comprehend the study and provide consent e.g., insufficient command of English in the absence of someone to adequately interpret. Patients were also excluded if they had a known lung, chest wall, neuromuscular or cardiac disease or abnormality that would confound the Spirometry test, peak flow and the asthma control questionnaire (ACQ) score. Finally, patients with a smoking history of more

than ten pack years were excluded from the study due to the fact that patients with high smoking pack years and low lung function may suggest they have undiagnosed chronic obstructive pulmonary disorder (COPD).

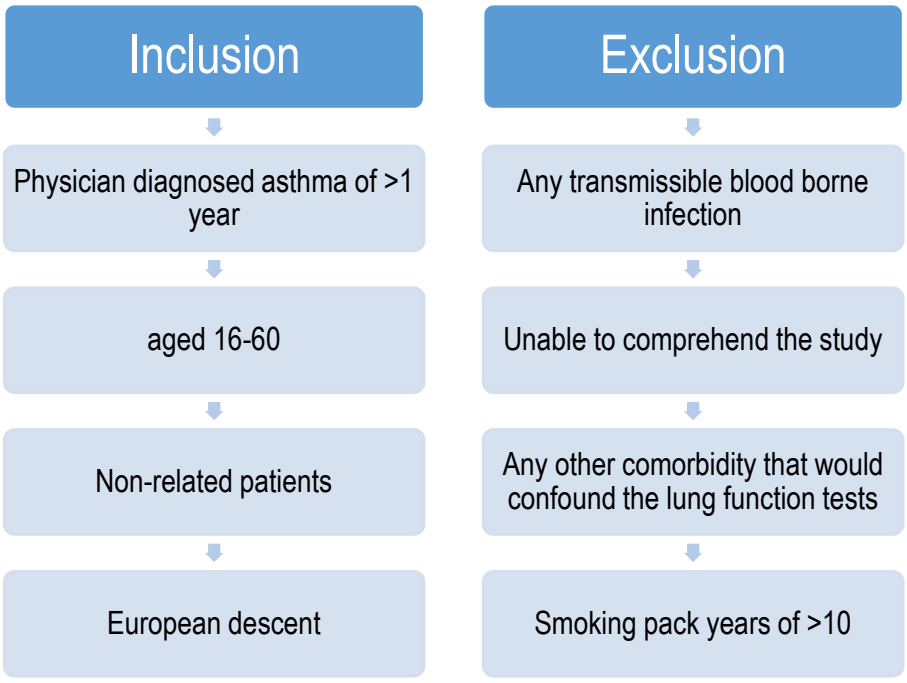


Figure 3.3.1 Overview of the inclusion and exclusion criteria for the serum biomarker study.

3.3.2 Collection of clinical and immunological data

Demographics such as gender, age, age of onset, height and weight were recorded along with clinical data including baseline lung function (FEV_1, FVC and PEF), smoking status, smoking pack years, current asthma medication, GINA classification and ACQ score. Other desirable measures where available were also recorded including total IgE levels, number of hospital admissions within the last year, comorbidities such as allergic rhinitis and allergic dermatitis and blood eosinophil counts.

3.3.3 Collection and processing of biological samples

As mentioned above previously a proportion of patients in the GASP cohort have blood and serum samples. During recruitment for these patients' two serum separating tubes (SST) of volume 8 mL were taken along with two EDTA tubes of 8 mL (if blood could not be taken a saliva sample was provided). These bloods were taken when the patients' asthma was stable pre-medication.

Sample processing

The two EDTA blood samples were for DNA analysis and these were not centrifuged but instead stored at -20ºC (These bloods were used for DNA extraction and genotyping in chapter 5) . Blood samples for serum were centrifuged at 3000 rpm for 10 minutes. The serum from the SST tubes were aliquot into Eppendorf tubes with 0.5ml serum per tube then all serum samples were stored at -20ºC. During sample transfer across the country, blood and sera were packaged in dry ice and on arrival they were recorded and transferred immediately in to the -80ºC freezer at the University of Nottingham Respiratory Medicine laboratories.

3.3.4 Luminex Assay Principle

Luminex is multiplex assay that uses colour-coded magnetic beads coated with analyte specific antibodies (Figure 3.3.4). The antibodies will recognise and bind to the different target analytes in the sample. The captured analytes are then detected using a cocktail of biotinylated detection secondary antibodies and a streptavidin-phycoerythrin conjugate. The sample is read on a dual-laser flow-based detection instrument. One laser classifies the bead and determines the analyte being detected. The second laser determines the magnitude of the streptavidin-phycoerythrin (PE) derived signal, which is directly proportional to the amount of analyte that's bound (R&D Systems, 2020).

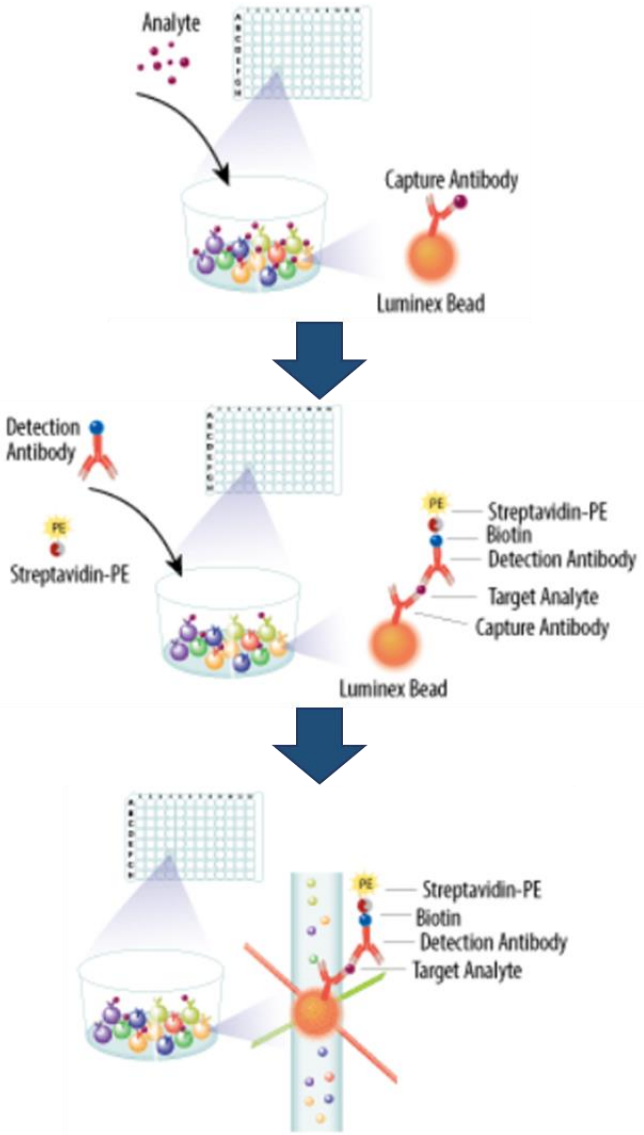


Figure 3.3.4 Luminex assay principal diagram: The serum is added to a mixture of color-coded beads, pre-coated with the analyte-specific capture antibodies. The antibodies bind to the analytes of interest. Biotinylated detection antibodies specific to the analytes of interest are added forming an antibody-antigen sandwich. PE-conjugated streptavidin is added and binds to the biotinylated detection antibodies. Beads are read on a dual-laser flow-based detection instrument (Bioplex). One laser classifies the bead and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. Taken from R&D systems 2021.

3.3.5 Luminex Protocol

R&D systems Magnetic Luminex® was used to measure the concentration of the small signalling proteins related to the Type 2 and non-Type inflammation pathways in the sera of asthma patients. 3 different Luminex kits were used in this study, Kit Lot Numbers: L128076, L123941 and L128077 with each kit containing a different panel of analytes (see appendix Table 7.2.12).

3.3.5.1 Wash Buffer

20ml of Wash Buffer Concentrate was added to 480 ml of deionized or distilled water to prepare a total volume 500ml of wash buffer.

3.3.5.2 Standards

Each standard cocktail was reconstituted with Calibrator Diluent RD6-52, the number of standard cocktails to reconstitute depended on the number of analytes chosen to measure in each kit (see appendix table 7.2.13a-c for the reconstitution volumes for the 3 Luminex kits). The cocktail standards were placed on a rotator for 15 minutes at gentle rotation. 100 µl of each reconstituted cocktail standard was added to an Eppendorf tube (standard 1) and Calibrator Diluent RD6-52 was added to make the final volume to 1 ml. 200 µl of Calibrator Diluent RD6-52 was added to a further 6 tubes (standard 2-7), and standard 1 was used to produce a 3-fold dilution series (see appendix figure 7.2.14 for dilution series diagram).

3.3.5.3 Microparticle Cocktail

The Microparticle Cocktail vial was centrifuged for 30 seconds at 1,000 g (1.0 RCF) and then gently vortexed to re-suspend the microparticles. 500 µl of the Microparticle Cocktail was diluted with 5 ml of Diluent RD2-1 in the opaque mixing bottle provided (Microparticle Cocktail is light sensitive) and then gently vortexed to re-suspend it.

3.3.5.4 Samples and Standards

An aliquot of sera for each patient was left out to thaw prior the start of experiment. Once thawed 50 µl of the sera for each patient sample was pipetted into the 96 well plate provided in duplicate, 50 µl of the 7 standards were also pipetted into separate wells in duplicate with 50 µl of Diluent RD6-52 added to the last 2 wells as a blank and finally 50 µl of the Microparticle Cocktail was added to all 96 wells. The plate was incubated at room temperature for 2 hours on a horizontal orbital shaker set at 800 rpm.

3.3.5.5 Washing

After the first 2-hour incubation period the plate was washed by attaching a magnetic device to the bottom of the plate and left to stand for 1 minute and the then liquid in the wells was removed with the magnetic plate still attached.

The magnetic plate was removed, 100 µl of Wash Buffer was added to each well, the magnetic plate was re-attached, the plate was left to stand for 1 minute and then liquid was removed with the magnetic plate still attached. This wash step was repeated 3 times.

3.3.5.6 Biotin Antibody Cocktail

The Biotin Antibody Cocktail vial was centrifuged for 30 seconds at 1,000 *g* and then gently vortexed. 500 µL the Biotin Antibody Cocktail was diluted using 5 ml of Diluent RD2-1 in a bijoux tube and then gently vortexed. 50 µL of the diluted Biotin Antibody Cocktail was added into each well and incubated at room temperature on a horizontal orbital shaker set at 800 rpm.

3.3.5.7 Washing

The plate was washed again as described in 2.2.2.5.

3.3.5.8 Streptavidin-PE

The Streptavidin-PE vial was centrifuged for 30 seconds at 1,000 g (1.0 RCF) and then gently vortexed. The Streptavidin-PE concentrate vial was diluted to 1x concentration by adding 220 µL of Streptavidin-PE concentrate to 5.35 ml of Wash Buffer. 50 µL of the diluted Streptavidin-PE was added to each well and incubated for 30 minutes at room temperature on the horizontal orbital shaker set at 800 rpm.

3.3.5.9 Washing

The plate was washed again as described in 2.2.2.5. After the final wash the magnetic plate was removed and the microparticles were re-suspended with 100 µl of Wash Buffer and incubated for 2 minutes at room temperature on the horizontal orbital shaker set at 800 rpm. The plate was read within 90 minutes on the Bioplex machine.

3.3.5.10 Calibrating the Bioplex 200 Machine

Prior to reading the plate the Bioplex 200 Machine was calibrated beforehand using the bioplex calibration kit and the calibration instructions were followed as on the screen.

3.3.5.11 Running the Samples on the Bioplex 200 Machine

Once the calibration test was passed, the Bioplex 200 machine was set up to run the samples. The appropriate analytes were selected for that kit with the appropriate catalogue number (see appendix table 7.2.12). Standards were also set up on the machine noting their maximum detection concentration (see appendix table 7.2.15) and their dilution factor. Discriminator gates were set to 8000 and 16500 and the Bioplex was set to read 50 events/beads with the sample size set to 50 µL.

3.3.6 Statistics

All statistical analysis were carried out on IBM SPSS Statistics 24 and StataSE 15. Details of specific statistical methods are described below.

3.3.6.1 Patient selection

To randomly select patients for the discovery and replication cohort the Stratarand command was used on StataSE 15, this is an unbiased function that randomly selected patients to go into GINA 3/4 and GINA 5 for the discovery and replication cohort.

3.3.6.2 Statistical analyses

To identify whether the continuous variables were either parametric or non-parametric, histograms were created to see if they were normally distributed. The mean was given for parametric data along with the standard deviation and the median with the range was given for non-parametric data. When two independent samples were being compared either a Student's t-test (parametric) or a Mann-Whitney U test (non-parametric) was used. When more than two independent samples were being compared either a one-way ANOVA (parametric) or a Kruskal-Wallis test (non-parametric) was carried out. For some of the variables where covariates had to be taken into account a univariate model was used to take into account age, gender height and weight etc. For the serum biomarker data all but periostin were not normally distributed and so data were Log transformed before being added to the univariate model. For testing the relationship between categorical variables, a Chi squared test was used. To look at the relationship between two continues variables that were non-parametric a Spearman's rank correlation was used

to get the R^2 value, for parametric data Pearson's correlation coefficient was used instead. Some of the biomarkers were adjusted for age and gender and so a univariate linear model was used to take into account these covariates.

3.4 Results

3.4.1 Demographics and Clinical Features of the Discovery Cohort

Table 3.4.1a describes the demographics and clinical features of the serum discovery cohort across GINA severity. For the discovery cohort there was 191 patients available in total in early 2019, with 61 in the mild group, 65 in the moderate/severe asthma group and 65 in the severe asthma group. Since the analysis for this discovery cohort was exploratory none of the p-values were corrected for multiple testing.

The majority of the cohort were female (66%) with the mean age of 41.7 years. Age was statistically significant across GINA severity with age increasing across severity (GINA 1 & 2: 39.2 years, GINA 3 & 4: 40.8 years and GINA 5: 44.8 years; p= 0.028). The mean asthma age of onset of the discovery cohort was 17.7 years however, this was not statistically different across the GINA groups. The mean BMI for the cohort was 30.8 suggesting that the majority of the patients were overweight, although not significant (p= 0.054) BMI was higher in the severe GINA group (32.88 kg/m^2) as reported by others (Moore et al., 2010). The majority of the cohort were never smokers (76.4%) with the rest being either ex-smokers (22.5%) or current smokers (1%) with a median smoking pack years of 4.

The mean FEV_1pp for the cohort was 81.64% which was statistically significant across the GINA group's p= <0.0001 with FEV_1pp decreasing as GINA severity increases (GINA 1 & 2: 88.6%, GINA 3 & 4: 84.42% and GINA 5: 72.95%). The mean FEV1/FVC for the cohort was 0.71 which was also statistically significant p= 0.037 with GINA, with the ratio being lowest in the GINA 5 group (GINA 1 & 2: 0.73, GINA 3 & 4: 0.73 and GINA 5: 0.67). Total IgE data was available for a proportion of the patients (n= 92) with the median blood IgE levels being 151.5 KU/L for just under half of the cohort, however this was not statistically significant across the GINA groups. 69.5% of the cohort has allergic rhinitis, this did not show to be statistically significant across the GINA groups. Additionally, 33% of the cohort has had a hospital admission in the last year, this was not statistically significant across the GINA groups. Lastly, the mean ACQ score for the cohort is 2.40 which showed to be statistically significant across GINA severity p=<0.0001 with the mean ACQ

score increasing as GINA severity increased (GINA 1 & 2: 1.91, GINA 3 & 4: 2.33 and GINA 5: 2.85). Table 3.4.1b outlines the medication the patients in the discovery cohort were taking.

Discovery	GINA 1 & 2 (Mild)	GINA 3 & 4 (Moderate/Severe)	GINA 5 (Severe)	P-Value	All patients
Subjects (n)	61	65	65	-	191
Females	37 (60.7%) N=61	46 (70.8%) N=65	43 (66.2%) N=65	0.488	126 (66.0%) N=191
Age seen Years (Years: Mean & S.D)	39.2 (11.55) N=61	40.8 (12.45) N=65	44.8 (11.05) N=65	**0.028**	41.7 (11.88) N=191
Age of onset Years (Years: Mean & S.D)	19.7 (16.41) N=57	16.22 (16.25) N=62	17.27 (14.56) N=60	0.415	17.7 (15.74) N=179
Age of Diagnosis <16	30 (50.8%) N=59	36 (57.1%) N=63	30 (47.6%) N=63	0.554	96 (51.9%) N=182
BMI (kg/m²: Mean & S.D)	29.89 (6.49) N=53	29.53 (6.48) N=60	32.88 (8.59) N=60	**0.054**	30.8 (7.40) N=173
Smoking status:	N=61	N=65	N=65		N=191
Never	44 (72.1%)	43 (66.2%)	59 (90.8%)		146 (76.4%)
Ex-smoker	16 (26.2%)	22 (33.9%)	5 (7.7%)	-	43 (22.5%)
Current smoker	1 (1.6%)	0 (0%)	1 (1.5%)		2 (1%)
Smoking pack years (Median & Min-Max)	3.63 (0.25-10) N=16	3.75 (0.5-10) N=22	6 (3-8) N=5	0.443	4 (0.25-10) N=43
FEV_1pp (Mean & S.D)	88.6 (16.62) N=55	84.42 (19.56) N=65	72.95 (16.95) N=65	**<0.0001**	81.64 (18.92) N=185
FEV1/FVCprbd Ratio (Mean & S.D)	0.73 (0.09) N=50	0.73 (0.11) N=65	0.67 (0.13) N=65	**0.037**	0.71 (0.12) N=182
Blood total IgE (KU/L: Median & Min-Max)	121 (2-4779) N=25	160 (5-4850) N=41	121 (2-4779) N=26	0.961	151.5 (2-4850) N= 92
Allergic rhinitis* (yes)	28 (59.6%) N=47	37 (77.1%) N=48	34 (69.4%) N=49	0.182	98 (69.5%) N=141
Hospital admissions:	N=58	N=65	N=65		N=188
Never	39 (67.2%)	46 (70.8%)	41 (63.1%)	0.647	126 (67%)
Ever	19 (32.8%)	19 (29.2%)	24 (36.9%)		62 (33%)
ACQ7 score (Mean & S.D)	1.91 (1.09) N=52	2.33 (1.14) N=65	2.85 (1.11) N=65	**<0.0001**	2.40 (1.17) N=182

Table 3.4.1a Demographic and clinical features of the discovery serum cohort stratified by GINA severity N=191: Data is presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). BMI: body mass index, FEV1pp: forced expiratory volume in 1 s percentage predicted; FEV_1/FVC: forced expiratory volume in 1 second/forced vital capacity ratio; IgE: Immunoglobulin E and ACQ: asthma control questionnaire. *Allergic rhinitis is doctor diagnosed or self-reported. Not corrected for multiple testing as this was exploratory.

Medication	Number of patients
SABA	169
LABA	105
ICS	154
OCS	56
Biologics (Omalizumab only)	12
Other	96

Table 3.4.1b Medication asthma patients were taking in the discovery cohort. Dosage for ICS and OCS were unavailable for the majority of the patients.

3.4.2 Luminex biomarker results for the discovery cohort

Table 3.4.2 shows the discovery cohort Luminex results for each of the analyte measured. The distribution of the data was first looked at using a histogram to check whether the biomarker data was parametric or non-parametric. All the biomarker data showed to be non-parametric (see appendix 7.2.1 for visualisation of the distribution curves for each biomarker). Therefore, non-parametric data was transformed prior to any statistical analysis. Table 3.4.2 displays the mean with standard deviation for the parametric data and the median, minimum and maximum for non-parametric data. The first column displays the number of patients with measurable data including those that fell below the standard curve, to which were they given a value of zero. Whereas the second column displays the number of subjects that fell within the standard curve to which the mean with standard deviation or median with minimum and maximum was only displayed for these patients. Out of the 191 patients in the discovery cohort periostin was the only biomarker that could be measured in all the 191 patients (median: 81454.57 pg/mL). Whereas 58 patients out of the 191 could only be measured for IL-5 (median: 3.53 pg/mL) and 128 out of 191 could only be measured for IL-13 (median: 276.93 pg/mL). Difficulty measuring IL-5 and IL-13 in serum has been seen in other studies before (Alam et al., 2017). Appendix 7.2.2 displays the table for a set of controls (n= 24) for comparison.

Analyte	Number of patients measured (incl. subjects below standard curve)	Number of patients measured (excl. subjects below standard curve)	Median or mean pg/mL	Min-max or Std Pg/mL
Eotaxin	187	185	154.7	4.67-2033.64
IL-4	187	145	32.43	0.65-7406.05
IL-6	191	185	1.24	0.01-69.15
IL17/17A	186	143	2.79	0.01-879.34
***Periostin**	191	191	81413.93	24542.32
TSLP	183	149	4.02	0.03-298.99
IFN-gamma	187	169	96.51	1.02-27009.65
IL-5	190	58	3.53	0.25-80.07
IL-13	162	128	276.93	24.24-8050.07
IL-33	185	184	12.24	0.09-3006.82
ST2	190	190	138914.86	8022.98-138914.86

Table 3.4.2 Discovery Serum Cohort Luminex Results N=191: Data is displayed as the median or mean* and the minimum-maximum (min-max) or standard deviation (std)* for each analyte in those subject that fell within the standard range.

3.4.3 Correlations between Type 2 and non-Type 2 biomarkers in the discovery cohort

Figure 3.4.3 is a heat map displaying the correlations coefficients (R^2) between each biomarker measured in the discovery cohort where a R^2 value of 0.5 or above was considered biologically significant. The R^2 values highlighted in bold indicate that they were statistically significant after Bonferroni correction p= 0.0009 (see appendix 7.2.3 for p-values for all the correlations between the biomarkers for the discovery cohort). IL-33 and IFN-gamma showed the strongest correlation out of all biomarkers measured (R^2= 0.769), IL-33 also demonstrated coordinated expression with TSLP (R^2= 0.570). Additionally, TSLP showed coordinated expression with Th2 cytokines IL-5 (R^2= 0.516),IL-4 (R^2= 0.462) and IL-13 (R^2= 0.437) and demonstrated coordinated expression with non-Th2 cytokines IL17/17A (R^2= 0.529) and IFN-gamma (R^2= 0.402). This suggests potentially how heterogeneous asthma patients are and their asthma maybe driven by multiple pathways rather than just the standard Th2 versus non-Th2 asthma. Periostin and ST2 showed the least coordinated expression between the other biomarkers, and many did not meet statistical significance after Bonferroni correction.

Discovery Cohort N=191											
Eotaxin	**0.161**	**0.229**	**0.194**	**0.210**	**0.139**	**0.312**	0.006	**0.242**	**0.084**	**0.234**	
IL4	0.003	**0.469**	**0.235**	**0.370**	**0.377**	**0.462**	0.038	**0.529**	**0.162**		
IL6	**0.072**	**0.209**	**0.097**	**0.212**	**0.130**	**0.157**	0.025	**0.096**			
IL17/17A	0.013	**0.239**	**0.138**	**0.264**	**0.171**	**0.287**	0.028				
Periostin	0.004	0.020	0.034	0.020	0.025	0.013					
TSLP	0.030	**0.570**	**0.437**	**0.516**	**0.402**						
IFN-gamma	0.013	**0.769**	**0.287**	**0.259**							
IL5	0.017	**0.392**	**0.377**								
IL13	0.006	**0.299**									
IL33	**0.074**										
ST2											
	ST2	IL33	IL13	IL5	IFN-gamma	TSLP	Periostin	IL17/17A	IL6	IL4	Eotaxin

Figure 3.4.3 Heat map of the correlations between each biomarker of the discovery cohort N=191: Spearman correlation was used to determine correlation coefficient between the biomarkers. Data is presented as the R^2 value for all the correlations between each analyte with red indicating a high R^2 value and green being low. An R^2 value of 0.5 and above is considered to be biologically significant. Values highlighted in bold indicate that they are statistically significant after Bonferroni correction p=0.0009.

3.4.4 Evaluating potential confounders for the biomarkers in the discovery cohort

Before carrying out analysis between biomarkers and the clinical features of asthma, the biomarkers were evaluated to see if age and gender were potential confounders. Age and gender were added to a univariate linear model to see if they were statistically significant (p= <0.05) between each biomarker, which would suggest that these influence biomarker levels in the sera. Table 3.4.4 displays the p-values for age and gender from the statistical model. Age and gender were confounders for IL-13 and TSLP, whereas age was a confounder for only IL-4 and IFN-γ and gender was a confounder for only eotaxin and ST2.

Biomarker	N	Age (p-value)	Gender (p-value)
Eotaxin	185	0.115	**$5.5x10^{-5}$**
IL-4	145	**0.031**	0.592
IL-6	185	0.793	0.633
IL17/17A	143	0.121	0.551
Periostin	191	0.150	0.179
TSLP	149	**0.018**	**0.021**
IFN-gamma	169	**0.051**	0.630
IL-5	58	0.031	0.851
IL-13	128	**0.033**	**0.015**
IL-33	184	0.071	0.351
ST2	190	0.536	**0.001**

Table 3.4.4 Confounders for the 11 biomarkers: age and gender were added to a univariate linear model to see if they were potential confounders. Data is presented as number (N) and p-value for each confounder from the statistical model, p-value of less than 0.05 is a confounder for that particular biomarker.

3.4.5 Relationship between asthma clinical features and Type 2 and non-Type 2 biomarkers in the discovery cohort

The relationship between the biomarkers and clinical features of asthma in the discovery cohort were then evaluated to see whether the biomarkers identified a subgroup of asthma patients. GINA severity, hospital admissions, ACQ score and lung function (FEV1pp and FEV_1/FVC) were selected to be tested with the biomarkers as they are common clinical characteristics of asthma which may be influenced by Type 2 and non-type 2 biomarkers.Figure 3.4.5 demonstrates that Type 2 and non-Type 2 serum biomarkers identified subgroups of patients in the discovery cohort (see appendix 7.2.4A-D which displays the relationship between the 11 biomarkers and 5 clinical features). IL17/17A, TSLP and ST2 were the only biomarkers that were statistically significant (p= <0.05) for GINA severity p= 0.043, 0.22 and $7.4x10^{-6}$; respectively but ST2 only met Bonferroni correction (p= 0.005) (see appendix 7.2.4 for the data). Figure 3.4.5A shows the serum levels of IL17/17a were lower in the GINA 3&4 and 5 group (3&4: 0.050 log_{10} pg/mL and 5: 0.057 log_{10} pg/mL) compared to the GINA 1&2 group (0.89 log_{10} pg/mL) p= 0.019 and 0.049 respectively. Figure 3.4.5B shows the levels of TSLP to be higher in the mild/moderate GINA 1&2 group (0.85 log_{10} pg/mL) compared to the severe asthma group GINA 5 (0.36 log_{10} pg/mL) p= 0.006. Figure 3.4.5C also shows the levels of soluble ST2 are considerably higher in the severe asthma group GINA 5 (4.03 log_{10} pg/mL) compared to the GINA 3&4 group (3.78 log_{10} pg/mL) p= $1.5x10^{-5}$. Soluble ST2 was only statistically significant for hospital admission. Figure 3.4.5D shows levels of soluble ST2 were higher in the hospital admissions group (ever) than those who had not had a hospital admission in the last year (Ever: 3.99 log_{10} pg/mL, never: 3.88 log_{10} pg/mL; p= 0.012). As for FEV_1pp and FEV_1/FVC IL-6 was the only biomarker that was statistically significant (p= 0.001 and 0.023; respectively) with FEV_1pp meeting Bonferroni correction. Figure 3.4.5E and F show a slight negative correlation with IL-6 and both FEV_1pp and FEV_1/FVC R^2= -0.056 and -0.029, respectively. Finally, IL-6 and IL17/17A were both statistically significant with ACQ score (p= 0.003 and 0.026; respectively) with IL-6 only meeting Bonferroni correction. Figure 3.4.5G and H both show a slightly positive correlation with ACQ score and both IL-6 and IL17/17A R^2= 0.049 and 0.036, respectively.

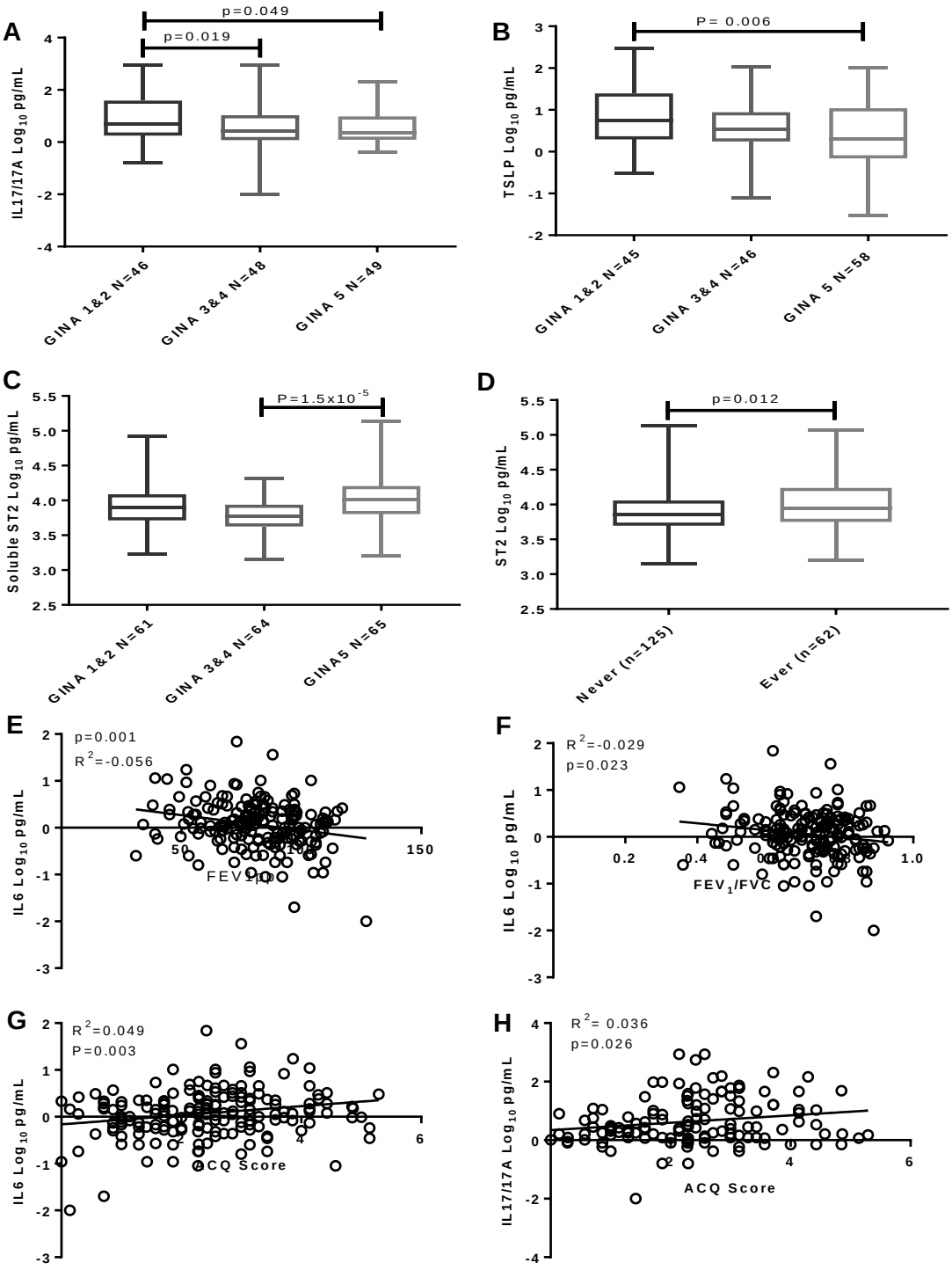


Figure 3.4.5 Biomarkers identified subgroups of patients in the discovery cohort N=191: the data displayed are those that met statistical significance of <0.05, data in A-D is displayed as min and max box plot. A: serum IL17/17A levels are lower in the GINA 3&4 and 5 group compared to the GINA 1&2 group p= 0.019 and 0.049 respectively. B: serum TSLP levels were lower in the GINA 5 group compared to the GINA 1& 2 group after adjusting for age and gender p= 0.006. C: serum soluble ST2 levels are higher in the GINA 5 group compared to the GINA 3 & 4 group after adjusting for gender p= $1.5x10^{-5}$. D: serum soluble ST2 levels were higher in the hospital admissions group (ever) than the never group after adjusting for gender p= 0.012. E: serum IL-6 shows a negative correlation with FEV_1pp R^2= -0.056, p= 0.001. F: serum IL-6 also shows a negative correlation with FEV_1/FVC R^2=-0.029, p= 0.036. G: serum IL-6 shows a positive correlation with ACQ score R^2= 0.049 and p= 0.003. H: serum IL17/17A levels also showed a positive correlation with ACQ score R^2=0.036, p= 0.026.

3.4.5 Demographics and clinical features of the replication cohort

A replication cohort was put together to see whether the results from the discovery cohort could be reproduced. However, it was difficult to put together a comparable replication cohort due to lack of GINA 1&2 and 5 patients in the GASP cohort at that present time and so there are some slight differences in demographical features across GINA groups. Table 3.4.6 describes the demographics and the clinical features of the replication serum cohort. 133 patients were selected for the replication cohort with 15 in the mild group, 59 in the moderate/severe group and 59 in the severe group, these numbers were lower than the discovery cohort.

The majority of the cohort were females (72.5%), and the average age of the cohort was 43.2 years and the mean age of onset being 19.3 years, these demographics were similar to the discovery cohort (Table 3.4.1). The mean BMI of the cohort was 30.25 kg/m^2 suggesting again that the majority of the cohort were overweight, however this was not statically significant like the discovery cohort. 71.4% of the cohort were non-smokers with the rest being either ex-smokers (24.8%) or current smokers (3.8%) with the median smoking pack years being 3 overall, displaying similar values for smoking.

The mean FEV_1pp for the replication cohort was 80.88% and the mean FEV_1FVC was 0.73, both of these lung function values demonstrated to be statistically significant across the GINA groups (p= 0.0002 and p= 0.018 respectively) with lung function being lower in the severe asthma group GINA 5 (FEV_1pp: 70.55% and FEV_1/FVC: 0.69) compared to the other GINA 1&2 (FEV_1pp: 86.19% and FEV_1FVC: 0.71) and GINA 3&4 (FEV_1pp: 88.46% and FEV_1FVC: 0.76). The discovery cohort was statistically significant across GINA (Table 3.4.1). However, there were some slight differences in lung function values across GINA between cohorts, the discovery cohort showed a decline across GINA severity whereas lung function values for GINA 1&2 in the replication cohort were lower than GINA 3&4. Over a third of the replication cohort (n= 75) had data for total serum

IgE levels however, there was no statistical significance across the GINA groups like the discovery cohort. From the proportion of patients that had

allergic rhinitis data and 59% (n= 105) of patients had allergic rhinitis, which was statically significant across the GINA groups (p= 0.021) with more patients in the GINA 3&4 group having allergic rhinitis (73.9%) than those in GINA 1& and 5 (53.6% and 45.7%; respectively). Hospital admissions also showed to be statistically significant across the GINA groups with 52.1% of the cohort having a hospital admission in the last year. Hospital admissions cases were considerably higher in the GINA 5 group (65.3%) than those in the GINA1&2 (38.5%) and GINA 3&4 (35.6%) p= 0.007, this was not however seen in the discovery cohort. Finally, for the two thirds of the cohort with ACQ score data (n= 97), the mean ACQ score was statistically higher in the GINA 5 group (3.17) than the GINA 1&2 and GINA 3&4 groups (1.46 and 1.95; respectively) p= <0.0001, this was also seen in the discovery cohort.

	GINA 1 & 2 (Mild)	GINA 3 & 4 (Moderate/Severe)	GINA 5 (Severe)	P-Value	All patients
Subjects n	15	59	59	-	133
Females	10 (66.7%) N=15	47 (79.7%) N=59	38 (66.7%) N=57	0.253	95 (72.5%) N=131
Age seen Years (Years: Mean & S.D)	40 (13.39) N=15	42.7 (10.48) N=59	44.4 (11.87) N=58	0.408	43.2 (11.44) N=132
Age of onset Years (Years: Mean & S.D)	17.7 (14.77) N=14	21.6 (17.39) N= 53	17.4 (15.61) N= 56	0.376	19.3 (16.32) N= 123
Age of Diagnosis <16	8 (57.1%) N=14	27 (47.4%) N=57	32 (55.2%) N=58	0.647	67 (51.9%) N=129
BMI (kg/m^2: Mean & S.D)	26.74 (4.32) N=13	30.28 (8.54) N= 54	31.34 (7.96) N= 40	0.197	30.25 (7.99) N= 107
Smoking status: Never Ex-smoker Current smoker	N=15 9 (60%) 5 (33.3%) 1 (6.7%)	N=59 47 (79.7%) 11 (18.6%) 1 (1.7%)	N=59 39 (66.1%) 17 (28.8%) 3 (5.1%)	N/A	N=133 95 (71.4%) 33 (24.8%) 5 (3.8%)
Smoking pack years (Median & Min-Max)	1.5 (0.6-10) N=5	2.75 (0.5-10) N=12	5.13 (0.25-10.0) N= 16	0.369	3 (0.25-10) N= 33
FEV_1pp (Mean & S.D)	86.19 (20.05) N=15	88.46 (23.16) N= 59	70.55 (22.56) N=51	**0.0002**	80.88 (24.0) N= 125
FEV1/FVCprbd Ratio (Mean & S.D)	0.71 (0.08) N=14	0.76 (0.1) N= 58	0.69 (0.14) N= 43	**0.018**	0.73 (0.12) N=115
Blood total IgE (KU/L: Median & Min-Max)	71.0 (3.2-890) N=7	41 (8-3528) N=26	96.5 (5-1513) N=18	0.754	75 (3.2-3528) N=51
***Allergic rhinitis (yes)**	7 (53.6%) N=13	34 (73.9%) N=46	21 (45.7%) N= 46	**0.021**	62 (59%) N=105
Hospital admissions: Never Ever	N=13 8 (61.5%) 5 (38.5%)	N=59 38 (64.4%) 21 (35.6%)	N= 49 17 (34.7%) 32 (65.3%)	**0.007**	N=121 63 (52.1%) 58 (47.9%)
Mean ACQ score (Mean & S.D)	1.46 (1.11) N=12	1.95 (1.04) N= 51	3.17 (1.35) N= 34	**<0.0001**	2.32 (1.32) N= 97

Table 3.4.6 Demographic and clinical features of the replication serum cohort stratified by GINA severity N=133: Data is presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). BMI: body mass index, FEV1pp: forced expiratory volume in 1 s percentage predicted; FEV_1/FVC: forced expiratory volume in 1 second/forced vital capacity ratio; IgE: Immunoglobulin E and ACQ: asthma control questionnaire. *Allergic rhinitis is doctor diagnosed or self-reported. Not corrected for multiple testing as this was exploratory.

3.4.7 Luminex Results for the Replication Cohort

Table 3.4.7 shows the replication cohort Luminex results for each of the analyte measured. The distribution of the data was first looked at using a histogram to check whether the biomarker data was parametric or non-parametric. All the biomarker data showed to be non-parametric except for periostin which showed a normal distribution (see appendix 7.2.5 for visualisation of the distribution curves for each biomarker). Therefore, non-parametric data will be transformed prior to any statistical analysis. Table 3.4.7 displays the mean with standard deviation for the parametric data and the median, minimum and maximum for non-parametric data. The first column displays the number of patients with measurable data including those that fell below the standard curve, to which were they given a value of zero. Whereas the second column displays the number of subjects that fell within the standard curve to which the mean with standard deviation or median with minimum and maximum was only displayed for these patients. Out of the 133 patients in the replication cohort eotaxin and periostin were the only biomarkers that could be measured in all the 133 patients (median: 173.33 and 82919.46 pg/mL; respectively). Whereas 50 patients out of the 133 could only be measured for IL-5 (median: 8.25 pg/mL) and 55 out of 133 could only be measured for IL-13 (median: 471.49 pg/mL). Similar difficulty in measuring IL-5 and IL-13 in serum was also observed in the discovery cohort. Additionally, when comparing the Luminex data from both cohorts, the values are generally higher in the replication cohort than the discovery. Analytes INF-γ, IL-5, IL-13, IL-33 and ST2 showed the biggest differences in median and min-max values which were higher in the replication cohort than the discovery cohort (Table 3.4.2).

Analyte	Number of patients measured (incl. subjects below standard curve)	Number of patients measured (excl. subjects below standard curve)	Median	Min-max
Eotaxin	133	133	173.33	11.49-2290.69
IL-4	133	126	26.27	1.93-546.01
IL-6	132	132	2.36	0.41-44.53
IL17/17A	132	74	3.36	0.18-952.43
Periostin	133	133	78385.71	17415.77-699421.52
TSLP	131	129	2.9	0.01-237.69
IFN-gamma	122	121	143.59	3.12-20897.89
IL-5	132	50	8.25	0.15-43.82
IL-13	122	55	471.49	34.85-4442.70
IL-33	123	122	36.22	1.01-6238.30
ST2	131	131	9519.55	1593.88-1117200

Table 3.4.7 Replication Serum Cohort Luminex Results N=133: Data is displayed as the median and the minimum-maximum (min-max) for each analyte in those subject that fell within the standard range.

3.4.8 Correlations between Type 2 and non-Type 2 biomarkers in the replication cohort

Figure 3.4.8 is a heat map displaying the correlations coefficients (R^2) between each biomarker measured in the replication cohort and a R^2 value of 0.5 or above was considered biologically significant. The R^2 values highlighted in bold indicate that they are statistically significant after bonferroni correction (see Table 7.2.6 for the all p-values between the biomarkers for the replication cohort). Again, like Figure 3.4.3, IL-33 and IFN-gamma showed to have the strongest correlation out of all biomarkers measured (R^2= 0.958), IL-33 also demonstrated coordinated expression with TSLP and IL-4 (R^2= 0.570 and R^2= 0.572 respectively) like in the discovery cohort. Additionally, like in the discovery cohort TSLP showed high coordinated expression with all 3 Th2 cytokines (IL-4, IL-5 and IL-13) (R^2= 0.783, R^2= 0.627 and R^2= 0.612 respectively). The Type 2 cytokines also demonstrated coordinated expression with each other IL-4 and IL-5 R^2= 0.598, IL-4 and IL-13 R^2= 0.621 and IL-5 and IL-13 R^2= 0.593. Some of the Type 2 cytokines showed coordinated expression with non-Type 2 biomarkers such as IL-4 and IFN-γ (R^2= 0.590) and IL-5 and IL17/17A (R^2= 0.576). Periostin and ST2 showed the least coordinated expression between the biomarkers, and many did not meet statistical significance after Bonferroni correction. Overall, the data is good agreement with data generated from the discovery cohort.

Replication Cohort N=133											
Eotaxin	**0.139**	**0.272**	**0.340**	**0.434**	**0.247**	**0.352**	0.050	**0.269**	**0.275**	**0.424**	
IL4	0.044	**0.572**	**0.621**	**0.598**	**0.590**	**0.783**	0.006	**0.457**	**0.497**		
IL6	0.021	**0.284**	**0.411**	**0.403**	**0.296**	**0.490**	0.003	**0.368**			
IL17/17A	0.018	**0.242**	**0.375**	**0.576**	**0.257**	**0.542**	0.000				
Periostin	0.000	0.003	0.016	0.007	0.004	0.001					
TSLP	0.052	**0.554**	**0.612**	**0.627**	**0.573**						
IFN-gamma	0.038	**0.958**	**0.437**	**0.375**							
IL5	0.019	**0.366**	**0.593**								
IL13	0.049	**0.432**									
IL33	**0.100**										
ST2											
	ST2	IL33	IL13	IL5	IFN-gamma	TSLP	Periostin	IL17/17A	IL6	IL4	Eotaxin

Figure 4.4.7 Heat Map of the Correlations between each Analyte of the Replication Cohort N=133: Data is presented as the R^2 value for all the correlations between each analyte with red indicating a high R^2 value and green being low. An R^2 value of 0.5 and above is considered to be biologically significant. Values highlighted in bold indicate that they are statistically significant after Bonferroni corrections P=0.0004.

3.4.8 Evaluating potential confounders for the biomarkers in the replication cohort

Like in the discovery cohort, the biomarkers were evaluated to see if age and gender were potential confounders before carrying out analysis between biomarkers and the clinical features of asthma. Age and gender were added to a univariate linear model to see if they were statistically significant (p= <0.05) between each biomarker, which would suggest that these influence biomarker levels in the sera. Table 3.4.8 displays the p-values for age and gender from the statistical model along with the numbers. Age and gender were confounders for IL-13 like in the discovery cohort, whereas age was the only confounder for TSLP. Gender was a confounder for eotaxin and ST2 like in the discovery cohort but gender was also a confounder for IL-4, IL-5 and periostin in the replication cohort.

Biomarker	N	Age (p-value)	Gender (p-value)
Eotaxin	131	0.646	**0.004**
IL-4	123	0.199	**0.029**
IL-6	130	0.150	**0.05**
IL17/17A	73	0.411	0.127
Periostin	131	0.514	**0.036**
TSLP	127	**0.048**	0.199
IFN-gamma	120	0.827	0.907
IL-5	49	0.135	0.619
IL-13	52	**0.037**	**0.044**
IL-33	121	0.382	0.352
ST2	129	0.708	**0.003**

Table 3.4.8 Confounders for the 11 biomarkers: age and gender were added to a univariate linear model to see if they were potential confounders. Data is presented as number (N) and p-value for each confounder from the statistical model, p-value of less than 0.05 is a confounder for that particular biomarker.

3.4.9 Clinical subsets of asthma patients identified in the discovery cohort were not replicated in the replication cohort

One of the key reasons of having the replication cohort was to see whether the clinical subsets of asthma patients identified in the discovery cohort could be replicated in the replication cohort to validate the results. In the discovery cohort GINA severity was associated with IL17/17A, TSLP and ST2 and hospital admissions was associated with ST2. Both lung function values were associated with IL-6 and ACQ score was associated with both IL-6 and IL17A. The replication cohort did not replicate any of these finding as all were not statistically significant (see appendix 7.2.7 for data).

3.4.10 Taking OCS only influences soluble ST2 levels in the sera

OCS and ICS affect levels of cytokines in the blood (Brattsand and Linden, 1996) by supressing the levels of them such IL-4, IL-5, IL13, IL-6 etc. In an additional subset of asthma patients (n= 42) from another asthma cohort (PROCLAIM) with full medication data the 11 biomarkers were also measured to see whether taking ICS or OCS influence the levels of the biomarkers in the sera (see appendix 7.2.8 for the Proclaim cohort demographics). Only one biomarker showed to be affected by the use of OCS, the rest of the biomarkers were not affected by the use of ICS and OCS (see appendix 7.2.9 for confounders for the biomarkers in the proclaim cohort and 7.2.10A and B for relationship between the serum biomarkers with ICS and OCS). Figure 3.4.10 shows after adjusting for age, soluble ST2 levels was significantly higher in patients that were taking OCS than those who were not (Not on OCS: 3.96 log_{10} pg/mL, taking OCS: 4.13 log_{10} pg/mL, p= 0.024). The relationship between OCS dosage and soluble ST2 levels were also analysed however, this was not statistically significant (see appendix 7.2.10C). Overall, the data suggests OCS and ICS do not influence biomarker levels in the serum.

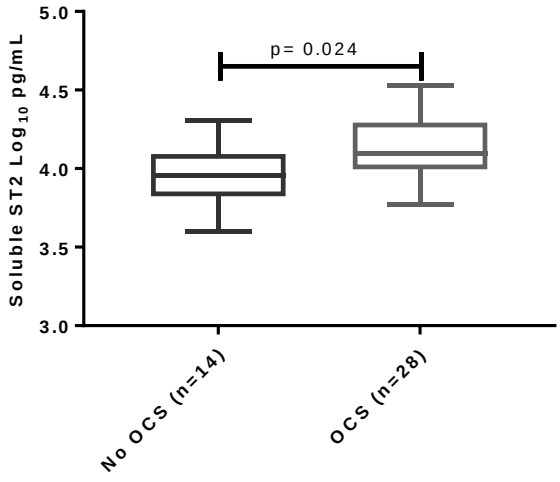


Figure 3.4.10 OCS influence serum soluble ST2 levels in asthma patients: univariate linear model was used to analyse the data and soluble ST2 was adjusted for age. Serum soluble ST2 levels are higher in those patients taking OCS compared to those not taking OCS p= 0.024.

3.4.11 Are immune cells co-ordinately expressed with the biomarkers in the sera?

The same subset of asthma patients from 3.4.10 that also had immune cell data, were used to see if the immune cells were correlated with any of the biomarkers. Figure 3.4.11 displays the correlation data with the R^2 and p-value between the biomarkers and immune cells that were statistically significant (p= 0.05) (see appendix 7.2.11 for all the correlation coefficient data between all the biomarkers and immune cells). Figure 3.4.11A shows a positive correlation with IL-4 and eosinophils (R^2= 0.281 and p= 0.048). Figure 3.4.11B shows a slight negative correlation with IL17/17A and lymphocytes (R^2= -0.219 and p= 0.021). In figure 3.4.11C IL-5 shows the strongest co-ordinate expression with neutrophils although the n numbers are low (R^2= 0.570, p= 0.05 and N= 7). Finally, figure 3.4.11D shows a positive correlation with soluble ST2 and basophils (R^2= 0.375 and p= 0.012).

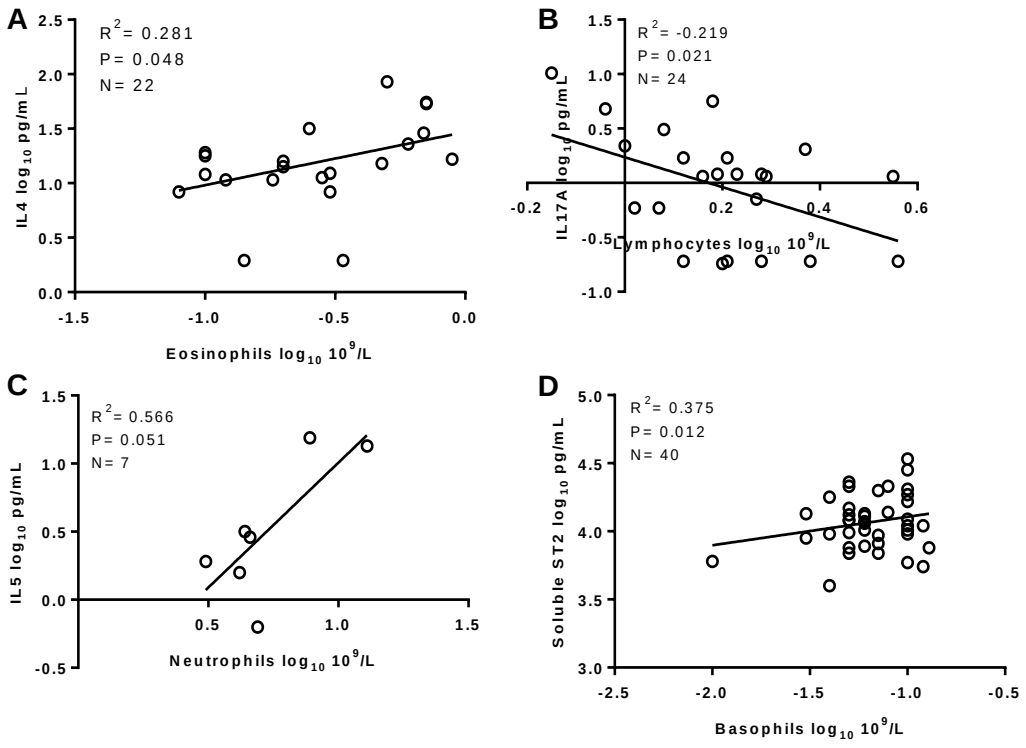


Figure 3.4.11 Correlations between the biomarkers and immune cells in a subset of asthma patient's n=42: linear regression model was used to determine the correlation coefficients between the immune cells and biomarkers highlighted in table 7.2.9 were adjusted for age in the model. A: eosinophils show a positive correlation with IL-4 R^2= 0.281, p= 0.048. B: Lymphocytes show a negative correlation with IL17/17A R^2= -0.208 and p= 0.025. C Neutrophils show a positive correlation with IL-5 17A R^2= 0.570 and p= 0.05. D: Basophils show a positive correlation with soluble ST2 R^2= 0.375 and p= 0.012. None of the biomarkers and immune cells met Bonferroni correction p= 0.0009.

3.5 Discussion

Little has been researched regarding analysing blood cytokines as potential inflammatory markers for identifying asthma endotypes and those studies that have used blood, had small sample sizes. The hypothesis of this chapter was cytokine and/or protein biomarkers in the blood of asthma patients can identify subsets of asthma patients with different inflammatory drivers and disease presentation. Most of the serum biomarkers were easily measured and some were influenced by age and gender. The key findings were many serum biomarkers were co-ordinately expressed with each other, particularly the biomarkers related to the type 2 inflammation as these had a R^2 of >0.05. However, the serum biomarkers showed limited correlation with clinical data as this could not be reproduced using the replication cohort. Additionally, a few serum biomarkers showed some correlation with eosinophils, neutrophils, lymphocytes and basophils in a smaller subset of asthma patients despite just reaching statistical significance.

Asthma analysis studies using blood, sputum and transcriptomics have identified inflammatory cytokine markers related to Th2 and non-Th2 inflammation which have helped in the identification of asthma stratification of biomarkers (Bigler et al., 2017) (Manise et al., 2013). Another study investigated associations between serum cytokine profiles and asthma outcomes and found that high levels of IL-1Ra and IL-10 were associated with a lower risk of future asthma exacerbations (OR 0.65, 95% CI: 0.29-1.47) and attacks in adults (OR 0.68, 95% CI: 0.4-1.16) (Akiki et al., 2017). Sputum data has also shown cytokines involved in the Th2 pathway were correlated with each other when measured in asthma patients. One study looked at the expression of IL-33 and TSLP in the airways of asthma patients and showed similar results to the data in this study that some of the Th2 cytokines and their mediators were correlated with each other (IL-33 & TSLP: r= 0.425, p= <0.0001 and IL-33 & IL-13: r= 0.426, p= <0.0001) (Li et al., 2018).

Both discovery and replication cohorts demonstrated similar clinical characteristics such as lower lung function and increased ACQ score across GINA severity which has been observed in previous studies (Thamrin et al., 2011). Lower lung function has been associated with severe asthma for a long

time (Dijkstra et al., 2006), this is due to severe asthma patients being more prone to exacerbations and less responsive to treatment (Bai et al., 2007) (Rogliani et al., 2020). A high ACQ (>1.5) score and low lung function goes hand in hand with severe asthma because their asthma is not well controlled despite being on high dose ICS and/or OCS. The main difference was the replication cohort showed the severe asthma patients had more hospital admissions. This relationship is commonly seen where disease severity is associated with exacerbations suggesting severe asthma patients have an increased risk of exacerbations (Suruki et al., 2017).

TSLP and IL-33 play important roles in allergic diseases such as asthma as they are known to drive disease pathology and so could be potential biomarkers for severe asthma (Kuruvilla et al., 2019). In this study TSLP and IL-33 were highly correlated with each other in both cohorts suggesting they are possibly in the same correlation pathway as each other. IL-33 and TSLP are both epithelial derived cytokines and are released in response to a stimulus (Li et al., 2018). Both IL-33 and TSLP may be induced by overlapping mechanisms as one study using air-liquid interface culture models of human bronchial epithelial cells (HBECs) of severe asthma patients co-cultured with monocyte-derived dendritic cells showed and increase release of IL-33 and TSLP (Gras et al., 2017). TSLP also showed coordinated expression with IL-5 in both cohorts, TLSP can activate T cells directly and induce dendritic cells to promote the differentiation of naive CD4+ T cells into Th2 pro-inflammatory cytokines such as IL-5 suggesting why they are strongly correlated with each other (Allakhverdi et al., 2007).

IFN-γ plays a role in the Th1 pathway and is produced by NK cells and $CD4^+$ Th1 T cells and when IFN-γ binds to its receptor triggering Jak/STAT signalling which activates immune effector genes and cause pro-inflammatory effects (Gajewski and Fitch, 1988) (Ivashkiv, 2018). In this study IFN-γ showed to have the strongest correlation with IL-33 in both cohorts. A study looking at the expression of IL-33 in airway smooth muscle cells in severe asthma demonstrated that IFN-γ increased IL-33 expression in the cells (Préfontaine et al., 2009). IFN-γ and IL-33 has also been demonstrated to be closely interlinked with atopic dermatitis as a study showed CD4+ cells that were

stimulate with IL-33 and IL-12 resulted in the increase of IFN-γ release (p= <0.001; n= 7) (Seltmann et al., 2013). Furthermore, IL-33 has shown to induce IFN-γ production from human natural killer cells in conjunction with IL-12 (Smithgall et al., 2008). This suggests that IL-33 may play an important role and amplify both Th1 and Th2 immune responses and would suggest why it maybe upregulated with IFN-γ during inflammation which was seen in this study.

IL-4 demonstrated coordinated expression with IL-17/17A in both cohorts. IL-4 is part of the Th2 pathway and IL-17/17A is part of the Th17 pathway, suggesting some patients may have multiple drivers of Th2 and Th17 inflammation. It has been proposed that TGF-β_1 is involved in Th2, Th17 and even Th1 cell differentiation (Zhang, 2018). Data has shown Th2 and Th17 derived cytokines, IL-4 and IL-17A both work with TGF-β_1 to promote airway remodelling in severe asthma patients (Ji et al., 2013). IL-4 also showed to be highly correlated with the other Th2 cytokines (IL-5 and IL-13) and the master regulatory cytokines of the Th2 pathway IL-33 and TSLP. IL-33 and TSLP are upstream of the Type 2 pathway and production of these cytokines causes a cascade of downstream events leading to the production and release of the Th2 cytokines (Fahy, 2015).

The data from this study suggests even in the blood the same cells are producing these cytokines at the same time as seen in the sputum in airway studies hence, many were highly correlated with each (Li et al., 2018). During an inflammatory response inflammatory cells such as Th2 cells, mast cells and basophils are recruited from the blood. Many of these inflammatory cells especially Th2 cells in the blood produce cytokines IL-4, IL-5 and IL-13 which are involved in inflammation of asthma (Chen et al., 2017). Current data has demonstrated this increase in Th2 cells in the blood during inflammation in asthma patients, with the % of CD4+ T cells expressing CRTh2 (a chemoattractant receptor-homologous molecule expressed on Th2 cells) being increased in exacerbating versus stable asthma patients (5.96% and 3.2% respectively; p= 0.003). The data also showed CD4+CRTh2+ T cells to be correlated with poor asthma control from the ACQ (R= 0.553, p= 0.026, n= 16) (Shrestha Palikhe et al., 2021). This indicates during inflammation Th2

cells, and the cytokines produced by these cells are being promoted at the same time in the blood as well as the lung. This suggests that measuring Th2 cytokines in the blood may be a useful biomarker for asthma severity, exacerbation and symptom control. Asthma transcriptomic studies have also shown high expression of Th2 associated genes. An asthma sputum transcriptomic study identified a Th2-high phenotypic cluster which was enriched with higher expression of IL-13/Th2 and ILC2 signatures and also associated with high sputum eosinophilia, severe asthma and OCS dependency (Kuo et al., 2017). Additionally, transcriptomic analysis studies from atopic asthma patients in blood have shown most differentially expressed genes were from the Th2 pathway (Jiang et al., 2019), further suggesting that genes relating to Th2 inflammation are also upregulated in the blood which would also explain why Th2 related cytokines are being produced in the blood as the same time as the airways. Additionally, IL-4 showed to be highly correlated with IFN-γ. As mentioned before IFN-γ is part of the Th1 pathway and IL-4 is a Th2 cytokine and released by Th2 cells down stream of the Th2 pathway. Even though they are not in the same pathway the data suggests that some patients may have multiple drivers of Th2 and Th1 inflammation. However, a previous study in the airways showed the levels of IL-4 and IFN-γ in exhaled breath in children with asthma showed that there was no correlation between both cytokines (r= 0.04, n= 37) (Shahid et al., 2002).

Periostin, Eotaxin and soluble ST2 did not show any correlation between any of the 11 biomarkers, this suggests that these biomarkers are independent from each other and the other biomarkers. IL-13 is known to induce the production of periostin and eotaxin downstream the Th2 pathway in the airway epithelial cells (Ito et al., 2018). Serum periostin has also been shown to be correlated with sputum IL-13 levels (r^2 = 0.33, p= 0.007, n= 41) (Konstantelou et al., 2015). Serum periostin was originally considered a potential biomarker at predicting responders and non-responders to new anti-IL-13 drug targets (Corren et al., 2011). However, periostin is a complicated biomarker for type 2 immunity as it has shown to have several characteristics correlating with asthma and also serum periostin levels have been shown to be diverse amongst asthma patients (Izuhara et al., 2016). In this study periostin also

showed no correlations between asthma clinical characteristics. The biomarker data did not identify any clinical subsets of asthma patients, even those clinical features found in the discovery cohort were not reproduced in the replication cohort. This suggests that serum biomarkers may not be a good predictor of clinical features of asthma.

In an additional subset of asthma patients ICS and OCS were looked to see if they influenced serum biomarker levels as it is known for steroids to supress levels of Th2 cytokines (Brattsand and Linden, 1996). Only soluble ST2 showed to be influenced by OCS which previous data has shown before (Watanabe et al., 2018). This suggests that high soluble ST2 levels may identify more severe asthma patients who are on OCS as also suggested in the data from the discovery cohort across GINA severity. In the same cohort correlation data between immune cells and the serum biomarkers showed some co-ordinate expression between a few of the immune cells and biomarkers. Most of these showed weak correlation and this was based on a small number of samples due to some of the biomarkers falling below the standard curve. Therefore, it is likely that these findings are false due to multiple simultaneous statistical tests and is unlikely to be reproducible.

In conclusion, many blood biomarkers showed correlated expression with each other particularly IL-33 and IFN-γ and many of the Th2 cytokines as their mediator's demonstrated coordinated expression with each other. The data also suggests that some patients may have multiple drivers of Th1, Th2 and Th17 inflammation. However, serum biomarkers showed that they were not good at identifying clinical subgroups of asthma patients as none of the data was reproducible. Nevertheless, patients who have high levels of multiple serum Th2 biomarkers may be more amenable to the new type 2 biologics coming into clinic and so could be potential biomarkers for these drugs.

Chapter 4- Can serum biomarkers help tailor new asthma monoclonal antibody therapy?

4.1 Introduction

As mentioned throughout there are new monoclonal antibody therapies currently in research, clinical trials or already available in clinic. Most of these monoclonal antibodies target specific aspects of the type 2 pathway (see chapter 1 figure 1.5.2 for what parts of the type 2 pathway these drugs target). However, it is still not well understood which asthma patients will benefit the most/respond the best to these new monoclonal antibodies. Furthermore, these new biologics can also cause adverse effects as clinical trial data reported 13% of patients of the 797 treated with Benralizumab had adverse effects which were mostly worsening of asthma (Bleecker et al., 2016). Therefore, it is important to identify which patients will benefit from these biologics to prevent any unnecessary adverse reactions. Blood/sputum eosinophil or IgE levels are the biomarkers used as one of the indicators of whether a patient may respond well to these biologics. Although measuring eosinophils has shown promising results in patients responding very well to these drugs by reducing exacerbations and improving lung function, it is however been reported that some patients do not respond to these drugs (Samant and Castro, 2019). There is currently no clear explanation why some patients do or do not respond to biologics. However, it is suggested high systemic inflammation plays a part in this with the frequency of non-respiratory symptoms in some severe asthma patients which may decrease the effectiveness of biologics (Hyland et al., 2019). Using other measurable biomarkers in the blood such as Th1, Th2 and Th17 cytokines may help identify patients who have asthma driven by one or more inflammation pathway. Additionally, this may help identify patients that would benefit from a particular monoclonal antibody drug that target these specific pathways. Here this chapter will examine the inflammatory profile in the blood of asthma patients and investigate the overlap in these inflammatory profiles (Th1, Th2 and Th17) across patients. The inflammatory profiling may then help tailor asthma therapy for a patient as it will provide information to suggest which biologic would be suitable for them.

4.1.1 Current Asthma Biomarkers Reflecting Type 2 Airway Inflammation

A variety of biological samples from different anatomical locations have been used as biomarkers for asthma treatment response and even diagnosis such as sputum, exhaled breath and blood (Wan and Woodruff, 2016) (Nekoee et al., 2020). Biomarkers from exhaled breath such as nitric oxide measurement can help identify patients most likely to benefit from inhaled corticosteroids (Price et al., 2013). Blood biomarkers can help identify patients who may benefit from targeted anti-monoclonal therapies such as high blood eosinophil levels which identify potential responders for new biologics like Mepolizumab (GINA 2020) see Table 4.1.1 for the eligibility criteria for the current biologics.

Biologic	Target	Biomarkers/ criteria for biologic
Omalizumab	IgE	Positive skin prick test Total IgE of 30-1500 IU/mL FEV_1 <80% Frequent daytime or night-time symptoms Multiple severe exacerbations Daily high dose of ICS and LABA
Mepolizumab	IL-5	Blood eosinophil of >300 cells/μL in the last 12 months >4 exacerbations in the last 12 months Continuous use of OCS in the last 6 months
Reslizumab	IL-5	Blood eosinophil of >400 cells/μL in the last 12 months >3 exacerbations in the last 12 months High doses of OCS and ICS
Benralizumab	IL-5R	Blood eosinophil count of >300 cells/μL >4 exacerbations in the last 12 months Non-responsive to standard treatment

Table 4.1.1 Current eligibility guidelines for each biologic (Bousquet et al., 2017) (Agache et al., 2021) (GINA, 2020).

The next section describes some of the current and common biomarkers used in clinical that reflects most specifically the Type 2 airway inflammation.

4.1.1.1 Fraction of Exhaled Nitric Oxide

Fractional exhaled nitric oxide (FeNO) has been used to predict response to biologics such as Omalizumab (anti-IgE) (Hanania et al., 2013) and Lebrikizumab (anti-IL-13). Data from an Omalizumab study demonstrated patients with high FeNO (≥19.5 ppb) had a 53% reduction in exacerbations ($p= 0.001$) compared to 16% reduction ($p= 0.45$) in patients with low FeNO (<19.5 ppb) after 48 weeks of treatment with Omalizumab. A Lebrikizumab treatment asthma study also showed 19% mean reduction in FeNO levels in

patients in the periostin high group than the periostin low group after 12 weeks with Lebrikizumab (Corren et al., 2011). FeNO is a quantitative method of measuring airway inflammation and is a marker of Type 2 inflammation. Nitric oxide is involved in physiological regulation of airway function and is produced by epithelial cells. Nitric oxide is produced in response to airway inflammation and in host defence against infection and is known to induce vasodilation in the arterioles, increase mucus secretion and indirect activation of Th2 cells, contributing to asthma physiopathology (Prado et al., 2011) (Menzies-Gow et al., 2020). FeNO has been shown to positively correlate with airway eosinophilia (r= 0.456; p= <0.0001) (Gao and Wu, 2018) but is thought to be independent of IL-5 (Haldar et al., 2009). FeNO has been known to be elevated in allergy related asthma (Pedrosa et al., 2012) as data has shown patients with allergic asthma exhibited a significant elevation of FeNO after 24 hours following allergen provocation with birch pollen (n= 13, p= <0.01) as well as an increased number of airway eosinophils (r= 0.68; p= <0.001) (Roos et al., 2014). This suggests that FeNO may be an important tool to monitor allergic asthma and may be used to limit allergic related exacerbations. FeNO can be used for asthma diagnosis as a FeNO level of >40 ppb in adults is considered a positive test for asthma however, a single positive test is insufficient to make an asthma diagnosis. A FeNO level of >50 ppb in adults is a strong indicator that a patient is likely to be responsive to ICS therapy (Menzies-Gow et al., 2020). High FeNO levels have been associated with a decline in lung function with one study demonstrating children and adolescents aged 6–18 years with persistent asthma had high FeNO levels (≥20 ppb) also associated with FEV_1pp decline (≥20 ppb: 83% and <20 ppb: 94%) (Soto-Ramos et al., 2013). Finally, FeNO has shown to predict exacerbations and poor asthma control. However, there are confounders that can affect FeNO measurement such as age, sex, weight, smoking and respiratory infections (Narendra et al., 2019).

4.1.1.2 Serum IgE

IgE is an essential mediator in the pathogenesis of atopic asthma and so serum specific and total IgE levels help identify patients with allergic asthma in combination with other clinical presentations. Specific IgE (i.e. IgE directed

against a specific allergen) levels are used to identify patients who may be eligible for the drug Omalizumab (Kim et al., 2017). Asthma patients with high serum IgE levels have demonstrated Omalizumab to be more effective at controlling asthma symptoms and reducing the need for systemic corticosteroids. One study demonstrated that the patients with a serum IgE level of >700 IU/mL had the biggest reduction in hospital visits and need for systemic steroids after treatment with Omalizumab compared with the lower serum IgE group (>700 IU/mL: hospital visits: p= 0.008 and systemic corticosteroids: p= <0.001) (Maselli et al., 2013). The current eligibility criteria for Omalizumab according to GINA (2020) sates that the patient is sensitised to inhaled allergen(s) on a skin prick test or specific IgE. However, IgE levels do not predict response to Omalizumab and they also have no pharmacodynamics properties. Additionally, elevated serum IgE levels are not necessarily specific to asthma, as they can be elevated due to other comorbidities such as atopic dermatitis (Narendra et al., 2019). (See chapter 1.5.2 for more on Omalizumab).

4.1.1.3 Sputum Eosinophils

Eosinophil counts can be taken from either the blood or sputum. It is thought that sputum eosinophil count is a more accurate in assessing eosinophil airway inflammation and may also provide additional information such as phenotyping, prognosis and response to ICS therapy (Westerhof et al., 2015). A sputum eosinophil count of ≥2% is suggestive of eosinophilic airway inflammation and elevated sputum eosinophil counts are associated with an increased risk of an exacerbation (Walsh et al., 2016). However, sputum eosinophil counts have some limitations such as they may be difficult to obtain from some patients as it is semi-invasive. Also, only specific and specialised laboratories can analysis these sputum samples (Narendra et al., 2019). Additionally, it has been shown that sputum eosinophils levels can vary throughout the day. Data from 392 sputum samples from severe asthma patients showed sputum eosinophil levels to be affected by the time of sampling as levels decreased over the course of the day (8-11 hrs: $0.7x10^9$ cells/L; 11-14 hrs: $0.5\ x10^9$ cells/L; 14-17 hrs: $0.4\ x10^9$ cells/L; 17-20 hrs: $0.3\ x10^9$ cells/L; p= 0.042) (Tavernier et al., 2018). Higher sputum eosinophil

levels in the morning compared to the evening were also seen in another study (37.4% vs 21.6% respectively; p= 0.002) (Durrington et al., 2018). This suggests that time of day may play an important implication for clinical practice. Sputum eosinophil levels have also helped guide a number of therapies such as anti-IL-5 therapy as IL-5 is involved in the recruitment of eosinophils. One study showed in a single dose of monoclonal antibody to IL-5 a decrease in sputum eosinophils levels after inhaled allergen challenge. 9 days after treatment of anti-IL-5, the percentage sputum eosinophils were 12.2% in the placebo group and lowered to 0·9% in the 10 mg/kg group (p= 0·0076) (Leckie et al., 2000). Another study has shown Mepolizumab to significantly lower sputum eosinophils by a factor of 7.1 compared to the placebo group (95% CI, 1.6-8.4; p= 0.002) (Haldar et al., 2009).

4.1.1.4 Blood Eosinophils

Blood eosinophils have been studied greatly as biomarkers for airway inflammation in asthma and is the most well-established blood biomarker for biological drugs (see Table 4.1.1 for current blood eosinophil criteria for each biological drug). Biomarkers measured in blood are relatively non-invasive and more straightforward than those measured from exhaled breath or directly from the airway. Studies have demonstrated that blood eosinophil counts of >300 cells/μL were associated with increased asthma exacerbations (Price et al., 2015) (Tran et al., 2014). Using blood eosinophil count may provide a useful biomarker to phenotype patients with Type 2 eosinophilic asthma. Blood eosinophil counts have demonstrated their effectiveness in identifying patients most likely to benefit from the targeted monoclonal antibody therapies such as Mepolizumab (anti-IL-5) or Benralizumab (anti-IL-5R) and also in monitoring subsequent treatment response (Pavord et al., 2017) (Ortega et al., 2014) (FitzGerald et al., 2016). To be considered for the Mepolizumab or Benralizumab drug, one of the criteria's the asthma patient must meet is having a blood eosinophil count of ≥300 cells/μL according to the GINA guidelines (2020). Blood eosinophils also have good pharmacodynamics properties as levels decrease in response to corticosteroids or anti-IL-5 therapies. For example Mepolizumab has shown to significantly decrease blood eosinophils levels by a factor of 6.6 compared to the placebo group (p=

<0.001) (Haldar et al., 2009). Nevertheless, blood eosinophils have some limitations, they can be affected by allergen exposure, infection and certain medications such as steroids. Also, blood eosinophil elevations are not necessarily specific to asthma as they can be elevated from allergic rhinitis, drug reactions etc. (Narendra et al., 2019).

In summary there have been many biomarkers that have been studied in asthma but only a few are used currently in clinic i.e., IgE, blood eosinophils and FeNO. Blood/serum biomarkers are relatively non-invasive and more straightforward than those measured from exhaled breath or directly from the airway. So far eosinophil blood count levels have shown to be a promising biomarker in treatment response to the anti-IL-5/IL-5R therapy. A biomarker should reflect disease mechanisms and predict the course, and response to certain therapies particularly severe asthma patients. Additionally, a biomarker should be easy to measure, reproducible and its measurement should not be confounded in the presence of other comorbidities. There are many biomarkers available that reflect the Type 2 pathway which are easily measured and predict some response to available targeted therapies which are used in clinic whilst others are still limited to research studies. There is also still an unmet need for biomarkers that reflect patient's that have non-Type 2 airway inflammation and as asthma is heterogeneous, different biomarkers will be needed to identify various subtypes of asthma.

4.1.2 Distinguishing inflammatory profiles and their overlap in order to suggest mono or combined therapy approaches with biologics

The key questions that will be studied in this chapter are, how independent are the inflammatory profiles and what is the extent of their overlap? Can an inflammatory signature associated with the different asthma patient groups from chapter 2 be identified i.e. age of onset and hospital admission? Due to lack of patients with both eosinophil and biomarker data, analysis could not be performed to identify an inflammatory signature with eosinophilic high patients. Following on from chapter 3, this chapter will utilise all the asthma patients from the GASP cohort that have serum data for all 11 biomarkers. Patients included in this analysis will be UK non-related asthma patients aged 16-60. Patients will be excluded if they are ex-smokers or current smokers who have

a smoking pack year of more than 10 due to high smoking pack years effecting lung function. Asthma patients in the upper quartile group of each biomarker will be used (Th1, Th2 and Th17 “high”) to investigate how independent these inflammatory profiles are and the extent of their overlap by using Venn diagrams. This may then help identify an asthma patient’s inflammatory profile and whether it is driven by one or multiple inflammatory pathways (Th1, Th2 and Th17) which will help tailor their asthma therapy by suggesting the most appropriate biological therapy. These inflammatory profiles will also be compared clinically to see if they identify any clinical features of asthma. Finally, an inflammatory signature, if any, associated with clinical features of asthma including age of onset and hospital admissions will be identified using all asthma patients with biomarker data.

4.2 Hypothesis

The hypothesis underlying this work is that cytokine and/or protein biomarkers in the blood of asthma patients may help identify patients with a specific inflammatory profile.

4.2.1 Aims

1. Compare patient subsets based on the inflammatory profiles Th1, Th2 and Th17.
2. Compare patient subsets based on the levels of targets of existing/new biologics to evaluate the utility of these drugs as single or combined therapy.
3. Identify the inflammatory signature associated with clinical features of asthma.

4.3 Methods

4.3.1 Inclusion Criteria

The patients involved in the study met the same inclusion and exclusion criteria as those in chapter 3 (see section 3.3.1 for further details). However, asthma patients that had serum biomarker data from the GASP cohort were all included (including both discovery and replication cohorts) in the analysis for this chapter.

4.3.2 Collection of clinical data and processing of biological samples

See section 3.3.2 for collection of clinical and immunological data and section 3.3.3 for the collection and processing of biological samples.

4.3.3 Luminex protocol

The biomarkers were measured in the sera using a Luminex assay which enables to measure multiple analytes at once using a small volume of serum. See section 3.3.4 for the Luminex assay principle and section 3.3.5 for the Luminex protocol.

4.3.4 Statistical analyses

All statistical analysis were carried out on IBM SPSS Statistics 24 and Venny 2.1.0 – BioinfoGP (online). Details of specific statistical methods are described.

Each biomarker was split into quartiles to compare patient subsets based on the levels of biomarkers i.e., upper quartiles. Venny 2.1.0 was used to create Venn diagrams to compare these groups. (Note: the quartiles for each biomarker included patients who fell below the standard curve and so were marked as zero)

To identify whether the continuous variables were either parametric or non-parametric, histograms were created with a bell-shaped curve to see if they were normally distributed. The mean was given for parametric data along with the standard deviation and the median with the range was given for non-parametric data. When two independent samples were being compared either a Student's t-test (parametric) or a Mann-Whitney U test (non-parametric) was used. When more than two independent samples were being compared either

a one-way ANOVA (parametric) or a Kruskal-Wallis test (non-parametric) was carried out. For some of the variables where covariates had to be taken into account a univariate model was used to take into account age, gender height and weight etc. For the serum biomarker data all but periostin were not normally distributed and so data was converted to Log_{10} before adding to the univariate model. For testing the relationship between categorical variables, a Chi squared test was used. To look at the relationship between two continues variables that were non-parametric a spearman's rank correlation was used to get the R^2 value, for parametric data Pearson's correlation coefficient was used instead. Some of the biomarkers were adjusted for age and gender and so a univariate linear model was used to take into account these covariates.

4.4 Results

4.4.1 Demographics, and clinical features of the serum cohort

Table 4.4.1a shows the overall demographics for the serum cohort. There was a total of 708 asthma patients that had serum biomarker available. These patients were mainly GINA 3-5 patients since recruitment centres were asked to primarily focus on moderate-severe patients. Not all the data was available for all the 708 patients' particularly immunological data. Based on 695 patients with age seen data the mean age of the cohort was 41 years (S.D: 11.84). The mean age of onset was 17 years (n= 639) and 71.2% (n= 702) of the cohort were predominately females. The median BMI of the cohort was 29.35 kg/m^2 (n= 595; 14.84-78.16), suggesting the serum cohort was obese (BMI= ≥25kg/m^2). 75.5% were non-smokers (n= 528) and the other 24.5% were either ex-smokers (22.7%, n= 159) or current smokers (1.7%, n= 12) with smoking pack years ranging from 0.5-10 (n= 156). The mean lung function values were 82.07% (S.D: 21.14, n= 669) for FEV_1pp and 0.73 (S.D: 0.12, n= 558) for FEV_1/FVC. A small portion of the cohort had blood total IgE levels and allergic rhinitis data. The median value for the blood total IgE levels were 119.0 KU/L (range: 2-4850 KU/L, n= 262) and 372 (70.1%) out of 531 patients had allergic rhinitis. Additional clinical features for the cohort were hospital admissions, ACQ score and GINA. Out of 641 asthma patients with hospital admissions data 36.5% (n= 234) had an exacerbation within the last year. The mean ACQ7 score was 2.25 (n= 587) suggesting that most of the cohort had unmanaged asthma symptoms (based on BTS guidelines). Finally, the majority of the serum cohort had moderate/severe asthma (GINA1/2: 11.3% [n= 76], GINA 3/4: 70.3% [n= 474] & GINA 5: 18.4% [n= 124]) which is expected as the cohort was designed to recruit predominantly moderate to severe asthma patients (Shrine et al., 2019). Table 4.4.1b outlines the medication the patients in the serum cohort were taking (where available).

Serum Cohort	
Subjects (n)	708
Gender (n)	702
Females	500 (71.2%)
Age seen (Years: Mean & S.D)	41.32 (11.84) N= 695
***Age of onset (Years: Mean & S.D)**	17.59 (15.92) N= 639
***Age of Diagnosis (n)**	667
<16	369 (55.3%)
16-34	176 (26.4%)
35-60	122 (18.3%)
BMI (kg/m^2: Mean & S.D)	29.35 (14.84-78.16) N= 595
Smoking status (n)	699
Never	528 (75.5%)
Ex-smoker	159 (22.7%)
Current smoker	12 (1.7%)
Smoking pack years (Median & Min-Max)	4.0 (0.05-10) N= 156
FEV_1pp (Mean & S.D)	82.07 (21.14) N= 669
FEV_1/FVCprbd Ratio (Mean & S.D)	0.73 (0.12) N= 558
Blood total IgE (KU/L: Median & Min-Max)	119.0 (2-4850) N= 262
Allergic rhinitis (n)**	531
(yes)	372 (70.1%)
Hospital admissions (n)	641
Yes	234 (36.5%)
ACQ7 score (Mean & S.D)	2.25 (1.21) N= 587
GINA (n)	674
1/2	76 (11.3%)
3/4	474 (70.3%)
5	124 (18.4%)

Table 4.4.1a Demographics, clinical and immunological features of the serum cohort n=708: Data are presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). FEV_1pp: forced expiratory volume in 1 second percentage predicted; FEV_1/FVC: forced expiratory volume in 1 second / forced vital capacity ratio; IgE: immunoglobulin E; ACQ: asthma control questionnaire; GINA: global initiative for asthma. *Age of onset- some only known as childhood onset not the actual age so marked as <16 therefore numbers vary for both variables. **Allergic rhinitis is doctor diagnosis and/or self-reported.

Medication	Number of patients
SABA	585
LABA	476
ICS	530
OCS	110
Biologics (Omalizumab only)	25
Other	675

Table 4.4.1b Medication asthma patients were taking in the whole serum cohort. Dosage for ICS and OCS were unavailable for the majority of the patients.

4.4.2 Luminex biomarker results for the whole serum cohort

Table 4.4.2 shows the whole serum cohort Luminex results for each of the analytes measured. The distribution of the data was first looked at using a histogram to check whether the biomarker data was normally distributed. All the biomarker data showed to be not normally distributed and so the data was transformed prior any statistical analysis. Table 3.4.2 displays the median, minimum and maximum has been displayed for the non-parametric data. The first column displays the number of patients with measurable data including those that fell below the standard curve, to which were they given a value of zero. Whereas the second column displays the number of subjects with measurable data only to which the median, minimum and maximum was only displayed for those patients. Out of the 708 patients in the serum cohort, periostin was the only biomarker that could measure all the 708 patients (median: 77536.63pg/mL). Whereas 247 patients out of the 708 could only be measured for IL-5 (median: 6.08 pg/mL) and 324 out of 708 could only be measured for IL-13 (median: 362.33 pg/mL). This suggests IL-5 and IL-13 are difficult to measure in the serum as the levels are too low for the assay to detect suggesting a more sensitive assay is needed to measure these cytokines.

Analyte	Amount measured (incl. subjects below standard curve)	Amount measured (excl. subjects below standard curve)	Median	Min-max
Eotaxin	703	697	165.38	4.67-2481.32
IL-4	703	631	29.67	0.65-7406.05
IL-6	707	698	1.94	0.01-178.55
IL17/17A	697	438	3.7	0.01-952.43
Periostin	708	708	77536.63	9219.04-699421.52
TSLP	689	642	3.20	0.01-298.99
IFN-gamma	671	652	135.76	1.02-29189.50
IL-5	705	247	6.08	0.15-257.93
IL-13	616	324	362.33	1.20-8050.07
IL-33	677	674	27.34	0.09-6238.30
ST2	699	699	9544.19	955.62-4010000.00

Table 4.4.2 Serum Cohort Luminex Results N= 708: Data is displayed as the median and the minimum-maximum (min-max) for each analyte in those subject that fell within the standard range.

4.4.3 Correlations between the type 2 and non-type 2 biomarkers in the whole serum cohort

Figure 4.4.3 shows the correlation between all the Type 2 and non-Type 2 biomarkers in the serum cohort. Like in chapter 2 an R^2 value of 0.5 or above was considered biologically significant and the R^2 values highlighted in bold indicate that they are statistically significant after Bonferroni correction (See appendix 7.3.1 for all the p-values between the biomarkers). As mentioned in the chapter 3 discovery and replication cohorts, IL-33 and IFN-gamma also showed to have the strongest correlation out of all biomarkers measured (R^2= 0.904). As seen in chapter 3 TSLP showed high correlation with IL-4, IL-5 and IL-33 (R^2= 0.677, R^2= 0.593 and R^2= 0.582; respectively) however, here TSLP also showed high correlation with IFN-γ (R^2= 0.549). In addition, like in chapter 3, IL-4 showed some coordinated expression with IL-33 (R^2= 0.540) however, in the whole serum cohort, IL-4 showed high correlation with IL-5 and IFN-γ (R^2= 0.513 and 0.518; respectively). Lastly, as in chapter 3 periostin and ST2 showed no correlation between any of the biomarkers and many did not reach statistical significance either.

	Eotaxin	IL4	IL6	IL17/17A	Periostin	TSLP	IFN-γ	IL5	IL13	IL33	ST2
Eotaxin											
IL4	**0.368**										
IL6	**0.263**	**0.366**									
IL17/17A	**0.279**	**0.480**	**0.216**								
Periostin	**0.034**	0.016	0.008	0.013							
TSLP	**0.397**	**0.677**	**0.361**	**0.424**	0.004						
IFN-γ	**0.262**	**0.518**	**0.265**	**0.225**	0.0002	**0.549**					
IL5	**0.370**	**0.513**	**0.365**	**0.408**	0.013	**0.593**	**0.375**				
IL13	**0.281**	**0.372**	**0.192**	**0.269**	**0.020**	**0.458**	**0.254**	**0.461**			
IL33	**0.298**	**0.540**	**0.296**	**0.239**	0.001	**0.582**	**0.904**	**0.398**	**0.240**		
ST2	**0.172**	**0.066**	**0.108**	**0.031**	0.007	**0.069**	**0.088**	**0.062**	**0.031**	**0.157**	
ST2	**Eotaxin**	**IL4**	**IL6**	**IL17/17A**	**Periostin**	**TSLP**	**IFN-γ**	**IL5**	**IL13**	**IL33**	**ST2**

Figure 4.4.3 Heat map of the correlations between each biomarker in the serum cohort N= 708: Data is presented as the R^2 value for all the correlations between each analyte with red indicating a high R^2 value and green being low. An R^2 value of 0.5 and above is considered to be biologically significant. Values highlighted in bold indicate that they are statistically significant after Bonferroni corrections p= 0.0004.

4.4.4 Overlap of Patients in the Top 25% of the Type 2 Cytokines IL-13, IL-4 and IL-5

Figure 4.4.4 shows the patients who were in the top 25% for the Type 2 cytokines IL-13, IL-4 and IL-5 and the percentage overlap between them all. See appendix 7.3.4 for each biomarker's lowest cut off point for the top quartile group. This data shows that 43.7% of the patients are high for all three cytokines which is in line with the literature that has shown that these cytokines are produced together by the same cell types (Walker and McKenzie, 2018). Whereas 6.3% are high for IL-13 & IL-4, 2.5% are high for IL-13 & IL-5 and 16% are high for IL-5 & IL-4. Only 12.2% were high for IL-13 alone, 8% for IL-4 alone and 11.3% for IL-5 high alone.

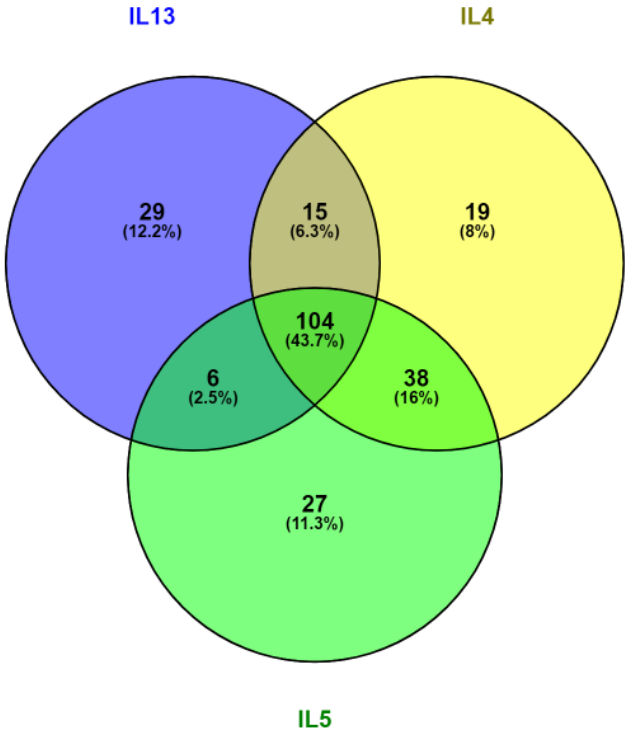


Figure 4.4.4 The overlap of IL-13, IL-4 and IL-5 high patients from patients with serum data N= 238: The data here is presented as patients in the top 25% for IL-13, IL-4 and IL-5 and their percentage overlap between the Type 2 cytokines.

4.4.5 Overlap of Patients in the Top 25% for Th1 (IFN-γ), Th2 (IL-13) and Th17 (IL17A) Inflammation

Figure 4.4.5 shows the patients who were in the top 25% for the biomarkers IFN-γ, IL-13 and IL17A which are associated with three different inflammation pathways Th1, Th2 and Th17 and the percentage overlap between them all. See appendix 7.3.4 for each biomarker lowest cut off point for the top quartile group. The data shows that 24.4% of the patients are high for all three biomarkers, whilst 12.5% are high for IL-13 & IL17A, 10% are high for IL-13 & IFN- γ and 12.2% are high for IFN- γ and IL17A. 15.5% of patients showed to be high for only IFN- γ, 15.5% were high for only IL17A and 10% showed to be high for only IL-13. The above data (Figure 4.4.4) clearly demonstrates a high concordant expression with all 3 Th2 cytokines as 43.7% were high for all 3 biomarkers. Whereas in contrast, nearly less than half (24.4%) were high for the three cytokines relating to the Th1, Th2 and Th17 pathway suggesting a less concordant expression between these three different inflammatory pathways.

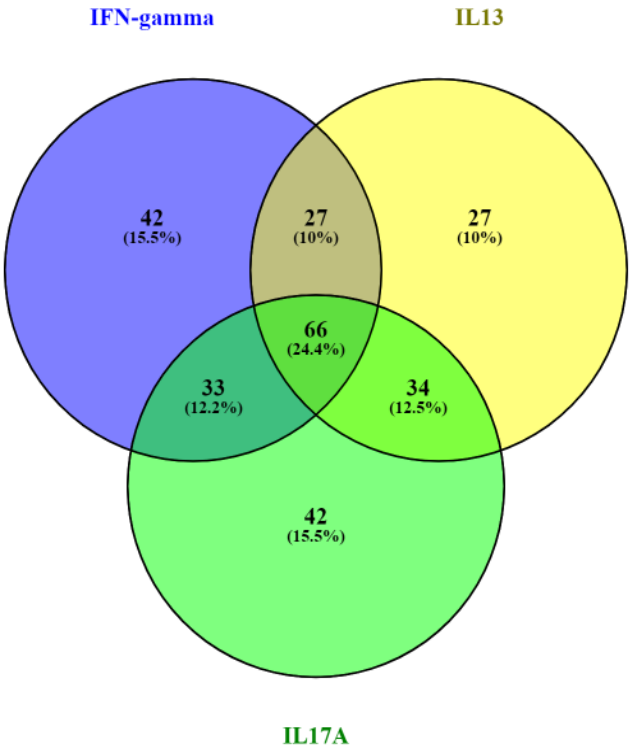


Figure 4.4.5 The overlap of IFN-γ, IL17A and IL-13 high patients from patients with serum data N= 271: The data here is presented as patients in the top 25% for IFN-γ, IL17A and IL-13 and their percentage overlap between the Th1, Th2 and Th17 cytokines.

4.4.6 Demographic and clinical features of patients who are Th1, Th2 or Th17 high

Table 4.4.6 displays the demographic and clinical features of patients who are either Th1 high, Th2 high or Th17 high. Patients who were in the top 25% for either IFN-γ, IL-13 or IL17A exclusively were used for determination of high Th1, high Th2 or high Th17 groups respectively. Patients who overlapped with either IFN-γ, IL-13 or IL17A were not include in the analysis. 42 patients were in the Th1 group, 27 in the Th2 group and 42 in the Th17 group. The data here shows that none of the demographical or clinical features were associated with any of the groups. The mean age for the Th1 high group was 41.2 years (S.D: 13.63) with 76.2% being females and the mean age of onset being 17 years (S.D: 13.69). For the Th2 high group the mean age was 36.0 years (S.D: 10.74) with 55.6% being females and the mean age of onset being 11.1 years (S.D: 14.19). Th17 high patients mean age was 42.1 years (S.D: 12.08) with 61.9% being females and the mean age of onset being 16.3 years (S.D: 16.58). None of these features were statically different across the groups despite the fact Th2 high patients mean asthma age of onset was childhood age. Lung function values were similar across the three groups (FEV_1pp: 81.46%, 86.91% and 83.78% respectively). Additionally, none of the other asthma features such as hospital admissions and ACQ score showed any significant differences across the groups.

Demographics and clinical data stratified based on asthma patients who are Th1, Th2 or Th17 high

	Th1 high	Th2 high	Th17 high	P value
Subjects (n)	42	27	42	
Age seen (years: Mean & S.D)	N=42 41.2 (13.63)	N=27 36.0 (10.74)	N=41 42.1 (12.08)	0.119
Age of onset (years: Mean & S.D)	N=39 17.0 (13.69)	N=24 11.1 (14.19)	N=40 16.3 (16.58)	0.288
Age of onset (n): **<16** **16-34** **35-60**	41 21 (51.2%) 14 (34.1%) 6 (14.6%)	25 20 (80%) 3 (12%) 2 (8%)	41 25 (61%) 8 (19.5%) 8 (19.5%)	0.128
Gender (n): **Female**	42 32 (76.2%)	27 15 (55.6%)	42 26 (61.9%)	0.169
BMI (Kg/m^2: Median & Min-Max)	N=30 27.95 (19.98-46.64)	N=25 30.10 (19.13-54.30)	N=35 29.07 (19.04-54.90)	0.823
Smoking status (n): **Never** **Ex-Smoker** **Current**	41 29 (70.7%) 11 (26.8%) 1 (2.4%)	27 21 (77.8%) 6 (22.2%) 0	42 36 (85.7%) 6 (14.3%) 0	0.424
FEV_1pp (Mean & S.D)	N=39 81.46 (20.19	N=26 86.91 (18.39)	N=40 83.78 (20.09)	0.565
FEV_1FVC (Mean & S.D)	N=29 0.71 (0.09)	N=23 0.73 (0.12)	N=35 0.72 (0.13)	0.880
Blood total IgE (KU/L: Median & Min-Max)	N=16 103.0 (19-2125)	N=10 104.5 (32-4850)	N=13 104.0 (5.20-2457)	0.949
*** Allergic rhinitis (n):** **Yes**	30 25 (83.3%)	19 15 (78.9%)	34 22 (64.7%)	0.206
Hospital admissions (n): **Yes**	34 12 (35.3%)	26 8 (30.8%)	40 14 (35%)	0.921
Mean ACQ Score 7 (Mean & S.D)	N=30 2.29 (1.37)	N=25 2.16 (1.28)	N=38 2.46 (1.13)	0.640
GINA (n): **1/2 (Mild)** **3/4 (Moderate/ Severe)** **5 (Severe)**	41 2 (4.9%) 32 (78%) 7 (17.1%)	25 5 (20%) 16 (64%) 4 (16%)	39 9 (23.1%) 19 (48.7%) 11 (28.2%)	0.059

Table 4.4.6 Demographics and clinical data of patents that are Th1 high, Th2 high or Th17 high n= 111: Data is presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). BMI: body mass index, FEV_1prbd: forced expiratory volume in 1 second pre-bronchial dilator, FVCprbd: forced vital capacity pre-bronchial dilator, FEV1pp: forced expiratory volume in 1 s percentage predicted; FVC: forced vital capacity; IgE: Immunoglobulin E, ACQ: asthma control questionnaire and GINA: Global Initiative for Asthma. *Allergic rhinitis is doctor diagnosis and self-reported. Bonferroni correction p= 0.004.

4.4.7 Overlap of Patients in the Top 25% for the Biomarkers that Target the Current Monoclonal Antibody Drugs Available in Clinic IL-13/IL-4, IL-5, IL-33 and TSLP

Figure 4.4.7 shows the patients who were in the top 25% for the biomarkers IL-13/IL-4 IL-5, TSLP and IL-33 and the percentage overlap between them all. See appendix Table 7.3.4 for each biomarker lowest cut off point for the top quartile group. These biomarkers are also associated with asthma monoclonal antibody drugs (Anti-IL-5: Mepolizumab, Reslizumab and Benralizumab, anti-IL-4/IL-13: Dupilumab, anti-TSLP: Tezepelumab and anti-IL-33: ANB020) which are either existing or new biologics that target primarily the type 2 pathway. Nearly a third of asthma patients (29.3%) out of 238 were high for all 4 biomarkers suggesting that these patients may benefit from any of these biologics or even benefit from combined therapy. Whereas only 3.3% were high for TLSP, IL-4/IL-13 and IL-33, 1.2% were high for IL-5, IL-33 and IL-4/IL-13, 10.2% were high for IL-5, TLSP and IL-4/IL-13 and 11.4% were high for TSLP, IL-33 and IL-5. 15.4% of patients were high for IL-33 only with 7.3% being only IL-5 high, 3.7% being TSLP high and 0.8% being IL-4/IL-13 high only. Overall, the data here shows the largest group is high for all 4 biomarkers therefore, asthma patients are predominantly present with multiple inflammatory profiles. Furthermore, patients may be suitable for multiple drug targets rather than just one suggesting there are multiple targets within and related to type 2 inflammation.

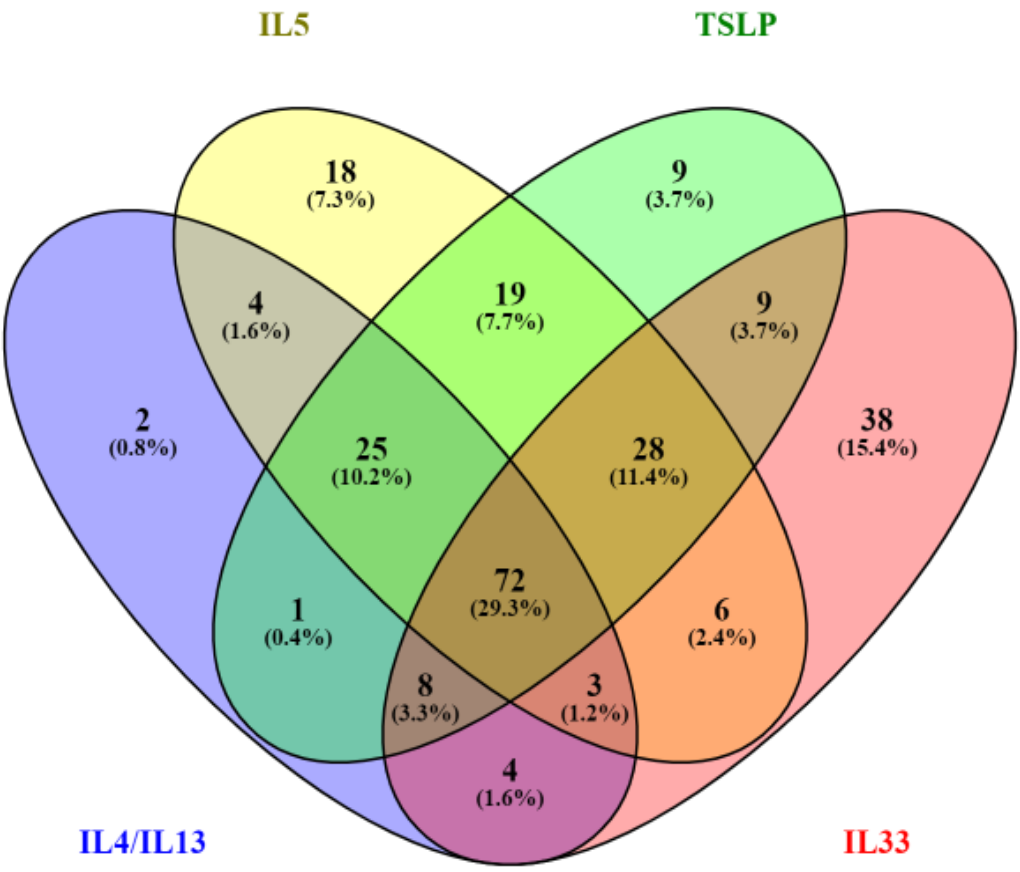


Figure 4.4.7 The overlap of IL-13/4, IL-5 and TSLP high patients from patients with serum data N= 238: The data here is presented as patients in the top 25% for IL-13/4, IL-5, IL-33 and TSLP and their percentage overlap between the biomarkers.

4.4.8 Is Asthma Age of Onset Associated with the Biomarkers in the serum?

To complement the exploratory analyses in chapter 3 investigating the relationship between blood biomarker levels and clinical and immunological features of asthma the biomarker levels were also examined in the three previously established asthma sub-phenotypes in chapter 2 (childhood vs adult-onset asthma and exacerbation prone vs stable). Here the biomarkers were examined with the three age of onset groups childhood (<16) vs adult onset (16-34 and >35) to see whether there was any association between age of onset and type 2 and non-type 2 biomarkers. Serum biomarker data was transformed to log_{10} prior analysis, and the appropriate biomarkers were adjusted for age and/or gender using a univariate analysis (see appendix 7.3.2 for which biomarkers were adjusted for age and gender). Table 4.4.8 shows the mean and standard deviation of all the serum biomarkers measured across the three age of onset categories. The data here shows that none of the biomarkers are statistically significant with asthma age of onset.

	<16	**16-34**	**>35**	**P value**
Subjects (n)	369	176	122	-
Eotaxin (log_{10} pg/mL)	2.2 (0.32) N= 359	2.23 (0.32) N= 172	2.24 (0.35) N= 118	0.802
IL-4 (log_{10} pg/mL)	1.56 (0.58) N= 334	1.51 (0.54) N= 160	1.62 (0.62) 2.25 N= 100	0.422
IL-6 (log_{10} pg/mL)	0.29 (0.47) N= 359	0.31 (0.44) N= 171	0.35 (0.52) N= 121	0.999
IL17/17A (log_{10} pg/mL)	0.60 (0.80) N= 237	0.61 (0.74) N= 102	0.77 (0.84) N= 79	0.258
Periostin (log_{10} pg/mL)	4.87 (0.16) N= 367	4.88 (0.15) N= 176	4.88 (0.19) N= 122	0.925
TSLP (log_{10} pg/mL)	0.58 (0.75) N= 343	0.54 (0.82) N= 160	0.62 (0.93) N= 100	0.826
IFN-y (log_{10} pg/mL)	2.24 (0.82) N= 344	2.16 (0.80) N= 163	2.20 (0.85) N= 105	0.576
IL-5 (log_{10} pg/mL)	0.72 (0.48) N= 138	0.84 (0.56) N= 56	0.81 (0.51) N= 43	0.272
IL-13 (log_{10} pg/mL)	2.58 (0.46) N= 177	2.48 (0.58) N= 78	2.71 (0.50) N= 52	0.070
IL-33 (log_{10} pg/mL)	1.60 (0.83) N= 354	1.48 (0.79) N= 168	1.48 (0.93) N= 113	0.189
ST2 (log_{10} pg/mL)	4.0 (0.36) N= 359	4.04 (0.40) N= 172	4.04 (0.42) N= 119	0.566

Table 4.4.8 Serum Biomarker Data Stratified Based on Age of Onset n= 667: Data is presented as mean & standard deviation log_{10} pg/mL A univariate analysis was used to analyse the data and as highlighted in table 7.3.2 these biomarkers were adjusted for age, gender or both.

4.4.9 Are hospital admissions associated with serum biomarkers?

Here the biomarkers were examined with the hospital admissions (never and ever) to see whether there was any association between hospital admissions and type 2 and non-type 2 biomarkers. Table 4.4.9 shows the mean and standard deviation of all the serum biomarkers measured across hospital admissions. Serum biomarker data was transformed to log_{10} prior analysis, and the appropriate biomarkers were adjusted for age and/or gender using a univariate analysis (see table 7.3.2 for which biomarkers were adjusted for age and gender). The data here shows that none of the Type 2 or non-Type 2 biomarkers are statistically significant with hospital admissions.

	Never	Ever	P Value
Subjects (n)	407	234	-
Eotaxin (log_{10} pg/mL)	2.23 (0.32) N= 396	2.19 (0.35) N= 228	0.526
IL-4 (log_{10} pg/mL)	1.58 (0.58) N= 360	1.52 (0.56) N= 206	0.274
IL-6 (log_{10} pg/mL)	0.28 (0.50) N= 398	0.32 (0.44) N= 228	0.162
IL17/17A (log_{10} pg/mL)	0.64 (0.82) N= 265	0.59 (0.72) N= 142	0.553
Periostin (log_{10} pg/mL)	4.88 (0.15) N= 406	4.86 (0.18) N= 233	0.232
TSLP (log_{10} pg/mL)	0.59 (0.79) N= 373	0.55 (0.81) N= 151	0.639
IFN-y (log_{10} pg/mL)	2.22 (0.82) N= 375	2.16 (0.83) N= 220	0.364
IL-5 (log_{10} pg/mL)	0.74 (0.50) N= 148	0.77 (0.51) N= 80	0.704
IL-13 (log_{10} pg/mL)	2.59 (0.53) N= 193	2.53 (0.46) N= 106	0.404
IL-33 (log_{10} pg/mL)	1.54 (0.86) N= 394	1.48 (0.80) N= 218	0.394
ST2 (log_{10} pg/mL)	4.0 (0.36) N= 399	4.01 (0.38) N= 225	0.303

Table 4.4.9 Serum Biomarker Data Stratified Based on Hospital Admissions: n= 641 Data is presented as mean & standard deviation log_{10} pg/mL A univariate analysis was used to analyse the data and were highlighted in table 7.3.2 these biomarkers were adjusted for age, gender or both.

4.4.10 Relationship between asthma clinical features and the biomarkers in the serum cohort

The relationship between the biomarkers and clinical features of asthma as described in chapter 3 were also evaluated in the whole serum cohort to increase power and to see if any of the results were replicated in section 3.4.5. Figure 4.4.10 demonstrates that some of the serum biomarkers identified clinical subgroups of patients in the serum cohort. Appendix 7.3.3A-D display the data for the relationship between 11 biomarkers and the clinical features. The results from chapter 3 in the discovery cohort demonstrated a relationship between IL-17/17A, TSLP and ST2 with GINA severity. The relationship with GINA severity was also observed here with TSLP and IL-17/17A p= 0.043 and 0.017; respectively (see appendix 7.3.3A). Looking in the whole serum cohort IL-6 was only statistically significant with GINA (p= $3.37x10^{-4}$) out of all the biomarkers and clinical features after Bonferroni correction p= <0.005. Figure 4.4.10 shows the levels of IL-6 to be higher in the GINA 3&4 group (0.34 log_{10} pg/mL) compared to the mild asthma group GINA 1&2 (0.13 log_{10} pg/mL) p= $4.51x10^{4}$.

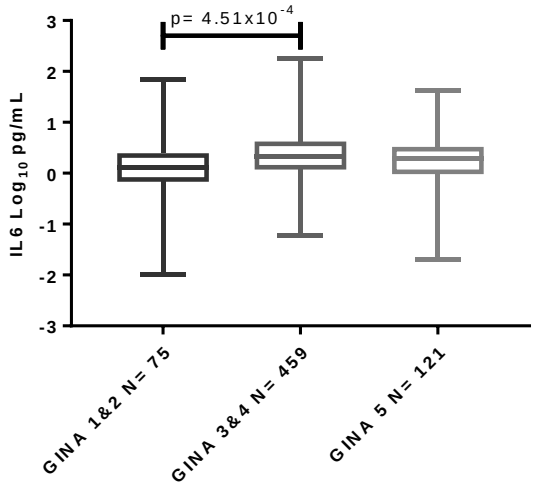


Figure 4.4.10 IL-6 levels were elevated in patients in GINA 3&4 than GINA 1&2 in the whole serum cohort N= 708: the data is displayed as min and max box plot. A: serum IL-6 levels were higher in GINA 3&4 compared to GINA 1&2 after adjusting for age p= $4.51x10^{-4}$ and after Bonferroni correction p= 0.005.

4.5 Discussion

Blood and airway biomarkers have been used to help identify which patients may benefit from new monoclonal antibody therapies such as Mepolizumab and Omalizumab. However, with new biologics coming into clinic it is important to know which patient will benefit from which biological target. Furthermore, more asthma monoclonal antibodies are being developed that target different inflammation pathways (Th1 and Th17) and so there will be a need for additional biomarkers to help identify what type of inflammation is driving a patient's asthma to know which therapy they will benefit from. There are also patients who still do not respond to these biological therapies so identifying the type of inflammation that is driving their asthma may indicate which biologic would be more suitable for them. Finally, biomarkers such as eosinophils have shown to be unreliable and inaccurate as their levels can fluctuate from day to day and even during time of day, suggesting more accurate biomarkers need to be identified to measure a patients inflammatory profile (Tavernier et al., 2018) (Durrington et al., 2018).

The hypothesis for this chapter was that cytokine and/or protein biomarkers in the blood of asthma patients may help identify patients with a specific inflammatory profile. The data in this study found that asthma patients predominated presented with multiple inflammatory profiles as the largest groups of asthma patients where high for multiple cytokines in the blood particularly Th2 cytokines. This demonstrates a particular subset of patients which may be responsive to a specific therapy or even combination therapy. Secondly, serum biomarker levels did not facilitate any of the previously described clinical subsets of asthma patients and even in the Th1, Th2 and Th17 "high" only patient groups. Finally, this study has shown that many serum biomarkers show correlated expression particularly those associated with Th2 as seen in chapter 3. In this chapter all 708 asthma patients with serum biomarker data were included in the analysis to increase the power. The correlations between the biomarkers from chapter 3 were repeated but using the whole serum cohort. The correlation coefficients results found in chapter 3 were again reproduced here in all 708 patients, with the strongest correlations

being mostly between the Type 2 cytokines and also IL-33 and IFN-γ (see discussion of the results in the chapter 3.5).

For the inflammatory profile approach, patients were split into quartiles for each biomarker and the top quartiles were used. Those biomarkers that had data that fell below the standard curve (IL-4, IL-5 and IL-13) were given a value of zero and so a top quartile group was able to be created with the patients that had measurable values. The data from this study suggests that multiple asthma patients are high for more than one inflammatory marker particularly those related to type 2 inflammation. IL-33 and TSLP are upstream of the Th2 pathway, and IL-33 and TSLP activate dendritic cells, basophils and ILC2 cells which induce the Type 2 adaptive immune response causing Th2 cytokines IL-4, IL-5 and IL-13 to be produced (Caminati et al., 2018). This would suggest why multiple patients maybe high for all three Th2 cytokines as these immune cells may be activated at the same time causing high levels of Th2 cytokines be released together.

When looking at the overlap between Th1, Th2 and Th17, the largest group was those high for all three suggesting there may not be distinct Th1, Th2 or Th17 inflammatory profiles in asthma patients but instead they can be presented with multiple inflammatory profiles. Cell and sputum studies have also shown this overlap of inflammatory pathways. One study showed that IL-23 and Th17 cells induced Th17 mediated neutrophilic inflammation but also upregulated Th2 cell mediated eosinophilic airway inflammation (Wakashin et al., 2008). IL-33 has also been shown to amplify activity in basophils to induce Th2 responses and in natural killer cells to induce IFN-γ for Th1 responses (Smithgall et al., 2008). Moreover, data has also shown CD4+ cells stimulated with IL-33 and IL-12 resulted in an increase of IFN-γ (Seltmann et al., 2013) (Smithgall et al., 2008) suggesting IL-33 may play an important role in amplifying both Th1 and Th2 immune responses.

Multiple sputum studies have tried determining the inflammatory expression profile in asthma patients such as type-2 versus non-type 2 (Baines et al., 2014) (Modena et al., 2017). Cluster analysis data of sputum cytokine-high profiles in 205 asthma patients, showed diversity in Th2-high (IL-4, IL-5 and

IL-13 cytokine high) asthma patients. Their results showed 4 out of 5 cluster analyses had a mixture of both type 2 and non-type 2 cytokines (Seys et al., 2017). Data from a sputum transcriptomic study also demonstrated a mixture of neutrophilic/eosinophilic inflammation was more prevalent in patients with severe asthma compared with mild/moderate asthma. Their data suggests both Th2 and non-Th2 pathways maybe interacting in some severe asthma patients (Rossios et al., 2018). The data from this study and previous studies suggest that multiple inflammatory pathways maybe activated at the same time in some asthma patients, indicating that these pathways may interact with each other or even have a common upstream driver. This may explain the overlap in cytokines for different inflammatory pathways in a large proportion of asthma patients particularly for all three pathways (24.4%). However, there was still a high proportion of patients who were high for only one of these biomarkers suggesting these patients are likely to predominantly have either Th1 (15.5%), Th2 (10%) or Th17 (15.5%) driven asthma. These findings highlight the heterogeneity in airway inflammation amongst asthma patients and that there may not be distinct Th1, Th2 or Th17 inflammation.

The current therapies target multiple components on the type 2 pathway i.e. Mepolizumab (anti-IL-5), Dupilumab (anti-IL-4/IL-13), Tezepelumab (anti-TSLP). The data indicates that these patients who overlapped for multiple Th2 cytokines (43.7%) may have type 2 driven inflammation and could benefit from several of these new therapeutic targets or even combination therapy. Whereas those which were high for either IL-4 (8%), IL-13 (12.2%) or IL-5 (11.3%) may benefit from monotherapy instead. Since the data showed large overlap of Th2 cytokines in a proportion of asthma patients, it could give a greater effect or be more beneficial to target upstream of the type 2 pathway i.e., IL-33 or TSLP. This study showed IL-33 and TSLP to be highly correlated with Th2 cytokines suggesting they are in the same mechanistic pathway as each other. Therefore, blocking upstream of the pathway may prevent downstream activation of Th2 cells and associated cytokines that contribute to the hallmarks of type 2 driven asthma such as elevated IgE or eosinophils levels. Also, recent clinical trials on anti-TSLP have suggested that inhibiting TSLP may have wider physiological effects than targeting individual Th2

cytokines as it showed to reduce blood eosinophil counts, serum IgE and FeNO levels after 4 weeks of treatment (Corren et al., 2017). Another point to consider when deciding whom may benefit from these drugs is that cytokines drive different features of asthma as mentioned above. For example IL-4 drives the class switching of B-cell immunoglobulin towards IgE (Paul, 2015) therefore patients with elevated levels of IgE may benefit more from anti-IL-4/IL-13 therapy. Eosinophil levels are also driven and activated by IL-5 (Pelaia et al., 2019) and so anti-IL-5 therapies are usually responded better by patients with high eosinophil levels. Furthermore, this high overlap in high for multiple cytokines associated with current and upcoming asthma monoclonal antibodies drugs, suggests that these patients may be more amenable to combination therapy. A mouse study demonstrated knocking out the IL-33 receptor ST2 increased TSLP production which stimulated ILC2 and mast cells to produce IL-13 and IL-9 leading to alterations in goblet cell function and mucus production in the airway cells. However, they saw a reduction of IL-5 indicating IL-33 is important for eosinophilic inflammation. The study suggested that even when blocking IL-33 asthma can still persist due to increased TSLP production causing airway physiological changes and when using an anti-TSLP antibody these changes were abrogated (Verma et al., 2018). Therefore, blocking TSLP in addition to IL-33 may be beneficial for those patients who do not respond to anti-IL-33 therapy alone suggesting combination therapy may be desirable for those patients who are high for both TSLP and IL-33.

Additional analysis was carried out to look at whether an inflammatory signature could be identified with clinical features of asthma including age of onset and hospital admissions. The data for age of onset and hospital admissions showed no association with the 11 biomarkers indicating that the sera may be not ideal at distinguishing previously described subgroups of asthma. Finally, the relationship between the biomarkers and clinical features of asthma were re-analysed using the whole serum cohort. Most of the associations found in chapter 3 were not replicated as they did not meet statistical significance with Bonferroni correction. Serum IL-6 only showed association with asthma severity (GINA) after Bonferroni correction, other

studies has also shown serum IL-6 levels to be elevated in moderate to severe adult asthma patients (Yokoyama et al., 1995). Overall, the data again suggests that serum biomarkers may not be useful at identifying inflammatory phenotype profiles of asthma patients even after increasing the power in the analysis. Potentially using sputum samples maybe a better way forward at identifying inflammatory phenotype profiles in asthma patients.

In conclusion patient serum levels of current drug targets in development showed some overlap. This suggests measuring a patient's inflammatory profile could help identify what type of inflammation a patient's asthma is driven by and could therefore help guide treatment and see who may benefit from new biologics or who may need combination therapy. However, serum biomarkers did not identify asthma clinical phenotypes suggesting using the sera to measure cytokines and signalling proteins may not be useful at identifying subsets of asthma patients. Potentially using sputum to measure a patient's inflammatory profile may be more beneficial as this is more direct measurement of the airways as described in the discussion from chapter 3. Nevertheless, the data confirmed data from previous studies that multiple Th2 cytokines are activated as they show high correlated expression and also multiple inflammation pathways may be activated in some patients.

Chapter 5- Do the Genetics Identified in the Moderate-Severe Asthma GWAS Drive Inflammation and Specific Clinical Features?

5.1 Introduction

A GWAS of moderate to severe asthma was carried out by Shrine et al in 2019 to identify genetic variants associated with moderate to severe asthma and to provide mechanistic insight using expression analyses in asthma patients. 25 genome-wide significant signals were discovered that were associated with the risk of developing moderate to severe asthma however, the *FLG* signal was excluded as it was influenced by atopy comorbidity. Many of these genes are in loci where the signals suggest genes/pathways that are involved in the innate and adaptive immune responses. Three of the signals identified were novel to moderate to severe asthma (*GATA3*, *MUC5AC* and *KIAA1109)* and the other 21 signals have previously been described in other asthma GWAS. To date, this is the largest GWAS carried out on moderate to severe asthma patients. This chapter will follow up these 24 genetic signals plus and additional signal, *FLG,* which was excluded in the GWAS due to the signal been driven by atopy. Patients from the GASP database that have genotyping data available for these 25 genetic signals as well as those with serum biomarker and clinical data will be utilised in this study. The first part of the study will look at whether the signals identified in the moderate-severe asthma GWAS drive Th1, Th2 or Th17 inflammation in the sera of asthma patients. The second part will look at whether the genetic signals drive the expression of different asthma phenotypes including age of onset, lung function, exacerbations, serum IgE, ACQ score, allergic rhinitis, atopic dermatitis and blood eosinophil levels. The aim is to identify the factors driving asthma phenotypes and characterise the inflammatory profiles of asthma patients. This may identify suitable biomarkers which will allow stratification of asthma patients and identify potential suitable biological treatment for severe asthma patients.

5.1.1 GWAS that have found Genetic Signals Related to the Type 2 Inflammation Pathway

There have been many successful findings of signals from asthma GWAS that the implicated candidate casual genes/loci are related to the type 2 inflammation pathway including *GATA3*, *STAT6*, *CD247*, *BACH2*, *TSLP*, *IL-33*, *IL1RL1*, *RAD50* and *CLEC16A* based on eQTLs (figure 5.1.1). Most of these genes and loci are more involved in innate immunity, however some are also involved in adaptive immunity and make potential therapeutic targets for asthma. Type 2 driven allergic inflammation is partly dependent on the transcription factor guanine adenine thymine adenine sequence-binding protein 3 (*GATA3*) (Krug et al., 2017) which has been identified in a number of asthma GWASs. *GATA3* is a protein coding gene that is important in regulating the Th2 cell production and promoting the secretion of Th2 cytokines (Hirota et al., 2011) (Demenais et al., 2018) (Shrine et al., 2019). *STAT6* (Signal transducer and activator of transcription 6) is another protein coding gene that regulates Th2 cell production and secretion of Th2 cytokines which has repeatedly been associated with increased susceptibility to asthma (Shrine et al., 2019). It has also been associated with increased risk of other Th2 mediated disease phenotypes such as allergy sensitisation and allergic rhinitis (Moffatt et al., 2010) (Bønnelykke et al., 2013). STAT6 activation leads to altered gene expression profiles in different cells involved in lung inflammation such as T and B lymphocytes, macrophages, airway epithelial cells and smooth muscle cells during an inflammatory response (Valladao et al., 2016).

Thymic stromal lymphopoietin (*TSLP*) is an epithelial derived cytokine and plays a crucial role in many atopic diseases such as asthma. It has been demonstrated that Th2 cellular adaptive response is induced by TSLP via a stimulus. TSLP induces the expression OX40 ligand on the dendritic cells which then stimulates the development of Th2 differentiated cells (Cianferoni and Spergel, 2014). Genetic signals round the gene encoding TSLP has been consistently featured and associated in asthma GWAS and it has been suggested that polymorphisms of the TSLP gene plays a key role in the induction of allergic inflammation (Ferreira et al., 2014) (Demenais et al., 2018)

(Shrine et al., 2019). *RAD50* homolog is adjacent to the IL-4, IL-13 cytokine locus and coded for a protein that functions in housekeeping for DNA double strand break repair, cell cycle checkpoint activation, telomere maintenance and meiotic recombination (Rojowska et al., 2014) (Sharma et al., 2021). This loci has been identified in several studies (Shrine et al., 2019) (Li et al., 2016) and has also be associated with asthma exacerbations and atopy (Bønnelykke et al., 2014) (Schaarschmidt et al., 2015). Li et al (2010) showed the *RAD50-IL13* region had the strongest association with multiple SNPs and it is thought that *RAD50* promotes the development of asthma by inducing the inflammatory cytokines secreted by the Th2 cells (Li et al., 2010).

IL-33 is a cytokine that belongs to the IL1 family, it is highly expressed by epithelial and endothelial cells and plays an important role in allergy and inflammation of asthma (Saluja et al., 2015). IL-33 functions as an alarmin to alert the immune system of epithelial damage during and infection or trauma (Moffatt et al., 2010). Additionally *IL1RL1,* the receptor of IL-33 (also known as ST2) is the gene encoding the Th2-promoting cytokines and regulates the Th2 pathway (Gordon et al., 2016). IL1RL1 levels are also known to be elevated in asthma via pro-inflammatory stimuli such as a virus and is thought to be involved in helper T cell function (Alvarez et al., 2019). Genetic signals spanning round the *IL-33* and *IL1RL1* gene have repeatedly been found in asthma GWAS (Ferreira et al., 2014) (Shrine et al., 2019). *IL1RL1* and *IL-33* are upstream of the Th2 pathway and GWASs have discovered that both variants have been strongly associated with the risk of asthma and blood eosinophil inflammation (Gudbjartsson et al., 2009) (Moffatt et al., 2010). One study looking at a rare IL-33 spliced acceptor variant (rs146597587) found that it produced less IL-33 protein and some of the protein formed lacked the ability to bind to the receptor and promote inflammation. This rare variant was associated with reduced blood eosinophils (β= -0.21 SD, p= 2.5×10^{-16}, N= 103,104) and reduced risk of asthma (OR= 0.47; 95%CI: 0.32-0.70, p= 1.8×10^{-4}, N cases= 6,465, N controls= 302,977) (Smith et al., 2017). This suggests the potential use of targeting the IL-33-IL1RL1 pathway as a therapeutic target for the management and control of asthma.

Using genetic approaches such as GWAS supports targeting the Type 2 pathway and with the rise in new biologics that target the Type 2 inflammatory pathway this has been confirmed. Genetics is now being used to understand asthma heterogeneity and has already explained some of asthma heterogeneity. With childhood and adult onset of asthma being the most commonly accepted phenotypes and many genetic variants specifically associated with childhood onset. This suggests heterogeneity is based, at least partly, on different genetic factors for asthma subtypes e.g. childhood vs adult asthma. Furthermore, using a patient's genetic profile could identify what is driving their asthma which then may indicate their response/suitability to new monoclonal antibody drugs.

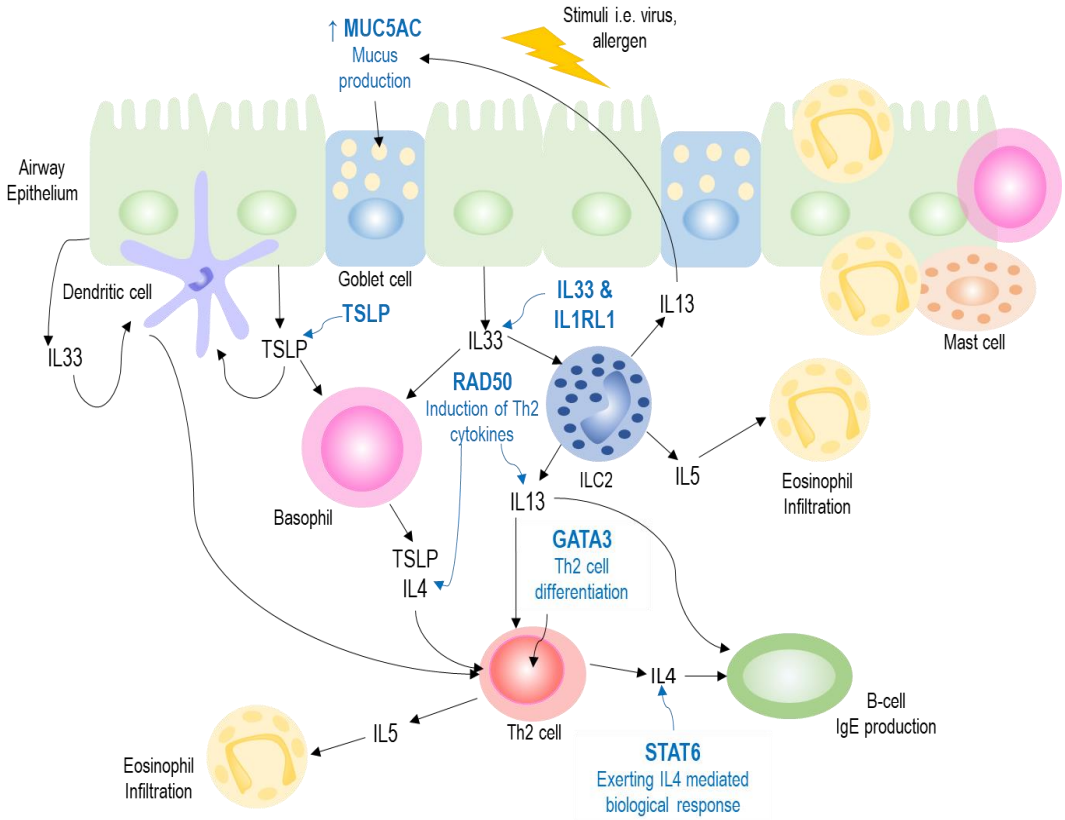


Figure 5.1.1 Candidate casual genes implicated by the genetic signals identified in the moderate-severe asthma GWAS and their contribution with the type 2 inflammation pathway: an environmental stimulus i.e. a virus causes the release of IL-33 and TSLP from epithelial cells. IL-33 and TSLP activate dendritic cells, basophils and ILC2 cells which induce Type 2 adaptive immune response, this causes Th2 cytokines IL-4, IL-5 and IL-13 to be produced. This response results pathological changes in the airways such as airway remodelling, increased mucus production and accumulation of inflammatory cells thus increasing the risk of an asthma exacerbation. Type 2 genes/loci that have been identified in asthma GWAS have been thought to affect the Type 2 pathway (genes and loci are highlighted in blue). Here highlight a few loci/genes that are involved in Th2 cell differentiation such as GATA3 and Th2 cytokine induction including RAD50 and STAT6. TSLP, IL-33 and IL1RL1 are involved in the production of cytokines TSLP and IL-33 which are upstream of the type 2 pathway.

5.1.2 Genetic Signals Identified from the Moderate-Severe Asthma GWAS

As mentioned above, the Shrine et al study on GWAS of moderate to severe asthma identified 25 genetic signals, three of them being novel to moderate to severe asthma and most relating to the Type 2 inflammatory pathway. To add greater understanding the 25 genetic signals (including the *FLG* signal) will be looked at to see whether they drive specific types of inflammation (Th1, Th2 and Th17) in asthma patients using the serum cytokines measured in the serum in chapter 3 and 4 as a marker of different types of inflammation. Additionally, this chapter will also investigate whether the 25 genetic signals lead to any Type 2 related clinical phenotypes including serum IgE, eosinophil levels, allergic rhinitis and allergic dermatitis. Shrine et al (2019) carried out a Moderate-to-severe asthma GWAS of European ancestry. There were 5135 cases and 25 675 controls for stage 1 (discovery), and 5414 cases and 21 471 controls for stage 2 (replication). In stage 1 a genome-wide association study of moderate-to-severe asthma was undertaken, and in stage 2 the independent variants that reached the significance threshold of p less than 1×10^{-6} in stage 1 were followed up. They identified 25 genome-wide significant signals of association with moderate-to-severe asthma, including several signals in innate or adaptive immune-response genes and three novel signals. However, after sensitivity analyses in stage 2, the *FLG* signal was excluded due to being driven partly by atopy. Table 5.1.2 describes the list of SNPs (including *FLG*) identified in the moderate to severe asthma GWAS from the meta-analysis that were replicated in both the discovery and replication cohort and the overall p-value that reached a genome-wide significance p-value of $5x10^{-8}$ or less.

Chr	Closest suggested Gene/Locus	Reported SNP	Risk Allele	Non-risk allele	RAF	P value Metanalysis
Chr1	*CD247*	rs7523907	T	C	54.08%	4.82E-09
Chr1	*FLG*	rs61816761	A	G	2.37%	1.18E-09
Chr2	*IL1RL1*	rs12479210	T	C	38.73 %	1.57E-29
Chr2	*D2HGDH*	rs34290285	G	A	74.26 %	2.24E-23
Chr4	*KIAA1109*	rs56026225	GATT	G	23.60 %	3.06E-09
Chr5	*TSLP*	rs1837253	C	T	74.16 %	1.95E-22
Chr5	*WDR36*	rs1438673	C	T	50.78 %	3.29E-13
Chr5	*C5orf56*	rs3749833	C	T	26.08%	5.60E-14
Chr5	*RAD50*	rs1986009	A	C	18.71 %	2.43E-15
Chr6	*HLA-DQA1*	rs776111176	A	AAT	14.85%	2.61E-16
Chr6	*HLA-DQB1*	rs9273410	A	C	55.30%	5.62E-32
Chr6	*BACH2*	rs367983479	CA	C	61.50 %	6.30E-11
Chr8	*MIR5708*	rs71266076	C	CT	36.93 %	6.53E-13
Chr9	*IL-33*	rs144829310	T	G	16.40 %	2.29E-20
Chr10	*GATA3*	rs10905284	C	A	42.94 %	1.76E-10
Chr10	*LOC101928272*	rs61840192	G	A	57.30%	8.33E-25
Chr11	*MUC5AC*	rs11603634	G	A	50.36 %	2.32E-08
Chr11	*C11orf30*	rs7936312	T	G	47.42 %	6.18E-24
Chr12	*RPS26*	rs7305461	A	C	44.61 %	1.01E-09
Chr12	*STAT6*	rs703816	C	T	43.41 %	3.69E-13
Chr15	*RORA*	rs10519068	G	A	87.25 %	1.84E-12
Chr15	*SMAD3*	rs72743461	A	C	23.60 %	4.52E-14
Chr16	*CLEC16A*	rs7203459	T	C	75.44 %	4.37E-18
Chr17	*IKZF3*	rs2941522	T	C	48.29 %	2.32E-12
Chr17	*ZNF652*	rs112502960	A	G	35.92%	4.12E-11

Table 6.1.2 Summary of results for the 25 sentinel SNPs associated with susceptibility to moderate to severe asthma in the meta-analysis: The table shows the chromosomal position (chr) for each signal reported, the gene/locus that is thought to be the closest to the sentinel SNP reported, the reported SNP in the moderate-severe asthma GWAS study, the risk allele associated with moderate-severe asthma for that particular SNP, the minor allele along with the risk allele frequency (RAF) associated with the SNP and the overall genome-wide significance p-value from the meta-analysis containing data from stages 1 and 2 (Shrine et al., 2019).

5.2 Hypothesis

Genetic risk factors identified for the development of moderate-severe asthma drive specific inflammation leading to disease expression.

5.2.1 Aims

- For the 25 genetic signals identified determine association between serum biomarkers related to Th1, Th2 and Th17 inflammation as well as clinical and immunological features of asthma.

5.3 Methods

Patients were selected from the GASP cohort of 4,205 individuals that had genotyping data, serum biomarker data and/or clinical data. Refer to chapter 2 section 2.3 for the patient inclusion criteria for the GASP cohort, chapter 3 section 3.3 for the inclusion/exclusion criteria for the serum and genetic analysis and chapter 2 section 2.3.1 for the inclusion criteria for the clinical phenotype and genetics analysis.

5.3.1 Selecting Proxy SNPs for the 25 Signals Identified in GWAS

Patients were genotyped for the 25 signals. However the SNPs identified in the GWAS were not directly genotyped and so proxy SNPs were chosen with the highest linkage disequilibrium (LD) to the sentinel SNP. Most of the SNPs reported in the moderate-severe asthma GWAS were imputed as 2,536 were from UK Biobank array data and the rest were either genotyped/extracted. Therefore, proxies were identified from online databases based on their R^2 value. R^2 is a measure of LD between two loci. These SNPs were then searched in the GWAS genotyped database to see if they had been genotyped in the moderate-severe asthma GWAS population. Online databases HaploReg v4.1 and LDlink 3.2.0 were used to search for proxies and the SNP with the highest R^2 value available on the GWAS genotyped database was used as the proxy SNP. HaploReg v4.1 is a database for exploring SNPs using LD information from the 1000 Genomes Project and LDlink 3.2.0 is a web-based application designed to easily and efficiently interrogate LD in population groups. Additionally, the association data for each proxy SNP was extracted from the GWAS stage 1 summary statistics database (see manuscript of (Shrine et al., 2019)). Appendix 7.4.1 and 7.4.2 shows the flow chart on how the proxy SNPs were found and their stage 1 summary statistics were extracted from the server. See table 6.3.1 for the proxy SNPs selected for genotyping.

Chr	Sentinel SNP from GWAS	Locus	SNP for Genotyping	Candidate causal genes	RAF EUR pop	Non-risk allele	Risk Allele	R^2	P Value (Stage 1)
Chr1	rs7523907	*CD247*	rs1773542	*CD247 & CREG1*	0.63	C	T	0.79	4.06E-09
Chr1	rs61816761	*FLG*	rs61816764	*FLG-AS1, THEM4, HRNR, FLG2 & FLG*	0.05	C	T	0.21	1.74E-03
Chr2	rs12479210	*IL1RL1*	rs1420101	*IL1RL1, IL18R1, IL18RAP & IL1R2*	0.34	C	T	0.97	1.23E-15
Chr2	rs34290285	*D2HGDH*	rs1106639	*D2HGDH & PDCD1*	0.74	A	G	0.79	2.15E-12
Chr4	rs560026225	*KIAA1109*	rs17388568	*KIAA1109, ADA1, IL2 & IL21*	0.29	G	A	0.66	2.59E-2
Chr5	rs1837253	*TSLP*	rs1837253	*TSLP & WDR36*	0.75	T	C	1	2.88E-18
Chr5	rs1438673	*WDR36*	rs3806933	*TSLP & WDR36*	0.55	T	C	0.72	1.36E-08
Chr5	rs3749833	*C5orf56*	rs10077785	*SLC22A5, SLC22A4, IL5, IRF1 & IRF1*	0.23	C	T	0.8	1.56E-10
Chr5	rs1986009	*RAD50*	rs2244012	*SLC22A5, RAD50, SLC22A4, IL5 &IRF1*	0.20	A	G	0.86	5.56E-06
Chr6	rs776111176	*HLA-DQA1*	rs532965	*HLA-DQA2, HLA-DQA1 & HLA-DQB1*	0.16	T	G	0.86	4.13E-18
Chr6	rs9273410	*HLA-DQB1*	rs9272629	*HLA-DQA2, HLA-DQA1 & HLA-DQB1*	0.57	G	T	0.55	9.39E-14
Chr6	rs367983479	*BACH2*	rs1847472	*BACH2 & MAP3K7*	0.63	A	C	0.88	1.13E-07
Chr8	rs71266076	*MIR5708*	rs13275219	*ZBTB10 & TPD52*	0.35	C	T	0.76	3.67E-08
Chr9	rs144829310	*IL33*	rs1342326	*ERMP1, IL33 & TPD52L3*	0.17	A	C	0.96	1.06E-11
Chr10	rs10905284	*GATA3*	rs10905279	*GATA3, GATA-AS1 & LINC00708*	0.40	G	A	0.69	4.93E-07
Chr10	rs61840192	*LOC101928272*	rs1444782	*GATA3 & GATA-AS1*	0.42	G	G	0.85	8.56E-14
Chr11	rs11603634	*MUC5AC*	rs28592983	*MUC5AC, MUC5B & MUC5B-AS1*	0.37	C	T	0.54	5.57E-05
Chr11	rs7936312	*C11orf30*	rs61893460	*LRRC32*	0.45	G	A	0.88	1.62E-08
Chr12	rs7305461	*RPS26*	rs10876864	*RAB5B, SUOX, RPS26 & ERBB3*	0.41	A	G	0.96	1.99E-08
Chr12	rs703816	*STAT6*	rs167769	*STAT6, NEMP1 & RBMS2*	0.33	C	T	0.82	8.61E-11
Chr15	rs10519068	*RORA*	rs10519067	*RORA & RORA-AS2*	0.86	A	G	0.99	3.84E-07
Chr15	rs72743461	*SMAD3*	rs17293632	*SMAD3, AAGAB & IQCH*	0.21	C	T	0.98	5.76E-11
Chr16	rs7203459	*CLEC16A*	rs36045143	*DEXI, RMI2 & CLEC16A*	0.75	G	A	0.98	3.83E-15
Chr17	rs2941522	*IKZF3*	rs9303277	*IKZF3, PGAP3, GSDMA, GSDMA & ORMDL3*	0.48	T	C	0.93	1.00E-07
Chr17	rs112502960	*ZNF652*	rs16948048	*GNGT2, PHOSPHO1 & ZNF652*	0.37	A	G	0.94	5.02E-08

Table 6.3.1 Table of proxy SNPs sent for genotyping to LGC Genomics: The table displays the chromosomal position (chr) for each signal reported, the gene/locus that is thought to be the closest to the sentinel SNP reported, the risk allele frequency (RAF) associated with the SNP along with the non-risk and the risk allele associated with moderate-severe asthma for that particular SNP, the sentinel SNP reported from the moderate to severe asthma GWAS study, the proxy SNP used for genotyping, the R^2 value between both SNPs and the p-value from the stage 1 summary statistics.

5.3.2 DNA Extraction and Genotyping by LGC

The genotyping was carried out by an external company LGC Genomics that provides services such as DNA sequencing and DNA/RNA extraction however, all quality control (QC) of the data and analysis was done in Nottingham. Blood samples were sent to LGC for DNA extraction and then genotyping or previously extracted DNA samples were normalised and then sent for genotyping. In order for the 25 target SNPs to be genotyped their sequences had to be sent to LGC. dbSNP was used to search for the 30 sequences which had to be 50 bases long either side of the SNP with the targeted SNP highlighted. dbSNP is a public-domain archive for a broad collection of simple genetic polymorphisms including SNPs, small scale deletions or insertions and retroposable element insertions and microsatellite repeat variations. See appendix 7.4.3 for the flow chart on looking up the 25 SNP sequences and 7.4.4 for the list of SNP IDs and their sequences. Once the DNA samples and the SNP information were sent to LGC Genomics, they were able to carry out the genotyping as described briefly below.

Kompetitive Allele-Specific PCR genotyping assay by LGC Genomics

LGC Genomics used Kompetitive Allele-Specific PCR (KASP) genotyping assay. The KASP assay mix contains three assay-specific non-labelled oligos: two allele-specific forward primers and one common reverse primer. The allele-specific primers each have a unique tail sequence that corresponds with a universal FRET (fluorescence resonant energy transfer) cassette; one labelled with FAM™ dye and the other with HEX™ dye. During thermal cycling, the relevant allele-specific primer binds to the template and elongates, thus attaching the tail sequence to the newly synthesised strand. The complement of the allele-specific tail sequence is then generated during subsequent rounds of PCR, enabling the FRET cassette to bind to the DNA. The FRET cassette is no longer quenched and emits fluorescence. Competitive allele-specific PCR achieves bi-allelic discrimination through the competitive binding of the two allele-specific forward primers. If the genotype at a given SNP is homozygous, only one of the two possible fluorescent signals will be generated. If the genotype is heterozygous, a mixed fluorescent signal will be generated.

5.3.3 Quality Control Checks of Genotyping data

Prior to any analysis, genotyping data was first QC checked to ensure there were no errors such as contamination during sample handling and abnormal genotyping calls (genotyping that does not fall within one of the three clusters explained below) from poor quality DNA sample. Other QC checks such as the minor allele frequency (MAF) in all the SNPs to ensure they were consistent with other European populations and Hardy Weinberg equilibrium (HWE) to indicate of any genotyping errors in the population. In addition to the genotyping sent by LGC, genotyping data from two other large UK datasets UK Biobank and Believe where also QC. LGC genotyping data was sent in two batches and so the four genotyping datasets LGC1, LGC2, UK biobank and Believe were QC separately and then all four datasets were combined together and QC.

5.3.3.1 Visualisation of the SNP Cluster Graphs

The first QC check was visualisation of each cluster graph from LGC genotyping results using the SNPviewer 2 software. LGC provided a cluster graph for each SNP per plate. Genotyping methods use a standard clustering method to “call” the SNP genotypes. A fluorescent signal from each individual DNA sample is represented as an independent data point on a cluster plot. For KASP, the X axis is used to plot the FAM fluorescence value and the Y axis is used to plot the HEX™ fluorescence value for each sample. A sample that is homozygous for the allele reported by the X-signal oligonucleotide will only generate X-signal fluorescence (blue data points in Figure 6.3.3.1) during the end-point genotyping reaction. This data point will be plotted close to the X axis, representing high X-signal and no Y-signal. A sample that is homozygous for the allele reported by the Y-signal oligonucleotide will only generate Y-signal fluorescence (red data points in Figure 6.3.3.1) during the end-point genotyping reaction. This data point will be plotted close to the Y axis, representing high Y-signal and no X-signal. A sample that is heterozygous will contain both the allele reported by the X-signal oligonucleotide and the allele reported by the Y-signal oligonucleotide. This sample will generate half as much X-signal fluorescence and half as much Y-signal fluorescence as the samples that are homozygous for these alleles. This

data point will be plotted in the centre of the plot, representing half X-signal and half Y-signal (green data points in Figure 6.3.3.1). To ensure the reliability of the results, an end-point genotyping reaction without any template DNA was included as a negative control. This is referred to as a no template control (NTC) and so will not generate any fluorescence and the data point will be plotted at the origin of the plot (black data point in Figure 6.3.3.1).

Each florescent tag in the allele on the cluster graph was checked to examine the clusters for homozygote 1, heterozygotes and homozygote 2. Additionally, the general formation and tightness of each cluster region was checked to see if any of the florescent tags fell out of the 3 cluster regions and if so, they would be excluded due to uncertainty. However, all the regions for each graph were tightly clustered with no points needing to be excluded. Figure 6.3.3.1 demonstrates a couple of examples of the cluster graphs.

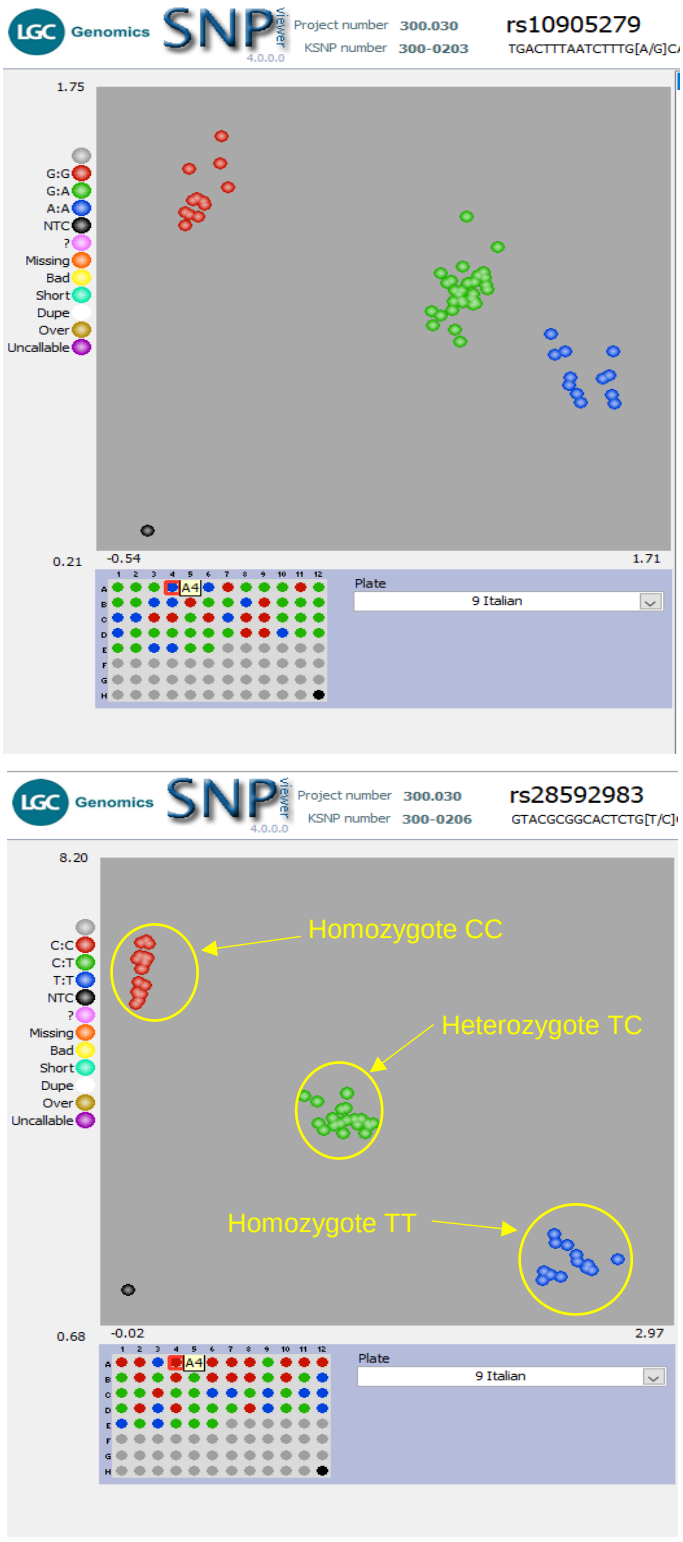


Figure 6.3.3.1 Cluster graphs from SNPs rs10905279 and rs28592983: both cluster graphs were viewed on the SNPviewer 2 software provided by LGC Genomics. Each fluorescence signal (red, green and blue) represents one of the three genotypes (homozygote 1, heterozygote and homozygote 2) and each point representing an individual patient. Black represents the no template control (NTC) which was water. Both graphs show tight clustering for the three different genotypes with no point falling too far out of the three regions.

5.3.3.2 Minor Allele frequency

The MAF is the frequency at which the second most frequent allele occurs in a population; this is useful in genetics as it informs which common or rare variants are present in the population. The MAF was checked in the four datasets separately first and then all four were combined. The MAF from the separate and combined datasets were compared to the MAF from the European population in the 1000 Genomes Project (Phase 3) on Ensembl. This was to ensure the second most common allele for each SNP was consistent in the European population and this studies population. Ensembl is an online source that provides the MAF percentages from the phase 3 1000 Genomes Project in the European population. The MAF was calculated by adding the total amount of each allele and then adding both allele's totals together. The smallest allele value was divided by the total value of both alleles and times by 100 to give the percentage of the minor allele. Table 5.3.3.2A displays the MAF from all four datasets separately and together plus the MAF from the 1000 Genomes Project European population for comparison. Table 5.3.3.2B displays the number of asthma patients that 0, 1 and 2 risk alleles for each SNP that were used in the analysis.

Locus	rs number	Risk allele	LGC 1	LGC 2	Believe	Biobank	All 4 combined	MAF (ensambl)
C5orf56	rs10077785	T	T= 24% C= 76%	T= 27% C= 73%	T= 25% C= 75%	T= 28% C= 72%	T= 27% C= 73%	T= 23% C= 77%
STAT6	rs167769	T	**C= 54%** **T= 46%**	**C= 57%** **T= 43%**	**C= 60%** **T= 40%**	**C= 57%** **T= 43%**	**C= 57%** **T= 43%**	**C= 67%** **T= 33%**
IL-33	rs1342326	C	A= 81% C= 19%	A= 80% C= 20%	A= 80% C= 20%	A= 81% C= 19%	A= 81% C= 19%	A= 83% C= 17%
RAD50	rs2244012	G	G= 22% A= 78%	G= 23% A= 77%	G= 19% A= 81%	G= 22% A= 78%	G= 22% A= 78%	G= 20% A= 80%
CD247	rs1773542	T	C= 38% T= 62%	C= 39% T= 61%	C= 40% T= 60%	C= 37% T= 63%	C= 38% T= 62%	C= 37% T= 63%
FLG	rs61816764	T	C= 94% T= 6%	C= 93% T= 7%	C=94% T=6%	C=93% T=7%	C=93% T=7%	C=95% T=5%
IL1RL1	rs1420101	T	**C= 38%** **T= 62%**	**C= 39%** **T= 61%**	C= 58% T= 42%	C= 58% T= 42%	**C= 52%** **T =48%**	**C= 66%** **T= 34%**
D2HGDH	rs1106639	G	A= 21% G= 79%	A= 24% G= 76%	A= 23% G= 77%	A= 22% G= 78%	A= 22% G= 78%	A= 26% G= 74%
HLA-DQA1	rs532965	G	**G= 22%** **T= 78%**	**G= 22%** **T= 78%**	**G= 22%** **T= 78%**	**G= 23%** **T= 77%**	**G= 23%** **T= 67%**	**G= 16%** **T= 84%**
HLA-DQB1	rs9272629	T	**G= 36%** **T= 64%**	**G= 40%** **T= 60%**	**N/A**	**G=36%** **T=64%**	G= 37% T= 63%	**G=43%** **T=57%**
BACH2	rs1847472	C	A= 33% C= 67%	A= 34% C= 66%	A= 34% C= 66%	A= 32% C= 68%	A= 33% C= 67%	A= 37% C= 63%
MIR5708	rs13275219	T	C= 64% T= 36%	C= 64% T= 36%	C= 64% T= 36%	C= 64% T= 36%	C= 64% T=36%	C= 65% T= 35%
GATA3	rs10905279	A	A= 40% G= 60%	A= 40% G= 60%	A= 41% G= 59%	A= 43% G= 57%	A= 41% G= 59%	A= 40% G= 60%
LOC101928272	rs1444782	G	A= 36% G= 64%	A= 38% G= 62%	A= 41% G= 59%	A= 38% G= 62%	A= 38% G= 62%	A= 42% G= 58%
MUC5AC	rs28592983	T	C= 64% T= 36%	C= 68% T= 32%	C= 66% T= 34%	C= 62% T= 38%	C= 64% T= 36%	C= 63% T= 37%
C11orf30	rs61893460	A	A= 50% G= 50%	A= 48% G= 52%	A= 48% G= 52%	A= 50% G= 50%	A= 49% G= 51%	A= 45% G= 55%
RPS26	rs10876864	G	A= 55% G= 45%	A= 55% G= 45%	A= 53% G= 47%	A= 57% G= 43%	A= 56% G= 44%	A= 59% G= 41%
RORA	rs10519067	G	A= 10% G= 90%	A= 9% G= 91%	A= 13% G= 87%	A= 11% G= 89%	A= 11% G= 89%	A= 14% G= 86%
CLEC16A	rs36045143	A	A= 76% G= 24%	A= 76% G= 24%	A= 76% G= 24%	A= 79% G= 21%	A= 78% G= 22%	A= 75% G= 25%
ZNF652	rs16948048	G	A= 61% G= 39%	A= 61% G= 39%	A= 63% G= 37%	A= 62% G= 38%	A= 62% G= 38%	A= 63% G= 37%
WDR36	rs3806933	C	C= 62% T= 38%	C= 58% T= 42%	C= 58% T= 42%	C= 59% T= 41%	C= 59% T= 41%	C= 55% T= 45%
TSLP	rs1837253	C	C= 72% T= 28%	C= 79% C= 21%	C= 75% T= 25%	C= 78% T= 22%	C= 78% T= 22%	C= 75% T= 25%
SMAD3	rs17293632	T	C= 76% T= 24%	C= 75% T= 25%	C= 73% T= 27%	C= 74% T= 26%	C= 74% T= 26%	C= 79% T= 21%
KIAA1109	rs17388568	A	A= 31% G= 69%	A= 29% G= 71%	A= 26% G= 74%	A= 27% G= 73%	A= 28% G= 78%	A= 29% G= 71%
IKZF3	rs9303277	C	C= 51% T= 49%	C= 53% T= 47%	C= 54% T= 46%	C= 51% T= 49%	C= 52% T= 48%	C= 48% T= 52%

Table 5.3.3.2A The minor allele frequency of the 25 SNPs from the 4 different datasets and all 4 combined: the two LGC datasets were genotyped for 25 SNPs whereas the Believe dataset was genotyped for 24 SNPs and the UK Biobank were genotyped for 25 SNPs thus N/A identifies that particular SNP was not genotyped for that dataset and so MAF was not available. Percentages highlighted in bold indicate that the MAF was more than a 5% difference between the datasets and the data from the 1000 genomes project.

Genetic signal	0 risk alleles	1 risk allele	2 risk alleles	Total
rs10077785	1645	1173	210	3028
rs167769	951	1507	566	3024
rs1342326	1953	958	113	3024
rs2244012	1864	1020	143	3027
rs1773542	470	1367	1185	3022
rs61816764	2661	361	4	3026
rs1420101	841	1428	751	3020
rs1106639	152	1043	1829	3024
rs532965	1773	1122	125	3020
rs9272629	433	957	1075	2465
rs1847472	326	1361	1332	3019
rs13275219	1210	1402	402	3014
rs10905279	1057	1492	475	3024
rs1444782	452	1422	1144	3018
rs28592983	1277	1332	409	3018
rs61893460	798	1457	756	3011
rs10876864	952	1490	576	3018
rs10519067	25	604	2401	3030
rs36045143	168	1026	1834	3028
rs16948048	1124	1458	439	3021
rs3806933	519	1431	1076	3026
rs1837253	144	1055	1829	3028
rs17293632	1633	1221	173	3027
rs17388568	1560	1224	233	3017
rs9303277	730	1428	823	2981

5.3.3.2B The number of patients that had 0, 1 or 2 risk alleles for each SNP and the total of patients that had genotyping for that particular SNP.

5.3.3.3 Hardy Weinberg Equilibrium

HWE is a mathematical equation used to calculate the genetic variation of a population at equilibrium. HWE demonstrates what happens between alleles and genotype frequencies under assumptions. It assumes that in the absence of mutation, migration, natural selection or genetic drift and under random mating in an extremely large population the frequency distribution of genotypes (homozygous dominant, heterozygous and homozygous recessive) remains constant. Under Hardy-Weinberg assumptions, allele and genotype frequencies can be estimated from one generation to the next. The Hardy Weinberg equation is used to measure whether the observed genotyping frequencies in a population differ from the frequencies predicted by the equation.

The HWE equation is expressed as:

$p^2 + 2pq + q^2 = 1$

p= the frequency of the dominant allele (i.e., A)

q= the frequency of the recessive allele (i.e., a)

so,

p^2 = frequency of AA (homozygous dominant)

2pq = frequency of Aa (heterozygous)

q^2 = frequency of aa (homozygous recessive)

The HWE equation checks that the allele and genotype frequencies in the population remains the same. If they do not meet HWE then potentially one more assumptions are invalid for that population. There are other explanations such methodological reasoning's to why they do not meet HWE including genotyping error, population stratification and biased selection of subjects from the population. Additionally, another explanation could be from the presence of a common deletion polymorphism and could be due to disease association (Balding, 2006).

The HWE equation was set up on excel as a macro to calculate the HWE of the genotypes. The frequency of the three different genotypes were first

counted and then entered into the formulae which then gave a p-value number. A p-value of >0.05 from the Fisher's Exact Test meant that population for that particular SNP was in HWE anything smaller means it was not in HWE. Bonferroni correction was used to correct for multiple testing which the statistically significant p value 0.05 was dived by the 25 SNPs used in the analysis which came to 0.002 and so after Bonferroni correction a p-value of 0.002 or higher was in HWE. Table 5.3.3.3 shows the HWE p-values and the MAF for all four genotyping data sets separately and all of them combined together. rs1420101 (*IL1RL1*), rs532965 (*HLA-DQA1*) and rs9272629 (*HLA-DQB1*) did not meet HWE and so these signals should be excluded from the analysis.

rs number	Locus	Risk allele	LGC 1	LGC 2	Believe	Biobank	All 4 combined	MAF (ensembl)
rs10077785	C5orf56	T	0.962552 T= 24% C= 76%	0.718426 T= 27% C= 73%	0.815118 T= 25% C= 75%	0.487674 T= 28% C= 72%	0.851469 T= 27% C= 73%	T= 23% C= 77%
rs167769	STAT6	T	0.367381 C= 54% T= 46%	0.287828 C= 57% T= 43%	0.904203 C= 60% T= 40%	0.767291 C= 57% T= 43%	0.724163 C= 57% T= 43%	**C= 67% T= 33%**
rs1342326	IL-33	C	0.459217 A= 81% C= 19%	0.258975 A= 80% C= 20%	0.821203 A= 80% C= 20%	0.276641 A= 81% C= 19%	0.937985 A= 81% C= 19%	A= 83% C= 17%
rs2244012	RAD50	G	0.838849 G= 22% A= 78%	0.277765 G= 23% A= 77%	0.650328 G= 19% A= 81%	0.751824 G= 22% A= 78%	0.820884 G= 22% A= 78%	G= 20% A= 80%
rs1773542	CD247	T	0.057384 C= 38% T= 62%	0.167497 C= 39% T= 61%	0.528087 C= 40% T= 60%	0.526328 C= 37% T= 63%	0.035533 C= 38% T= 62%	C= 37% T= 63%
rs61816764	FLG	T	0.284165 C= 94% T= 6%	0.304504 C= 93% T= 7%	**C=94% T=6%**	**C=93% T=7%**	0.96 C= 94% T= 6%	C=95% T=5%
rs1420101	IL1RL1	T	0.075355 C= 38% T= 62%	0.226029 C= 39% T= 61%	0.562119 C= 58% T= 42%	0.853435 C= 58% T= 42%	**0.00031 C= 52% T =48%**	**C= 66% T= 34%**
rs1106639	D2HGDH	G	0.66217 A= 21% G= 79%	0.078583 A= 24% G= 76%	0.373569 A= 23% G= 77%	0.540547 A= 22% G= 78%	0.417075 A= 22% G= 78%	A= 26% G= 74%
rs532965	HLA-DQA1	G	0.681739 G= 22% T= 78%	0.156015 G= 22% T= 78%	**0.017604** G= 22% T= 78%	**0.015361** G= 23% T= 77%	**0.000653** G= 23% T= 67%	**G= 16% T= 84%**
rs9272629	HLA-DQB1	T	**1.72E-19 G= 36% T= 64%**	**6.68E-08 G= 40% T= 60%**	**N/A**	**G=36% T=64%**	**5.02E-25** G= 38% T= 62%	**G=43% T=57%**
rs1847472	BACH2	C	0.779551 A= 33% C= 67%	0.713625 A= 34% C= 66%	0.890287 A= 34% C= 66%	0.901993 A= 32% C= 68%	0.826259 A= 33% C= 67%	A= 37% C= 63%
rs13275219	MIR5708	T	0.833248 C= 64% T= 36%	0.328447 C= 64% T= 36%	0.519346 C= 64% T= 36%	0.583239 C= 64% T= 36%	0.700702 C= 64% T=36%	C= 65% T= 35%
rs10905279	GATA3	A	0.856509 A= 40% G= 60%	0.978921 A= 40% G= 60%	**0.041087** A= 41% G= 59%	0.730105 A= 43% G= 57%	0.534918 A= 41% G= 59%	A= 40% G= 60%
rs1444782	LOC101928272	G	0.310364 A= 36% G= 64%	0.949623 A= 38% G= 62%	0.583501 A= 41% G= 59%	0.715801 A= 38% G= 62%	0.807957 A= 38% G= 62%	A= 42% G= 58%
rs28592983	MUC5AC	T	0.533728 C= 64% T= 36%	0.461285 C= 68% T= 32%	0.114034 C= 66% T= 34%	0.124571 C= 62% T= 38%	0.017258 C= 64% T= 36%	C= 63% T= 37%
rs61893460	C11orf30	A	0.938227 A= 50% G= 50%	0.625067 A= 48% G= 52%	**0.02124** A= 48% G= 52%	0.929717 A= 50% G= 50%	0.370428 A= 49% G= 51%	A= 45% G= 55%
rs10876864	RPS26	G	0.247822 A= 55% G= 45%	0.639331 A= 55% G= 45%	0.510722 A= 53% G= 47%	0.826998 A= 57% G= 43%	0.886883 A= 56% G= 44%	A= 59% G= 41%
rs10519067	RORA	G	0.283002 A= 10% G= 90%	0.841656 A= 9% G= 91%	0.604738 A= 13% G= 87%	0.675082 A= 11% G= 89%	0.703008 A= 11% G= 89%	A= 14% G= 86%

rs36045143	**CLEC16A**	A	0.865647 A= 76% G= 24%	0.221684 A= 76% G= 24%	0.45984 A= 76% G= 24%	0.752179 A= 79% G= 21%	0.457211 A= 78% G= 22%	A= 75% G= 25%
rs16948048	**ZNF652**	G	0.952257 A= 61% G= 39%	0.107025 A= 61% G= 39%	0.557583 A= 63% G= 37%	0.460621 A= 62% G= 38%	0.16362 A= 62% G= 38%	A= 63% G= 37%
rs3806933	**WDR36**	C	0.874252 C= 62% T= 38%	0.152287 C= 58% T= 42%	0.842352 C= 58% T= 42%	0.270817 C= 59% T= 41%	0.181572 C= 59% T= 41%	C= 55% T= 45%
rs1837253	**TSLP**	C	0.42548 C= 72% T= 28%	0.832743 C= 79% C= 21%	0.950295 C= 75% T= 25%	0.086891 C= 78% T= 22%	0.476152 C= 78% T= 22%	C= 75% T= 25%
rs17293632	**SMAD3**	T	0.253988 C= 76% T= 24%	0.491381 C= 75% T= 25%	**0.000231** C= 73% T= 27%	0.461039 C= 74% T= 26%	0.005205 C= 74% T= 26%	C= 79% T= 21%
rs17388568	**KIAA1109**	A	0.472142 A= 31% G= 69%	**0.02545** **A= 29%** **G= 71%**	0.97983 A= 26% G= 74%	0.33713 A= 27% G= 73%	0.264665 A= 28% G= 78%	A= 29% G= 71%
rs9303277	**IKZF3**	C	0.531192 C= 51% T= 49%	0.130447 C= 53% T= 47%	0.873536 C= 54% T= 46%	**0.01927** C= 51% T= 49%	0.038151 C= 52% T= 48%	C= 48% T= 52%

Table 5.3.3.3 Hardy Weinberg equilibrium p-value and minor allele frequency for 31 SNPs from 4 different datasets and all 4 datasets combined together: the 2 LGC datasets were genotyped for 30 SNPs whereas the Believe and the UK Biobank were genotyped for 25 SNPs thus N/A identifies that particular SNP was not genotyped for that dataset of patients. Values highlighted in bold indicates that the p-value did not meet statistical significance after bonferroni correction (Bonferroni correction p=0.002 for 25 SNPs), a value lower than the p-value is classed as not meeting HWE. As for the MAF, it had to match or be near the MAF using the 1000 Genomes Project Phase 3 allele frequencies from the European population.

5.3.4 Selecting Biomarkers that Represent Th2, Th1 and Th17 Inflammation and Clinical Features that Reflect Type 2 Asthma Phenotypes

For the serum and genetic analysis, not all 11 biomarkers were analysed due to the previous chapters showing that there was correlated expression of multiple biomarkers that represent type 2 inflammation. Therefore, a single biomarker was chosen to represent this group. Based on the correlation data in the whole serum cohort in chapter 4, the most independent biomarkers were chosen (IL-4, IL-33, IL-17A, IFN-gamma, soluble ST2 and periostin) to look at whether the genetic signals drive different type of inflammation captured by these 6 biomarkers. IFN-γ, IL17A, IL-4, IL-33, soluble ST2 and periostin represent either the Th1, Th2 or Th17 pathway and were also the most easily measured in the sera compared to other biomarkers. IFN-γ represents the Th1 pathway, IL17A represents the Th17 pathway, IL-4 is one of the Th2 cytokines in the Th2 pathway and IL-33 is upstream of the Th2 pathway. Even though soluble ST2 and periostin did not correlate with each other or any of the other biomarkers, they demonstrated to be independent. Soluble ST2 is the receptor for IL-33 and so part of the Th2 pathway and periostin is downstream product of the Th2 pathway of IL-13.

In addition, clinical phenotypes related to asthma, particularly ones relating to type 2 inflammation including atopy (allergic dermatitis and allergic rhinitis), serum IgE levels and blood eosinophils were looked at to see if these genetic signals lead to these clinical features in asthma patients. These clinical features were selected based on asthma GWAS literature search (see Table 5.3.4) and asthma phenotypes identified in cluster analysis studies.

Genes/Loci associated	Associated phenotype	GWAS study reference
TEX41/ACVR2A (adult onset specific) *GSDMB*, *ZPBP2*, *ORMDL3* (childhood onset specific) *ORMDL3, GSDMA* or *GSDMB* (childhood specific) *DENND1B* (childhood specific) *USP38* and *GAB1* (adult onset specific)	Age of onset	(Pividori et al., 2019) (Ferreira et al., 2019) (Moffatt et al., 2007) (Sleiman et al., 2010) (Hirota et al., 2011)
IL18R, IL1RL1, IL33, SMAD3, RORA, IL13, HLA-DQ LINC00469, CCDC82, TBX18, SOBP	IgE	(Moffatt et al., 2010) (Levin et al., 2013)
PLAUR	Lung Function	(Barton et al., 2009)
FLG, IL4/KIF3A, EFHC1, OVOL1, C11orf30, TMTC2/SLC6A15 and *IKZF3* (specific to atopic march) *EDC (FLG), XIRP2, RAD50/IL13/KIF3A, DMRTA1, RNF111* (atopic dermatitis specific) *HLA-DQB1, TLR1, WDR36, IL1RL1, LRRC32, GSDMA, TSLP, IL33, ZBTB10, SMAD3, CLEC16A/DEX1* (allergic rhinitis specific)	Atopy	(Marenholz et al., 2015) (Schaarschmidt et al., 2015) (Ferreira et al., 2014)
CACNA2D3-WNT5A *SNOZ247/LINC01913*	Exacerbations	(Hernandez-Pacheco et al., 2021)
IL1RL1, WDR36/ TSLP, RAD50, IL33, MYB	Eosinophils	(Gudbjartsson et al., 2009)

Table 5.3.4 Selected Genome-Wide Association studies for Asthma and the Associated Phenotype and the Suggested Causative Candidate genes/loci Involved

5.3.5 Statistics

All statistical analysis were carried out on IBM SPSS Statistics 24 and G*Power for power calculations. Details of specific statistical methods are described below.

5.3.5.1 Statistical Analysis

As the main question was to find out whether carrying the risk allele drives the serum biomarker levels and lead to type 2 clinical phenotypes, for the analysis the three genotype groups were labelled 0, 1 or 2 depending on how many risk alleles they carried (0= no risk alleles, 1= 1 risk allele and 2= 2 risk alleles). However, due to some of the numbers being very low in some of the genotype groups (if n=<10) especially where the MAF was less than 5%, that genotype group had to be combined with heterozygous group therefore some signals only had 2 groups to be analysed.

The distribution of data for the continual variables were looked at first using a histogram to see whether they need to be transformed if they were not

normally distributed. Serum biomarkers IL-4, IL-33, IL17A, IFN-γ and soluble ST2, ACQ score, blood eosinophils and serum IgE levels were not normally distributed so the data was normalised using either to Log_{10} before the data was analysed (see chapter 4 section 4.4.2 for distribution of serum biomarker data). The mean value and the standard deviation were given for continuous data along with the N value. A general univariate model was used to analyse serum biomarkers periostin and soluble ST2 and clinical features IgE, FEV_1 and FVC in order to correct for covariates including age, gender, height and weight with the genetic signals since these are known confounders for these variables (see appendix 7.3.4 for the evaluation of potential confounders for biomarkers in the serum). Therefore, adjusting for these will generally improve the precision of the analyses and effect. For the other continuous variables that did not need adjusting a one-way ANOVA or an independent t-test was used to analyse the data depending on if two or three independent samples were being compared. For categorical variables a Chi squared test was first used to give a descriptive analysis i.e., percentages and numbers between the genotype groups. Logistic regression as further analysis to adjusted for any covariates including age and gender and/or to discover the effects between the risk allele groups. Where available for some categorical variables, continuous data was available for these variables and so a one-way ANOVA.

Correction for multiple testing was used throughout the analysis using Bonferroni as this will give confidence in the results and get rid of any finding that are likely not to lead to anything.

5.3.5.2 Post hoc power analysis

A post hoc power analysis to aid interpretation of the results and to observe how well powered the study was to have confidence in the results. For example, whether an insignificant finding was just under powered or a genuine insignificant finding that was well powered therefore, it gives confidence that it could not be replicated in another separate population. An effect size of 0.85 or more suggests the study is well powered a small value signifies that the study needs a bigger sample size.

An ANOVA, T-test, logistic regression or Chi-squared post hoc power analysis was also calculated using G*power (version 3.1.9.7) or selecting the power output on SPSS for the general univariate model for both serum and clinical features analysis with the genetic signals. G* power is a statistical software package to calculate statistical power for wide range of statistical tests including t-tests, chi squared tests, logistic regression etc.

5.4 Results

5.4.1 Demographics of patients used in the genetic analysis

Table 5.4.1 shows the overall demographics of the patients used in the inflammatory profile analysis (n= 670) and the clinical phenotype and genetic analysis (n= 3,037). Not all the data was available for both groups particularly the patients with serum and genetic data where there was very minimal immunological and clinical data. Both groups were mainly GINA 3-5 patients (moderate-severe asthma patients), with an age of onset of 17.5 years (S.D:11.73) in the serum and genetics group and 18.7 years (S.D: 11.45) in the clinical and genetics group. There was also 71.8% of females in the in the serum and genetics group and 67.5% in the clinical and genetics group. A high BMI for both groups were seen 29.35 kg/m^2 (n= 560; 14.84-78.16) and 28.45 kg/m^2 (n= 2,048; 14.84-78.16). 78.5% are non-smokers in the serum and genetics group (n= 503) and 64.6% in the clinical and genetics group (n= 1,576). The mean FEV_1pp and FEV_1/FVC in the clinical and genetics group was 80.49% (S.D: 22.33, n= 2,220) and 0.71 (S.D: 0.13, n= 2,203) and 82.12% (S.D: 21.15, n= 635) and 0.73 (S.D: 0.12, n= 527) in the serum and genetics group. A small portion of the serum and genetics group had total IgE data with the median value being 116 KU/L (range: 2-4850, n= 253) whereas a third of the clinical and genetics group had total IgE data 118 KU/L (range: 0.6-4,900, n= 1,325). Additional clinical features for both groups included hospital admissions and ACQ score. 36.8% (n= 225) of asthma in the serum and genetics group and 33.3% (n= 603) of asthma patients in the clinical and genetics group had been to hospital in the last year. The mean ACQ7 score for the serum and genetics group was 2.25 (S.D: 1.22, n= 533) and 1.96 (S.D: 1.25, n= 837) for the clinical and genetics group.

	Serum & Genetics Patients	Clinical & Genetics Patients
Subjects (n)	670	3,037
Age seen (years: Mean & S.D)	41.3 (11.73) (n= 661)	43.1 (11.45) (n= 2,597)
***Age of onset (years: Mean & S.D)**	17.5 (15.94) (n= 607)	18.7 (15.61) (n= 1,766)
***Age of onset (n):**	632	1,766
<16	351 (55.5%)	896 (50.7%)
16-34	165 (26.1%)	513 (29%)
>35	116 (17.3%)	357 (11.8%)
Gender (n):	667	2849
Female	479 (71.8%)	1,922 (67.5%)
BMI (Kg/m^2: Median & Min-Max)	29.35 (14.84-78.16) (n= 560)	28.45 (14.84-78.16) (n= 2,048)
Smoking status (n):	664	2,438
Never	503 (75.8%)	1,576 (64.6%)
Ex-Smoker	151 (22.7%)	543 (22.3%)
Current	10 (1.5%)	319 (13.1%)
Smoking Pack Years (Median & Min-Max)	4 (0.05-10) (n= 146)	7 (0.03-161) (n= 645)
FEV_1pp (Mean & S.D)	82.12 (21.15) (n= 635)	80.49 (22.33) (n= 2,220)
FEV_1FVC (Mean & S.D)	0.73 (0.12) (n= 527)	0.71 (0.13) (n= 2203)
Peak Flow (L/min: Mean & S.D)	366.72 (135.12) (n= 91)	388.70 (130.54) (n= 1140)
Blood total IgE (KU/L: Median & Min-Max)	116 (2-4,850) (n= 253)	118 (0.60-4,900) (n= 1,325)
Blood Eosinophils (x10^9/L: Median & Min-Max)	-	0.21 (0.0-5.42) (n= 731)
** **Allergic rhinitis (n):**	506	1,520
Yes	355 (70.2%)	926 (60.9%)
****Atopic dermatitis (n):**	-	756
Yes		259 (34.3%)
Hospital admissions (n):	612	904
Never	387 (63.2%)	603 (66.7%)
Ever	225 (36.8%)	301 (33.3%)
Mean ACQ Score 7 (Mean & S.D)	2.25 (1.22) (n= 553)	1.96 (1.25) (n= 837)
GINA (n):	640	1415
1 (Mild)	13 (2%)	90 (6.4%)
2 (Mild/Moderate)	60 (9.4%)	196 (13.9%)
3/4 (Moderate/ Severe)	445 (69.5%)	956 (67.6%)
5 (Severe)	122 (19.1%)	173 (12.2%)

5.4.1 Demographics and clinical features of Asthma Patients used in the Inflammatory Profile Analysis and the Clinical & Genetic Analysis: Data is presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). FEV_1pp: forced expiratory volume in 1 second percentage predicted; FEV_1/FVC: forced expiratory volume in 1 second / forced vital capacity ratio; IgE: immunoglobulin E; ACQ: asthma control questionnaire; GINA: global initiative for asthma. *Age of onset- some only known as childhood onset not the actual age so marked as <16 therefore numbers vary for both variables. **Allergic rhinitis and atopic dermatitis is doctor diagnosis and/or self-reported. – indicates there was not enough data available.

5.4.2 Genetics do not drive the expression levels of the biomarkers in the serum of asthma patients

Six biomarkers were chosen to be analysed because these biomarkers identified different types of inflammation including Th1, Th2 and Th17. Table 5.4.2 shows the data for the serum biomarkers and the genetic signals that reached statistically significant value of <0.05. The mean biomarker measurement, standard deviation and the number in each genotype group are displayed for each genetic signal. Figure 6.4.1 displays the statistical differences between the risk allele groups for the genetic signal that had a p-value of <0.05. The data showed (Figure 5.4.2.A) that asthma patients carrying 1 or 2 risk alleles for the rs61816764 SNP (*FLG*) had slightly higher IL-4 serum levels than those carrying no risk alleles (0: 1.54 pg/mL log_{10} & 1 & 2: 1.69 pg/mL log_{10}; p=0.044). Additionally, asthma patients carrying 2 risk alleles for the rs532965 SNP (*HLA-DQA1*) (Figure 5.4.2.B) had higher levels of IL-33 in the sera (1.64 pg/mL log_{10}) then those carrying no risk alleles (1.46 pg/mL log_{10}) p=0.043. Finally, levels of periostin where higher in patients carrying 0 risk alleles for rs13275219 (*MIR5708*) (84677.35 pg/mL) and 1 or 2 risk alleles (77116.93 pg/mL and 80586.88 pg/mL; respectively) (Figure 5.4.2.C). However, these three genetic signals did not meet Bonferroni correction and rs532965 showed to be well powered suggesting that this particular signal may not drive levels of IL-33 in the serum of asthma patients.

Biomarker	SNP	Locus	0	1	2	P-value	Power
IL-4 log_{10} pg/mL	rs61816764	*FLG**	N= 517 1.54 (0.57)	N= 72 1.69 (0.54)		0.044	0.52
IL-33 log_{10} pg/mL	rs532965	*HLA-DQA1*	N=375 1.46 (0.84)	N=219 1.63 (0.84)	N=36 1.64 (0.78)	0.043	1.0
Periostin pg/mL	rs13275219	*MIR5708*	N=261 84677.35 (46815.73)	N=316 77116.93 (26388.46)	N=84 80586.88 (27511.64)	0.029	0.66

Table 5.4.2 Genetics signals identified in the moderate to severe asthma GWAS study that showed some changes in serum levels between genotype risk groups: data is presented as mean and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. Univariate model was used to correct for gender for IL-4 and periostin and to analyse the 3 or 2 genotype groups for IL-33 a one way-ANOVA was used. *Two risk allele groups were combined together due to the n's being low in one of the groups. Power was also computed and a value more than 0.85 is considered a well powered study. These results did not meet Bonferroni correction p=0.002 but had a p-value of 0.05 or less.

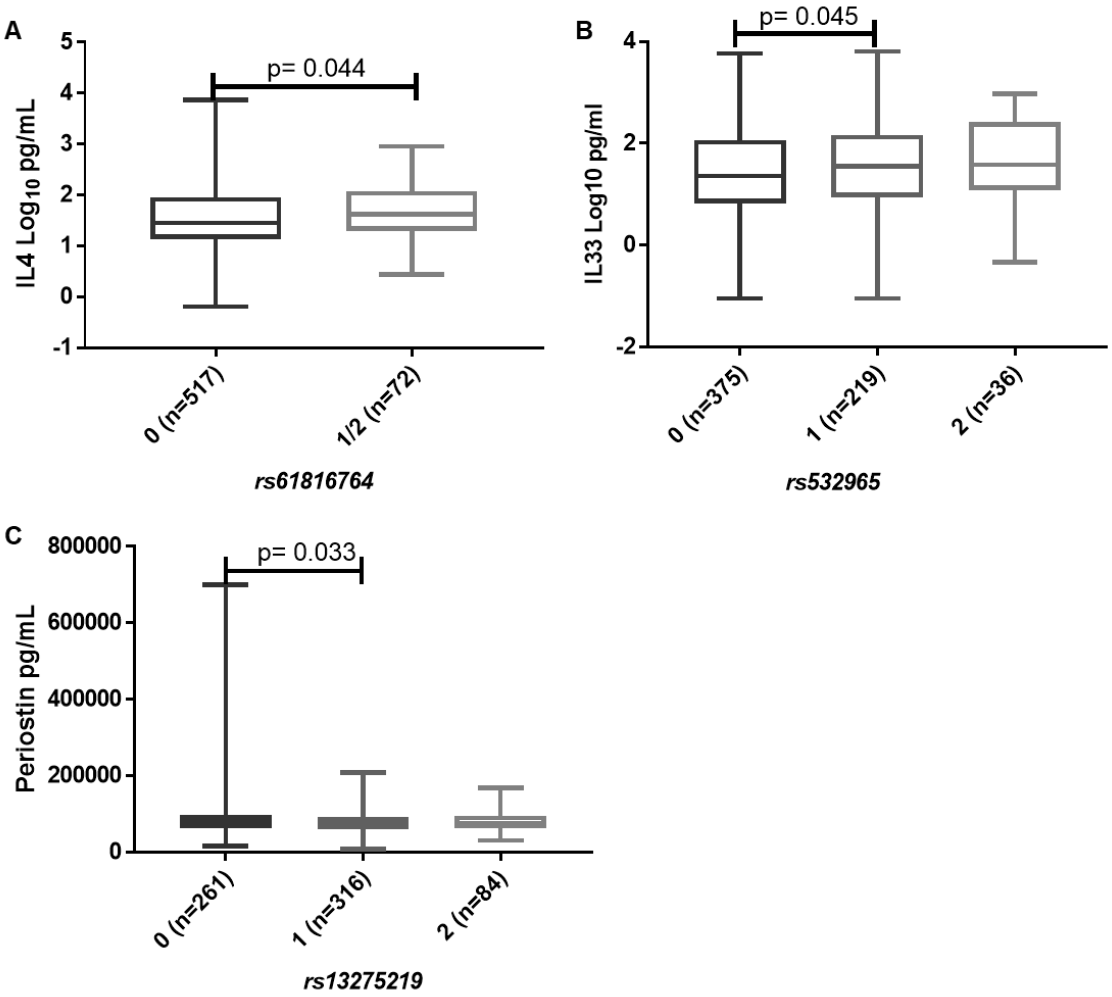


Figure 5.4.2 Three genetics signals identified in the moderate to severe asthma GWAS study that showed a modest association with biomarker levels between genotype risk groups: A: IL-4 serum levels are higher if patients who carried 1 or 2 risk alleles for FLG compared to those that carried no risk allele p=0.044. B: IL-33 serum levels were higher in patients who carried 1 risk allele for HLA-DQA1 than those who carried 0 risk alleles p=0.045. C: Periostin levels were higher in patients who carried 0 risk alleles for MIR5708 than those carrying 1 risk allele p=0.033.

5.4.3 Childhood onset asthma showed association with previously known signals

Age of onset was looked at to see whether carrying 1 or 2 risk alleles for the 25 genetic signals were associated with childhood or adult asthma onset. In the first set of analyses, age of onset was looked at between the three different age of onset groups and so a chi-squared was used. This gave a categorical analysis i.e., percentages between all age of onset groups and the three risk allele groups of all the 25 genetic signals.

The data showed that several of genetic signals were associated between the different age of onset groups (Table 5.4.3A) particularly genetic signals rs61816764 (*FLG*) (p= 0.0001) and rs9303277 (*IKFZ3*) (p= $1.0x10^{-5}$) where they met Bonferroni correction (p= <0.0002). However, the p-value from a chi-squared test does not show where the direction of effect is coming from since there are more than two groups. Therefore, the age of onset variable was then analysed as a continual variable for the signals that had a p-value of <0.05 in the previous analysis. The age of onset data in table 5.4.3B was analysed using an ANOVA on the mean age, standard deviation and the number in each genotype group are displayed for each genetic signal. Overall, the data showed that patients carrying 1 or/& 2 risk alleles for genetic signals rs2244012 (*RAD50*), rs61816764 (*FLG*) and rs9303277 (*IKZF3*) had a lower asthma age of onset than those who carried 0 risk alleles (p= 0.018, $2.67x10^{-4}$ and $2.20x10^{-7}$; respectively). Figure 5.4.3 shows the statistical differences between the risk allele groups for the three signals that had a p-value of <0.05. In figure 5.4.3A asthma patients carrying 1 risk allele (17.34 years) for signal rs2244012 (*RAD50*) had lower age of onset than those carrying 0 risk alleles (19.53 years) p= 0.017. Figure 5.4.3B shows patients who carry 1/2 risk alleles for the rs61816764 signal (*FLG*) have childhood asthma with lower age of onset (15.02 years) and those who carry 0 risk alleles are more likely to have adult asthma age of onset (19.2 years, p= 0.0003). Figure 5.4.3C shows patients who carry 1 (18.2 years) or 2 (16.75 years) risk alleles for signal rs9303277 (*IKZF3*) signal have lower asthma age of onset compared to patients who carry 0 risk alleles (22.23 years) (p=<0.0001; respectively) who have older age of onset. Both genetic signals rs61816764 (*FLG*) and

rs9303277 (*IKZF3*) were statistically significant even after Bonferroni correction whereas signal rs2244012 (*RAD50*) did not meet Bonferroni correction. The genes/loci that are suggested to be related to signals rs61816764 and rs9303277 have previously been reported and associated in other childhood onset asthma GWAS (Moffatt et al., 2007) (Halapi et al., 2010) (Marenholz et al., 2015).

	Age of Onset Groups			
Locus	**0**	**1**	**2**	**P-value**
RAD50 (rs2244012)	N= 918 <16: 429 (46.7%) 16-34: 293 (31.9%) 35-60: 196 (21.4%)	N= 474 <16: 256 (54%) 16-34: 125 (26.4%) 35-60: 93 (19.6%)	N= 76 <16: 38 (50%) 16-34: 28 (36.8%) 35-60: 10 (13.2%)	**0.038**
FLG (rs61816764)	N=1544 <16: 756 (49%) 16-34: 463 (30%) 35-60: 325 (21%)	N= 211 <16: 136 (64.5%) 16-34: 45 (21.3%) 35-60: 30 (14.2%)		**0.0001***
RORA (rs10519067)	N=373 <16: 184 (49.3%) 16-34: 96 (25.7%) 35-60: 93 (24.9%)		N= 1387 <16: 710 (51.2%) 16-34: 413 (29.8%) 35-60: 264 (19%)	**0.032***
ZNF652 (rs16948048)	N= 665 <16: 313 (47.1%) 16-34: 202 (30.4%) 35-60: 150 (22.6%)	N= 834 <16: 439 (52.6%) 16-34: 227 (27.2%) 35-60: 168 (20.1%)	N= 255 <16: 137 (53.7%) 16-34: 80 (31.4%) 35-60: 38 (14.9%)	**0.041**
KIAA1109 (rs17388568)	N= 896 <16: 459 (51.2%) 16-34: 240 (26.8%) 35-60: 197 (22%)	N= 723 <16: 369 (51%) 16-34: 228 (31.5%) 35-60: 126 (17.4%)	N= 130 <16: 59 (45.4%) 16-34: 38 (29.2%) 35-60: 33 (25.4%)	**0.045**
IKZF3 (rs9303277)	N= 414 <16: 168 (40.6%) 16-34: 132 (31.9%) 35-60: 114 (27.5%)	N= 822 <16: 430 (52.3%) 16-34: 241 (29.3%) 35-60: 151 (18.4%)	N= 479 <16: 270 (56.4%) 16-34: 125 (26.1%) 35-60: 84 (17.5%)	**$1.0x10^{-5}$**

Table 5.4.3A Six genetics signals identified in the moderate to severe asthma GWAS study that showed nominal association with asthma age of onset: data is presented as percentage and n's between the age of onset groups across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. Chi-squared test was used to analyse both these categorical groups. *Two genotyping groups were combined together due to the n's being low in one of the groups. Some of the signals however did not meet Bonferroni correction p= 0.002.

	Age of onset				
Locus	**0**	**1**	**2**	**P-value**	**Power**
RAD50 (rs2244012)	19.53 (S.D 15.51) N=1079	17.34 (S.D 15.91) N=592	17.59 (S.D 14.10) N=86	0.018	1.0
FLG (rs61816764)	19.20 (S.D 15.63) N=1544	15.02 (S.D 15.18) N=211		**$2.67x10^{-4}$**	1.0
RORA (rs10519067)	18.43 (S.D 15.84) N=373		18.43 (S.D 15.57) N=1387	0.156	1.0
ZNF652 (rs16948048)	19.69 (S.D 15.74) N=665	18.43 (S.D 15.65) N=834	17.09 (S.D 15.02) N=255	0.06	1.0
KIAA1109 (rs17388568)	18.99 (S.D 16.09) N=896	18.08 (S.D 14.90) N=723	20.70 (S.D 16.46) N=130	0.168	1.0
IKZF3 (rs9303277)	22.32 (S.D 15.82) N=414	18.20 (S.D 15.36) N=822	16.75 (S.D 15.46) N=479	**$2.20x10^{-7}$**	1.0

Table 5.4.3B *FLG* and *IKZF3* signals showed strong association with age of onset: data is presented as mean and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. One-ANOVA or an independent T-test was used to analyse the age of onset as a linear variable with the genetic signals. *Two genotyping groups were combined together due to the n's being low in one of the groups. Some of the signals from the previous analysis in 6.4.3A however did not meet Bonferroni correction p=0.008.

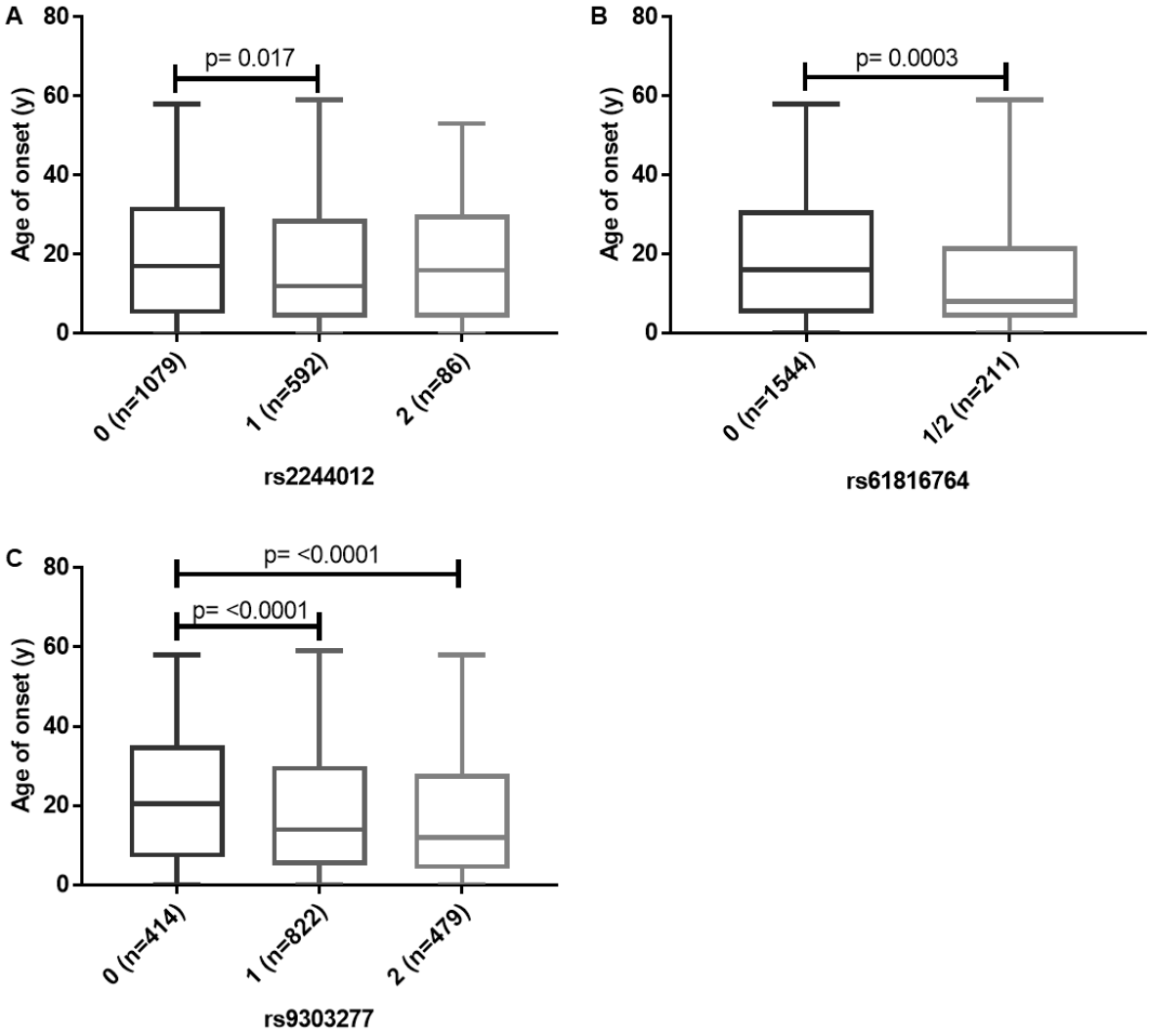


Figure 5.4.3 rs61816764 and rs9303277 showed association with age of onset: A: age of onset was lower in patients who carried 1risk allele for RAD50 compared to those that carried no risk allele p=0.017. B: age of onset was lower in patients who carried 1/2 risk alleles for FLG than those who carried 0 risk alleles p=0.0003. C: age of onset was lower in patients who carried 1 or 2 risk alleles for IKZF3 than those carrying 0 risk alleles p=<0.0001.

5.4.4 rs9303277 and rs28592983 showed association with FEV_1 or FVC whereas rs17293632 and rs9272629 showed association with Peak Expiratory Flow

Moderate-severe asthma risk alleles were tested for association with lung function (FEV_1 and FVC pre bronchodilator), the hypothesis being that they may driver a lower lung function leading to disease severity. FEV_1 and FVC were corrected for age, gender, height and weight in a univariate linear model in the analysis. Table 5.4.4 displays the genetic signals that met statistical significance of p= <0.05 for the three lung function measurements but they did not meet correction for multiple testing (p= 0.002). The mean, standard deviation and numbers in each genotype risk group are displayed in the table along with the p-value and power for signals rs9303277 (*IKZF3*), rs28592983 (*MUC5AC*), rs9272629 (*HLA-DQB1*) and rs17293632 (*SMAD3*). *rs9303277 (IKZF3)* just reached statistical significance with FEV_1prbd (p= 0.054). FEV_1 is lower in patients carrying 1 risk allele (2.46 L) compared to patients who carry 0 risk alleles (2.53 L) (p= 0.018) as seen in figure 5.4.4A. Table 5.4.4 shows that rs28592983 (*MUC5AC*), is statistically significant with FVCprbd (p= 0.015). FVCprbd is shown to decrease when patients carry 1 risk allele (3.43 L) compared to those who carry 0 risk alleles (3.56 L) (p= 0.004) as displayed in figure 5.4.4B. PEF was also shown to be statistically significant with rs9272629 (*HLA-DQB1*) and rs17293632 (*SMAD3*) (p= 0.033 and 0.031; respectively) (Table 5.4.4). Figure 5.4.4C shows when patients carry 1 risk allele for rs9272629 (*HLA-DQB1*) PEF decreases compared to the patients carrying 0 risk alleles (1= 376.44 L/min and 0= 406.98 L/min; p= 0.046). However, figure 5.4.4D demonstrates that PEF increases in patients carrying 1 risk allele for rs17293632 (*SMAD3*) compared to patients carrying 0 risk alleles (1= 400.77 L/min and 0= 379.66 L/min; p= 0.023).

Lung Function	Locus	0	1	2	P-value	Power
FEV_1prbd/L	*IKZF3* (rs9303277)	2.53 (0.81) N= 459	2.46 (0.84) N= 891	2.48 (0.87) N= 522	0.054	0.57
FVCprbd/L	*MUC5AC* (rs28592983)	3.56 (1.0) N= 804	3.43 (0.99) N= 826	3.43 (0.98) N= 244	0.015	0.74
PEF (L/min)	*HLA-DQB1* (rs9272629)	406.98 (134.03) N= 147	376.44 (124.18) N= 371	394.61 (137.60) N= 401	0.033	1.0
PEF (L/min)	*SMAD3* (rs17293632)	379.66 (122.55) N= 616	400.77 (139.81) N= 464	387.18 (130.43) N= 57	0.031	1.0

Table 5.4.4 Asthma genetic signal that shows some changes in lung function between the genotype risk groups: data is presented as mean and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A univariate analysis model corrected for age, gender, height and weight was used to analyse FEV_1prbd and FVCprbd and a one-way ANOVA was used to analyse PEF with the genetic signals. Bonferroni correction p= 0.002.

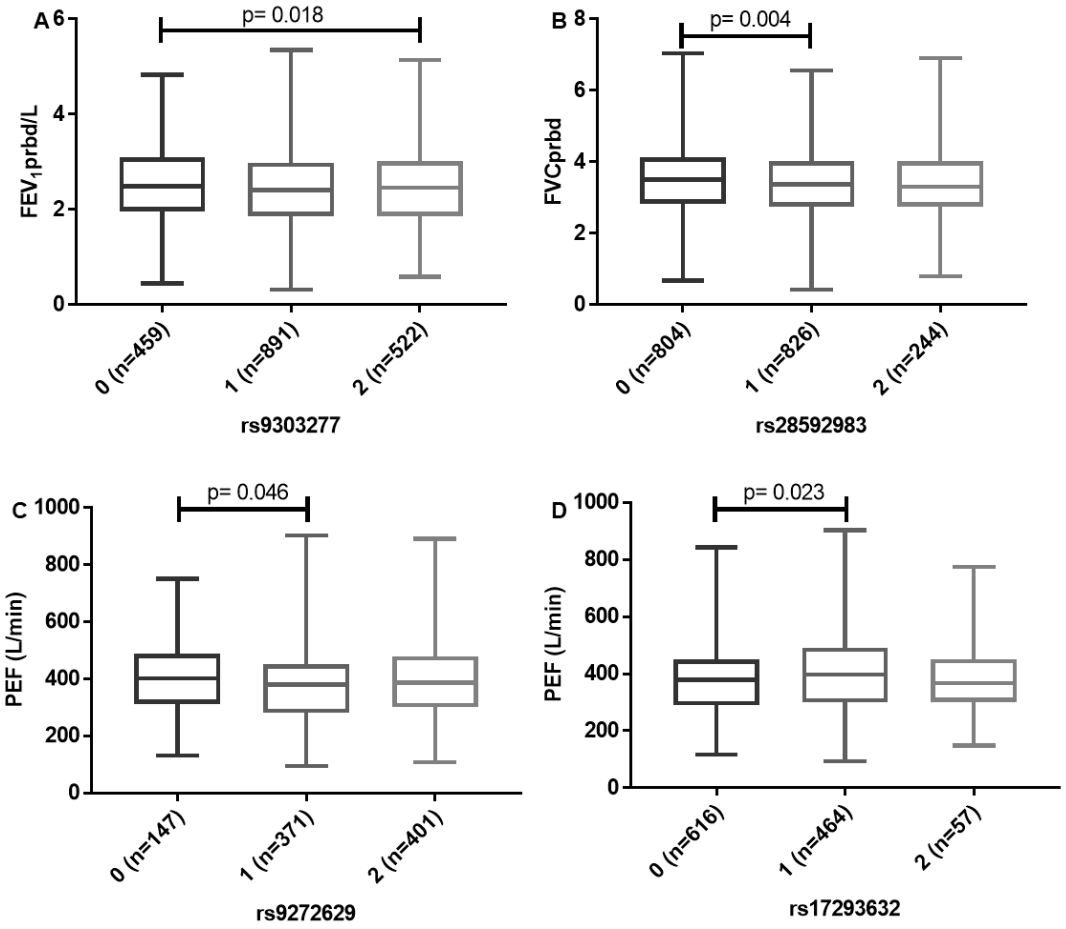


Figure 5.4.4 IKZF3, MUC5AC, HLA-DQB1 and SMAD3 signals showed nominal changes in lung function between the two risk allele groups and 0 risk allele group: A: FEV_1 was lower in patients who carried 1 risk allele for rs9303277 compared to those that carried 0 risk alleles p= 0.018. B: FVC was lower in patients who carried 1 risk allele for rs28592983 than those who carried 0 risk alleles p= 0.004. C: PEF was lower in patients who carried 1 risk allele for rs9272629 than those carrying 0 risk alleles p= 0.046. D: PEF was higher in patients who carried 1 risk allele for rs17293632 than those who carried 0 risk alleles p= 0.023.

5.4.5 Several Asthma Genetic Risk Alleles are Associated with Levels of IgE in the Blood of Asthma Patients

Moderate-severe asthma risk alleles were tested for association with blood IgE levels, the hypothesis being that they may driver higher blood IgE leading to disease severity. All these signals in table 5.4.5 were significant at the p= <0.05 level but did not meet correction for multiple testing. Table 5.4.5 displays the mean, standard deviation and numbers in each genotype risk group with the p-value and power for genetic signals rs100077785 (*C5orf56*), rs167769 (*STAT6*), rs2244012 (*RAD50*), rs1106639 (*D2HGDH*), rs61893460 (*C11orf30*), rs10519067 (*RORA*) and rs1837253 (*TSLP*).

The rs10077785 (*C5orf56*) moderate-severe asthma risk allele was associated with elevated serum IgE (p= 0.037), figure 5.4.5A shows patients carrying 1 risk allele for rs10077785 have increased blood IgE levels compared to the patients who carry 0 risk alleles (1= 2.12 KU/LLog$_{10}$ and 0= 2.03 KU/LLog$_{10}$; p= 0.014). The rs167769 *(STAT6)* moderate-severe asthma risk allele was associated with elevated serum IgE (p= 0.007) and figure 5.4.5B shows patients carrying 2 risk alleles (2.15 KU/LLog$_{10}$) or 1 risk allele (2.08 KU/LLog$_{10}$) for rs167769 have statistically higher blood IgE levels than those carrying 0 risk alleles (0= 1.99 KU/LLog$_{10}$) (p= 0.014 and p= 0.004; respectively). The rs2244012 (*RAD50*) moderate-severe asthma risk allele was associated with elevated serum IgE (p= 0.028) with figure 5.4.5C showing IgE levels being higher in patients carrying 1 risk allele compared to those carrying 0 risk alleles (1= 2.14 KU/LLog$_{10}$ and 0= 2.03 KU/LLog$_{10}$; p= 0.01) for rs2244012. In table 5.4.5 the rs1106639 (*D2HGDH*) moderate-severe asthma risk allele was associated with elevated serum IgE (p= 0.049), however there was no statistical significance between the risk allele groups. The rs61893460 (*C11orf30*) moderate-severe asthma risk allele was associated with elevated serum IgE (p= 0.009) and figure 5.4.5D shows patients that carried 2 risk alleles (2.14 KU/LLog$_{10}$) or 1 risk allele (2.07 KU/LLog$_{10}$) for rs61893460 have statistically higher blood IgE levels than those carrying 0 risk alleles (0= 1.99 KU/LLog$_{10}$) (p= 0.026 and p= 0.003; respectively). The rs10519067 (*RORA*) moderate-severe asthma risk allele was associated with elevated serum IgE (p= 0.032) with figure 5.4.5E showing IgE levels being higher in patients

carrying 2 risk alleles compared to those carrying 0 risk alleles (2= 2.08 KU/LLog$_{10}$ and 0= 1.60 KU/LLog$_{10}$; p= 0.026). Finally, the rs1837253 (*TSLP*) moderate-severe asthma risk allele was associated with elevated serum IgE (p= 0.039) and figure 5.4.5F shows patients carrying 2 risk allele for rs1837253 have increased blood IgE levels compared to the patients who carry 0 risk alleles (2= 2.09 KU/LLog$_{10}$ and 0= 1.86 KU/LLog$_{10}$; p= 0.015). Several of these signals that are associated with IgE levels are in good agreements with other studies that have found signals in the same gene/loci associated with elevated blood IgE levels when carrying the risk allele for asthma (Li et al., 2012) (Zhu et al., 2018b) (Laulajainen-Hongisto et al., 2020).

	Blood IgE (KU/L Log$_{10}$)				
Locus	**0**	**1**	**2**	**P-value**	**Power**
C5orf56 (rs10077785)	2.03 (0.70) N=684	2.12 (0.70) N=532	2.00 (0.76) N=100	0.037	0.63
STAT6 (rs167769)	1.99 (0.71) N=438	2.08 (0.70) N=632	2.15 (0.69) N=245	0.007	0.81
RAD50 (rs2244012)	2.03 (0.69) N=800	2.14 (0.74) N=452	2.0 (0.71) N=65	0.028	0.66
D2HGDH (rs1106639)	1.93 (0.68) N=61	2.01 (0.69) N=465	2.10 (0.71) N=791	0.049	0.59
C11orf30 (rs61893460)	1.98 (0.71) N=363	2.07 (0.68) N=610	2.14 (0.73) N=337	0.009	0.79
RORA (rs10519067)	1.60 (0.56) N=12	2.03 (0.72) N=268	2.08 (0.70) N=1037	0.032	0.65
TSLP (rs1837253)	1.86 (0.76) N=59	2.04 (0.72) N=459	2.09 (0.68) N=797	0.039	0.62

Table 5.4.5 Asthma genetic signal that were nominally associated with serum IgE levels: data is presented as mean (log$_{10}$) and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A univariate analysis model corrected for age and gender was used to analyse IgE with the genetic signals. Bonferroni correction p= 0.002.

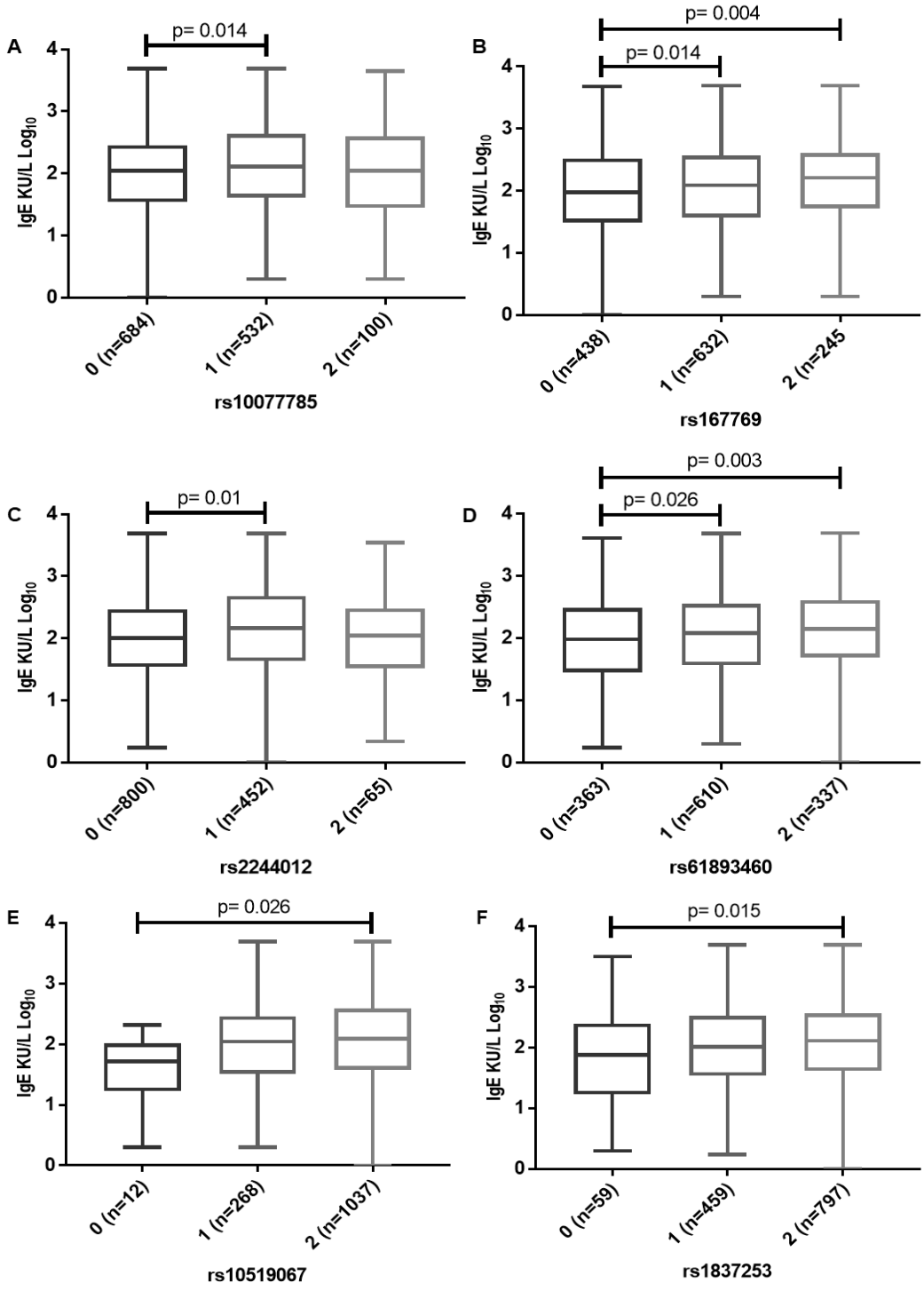


Figure 5.4.5 several asthma genetic signals were associated with increased levels of serum IgE: A: IgE levels were higher in patients who carried 1 risk allele for *C5orf56* compared to those that carried 0 risk alleles (p = 0.014). B: IgE levels were higher in patients who carried 1 or 2 risk alleles for *STAT6* than those who carried 0 risk alleles (p= 0.014 and p= 0.004; respectively). C: IgE was higher in patients who carried 1 risk allele for *RAD50* than those carrying 0 risk alleles (p= 0.01). D: IgE was higher in patients who carried 1 or 2 risk alleles for *C11orf30* than those who carried 0 risk alleles (p= 0.026 and p= 0.003; respectively). E: IgE levels were higher in patients that carried 2 risk alleles for *RORA* than those who carried 0 risk alleles (p= 0.026). F: IgE levels were higher in patients that carried 2 risk alleles for *TSLP* than those who carried 0 risk alleles (p= 0.015). A univariate analysis model corrected for age and gender was used to analyse IgE with the genetic signals.

5.4.6 Signals rs1847472 and rs1444782 are Associated with Symptom Score in Asthma Patients

Moderate-severe asthma risk alleles were tested for association with ACQ score, the hypothesis being that they may drive higher ACQ score leading to disease severity. All these signals in table 5.4.6 were significant at the p= <0.05 level but did not meet correction for multiple testing. Table 5.4.6 displays the mean, standard deviation and numbers in each genotype risk group with the p-value and power for genetic signals rs1847472 (*BACH2*) and rs1444782 (*LOC101928272*). rs1847472 (*BACH2*) shows to be statistically significant with ACQ score (p= 0.016). Figure 5.4.6A shows patients carrying 2 risk alleles for rs1847472 (*BACH2*) have an increased ACQ score compared to the patients who carry 1 risk allele (2= 1.35 and 1= 1.25; p= 0.022). rs1444782 (*LOC101928272*) was also shown to be statistically significant with ACQ score (p=0.004). Figure 5.4.6B shows patients that carried 1 risk allele (1.35) or 2 risk alleles (1.31) for rs1444782 (*LOC101928272*) have a statistically higher ACQ score than those carrying 0 risk alleles (1.16) (p= 0.002 and p= 0.023; respectively).

	ACQ Score (sqrt)				
Locus	**0**	**1**	**2**	**P-value**	**Power**
BACH2 (rs1847472)	1.37 (0.53) N=85	1.25 (0.51) N=376	1.35 (0.49) N=369	0.016	1.0
LOC101928272 (rs1444782)	1.16 (0.52) N=107	1.35 (0.49) N=419	1.31 (0.52) N=302	0.004	1.0

Table 5.4.5 Asthma genetic signal that are associated with ACQ symptom score: data is presented as mean (square root) and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A one-way ANOVA was used to analyse the mean ACQ score with the asthma genetic signals. Bonferroni correction p=0.002.

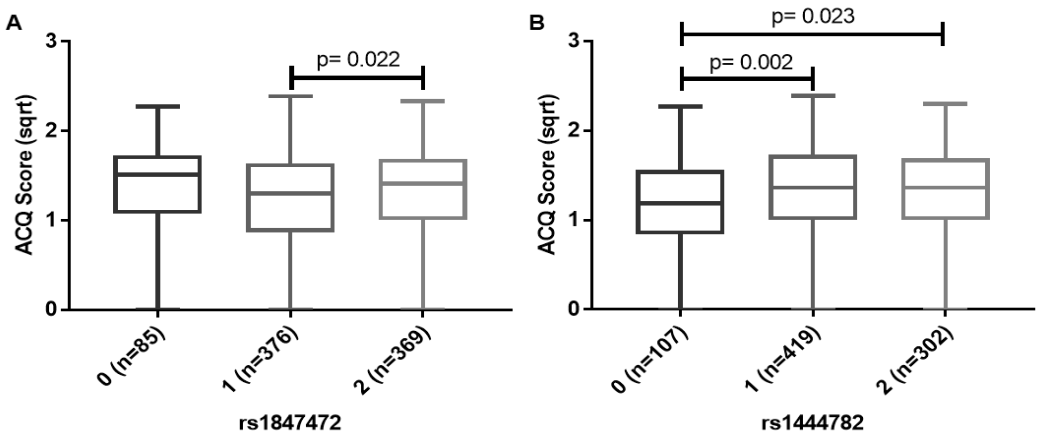


Figure 5.4.6 rs1847472 and rs1444782 shows changes in ACQ score across asthma risk allele groups: A: ACQ score was higher in patients who carried 2 risk alleles for rs1847472 compared to those that carried 1 risk allele p=0.022. B: ACQ score was higher in patients who carried 1 or 2 risk alleles for rs1444782 than those who carried 0 risk alleles p=0.002 and p=0.023; respectively.

5.4.7 The risk of having a hospital admission Increases when carrying 1 or more risk allele for genetic signals rs100077785, rs61893460 and rs17293632

Moderate-severe asthma risk alleles were tested for association with hospital admissions, the hypothesis being that they may drive hospital admissions leading to disease severity. In the first set of analyses, a chi-squared test was used to analyse hospital admissions with the risk allele groups for the 25 genetic signals. Genetic signals rs100077785 (*C5orf56*), rs61893460 (*C11orf30*) and rs17293632 (*SMAD3*) in table 5.4.7A met statistical significance with hospital admissions (p= <0.05) but did not meet correction for multiple testing (p= <0.002).

Table 5.4.7A displays the percentages, number of patients that have had a hospital admission in the risk and non-risk allele groups, the p-value and power. rs100077785 (*C5orf56*), rs61893460 (*C11orf30*) and rs17293632 (*SMAD3*) moderate-severe asthma risk alleles were associated with increased number of hospital admissions (p= 0.022, p= 0.008 and p= 0.023; respectively). However, the p-value from a chi-squared test only gives a descriptive analysis of the results therefore, a logistic regression was then preformed to discover the direction of effect in relation to the risk alleles for rs100077785, rs61893460 and rs17293632 on the likelihood that asthma patients have a hospital admission. Table 5.4.7B displays the uncorrected and corrected odds ratio and p-value for rs100077785, rs61893460 and rs17293632. The model was corrected for age and the reference group was the 0 risk allele group. The data shows that the logistic regression model for rs100077785 (*C5orf56*) was statistically significant when corrected for age (p= 0.02), the model also explained that asthma patients carrying 2 risk alleles were 2.13 (95% C.I: 1.24-3.65) times more likely to have a hospital admission than those who carry 0 risk alleles (Figure 5.4.7A) (p= 0.006). The logistic regression model for rs61893460 (*C11orf30*) was statistically significant when corrected for age (p= 0.006). The model also explained that asthma patients carrying 1 risk allele were 1.69 (95% C.I: 1.16-2.43) times more likely to have a hospital admission than those who carry 0 risk alleles (figure 5.4.7B) (p= 0.005). Whereas patients carrying 2 risk alleles were 1.13 (95% C.I: 0.74-1.72)

times more likely to have a hospital admission (Figure 5.4.7B), however this was not statistically significant. Finally, the logistic regression model for rs17293632 (*SMAD3*) was statistically significant when corrected for age (p= 0.02), the model also explained that asthma patients carrying 2 risk alleles were 2.27 (95% C.I:1.28-4.05) times more likely to have a hospital admission than those who carry 0 risk alleles (Figure 5.4.7C) (p= 0.005).

	Hospital Admissions				
Locus	**0**	**1**	**2**	**P-value**	**Power**
C5orf56 (rs100077785)	153 (30.7%) N=496	117 (34.9%) N=330	30 (47.6%) N=63	0.022	1.0
C11orf30 (rs61893460)	59 (27.7%) N=209	173 (29.8%) N=549	66 (29.7%) N=220	0.008	1.0
SMAD3 (rs17293632)	153 (31.5%) N=480	117 (32.8%) N=356	27 (50%) N=53	0.023	1.0

Table 5.4.7A Asthma genetic signals that show some changes in hospital admissions between the risk allele groups: data is presented as percentage and N's for the patients who had a hospital admission in the last year and the total number of patients who had hospital admissions data across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma.. A chi-squared test was used to analyse these categorical groups. Bonferroni correction p=0.002.

	Uncorrected			Corrected		
	N	**O.R (95% C.I)**	**p-value**	**N**	**O.R (95% C.I)**	**p-value**
C5orf56 (rs100077785)						
0 risk alleles	498	(ref)	0.023	496	(ref)	0.02*
1 risk allele	335	1.21 (0.90-1.63)	0.204	330	1.19 (0.88-1.60)	0.267
2 risk alleles	63	2.05 (1.21-3.48)	0.008	63	2.13 (1.24-3.65)	0.006
C11orf30 (rs61893460)						
0 risk alleles	213	(ref)	0.009	209	(ref)	0.006*
1 risk allele	550	1.63 (1.14-2.33)	0.007	549	1.69 (1.17-2.43)	0.005
2 risk alleles	222	1.104 (0.73-1.67)	0.640	220	1.13 (0.74-1.72)	0.586
SMAD3 (rs17293632)						
0 risk alleles	485	(ref)	0.027	480	(ref)	0.02*
1 risk allele	357	1.06 (0.79-1.42)	0.706	356	1.06 (0.79-1.37)	0.698
2 risk alleles	54	2.17 (1.23-3.83)	0.007	53	2.27 (1.28-4.05)	0.005

Table 5.4.7B Logistic regression model of predictors of asthma patient having a hospital admission: data is presented for signals rs100077785, rs61893460 and rs17293632. The 0 risk allele is the reference group for each signal and age was the covariant for the corrected model. The number for each group is given along with the uncorrected and corrected odds ratio (O.R), 95% confidence interval (C.I) and p-value. *The overall p-value for the logistic regression model.

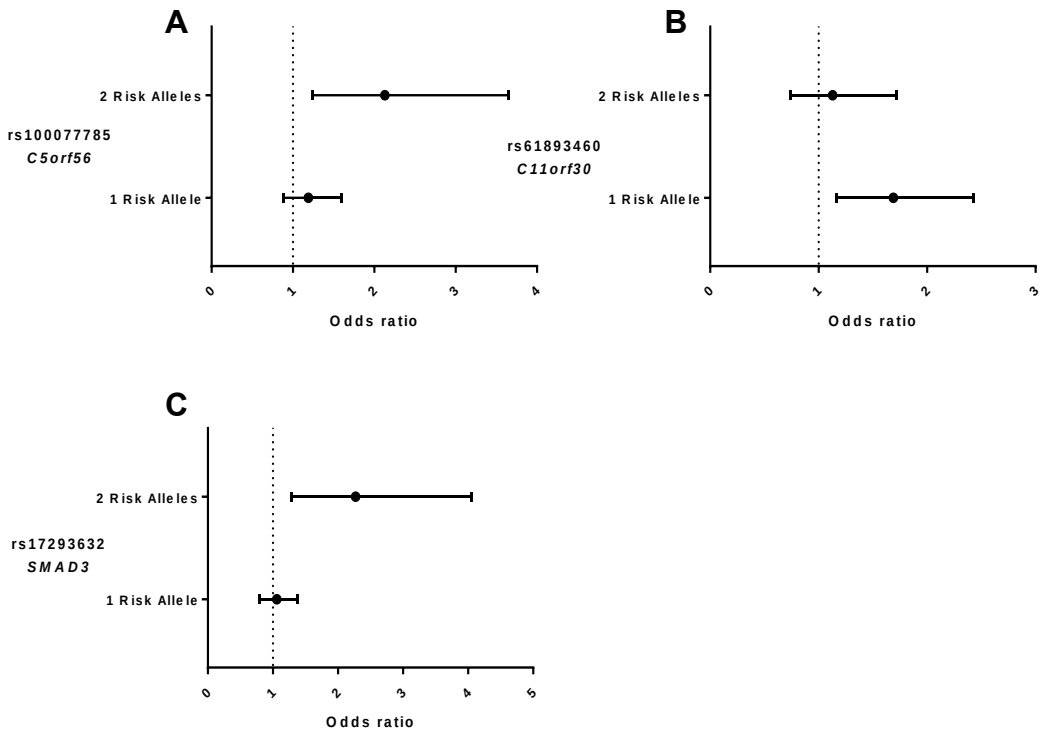


Figure 5.4.7 Odds ratio plot of the genetic signals that are associated with an asthma patient having a hospital admission: the point in the middle indicates the odds ratio (OR) for each risk allele (adjusted), the whiskers wither side indicate the 95% confidence intervals (C.I) and the dotted line through 1 indicates the reference point (0 risk allele group is the reference group) to see if there is any difference between the 0 risk alleles and 1 or 2 risk alleles.

5.4.8 Patients who carry 1/2 Risk Alleles for the Genetic Signal rs61816764 have an Increased Odds Ratio of having Atopic Dermatitis as a Comorbidity

Allergic dermatitis and allergic rhinitis are common comorbidities associated with asthma. Moderate-severe asthma risk alleles were tested for association atopic dermatitis and allergic rhinitis as a comorbidity, the hypothesis being that they may drive these comorbidities leading to disease severity. Both were analysed to see whether carrying 1 or 2 risk alleles for the 25 genetic signals were associated with having any of these atopic comorbidities. In the first set of analyses, a chi-squared test was used to analyse the allergic dermatitis and rhinitis data with the risk allele groups for the 25 genetic signals. None of the 25 genetic signals showed any association with allergic rhinitis. Table 5.4.8A displays the percentages, number of patients that have atopic dermatitis in the risk and non-risk allele groups, the p-value and power for rs61816764 (*FLG*) which was the only statistically significant (p= <0.05) signal with atopic dermatitis but, it did not meet correction for multiple testing. The data from the chi-squared test shows that atopic dermatitis was associated between the risk allele groups for rs61816764 (*FLG*) (p= 0.013). Since, the p-value from a chi-squared test only gives a descriptive analysis of the results, a logistic regression was then preformed to discover the effect of carrying 1 or 2 risk alleles for rs61816764 (*FLG*) on the likelihood that asthma patients have atopic dermatitis. Table 5.4.8B displays the odds ratio and p-value for rs61816764 (*FLG*) with the reference group being the 0 risk allele. The model explains that asthma patients carrying 1/2 risk alleles were 1.74 (95% C.I: 1.12-2.69) times more likely to have atopic dermatitis than those who carry 0 risk alleles (figure 5.4.8) (p= 0.013).

	Atopic Dermatitis				
Locus	**0**	**1**	**2**	**P-value**	**Power**
FLG	N=658	N=94		0.013	1.0
(rs61816764)	215 (32.7%)	43 (45.7%)			

Table 5.4.8A rs61816764 *(FLG)* shows association with allergic dermatitis: data is presented as percentage and n's for the patients who had have allergic dermatitis and the total number of patients who have allergic dermatitis data across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A chi-squared test was used to analyse both these categorical groups. Bonferroni correction p=0.002.

	N	O.R (95% C.I)	P-value
***FLG* (rs61816764)**			
0 risk alleles	658	(ref)	0.013
1/2 risk alleles	94	1.74 (1.12-2.69)	0.013

Table 5.4.8B Logistic regression model of predictors of asthma patient having allergic dermatitis: data is presented for signals *FLG.* 0 risk alleles are the reference group, N's for each group along with the odds ratio (O.R), 95% confidence interval (C.I) and p-value.

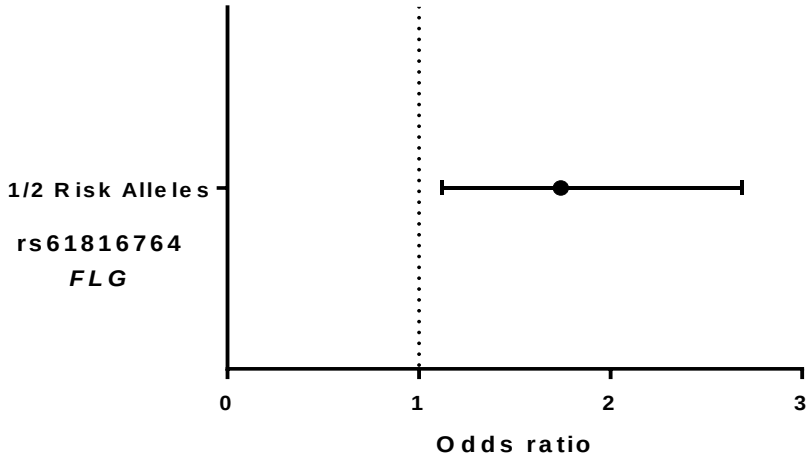


Figure 5.4.8 Odds ratio plot for rs61816764 that is associated with an asthma patient having atopic dermatitis: the point in the middle indicates the odds ratio (OR) for each risk allele (adjusted), the whiskers wither side indicate the 95% confidence intervals (C.I) and the dotted line through 1 indicates the reference point (0 risk allele group is the reference group) to see if there is any difference between the 0 risk alleles and 1/2 risk alleles.

5.4.9 Patients carrying asthma risk alleles for rs1342326 (*IL-33*) and rs36045143 (*CLEC16A*) may have a high blood eosinophil count

Eosinophilic and non-eosinophilic asthma have been identified as major asthma subgroups. The role of the 25 genetic variants were examined to see whether carrying 1 or 2 risk alleles were associated with blood eosinophil levels stratified based on the blood eosinophil count (≥300 cells/μL) according to GINA (GINA, 2020). In the first set of analyses, a chi-squared test was used to analyse the two eosinophil groups with the three risk allele groups for the 25 genetic signals. Table 5.4.9A shows the genetic signals for rs1342326 (*IL-33*) and rs36045143 (*CLEC16A*) that met statistically significant (p= <0.05) but did not meet correction for multiple testing. Table 5.4.9A displays the percentages and number for patients in the >300 μL group across the risk allele group along with the p-value. These data shows that blood eosinophils have some association between the risk allele groups for rs1342326 (*IL-33*) and rs36045143 (*CLEC16A*) (p= 0.031 and p= 0.033; respectively). The p-value from a chi-squared test only gives a descriptive analysis of the results. The two eosinophil groups were then analysed as a using logistic regression to discover the effect of carrying 1 or 2 risk alleles for rs1342326 (*IL-33*) and rs36045143 (*CLEC16A*) on the likelihood that asthma patients have a high eosinophil count. Age and gender were checked as covariates for blood eosinophils but did not come up as statistically significant in the model. Table 5.4.9B displays the odds ratio and p-value for rs1342326 (*IL-33*) and rs36045143 (*CLEC16A*) with the reference group being the 0 risk allele. The model explains that asthma patients carrying 2 risk alleles for rs1342326 (*IL-33*) were 2.43 (95% C.I: 1.15-5.13) times more likely to have a high blood eosinophil level of more than >300 cells/μL than those who carry 0 risk alleles (figure 5.4.9) (p= 0.02). For rs36045143 (*CLEC16A*) signal, the model shows that asthma patients carrying 1 risk allele were 2.58 (95% C.I: 1.09-6.11) times more likely to have a high blood eosinophil level of more than >300 cells/μL than those who carry 0 risk alleles (p= 0.031) (figure 5.4.9). Additionally, those patients who carry 2 risk alleles are 2.91 (95% C.I: 1.09-6.11, 1.25-6.75) times more likely to have a high blood eosinophil level of more than >300 cells/μL than those who carry 0 risk alleles (p= 0.013) (figure 8.4.9).

	Eosinophils >300 cells/μL				
Locus	**0**	**1**	**2**	**P-value**	**Power**
IL-33 **(rs1342326)**	165 (35%) N=472	93 (40.6%) N=229	17 (56.7%) N=30	0.031	1.0
CLEC16A **(rs36045143)**	7 (18.4%) N=38	88 (36.8%) N=239	180 (39.6%) N=454	0.033	0.99

Table 5.4.9A the genetics signals rs1342326 (*IL-33*) and rs36045143 (*CLEC16A*) moderate association with the higher blood eosinophil count group (>300 cells/μL): data is presented as percentage and n's for the patients who had a blood eosinophil count of >300 μL and then the total number of patients who had a blood eosinophil count across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A chi-squared test was used to analyse both these categorical groups. Bonferroni correction p=0.002.

	N	**O.R (95% C.I)**	**P-value**
IL-33 **(rs1342326)**			
0 risk alleles	165	(ref)	0.035
1 risk allele	93	1.27 (0.92-1.76)	0.146
2 risk alleles	17	2.43 (1.15-5.13)	0.02
CLEC16A **(rs36045143)**			
0 risk alleles	38	(ref)	0.042
1 risk allele	238	2.58 (1.09-6.11)	0.031
2 risk alleles	452	2.91 (1.25-6.75)	0.013

Table 5.4.8B Logistic regression model of predictors of asthma patient having a high blood eosinophil count (>300 cells/μL): data presented for signals rs1342326 (*IL-33*) and rs36045143 (*CLEC16A*). 0 risk alleles is the reference group, N's for each group along with the odds ratio (O.R), 95% confidence interval (C.I) and p-value.

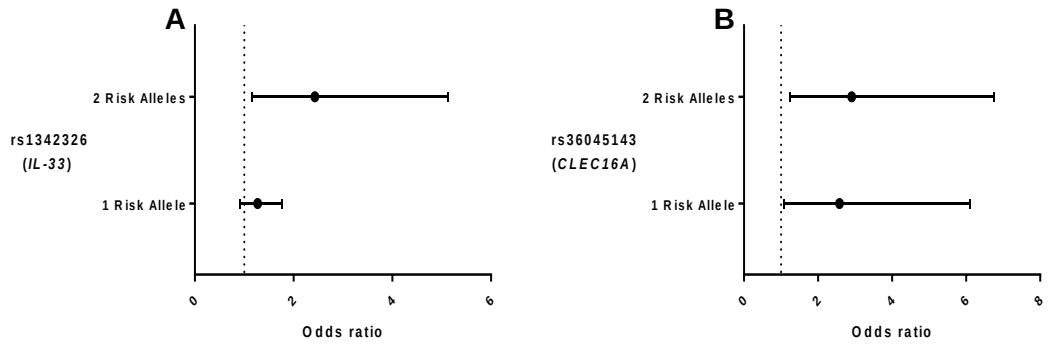


Figure 5.4.9 Odds ratio plot for rs1342326 (*IL-33*) and rs36045143 (*CLEC16A*) that are associated with an asthma patient having a high blood eosinophil level of >300 cell/μL: the point in the middle indicates the odds ratio (OR) for each risk allele (adjusted), the whiskers wither side indicate the 95% confidence intervals (C.I) and the dotted line through 1 indicates the reference point (0 risk allele group is the reference group) to see if there is any difference between the 0 risk alleles and 1/2 risk alleles.

5.5 Discussion

The aims of this chapter were to identify whether the 25 genetic signals identified in the moderate to severe asthma GWAS as asthma risk alleles drive specific types of inflammation (Th1, Th2 and Th17) in asthma patients using the serum cytokines and whether they lead to clinical Type 2 phenotypes. The main findings were that patients carrying the asthma risk alleles for genetic signals rs61816764, rs532965 and rs13275219 had nominally statistically significant higher serum IL-4, IL-33 and periostin levels respectively. However, they did not meet correction for multiple testing. The asthma risk alleles for several of the genetic signals such as rs9303277, rs28592983, rs10077785, rs167769, rs2244012, rs1106639, rs61893460, rs61816764, rs1342326 and rs36045143 were associated with clinical phenotypes including age of onset, serum IgE, allergic dermatitis, lung function, blood eosinophil levels, hospital admissions and asthma control. Asthma patients that carried 1 or 2 risk alleles for rs9303277 and rs61816764 were both associated with early onset of asthma and were the only signals that met correction for multiple testing. Therefore, based on the analyses I have shown that signals previously associated with moderate to severe asthma may drive several Type 2 clinical phenotypes of asthma including age of onset, IgE levels, blood eosinophils and atopic dermatitis but do not drive Th1, Th2 or Th17 inflammation in the blood of asthma patients.

Severe asthma GWAS have identified many signals that are implicated in genes/loci involved in the Type 2 immune response such as *RAD50*, *STAT6*, *C5orf56*, *IL1R1* and *TSLP* (Shrine et al., 2019) (Li et al., 2010) (Wan et al., 2012). Studies have not confirmed the candidate causal genes related to the Type 2 pathway that drive Th2 cytokines, receptors and small signalling molecules in the sera of asthma patients. However, if the genetic signals from Shrine et al study are associated with different types of inflammation i.e., Th1, Th2 and Th17 then it may provide mechanistic understanding and the potential for stratified medicine. The data from the genetic signals and biomarkers reflecting Th1, Th2 and Th17 inflammation showed very little to no association between the asthma risk allele groups and the biomarker levels. As mentioned, genetic signals rs61816764, rs532965 and rs13275219 showed minimal

association with serum biomarkers IL-4, IL-33 and periostin; respectively. rs61816764 and rs13275219 was also not well powered (<0.85) suggesting potentially increasing the power a bigger significant difference may be seen between the expression levels across the risk allele groups. Thus, it does not exclude the fact that these two signals do not drive the levels of these biomarkers in the sera of asthma patients. However, the levels of these biomarkers across asthma risk allele were very minimal and they did not meet correction for multiple testing. The data in this chapter and chapters 3 and 4 suggests that the inflammatory markers in the blood may not be driven by genetic signals related to moderate-severe asthma and do not relate to ongoing clinical and immunological measured associated with asthma therefore may be poor markers of ongoing pathogenesis. Cytokine levels in the serum may also not be a good reflection of measuring an asthma patient's inflammatory profile instead, cytokines levels in the sputum could be a better indication of whether these genetic signals drive the cytokine expression since it is a more direct measurement of the airways compared to peripheral blood.

A study looking at cytokine profiles in the serum and sputum of asthma and COPD patients found more notable differences between inflammatory mediators in the sputum compared to the serum (Cao et al., 2012). Additional data has shown in severe exacerbations in moderate to severe asthma patients that measured inflammatory markers in sputum and serum found inflammatory mediators in the sputum to be the strongest identifiers of an exacerbation (Ghebre et al., 2019). These studies indicate that sputum maybe a more relevant site of airway inflammation than serum. Furthermore, measuring mRNA in the sputum of asthma patients may also be another approach to examine the airway inflammation as one study showed a predominance of Th2 cytokines IL-4, IL-5 and IL-13 in allergic asthma compared to non-allergic asthma (p= 0.03, p= 0.02 and p= 0.0002; respectively) as well as increase levels of IFN-γ in severe asthma than mild asthma patients (p= <0.01) (Truyen et al., 2006). Some studies have also shown genetic variants related to asthma to be associated with mRNA level in lung tissue. One study looking at genetic variants in the *IL-33* locus found two of the signals to be associated with IL-33 mRNA in the bronchial epithelial cells

in vivo when carrying the risk allele for asthma (rs992969: p= $8.30x10^{-12}$ and rs442246: p= 0.0298) (Ketelaar et al., 2021). Another study looking at the phenotypic and functional translation of *IL1RL1* locus (receptor of IL33) polymorphisms in asthmatic airway epithelium identified genetic signal rs995514 to contribute to eosinophilic phenotypes in asthma when carrying the asthma risk allele (Portelli et al., 2020). These studies suggest that measuring cytokines in the airways may be a better indicator at looking at airway inflammation. Furthermore, current data has shown asthma associated SNPs in loci such as *IL-33* have an effect on mRNA and protein levels in lung tissue which suggests that there may be multiple more signals identified in asthma GWAS that drive inflammation in the airways. Both studies from Ketelaar et al and Portelli et al overlap with my study as they use the same patients from the GASP cohort and so are not independent.

The 25 genetic signals were then looked to see if they lead to any clinical type 2 phenotype. Signals rs9303277 and rs61816764 were the only 2 signals out of the study that met Bonferroni correction. The data demonstrated rs9303277 to be highly associated with earlier age of onset when carrying 1 or 2 risk alleles compared to those carrying 0 risk alleles for moderate-severe asthma p= $2.20x10^{-7}$. According to open target genetics the variant-gene (V2G) score suggests that potential candidate causative genes for signal for rs9303277 is *IKZF3* (IKAROS family zinc finger 3) (V2G= 0.3), *GSDMA/B* (gasdermin-like) (V2G= 0.25) and *ORMDL3* (orosomucoid 1-like 3) (V2G= 0.25). SNPs around the *IKZF3* locus have also been previously reported as one of the gene regions associated with susceptibility of asthma (Yan et al., 2017). The SNP reported in Yan et al study (rs907092) is also in high LD with the proxy SNP from the moderate to severe GWAS (rs9303277) r^2= 0.95 and their SNP also showed the most significant association with asthma (OR: 0.71 p= 1.2×10^{-12}). Polymorphisms around genes *GSDMB* and *ORMDL3* have particularly been associated with childhood onset (Moffatt et al., 2007) (Zhao et al., 2015) (Wu et al., 2009). Wu et al study genotyped rs4378650 in *ORMDL3* and rs7216389 in *GSDML* in children (age 4-17) and found individuals carrying the one or two copies of the C allele of rs4378650 or the T allele of rs7216389 had increased risk of asthma [OR: 1.73, 95%CI 1.19–2.53, p= 0.003 and OR: 1.64, 95%CI

1.12–2.38, p= 0.009; respectively). The SNPs reported in Wu et al study (rs4378650 and rs7216389) is also in high LD with the sentinel SNP from the moderate to severe GWAS (rs2941522) r^2= 0.74 and 0.75 respectively. My data and previous data support the fact SNPs in these in these loci/genes may contribute to childhood asthma.

SNPs around the *FLG* (filaggrin) loci have also appeared in many asthma GWAS and has shown to be the variant with the greatest effect size on childhood asthma as well as being associated with atopy (Zhu et al., 2018b) (Johansson et al., 2019) (Pividori et al., 2019). Pividori et al study showed signal rs61816761 near the *FLG* gene region to have the largest effect on childhood onset when carrying both risk alleles (OR: 1.97, CI 1.82-2.13; p= $1.88x10^{-65}$) compared to adult onset (OR: 1.02, CI 0.95-1.08; p= 0.188). My data demonstrated similar findings that patients carrying 1/2 risk alleles for rs61816764 to which had lower age of onset (15.02 years) than those who carried 0 risk alleles who had more adult asthma age of onset (19.2 years) p= $2.67x10^{-4}$. The *FLG* gene region is suggested to be the potential candidate causative gene according to open target genetics with a V2G score of 0.09. This supports the fact that SNPs at the *FLG* locus may contribute to childhood asthma. Additionally, other studies have found signals in the *FLG* loci to be associated with allergic dermatitis (OR: 3.20, CI: 1.46-7.02; p= 0.004) (Bisgaard et al., 2009) and atopic march (OR: 1.61, CI: 1.41-1.89; p= $8.5x10^{-11}$) (Marenholz et al., 2015). *FLG* is thought to be essential for epidermal differentiation and maintaining essential barrier function (Palmer et al., 2006) (Marenholz et al., 2015). Furthermore, my study also showed rs61816764 (*FLG*) to be associated with allergic dermatitis with the odds of having allergic dermatitis increasing by 1.74 (CI: 1.12-2.69) when carrying 1/2 risk alleles. It has also been shown that single nucleotide variant rs61816761 produces a premature stop codon in the *FLG* gene known as R501X causing an amino acid deletion which has been associated with atopic dermatitis and asthma (Ramirez Aponte and Martinez Cruzado, 2019). According to the online database LD Link, the proxy SNP from the moderate-severe asthma GWAS was in low linkage disequilibrium (R^2= 0.19) with rs61816761 suggesting both these polymorphisms are independent from each other. This highlights the

importance *FLG* has with the association and overlap between asthma and allergic diseases particularly in childhood onset of the disease as these patients are known to be more atopic than adult onset.

Overall, it is known that childhood onset is associated with the most independent loci compared with other defined groups of asthma such as adult onset and severe asthma which are associated with fewer genes. There is also a significant overlap between asthma and other allergic diseases particularly with respect to childhood onset of asthma which involves genes that reflect the importance of epithelial barrier function. Disruption in the epithelial barrier function via allergens viruses etc. is suggested to be the pathophysiology of airway inflammation for allergic asthma, particularly in infant/childhood. Pividori et al's study also found childhood onset loci were enriched for genes with the highest expression in the skin highlighting how childhood onset supports the idea that childhood asthma is due to impaired barrier function in the skin and other epithelial surfaces (Pividori et al., 2019). Atopic march describes the disease progression from atopic dermatitis to allergic airway disease in childhood and it is associated with severe and persistent allergic characteristics. The understanding of atopic march was through the discovery of filaggrin loss-of-function mutations that provided genetic evidence linking skin barrier deficiency to eczema and subsequent asthma development (Marenholz et al., 2006). It been shown that predominantly eczema loci increase the risk for the atopic march, suggesting that eczema plays an important role in the development of asthma after eczema (Marenholz et al., 2015). The fact that this thesis identified the signal in the *FLG* region to be associated with early age of onset and allergic dermatitis supports atopic march and the idea of compromised epithelial barriers promoting sensitisation to airway allergens leading to asthma in early life.

IgE usually measures allergic disease and is a hallmark of type 1 hypersensitivity, IgE also has the ability to influence the function of several immune and structural cells involved in the pathogenesis of allergic inflammation such as asthma. Total IgE measures the overall quantity of IgE in the blood whereas specific IgE measures allergen specific IgE antibodies.

In asthma patients there is usually an increase in total and specific IgE levels in the serum (Matucci et al., 2018). Increased serum IgE is one of the key characterisations in some asthma patients, particularly in those with Th2 high/allergic asthma and is also associated with other allergic disease such as hay fever and eczema. Despite not meeting Bonferroni correction, several genetic signals including rs100077785, rs167769, rs2244012, rs1106639, rs61893460, rs10519067 and rs1837253 were associated with serum IgE levels ($p = <0.05$). These signals showed that serum IgE levels increased when carrying one or more moderate-severe asthma risk alleles compared to carrying 0 risk alleles even after adjusting for age and gender. Signals rs167769 and rs61893460 showed the strongest association with IgE levels. When looking at open target genetics it is likely that rs167769 signal is being driven by *STAT6* as the variant to gene (V2G) score is 0.3, as for rs61893460 it is likely that this signal is being driven by *LRRC32* (Leucine Rich Repeat Containing 32) with a V2G score of 0.17. A study reported multiple signals in the *C11orf30-LRRC32* gene region to be associated with serum IgE levels, suggesting *C11orf30* and *LRRC32* may potentially regulate serum IgE levels (Li et al., 2012). Other signals in the *C11orf30-LRRC32* region have also been associated with atopic dermatitis (Esparza-Gordillo et al., 2009) (Paternoster et al., 2015) and in the atopic march (Marenholz et al., 2015). *C11orf30* encodes the EMSY protein and is suggested to play a role in epithelial barrier function (Esparza-Gordillo et al., 2009) and atopic march is involved in early sensitisation through dysfunction skin barrier during infancy (eczema) which potentially may lead to atopic childhood asthma. The data in this study and previous studies suggests signals round the *C11orf30-LRRC32* region is likely to be involved in atopic diseases including asthma and may affect the biological pathway involved in sensitisation through epithelial barrier dysfunction and regulation of serum IgE levels. Furthermore, polymorphisms in the *STAT6* gene region have previously been confirmed as susceptibility locus modulating IgE levels (Weidinger et al., 2004). *STAT6* belongs to the Signal Transducer and Activator of Transcription family of proteins. It is essential for number of responses in T cells such as the development of Th2 cells, IL-4-stimulated proliferative responses and in B cells STAT6 promotes immunoglobulin class switching to IgE and IgG1 (Goenka and Kaplan, 2011).

A mouse study showed that levels of serum IgE were reduced in STAT6$^{-/-}$ mice when they were sensitised with an antigen (Shimoda et al., 1996). mRNA levels of *STAT6* have also shown some positively correlation with serum IgE levels (rho= 0.077 p= 0.038) (Antczak et al., 2016). The data in this study and previous studies is consistent with the fact that *STAT6* may be one of the key signals in activating and promoting IgE production and suggests that it may also regulate the levels if IgE produce in the serum of asthma patients.

Other studies have also identified signals that have also been highly associated with the Th2 inflammation in allergic asthma and other allergic diseases including IgE mediated (Guo et al., 2020) (Li et al., 2010) (Zhu et al., 2018b) (Laulajainen-Hongisto et al., 2020). My study demonstrated that rs10519067 is associated with IgE levels and according to open target genetics it is likely that the signal is being driven by *RORA* (Related Orphan Receptor A) with a V2G score of 0.05. *RORA* has been demonstrated to regulate Th2 cell responses in airway allergic inflammation. A recent *in vivo* study using an allergic asthma in IL-4 reporter mice model found that Th2 cells in the lung expressed higher levels of RORA than those in the lymph nodes, and treatment with a RORA agonist resulted in diminished Th2 cell responses. Additionally, they also reported that when the mice were challenged with house dust mite, they exhibited an increase in Th2 cells in airways and eosinophilic inflammation compared to control mice (Lee et al., 2020). rs2244012 was also associated with IgE levels and open target genetics shows evidence that it is likely the signal is being driven by a combination of *SLC22A4* (V2G= 0.19)*, SLC22A5* (V2G= 0.23) as well as genes in the Th2-cytokine locus including *RAD50* (V2G= 0.19), *IL4/IL5/IL13 (*V2G= 0.07-0.9). Many of these genes in the Th2 cytokine locus have been previously linked with total IgE, this suggests it is likely to be a combination of genes that is driving the rs2244012 signal. A GWAS on total IgE identified multiple polymorphisms within the *RAD50* gene on chromosome 5q31 that were consistently associated with IgE levels (p= 6.28×10^{-7}-4.46×10^{-8}) and increased the risk for atopic eczema and asthma. *RAD50* encodes for a DNA repair protein that is involved in a DNA double-strand break repair (Weidinger et al., 2008) and is suggested to play a role in regulation of the Th2 cytokine gene

transcription (Lee et al., 2003). My data has shown that several signals were in gene regions that have been previously replicated in other studies with serum IgE levels as well as asthma and atopy. It is well known that IgE is related to allergic asthma and type 2 inflammation and multiple asthma GWAS have repeatedly reported the same signals that are associated with the type 2 inflammation pathway and even shown association with IgE levels. This has led on to studies using approaches to suggest how these signals are regulating the type 2 pathway and even IgE production including how different expression levels of these signals regulate serum IgE levels. My study demonstrated that several of the genetic signals may drive IgE production in the type 2 inflammation pathway and even regulate levels of IgE as my data showed carrying one or 2 risk alleles for moderate to severe asthma levels increased.

A few GWAS on asthma exacerbations have provided insight on how genetic variants may predispose to asthma exacerbations which is a problematic feature in severe asthma patients. A GWAS on childhood asthma with severe exacerbations identified five signals in gene regions of rs2305480 (*GSDMB*), rs928413 (*IL33*), rs6871536 (*RAD50*), rs1558641 (*IL1RL1*) and rs6967330 (*CDHR3*) associated with exacerbations in childhood asthma. They also showed that, when stratified based on number of hospital admission, the odds ratio increased with increased number of hospital admission for all five signals (Bønnelykke et al., 2014). A more recent GWAS of asthma exacerbation identified a signal rs12946510 located within the *GRB7/IKZF3* region to be associated with severe exacerbations (OR for T allele= 0.71, 95%CI 0.64–0.79, p= 1.12×10^{-10}), they also suggested because *GRB7/IKZF3* gene regulates the development of B lymphocytes, it is likely to impact the immune response in these subjects with asthma exacerbations (Herrera-Luis et al., 2021). For my study, signals rs10077785 (*SLC22A5*), rs61893460 *(LRRC32)* and rs17293632 (*SMAD3*) were associated with hospital admissions, none of the signals were in gene regions that were previously reported in the above studies. My data showed patients that carried 1 or 2 risk alleles for the moderate-severe asthma signals rs10077785, rs61893460 and rs17293632 had an increased odds ratio of having a hospital admission compared to those who carry 0 risk alleles (rs10077785: OR for 2 risk alleles= 2.13, 95%CI: 1.24-

3.65; rs61893460: OR for 1 risk allele= 1.69, 95%CI: 1.17-2.43; rs17293632: OR for 2 risk alleles 2.27, 95%CI: 1.28-4.05). When looking at open target genetics it is likely that rs17293632 is likely to be driving *SMAD3* expression with a V2G score of 0.33. *SMAD3* is a protein coding gene that's involved in transforming growth factor-beta (TGF-β) signalling with TGF- β levels shown to increase in the asthmatic airways. It has been suggested that SMAD3 is involved in type 2 immune response. One study showed that in SMAD3$^{-/-}$ mice, mRNA and protein expression of Th2 cytokines were decreased in the lung including IL-13 and IL-5 compared to the wild type mice (Anthoni et al., 2007). As mentioned previously *LRRC32* is suggested to play role in epithelial barrier function and IgE production but it is also essential for inflammatory T cell function and activation (Li et al., 2012). Many of these genes have shown to be involved in the type 2 immune responses and allergy (Li et al., 2012) (Zhu et al., 2018b), my data and previous data have shown many of these signals are involved and regulate type 2 responses suggesting that these signals may impact type 2 immune responses in moderate-severe asthma patients increasing their risk to a hospital admission.

Blood eosinophil counts have been used to identify potential subsets of asthma including those that may be more amenable to the recently introduced biologics e.g., Mepolizumab. Previously data has suggested elevated blood eosinophil levels are at least in part driven by genetic mechanisms. One study identified a SNP in the *IL-33* region rs992969 to be associated with blood eosinophil levels, asthma and eosinophilic asthma (Ketelaar et al., 2021). A second study showed several polymorphisms associated with blood eosinophil counts that reached genome-wide significance (2q12 rs1420101, 2q13 rs12619285, 3q21 rs4857855, 5q31 rs4143832 and 12q24 rs3184504). A SNP at *IL-33* gene region rs3939286 indicated association with blood eosinophil counts ($p= 1.8x10^{-5}$) and also atopic asthma (OR: 1.12, 95% CI: 1.07-1.17; $p= 2.2x10^{-5}$) (Gudbjartsson et al., 2009). In the current study we aimed to replicate and expand what genetic signals from the moderate-severe asthma GWAS may be driving blood eosinophil levels in asthma patients. This study found signals rs1342326 and rs36045143 to be associated with the high blood eosinophil group (>300 µL) when carrying 1 or 2 risk alleles. rs1342326

is a signal in the *IL-33* region (V2G= 0.09) and my data showed when carrying both risk alleles had a 2.43 (CI: 1.15-5.13) odds ratio of having a high blood eosinophil levels. IL-33 and its receptor ST2 is suggested to play an important role in eosinophilic inflammation and monoclonal antibodies targeting ST2 have shown to inhibit eosinophil responses to IL-33 (Cherry et al., 2008). This suggests that targeting IL-33 or its receptor may be beneficial for patients with high eosinophilic asthma and may. Additionally, a GWAS has previously reported a SNP rs12924112 near the *CLEC16* gene region to be a risk factor for eosinophilic esophagitis, a chronic inflammatory disease of the oesophagus which is associated with a very high eosinophil count (Kottyan et al., 2019) (O'Shea et al., 2021). Open target genetics suggests that the signal is likely to be driving *CLEC16* expression (V2G= 0.13). It is known that *CLEC16A* plays a key role in B cell function, (Rijvers et al., 2020) and has been shown that peripheral B cell counts have been correlated with eosinophil counts (r=0.22; p=<0.05) (Kuang et al., 2019) suggesting *CLEC16* could be involved in the regulation of eosinophils. Both these signals suggest from previous data that they may be involved in eosinophil inflammation in asthma patients.

This study had also some novel findings as the genetic signals were also looked to see if they associated with ACQ symptom score. Here signals rs1847472 and rs1444782 showed association with a low symptom score when carrying 1 or 2 risk alleles for these signals but did not meet correction for multiple testing. These two signals may indicate poor asthma control when patients carry the risk alleles for these signals however, these changes are minimal suggesting these results are false due to multiple simultaneous statistical tests and is unlikely to be reproducible. Therefore, it is likely these signals may not indicate asthma symptom control. Lastly, signal rs28592983 which is thought to be driving *MUC5AC* expression (V2G= 0.09) was one of the novel signals found in Shrine et al study, *MUC5AC* is a mucin gene and is associated with the production and secretion of mucus. It has been shown that airway mucus hypersecretion compromises lung function which in turn may lead to exacerbation in asthma patients (Bonser and Erle, 2017). The data demonstrated that rs28592983 was associated with lower FVC when carrying

the 1 risk allele compared to the 0 risk allele group after adjusting for age, gender, height and weight. Even though this did not meet Bonferroni correction, it suggests that carrying the risk variant for rs28592983 may cause a decline in lung function due to obstruction of excess mucus production. Data has also shown a reduced FVC being correlated with asthma severity (r= -0.25; p= 0.01) particularly in women, it was suggested that FVC may provide information over FEV_1 about airway obstruction in severe asthma patients (Castelluccio et al., 2015) as airway obstruction has been seen in 50% of severe asthma patients (Lee et al., 2007). Signals rs9303277, rs9272629 and rs17293632 also were associated with lower lung function levels when carrying the risk allele. The reduction in these lung function values in these three signals and rs28592983 were only minimal therefore, it is likely that these signals may not cause change in lung function. Again, it is likely that these findings are false due to multiple simultaneous statistical tests and is unlikely to be reproducible.

To conclude, the data from this study has shown that the genetic risk factors for the development of moderate-severe asthma are not associated with specific inflammatory profiles in the blood on asthma patients. However, the genetic risk factors for the development of moderate-severe asthma potentially drive clinical and immunological features of asthma such as age of onset, serum IgE levels and atopy. Most of the proxies found where in high LD with the original SNP identified with most being above 0.7. rs28592983 and rs61816764 were the only two SNPs in low LD with the original SNP and so may impact the results as it suggests they may be independent from each other but still suggests some coinheritance. Many of these potential candidate casual genes are involved in type 2 inflammatory pathway to which reflect typical type 2 clinical phenotype that are already known such as childhood onset and atopy. This therefore provides greater insight and the scope for stratified medicine for asthma patients using genetics.

Chapter 6- General Discussion

6.1 Hypothesis and Aims

The three main hypotheses of this thesis were that; i) a greater understanding of clinical and immunological features of asthma will identify specific sub-groups of asthma that provide insight into the drivers of age of onset, exacerbations and eosinophilic asthma, ii) serum cytokines represent an accessible measure to further characterise asthma patients and this profile is related to age of onset, exacerbations and eosinophilic asthma and iii) genetic risk factors identified for the development of moderate-severe asthma drive specific inflammatory profiles in asthma and/or specific clinical features of disease and that any of these or a combination may facilitate a more personalised approach to asthma management i.e. stratified medicine. The main findings from this thesis were that childhood onset of asthma is more likely to be atopic than adult onset of asthma who are more likely to smoke, be heavier and have lower lung function. Patients who had a hospital admission within the last 12 months had a high GINA score, reduced lung function and high ACQ score compared to those who were never admitted. Patients who were eosinophilic high did not identify as a clinically distinct sub-phenotype of patients. I was able to measure a panel of pro-inflammatory cytokines and biomarkers in the blood of asthma patients. They did not identify asthma clinical phenotypes but many showed high correlated expression with each other and there was a high percentage overlap with multiple inflammatory cytokines. There was also a weak relationship between the cytokines measured in the sera and the blood cell counts potentially due to low numbers. The genetic risk factors for the development of moderate-severe asthma were not associated with specific inflammatory profiles in the blood on asthma patients but were associated with the risk of developing clinical and immunological features of asthma including age of onset, serum IgE levels and atopy. These findings have further added to the field of asthma heterogeneity and suggest that a patient's asthma could be driven by multiple pathways. The data also suggests that genetics may be the way forward in personalised medicine as the genetic variants could predict a specific

phenotype of asthma which might respond better to a particular type of biological therapy.

Cluster analysis of clinical data and inflammatory profiling using cytokine and mRNA expression levels in the sputum and blood of asthma patients has helped identify phenotypes and endotypes which has provided a framework and is used today in the diagnosis and treatment of asthma (Moore et al., 2010) (Akiki et al., 2017). Furthermore, GWAS studies have identified hundreds of genetic signals suggested to be associated in the development of asthma including moderate to severe asthma with many of the implicated candidate causal genes where the signals suggests genes/pathways that are involved in the innate and adaptive immune responses (Wan et al., 2012) (Ferreira et al., 2017) (Shrine et al., 2019). In addition to this, monoclonal antibody drugs have been developed that target components of the type 2 inflammatory pathway to which clinical trials have already shown convincing results in the reduction of exacerbations and improvement of asthma symptoms in some severe asthma patients. However, it is still unknown is which asthma patients will benefit from these new drugs and are the effects of administering multiple drugs additive. Therefore, identifying what type of inflammation is present in specific asthma patients by using their clinical, blood and genetic profile could help tailor asthma therapy and identify who may benefit from the new monoclonal antibody drugs.

6.2 Main Findings

6.2.1 Age of Onset- Lessons Learnt

Age of onset is one of the main phenotypes recognised in asthma heterogeneity today which was identified by observation a long time ago and more formally recognised in more scientific studies subsequently e.g. cluster analysis. Studies have also looked at clinical, immunological and genetic markers with age of onset separately where they found early age of onset of asthma to be highly associated with atopy, high total IgE and eosinophilia (Miranda et al., 2004) (Tan et al., 2016) (Chaudhuri et al., 2016) (Froidure et al., 2016) (Hill and Spergel, 2018) (Trivedi and Denton, 2019). GWAS studies on childhood asthma have also repeatedly found genetic signals near genes including *ORMDL3, GSDMB* and *FLG* (Moffatt et al., 2007) (Pividori et al., 2018). The clinical data from the GASP cohort supports previous findings that patients with childhood onset of asthma are typically atopic, with higher total serum IgE and prevalence of atopic dermatitis and rhinitis. This also supports the atopic march multi-morbidity hypothesis as a subtype of asthma with an allergic foundation. Whereas the late age of onset patients typically had a high BMI, lower lung function, a higher prevalence of smokers and less allergic comorbidities such as atopic dermatitis and rhinitis. Deep phenotyping patients helps characterise and understand what may be driving a patient's asthma such as whether they have atopic or non-atopic atopic asthma which can help with deciding a suitable therapy may for patients. However, the serum biomarkers did not help in identifying an inflammatory signature associated with age of onset. Following up the genetic signals from the moderate-severe asthma GWAS (Shrine et al., 2019), the candidate causative genes for signals rs9303277 (*IKZF3/GSDMA/ORMDL3*) and rs61816764 (*FLG*) showed the strongest effect size with age of onset when carrying both risk alleles for moderate-severe asthma compared with all the genetic signals and clinical features.

Signals around the *IKZF3* gene have also previously been reported in age of onset GWAS studies (Moffatt et al., 2007) (Halapi et al., 2010) (Marenholz et al., 2015). The data in this thesis showed rs61816764 (*FLG*) to be associated with increased odds of having allergic dermatitis as a comorbidity when

carrying the risk allele for moderate to severe asthma which has been previously been described (Zhu et al., 2018b) (Johansson et al., 2019) (Pividori et al., 2019). *FLG* has been shown to be essential for epidermal differentiation and maintaining essential barrier function as well as being associated with allergic dermatitis and atopic march (Palmer et al., 2006) (Marenholz et al., 2015). There is a big overlap in genes with childhood asthma and allergic diseases in which the genes involved reflect epithelial barrier function with *FLG* making a protein that is a crucial gene for making essential barrier function. At the same time atopic march is due to impaired barrier function in epithelial surfaces and the understanding of atopic march was through the discovery of a loss of function of *FLG* (R501X) through a mutation which provided evidence linking skin barrier deficiency to eczema and subsequently the development of asthma (Marenholz et al., 2006). This makes *FLG* an ideal therapeutic target for patients with atopic diseases particularly eczema associated asthma. *FLG* highlights how it may have an important association and/or early intervention in multiple allergic conditions and also childhood onset of asthma.

TSLP has been shown to downregulate *FLG* expression in human skin and acts as a sensory neuronal activator, resulting in itching (Wallmeyer et al., 2017) suggesting that targeting TSLP may be an important therapeutic target for reducing asthma in patients with atopic dermatitis who carry 1 or 2 risk alleles for *FLG* signal and also TSLP may be a good marker for atopy. TSLP acts on a broad range of immune cell types such as DCs, basophils and mast cells and is also highly produced by epithelial cells lining the barrier surfaces of the lungs and even skin and gut through a stimuli such as an allergen (West et al., 2012) (see chapter 1.2.1.1 for more on TSLP). Data has shown that during an allergen-induced asthmatic response an anti-TSLP monoclonal antibody significantly reduced sputum eosinophil levels (4.1±2.3% at baseline to 0.4±0.1% on day 41 and 0.4±0.1% on day 83, p= <0.05) suggesting patients with allergen induced asthma/atopic asthma may benefit from an anti-TSLP drug (Gauvreau et al., 2014). A recent study found Tezepelumab significantly reduced annual exacerbations and improved lung function in patients who had allergic asthma or non-allergic asthma i.e. it was effective in patients with high

and low eosinophils, indicating Tezepelumab may be beneficial in a broad population of severe asthma patients (Corren et al., 2021).

6.2.2 Drivers of exacerbation

Many patients are prone to exacerbate despite taking high dose inhaled corticosteroids and so it is of interest to know how to clinically, immunologically and genetically characterise these patients to understand what drives their exacerbations. The clinical data from this thesis showed the main predictors of asthma related hospital admissions were poor asthma symptom control, low lung function and severe asthma as defined by GINA. Previous studies have demonstrated these clinical characteristics in asthma patients prone to exacerbations further adding to the field (Suruki et al., 2017), (Ortega et al., 2018) (Uematsu et al., 2018) (Sprio et al., 2020).

A cluster analysis study in exacerbation prone adults identified four sub phenotypes two of these groups were identified as the exacerbation-prone asthmatics. Interestingly both of those groups were associated with poor lung function, but one group was also associated with atopy and other group was not associated with atopy (Sprio et al., 2020). The hospital admission prone asthma patients from the GASP cohort did not show any association with atopy but still suggests that these exacerbation prone asthma patients reflect clinical characteristics of exacerbation prone asthma patients who are non-atopic.

The genetic data identified signals rs10077785 (*SLC22A5*), rs61893460 *(LRRC32)* and rs17293632 (*SMAD3*) associated with increased odds of a hospital admission when carrying the risk alleles for moderate-severe asthma. The potential candidate causative genes for these signals are suggested to be associated with the type 2 inflammatory pathway particularly with Th2 cytokine production and also in allergic asthma and other allergic diseases (Guo et al., 2020) (Li et al., 2010) (Zhu et al., 2018b) (Laulajainen-Hongisto et al., 2020) (Marenholz et al., 2015). This highlights how inflammation is driving an exacerbation especially by type 2 immune responses as Th2 cytokines in the lungs cause the airway smooth muscle to contract, narrowing the airways and hence initiating shortness of the breath, wheezing, excess mucus production and plugging and chest tightness resulting in an exacerbation. However, the

serum biomarkers in this thesis did not show reproducible association with hospital admissions even after looking at levels of soluble ST2 in the two cohorts or when the data was combined, this could potentially be due to the serum biomarkers not reflecting airway inflammation.

Current data has shown some evidence in blood biomarkers predicting exacerbation frequency with one cross sectional study showing plasma IL-6 levels being significantly associated with exacerbation-prone asthma patients who were characterised with high BMI and old age compared with exacerbation-resistant and exacerbation-intermittent asthma patients (3.3 pg/mL, 1.9 pg/mL and 2.8 pg/mL; respectively n= 406, p= <0.001). They also found in a longitudinal analysis, blood eosinophils and IL-6 to be predictors of exacerbations over a 3 year period in exacerbation prone patients (p= 0.011 and p= 0.008, respectively n= 291) (Peters et al., 2020), indicating IL-6 and eosinophils maybe a good biomarker to predict future exacerbations. Furthermore, IL-6 maybe a driver of exacerbations and so patients with high levels of IL-6 may be suitable for an anti-IL-6 therapy to reduce exacerbations. One study showed elevated IL-6 was associated with lower FEV_1 in patients with mixed eosinophilic-neutrophilic bronchitis and by knocking out or antibody-mediated neutralisation, IL-6 signalling in mice revoked elevations of eosinophil and neutrophil recruiting cytokines/chemokines and allergen-induced airway inflammation (Chu et al., 2015). This suggests how therapies targeting IL-6 maybe ideal for asthma patients with combined eosinophilic and neutrophilic bronchitis who are prone to exacerbations. Fairly recent data has also shown how lung epithelial IL-6 trans-signalling (IL6-TS) activation in the absence on type 2 airway inflammation defined a novel subset of asthma patients that were associated with frequent exacerbation, eosinophilia, airway inflammation, remodelling and epithelial integrity. Activation of IL6-TS in their air liquid interface cultures reduced epithelial integrity and induced a specific gene signature enriched in genes associated with airway remodelling including matrix metalloproteinase (*MMP1, MMP2* and *MMP3*), *IL33* and chitinase-like proteins (*CHI3L1/YKL-40* and *CHI3L2/YKL-39*). This novel IL-6TS–high asthma subset opens up a stratified medicine approach to treating a significant unmet medical need in patients with severe asthma.

It is also suggested that clinical characteristics such as high BMI and older age increases systemic inflammation and white blood cells therefore increasing markers in the periphery such as IL-6 and eosinophils. Despite the asthma patients who had an exacerbation in the last year in this thesis displaying similar clinical characteristics, IL-6 levels were not elevated in the sera of these patients. This suggests that not all patients who are prone to exacerbations have systemic inflammation but instead have type 2 driven inflammation or potentially overlapping of both. Overall, the exacerbation data suggests that genetic profiling may indicate and/or predict which patients are more likely to exacerbate and even may benefit from the type 2 biological treatment since many of these signals are involved in the production of Th2 cytokines.

6.2.3 Drivers of Eosinophilic asthma

Since more new biologics such as Tezepelumab that target components of the type 2 pathway are being introduced into clinic, blood eosinophil levels are being used as a biomarker to see whether patients will be suitable for the drug. For patients to be put on Mepolizumab, they must have had a blood eosinophil count of >300 cells/µL within the last 12 months. So it would be of interest to know what clinical, immunological and genetic characteristics drive eosinophilic versus non-eosinophilic asthma. Despite high blood eosinophil counts having been associated with atopic diseases such as atopic dermatitis and rhinitis (Froidure et al., 2016), only high serum IgE levels were associated with the high eosinophil group of patients. This suggests that eosinophils may not be useful in identifying distinct asthma subsets on a clinical and genetic level as most of the associations found were very minimal.

SARP and U-BIOPRED have both shown via cluster analysis that the clinical features in patients displaying eosinophilia are not robust and are very heterogeneous as there were multiple eosinophil high cluster groups (Moore et al., 2010) (Loza et al., 2016). This suggests there may be several endotypes driving multiple clinical phenotypes and that patients may have overlapping endotypes which results in asthma heterogeneity. Data has shown how eosinophil levels can fluctuate day by day and even throughout the day with levels being higher in the morning (Tavernier et al., 2018) (Durrington et al., 2018). This fluctuation could be a reason why minimal associations were found

in this study from using cross sectional eosinophil levels. Potentially measuring each patient's blood eosinophil levels at the same time point may give a better indication on whether eosinophilia can identify subsets of asthma patients and also identify what biomarkers and genetic signals drives eosinophilia.

Furthermore, the genetic data showed the SNP in the *IL-33* region to be associated with high blood eosinophil levels when carrying 1 or 2 risk alleles for moderate to severe asthma. Current data has also recently demonstrated this finding that a SNP in the *IL-33* region was associated with high blood eosinophil levels (Ketelaar et al., 2021). This suggests that targeting IL-33 or its receptor may be beneficial for patients with high eosinophilic asthma but what is not understood is whether this will equate to clinical improvement for these patients. IL-33 is understood to act directly on eosinophils and regulate their activation and survival. Data has shown IL-5 and IL-33 has an influence on activating eosinophils, like with IL-5 eosinophil stimulation with IL-33 resulted in an increase expression of cell surface markers such as CD18, CD11b and ICAM-1 (Angulo et al., 2019). IL-33 is released from the airway epithelial cells and other cells such as the endothelium during an environmental stimuli, this leads to downstream Th2 signalling causing lymphocytes and ILC2s to release cytokines IL-4, IL-5 and IL13 driving inflammation by causing eosinophil migration to the airways. IL-5 was originally thought to control eosinophil activation but now data suggests that elevated IL-33 levels may be involved too, strengthening the argument for use of IL-33 as a therapeutic target due for eosinophilic inflammation.

Press release data from a phase 2 clinical trial (n= 25) by AnaptysBio anti-IL-33 drug has shown its effectiveness in improving FEV_1 by 15%, decrease in ACQ5 by -0.67 and decreasing blood eosinophil levels by -40% by day 64 (AnaptysBio, 2019). However, within the last year a few companies are moving out of targeting IL-33 and its receptor ST2 due to poor clinical data results in comparison to other anti-IL-33 data from competing companies (FierceBiotech, 2020), this could potentially be from not targeting the right patients that will respond best to the anti-IL33 drug. Like the development of Mepolizumab in which when asthma patients were enrolled in the study they were not selected in anyway and the initial clinical data on its effects on certain

clinical out puts such as lung function were disappointing which brought the drug to a halt. However, over time it became clear that Mepolizumab was more effective against other clinical end points particularly exacerbations in severe asthma patients that had high eosinophil levels to begin with. Moreover, a further understanding of asthmas heterogeneity over the years has led to identification of an appropriate target population for example the anti-IgE Xolair was the first drug to give to asthma patients in a stratified way by measuring IgE in the serum and also eosinophils is now a predictive biomarker for Mepolizumab (Pavord et al., 2021). This shows how it is important in identifying subsets of patients that may be more amenable to certain biological treatments and leads on to how this thesis tried to identify subsets of asthma patients based on the inflammatory profiles Th1, Th2 and Th17 as well as levels of targets of existing/new biologics.

6.2.4 Inflammatory signature within asthma patients using sera

Using sera has been studied less extensively to measure cytokines to use as potential biomarkers for tailoring asthma biological drug therapy would be less invasive than sputum and also easier to measure. The serum correlation data showed that many of the type 2 biomarkers were highly correlated with each other suggesting they are in the same mechanistic pathway as each other. In addition, some of the non-type 2 biomarkers including IFN-γ was strongly correlated with the type 2 biomarkers like IL-33 suggesting that the regulation of production of cytokines does not fall into distinct groups such as type 2 or non-type 2. IL-33 is now thought to be an important inducer in Th1 cytokines as IL-33 has been shown to enhance Th1 responses controlled by IFN-γ in conjunction with IL-12. Another study demonstrated evidence for a regulatory loop between IFN-γ and IL-33 in skin inflammation. They showed IFN-γ induced IL-33 in keratinocytes and IL-33 acted on activated CD4+ T cells to further increase the release of IFN-γ which then drove skin inflammation (Seltmann et al., 2013). One study demonstrated this as IL-33 promoted Th1 cell development via myeloid differentiation factor 88 and synergized with IL-12 to enhance ST2 and IL-12R expression in human CD4+ T cells (Komai-Koma et al., 2016). This has made an IL-33 and its receptor ST2 a therapeutic target of interest with several currently in clinical development, as these

studies have highlighted the potential importance of targeting an upstream cytokine which may affect multiple pathways and also disease activity more broadly.

Furthermore, it even proposes that patients may even have their asthma driven by more than one inflammatory pathway, which can also be seen in the overlap of patients in the top 25% for Th1, Th2 and Th17 biomarkers. Even though there was less coordinate expression between the Th1, Th2 and Th17 biomarkers there was still a significant overlap between these different biomarkers (24.4%) demonstrating that their asthma may be driven my multiple pathways and the concept of Th1, Th2 and Th17 is simplistic and does not hold up *in vivo*. The data also suggests that these patients may benefit from combination therapy combination therapy by blocking multiple inflammatory pathways that's driving their asthma. Additional data showed that a high percentage of patients overlapped for multiple biomarkers that are biological targets that are currently in clinic or in phase 3 trials. Moreover, since several cytokines may be elevated simultaneously in the same patient, potentially blocking multiple pathways or cytokines may be a better strategy than biological monoclonal antibodies blocking only one cytokine. This may be why in most clinical trials of anti-IL5 such as Mepolizumab even those the patients meet the criteria for the drug a proportion of patients (~25%) do not respond well to the drug (Drick et al., 2018) therefore these patients may benefit from combination therapy.

Those who are high for IL-33 and TSLP may benefit from anti-IL-33 and Tezepelumab as blocking the two main upstream mediators of the type 2 pathway may be more beneficial at preventing activation of the type 2 pathway and its downstream effects including airway remodelling, increase mucus production and smooth muscle contraction as mentioned previously. Data has also shown Tezepelumab to be effective in both eosinophilic high and low patients (n= 529 assigned the Tezepelumab and n= 532 assigned the placebo), patients with a blood eosinophil count of <300 cells/ µL the annual exacerbation rate was 1.02 (95% CI: 0.84-1.23) with Tezepelumab and 1.73 (95% CI: 1.46-2.05) with placebo (Menzies-Gow et al., 2021). The drug inhibits TSLP binding to its heterodimer receptor therefore blocking a range of

inflammatory cytokines and markers including IgE, IL-4, IL-13 and IL-5 which then impacts a broad range of inflammatory responses including the production and infiltration of eosinophils. This suggests that Tezepelumab could supress multiple inflammatory pathways and so has a broader physiological effects which suggests why those with low eosinophil counts benefited from the drug too.

It is likely that patients with very severe uncontrolled asthma may require therapy that targets multiple pathways however, very little has been researched into combination therapy. One case study of a 55 year old female taking combination of Mepolizumab and Omalizumab for severe eosinophilic (blood eosinophil 400 cells/μL) and allergic asthma (IgE 132 IU/mL) showed in notable improvement after 24 months. Her FEV_1pp reaching normal range from 38% to 96% and her OCS dosage was reduced from 20 mg to 2.5 mg per day, no side effects were observed in this patient (Domingo et al., 2020). For patients who only partially respond to either biologic as well as with the right phenotyping and where there is a biological overlap i.e. high eosinophils and IgE, combination therapy could be considered. However, the efficacy and safety should be considered beforehand for combination treatment as this has yet been studied on a larger scale. The data in this thesis suggests that cytokine profiling in the blood has highlighted significant biological overlap which could indicate that patients with severe asthma uncontrolled asthma may benefit from combination therapy as their asthma may be driven by multiple inflammatory pathways. Most new asthma treatment is focused on single biological therapies, for those patients who asthma remains uncontrolled with high dose ICS and/or OCS they will be ineligible for biological add on therapy due to their phenotyping and inflammation endotype (non-type 2). There are however some alternative treatment to help alleviate their symptoms for example a trial of macrolide therapy (a group of antibiotics) is suggested to help reduce asthma exacerbations in symptomatic uncontrolled asthma patients with and without eosinophilia (Holguin et al., 2020).

Despite having the power to look at whether an inflammatory signature could be identified within different asthma patient groups such as disease severity none of the clinical features that were statistically significant with the type 2 or

non-type 2 biomarkers in the discovery cohort could not be replicated in the replication cohort and even after using all the 708 patients with serum data. For example in the discovery cohort there was elevated serum soluble ST2 levels in patients with severe asthma based on GINA which has previously been seen in other studies (Watanabe et al., 2018) however this could not be replicated in the replication cohort. This was also a similar case when looking at whether the genetic signals in the moderate to severe GWAS drove any of the levels of the type 2 and non-type 2 biomarkers in the sera as very minimal association was also seen between the genetic signals and biomarker levels. This could potentially be due to cytokines and small signalling proteins measured in the serum not being a true reflection of airway inflammation and also because of how heterogeneous asthma is which may have showed no association. At the same time these patients had stable asthma which may be a reason none of the genetic signals and biomarkers were correlated, potentially looking in a cohort of exacerbation prone asthma patients may have shown some association. Despite none of the signals showed to drive any of the biomarker levels to my knowledge this is the first study to look at whether genetic signals from an asthma GWAS drive the levels of type 2 and non-type 2 biomarkers in the sera of asthma patients. However, my data suggests that serum biomarkers are not ideal at identifying subsets of asthma patients and potentially using sputum maybe ideal as it is a more direct measurement of airway inflammation. One study using 59 asthma patients identified a 6-gene (*CLC*, *CPA3*, *DNASE1L3*, *IL1B*, *ALPL*, and *CXCR2*) expression biomarker signature derived from sputum that distinguishes asthma inflammatory phenotypes such as exacerbations and predicting IC treatment response (Baines et al., 2014). Furthermore, Seys et al study identified 5 asthma phenotypes based on sputum cytokine-high expression (sputum mRNA expression levels of that particular cytokine were outside the 90th percentile value of the control group). Their data demonstrated that half of the patients had a classical Th2 “high” sputum cytokine profiling. However when unsupervised cluster analysis was applied, *a priori* classification of type 2 versus non-type 2 molecular phenotype did not show up (Seys et al., 2017). Previous data has demonstrated the heterogeneity in airway inflammation amongst asthma patients and that using cytokine profiling particularly in

sputum maybe more useful in tailoring asthma biological therapy as it will identify what type of inflammation is presented in specific asthma patients.

Overall the data from this thesis and previous data mentioned throughout this thesis has shown how asthma has multiple phenotypes that can be partly identified by using biomarkers such as eosinophils. However, what can be appreciated is the pathology and genetic mechanisms are complex and not completely understood making it challenging to choose suitable biomarkers to stratify patients especially now more biologics are entering into clinic. The use of biomarkers and further understanding of the underlying disease mechanism will also help define the different asthma endotypes. Measuring biomarkers in the sputum, blood, exhaled breath were/are commonly used. Nevertheless, in last few years from the advancement of GWAS studies detecting pathways and omics are being used to understand pathological pathways and even detect new therapy targets. Promising results have been seen so far which have been demonstrated throughout this thesis and ongoing proteomic studies have even identified new molecular factors including IFN-γ, CXCL17 and TNF-β that are elevated in severe asthma patients compared to mild-moderate patients (Kuruvilla et al., 2019). With the multiple biomarkers used by researchers and clinicians each having their own advantages and limitations, most biomarkers are not routinely used because they are not commercially available or are too expensive. This remains a problem as more advanced biomarkers are needed to help accurately detect response to new biologics or ICS/OCS treatment and identify disease endotypes in patients particularly non-type 2 inflammation. The right combination of phenotypes and use biomarkers from either blood, sputum, omics etc. may be the way forward in better understanding the disease and individual personalised medicine.

6.3 Limitations of the Study

This study has several limitations. One limitation is that this thesis is hypothesis driven rather than hypothesis generating as known biomarkers and genetic variants that's has previously been known were measured, rather than identifying new measurable biomarkers for asthma. Secondly, the amount of data for each of the variables are not complete and vary quite significantly, this is due to either missing data, the data may be unavailable for the patient as they may not have had a full blood counts done during their visit or they unwilling to have it done. Skin prick data to define atopy was not available for the majority of the patients. Also GASP was a consortium to bring together existing cohorts and so by design will have variable measures on each patient.

A third limitation was that OCS and ICS were not well recorded for each patient and so could not be taken into account in the analyses however, in the small subset of asthma patients only showed soluble ST2 levels to be influenced by OCS. Also due to the lack of ICS and OCS dosage data it was not possible to correctly categorise patients into either GINA 3 or 4 so these asthma patients were combined together to form a moderate to severe asthma group. Additionally, the GASP is predominately moderate to severe asthma cohort and so there was a lack of GINA 1 and 2 patients. Therefore, both GINA 1 and 2 groups were joined together and classified as a mild/moderate asthma group for the serum part of the study. In addition to this, it was difficult to reproduce an exact replication of the discovery cohort due to low numbers of mild/moderate asthma patients out of the whole serum cohort.

Some of the biomarkers were difficult to measure in the serum as they fell below the standard curve particularly IL-5 and IL-13. This has been previously reported in other studies that these cytokines are difficult to measure due to multiple factors such as cytokines having short half-lives and drug dosage having an effect on their levels particularly steroids (Aziz, 2015). A study exploring the relationship between neutrophils and 244 biomarkers of refractory asthma (refractory asthma n=60 and non-refractory asthma n=30) using a multiplex assay, were unable to detect 29 out of the 244 biomarkers including IL-4, IL-5 and IL-13 in the serum (Alam et al., 2017). This suggests that a more sensitive assay maybe needed to detect the low levels of the

cytokines in the serum. Using a more sensitive Luminex assay to measure the cytokines in the patients that fell below the standard curve could identify a clinical subgroup of asthma patients. Additionally, a few biomarkers could also not be read and that was due to factors such as aggregation of the beads or insufficient bead count causing a particular biomarker not to be read by the bioplex machine.

The final limitation is that serum is not necessarily a direct measurement of the airways and so these levels may not directly reflect inflammation of the airways. Having a subset of patients with sputum samples as well would have established whether there are any differences in biomarker levels in the serum and sputum. One study that investigated serum and sputum cytokine profiles in asthma and COPD patients indicated that inflammatory mediators in the sputum were more significant than those observed in the serum (Cao et al., 2012). This suggests that sputum samples may be more sensitive and representative than serum for examination of the airways for inflammatory response as it is a more direct measurement. The limitation to using sputum however is, it is a more invasive procedure and also some assays require a large volume of sputum sample which may not be possible for some patients to produce a large sample size. This study also had a lack of eosinophil data and so it was not possible to look at the effects of eosinophil levels with the serum biomarker data in the asthma patients. Moreover, there was very small sample size when looking at the correlations between the immune cells and biomarkers and so it only gave small statistical significance between a few of them and so it cannot be said with certainty that these findings are correct. Having a larger sample size may give a better indication of how the immune cells and biomarkers are correlated with each other. However, if these findings are true their level of disconnect could be from the cytokines degrading during blood processing or the cytokines are being released elsewhere for example, IL-33 has been shown to be upregulated by endothelial cells and also involved in angiogenesis (Fahey and Doyle, 2019).

6.4 Future Directions

Another way to identify subsets of asthma patients and identify patients with a specific inflammatory profile would be to look at these biomarkers in the sputum of asthma patients as well as the serum to see how the results will both compare and whether they correlated with each other. This would give a better indication to see whether using sera would be more useful in measuring biomarkers if the biomarker levels would match the sputum levels. However, a more sensitive Luminex assay would be used to detect the levels of IL-5 and IL-13 in those patients that fell below the standard curve. This would then allow me to compare those who are “high” and “low” for the serum biomarker related the Th1, Th2 and Th17 inflammation. As mentioned previously using sputum would give a more direct reflection of airway inflammation and previous sputum studies have already identified cytokine clustering with mRNA expression levels (Seys et al., 2017). Furthermore, using sputum biomarker data may even give a better indication to whether the signals identified in the moderate to severe asthma GWAS drive the expression of these biomarkers in the sputum.

Another way identify patients with a specific inflammatory profile would be to use analyse similar panel of cytokines in the sputum and/or blood with real-time PCR or exploratory RNA-sequencing to look at their mRNA expression in certain cell populations such as macrophages, neutrophils, CD4 cells etc. and carryout a similar analysis to this thesis. Additionally, running a cluster analysis to identify different inflammatory profiles in these patients. This is technique may be more advantageous as it is more exploratory due to the fact it does not make any distinction between dependent and independent variables but instead it discovers patterns in data without providing an explanation or interpretation.

This mRNA data would also allow to look at more specific questions such as exacerbation prone patients versus those that do not exacerbate or childhood onset versus adult onset of asthma to see how they differ on an mRNA level.

Also looking at biomarker levels over the course of the disease from onset longitudinally and look how the levels different when the patient is stable verses when exacerbating.

Finally, having a data set of patients who are on different biological therapies to see how their cytokine levels differs between those who are not on biological therapies. It could also tell us which other cytokines or small signalling protein are being targeted downstream by these new biologics. Additionally, having patients with cytokine data before and after biological therapy could also give an indication of how these patients respond to the drugs on a cytokine and clinical level.

6.5 Final Conclusion

The data from this thesis adds further to the field proving additional evidence of asthma heterogeneity as many of the clinical features identified from the sub-phenotype groups in this thesis have previously been recognised in other asthma cohorts and studies (Fitzpatrick et al., 2011) (Fleming et al., 2015) (Suruki et al., 2017), (Ortega et al., 2018) (Uematsu et al., 2018). The genetic signals from the moderate-severe asthma GWAS demonstrated the genetic risk factors for moderate-severe asthma may drive type 2 clinical phenotypes of asthma with many of the potential candidate casual genes being involved in type 2 inflammation. Several of these genes have previously been associated with some of the clinical features identified in this thesis. The data suggested serum biomarkers may not be useful at identifying asthma clinical phenotypes and the genetic risk factors for the development of moderate-severe asthma did not drive serum biomarker levels. Despite the signals not driving serum biomarker levels this data is novel and new to the field of asthma. Serum biomarkers also demonstrated asthmas heterogeneity on an inflammatory profile level as seen in previous studies (Seys et al., 2017) (Akiki et al., 2017). Many biomarkers showed correlated expression with each other suggesting these cytokines and signalling proteins may be produced by the same inflammatory cells in the blood and suggest maybe a patient's asthma is driven by more than one inflammatory pathway. Additionally, a large proportion of patients overlapped for Th1, Th2 and Th17 cytokines and similar for those high in targets for biologics suggesting pathways may interact with each other or even have a common upstream driver. Therefore, targeting upstream may be more beneficial for these patients or even combination therapy in very severe asthma cases. Most of the biomarkers were measurable in the sera and showed their utility in measuring a patient's inflammatory profile which could be useful in guiding asthma treatment and identifying who may benefit from new biologics than using blood eosinophils since levels fluctuate throughout the day (Tavernier et al., 2018) (Durrington et al., 2018).

In summary, this thesis adds further to the scientific field demonstrating asthma heterogeneity and how it is driven by multiple overlapping clinical and

immunological features. Using genetic and clinical phenotype data has shown that many of the candidate causal genes are involved in type 2 inflammatory pathway which reflect typical type 2 clinical phenotype. Using a patient's genetic profile and phenotype seems to provide a greater scope and insight and may be the way forward to better understanding the disease for stratified medicine for asthma.

7 Appendices

7.1 Appendix 1: Chapter 2

7.1.1 Demographics and clinical features from a cohort of control patients

Subjects (n)	**823**
Age seen (years: Mean & S.D)	48.1 (8.32) (n= 823)
Gender (n): **Female**	823 436 (53%)
Height (m: Mean & S.D)	1.69 (0.09) (n=823)
Weight (kg: Mean & S.D)	75.22 (14.49) (n=823)
BMI (Kg/m^2: Median & Min-Max)	25.79 (18.11-44.25) (n=823)
Smoking status (n): **Never** **Ex-Smoker** **Current**	823 486 (59.1%) 188 (22.8%) 149 (18.1%)
Smoking Pack Years (Median & Min-Max)	12 (0.05-217.50) (n=304)
FEV_1FVC (Mean & S.D)	0.79 (0.05) (n=822)
Blood total IgE (KU/L: Median & Min-Max)	28.0 (1.41-2001.0) (n=746)

7.1.1 Demographics and clinical features from a cohort of control patients (n= 823): Data is presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%).FEV_1/FVC: forced expiratory volume in 1 second / forced vital capacity ratio; IgE: immunoglobulin E.

7.2 Appendix 2: Chapter 3

7.2.1 Distribution of the biomarkers for the discovery cohort

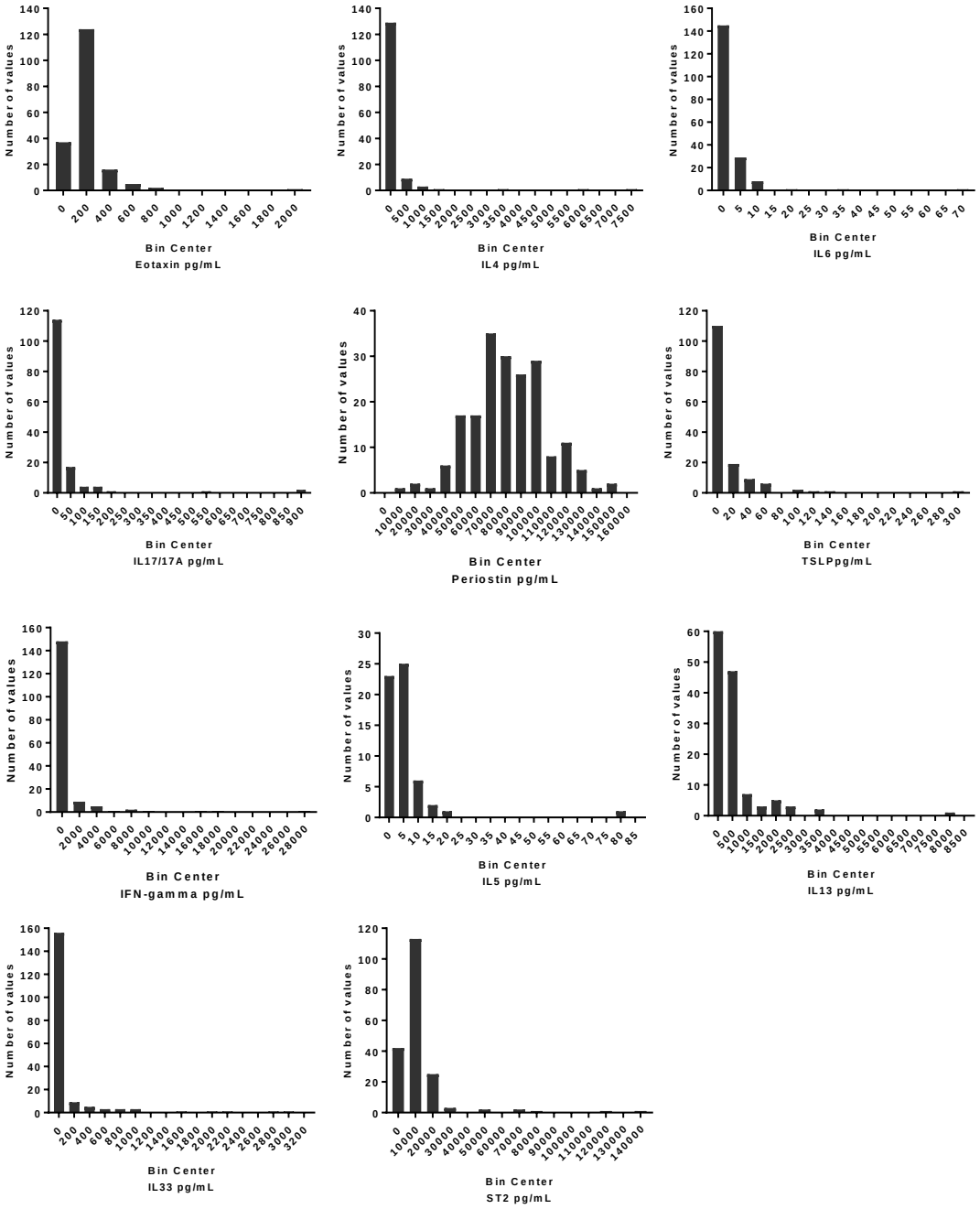


Figure 7.2.1 Distributions of the biomarker data from the discovery cohort: the x axis displays the biomarker concentration in groupings (bin) and the y axis displays the number of values in each bin grouping. Most of the biomarkers display a non-parametric distribution and so will be transformed prior analysis.

7.2.2 Luminex biomarker results for the controls

Analyte	Amount measured (incl. subjects below standard curve)	Amount measured (excl. subjects below standard curve)	Median Pg/mL	Min-max
Eotaxin	21	21	142.02	59.72-75707.47
IL-4	20	18	345.63	21.9-4391.99
IL-6	18	17	2.44	0.25-65.53
IL17/17A	21	20	70.27	2.06-758807.77
Periostin	24	24	23761.66	23761.66-140860.1
TSLP	21	19	8.87	0.14-166.34
IFN-gamma	20	18	189.67	1.75-16942.61
IL-5	15	13	3.96	0.54-110.19
IL-13	21	18	561.25	209.21-1774.51
IL-33	20	19	29.47	1.85-2456.16
ST2	22	21	7295.48	2827.32-316695.5

Table 7.2.2 Control Luminex Results N=24: Data is displayed as the median and the minimum-maximum (min-max) or standard deviation for each analyte in those subject that fell within the standard range.

7.2.3 Correlations coefficients between biomarkers in the discovery cohort

Biomarkers	N	R^2	P-value
Eotaxin & IL-4	187	0.234	<0.0001
Eotaxin & IL-6	187	0.084	<0.0001
Eotaxin & IL17A	186	0.242	<0.0001
Eotaxin & Periostin	187	0.006	0.306
Eotaxin & TSLP	179	0.312	<0.0001
Eotaxin & IFN-γ	187	0.139	<0.0001
Eotaxin & IL-5	186	0.210	<0.0001
Eotaxin & IL-33	181	0.229	<0.0001
Eotaxin & ST2	186	0.161	<0.0001
Eotaxin & IL-13	160	0.194	<0.0001
IL-4 & IL-6	187	0.162	<0.0001
IL-4 & IL17A	**186**	**0.529**	**<0.0001**
IL-4 & Periostin	187	0.038	0.008
IL-4 & TSLP	179	0.462	<0.0001
IL-4 & IFN-γ	187	0.377	<0.0001
IL-4 & IL-5	186	0.370	<0.0001
IL-4 & IL-33	181	0.469	<0.0001
IL-4 & ST2	186	0.003	0.425
IL-4 & IL-13	160	0.235	<0.0001
IL-6 & IL17A	186	0.096	<0.0001
IL-6 & Periostin	191	0.025	0.029
IL-6 & TSLP	183	0.157	<0.0001
IL-6 & IFN-γ	187	0.130	<0.0001
IL-6 & IL-5	190	0.212	<0.0001
IL-6 & IL-33	185	0.209	<0.0001
IL-6 & ST2	190	0.072	<0.0001
IL-6 & IL-13	162	0.097	<0.0001
IL17A & Periostin	186	0.028	0.024
IL17A & TSLP	178	0.287	<0.0001
IL17A & IFN-γ	186	0.171	<0.0001
IL17A & IL-5	185	0.264	<0.0001
IL17A & IL-33	180	0.239	<0.0001
IL17A & ST2	185	0.013	0.130
IL17A & IL-13	159	0.138	<0.0001
Periostin & TSLP	183	0.013	0.117
Periostin & IFN-γ	187	0.025	0.031
Periostin & IL-5	190	0.020	0.051
Periostin & IL-33	185	0.020	0.056
Periostin & ST2	190	0.004	0.389
Periostin & IL-13	162	0.034	0.019
TSLP & IFN-γ	179	0.402	<0.0001
TSLP & IL-5	**183**	**0.516**	**<0.0001**
TSLP & IL-33	**179**	**0.570**	**<0.0001**
TSLP & ST2	182	0.030	0.020
TSLP & IL-13	154	0.437	<0.0001
IFN-γ & IL-5	186	0.259	<0.0001
IFN-γ & IL-33	**181**	**0.769**	**<0.0001**
IFN-γ & ST2	186	0.013	0.127
IFN-γ & IL-13	160	0.287	<0.0001
IL-5 & IL-33	185	0.392	<0.0001
IL-5 & ST2	189	0.017	0.075
IL-5 & IL-13	161	0.377	<0.0001
IL-33 & ST2	185	0.074	<0.0001
IL-33 & IL-13	156	0.299	<0.0001
ST2 & IL-13	161	0.006	0.320

Table 7.2.3 Correlation coefficients between the biomarkers for the discovery cohort n=191: Data shown is the N (number), R^2 value and the p-value between all 11 biomarkers. Bonferroni correction p=0.0004

7.2.4 Relationship between the biomarkers and clinical features of asthma in the discovery cohort

Biomarker	GINA 1&2	GINA 3&4	GINA 5	P-value
Eotaxin	2.18 (0.37) N=57	2.12 (0.33) N=63	2.25 (0.24) N=65	0.055
IL-4	1.81 (0.73) N=45	1.48 (0.61) N=49	1.52 (0.64) N=51	0.079
IL-6	0.78 (0.58) N=60	0.03 (0.47) N=61	0.87 (0.49) N=64	0.824
IL17/17A	0.89 (0.88) N=46	0.50 (0.82) N=48	0.57 (0.65) N=49	**0.043**
Periostin	81742.98 (25895.46) N=61	80209.21 (23330.31) N=65	82309.85 (24754.67) N=65	0.882
TSLP	0.85 (0.71) N=45	0.58 (0.57) N=46	0.36 (0.84) N=58	**0.022**
IFN-gamma	2.08 (1.0) N=53	1.93 (0.79) N=57	2.02 (0.86) N=59	0.590
IL-5	0.63 (0.51) N=22	0.41 (0.42) N=16	0.43 (0.37) N=20	0.221
IL-13	2.49 (0.53) N=38	2.53 (0.42) N=45	2.40 (0.42) N=45	0.483
IL-33	1.35 (0.97) N=59	1.09 (0.71) N=63	1.26 (0.79) N=62	0.196
ST2	3.94 (0.35) N=61	3.78 (0.27) N=64	4.03 (0.33) N=65	**$7.4x10^{-6}$**

Table 7.2.4A Relationship between the biomarkers across GINA severity in the discovery cohort n=191: all biomarkers except periostin were transformed to the log_{10} prior analysis. A univariate linear model was used to analyse the data and the biomarkers highlighted from table 3.4.4 were adjusted for age, gender or both as well. The data is presented as mean, standard deviation, number (n) along with the p-value from the univariate model. IL17/17A, TSLP and ST2 were statistically significant p= <0.05.

Biomarker	Never Hospital Admission	Ever Hospital Admission	P-value
Eotaxin	2.19 (0.25) N=120	2.17 (0.40) N=62	0.953
IL-4	1.60 (0.68) N=95	1.56 (0.65) N=48	0.622
IL-6	0.03 (0.50) N=121	0.17 (0.53) N=61	0.075
IL17/17A	0.62 (0.77) N=96	0.67 (0.84) N=44	0.706
Periostin	81255.51 (24177.81) N=126	81613.01 (25861.09) N=62	0.926
TSLP	0.55 (0.74) N=104	0.63 (0.77) N=44	0.511
IFN-gamma	2.02 (0.84) N=108	1.98 (0.97) N=59	0.607
IL-5	0.50 (0.41) N=39	0.52 (0.54) N=18	0.883
IL-13	2.48 (0.44) N=90	2.44 (0.49) N=37	0.835
IL-33	1.22 (0.81) N=121	1.25 (0.87) N=60	0.824
ST2	3.88 (0.31) N=125	3.99 (0.37) N=62	**0.012**

Table 7.2.4B Relationship between the biomarkers with hospital admissions in the discovery cohort n=191: all biomarkers except periostin were transformed to the $\log_{10}$ prior analysis. A univariate linear model was used to analyse the data and the biomarkers highlighted from table 3.4.4 were adjusted for age, gender or both as well. The data is presented as mean, standard deviation, number (n) along with the p-value from the univariate model. ST2 was statistically significant with GINA p= <0.05.

	Lung function			
Biomarker	**FEV1pp**	**P-value**	**FEV1/FVC**	**P-value**
Eotaxin	-0.095 N=179	0.198	-0.079 N=177	0.778
IL-4	-0.036 N=139	0.388	0.029 N=138	0.774
IL-6	-0.056 N=185	**0.001**	-0.029 N=176	**0.023**
IL17/17A	-0.008 N=139	0.279	-0.0006 N=137	0.772
Periostin	-0.002 N=185	0.537	-0.016 N=182	0.088
TSLP	0.074 N=145	0.661	0.069 N=143	0.637
IFN-gamma	-0.023 N=163	0.766	-0.02 N=161	0.980
IL-5	-0.03 N=56	0.199	-0.015 N=54	0.381
IL-13	-0.081 N=124	0.384	-0.073 N=122	0.565
IL-33	0.001 N=178	0.668	0.0003 N=175	0.809
ST2	-0.061 N=184	0.603	-0.069 N=181	0.750

Table 7.2.4C Relationship between the biomarkers with lung function (FEV_1pp and FEV_1/FVC) in the discovery cohort n=191: all biomarkers except periostin were transformed to the log_{10} prior analysis. A regression linear model was used to analyse the data and the biomarkers highlighted from table 3.4.4 were adjusted for age, gender or both as well. The data is presented as R^2, number (n) along with the p-value from the univariate model. IL-6 was statistically significant with both FEV_1pp and FEV_1/FVC p= <0.05.

Biomarker	ACQ	P-value
Eotaxin	0.097 N=177	0.189
IL-4	0.046 N=137	0.168
IL-6	0.049 N=176	**0.003**
IL17/17A	0.036 N=137	**0.026**
Periostin	0.005 N=182	0.321
TSLP	-0.077 N=142	0.993
IFN-gamma	-0.025 N=160	0.661
IL-5	0.021 N=55	0.289
IL-13	0.099 N=123	0.086
IL-33	-0.002 N=175	0.529
ST2	0.058 N=181	0.585

Table 7.2.4D Relationship between the biomarkers ACQ score in the discovery cohort n=191: all biomarkers except periostin were transformed to the $\log_{10}$ prior analysis. A regression linear model was used to analyse the data and the biomarkers highlighted from table 3.4.4 were adjusted for age, gender or both as well. The data is presented as R^2, number (n) along with the p-value from the univariate model. IL-6 and IL17/17A were statistically significant with ACQ score p= <0.05.

7.2.5 Distribution of biomarkers for the replication cohort

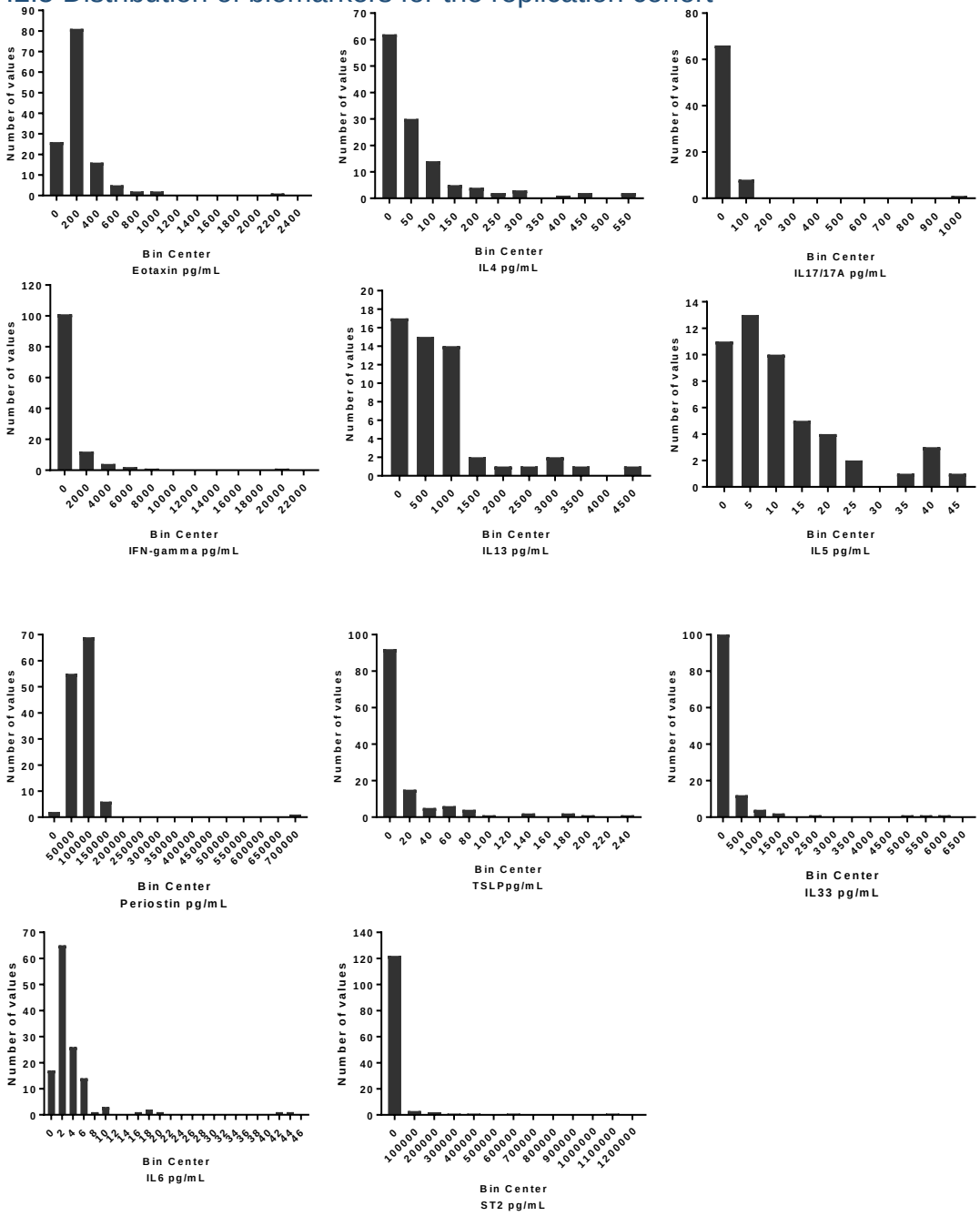


Figure 7.2.5 Distributions of the biomarker data from the replication cohort: the x axis displays the biomarker concentration in groupings (bin) and the y axis displays the number of values in each bin grouping. Most of the biomarkers display a non-parametric distribution and so will be transformed prior analysis.

7.2.6 Correlations coefficients between biomarkers in the replication cohort

Biomarkers	N	R^2	p-value
Eotaxin & IL-4	133	0.424	<0.0001
Eotaxin & IL-6	132	0.275	<0.0001
Eotaxin & IL17A	132	0.269	<0.0001
Eotaxin & Periostin	133	0.050	0.010
Eotaxin & TSLP	131	0.352	<0.0001
Eotaxin & IFN-γ	122	0.247	<0.0001
Eotaxin & IL-5	132	0.434	<0.0001
Eotaxin & IL-33	123	0.272	<0.0001
Eotaxin & ST2	131	0.139	<0.0001
Eotaxin & IL-13	121	0.340	<0.0001
IL-4 & IL-6	132	0.497	<0.0001
IL-4 & IL17A	132	0.457	<0.0001
IL-4 & Periostin	133	0.006	0.375
IL-4 & TSLP	**131**	**0.783**	**<0.0001**
IL-4 & IFN-γ	**122**	**0.590**	**<0.0001**
IL-4 & IL-5	**123**	**0.598**	**<0.0001**
IL-4 & IL-33	**123**	**0.572**	**<0.0001**
IL-4 & ST2	131	0.044	0.016
IL-4 & IL-13	**121**	**0.621**	<0.0001
IL-6 & IL17A	**131**	**0.368**	**<0.0001**
IL-6 & Periostin	132	0.003	0.506
IL-6 & TSLP	130	0.490	<0.0001
IL-6 & IFN-γ	122	0.296	<0.0001
IL-6 & IL-5	131	0.403	<0.0001
IL-6 & IL-33	122	0.284	<0.0001
IL-6 & ST2	130	0.021	0.099
IL-6 & IL-13	120	0.411	<0.0001
IL17A & Periostin	132	0.0001	0.916
IL17A & TSLP	**130**	**0.542**	**<0.0001**
IL17A & IFN-γ	121	0.257	<0.0001
IL17A & IL-5	**131**	**0.576**	**<0.0001**
IL17A & IL-33	122	0.242	<0.0001
IL17A & ST2	130	0.018	0.128
IL17A & IL-13	120	0.375	<0.0001
Periostin & TSLP	131	0.001	0.764
Periostin & IFN-γ	122	0.004	0.469
Periostin & IL-5	132	0.007	0.333
Periostin & IL-33	123	0.003	0.563
Periostin & ST2	131	0.0003	0.846
Periostin & IL-13	121	0.016	0.173
TSLP & IFN-γ	**121**	**0.573**	**<0.0001**
TSLP & IL-5	**131**	**0.627**	**<0.0001**
TSLP & IL-33	**122**	**0.554**	**<0.0001**
TSLP & ST2	129	0.052	0.010
TSLP & IL-13	**119**	**0.612**	**<0.0001**
IFN-γ & IL-5	122	0.375	<0.0001
IFN-γ & IL-33	**120**	**0.958**	**<0.0001**
IFN-γ & ST2	122	0.038	0.032
IFN-γ & IL-13	111	0.437	<0.0001
IL-5 & IL-33	123	0.366	<0.0001
IL-5 & ST2	130	0.019	0.120
IL-5 & IL-13	120	0.593	<0.0001
IL-33 & ST2	123	0.100	<0.0001
IL-33 & IL-13	112	0.432	<0.0001
ST2 & IL-13	119	0.049	0.016

Table 7.2.6 Correlation coefficients between the biomarkers for the replication cohort n=133: Data shown is the number (n) R^2 value and the p-value between all 11 biomarkers. Bonferroni correction p=0.0004

7.2.7 Relationship between the biomarkers and clinical features of asthma found in the discovery cohort repeated in the replication cohort

Biomarker	GINA 1&2	GINA 3&4	GINA 5	P-value
IL17/17A	0.89 (0.84) N=9	0.68 (0.73) N=32	0.55 (0.68) N=34	0.439
TSLP	0.63 (1.13) N=14	0.65 (0.78) N=59	0.51 (0.77) N=55	0.748
Soluble ST2	3.96 (0.32) N=15	4.06 (0.47) N=57	4.10 (0.40) N=57	0.505

Table 7.2.7A Relationship between the IL17/17A, TSLP and soluble ST2 across GINA severity could not replicated in the replication cohort: the biomarkers were transformed to the log_{10} prior analysis. A univariate linear model was used to analyse the data and the biomarkers highlighted from table 3.4.8 were adjusted for age, gender or both as well. The data is presented as mean, standard deviation, number (n) along with the p-value from the univariate model. IL17/17A, TSLP and soluble ST2 were not statistically significant with GINA p= <0.05.

Biomarker	Never Hospital Admission	Ever Hospital Admission	P-value
Soluble ST2	4.08 (0.48) N=62	4.03 (0.35) N=56	0.434

Table 7.2.7B Relationship between soluble ST2 with hospital admissions could not be replicated in the replication cohort: soluble ST2 was transformed to the log_{10} prior analysis. A univariate linear model was used to analyse the soluble ST2 and hospital admissions and was also adjusted for gender. The data is presented as mean, standard deviation, number (n) along with the p-value from the univariate model. Soluble ST2 was not statistically significant with hospital admissions p= <0.05.

	Lung function			
Biomarker	FEV1pp	P-value	FEV1/FVC	P-value
IL-6	-0.038 N=123	0.372	-0.035 N=114	0.497

Table 7.2.7C Relationship between the IL-6 and FEV_1pp and FEV_1/FVC could not be replicated in the replication cohort: IL-6 was transformed to the log_{10} prior analysis. A regression linear model was used to analyse IL-6 and FEV_1pp and FEV_1/FVC and was also adjusted for gender. The data is presented as R^2, number (n) along with the p-value from the univariate model. IL-6 was not statistically significant with both FEV_1pp and FEV_1/FVC p= <0.05.

Biomarker	ACQ	P-value
IL-6	0.023 N=96	0.329
IL17/17A	-0.134 N=58	0.317

Table 7.2.7D Relationship between IL-6 and IL17/17 and ACQ score could not be replicated in the replication cohort: IL-6 and IL17/17A were transformed to the log_{10} prior analysis. A regression linear model was used to analyse the data and IL-6 was adjusted for gender. The data is presented as R^2, number (n) along with the p-value from the univariate model. IL-6 and IL17/17A were not statistically significant with ACQ score p= <0.05.

7.2.8 Proclaim cohort demographics

	Proclaim Cohort
Subjects (n)	42
Age seen (years: Mean & S.D)	51 (15.6) N=42
Age of onset (years: Mean & S.D)	29.02 (23.0)
Gender (n): **Female**	26 (61.9%) N=42
Smoking status (n): **Never** **Ex-Smoker** **Current**	42 18 (42.9%) 20 (47.6%) 4 (9.5%)
Smoking Pack Years (Median & Min-Max)	17.18 (0.25-52.50) N=24
FEV_1pp (Mean & S.D)	64.26 (21.17) N=34
FEV_1FVC (Mean & S.D)	0.67 (0.12) N=34
ACQ7 score (Mean & S.D)	3.51 (1.17) N=33

Table 7.2.8 Demographics of the Proclaim cohort n=42: Data is presented as mean & standard deviation (S.D), median & minimum and maximum (Min-Max) or Number (n) & percentage (%). FEV1pp: forced expiratory volume in 1 s percentage predicted; FEV_1/FVC: forced expiratory volume in 1 second/forced vital capacity ratio; and ACQ: asthma control questionnaire.

7.2.9 Evaluating age and gender as confounders for the Proclaim cohort

Biomarker	N	Age (p-value)	Gender (p-value)
Eotaxin	42	0.391	0.551
IL-4	25	**0.034**	0.765
IL-6	40	0.315	0.732
IL17/17A	24	0.151	0.551
Periostin	42	0.576	0.684
TSLP	32	**0.049**	0.756
IFN-gamma	35	0.367	0.511
IL-5	7	0.908	0.949
IL-13	10	0.178	0.986
IL-33	41	0.719	0.145
ST2	42	**0.003**	0.688

Table 7.2.9 Confounders for the 11 biomarkers for the Proclaim cohort: age and gender were added to a univariate linear model to see if they were potential confounders. Data is presented as number (N) and p-value for each confounder from the statistical model, p-value of less than 0.05 is a confounder for that particular biomarker.

7.2.10 Relationship between ICS and OCS with the biomarkers from the Proclaim cohort

Biomarker pg/mL	Not on ICS (n=28)	Taking ICS (n=14)	P-value
IL-4 (Log_{10})	1.25 (0.42) N=16	1.21 (0.42) N=9	0.811
IL-6 (Log_{10})	0.28 (0.37) N=27	0.11 (0.33) N=13	0.161
IL17A (Log_{10})	0.09 (0.53) N=15	-0.22 (0.43) N=9	0.158
TSLP (Log_{10})	-0.03 (0.61) N=22	0.22 (0.40) N=10	0.160
IFN-gamma (Log_{10})	1.71 (0.56) N=24	1.91 (0.78) N=11	0.406
IL-5 (Log_{10})	0.52 (0.55) N=6	0.46 N=1	0.934
IL-13 (Log_{10})	2.43 (0.36) N=8	2.53 (0.02) N=2	0.736
IL-33 (Log_{10})	1.05 (0.68) N=27	0.95 (1.10) N=14	0.736
Soluble ST2 (Log_{10})	4.06 (0.20) N=28	4.09 (0.22) N=14	0.979
Eotaxin	199.10 (100.37) N=28	185.11 (73.63) N=14	0.647
Periostin	73872.09 (20082.87) N=28	76360.31 (16676.83) N=14	0.692

Table 7.2.10A Relationship between ICS and the biomarkers n=42: some of the biomarker data was transformed to the log_{10} prior analysis and a univariate linear model was used to analyse the data and the biomarkers highlighted from table 7.2.9 were adjusted for age. The data is presented as mean, standard deviation, number (n) along with the p-value from the univariate model. No of the biomarkers met statistical significance with ICS p= <0.05.

Biomarker pg/mL	Not on OCS (n=14)	Taking OCS (n=28)	P-value
IL-4 (Log_{10})	1.05 (0.52) N=8	1.33 (0.33) N=17	0.162
IL-6 (Log_{10})	0.24 (0.21) N=12	0.21 (0.41) N=28	0.830
IL17A (Log_{10})	0.02 (0.49) N=5	-0.04 (0.53) N=19	0.824
TSLP (Log_{10})	-0.12 (0.51) N=9	0.12 (0.57) N=23	0.273
IFN-gamma (Log_{10})	1.74 (0.69) N=10	1.79 (0.62) N=25	0.838
IL-5 (Log_{10})	0.46 N=1	0.52 (0.55) N=6	0.934
IL-13 (Log_{10})	2.18 (0.25) N=3	2.57 (0.28) N=7	0.068
IL-33 (Log_{10})	0.96 (0.82) N=13	1.04 (0.86) N=28	0.790
Soluble ST2 (Log_{10})	3.96 (0.19) N=14	4.13 (0.19) N=28	**0.024**
Eotaxin	162.12 (89.71) N=14	210.59 (89.81) N=28	0.107
Periostin	71721.03 (24180.52) N=14	76191.74 (15841.07) N=28	0.475

Table 7.2.10B Relationship between OCS and the biomarkers n=42: some of the biomarker data was transformed to the log_{10} prior analysis and a univariate linear model was used to analyse the data and the biomarkers highlighted from table 7.2.9 were adjusted for age. The data is presented as mean, standard deviation, number (n) along with the p-value from the univariate model. Soluble ST2 was the only biomarker meeting statistical significance with OCS p= <0.05.

	OCS (sqrt) mg		
	N	**R^2 (n)**	**P-value**
Soluble ST2 (Log_{10}) pg/mL	28	0.308	0.751

Table 7.2.10C Correlation between soluble ST2 and OCS dosage: OCS dosage was transformed to the square root (sqrt) prior analysis. Soluble ST2 and OCS dosage was analysed using a linear regression model with soluble ST2 being adjusted for age. The data is presented as number (N), correlation coefficient (R^2) and the p-value from linear regression model. Soluble ST2 was not statistical significant with OCS dosage p= <0.05.

7.2.11 Relationship between the biomarkers and immune cells in the Proclaim cohort

	Neutrophils 10^9/L (Log_{10})	Lymphocytes 10^9/L (Log_{10})	Monocytes 10^9/L (Log_{10})	Eosinophils 10^9/L (Log_{10})	Basophils 10^9/L (Log_{10})
Eotaxin pg/mL (Log_{10})	R^2= 0.068 P= 0.095 N= 42	R^2= 0.077 P= 0.075 N= 42	R^2= 0.021 P= 0.364 N= 42	R^2= 0.045 P= 0.211 N= 39	R^2= 0.052 P= 0.158 N= 40
IL-4 pg/mL (Log_{10})	R^2= 0.259 P= 0.170 N= 25	R^2= 0.216 P= 0.409 N= 25	R^2= -0.201 P= 0.609 N= 25	**R^2= 0.281 P= 0.048 N= 22**	R^2= 0.314 P= 0.091 N= 23
IL-6 pg/mL (Log_{10})	R^2= 0.026 P= 0.317 N= 40	R^2= 0.006 P= 0.629 N= 40	R^2=0.089 P=0.061 N= 40	R^2=0.038 P=0.247 N=37	R^2=0.0773 P=0.091 N=38
IL17A pg/mL (Log_{10})	R^2= 0.01 P= 0.642 N= 24	**R^2= -0.219 P= 0.021 N= 24**	R^2= 0.005 P= 0.735 N= 24	R^2= 0.058 P= 0.296 N= 21	R^2= 0.007 P= 0.712 N= 23
TSLP pg/mL (Log_{10})	R^2= 0.188 P= 0.196 N= 32	R^2= -0.196 P= 0.162 N= 32	R^2= -0.242 P= 0.056 N= 32	R^2= 0.093 P= 0.212 N= 29	R^2= 0.233 P= 0.102 N= 30
IFN-gamma pg/mL (Log_{10})	R^2= 0.001 P= 0.878 N= 35	R^2= -0.048 P= 0.207 N= 35	R^2 =-0.017 P =0.452 N= 35	R^2= 0.112 P= 0.061 N= 32	R^2= 0.044 P= 0.241 N= 33
IL-5 pg/mL (Log_{10})	**R^2= 0.566 P= 0.051 N= 7**	R^2= -0.093 P= 0.506 N= 7	R^2= 0.003 P= 0.902 N= 7	R^2= 0.001 P= 0.945 N= 6	R^2= 0.108 P= 0.472 N= 7
IL-13 pg/mL (Log_{10})	R^2= 0.291 P= 0.108 N= 10	R^2= -0.105 P= 0.361 N= 10	R^2 =0.081 P= 0.427 N= 10	R^2= 0.009 P= 0.822 N= 8	R^2 =0.303 P= 0.100 N= 10
IL-33 pg/mL (Log_{10})	R^2= 0.014 P= 0.462 N= 41	R^2= -0.01 P= 0.532 N= 41	R^2= -0.001 P= 0.814 N= 41	R^2= 0.05 P= 0.176 N= 38	R^2 =0.058 P= 0.140 N= 39
Periostin pg/mL	R^2= -0.046 P= 0.173 N= 42	R^2= 0.005 P= 0.673 N= 42	R^2= -0.003 P= 0.727 N= 42	R^2= -0.012 P= 0.501 N= 39	R^2= -0.011 P= 0.514 N= 40
Soluble ST2 pg/mL (Log_{10})	R^2= 0.227 P= 0.498 N= 42	R^2= 0.223 P= 0.627 N= 42	R^2= 0.218 P= 0.975 N= 42	R^2= -0.236 P= 0.571 N= 40	**R^2= 0.375 P= 0.012 N= 40**

Table 7.2.11 Correlations between the biomarkers and immune cells in the Proclaim cohort n=42: the immune cell data some of the biomarker data was transformed to the log_{10} prior analysis. A linear regression model was used to analyse the data and the biomarkers highlighted from table 7.2.9 were adjusted for age. The data is presented as R^2, p-value from the linear regression model and the number (n). The data highlighted in bold met statistical significance p= <0.05.

7.2.12 Luminex kit lot number

Kit Lot Number	Analytes Measured
L128076	IL-13, IL-6, TSLP, IL-5, IL-33, Periostin, ST2, IL-4, IL17/IL17A (IL17A), IFN-γ and CCL11/Eotaxin (Eotaxin)
L123941	IL-13, IL-6, TSLP, IL-5, IL-33, Periostin and ST2
L128077	IL-4, IL17/IL17A (IL17A), IFN-γ and CCL11/Eotaxin (Eotaxin)

Table 7.3.12 Analytes measured in each Luminex kit

7.2.13 Reconstitution volume for each Luminex kit

Standard	Lot No	Reconstitution Volume
J	1496225	0.25 ml
A	1526368	0.2 ml
B	1511314	0.25 ml
G	1517060	0.25 ml
C	1509768	0.225 ml
4	1457378	0.2 ml

Table 7.2.13a the reconstitution volume for the 6 standards for the Luminex kit lot Number L128076 human assay: each standard cocktail was reconstituted with Calibrator Diluent RD6-52.

Standard	Lot No	Reconstitution Volume
G	1473632	0.25 ml
C	1487330	0.25 ml
B	1491801	0.25 ml
A	1493203	0.225 ml
4	1457378	0.2 ml

Table 7.2.13b the reconstitution volume for the 5 standards for the Luminex kit lot Number L123941 human assay: each standard cocktail was reconstituted with Calibrator Diluent RD6-52.

Standard	Lot No	Reconstitution Volume
J	1496225	0.25 ml
A	1526368	0.2 ml
B	1511314	0.25 ml

Table 7.2.13c the reconstitution volume for the 3 standards for the Luminex kit lot Number L128077human assay: each standard cocktail was reconstituted with Calibrator Diluent RD6-52.

7.2.14 Luminex dilution series for the standards

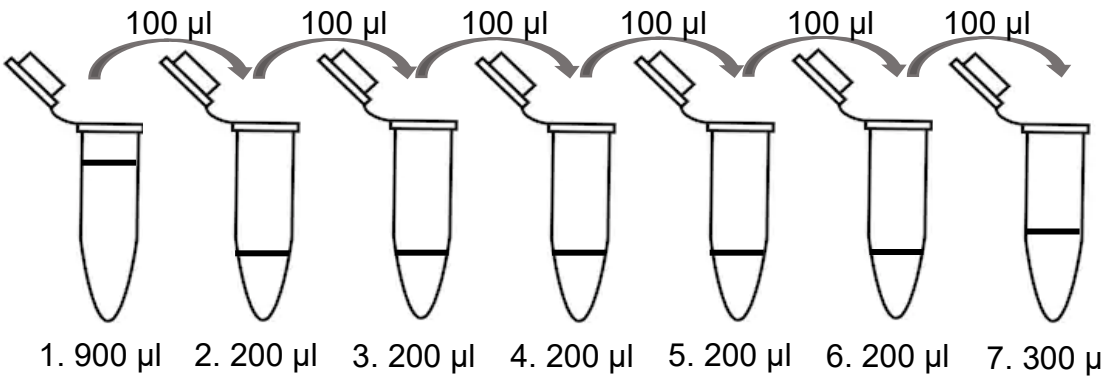


Figure 7.2.14 3-fold dilution series of the standards: 100 µL of each reconstituted cocktail was added into standard 1 and Calibrator Diluent RD6-52 was added to make the final volume up to 1 mL and mixed by vortex. 200 µL of Calibrator Diluent RD6-52 was added to standards 2-7. Standard 1 was used to produce a 3-fold dilution series by removing 100 µL out of standard 1 into standard 2 and then 100 µL out of standard 2 into standard 3 and so on as shown above. Between each addition of 100 µL the standard was mixed by vortex.

7.2.15 Maximum detection limit for each analyte measured

Analyte	Maximum detection limit pg/mL
Eotaxin	14,750
IL-4	3,380
IL-6	1,400
IL17/17A	2,870
***Periostin**	351,020
TSLP	710
IFN-gamma	12,330
IL-5	1,440
IL-13	95,060
IL-33	2,660
ST2	95,470

Table 7.2.15 standard set up for each analyte on the bioplex 200 machine: maximum concentration was entered along with the dilution fold of 3.

7.3 Appendix 3: Chapter 4

7.3.1 Correlations between the biomarkers in the serum cohort

Biomarkers	N	R^2	P-value
Eotaxin & IL-4	702	0.368	<0.0001
Eotaxin & IL-6	702	0.263	<0.0001
Eotaxin & IL17A	696	0.279	<0.0001
Eotaxin & Periostin	703	0.034	<0.0001
Eotaxin & TSLP	684	0.397	<0.0001
Eotaxin & IFN-γ	670	0.262	<0.0001
Eotaxin & IL-5	700	0.370	<0.0001
Eotaxin & IL-33	672	0.298	<0.0001
Eotaxin & ST2	694	0.172	<0.0001
Eotaxin & IL-13	613	0.281	<0.0001
IL-4 & IL-6	702	0.366	<0.0001
IL-4 & IL17A	**696**	0.480	<0.0001
IL-4 & Periostin	703	0.016	0.001
IL-4 & TSLP	**684**	**0.677**	**<0.0001**
IL-4 & IFN-γ	**670**	**0.518**	**<0.0001**
IL-4 & IL-5	**700**	**0.513**	**<0.0001**
IL-4 & IL-33	**672**	**0.540**	**<0.0001**
IL-4 & ST2	694	0.066	<0.0001
IL-4 & IL-13	614	0.372	<0.0001
IL-6 & IL17A	696	0.216	<0.0001
IL-6 & Periostin	707	0.008	0.021
IL-6 & TSLP	688	0.361	<0.0001
IL-6 & IFN-γ	671	0.265	<0.0001
IL-6 & IL-5	704	0.365	<0.0001
IL-6 & IL-33	676	0.296	<0.0001
IL-6 & ST2	698	0.108	<0.0001
IL-6 & IL-13	615	0.192	<0.0001
IL17A & Periostin	697	0.013	0.003
IL17A & TSLP	680	0.424	<0.0001
IL17A & IFN-γ	667	0.225	<0.0001
IL17A & IL-5	694	0.408	<0.0001
IL17A & IL-33	669	0.239	<0.0001
IL17A & ST2	689	0.031	<0.0001
IL17A & IL-13	607	0.269	<0.0001
Periostin & TSLP	689	0.004	0.098
Periostin & IFN-γ	671	0.0002	0.698
Periostin & IL-5	705	0.013	0.002
Periostin & IL-33	677	0.001	0.438
Periostin & ST2	699	0.007	0.028
Periostin & IL-13	616	0.020	0.0004
TSLP & IFN-γ	**657**	**0.549**	**<0.0001**
TSLP & IL-5	**688**	**0.593**	**<0.0001**
TSLP & IL-33	**665**	**0.582**	**<0.0001**
TSLP & ST2	680	0.069	<0.0001
TSLP & IL-13	599	0.458	<0.0001
IFN-γ & IL-5	669	0.375	<0.0001
IFN-γ & IL-33	**662**	**0.904**	**<0.0001**
IFN-γ & ST2	670	0.088	<0.0001
IFN-γ & IL-13	584	0.254	<0.0001
IL-5 & IL-33	676	0.398	<0.0001
IL-5 & ST2	696	0.062	<0.0001
IL-5 & IL-13	613	0.461	<0.0001
IL-33 & ST2	677	0.157	<0.0001

IL-33 & IL-13	588	0.240	<0.0001
ST2 & IL-13	607	0.031	<0.0001

Table 7.3.1 Correlation data between all 11 biomarkers in the serum cohort n=708: Data shown as the numbers, correlation coefficients (R2) and p-value between all 11 biomarkers. Bonferroni correction p=0.0004.

7.3.2 Evaluating potential confounders for the biomarkers in the serum cohort

Biomarker	N	Age (p-value)	Gender (p-value)
Eotaxin	682	**0.001**	**5.48×10^{-7}**
IL-4	618	0.621	**0.054**
IL-6	683	**0.010**	0.522
IL17/17A	426	0.792	0.968
Periostin	693	0.134	**0.031**
TSLP	627	0.307	**0.042**
IFN-gamma	638	0.781	0.799
IL-5	244	0.385	0.948
IL-13	320	0.565	**0.026**
IL-33	661	0.515	0.463
ST2	684	**0.037**	**3.34×10^{-7}**

Table 7.3.2 Confounders for the 11 biomarkers: age and gender were added to a univariate linear model to see if they were potential confounders. Data is presented as number (N) and p-value for each confounder from the statistical model, p-value of less than 0.05 is a confounder for that particular biomarker.

7.3.3 Relationship between the biomarkers and clinical features of asthma in the whole serum cohort

Biomarker	GINA 1&2	GINA 3&4	GINA 5	P-value
Eotaxin	2.32 (0.34) N= 72	2.22 (0.34) N= 458	2.25 (0.27) N= 122	0.559
IL-4	1.77 (0.70) N= 59	1.55 (0.54) N= 433	1.51 (0.56) N= 104	**0.012**
IL-6	0.13 (0.56) N= 75	0.34 (0.46) N= 459	0.24 (0.45) N= 121	**$3.37x10^{-4}$**
IL17/17A	0.89 (0.56) N= 55	0.64 (0.79) N= 269	0.56 (0.66) N= 83	**0.043**
Periostin	4.89 (0.13) N= 76	4.87 (0.26) N= 351	4.88 (0.19) N= 122	0.599
TSLP	0.80 (0.83) N= 59	0.59 (0.78) N= 434	0.43 (0.81) N= 112	**0.017**
IFN-gamma	2.16 (1.01) N= 67	2.26 (0.78) N= 441	2.09 (0.8) N= 112	0.128
IL-5	0.72 (0.50) N= 29	0.81 (0.48) N= 169	0.61 (0.54) N= 41	0.057
IL-13	2.51 (0.53) N= 57	2.60 (0.52) N= 205	2.50 (0.44) N= 64	0.179
IL-33	1.43 (0.97) N= 73	1.59 (0.81) N= 451	1.42 (0.83) N= 116	0.065
ST2	3.94 (0.34) N= 76	4.02 (0.39) N= 456	4.06 (0.36) N= 122	0.091

Table 7.3.3A Relationship between the biomarkers across GINA severity in the serum cohort n= 708: all biomarkers were transformed to the $\log_{10}$ prior analysis. A univariate linear model was used to analyse the biomarkers and hospital admissions and some of the biomarkers were adjusted for age and/or gender based on the data in table 7.3.2. The data is presented as mean, standard deviation, number (n) along with the p-value from the univariate model. IL-4, IL-6, IL17/17A and TSLP were statistically significant p= <0.05 with IL-6 only meeting Bonferroni correction p= 0.005.

Biomarker	Never Hospital Admission	Ever Hospital Admission	P-value
Eotaxin	2.23 (0.32) N= 396	2.19 (0.35) N= 228	0.526
IL-4	1.58 (0.58) N= 360	1.52 (0.56) N= 206	0.274
IL-6	0.28 (0.50) N= 398	0.32 (0.44) N= 228	0.162
IL17/17A	0.64 (0.82) N= 265	0.59 (0.72) N= 142	0.553
Periostin	4.88 (0.16) N= 406	4.86 (0.18) N= 233	0.232
TSLP	0.59 (0.79) N= 373	0.55 (0.81) N= 205	0.639
IFN-gamma	2.22 (0.82) N= 375	2.16 (0.83) N= 220	0.364
IL-5	0.74 (0.50) N= 148	0.77 (0.51) N= 80	0.704
IL-13	2.59 (0.53) N= 193	2.53 (0.46) N= 106	0.404
IL-33	1.54 (0.86) N= 394	1.48 (0.80) N= 218	0.394
ST2	4.0 (0.36) N= 399	4.01 (0.38) N= 225	0.303

Table 7.3.3B Relationship between biomarkers with hospital admissions in the serum cohort n= 708: biomarkers were transformed to the $\log_{10}$ prior analysis. A univariate linear model was used to analyse the biomarkers and hospital admissions and some of the biomarkers were adjusted for age and/or gender based on the data in table 7.3.2. The data is presented as mean, standard deviation, number (n) along with the p-value from the univariate model. None of the biomarkers were statistically significant with hospital admissions p= <0.05.

	Lung function			
Biomarker	**FEV1pp**	**P-value**	**FEV1/FVC**	**P-value**
Eotaxin	0.048 N= 650	0.543	0.057 N= 546	0.962
IL-4	0.005 N= 592	0.615	0.01 N= 490	0.905
IL-6	-0.011 N= 652	0.095	-0.007 N= 547	0.923
IL17/17A	0.001 N= 418	0.463	-0.002 N= 356	0.507
Periostin	-0.006 N= 667	0.189	-0.009 N= 556	0.080
TSLP	0.006 N= 605	0.305	-0.01 N= 501	0.775
IFN-gamma	-0.002 N= 614	0.890	-0.002 N= 515	0.860
IL-5	-0.001 N= 236	0.412	-0.005 N= 201	0.877
IL-13	-0.013 N= 306	0.206	-0.015 N= 273	0.559
IL-33	0.001 N= 637	0.598	-0.002 N= 531	0.991
ST2	-0.036 N= 651	0.766	-0.051 N= 545	0.290

Table 7.3.3C Relationship between the biomarkers with lung function (FEV_1pp and FEV_1/FVC) in the serum cohort n= 708: all biomarkers were transformed to the $\log_{10}$ prior analysis. A regression linear model was used to analyse the data and some of the biomarkers were adjusted for age and/or gender based on the data in table 7.3.2. The data is presented as R^2, number (n) along with the p-value from the univariate model. None of the biomarkers were statistically significant with both FEV_1pp and FEV_1/FVC p= <0.0

Biomarker	ACQ	P-value
Eotaxin	0.048 N= 573	0.633
IL-4	0.005 N= 516	0.658
IL-6	0.012 N= 572	0.074
IL17/17A	-0.003 N= 386	0.954
Periostin	-0.001 N= 586	0.630
TSLP	-0.005 N= 526	0.838
IFN-gamma	$8.4x10^{-5}$ N= 541	0.329
IL-5	-0.004 N= 209	0.720
IL-13	0.025 N= 276	**0.044**
IL-33	-0.003 N= 559	0.112
ST2	0.047 N= 572	0.344

Table 7.3.3D Relationship between the biomarkers ACQ score in the serum cohort n= 708: all biomarkers were transformed to the log_{10} prior analysis. A regression linear model was used to analyse the data and some of the biomarkers were adjusted for age and/or gender based on the data in table 7.3.2. The data is presented as R^2, number (n) along with the p-value from the univariate model. IL-13 was only statistically significant with ACQ score p= <0.05.

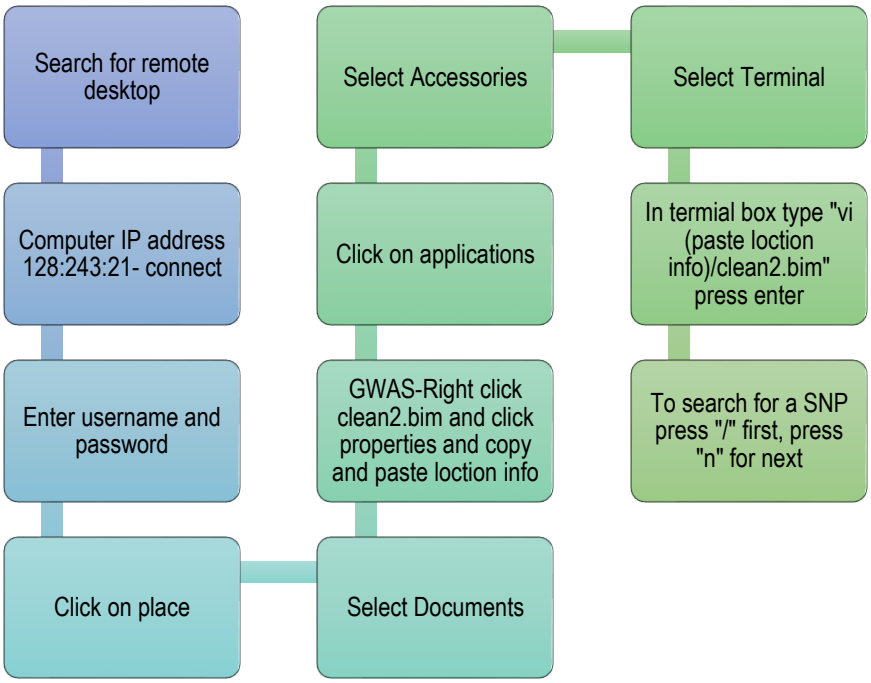
7.3.4 The Lowest Cut-off Point for the Biomarker Quartile Groups

Biomarker	Top 25% lowest cut off point
IL-4	≥75.51 pg/mL
IL-5	≥3.48 pg/mL
IL-13	≥398.72 pg/mL
IL-33	≥12 pg/mL4.65
TSLP	≥11.54 pg/mL
IL-17A	≥6.43 pg/mL
IFN-y	≥522.88 pg/mL

Table 7.3.4 the biomarkers lowest cut off point for each top quartile group

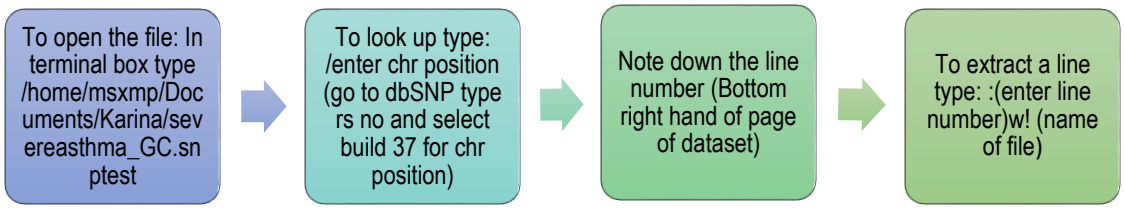
7.4 Appendix 4: Chapter 5

8.4.1 Accessing and Searching GWAS Genotyped SNPs



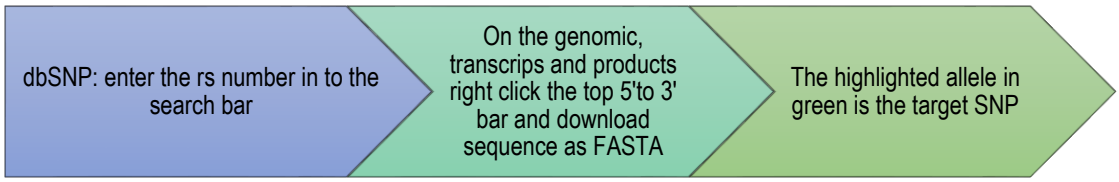
8.4.1 Flow chart of how the genotyped SNPs from the moderate-severe asthma GWAS were searched for on the database after looking for potential proxy SNPs on the haploreg database.

8.4.2 Opening, Searching and Extracting GWAS Summary Stage 1 Statistics



8.4.2 Flow chart of how the moderate-severe asthma GWAS summary stage 1 statistics were searched for and extracted from the database.

8.4.3 Look up and Downloading SNP Sequences



8.4.3 Flow chart of how the SNP sequences were searched for and downloaded to send them to LCG Genomics for genotyping the 25 signals on all the bloods and DNA.

8.4.4 Look up of the of the minor allele frequencies on Ensembl

8.4.4 Flow chart of how the minor allele frequencies for the European population were searched for on Ensembl (100 Genomes Project Phase 3 allele frequencies)

8.4.5 SNP Sequences for LGC

SNP ID	Sequence
rs1773542	TTTTGTGGAGGAAAATCAACTTTGAACATTCAAGAAAAAAGACCCCATTG[T/C]AATGATTGGTTAGAATTTGATCTGGATCTACATAGATTCATTTTCTAAAACTATTCAA
rs61816764	GAACACTCAAATTGCTTCTAATTAATTCCTCAGCACTAAAAATAATGCTG[C/T]GACGAACATCCTCTTACATATGTCCATTATGGATCTCTGGGAGACATTTTTGGGGGTC
rs1420101	TTTTGTGGAGGAAAATCAACTTTGAACATTCAAGAAAAAAGACCCCATTG[T/C]AATGATTGGTTAGAATTTGATCTGGATCTACATAGATTCATTTTCTAAAACTATTCAA
rs1106639	GCCTCTGCTCACTCTGCCTTCCTTGCTCACTTCTAGAGAGTCCGTTTTAC[G/A]TCCTCATCGAGACTTCAGGCTCCAACGCAGGCCATGACGCTGAGAAGCTGGGCCACTT
rs9272629	GTTTGTTCCTTAATAGTAGACCATGCCTGATTGGTGTTTTACACATCTCC[G/T]GCTATGTCTGACACTTGTGGATGCTCAGAAAGTGGGGAAGGAAGGAAAGATACGATGG
rs17388568	TATCTAAGTCATCGGATTAATTCCATGTCACCTTTGGAACATTTATCATA[G/A]TAACGCCAATGTCTACCCTGTGGATTTTAGTGGCTTCTATTATTAAGAACTGTGAACT
rs1837253	TTGACTCACATAATGGTTACTTTGTAAAAGATCCTTTTATACATAAACAA[T/C]GTGTCTAAACTATGAAGCAAATTACACAATAGAGTCCAGTAGGTGAATAGATTTCTTG
rs3806933	CACCTGCTAGAAAGTGCTAGAGGACTCTTCAGCCCCTAAGGTGCCCCTAG[C/T]CACCAAGAGTAGGCGTTTTTCCTGAAATTGATATGGGAAATTTGAAAAGTAGGGTTTT
rs10077785	AGGGGGAGGTGTCCCACATGGCACACTCTCCCTAGCCCTTGCTTTGCCTC[C/T]GTTACCTACATAGATGGGCTTCACAGCCTCTTCCAGATAGGCAGGGCACTGAGCTCTG
rs2244012	TCCCTCATGGGATGCTTTTCCATTCTGTATACACTTTGACTTCTGGTGCT[A/G]GATCATGTCCTCATTCCCCGCCCTTCTCTGATACCAGGTGGCCTCTTGCAGGGATGCC
rs532965	AGATACCATATTTTGTAGTTGTGAAGCAGAGTAAAGACTGAGACTCTTAG[T/G]ACTTAGTATTGGTGGGGGAGAAGAGGTTACCTGATATTTTTCATGCCTCTGTGGGAAG
rs1847472	TGAAAAATAAATCCCCAAACATAATTCTTCTGGCAAAATATTAAATCCTA[C/A]CGAAGAAGGAGGCTTTCAGGGAAACAGTCCAAATAAACCTTTTAATTTGTTTAATGTT
rs13275219	TGTTTTCTTCTCGTCCTTGACCATGATAGCCACTTATCAGTCATGTGCTA[T/C]GTCTTAGGACAGAATCGAGATTTACCTGCCGTCTTTCTTTTCCACTCACCCTCCTGGT
rs1342326	AAATGAAGTATTTGGAGTCCAAAAGATATAAATAAGAATAAGAGGTCATG[A/C]TGGTGTCTTCATGAGAAAAGATTGGGAATTAAAGATGTTTATGCTGAAATAGAATTAT
rs10905279	AGAAGGAAAACTTTCTAGGGATGGAAATGTCCACATGACTTTAATCTTTG[A/G]CAATCCTGGATATCGAGGCTTTATTCTTTCTGATCTTTGCCCCCTTCTCCAACTTTCT
rs1444782	AAATTGTGGGTGGGTAGGCAAATAGTGTAATATAATTCAGTGACATTTAC[G/A]TTCATGAGTCCAGTTGGCAAAAGGCATCGTATTTTTCACTAAGTGTAGGAAGAATCAT
rs28592983	ACGGACCTGGATGTGGTGTGCTCCAGCCTGGAGCTGTACGCGGCACTCTG[T/C]GCGTCCCACGACATCTGCATCGATTGGAGAGGCCGGACCGGCCACATGTGCCGTGAGT
rs61893460	GCTGGCATTGGTAGGGCCCACCTGGAGGCTGGTGGGTGGTGAAATGAGCC[G/A]GGGCTTGCTTGCGCACAGAGCTCCCGTTCTTCCCTTGGGTTGTGGTAAACCCAGTCCC
rs10876864	TTAGATTGCAAACTGGGTGCAGATTAAAGGCTAGTTTTTGGGTTACCTTC[G/A]GCAGACGATGGTGCCTTTCGGATTTGAATCTTTCGGCGCTCGGCTACTCTCCCCTAGC
rs167769	TCTCCCAGGCCTCTCAGAGGGATGGAAAATGGAGAGGTCCCTTCTCTGGA[C/T]GTTTCTGCTCCAAGACCTTCATGCCCCTCTTTCCTCCAGTGCCCACTTACCCCAGCTC
rs10519067	TTGCTGGCTCGGCTAGAATTGAGTGTAGACAGCACCCACGATATTGTGAT[G/A]ACCAACCAGCTTGAGATGAAGGCACTCATGACCAGCTTGCAGGAGCAGGCCTCGTCCC
rs17293632	GCATGTTATGCAGCACTTGTGACATTGTTTTATCTCTCTAGCCGGTGACT[C/T]ATAATTAAGGAAGTCAAAACTGTGCAATTAACACAAAACCTCCTCATTTGCCCTCTTC
rs36045143	GCTGGTTTTATGTTGATGGCTCTCACCCTCACAGAATGTTCAGCTTCACG[A/G]GGGATTGTGCGTGCGCCTGGCCAAGGAGGGGGGTCAGTGGCGGCCAGTATGTGGGGGT
rs9303277	AAACAAAACTCCAAAGTTATCTGTGTTCAGTGTCATGCGATGAATTAAAA[C/T]AGGGCTGTGTTAAAATAGTGACTGAATTGTCAGGAAAAGCTTTTCTGAAGAAATGACA
rs16948048	AGTTAACAAGGTAGCATTTATCCGAGTTCAGAGACAAGAGAGTGTCCATA[A/G]TTGAAAGTGTTTAGGGTACAGGGAGAGGAAATGAACCCGGCAAGGTGAAGGCCTGGGA

8.4.5 The 25 SNP sequences sent to LGC Genomics for genotyping that were taken from dbSNP.

8.4.6 Results of 25 genetic signals and IFN-γ serum

Locus	0	1	2	P-value	Power
rs10077785 ***C5orf56***	N=331 2.21 (0.81)	N=241 2.19 (0.83)	N=41 2.24 (0.91)	0.929	0.35
rs167769 ***STAT6***	N=190 2.27 (0.89)	N=298 2.16 (0.80)	N=124 2.19 (0.76)	0.353	1.0
rs1342326 ***IL-33***	N=395 2.19 (0.81)	N=201 2.24 (0.85)	N=15 1.97 (0.68)	0.435	1.0
rs2244012 ***RAD50***	N=375 2.22 (0.80)	N=207 2.18 (0.85)	N=30 2.19 (0.86)	0.868	0.89
rs1773542 ***CD247***	N=112 2.17 (0.79)	N=275 2.17 (0.81)	N=224 2.26 (0.84)	0.429	1.0
rs61816764 ***FLG****	N=533 2.18 (0.82)	N=78 2.34 (0.81)		0.106	0.30
rs1420101 ***IL1RL1***	N=112 2.18 (0.80)	N=272 2.16 (0.81)	N=229 2.25 (0.85)	0.438	1.0
rs1106639 ***D2HGDH***	N=27 2.03 (0.88)	N=199 2.26 (0.84)	N=384 2.18 (0.81)	0.300	1.0
rs532965 ***HLA-DQA1***	N=362 2.15 (0.82)	N=212 2.26 (0.82)	N=36 2.35 (0.85)	0.143	1.0
rs9272629 ***HLA-DQB1***	N=114 2.23 (0.82)	N=186 2.18 (0.78)	N=301 2.21 (0.85)	0.830	0.99
rs1847472 ***BACH2***	N=63 2.27 (0.85)	N=274 2.21 (0.88)	N=274 2.17 (0.75)	0.677	1.0
rs13275219 ***MIR5708***	N=241 2.21 (0.82)	N=291 2.19 (0.83)	N=78 2.20 (0.79)	0.955	0.16
rs10905279 ***GATA3***	N=223 2.23 (0.84)	N=304 2.19 (0.74)	N=85 2.15 (0.86)	0.736	1.0
rs1444782 ***LOC101928272***	N=70 2.23 (0.70)	N=316 2.17 (0.85)	N=224 2.22 (0.81)	0.761	1.0
rs28592983 ***MUC5AC***	N=255 2.23 (0.83)	N=278 2.18 (0.87)	N=75 2.18 (0.64)	0.789	1.0
rs61893460 ***C11orf30***	N=154 2.32 (0.77)	N=297 2.19 (0.85)	N=151 2.14 (0.81)	0.146	1.0
rs10876864 ***RPS26***	N=174 2.13 (0.88)	N=319 2.23 (0.80)	N=117 2.23 (0.81)	0.440	1.0
rs10519067 ***RORA***	N=109 2.21 (0.88)		N=503 2.20 (0.81)	0.908	0.05
rs36045143 ***CLEC16A***	N=40 2.11 (0.75)	N=213 2.14 (0.82)	N=360 2.25 (0.83)	0.230	1.0
rs16948048 ***ZNF652***	N=217 2.25 (0.84)	N=301 2.18 (0.85)	N=91 2.13 (0.67)	0.442	1.0
rs3806933 ***WDR36***	N=89 2.25 (0.82)	N=282 2.16 (0.85)	N=242 2.22 (0.79)	0.529	1.0
rs1837253 ***TSLP***	N=31 2.37 (0.90)	N=206 2.20 (0.76)	N=376 2.18 (0.85)	0.477	1.0
rs17293632 ***SMAD3***	N=331 2.22 (0.84)	N=239 2.18 (0.79)	N=42 2.11 (0.92)	0.631	1.0
rs17388568 ***KIAA1109***	N=292 2.17 (0.81)	N=272 2.20 (0.83)	N=48 2.39 (0.86)	0.217	1.0
rs9303277 ***IKZF3***	N=143 2.14 (0.88)	N=275 2.25 (0.78)	N=165 2.20 (0.79)	0.363	1.0

Table 8.4.5 Genetics signals identified in the moderate to severe asthma GWAS study did not drive the levels of IFN-γ in the serum of asthma patients: data is presented as mean $\log_{10}$ pg/mL and standard deviation. ANOVA was used to analyse the 3 genotype groups. *Two genotyping groups were combined together due to the n's being low in one of the groups therefore independent T-tests was used analyses. Power was also computed and a value more than 0.8 is considered a well powered study.

8.4.7 Results of 25 genetic signals and IL17A

Locus	0	1	2	P-value	Power
rs10077785 ***C5orf56***	N=216 0.66 (0.74)	N=160 0.65 (0.83)	N=29 0.66 (0.77)	0.996	0.05
rs167769 ***STAT6***	N=129 0.71 (0.81)	N=200 0.64 (0.75)	N=77 0.58 (0.80)	0.462	1.0
rs1342326 ***IL-33***	N=262 0.69 (0.81)	N=134 0.59 (0.71)	N=9 0.32 (0.91)	0.221	1.0
rs2244012 ***RAD50***	N=248 0.67 (0.78)	N=137 0.64 (0.80)	N=21 0.53 (0.67)	0.701	1.0
rs1773542 ***CD247***	N=66 0.68 (0.83)	N=178 0.62 (0.81)	N=159 0.68 (0.74)	0.762	1.0
rs61816764 ***FLG****	N=350 0.64 (0.79)	N=55 0.74 (0.72)		0.398	0.14
rs1420101 ***IL1RL1***	N=67 0.70 (0.82)	N=177 0.62 (0.81)	N=163 0.67 (0.74)	0.736	1.0
rs1106639 ***D2HGDH***	N=16 0.68 (0.83)	N=135 0.68 (0.77)	N=252 0.65 (0.78)	0.934	0.21
rs532965 ***HLA-DQA1***	N=248 0.63 (0.79)	N=133 0.71 (0.74)	N=22 0.60 (0.94)	0.608	1.0
rs9272629 ***HLA-DQB1***	N=81 0.76 (0.74)	N=131 0.63 (0.77)	N=184 0.65 (0.80)	0.461	1.0
rs1847472 ***BACH2***	N=43 0.72 (0.77)	N=175 0.73 (0.77)	N=187 0.57 (0.79)	0.160	1.0
rs13275219 ***MIR5708***	N=157 0.66 (0.73)	N=193 0.66 (0.81)	N=56 0.58 (0.81)	0.785	0.99
rs10905279 ***GATA3***	N=145 0.71 (0.68)	N=206 0.64 (0.81)	N=54 0.54 (0.86)	0.376	1.0
rs1444782 ***LOC101928272***	N=52 0.73 (0.85)	N=202 0.60 (0.79)	N=149 0.71 (0.74)	0.343	1.0
rs28592983 ***MUC5AC***	N=174 0.67 (0.76)	N=178 0.67 (0.80)	N=51 0.57 (0.75)	0.683	1.0
rs61893460 ***C11orf30***	N=107 0.67 (0.75)	N=189 0.61 (0.85)	N=104 0.72 (0.68)	0.534	1.0
rs10876864 ***RPS26***	N=119 0.63 (0.82)	N=213 0.65 (0.75)	N=74 0.71 (0.81)	0.801	0.98
rs10519067 ***RORA***	N=84 0.74 (0.86)		N=321 0.63 (0.76)	0.305	0.17
rs36045143 ***CLEC16A***	N=23 0.77 (0.91)	N=141 0.66 (0.72)	N=242 0.64 (0.80)	0.758	1.0
rs16948048 ***ZNF652***	N=146 0.68 (0.78)	N=205 0.66 (0.77)	N=53 0.58 (0.80)	0.710	1.0
rs3806933 ***WDR36***	N=60 0.79 (0.77)	N=179 0.66 (0.76)	N=167 0.60 (0.81)	0.270	1.0
rs1837253 ***TSLP***	N=24 0.65 (0.70)	N=130 0.68 (0.75)	N=253 0.64 (0.80)	0.912	0.36
rs17293632 ***SMAD3***	N=220 0.63 (0.80)	N=159 0.71 (0.78)	N=26 0.57 (0.57)	0.517	1.0
rs17388568 ***KIAA1109***	N=196 0.63 (0.76)	N=173 0.71 (0.79)	N=35 0.57 (0.83)	0.450	1.0
rs9303277 ***IKZF3***	N=95 0.70 (0.94)	N=179 0.67 (0.71)	N=106 0.66 (0.77)	0.940	0.17

Table 8.4.7 Genetics signals identified in the moderate to severe asthma GWAS study did not drive the levels of IL17A in the serum of asthma patients: data is presented as mean $\log_{10}$ pg/mL and standard deviation. ANOVA was used to analyse the 3 genotype groups. *Two genotyping groups were combined together due to the n's being low in one of the groups therefore independent T-tests was used analyses. Power was also computed and a value more than 0.8 is considered a well powered study.

8.4.8 Results of 25 genetic signals and IL-4

Locus	0	1	2	P-value	Power
rs10077785 ***C5orf56***	N=324 1.54 (0.57)	N=234 1.57 (0.59)	N=38 1.58 (0.51)	0.792	1.0
rs167769 ***STAT6***	N=179 1.60 (0.60)	N=298 1.54 (0.57)	N=117 1.53 (0.55)	0.508	1.0
rs1342326 ***IL-33***	N=382 1.57 (0.60)	N=195 1.55 (0.52)	N=16 1.37 (0.54)	0.393	1.0
rs2244012 ***RAD50***	N=372 1.55 (0.58)	N=192 1.56 (0.57)	N=30 1.57 (0.50)	0.974	0.08
rs1773542 ***CD247***	N=108 1.54 (0.55)	N=268 1.52 (0.60)	N=216 1.60 (0.55)	0.262	1.0
rs61816764 ***FLG****	N=519 1.54 (0.58)	N=73 1.68 (0.54)		0.044	0.54
rs1420101 ***IL1RL1***	N=110 1.52 (0.56)	N=265 1.61 (0.60)	N=220 1.6 (0.55)	0.259	1.0
rs1106639 ***D2HGDH***	N=25 1.36 (0.54)	N=192 1.56 (0.55)	N=375 1.56 (0.59)	0.224	1.0
rs532965 ***HLA-DQA1***	N=354 1.56 (0.57)	N=207 1.56 (0.58)	N=31 1.45 (0.55)	0.574	1.0
rs9272629 ***HLA-DQB1***	N=115 1.63 (0.58)	N=180 1.57 (0.54)	N=289 1.53 (0.59)	0.241	1.0
rs1847472 ***BACH2***	N=58 1.65 (0.60)	N=271 1.56 (0.57)	N=264 1.53 (0.57)	0.326	1.0
rs13275219 ***MIR5708***	N=229 1.55 (0.55)	N=285 1.56 (0.59)	N=79 1.56 (0.59)	0.984	0.06
rs10905279 ***GATA3***	N=220 1.58 (0.58)	N=290 1.54 (0.57)	N=84 1.53 (0.58)	0.651	1.0
rs1444782 ***LOC101928272***	N=75 1.62 (0.60)	N=299 1.57 (0.56)	N=218 1.57 (0.56)	0.392	1.0
rs28592983 ***MUC5AC***	N=254 1.56 (0.59)	N=265 1.54 (0.56)	N=72 1.57 (0.53)	0.926	0.37
rs61893460 ***C11orf30***	N=158 1.61 (0.54)	N=273 1.53 (0.63)	N=154 1.55 (0.51)	0.369	1.0
rs10876864 ***RPS26***	N=174 1.56 (0.62)	N=305 1.56 (0.56)	N=113 1.55 (0.55)	0.997	0.05
rs10519067 ***RORA***	N=107 1.65 (0.64)		N=487 1.54 (0.56)	0.067	0.42
rs36045143 ***CLEC16A***	N=35 1.49 (0.65)	N=209 1.55 (0.57)	N=351 1.57 (0.57)	0.743	1.0
rs16948048 ***ZNF652***	N=216 1.61 (0.57)	N=284 1.53 (0.58)	N=93 1.49 (0.55)	0.132	1.0
rs3806933 ***WDR36***	N=92 1.58 (0.49)	N=276 1.53 (0.60)	N=227 1.58 (0.57)	0.595	1.0
rs1837253 ***TSLP***	N=32 1.57 (0.44)	N=198 1.55 (0.52)	N=365 1.56 (0.61)	0.950	0.19
rs17293632 ***SMAD3***	N=309 1.55 (0.59)	N=242 1.57 (0.57)	N=43 1.56 (0.48)	0.954	0.16
rs17388568 ***KIAA1109***	N=280 1.53 (0.53)	N=269 1.57 (0.61)	N=46 1.62 (0.61)	0.566	1.0
rs9303277 ***IKZF3***	N=134 1.58 (0.65)	N=273 1.54 (0.55)	N=164 1.57 (0.56)	0.829	0.98

Table 8.4.8 Genetics signals identified in the moderate to severe asthma GWAS study did not drive the levels of IL-4 in the serum of asthma patients: data is presented as mean $\log_{10}$ pg/mL and standard deviation. ANOVA was used to analyse the 3 genotype groups. *Two genotyping groups were combined together due to the n's being low in one of the groups therefore independent T-tests was used analyses. Power was also computed and a value more than 0.8 is considered a well powered study.

8.4.9 Results of 25 genetic signals and IL-33

Locus	0	1	2	P-value	Power
rs10077785 ***C5orf56***	N=347 1.53 (0.86)	N=245 1.52 (0.81)	N=41 1.67 (0.88)	0.588	1.0
rs167769 ***STAT6***	N=193 1.62 (0.88)	N=315 1.49 (0.86)	N=125 1.49 (0.72)	0.198	1.0
rs1342326 ***IL-33***	N=410 1.58 (0.85)	N=209 1.49 (0.69)	N=14 1.49 (0.69)	0.431	1.0
rs2244012 ***RAD50***	N=392 1.56 (0.85)	N=209 1.48 (0.82)	N=32 1.50 (0.91)	0.494	1.0
rs1773542 ***CD247***	N=114 1.47 (0.77)	N=282 1.52 (0.82)	N=235 1.57 (0.89)	0.535	1.0
rs61816764 ***FLG****	N=552 1.51 (0.84)	N=78 1.66 (0.83)		0.150	0.31
rs1420101 ***IL1RL1***	N=114 1.50 (0.78)	N=279 1.50 (0.82)	N=241 1.58 (0.90)	0.527	1.0
rs1106639 ***D2HGDH***	N=27 1.38 (0.88)	N=205 1.55 (0.85)	N=399 1.53 (0.83)	0.626	1.0
rs532965 ***HLA-DQA1***	N=375 1.46 (0.84)	N=219 1.63 (0.84)	N=36 1.64 (0.78)	0.043	1.0
rs9272629 ***HLA-DQB1***	N=121 1.53 (0.87)	N=193 1.52 (0.84)	N=307 1.55 (0.84)	0.885	0.79
rs1847472 ***BACH2***	N=62 1.60 (0.90)	N=287 1.54 (0.89)	N=283 1.50 (0.77)	0.681	1.0
rs13275219 ***MIR5708***	N=250 1.58 (0.88)	N=299 1.50 (0.81)	N=82 1.52 (0.81)	0.526	1.0
rs10905279 ***GATA3***	N=235 1.56 (0.88)	N=314 1.53 (0.83)	N=83 1.44 (0.77)	0.553	1.0
rs1444782 ***LOC101928272***	N=75 1.63 (0.83)	N=322 1.49 (0.84)	N=233 1.56 (0.83)	0.333	1.0
rs28592983 ***MUC5AC***	N=267 1.56 (0.85)	N=282 1.53 (0.87)	N=79 1.47 (0.69)	0.661	1.0
rs61893460 ***C11orf30***	N=159 1.59 (0.87)	N=303 1.50 (0.83)	N=160 1.56 (0.83)	0.537	1.0
rs10876864 ***RPS26***	N=183 1.55 (0.89)	N=333 1.52 (0.84)	N=115 1.52 (0.78)	0.940	0.26
rs10519067 ***RORA***	N=10 1.55 (0.94)	N=105 1.57 (0.87)	N=518 1.52 (0.84)	0.882	0.81
rs36045143 ***CLEC16A***	N=39 1.44 (0.75)	N=224 1.49 (0.83)	N=371 1.57 (0.86)	0.441	1.0
rs16948048 ***ZNF652***	N=226 1.59 (0.87)	N=314 1.49 (0.87)	N=90 1.54 (0.67)	0.371	1.0
rs3806933 ***WDR36***	N=90 1.57 (0.84)	N=294 1.50 (0.97)	N=250 1.55 (0.81)	0.724	1.0
rs1837253 ***TSLP***	N=31 1.63 (0.87)	N=218 1.53 (0.79)	N=385 1.53 (0.87)	0.801	1.0
rs17293632 ***SMAD3***	N=338 1.55 (0.84)	N=250 1.51 (0.83)	N=45 1.51 (0.95)	0.808	1.0
rs17388568 ***KIAA1109***	N=306 1.54 (0.84)	N=277 1.50 (0.84)	N=48 1.71 (0.83)	0.285	1.0
rs9303277 ***IKZF3***	N=145 1.47 (0.91)	N=284 1.61 (0.81)	N=170 1.55 (0.79)	0.250	1.0

Table 8.4.9 Genetics signals identified in the moderate to severe asthma GWAS study did not drive the levels of IL-33 in the serum of asthma patients: data is presented as mean $\log_{10}$ pg/mL and standard deviation. ANOVA was used to analyse the 3 genotype groups. *Two genotyping groups were combined together due to the n's being low in one of the groups therefore independent T-tests was used analyses. Power was also computed and a value more than 0.8 is considered a well powered study.

8.4.10 Results of 25 genetic signals and periostin

Locus	0	1	2	P-value	Power
rs10077785	N=365	N=255	N=43	0.521	0.16
C5orf56	82018.12 (42295.35)	78650.42 (26626.25)	79760.99 (25365.11)		
rs167769	N=202	N=333	N=128	0.785	0.09
STAT6	81114.55 (29246.21)	81106.65 (42497.04)	78271.36 (26324.26)		
rs1342326	N=432	N=214	N=16	0.443	0.19
IL-33	81878.74 (40624.41)	78492.97 (25660.44)	73840.0 (19627.60)		
rs2244012	N=409	N=221	N=33	0.785	0.09
RAD50	81068.19 (41298.62)	79070.44 (24912.53)	82605.43 (27767.08)		
rs1773542	N=118	N=296	N=247	0.709	0.11
CD247	82335.14 (25197.07)	79310.0 (28650.39)	81389.85 (46884.25)		
rs61816764	N=577	N=83		0.929	0.051
FLG*	80655.17 (37076.47)	80133.71 (28792.59)			
rs1420101	N=118	N=213	N=253	0.415	0.20
IL1RL1	83798.89 (25592.94)	78586.53 (28282.39)	81284.18 (46624.99)		
rs1106639	N=28	N=213	N=420	0.471	0.18
D2HGDH	88881.73 (28670.57)	79546.74 (25403.6)	80521.06 (40716.32)		
rs532965	N=397	N=225	N=38	0.561	0.15
HLA-DQA1	80898.66 (40005.57)	81245.88 (29821.89)	74550.87 (25846.82)		
rs9272629	N=130	N=203	N=317	0.575	0.14
HLA-DQB1	83411.44 (60158.30)	78986.61 (26138.58)	80426.65 (27995.72)		
rs1847472	N=65	N=301	N=296	0.436	0.19
BACH2	83189.58 (28658.86)	81842.08 (44764.29)	78843.96 (26237.38)		
rs13275219	N=261	N=316	N=84	0.029	0.66
MIR5708	84677.35 (46815.73)	77116.93 (26388.46)	80586.88 (27511.64)		
rs10905279	N=247	N=325	N=90	0.844	0.08
GATA3	79743.16 (27449.88)	81310.86 (43021.83)	80363.06 (27576.7)		
rs1444782	N=82	N=335	N=243	0.194	0.35
LOC101928272	87243.44 (73434.24)	78890.52 (25144.36)	80911.94 (28969.28)		
rs28592983	N=281	N=293	N=84	0.642	0.12
MUC5AC	80124.31 (27066.15)	80188.02 (27883.19)	83498.10 (71664.90)		
rs61893460	N=170	N=314	N=168	0.490	0.17
C11orf30	80075.44 (24952.37)	79332.64 (26778.56)	83389.34 (55597.21)		
rs10876864	N=195	N=344	N=122	0.054	0.57
RPS26	77590.59 (26616.55)	84008.17 (43088.09)	75579.25 (25232.67)		
rs10519067	N=10	N=113	N=540	0.302	0.10
RORA	90686.75 (26659.95)	79554.03 (28069.51)	80561.31 (37667.84)		
rs36045143	N=41	N=230	N=393	0.373	0.22
CLEC16A	72721.83 (27937.06)	81805.58 (27323.75)	80731.22 (40840.68)		
rs16948048	N=234	N=326	N=100	0.428	0.20
ZNF652	82772.43 (48912.21)	80024.33 (27213.83)	77188.56 (24515.10)		
rs3806933	N=95	N=315	N=254	0.917	0.06
WDR36	81756.99 (26323.09)	79630.84 (28014.84)	81287.54 (46551.13)		
rs1837253	N=33	N=226	N=405	0.535	0.16
TSLP	85614.18 (25492.75)	79047.56 (26348.41)	80981.2 (41090.14)		
rs17293632	N=353	N=261	N=49	0.463	0.18
SMAD3	79420.17 (27497.16)	82270.6 (46393.71)	79937.47 (26276.24)		
rs17388568	N=317	N=293	N=51	0.741	0.10
KIAA1109	80599.97 (26907.99)	81031.64 (45296.33)	77746.34 (24918.66)		
rs9303277	N=152	N=296	N=179	0.540	0.15
IKZF3	81909.45 (28635.04)	82780.85 (44922.55)	78875.87 (25161.69)		

Table 8.4.10 Genetics signals identified in the moderate to severe asthma GWAS study did not drive the levels of periostin in the serum of asthma patients: data is presented as mean pg/mL and standard deviation. Univariate model was used to correct for gender and to analyse the 3 or 2 genotype groups. *Two genotyping groups were combined together due to the n's being low in one of the groups. Power was also computed and a value more than 0.8 is considered a well powered study.

8.4.11 Results of 25 genetic signals and soluble ST2

Locus	0	1	2	P-value	Power
rs10077785 ***C5orf56***	n=355 4.01 (0.39)	N=248 4.02 (0.39)	n=43 4.01 (0.32)	0.203	0.08
rs167769 ***STAT6***	N=199 4.04 (0.39)	N=321 4.03 (0.42)	N=126 3.93 (0.27)	0.048	0.62
rs1342326 ***IL-33***	N=421 4.03 (0.39)	N=210 3.99 (0.37)	N=14 3.90 (0.30)	0.274	0.28
rs2244012 ***RAD50***	N=399 4.02 (0.40)	N=214 4.01 (0.31)	N=33 4.02 (0.59)	0.964	0.06
rs1773542 ***CD247***	N=116 3.99 (0.41)	N=289 4.01 (0.34)	N=239 4.02 (0.41)	0.800	0.09
rs61816764 ***FLG****	N=565 4.01 (0.38)	N=78 4.02 (0.41)		0.900	0.05
rs1420101 ***IL1RL1***	N=117 4.00 (0.41)	N=285 4.00 (0.34)	N=245 4.03 (0.41)	0.635	0.12
rs1106639 ***D2HGDH***	N=27 4.0 (0.22)	N=207 4.0 (0.36)	N=410 4.02 (0.40)	0.862	0.07
rs532965 ***HLA-DQA1***	N=384 4.02 (0.40)	N=223 4.01 (0.37)	N=36 4.07 (0.33)	0.685	0.11
rs9272629 ***HLA-DQB1***	N=125 4.03 (0.38)	N=200 4.00 (0.41)	N=308 4.02 (0.37)	0.910	0.06
rs1847472 ***BACH2***	N=64 3.98 (0.32)	N=293 4.03 (0.39)	N=288 4.00 (0.39)	0.515	0.16
rs13275219 ***MIR5708***	N=252 4.02 (0.39)	N=310 4.02 (0.40)	N=82 3.99 (0.28)	0.680	0.11
rs10905279 ***GATA3***	N=243 4.03 (0.41)	N=316 4.01 (0.35)	N=86 3.98 (0.43)	0.495	0.17
rs1444782 ***LOC101928272***	N=78 4.03 (0.44)	N=327 3.99 (0.37)	N=238 4.03 (0.39)	0.569	0.14
rs28592983 ***MUC5AC***	N=273 4.01 (0.39)	N=287 4.03 (0.37)	N=81 3.97 (0.42)	0.637	0.12
rs61893460 ***C11orf30***	N=163 4.06 (0.47)	N=307 3.99 (0.34)	N=165 4.02 (0.36)	0.089	0.49
rs10876864 ***RPS26***	N=187 4.03 (0.40)	N=340 4.02 (0.39)	N=117 3.97 (0.33)	0.543	0.15
rs10519067 ***RORA***	N=10 4.10 (0.26)	N=107 4.05 (0.34)	N=529 4.01 (0.39)	0.528	0.16
rs36045143 ***CLEC16A***	N=40 4.00 (0.23)	N=225 4.00 (0.43)	N=382 4.02 (0.37)	0.693	0.11
rs16948048 ***ZNF652***	N=227 4.01 (0.40)	N=320 4.00 (0.32)	N=96 4.07 (0.52)	0.128	0.43
rs3806933 ***WDR36***	N=93 4.00 (0.39)	N=304 4.02 (0.41)	N=250 4.01 (0.35)	0.764	0.09
rs1837253 ***TSLP***	N=33 4.08 (0.37)	N=220 3.99 (0.36)	N=394 4.02 (0.40)	0.420	0.20
rs17293632 ***SMAD3***	N=345 4.00 (0.34)	N=253 4.02 (0.43)	N=48 4.07 (0.42)	0.289	0.27
rs17388568 ***KIAA1109***	N=310 4.03 (0.42)	N=284 3.98 (0.31)	N=50 4.10 (0.48)	0.102	0.47
rs9303277 ***IKZF3***	N=148 4.03 (0.37)	N=291 4.02 (0.42)	N=172 4.00 (0.34)	0.639	0.12

Table 8.4.11 Genetics signals identified in the moderate to severe asthma GWAS study did not drive the levels of soluble ST2 in the serum of asthma patients: data is presented as mean $\log_{10}$ pg/mL and standard deviation. Univariate model was used to correct for age and gender and to analyse the 3 or 2 genotype groups. *Two genotyping groups were combined together due to the n's being low in one of the groups. Power was also computed and a value more than 0.8 is considered a well powered study.

8.4.12 Association of Age of Onset with the Moderate-Severe Asthma Genetic Signals

Rs number	0	1	2	P-value
rs10077785 (*C5orf56*)	N=976 <16: 484 (49.6%) 16-34: 291 (29.8%) 35-60: 201 (20.6%)	N=651 <16: 340 (52.2%) 16-34: 180 (27.6%) 35-60: 131 (20.1%)	N= 131 <16: 67 (51.1%) 16-34: 30 (29.8%) 35-60: 25 (19.1%)	0.853
rs167769 (*STAT6*)	N=582 <16: 284 (48.8%) 16-34: 158 (27.1%) 35-60: 140 (24.1%)	N= 861 <16: 438 (50.9%) 16-34: 259 (301%) 35-60: 164 (19%)	N= 313 <16: 171 (54.6%) 16-34: 90 (28.8%) 35-60: 52 (16.6%)	0.056
rs1342326 (*IL33*)	N= 1128 <16: 558 (49.5%) 16-34: 339 (30.1%) 35-60: 231 (20.5%)	N= 566 <16: 302 (53.4%) 16-34: 155 (27.4%) 35-60: 109 (19.3%)	N= 61 <16: 30 (49.2%) 16-34: 15 (24.6%) 35-60: 16 (26.2%)	0.422
rs2244012 (*RAD50*)	N= 918 <16: 429 (46.7%) 16-34: 293 (31.9%) 35-60: 196 (21.4%)	N= 474 <16: 256 (54%) 16-34: 125 (26.4%) 35-60: 93 (19.6%)	N= 76 <16: 38 (50%) 16-34: 28 (36.8%) 35-60: 10 (13.2%)	**0.038**
rs1773542 (*CD247*)	N= 291 <16: 140 (48.1%) 16-34: 87 (29.9%) 35-60: 64 (22%)	N= 803 <16: 407 (50.7%) 16-34: 235 (29.3%) 35-60: 161 (20%)	N= 660 <16: 343 (52%) 16-34: 186 (28.2%) 35-60: 131 (19.8%)	0.853
rs61816764 (FLG)	N=1544 <16: 756 (49%) 16-34: 463 (30%) 35-60: 325 (21%)	N= 211 <16: 136 (64.5%) 16-34: 45 (21.3%) 35-60: 30 (14.2%)		**0.0001**
rs1420101 (*IL1RL1*)	N= 462 <16: 224 (48.5%) 16-34: 146 (31.6%) 35-60: 92 (19.9%)	N= 824 <16: 414 (50.2%) 16-34: 235 (28.5%) 35-60: 175 (21.2%)	N= 465 <16: 250 (53.8%) 16-34: 128 (27.5%) 35-60: 87 (18.7%)	0.434
rs1106639 (*D2HGDH*)	N= 84 <16: 46 (54.8%) 16-34: 21 (25%) 35-60: 17 (20.2%)	N= 624 <16: 304 (48.7%) 16-34: 187 (30%) 35-60: 133 (21.3%)	N= 1046 <16: 541 (51.7%) 16-34: 300 (28.7%) 35-60: 205 (19.6%)	0.692
rs532965 (*HLA-DQA1*)	N= 1018 <16: 510 (50.1%) 16-34: 296 (29.1%) 35-60: 212 (20.8%)	N= 656 <16: 331 (50.5%) 16-34: 198 (30.2%) 35-60: 127 (19.4%)	N= 77 <16: 45 (58.4%) 16-34: 16 (20.8%) 35-60: 16 (20.8%)	0.475
rs9272629 (*HLA-DQB1*)	N= 284 <16: 133 (46.8%) 16-34: 76 (26.8%) 35-60: 75 (26.4%)	N= 554 <16: 289 (52.2%) 16-34: 160 (28.9%) 35-60: 105 (19%)	N= 645 <16: 187 (46.6%) 16-34: 133 (33.2%) 35-60: 81 (20.2%)	0.380
rs1847472 (*BACH2*)	N=197 <16: 107 (54.3%) 16-34: 58 (29.4%) 35-60: 32 (16.2%)	N=788 <16: 388 (49.2%) 16-34: 235 (29.8%) 35-60: 165 (20.9%)	N=768 <16: 337 (52.2%) 16-34: 189 (29.3%) 35-60: 119 (18.4%)	0.072
rs13275219 (*MIR5708*)	N=699 <16: 359 (51.4%) 16-34: 197 (28.2%) 35-60: 143 (20.5%)	N=828 <16: 409 (49.4%) 16-34: 250 (30.2%) 35-60: 169 (20.4%)	N=226 <16: 123 (54.4%) 16-34: 60 (26.5%) 35-60: 43 (19%)	0.704
rs10905279 (*GATA3*)	N= 604 <16: 330 (54.6%) 16-34: 155 (25.7%) 35-60: 119 (19.7%)	N= 886 <16: 429 (48.4%) 16-34: 273 (30.8%) 35-60: 184 (20.8%)	N= 266 <16: 132 (49.6%) 16-34:81 (30.5%) 35-60: 53 (19.9%)	0.162
rs1444782 (*LOC101928272*)	N= 257 <16: 138 (53.7%)	N= 850 <16: 440 (51.8%)	N= 646 <16: 312 (48.3%)	0.419

	16-34: 74 (28.8%) 35-60: 45 (17.5%)	16-34: 234 (27.5%) 35-60: 176 (20.6%)	16-34: 199 (30.8%) 35-60: 135 (20.9%)	
rs28592983 (*MUC5AC*)	N= 746 <16: 390 (52.3%) 16-34: 200 (26.8%) 35-60: 156 (20.9%)	N= 761 <16: 381 (50.1%) 16-34: 238 (31.3%) 35-60: 142 (18.7%)	N= 244 <16: 116 (47.5%) 16-34: 71 (29.1%) 35-60: 57 (23.4%)	0.217
rs61893460 (*C11orf30*)	N= 468 <16: 217 (46.4%) 16-34: 155 (33.1%) 35-60: 96 (20.5%)	N= 834 <16: 436 (52.3%) 16-34: 226 (27.1%) 35-60: 172 (20.6%)	N= 444 <16: 232 (52.3%) 16-34: 126 (28.4%) 35-60: 86 (19.4%)	0.169
rs10876864 (*RPS26*)	N= 384 <16: 201 (52.3%) 16-34: 111 (28.9%) 35-60: 72 (18.8%)	N= 552 <16: 277 (50.2%) 16-34: 161 (29.2%) 35-60: 114 (20.7%)	N= 342 <16: 174 (50.9%) 16-34: 101 (29.5%) 35-60: 67 (19.6%)	0.993
rs10519067 (*RORA*)	N=373 <16: 184 (49.3%) 16-34: 96 (25.7%) 35-60: 93 (24.9%)		N= 1387 <16: 710 (51.2%) 16-34: 413 (29.8%) 35-60: 264 (19%)	**0.032**
rs36045143 (*CLEC16A*)	N= 111 <16: 50 (45%) 16-34: 33 (29.7%) 35-60: 28 (25.2%)	N= 597 <16: 301 (50.4%) 16-34: 173 (29%) 35-60: 123 (20.6%)	N= 1050 <16: 541 (51.5%) 16-34: 304 (29%) 35-60: 205 (19.5%)	0.638
rs16948048 (*ZNF652*)	N= 665 <16: 313 (47.1%) 16-34: 202 (30.4%) 35-60: 150 (22.6%)	N= 834 <16: 439 (52.6%) 16-34: 227 (27.2%) 35-60: 168 (20.1%)	N= 255 <16: 137 (53.7%) 16-34: 80 (31.4%) 35-60: 38 (14.9%)	**0.041**
rs3806933 (*WDR36*)	N= 301 <16: 158 (52.5%) 16-34: 87 (28.9%) 35-60: 56 (18.6%)	N= 822 <16: 411 (50%) 16-34: 238 (29%) 35-60: 173 (21%)	N= 635 <16: 323 (50.9%) 16-34: 185 (29.1%) 35-60: 127 (20%)	0.916
rs1837253 (*TSLP*)	N= 89 <16: 45 (50.6%) 16-34: 24 (27%) 35-60: 20 (22.5%)	N= 613 <16: 306 (49.9%) 16-34: 187 (30.5%) 35-60: 120 (19.6%)	N= 1058 <16: 543 (51.3%) 16-34: 299 (28.3%) 35-60: 216 (20.4%)	0.857
rs17293632 (*SMAD3*)	N=947 <16: 477 (50.4%) 16-34: 268 (28.3%) 35-60: 202 (21.3%)	N=707 <16: 350 (49.5%) 16-34: 219 (31%) 138 (19.5%)	N=104 <16: 64 (61.5%) 16-34: 24 (23.1%) 35-60: 16 (15.4%)	0.139
rs17388568 (*KIAA1109*)	N= 896 <16: 459 (51.2%) 16-34: 240 (26.8%) 35-60: 197 (22%)	N= 723 <16: 369 (51%) 16-34: 228 (31.5%) 35-60: 126 (17.4%)	N= 130 <16: 59 (45.4%) 16-34: 38 (29.2%) 35-60: 33 (25.4%)	**0.045**
rs9303277 (*IKZF3*)	N= 414 <16: 168 (40.6%) 16-34: 132 (31.9%) 35-60: 114 (27.5%)	N= 822 <16: 430 (52.3%) 16-34: 241 (29.3%) 35-60: 151 (18.4%)	N= 479 <16: 270 (56.4%) 16-34: 125 (26.1%) 35-60: 84 (17.5%)	**$1.0x10^{-5}$**

Table 8.4.12 Genetic signals identified in the moderate to severe asthma GWAS and their association with asthma age of onset: data is presented as percentage and n's between the age of onset groups across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. Chi-squared test was used to analyse both these categorical groups. *Two genotyping groups were combined together due to the n's being low in one of the groups. Some of the signals however did not meet Bonferroni correction p= 0.002.

8.4.13 Association of FEV_1 with the Moderate-Severe Asthma Genetic Signals

Rs number	0	1	2	P-value	Power
rs10077785 (*C5orf56*)	N=1021 2.50 (0.83)	N=756 2.46 (0.85)	N=135 2.42 (0.84)	0.253	0.30
rs167769 (*STAT6*)	N=606 2.50 (0.84)	N=951 2.47 (0.84	N=353 2.49 (0.82)	0.782	0.09
rs1342326 (*IL33*)	N=1232 2.47 (0.84)	N=602 2.49 (0.82)	N=74 2.56 (0.93)	0.902	0.07
rs2244012 (*RAD50*)	N=1178 2.45 (0.82)	N=643 2.54 (0.85)	N=91 2.46 (0.96)	0.172	0.37
rs1773542 (*CD247*)	N=296 2.55 (0.88)	N=866 2.46 (0.83)	N=745 2.47 (0.83)	0.606	0.13
rs61816764 (FLG)	N=1694 2.47 (0.84)	N=202 2.57 (0.85)	N=13 2.22 (0.95)	0.354	0.23
rs1420101 (*IL1RL1*)	N=533 2.48 (0.87)	N=912 2.44 (0.82)	N=463 2.55 (0.84)	0.328	0.25
rs1106639 (*D2HGDH*)	N=95 2.50 (0.81)	N=667 2.45 (0.82)	N=1148 2.50 (0.85)	0.301	0.26
rs532965 (*HLA-DQA1*)	N=1141 2.50 (0.84)	N=683 2.46 (0.85)	N=83 2.38 (0.77)	0.076	0.52
rs9272629 (*HLA-DQB1*)	N=314 2.51 (0.83)	N=691 2.38 (0.81)	N=793 2.41 (0.86)	0.074 *excl weight	0.52
rs1847472 (*BACH2*)	N=207 2.51 (0.81)	N=851 2.50 (0.87)	N=849 2.45 (0.81)	0.653	0.12
rs13275219 (*MIR5708*)	N=779 2.47 (0.82)	N=876 2.47 (0.84)	N=252 2.53 (0.85)	0.541	0.15
rs10905279 (*GATA3*)	N=677 2.49 (0.83)	N=938 2.50 (0.83)	N=298 2.41 (0.89)	0.256	0.30
rs1444782 (*LOC101928272*)	N=265 2.49 (0.83)	N=921 2.48 (0.85	N=717 2.47 (0.83)	0.716	0.10
rs28592983 (*MUC5AC*)	N=821 2.5 (0.85)	N=837 2.43 (0.83)	N=249 2.47 (0.73)	0.129	0.42
rs61893460 (*C11orf30*)	N=494 2.45 (0.81)	N=927 2.48 (0.85)	N=479 2.49 (0.83)	0.724	0.10
rs10876864 (*RPS26*)	N=607 2.51 (0.82)	N=947 2.47 (0.85)	N=351 2.45 (0.84)	0.212	0.33
rs10519067 (*RORA*)	N=15 2.26 (0.81)	N=376 2.54 (0.83)	N=1524 2.47 (0.84)	0.446	0.19
rs36045143 (*CLEC16A*)	N=111 2.47 (0.85)	N=635 2.49 (0.80)	N=1168 2.47 (0.86)	0.441	0.19
rs16948048 (*ZNF652*)	N=708 2.50 (0.83)	N=930 2.49 (0.85)	N=268 2.41 (0.80)	0.909	0.07
rs3806933 (*WDR36*)	N=333 2.48 (0.83)	N=885 2.47 (0.85)	N=692 2.49 (0.83)	0.597	0.14
rs1837253 (*TSLP*)	N=85 2.61 (0.83)	N=684 2.48 (0.88)	N=1144 2.47 (0.81)	0.468	0.18
rs17293632 (*SMAD3*)	N=1028 2.48 (0.83)	N=777 2.48 (0.85)	N=106 2.44 (0.85)	0.543	0.15
rs17388568 (*KIAA1109*)	N=994 2.46 (0.85)	N=765 2.48 (0.83)	N=148 2.56 (0.85)	0.071	0.53
rs9303277 (*IKZF3*)	N=459 2.53 (0.81)	N=891 2.46 (0.84)	N=522 2.48 (0.87)	**0.054**	0.57

Table 8.4.13 Genetic signals identified in the moderate-severe asthma GWAS and their association with FEV_1prbd: data is presented as mean and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A univariate analysis model corrected for age, gender, height and weight was used to analyse FEV_1prbd with the genetic signals. Bonferroni correction p= 0.002.

8.4.14 Association of FVC with the Moderate-Severe Asthma Genetic Signals

Rs number	0	1	2	P-value	Power
rs10077785 (*C5orf56*)	N=1003 3.48 (0.98)	N=741 3.51 (1.04)	N=135 3.43 (0.94)	0.634	0.13
rs167769 (*STAT6*)	N=597 3.50 (0.99)	N=930 3.49 (1.0)	N=350 3.47 (0.99)	0.257	0.29
rs1342326 (*IL33*)	N=1214 3.47 (1.0)	N=589 3.52 (0.95)	N=72 3.61 (1.11)	0.943	0.06
rs2244012 (*RAD50*)	N=1156 3.45 (0.95)	N=634 3.56 (1.04)	N=89 3.49 (0.81)	0.155	0.39
rs1773542 (*CD247*)	N=288 3.58 (1.04)	N=850 3.45 (0.99)	N=736 3.48 (0.97)	0.532	0.16
rs61816764 (FLG)	N=1665 3.47 (0.98)	N=198 3.63 (1.08)	N=13 3.09 (0.84)	0.163	0.38
rs1420101 (*IL1RL1*)	N=524 3.53 (1.03)	N=898 3.44 (0.97)	N=453 3.53 (0.99)	0.209	0.33
rs1106639 (*D2HGDH*)	N=94 3.41 (0.93)	N=659 3.42 (0.99)	N=1124 3.53 (1.0)	0.182	0.36
rs532965 (*HLA-DQA1*)	N=1117 3.49 (0.99)	N=676 3.49 (1.01)	N=81 3.38 (0.88)	0.166	0.38
rs9272629 (*HLA-DQB1*)	N=285 3.54 (1.0)	N=624 3.40 (0.94)	N=711 3.47 (1.02)	0.468	0.18
rs1847472 (*BACH2*)	N=205 3.48 (0.94)	N=833 3.50 (1.0)	N=836 3.48 (1.0)	0.899	0.07
rs13275219 (*MIR5708*)	N=767 3.48 (0.97)	N=859 3.47 (1.0)	N=248 3.56 (1.01)	0.243	0.31
rs10905279 (*GATA3*)	N=664 3.50 (0.99)	N=923 3.48 (0.97)	N=293 3.48 (1.05)	0.462	0.18
rs1444782 (*LOC101928272*)	N=258 3.50 (0.96)	N=903 3.49 (1.01)	N=710 2.48 (0.97)	0.656	0.12
rs28592983 (*MUC5AC*)	N=804 3.56 (1.0)	N=826 3.43 (0.99)	N=244 3.43 (0.98)	**0.015**	0.74
rs61893460 (*C11orf30*)	N=488 3.48 (0.99)	N=907 3.48 (1.0)	N=473 3.50 (0.99)	0.164	0.38
rs10876864 (*RPS26*)	N=593 3.49 (0.97)	N=935 3.50 (1.02)	N=344 3.45 (0.97)	0.737	0.10
rs10519067 (*RORA*)	N=15 2.96 (0.89)	N=368 3.55 (1.05)	N=1500 3.48 (0.98)	0.885	0.07
rs36045143 (*CLEC16A*)	N=109 3.41 (0.96)	N=627 3.48 (0.95)	N=1145 3.50 (1.02)	0.941	0.06
rs16948048 (*ZNF652*)	N=697 3.48 (0.95)	N=913 3.51 (1.03)	N=264 3.43 (0.94)	0.549	0.15
rs3806933 (*WDR36*)	N=329 3.44 (0.95)	N=875 3.47 (1.0)	N=674 3.53 (1.0)	0.183	0.36
rs1837253 (*TSLP*)	N=84 3.49 (0.95)	N=672 3.47 (1.04)	N=1124 3.50 (0.97)	0.676	0.11
rs17293632 (*SMAD3*)	N=1005 3.48 (0.98)	N=768 3.49 (1.01)	N=105 3.53 (1.02)	0.884	0.07
rs17388568 (*KIAA1109*)	N=979 3.47 (1.01)	N=752 3.50 (0.97)	N=143 3.54 (1.01)	0.314	0.26
rs9303277 (*IKZF3*)	N=451 3.53 (0.99)	N=874 3.45 (0.97)	N=514 3.51 (1.04)	0.099	0.47

Table 8.4.14 Genetic signals identified in the moderate to severe asthma GWAS and their association with FVCprbd: data is presented as mean and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A univariate analysis model corrected for age, gender, height and weight was used to analyse FVCprbd with the genetic signals. Bonferroni correction p= 0.002.

8.4.15 Association of Peak Expiratory Flow with the Moderate-Severe Asthma Genetic signals

Rs number	0	1	2	P-value	Power
rs10077785 (*C5orf56*)	N=610 385.02 (129.85)	N=445 395.58 (132.67)	N=83 378.27 (125.34)	0.325	1.0
rs167769 (*STAT6*)	N=347 397.06 (134.39)	N=581 386.30 (131.84)	N=207 382.69 (120.47)	0.359	1.0
rs1342326 (*IL33*)	N=737 388.74 (131.66)	N=355 388.60 (127.41)	N=45 393.34 (138.15)	0.973	0.12
rs2244012 (*RAD50*)	N=653 380.60 (129.51)	N=318 384.65 (127.70)	N=45 363.29 (132.40)	0.575	1.0
rs1773542 (*CD247*)	N=173 389.93 (141.58)	N=531 388.43 (129.89)	N=431 387.48 (126.40)	0.978	0.09
rs61816764 (FLG)	N=1002 388.31 (129.72)	N=134 388.30 (133.76)		1.0	0.05
rs1420101 (*IL1RL1*)	N=341 385.26 (125.07)	N=557 387.68 (134.01)	N=236 396.51 (130.35)	0.573	1.0
rs1106639 (*D2HGDH*)	N=58 392.38 (120.83)	N=412 391.97 (132.08)	N=668 386.03 (130.59)	0.748	1.0
rs532965 (*HLA-DQA1*)	N=676 387.07 (127.48)	N=411 391.48 (136.04)	N=50 387.10 (130.15)	0.862	1.0
rs9272629 (*HLA-DQB1*)	N=147 406.98 (134.03)	N=371 376.44 (124.18)	N=401 394.61 (137.60)	**0.033**	1.0
rs1847472 (*BACH2*)	N=112 412.24 (131.93)	N=516 386.44 (135.01)	N=508 385.08 (125.33)	0.124	1.0
rs13275219 (*MIR5708*)	N=452 390.62 (130.80)	N=545 387.55 (128.88)	N=138 388.24 (136.53)	0.932	0.55
rs10905279 (*GATA3*)	N=387 387.11 (127.58)	N=554 386.12 (126.97)	N=199 398.96 (145.42)	0.472	1.0
rs1444782 (*LOC101928272*)	N=167 396.80 (124.03)	N=516 382.07 (128.72)	N=449 392.78 (134.87)	0.300	1.0
rs28592983 (*MUC5AC*)	N=486 390.44 (130.73)	N=503 385.05 (130.56)	N=148 396.19 (130.42)	0.617	1.0
rs61893460 (*C11orf30*)	N=301 380.48 (127.48)	N=552 393.40 (138.00)	N=282 389.39 (118.14)	0.384	1.0
rs10876864 (*RPS26*)	N=373 392.70 (124.62)	N=547 386.30 (134.03)	N=215 389.17 (132.47)	0.767	1.0
rs10519067 (*RORA*)	N=15 385.53 (131.53)	N=225 400.02 (124.44)	N=900 385.92 (132.00)	0.348	1.0
rs36045143 (*CLEC16A*)	N=64 392.49 (125.34)	N=392 393.77 (130.37)	N=683 385.55 (131.26)	0.594	1.0
rs16948048 (*ZNF652*)	N=436 391.10 (130.27)	N=556 384.07 (131.50)	N=145 400.77 (126.53)	0.352	1.0
rs3806933 (*WDR36*)	N=214 383.60 (125.97)	N=505 385.89 (128.15)	N=418 394.19 (135.72)	0.524	1.0
rs1837253 (*TSLP*)	N=47 405.95 (109.18)	N=420 384.20 (135.60)	N=671 390.22 (128.87)	0.495	1.0
rs17293632 (*SMAD3*)	N=616 379.66 (122.55)	N=464 400.77 (139.81)	N=57 387.18 (130.43)	**0.031**	1.0
rs17388568 (*KIAA1109*)	N=616 388.76 (134.34)	N=443 390.55 (127.71)	N=77 374.72 (117.51)	0.617	1.0
rs9303277 (*IKZF3*)	N=279 395.58 (137.00)	N=537 384.62 (123.41)	N=316 389.33 (137.12)	0.522	1.0

Table 8.4.15 Genetic signals identified in the moderate-severe asthma GWAS and their association with PEF: data is presented as mean and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe

asthma. A one-way ANOVA was used to analyse PEF with the genetic signals. Bonferroni correction p= 0.002.

8.4.16 Association of Serum IgE with the Moderate-Severe Asthma Genetic Signals

Rs number	0	1	2	P-value	Power
rs10077785 (*C5orf56*)	N=684 2.03 (0.70)	N=532 2.12 (0.70)	N=100 2.00 (0.76)	**0.037**	0.63
rs167769 (*STAT6*)	N=438 1.99 (0.71)	N=632 2.08 (0.70)	N=245 2.15 (0.69)	**0.007**	0.81
rs1342326 (*IL33*)	N=860 2.05 (0.70)	N=409 2.11 (0.72)	N=47 1.96 (0.62)	0.126	0.43
rs2244012 (*RAD50*)	N=800 2.03 (0.69)	N=452 2.14 (0.74)	N=65 2.0 (0.71)	**0.028**	0.66
rs1773542 (*CD247*)	N=191 2.08 (0.70)	N=606 2.05 (0.69)	N=517 2.07 (0.73)	0.980	0.05
rs61816764 (FLG)	N=1147 2.04 (0.69)	N=156 2.21 (0.73)	N=12 2.06 (0.73)	0.058	0.56
rs1420101 (*IL1RL1*)	N=378 2.07 (0.68)	N=643 2.07 (0.72)	N=292 2.01 (0.71)	0.172	0.37
rs1106639 (*D2HGDH*)	N=61 1.93 (0.68)	N=465 2.01 (0.69)	N=791 2.10 (0.71)	**0.049**	0.59
rs532965 (*HLA-DQA1*)	N=776 2.09 (0.71)	N=481 2.02 (0.69)	N=57 1.95 (0.72)	0.061	0.55
rs9272629 (*HLA-DQB1*)	N=185 2.00 (0.71)	N=444 2.12 (0.71)	N=484 2.08 (0.69)	0.097	0.47
rs1847472 (*BACH2*)	N=131 2.05 (0.74)	N=580 2.05 (0.71)	N=603 2.07 (0.68)	0.713	0.10
rs13275219 (*MIR5708*)	N=518 2.02 (0.71)	N=613 2.08 (0.71)	N=178 2.12 (0.67)	0.328	0.25
rs10905279 (*GATA3*)	N=446 2.04 (0.70)	N=666 2.05 (0.72)	N=204 2.15 (0.66)	0.104	0.46
rs1444782 (*LOC101928272*)	N=186 2.07 (0.70)	N=617 2.09 (0.70)	N=509 2.03 (0.70)	0.406	0.21
rs28592983 (*MUC5AC*)	N=562 2.06 (0.69)	N=580 2.09 (0.70)	N=173 1.98 (0.75)	0.322	0.251
rs61893460 (*C11orf30*)	N=363 1.98 (0.71)	N=610 2.07 (0.68)	N=337 2.14 (0.73)	**0.009**	0.79
rs10876864 (*RPS26*)	N=401 2.04 (0.72)	N=655 2.07 (0.69)	N=258 2.08 (0.70)	0.627	0.13
rs10519067 (*RORA*)	N=12 1.60 (0.56)	N=268 2.03 (0.72)	N=1037 2.08 (0.70)	**0.032**	0.65
rs36045143 (*CLEC16A*)	N=69 1.87 (0.67)	N=429 2.06 (0.69)	N=818 2.08 (0.71)	0.158	0.39
rs16948048 (*ZNF652*)	N=471 2.0 (0.68)	N=654 2.08 (0.73)	N=189 2.11 (0.68)	0.139	0.41
rs3806933 (*WDR36*)	N=236 2.01 (0.77)	N=599 2.07 (0.69)	N=481 2.08 (0.68	0.446	0.19
rs1837253 (*TSLP*)	N=59 1.86 (0.76)	N=459 2.04 (0.72)	N=797 2.09 (0.68)	**0.039**	0.62
rs17293632 (*SMAD3*)	N=695 2.08 (0.71)	N=540 2.04 (0.70)	N=82 2.04 (0.68)	0.587	0.14
rs17388568 (*KIAA1109*)	N=683 2.08 (0.71)	N=532 2.04 (0.70)	N=97 2.10 (0.68)	0.515	0.16
rs9303277 (*IKZF3*)	N=316 2.09 (0.69)	N=612 2.05 (0.71)	N=371 2.06 (0.70)	0.736	0.10

Table 8.4.16 Genetic signals identified in the moderate-severe asthma GWAS and their association with serum IgE levels: data is presented as mean (log_{10}) and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A univariate analysis model corrected for age and gender was used to analyse IgE with the genetic signals. Bonferroni correction p= 0.002.

8.4.17 Association of ACQ Score with the Moderate-Severe Asthma Genetic Signals

Rs number	0	1	2	P-value	Power
rs10077785 (*C5orf56*)	N=457 1.31 (0.51)	N=314 1.30 (0.51)	N=59 1.34 (0.14-5.28)	0.851	0.99
rs167769 (*STAT6*)	N=266 1.31 (0.50)	N=406 1.33 (0.50)	N=158 1.25 (0.55)	0.224	1.0
rs1342326 (*IL33*)	N=540 1.30 (0.50))	N=270 1.32 (0.51)	N=20 1.31 (0.49)	0.838	1.0
rs2244012 (*RAD50*)	N=506 1.31 (0.50)	N=291 1.30 (0.52)	N=34 1.33 (0.47)	0.962	0.16
rs1773542 (*CD247*)	N=144 1.32 (0.48)	N=380 1.27 (0.52)	N=304 1.36 (0.50)	0.071	1.0
rs61816764 (FLG)	N=731 1.32 (0.50)	N=98 1.23 (0.50)		0.091	0.39*
rs1420101 (*IL1RL1*)	N=154 1.28 (0.49)	N=387 1.29 (0.52)	N=288 1.36 (0.50)	0.117	1.0
rs1106639 (*D2HGDH*)	N=45 1.27 (0.47)	N=277 1.27 (0.53)	N=506 1.33 (0.49)	0.309	1.0
rs532965 (*HLA-DQA1*)	N=506 1.28 (0.51)	N=282 1.33 (0.49)	N=42 1.38 (0.57)	0.265	1.0
rs9272629 (*HLA-DQB1*)	N=173 1.26 (0.52)	N=267 1.30 (0.54)	N=357 1.36 (0.46)	0.055	1.0
rs1847472 (*BACH2*)	N=85 1.37 (0.53)	N=376 1.25 (0.51)	N=369 1.35 (0.49)	**0.016**	1.0
rs13275219 (*MIR5708*)	N=331 1.29 (0.51)	N=394 1.33 (0.51)	N=103 1.30 (0.48)	0.647	1.0
rs10905279 (*GATA3*)	N=302 1.33 (0.48)	N=408 1.30 (0.53)	N=121 1.27 (0.48)	0.525	1.0
rs1444782 (*LOC101928272*)	N=107 1.16 (0.52)	N=419 1.35 (0.49)	N=302 1.31 (0.52)	**0.004**	1.0
rs28592983 (*MUC5AC*)	N=382 1.30 (0.50)	N=345 1.32 (0.51)	N=101 1.32 (0.52)	0.873	0.94
rs61893460 (*C11orf30*)	N=189 1.28 (0.50)	N=424 1.31 (0.50)	N=209 1.34 (0.53)	0.420	1.0
rs10876864 (*RPS26*)	N=263 1.28 (0.50)	N=426 1.33 (0.49)	N=140 1.29 (0.55)	0.382	1.0
rs10519067 (*RORA*)	N=157 1.31 (0.49)		N=675 1.31 (0.51)	0.959	0.05*
rs36045143 (*CLEC16A*)	N=53 1.26 (0.50)	N=300 1.29 (0.53)	N=479 1.33 (0.50)	0.482	1.0
rs16948048 (*ZNF652*)	N=299 1.29 (0.50)	N=413 1.32 (0.50)	N=116 1.32 (0.54)	0.726	1.0
rs3806933 (*WDR36*)	N=131 1.22 (0.53)	N=388 1.34 (0.50)	N=313 1.30 (0.50)	0.066	1.0
rs1837253 (*TSLP*)	N=51 1.27 (0.52)	N=295 1.27 (0.50)	N=488 1.33 (0.51)	0.196	1.0
rs17293632 (*SMAD3*)	N=454 1.30 (0.50)	N=329 1.31 (0.50)	N=48 1.35 (0.56)	0.768	1.0
rs17388568 (*KIAA1109*)	N=410 1.30 (0.50)	N=354 1.31 (0.52)	N=64 1.37 (0.52)	0.536	1.0
rs9303277 (*IKZF3*)	N=186 1.30 (0.51)	N=378 1.30 (0.49)	N=226 1.30 (0.53)	0.991	0.05

Table 8.4.17 Genetic signals identified in the moderate-severe asthma GWAS and their association with ACQ symptom score: data is presented as mean (square root) and standard deviation across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying

2 risk alleles for moderate-severe asthma. A one-way ANOVA was used to analyse the mean ACQ score with the asthma genetic signals. Bonferroni correction p=0.002.

8.4.18 Association of Hospital Admissions with the Moderate-Severe Asthma Genetic Signals

Rs number	0	1	2	P-value
rs10077785 (*C5orf56*)	N=496 153 (30.7%)	N=330 117 (34.9%)	N=63 30 (47.6%)	**0.02**
rs167769 (*STAT6*)	N=279 98 (35%)	N=441 149 (33.4%)	N=169 53 (31.2%)	0.586
rs1342326 (*IL33*)	N=576 191 (32.9%)	N=286 97 (33.7%)	N=25 8 (30.8%)	0.901
rs2244012 (*RAD50*)	N=533 176 (32.8%)	N=317 110 (34.4%)	N=37 13 (35.1%)	0.946
rs1773542 (*CD247*)	N=149 49 (32.7%)	N=400 140 (34.9%)	N=337 108 (31.6%)	0.574
rs61816764 (FLG)	N=783 264 (33.7%)	N=113 35 (31%)		0.563
rs1420101 (*IL1RL1*)	N=152 52 (34%)	N=409 142 (34.6%)	N=326 (31.4%)	0.686
rs1106639 (*D2HGDH*)	N=46 12 (26.1%)	N=296 99 (33.2%)	N=544 188 (34.2%)	0.576
rs532965 (*HLA-DQA1*)	N=531 172 (32.1%	N=314 106 (33.7%)	N=42 19 (44.2%)	0.198
rs9272629 (*HLA-DQB1*)	N=185 58 (31.4%)	N=281 94 (33.1%)	N=385 (34.4%)	0.798
rs1847472 (*BACH2*)	N=93 34 (36.2%)	N=405 134 (32.8%)	N=389 130 (33.2%)	0.858
rs13275219 (*MIR5708*)	N=354 125 (35%)	N=417 143 (34%)	N=114 29 (25.5%)	0.091
rs10905279 (*GATA3*)	N=328 109 (33%)	N=434 141 (32.2%)	N=126 49 (38.3%)	0.529
rs1444782 (*LOC101928272*)	N=109 38 (34.8%)	N=442 145 (32.4%)	N=334 115 (34.3%)	0.717
rs28592983 (*MUC5AC*)	N=392 123 (31.2%)	N=385 130 (33.3%)	N=107 44 (41.1%)	0.129
rs61893460 (*C11orf30*)	N=209 59 (27.7%)	N=549 173 (29.8%)	N=220 66 (29.7%)	**0.006**
rs10876864 (*RPS26*)	N=267 87 (32.1%)	N=455 153 (33.5%)	N=164 59 (35.8%)	0.692
rs10519067 (*RORA*)	N=169 46 (27.2%)		N=729 253 (34.7%)	0.063
rs36045143 (*CLEC16A*)	N=55 18 (32.7%)	N=321 96 (29.6%)	N=516 186 (35.8%)	0.299
rs16948048 (*ZNF652*)	N=317 108 (33.8%)	N=438 152 (34.5%)	N=130 36 (27.3%)	0.312
rs3806933 (*WDR36*)	N=143 42 (29.2%)	N=417 141 (33.7%)	N=330 116 (34.7%)	0.468
rs1837253 (*TSLP*)	N=47 19 (40.4%)	N=310 98 (31.2%)	N=535 182 (33.8%)	0.328
rs17293632 (*SMAD3*)	N=480 153 (31.5%)	N=356 117 (32.8%)	N=53 27 (50%)	**0.02**
rs17388568 (*KIAA1109*)	N=414 139 (33.2%)	N=395 133 (33.5%)	N=75 24 (32%)	0.947
rs9303277 (*IKZF3*)	N=197 64 (32.3%)	N=404 134 (32.9%)	N=247 86 (34.4%)	0.939

Table 8.4.18 Genetic signals identified in the moderate to severe asthma GWAS and their association with hospital admissions: data is presented as percentage and N's for the patients who had a hospital admission in the last year and the total number of patients who had hospital admissions data across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles

for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma.. A chi-squared test was used to analyse these categorical groups. Bonferroni correction p=0.002.

8.4.19 Association of Atopic Dermatitis with the Moderate-Severe Asthma Genetic Signals

Rs number	0	1	2	P-value
rs10077785 (*C5orf56*)	N=412 146 (35.4%)	N=286 101 (35.3%)	N=56 11 (19.6%)	0.058
rs167769 (*STAT6*)	N=252 85 (33.7%)	N=373 129 (34.6%)	N=127 43 (33.9%)	0.973
rs1342326 (*IL33*)	N=476 170 (35.7%)	N=248 80 (32.3%)	N=30 9 (30%)	0.570
rs2244012 (*RAD50*)	N=471 155 (32.3%)	N=250 86 (34.4%)	N=33 16 (48.5%)	0.188
rs1773542 (*CD247*)	N=122 44 (36.1%)	N=357 115 (32.2%)	N=274 98 (35.8%)	0.573
rs61816764 (FLG)	N=658 215 (32.7%)	N=94 43 (45.7%)		**0.013**
rs1420101 (*IL1RL1*)	N=236 81 (34.3%)	N=381 138 (36.2%)	N=134 38 (28.4%)	0.256
rs1106639 (*D2HGDH*)	N=41 16 (39%)	N=268 81 (30.2%)	N=445 162 (36.4%)	0.197
rs532965 (*HLA-DQA1*)	N=430 142 (33%)	N=296 108 (36.5%)	N=28 8 (28.6%)	0.510
rs9272629 (*HLA-DQB1*)	N=75 35 (46.7%)	N=222 89 (40.1%)	N=220 99 (45.0%)	0.459
rs1847472 (*BACH2*)	N=81 25 (30.9%)	N=354 131 (37%)	N=319 102 (32%)	0.310
rs13275219 (*MIR5708*)	N=297 93 (31.3%)	N=360 130 (36.1%)	N=97 36 (37.1%)	0.361
rs10905279 (*GATA3*)	N=250 100 (40%)	N=384 119 (31%)	N=122 40 (32.8%)	0.061
rs1444782 (*LOC101928272*)	N=112 30 (26.8%)	N=376 138 (36.7%)	N=264 89 (33.7%)	0.149
rs28592983 (*MUC5AC*)	N=341 115 (33.7%)	N=305 100 (32.8%)	N=107 42 (39.3%)	0.468
rs61893460 (*C11orf30*)	N=202 66 (31.7%)	N=354 115 (32.5%)	N=197 75 (38.1%)	0.372
rs10876864 (*RPS26*)	N=263 92 (35%)	N=350 125 (35.7%)	N=140 41 (29.3%)	0.381
rs10519067 (*RORA*)	N=166 52 (31.3%)		N=590 207 (35.1%)	0.367
rs36045143 (*CLEC16A*)	N=47 12 (25.5%)	N=238 79 (33.2%)	N=470 167 (35.5%)	0.359
rs16948048 (*ZNF652*)	N=309 101 (32.7%)	N=339 120 (35.4%)	N=107 37 (34.6%)	0.764
rs3806933 (*WDR36*)	N=143 46 (32.2%)	N=353 123 (34.8%)	N=258 90 (34.9%)	0.830
rs1837253 (*TSLP*)	N=37 8 (21.6%)	N=272 91 (33.5%)	N=447 160 (35.8%)	0.205
rs17293632 (*SMAD3*)	N=394 140 (35.5%)	N=323 105 (32.5%)	N=38 14 (36.8%)	0.659
rs17388568 (*KIAA1109*)	N=415 135 (32.5%)	N=292 105 (36%)	N=47 18 (38.3%)	0.531
rs9303277 (*IKZF3*)	N=180 70 (38.9%)	N=354 108 (30.5%)	N=216 78 (36.1%)	0.119

Table 8.4.19 Genetic signals identified in the moderate to severe asthma GWAS and their association with atopic dermatitis: data is presented as percentage and n's for the patients with allergic dermatitis and the total number of patients with allergic dermatitis data across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe

asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A chi-squared test was used to analyse both these categorical groups. Bonferroni correction p=0.002.

8.4.20 Association of Allergic Rhinitis with the Moderate-Severe Asthma Genetic Signals

Rs number	0	1	2	P-value
rs10077785 (*C5orf56*)	N=828 500 (60.4%)	N=574 358 (62.4%)	N=110 66 (60%)	0.733
rs167769 (*STAT6*)	N=512 308 (60.2%)	N=733 449 (61.3%)	N=265 164 (61.9%)	0.878
rs1342326 (*IL33*)	N=981 592 (60.3%)	N=481 300 (62.4%)	N=48 31 (64.6%)	0.668
rs2244012 (*RAD50*)	N=782 441 (56.4%)	N=405 257 (63.5%)	N=55 30 (54.5%)	0.053
rs1773542 (*CD247*)	N=246 143 (58.1%)	N=685 431 (62.9%)	N=578 346 (59.9%)	0.329
rs61816764 (FLG)	N=1343 823 (61.3%)	N=156 91 (58.3%)	N=10 6 (60%)	0.773
rs1420101 (*IL1RL1*)	N=393 222 (56.5%)	N=722 446 (61.8%)	N=393 251 (63.9%)	0.086
rs1106639 (*D2HGDH*)	N=74 48 (64.9%)	N=530 314 (59.2%)	N=906 559 (61.7%)	0.512
rs532965 (*HLA-DQA1*)	N=881 553 (62.8%)	N=561 331 (59%)	N=66 38 (57.6%)	0.299
rs9272629 (*HLA-DQB1*)	N=225 142 (63.1%)	N=463 298 (64.4%	N=548 353 (64.4%)	0.936
rs1847472 (*BACH2*)	N=174 110 (63.2%)	N=675 401 (59.4%)	N=661 411 (62.2%)	0.481
rs13275219 (*MIR5708*)	N=601 373 (62.1%)	N=713 433 (60.7%)	N=192 110 (57.3%)	0.498
rs10905279 (*GATA3*)	N=539 349 (64.7%)	N=761 453 (59.5%)	N=211 122 (57.8%)	0.092
rs1444782 (*LOC101928272*)	N=212 112 (52.8%)	N=744 464 (62.4%)	N=553 347 (62.7%)	0.027
rs28592983 (*MUC5AC*)	N=659 409 (62.1%)	N=643 396 (61.6%)	N=203 112 (55.2%)	0.192
rs61893460 (*C11orf30*)	N=382 228 (59.7%)	N=728 439 (60.3%)	N=392 248 (63.3%)	0.531
rs10876864 (*RPS26*)	N=482 292 (60.6%)	N=742 742 (63.1%)	N=282 157 (55.7%)	0.094
rs10519067 (*RORA*)	N=10 5 (50%)	N=304 192 (63.2%)	N=1200 727 (60.6%)	0.551
rs36045143 (*CLEC16A*)	N=92 56 (60.9%)	N=504 313 (62.1%)	N=918 555 (60.5%)	0.830
rs16948048 (*ZNF652*)	N=563 339 (60.2%)	N=728 447 (61.4%)	N=218 134 (61.5%)	0.898
rs3806933 (*WDR36*)	N=268 154 (57.5%)	N=702 429 (61.1%)	N=543 341 (62.8%)	0.341
rs1837253 (*TSLP*)	N=79 44 (55.7%)	N=544 352 (64.7%)	N=892 529 (59.3%)	0.076
rs17293632 (*SMAD3*)	N=802 497 (62%)	N=623 373 (59.9%)	N=87 52 (59.8%)	0.703
rs17388568 (*KIAA1109*)	N=770 456 (59.2%)	N=620 383 (61.8%)	N=118 80 (67.8%)	0.177
rs9303277 (*IKZF3*)	N=350 206 (58.9%)	N=709 434 (61.2%)	N=424 263 (62%)	0.648

Table 8.4.20 Genetic signals identified in the moderate to severe asthma GWAS and their association with allergic rhinitis: data is presented as percentage and n's for the patients with allergic rhinitis and the total number of patients with allergic rhinitis data across the risk allele groups for moderate-severe asthma with 0 indicating carrying no risk alleles for moderate-severe asthma, 1

carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A chi-squared test was used to analyse both these categorical groups. Bonferroni correction p=0.002.

8.4.21 Association of Blood Eosinophils with the Moderate-Severe Asthma Genetic Signals

Rs number	0	1	2	P-value
rs10077785 (*C5orf56*)	N= 360 137 (38.1%)	N=310 111 (35.8%)	N=61 27 (44.3%)	0.447
rs167769 (*STAT6*)	N=240 96 (40%)	N=347 129 (37.2%)	N= 143 49 (34.3%)	0.524
rs1342326 (*IL33*)	N=472 165 (35%)	N=229 93 (40.6%)	N=30 17 (56.7%)	**0.031**
rs2244012 (*RAD50*)	N=419 150 (35.8%)	N=256 100 (39.1%)	N=44 20 (45.5%)	0.374
rs1773542 (*CD247*)	N=99 32 (32.3%)	N=321 125 (38.9%)	N=310 117 (37.7%)	0.491
rs61816764 (FLG)	N=642 248 (38.6%)	N=88 27 (30.7%)		0.149
rs1420101 (*IL1RL1*)	N=238 91 (38.2%)	N=349 126 (36.1%)	N=143 58 (40.6%)	0.636
rs1106639 (*D2HGDH*)	N=37 8 (21.6%)	N=251 92 (36.7%)	N=443 175 (39.5%)	0.09
rs532965 (*HLA-DQA1*)	N=426 150 (35.2%)	N=273 111 (40.7%)	N=31 14 (45.2%)	0.237
rs9272629 (*HLA-DQB1*)	N=83 35 (42.2%)	N=247 85 (34.4%)	N=265 265 (42.6%)	0.225
rs1847472 (*BACH2*)	N=68 26 (38.2%)	N=333 117 (35.1%)	N=328 130 (39.6%)	0.135
rs13275219 (*MIR5708*)	N=287 106 (36.9%)	N=340 123 (36.2%)	N=101 44 (43.6%)	0.391
rs10905279 (*GATA3*)	N=262 100 (38.2%)	N=350 128 (36.6%)	N=119 47 (39.5%)	0.829
rs1444782 (*LOC101928272*)	N=121 49 (40.5%)	N=346 132 (38.2%)	N=261 94 (36%)	0.689
rs28592983 (*MUC5AC*)	N=304 113 (37.2%)	N=329 126 (38.3%)	N=98 36 (36.7%)	0.940
rs61893460 (*C11orf30*)	N=218 72 (33%)	N=333 131 (39.3%)	N=179 72 (40.2%)	0.235
rs10876864 (*RPS26*)	N=227 91 (40.1%)	N= 367 128 (34.9%)	N=135 56 (41.5%)	0.270
rs10519067 (*RORA*)	N=153 60 (39.2%)		N=578 215 (37.2%)	0.647
rs36045143 (*CLEC16A*)	N=38 7 (18.4%)	N=239 88 (36.8%)	N=454 180 (39.6%)	**0.033**
rs16948048 (*ZNF652*)	N=257 94 (36.6%)	N=367 129 (35.1%)	N=104 49 (47.1%)	0.08
rs3806933 (*WDR36*)	N=131 51 (38.9%)	N=329 125 (38%)	N=270 99 (36.7%)	0.896
rs1837253 (*TSLP*)	N=29 13 (44.8%)	N=254 87 (34.3%)	N=446 173 (38.8%)	0.346
rs17293632 (*SMAD3*)	N=374 153 (40.9%)	N=314 104 (33.1%)	N=42 18 (42.9%)	0.085
rs17388568 (*KIAA1109*)	N=397 153 (38.5%)	N=282 98 (34.8%)	N=50 23 (46%)	0.269
rs9303277 (*IKZF3*)	N=183 74 (40.4%)	N=348 134 (38.5%)	N=198 67 (33.8%)	0.380

Table 8.4.21 Genetic signals identified in the moderate to severe asthma GWAS and their association with blood eosinophil count: data is presented as percentage and n's for the patients who had a blood eosinophil count of >300 µL and then the total number of patients who had a blood eosinophil count across the risk allele groups for moderate-severe asthma with 0 indicating carrying no

risk alleles for moderate-severe asthma, 1 carrying 1 risk allele for moderate-severe asthma and 2 carrying 2 risk alleles for moderate-severe asthma. A chi-squared test was used to analyse both these categorical groups. Bonferroni correction p=0.002.

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