EXPLORING THE HETEROGENEITY OF ASTHMA EXACERBATIONS

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Abstract

Asthma is a chronic inflammatory disease of the airways that manifests due to a complex interaction between genetics and the environment. It leads to variable symptoms and airway obstruction.

A decline in lung function can be associated with worsening asthma symptoms that are not alleviated by the asthmatic's usual medication. This is the basis of asthma exacerbations. Despite our understanding of asthma as a heterogeneous disease process, most exacerbations are therapeutically managed one-dimensionally with oral corticosteroids (OCS).

Aims:

This thesis focuses on asthma exacerbations and will explore them through quantitative and laboratory-based studies.

Methods:

A longitudinal cohort study was conducted to explore the biological heterogeneity of asthma exacerbations. Participants were phenotyped at baseline and then at the point of an exacerbation through a combination of clinical, physiological, and clinically relevant biomarkers of airway inflammation. The cohort was longitudinally followed up over three years. Viruses are the most common cause of asthma exacerbation, and the proportion of exacerbations related to viral aetiology was examined. The heterogeneity of exacerbations was explored by characterising the gene expression at the point of an exacerbation through RNA sequencing.

Results:

Asthma exacerbations were associated with both T2-high and T2-low inflammation. Small airway Disease is a significant contributing factor to asthma exacerbations and has the potential to help distinguish asthma

exacerbations that require a course of steroids. Longitudinal assessment suggests that the inflammatory profile of asthma exacerbations is stable.

Viral PCR performed on nasal brush samples at baseline and during an exacerbation has identified that 80% of asthma exacerbations that required a course of steroids had a virus present. A virus at exacerbation was associated with worsening peripheral resistance of the smaller airways R5R20 1.30cmH20/L/S (p=0.03).

Transcriptomic analysis of nasal epithelial cells was obtained at baseline and the point of an exacerbation and identified enrichment of immune response pathways. Ingenuity Pathway Analysis (IPA) has identified gene modules associated with T1 and T2 inflammation and airway remodelling associated with asthma exacerbations.

Conclusions:

There is evidence of heterogeneity in asthma exacerbations, with T2-high and T2-low exacerbations associated with similar physiological reductions in lung function. Longitudinal follow-up of the APEX cohort has revealed stability within the identified phenotypes.

Thesis: Aim and Outline

Asthma is now well-recognised as a heterogeneous disease that can be a significant burden for those affected. It has also contributed to high healthcare costs. Asthma exacerbations are a common phenomenon that can lead to hospitalisation and can pose a risk of mortality.
Viruses are known to be the most common cause of asthma exacerbations, and upper respiratory tract symptoms are often described before the onset of an exacerbation. A growing number of targeted treatments have been developed to maintain asthma control. Despite the understanding of heterogeneity in asthma and the variety of triggers that lead to an asthma exacerbation, for most severe asthma exacerbations, the treatment is predominately one-dimensional with oral corticosteroids, which are known to have considerable side effects. The way we address and manage asthma exacerbations or exacerbations needs updating with the initial recognition that there could be heterogeneity in asthma exacerbations both biologically but also in the experience of those who suffer from exacerbation themselves. Through the advent of molecular-based testing, we can phenotype asthma exacerbations in a greater depth than ever before.

This thesis aims to further our understanding of the heterogeneity of asthma exacerbations. By delving into the phenotypical basis of their occurrence through a combination of inflammatory biomarkers and gene expression measurements, we aim to understand the complexities of this condition. Asthma exacerbations, primarily caused by viruses, will be further categorised based on their viral aetiology. Moreover, we will explore the impact of these exacerbations on human gene expression through RNA sequencing and a series of analyses, thereby providing a comprehensive understanding of the characteristic clinical manifestations.

Statement of work performed.

My role as a clinical research fellow at the Nottingham Respiratory Research Unit was from August 2019 until July 2023. I returned to clinical practice in August 2023. The asthma phenotyping exacerbations (APEX) study protocol was written and ethically approved before I began to work on the study. Alongside Karen Shaw and Robert Needham, I conducted Baseline and Annual Visits involving consenting, sample collection and performance of Impulse Oscillometry (IOS), spirometry, and sputum induction. I was also involved in processing some of the sputum samples and blood. The recruitment process was a team effort, with visits to the wards at Nottingham City Hospital to recruit patients for the study. With patients' consent, I checked medical records to check their diagnoses. I also established Kingsmill Hospital as a Participant Identification Centre (PIC) and Pilgrim Hospital as a research site.
I solely assessed and triaged all exacerbation visits until July 2023, when I returned to clinical practice. After this period, a clinician I trained in exacerbation visits oversaw exacerbations. I performed all statistical analyses in this thesis.

I performed all DNA/RNA extractions of Nasal Brush samples at baseline, subsequent exacerbations, and annual events until May 2023, tutored by Dr. Micheal Portelli. Of these samples, I conducted all the qPCR tests and synthesized cDNA. Dr. Robert Hall kindly tutored me in the methodology.
I plated the RNA samples, which underwent sequencing by Oxford Genomics (Oxford). I analysed the RNA sequencing data under the direct supervision and guidance of Dr. Robert Hall. I am not experienced in R coding, and I thank Dr. Hall for supporting this analysis and creating PCA plots, Volcano Plots, and Heat Maps. I performed the Ingenuity Pathway Analysis (IPA) independently.

During my PhD, I performed two additional studies. The first study was exploring the impact of asthma exacerbations (IMPAX). This is a

qualitative study conceptualised by Dr Matthew Martin. I performed 27/30 interviews. I undertook all analyses. The manuscript is undergoing revision after being submitted to the Journal of Asthma. (not included in this thesis)

The second study compared coached supervised in-clinic spirometry versus supervised coached spirometry at home (COMPAIR) with an analysis to assess the utility of Artificial Intelligence (AI) in overreading. I wrote the protocol and supervised the study. I performed all analyses, and wrote the study report, and recently presented my findings at the ATS conference in 2024. The manuscript is currently being prepared (not included in this thesis).

COVID-19 impact statement.

The COVID-19 pandemic began towards the start of the APEX study. The study commenced in October 2019 and paused in January 2020. As a result, recruitment into the study and exacerbation visits had to be paused for seven months. I was seconded into secondary care during this period to help with the efforts on the front line. As a result, the study had to be extended to account for this period. The study finished in February 2024.

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These studies would not have been achieved if it hadn't been for the 305 patients who took part in APEX. I thank them for their time, patience, and trust in our team to look after them during periods of uncertainty. It was a privilege to look after them, and the education they provided me is what I will take forward in my clinical care. The large volume of data generated from the various studies would not have been possible to organise without the help of Glenn Hearson, who helped set up the databases to capture data for the studies.

On a personal note, I would like to thank my late father, Dr Gorakh Prasad Pradhan and my late mother, Snay Lata Pradhan. They always supported and believed in me even when I was in doubt. I want to dedicate the work completed in this thesis to my parents. I finally would like to thank my wife, Kalyani, for being there for me throughout this journey and for her unwavering support. I also must mention my four older sisters, two nieces, and three nephews for being them.

List of abbreviations

ACh Acetylcholine
ACQ Asthma Control Questionnaire
AHR Airway Hyperresponsiveness
ASM Airway Smooth Muscle
ATS American Thoracic Society
Ax Area under reactance curve
BHR Bronchial Hyperresponsiveness
BTS British Thoracic Society
APEX Asthma: Phenotyping Exacerbations
CRP C-Reactive Protein
DC Dendritic cells
DEG Differentially Expressed Genes
DNA Deoxyribonucleic acid
ERS European Respiratory Society
FEV1 Forced Expiratory Vital Capacity in 1 second
FVC Forced Vital Capacity
FBC Full Blood Count
GINA Global Initiative for Asthma
HMV Human metapneumoviruses.
HP1 Human Parainfluenza Virus 1
HP3 Human Parainfluenza Virus 3
ICS Inhaled corticosteroid
ILC Innate Lymphoid Cells
INF Interferon
IOS Impulse Oscillometry
LABA Long-acting beta agonist
LAMA Long-acting Muscarinic antagonists
NHS National Health Service.
NICE The National Institute for Health and Care Excellence
OCS Oral Corticosteroids
PAAP Personalised Asthma Action Plan.
PCR Polymerase Chain Reaction
R5 Total resistance
R20 Central resistance
R520 Peripheral resistance
RNA Ribonucleic Acid
RV Human Rhinovirus
RSV Respiratory Syncytial Virus
SABA Short-Acting Beta Agonist.
SIGN Scottish Intercollegiate Guidelines Network
SPT Skin Prick testing

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Chapter 1: The Background to asthma exacerbations

Introduction

Asthma and Asthma Exacerbations

Asthma is a common chronic disease affecting the airways.[1] It is now widely recognised as a heterogenous condition caused by a complex interaction between genetics and the environment in response to various triggers.[2,3] It can be associated with significant symptoms that can be episodic or persistent and, as a result, can affect the daily life of asthma patients.

Several conditions share similar symptoms to asthma, and there is currently no gold-standard test to diagnose asthma. This can, in some cases, make the diagnosis difficult to make. Asthma management involves inhaled corticosteroids (ICS) alone or combined with a bronchodilator to achieve asthma control. In severe cases, the patient may require biological therapy or daily steroids. The focus of asthma management is symptom control and prevention of asthma exacerbations. There is currently no cure for asthma.

Asthma can be associated with a significant deterioration in the patient's usual symptoms that are not resolved by their regular medication. In combination, there is also a reduction in lung function, which requires a change in treatment or current management to prevent serious consequences such as hospitalisation or, even worse, death. The triggering factors for such events vary, but viruses contribute significantly to the aetiology of exacerbations.

These events are known as asthma exacerbations, sometimes called "asthma attacks" or "flares." The severity of exacerbations can vary, as can the response to treatment, usually a course of oral corticosteroids. Asthma has significant health economic costs to the National Health

Service (NHS), too, and can be associated with morbidity. This chapter reviews key clinically relevant concepts related to asthma and asthma exacerbations.

Definitions

Asthma has been defined by the Global Initiative for Asthma (GINA) as:

"A heterogeneous disease usually characterised by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation".

GINA 2023

This definition encompasses the complexity of this disease and the variability associated with asthma. It forces asthma to be considered an "umbrella term" like "anaemia" and "cancer."

The definition of a severe exacerbation is currently defined as:

"*Events that require urgent action on the part of the patient and the physician to prevent a severe outcome, such as hospitalisation or death* [4]*."*

ATS/ERS joint statement 2009.

Moderate exacerbations are defined as "…events that are troublesome to the patient, and that prompt a need for a change in treatment, but that are not severe. These events are clinically identified by being outside the patient's usual range of day-to-day asthma variation". [4] Moderate exacerbations are events that do not require a course of oral corticosteroids or a hospital admission. Mild exacerbations currently do not have a definition, as the current thought is that they could represent a loss of asthma control rather than an exacerbation and may be just outside a patient's typical day-to-day symptoms should they experience them. [1]

There is currently no commonly accepted definition of an asthma exacerbation. [1] The definition of an exacerbation lacks objectivity. It relies on assessing symptoms and lung function, often a peak-flow measurement, to gauge when an exacerbation is occurring.
An asthma exacerbation is usually physician-diagnosed, but there could be physician-to-physician variability in that diagnosis. In some cases, this assessment is in the hands of the patient to decide when to commence their rescue pack of steroids. There is a much-needed shift from this subjective diagnosis to one that relies on parameters or biomarkers to help achieve the diagnosis of an asthma exacerbation. Phenotyping asthma exacerbations could be the approach towards this.

The definition of an asthma exacerbation is retrospective for clinical trials and is often used as an essential clinical endpoint. It is defined as:

1) The use of systemic steroids, or an increase from a stable maintenance dose, for at least three days. For consistency, courses of corticosteroids separated by one week or more should be treated as separate severe exacerbations.

2) A hospitalisation or emergency room visit because of asthma requiring systemic corticosteroids. [4]

Epidemiology of asthma & health costs.

Asthma is a non-communicable disease with a worldwide impact on health. It is very common, with an estimated 339 million people worldwide affected by asthma.[5] This is likely to be underestimated due to countries' differing capabilities in correctly coding asthma diagnoses. The prevalence of atopic sensitisation is expected to increase in the coming decades, causing this number to grow even further.[6]
There is an estimated prevalence of asthma in adults at 4.3%.[5] Asthma is more common in more economically developed countries, such as

those in the Western hemisphere and areas of urbanisation. [7] The prevalence in less economically developed countries has been reported to be as low as ≤1%. [8] However, as less economically developed countries adopt Western lifestyles – the prevalence is increasing.[9]

In the UK, over 9.8 million reported symptoms of asthma that a physician had diagnosed. [10] 5.4 million patients are currently receiving treatment for their asthma. The incidence of new asthma diagnoses is 160,000 per year. The UK is reported to have one of the highest prevalence of asthma in Europe.[11]

Epidemiology of asthma exacerbations

Asthma exacerbation rates are challenging to assess due to differing definitions of this important outcome in studies on a national and global scale. Studies have assessed exacerbation rates in those with mild, moderate, and severe asthma by evaluating the utility of combination inhalers. [12] [13] Asthma exacerbations occur across the range of severity but occur more commonly in those with severe asthma [14] [15] and can occur despite the patient being on high-dose inhaled corticosteroids (ICS). [16] Severe asthma is defined by ATS/ERS as “asthma which requires treatment with guidelines suggested medication for GINA steps 4-5 or systemic corticosteroid therapy.”[17]

For the majority of those with mild and moderate asthma, ICS on its own or often in combination with a bronchodilator is sufficient to prevent and reduce the frequency and risk of an asthma exacerbation. [18]

A Clinical Practice, Research Datalink study, assessed exacerbation rate according to GINA-based therapy in the US and UK. [19] The study demonstrated that the exacerbation rate increased with disease severity. However, each step was associated with exacerbations, suggesting that those with mild asthma are still at risk of asthma exacerbations. Studies have also supported this finding. [20] (Figure 1.1)

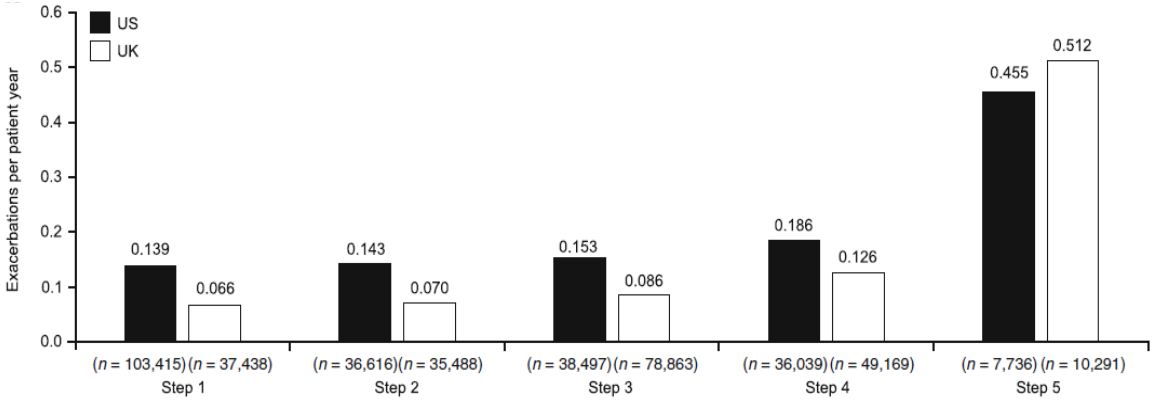


Figure 1. 1: Exacerbation events per patient-year according to GINA [19]

After adjusting for covariates, the study found that patients with a previous asthma exacerbation that required ED/hospital visits had a 32% increased risk of a subsequent exacerbation. (Figure 2)

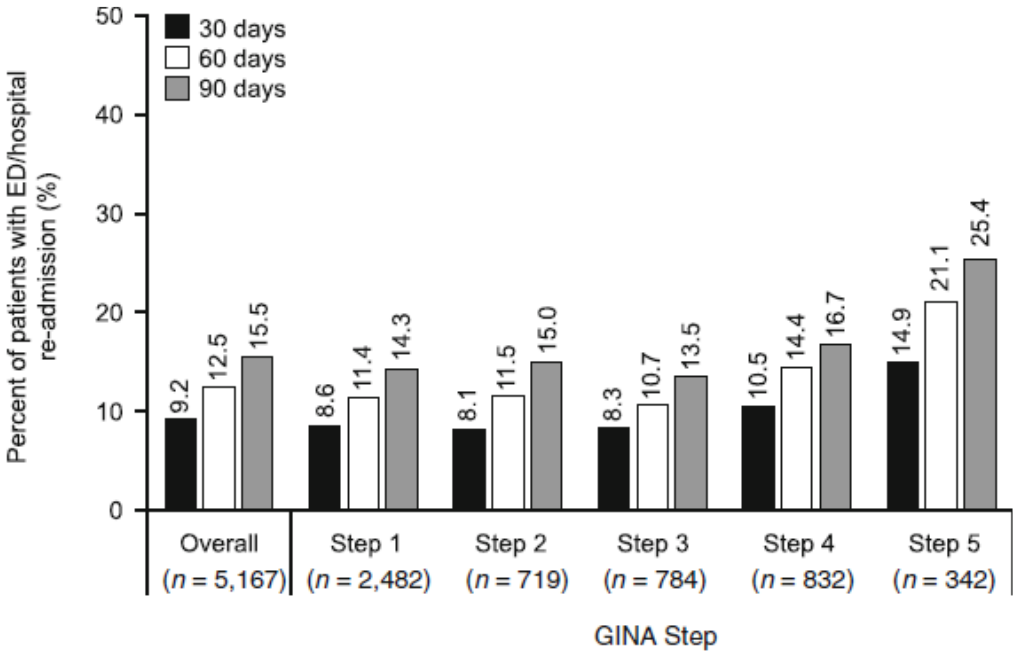


Figure 1. 2: Proportion of patients with an asthma-related ED/hospital re-admission after being discharged for an asthma-related ED/hospital admission [19]

Images obtained from Suruki et al: The frequency of asthma exacerbations and healthcare utilisation in patients with asthma from the UK and USA. BMC Pulmonary Medicine 2017;17(1):74.

The proportion of patients with asthma-related readmission after 30 days almost doubled from GINA Step 3 to Step 5 (8.3% vs 14.9%). Asthma exacerbations have also been identified to be associated with progressive longitudinal decline in lung function.[21]

Cost of asthma care

Asthma has high economic costs, both directly and indirectly. Asthma has a significant impact on the NHS, with 6.3 million (2-3%) of primary care consultations attributed to asthma. The burden on secondary is substantial, with 93,000 hospital admissions and 200,000 bed days per year in the UK. The severity of asthma exacerbations involved 1800 intensive care episodes. [10]

Asthma significantly impacts quality of life, with 36,800 disability claim allowances. The estimated cost for asthma was £1.1 billion: 74% was attributed to primary care, 12% to hospital care, and 13% to disability claims. [10].

This could be underestimated owing to different coding entries for first presentations, such as “wheeze” or “cough.” This data highlights the enormous costs associated with asthma exacerbations and the need for greater effort in preventing them.

This data highlights the need for further recognition of asthma as a serious condition and its broader impact on the healthcare system in terms of health resources. It also calls for asthma to be considered a priority condition in strategies to prevent asthma exacerbations, which can only happen with a change in practice.

Mortality of asthma

Asthma is attributed to 1 in every 250 deaths globally – an estimated 180,000 deaths per year. [7].

The number of asthma deaths has declined since the 1950s. The National Review of Asthma Deaths (NRAD) was the first review of

asthma deaths in the UK to evaluate the circumstances regarding asthma deaths. The report collated data across 297 hospitals and data accessed from primary and secondary care. Data was collected between February 2012 and January 2013. A total of 195 deaths were attributed to asthma.

The report identified several gaps in care. The follow-up of these patients was sub-optimal, with less than 50% of the identified deaths having an asthma review in primary care in the year prior to death, and just over a fifth of the reported deaths had a personal asthma action plan (PAAP).
The report highlighted that 39% of the asthma deaths had severe asthma, 49% had moderate asthma and 9% had mild asthma. This could suggest that these groups had poorly controlled asthma, but also, they could have been undertreated and misclassified. The UK, in comparison to other countries, has a worse death rate, and it is almost 50% higher than the average death rate in the European Union.

Susceptibility to the development of Asthma.

The development of asthma is often a combination of host factors, which are primarily genetic, and the environment in which the person is living. The host-environment interaction is a dynamic and complex process that changes with time.
The interest in understanding genetic risk factors for asthma and exacerbations has increased,[22] with the hope that exploring the genetic basis of asthma could explain, in part, the mechanisms of the manifestations associated with asthma. Asthma has been found to run in families, but the mode of inheritance is complex and polygenic. The genetic aspect determining whether you develop asthma or not is termed hereditability. In monozygotic twins, the concordance for asthma development is 75% and not 100% - highlighting the role of other contributing factors, such as the environment. [23] The hereditability of asthma is estimated to be 35%-95%[24,25]
The largest genome-wide association study to date identified many variants, candidate genes, and pathways that may be important in

susceptibility to asthma, with childhood-onset disease having a clear role in genetics versus the environment. [26] Nasal transcriptomic expression of epithelial cells concerning asthma exacerbations will be covered in detail in the background section of Chapter 6. The concept of “Atopic March” on the development of allergic diseases in childhood has been established, with the prevalence of atopy in children with asthma being 51.6%.[27,28]

Clinical features of Asthma and Asthma Exacerbations

Airflow Limitation

Airflow limitation is a characteristic feature of asthma and asthma exacerbations. Exaggerated vagal activity is a hallmark mechanism associated with asthma. Acetylcholine binds to anticholinergic receptors, which causes contraction of the smooth muscle of the airway.[29] Its increased expression causes increased airway tone, which causes neuronal plasticity. This leads to additional release of ACh thus creating a vicious circle.[30].

Airflow obstruction is defined as having an FEV1/FVC ratio <0.70. This may be underestimated in children and young adults and overestimated in males aged>40 years and females >50 years.[31] The ratio decreases with advancing age. Furthermore, airflow limitation is influenced by the circadian rhythm and tends to be worse in the morning. Because it is a dynamic measurement, a normal value at a point in time may not necessarily exclude a diagnosis of asthma.[32]

In asthma, an increase in FEV1 of >12% and a 200ml improvement in lung function after inhalation of a bronchodilator is suggestive of airflow limitation with reversibility. [33].

Small airways disease (SAD) is common in asthma and has been identified as a risk factor for the development of asthma exacerbations.[34]

There are no normal ranges for forced expiratory flow at 25-75% of FVC (FEF25-75), commonly used to measure SAD .[35] An alternative way to measure small airway resistance is Impulse Oscillometry (IOS). According to studies, this non-invasive method appears more sensitive in detecting small airway disease than FEF25-75.[35] However, its reference thresholds are very heterogeneous.[36] The presence of small airways disease is estimated to be present in 50-60% of patients with asthma and is more prevalent in the more severe GINA steps. [37]

Airway hyperresponsiveness (AHR)

Airway hyperresponsiveness (AHR) is a prominent feature of asthma across all asthma phenotypes.[38] It was initially described in 1946 after airways were exposed to methacholine and histamine, which are known to provoke bronchoconstriction.[39] It results in the airways narrowing secondary to stimuli known to cause constriction. [40]

Mast cells play a key role in airway hyperresponsiveness, and their level has been shown to correlate well with that of AHR. AHR is associated with an increased risk of asthma exacerbations. [41] During an asthma exacerbation, AHR is increased. [39]
Clinically measuring AHR can help diagnose asthma in the presence of normal spirometry.[42] The mannitol test assesses airway hyperresponsiveness through indirect bronchial provocation and activation of mast cells, resulting in bronchoconstriction.

Thymic stromal lymphopoietin (TSLP) and IL-33 are released by epithelial cells within the airway, which are then recognised by mast cells.[43] Indirect AHR to mannitol is reduced by blockade of TSLP.[44] Inhaled corticosteroids are effective in the suppression of AHR and mast cell infiltration into the airways. [45] A prospective study of 50 steroid naïve patients with features of AHR who were split with airway inflammation defined as Fractional exhaled nitric oxide (FeNO) high and FeNO low.

After commencing ICS, there was an improvement in AHR and a reduction in levels of TSLP and IL-33 regardless of the FeNO status. In the FeNO high group, levels of FeNO correlated with mast cell concentration in the epithelial and smooth muscle cells. [45]

Small Airways Disease

Impulse Oscillometry (IOS)

The importance of small airway disease in asthma has become increasingly recognised in asthma.[34] The smaller airways are defined as airways less than 2mm in calibre and occur after generation 8 of the bronchial tree.[34] Due to the large number of divisions that occur after generation 8, the mucosal surface area covered after generation 8 is significantly larger than that of the area covered by the proximal airways.

IOS is a non-invasive technique that can quantify the role of small airways disease within asthma over time.[46] Spirometry and Peak-Flow meters cannot distinguish between large and small airways disease, with FEV_1 and PEFR mainly reflecting the larger airways. FEF25-75 potentially reflects small airway function [47] but has been found to have poor reproducibility [48] and is effort—and volume-dependent in effectively reaching the residual volume. If this does not occur, the expiratory flow rate can be underestimated.

IOS has been used for decades in research but has now started to be recognised as having a role in clinical practice. The technique is relatively simple and quick and, therefore, is useful in certain age groups, such as those of preschool age, the elderly, and those with disabilities.

Interpretation of Airwaves Oscillometry

Respiratory impedance (Zrs) is a relationship of pressure (cmH_20/kPa) to flow ($L^{-1}s$). It has two components: resistance, which is the resistance to airflow (R), and compliance/ reactance, an indicator of elastic recoil in the airway (X). [49](Figure 1.3). Oscillometry is a method of measuring impedance across a range of frequency pressure waves. [49]

In the diagram below, the impedance is plotted against the frequency. The solid line represents the resistance line, which varies depending on frequency. At low frequencies, for example, at 5Hz, the resistance is much higher than at a frequency of 20Hz. This is because lower-frequency waves can penetrate deeper into the airways, and higher frequencies can only travel a much shorter distance. R5 represents the resistance of the whole airways from the trachea to generation 23 of the bronchial tree. R20 represents the resistance of the central airwaves to generation 8 of the bronchial tree. Therefore, subtracting R20 from the peripherak airway resistance of R5 produces the R5-R20 value, which represents the peripheral airway resistance. In small airways disease, the peripheral airway resistance becomes greater in the lower frequencies.

The dashed line in Figure 1.3 represents the reflective capacitance wave produced from the elastic recoil of the airways and is denoted as the reactance (X). Reactance is informative of the elastic compliance of the airways. There are three measures which are attained. The first is the reactance at a low frequency of 5 Hz (X5). This represents the elasticity of the small airways. IOS is unable to measure frequencies smaller than 5 Hz. [50] At higher frequencies, representing the conducting airways, the resonant frequency is the point at which the reflective capacitance reaches zero and is at the point where capacitance and inertance offset each other. AX is represented from 5 Hz to where reactance crosses R/X = 0, the area under the reactance curve—AX and Fres increase in obstructive lung diseases such as asthma and COPD.

In summary, evidence of small airway obstruction is suggested by a raised resistance at 5 Hz associated with a raised resistance change from 5 to 20 Hz and increased reactance. A high R5-R20 has been previously described as higher than 0.08 kPa/L.s [51].

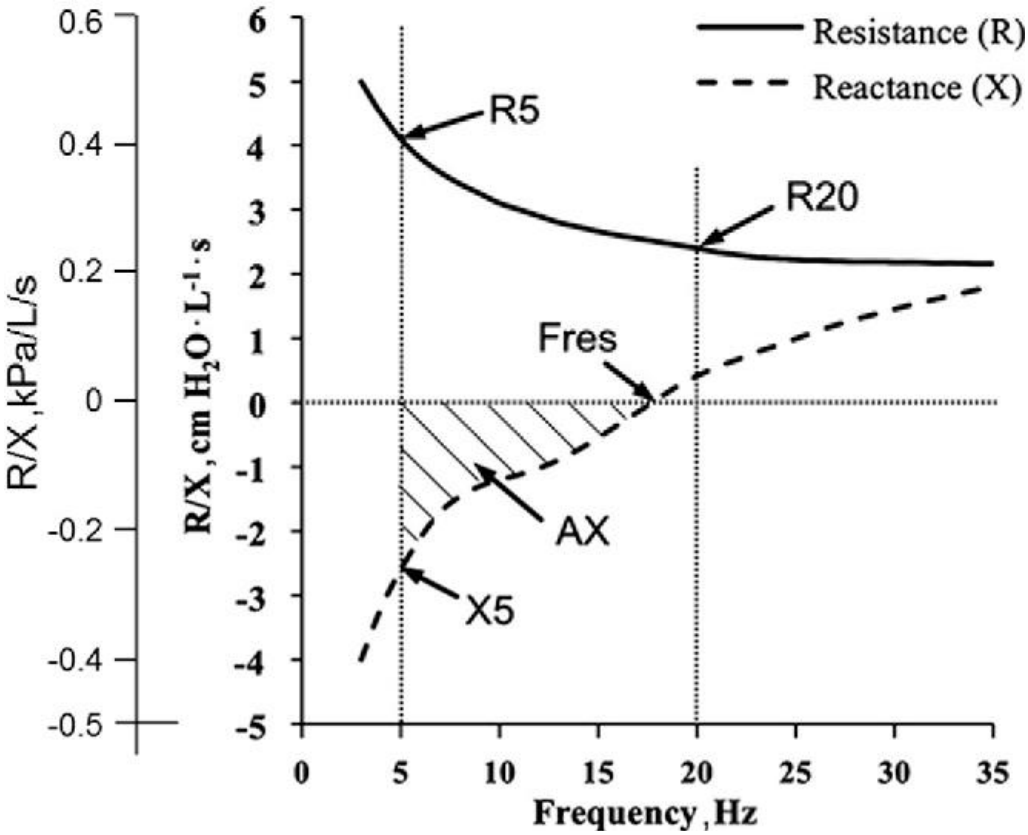


Figure 1. 3: Graphical illustration of IOS measurements.

Lipworth et al. Annals of Allergy, Asthma Immunology 2017 Jun; 118 (6): 664-671

The normal ranges for IOS have differed regarding resistance and reactance across studies. This depended on the primary outcomes of interest and the level of control, such as stable, uncontrolled or perceived asthma exacerbation. As such, this creates significant heterogeneity in the cut-off values. Vogel and colleagues, in 1994 46, performed a sizeable epidemiological study that formed the basis of reference ranges. This study was based on a population in an industrial city, some of whom were smokers. This contrasts with the guidance produced by ATS for measuring lung function reference ranges [52]. More recently, the

Thorasys®(Montreal, Canada), which is the device used in this study to measure IOS, uses reference values from Oostveen and colleagues,[49] [53] who collected impedance data at 4 to 6 Hz in 368 healthy Caucasian subjects between the ages of 18-80 across five centres. Those with a pack-year greater than ten were excluded from the study. Normative values based on height, weight, smoking status, ethnicity and age were produced.

The Role of Airway Oscillometry in Asthma

Most asthmatics do respond and show an improvement in lung function when prescribed ICS. However, a proportion of asthmatics who have improvement in symptoms is not reflected in their spirometry. [54] This could be explained by the involvement of the small airways in asthma, with a "small airway asthma phenotype" being recognised. [55] Studies have also demonstrated a correlation between asthma control and IOS. [56] [57] [58] [59] [60]

IOS has been successful in demonstrating airway reversibility.[61] After bronchodilator administration, the heterogeneity of airway resistance is reduced according to frequency. This is also replicated in airway reactance, with a reduction in the AX. [61]

In an extensive study of 378 patients across BTS steps 2,3,4; the role of small airway disease across different asthma severities was explored. [51] The respective mean FEV1% predicted in each group were 90%, 86% and 84%.[51] However, in step 2, despite having a preserved FEV1, 65% had an abnormal R5-R20 value and abnormal peripheral airway resistance.

Similar findings were identified in a cohort of over 300 patients with mild-moderate asthma on BTS therapy 2-4, with a mean predicted FEV1 of 97%. 45% of those demonstrated abnormal peripheral resistance. During the follow-up period, those patients who had raised peripheral resistance required more salbutamol and greater oral steroid use.[62]

The utility of IOS has been demonstrated with its ability to explain the deposition of ultrafine inhaled particles within the smaller airways. IOS has also been shown to be responsive to treatment initiation of ICS with a 50% reduction in R5-R20 with hydrofluoroalkane-beclomethasone dipropionate (HFA-BDP) after 12 weeks. HFA-BDP has a small particle size of 1.0 micrometres, so it is small enough to enter the smaller airways. However, compared to chlorofluorocarbon-beclomethasone dipropionate (CFC-BDP), which contains particles of a greater size of approximately 4.0 micrometres, there was no significant change in R5-R20 after 12 weeks. [63]

IOS has also been shown to be related to airway inflammation.[64] In a cohort of over 110 patients with asthma on a mean dose of 644µg of ICS, AX, R5-R20 were higher in those with a high eosinophil count (>300 cells per microlitre) in comparison to those with a low eosinophil count (<300 cells per microlitre); with high eosinophil acting as the marker of type 2 inflammation. [64]FeNO and IgE levels were measured to assess the validity of the cohorts' differences based on eosinophils as a marker of T2 inflammation. As expected, the higher eosinophil group had greater IgE ($p=0.02$) and FeNO levels ($p=0.045$).

Airway inflammation

History of Endotyping

Endotyping defines the underlying pathophysiological mechanism associated with a disease, such as asthma.[65] The first concept of endotyping asthma began in 1947 when Rackemann and colleagues [66] developed a working classification of asthma, beginning before age 30, as “extrinsic” or allergy and “intrinsic” or bacterial. Allergic asthma is typically diagnosed when symptoms are triggered by exposure to an allergen. Atopy can be confirmed by testing reactivity through skin-prick tests/ and or measuring the serum levels of IgE. The concepts initially identified by Rakemann have been disproven, [67] with the identification that both groups share common immunopathological mechanisms involving IL-5. [67]

The classification of asthma according to T-lymphocyte helper cells – T-helper 1 (Th1) and T-helper 2 (Th2) and subsequent cytokine release from bronchial biopsies and bronchoalveolar (BAL) fluid was then developed. [68] Robinson and colleagues identified the presence of IL-3,4,5 and Granulocyte-macrophage colony-stimulating factor(GM-CSF) via the activation of Th2 cells in atopic patients with asthma in Bronchoalveolar Lavage (BAL) fluid. [68]

This has given rise to the concept of Th2-high and Th2-low asthma.[69] Through gene expression of bronchial biopsies, Woodruff and colleagues demonstrated two distinct groups divided by the presence or absence of IL-5 and IL-13. They also showed how Th2-high inflammation conferred a better response to ICS than Th2-low inflammation. [69]

However, we know with the identification of innate lymphoid cells (ILCs) that their contribution to the pathogenesis of asthma is of great importance. This has, therefore, caused a change in terminology to account for this, with Th2-high becoming type 2 high (T2-high) and Th2-

low becoming type 2 low (T2-low) due to their involvement in T2 inflammation[70] T2-high asthma encompasses the terms “eosinophilic asthma” and “allergic” and “non-allergic”—identified phenotypes. T2-high asthma is associated with the following biomarkers: elevated IgE, FeNO, and blood eosinophilia. T2-low/T1 asthma is associated with increased IL-17 expression in CD4/CD8 cells. [71]The mechanisms contributing to this are poorly understood and there are currently no biomarkers for this phenotype.[72]

Proposed mechanisms of T2-high asthma

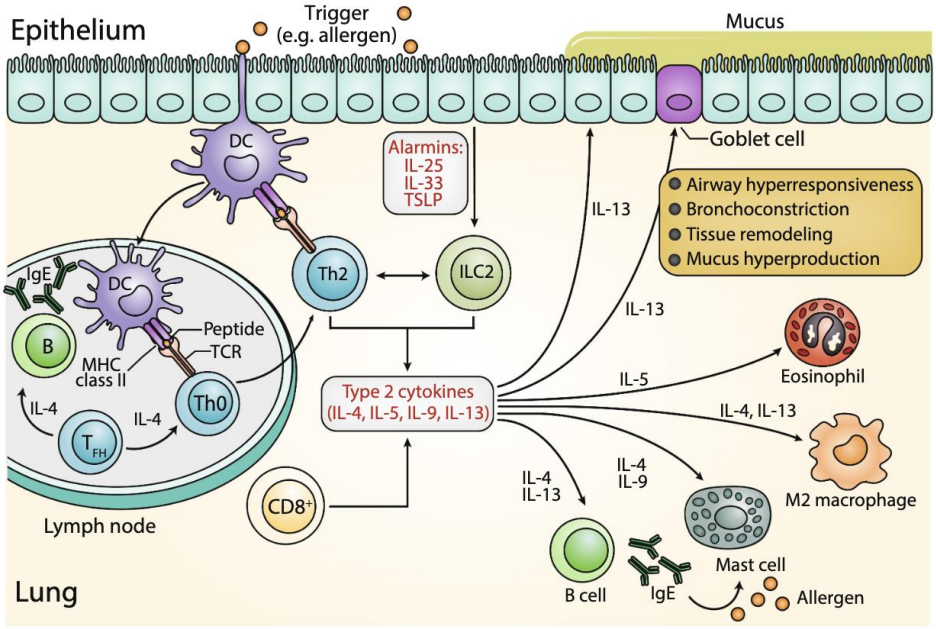


Figure 1. 4: Pathophysiology of T2- high asthma.

Walker, J., McKenzie, A. TH2 cell development and function. Nat Rev Immunol 18, 121–133 (2018).

Th2-lymphocytes and ILC2 are key in T2-high asthma. They produce a cytokine response driven by IL-4,5,9,13, prostaglandin D2 (PGD2), and eosinophils. T2-high asthma develops after airway epithelium cells are exposed to different aeroallergens. This initial exposure stimulates the release of IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) cytokines. IL-33 and TSLP lead to the release of dendritic cells (DCs).[73]

DCs can act as antigen-presenting cells (APC) and recognise the allergen, resulting in Th2 development.[74] Th2 cells cause a release of cytokines related to T2 inflammation and the production of IgE. IgE cross-links with mast cells, causing mast cells degranulation. [75] This leads to the contraction of airway smooth muscle.

These T2 cytokines are responsible for eosinophils' recruitment, activation, maturation, and survival. IL-4 and 13 are responsible for nitric oxide production and is measured as fractional exhaled nitric oxide (FeNO). [75] Airway remodelling due to structural changes can occur with goblet cell metaplasia, resulting in mucus hypersecretion and mucus plugs. [76]

Proposed mechanisms of T2-low asthma

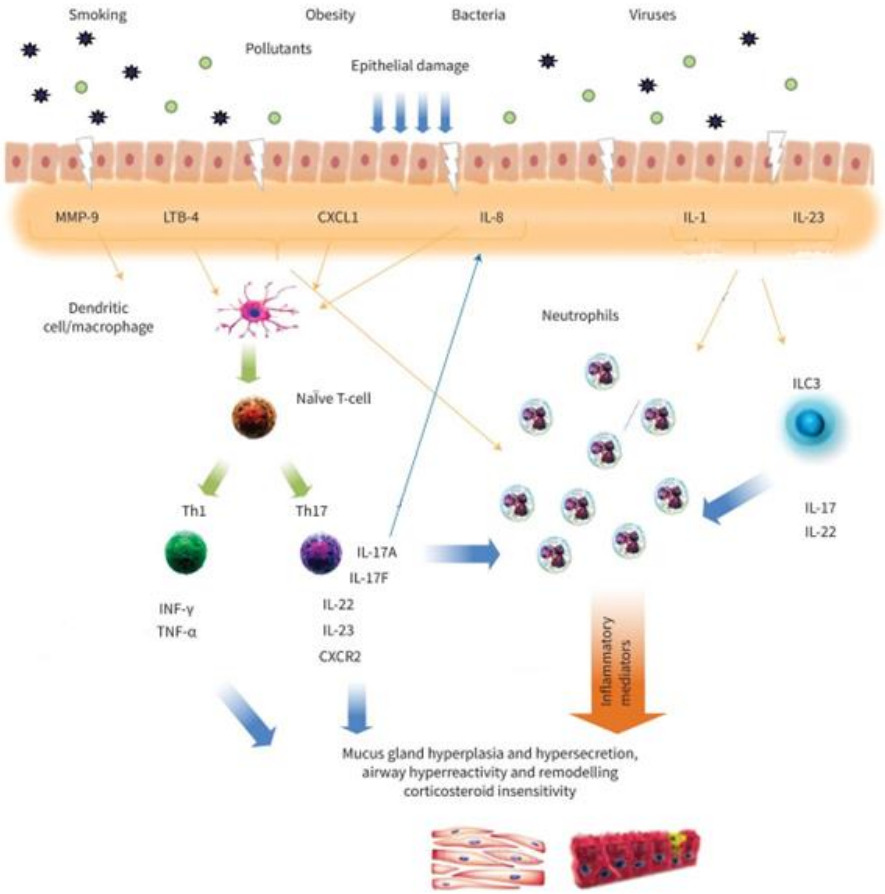


Figure 1. 5: Potential pathways of T2-low asthma.

Image obtained from: Kyriakpopoulos et al. Identification and treatment of T2-low asthma in the era of biologics. ERJ Open Research 2021 7: 00309-2020.

There currently is no standardised definition of T2-low asthma; clinically, it is the absence of raised biomarkers associated with T2-high asthma, such as FeNO and raised blood eosinophils. Mechanisms related to T2 low asthma are poorly understood. Inflammatory pathways thought to contribute are the T1 and T3 immune response. T1 pathways are responsible for antiviral pathways, which will be described in detail in Chapter 6, but provide an interferon response. Th17 and ILC3 mediate T3 immunity and releases IL-17. These pathways are thought to contribute to steroid resistance and airway remodelling that occurs that can be associated with T2-low asthma [77] Ricciardolo and colleagues explored T1-associated inflammation in asthma exacerbations by comparing bronchial and nasal biopsies from patients with severe asthma, mild asthma, and controls. After performing immunohistochemistry on the samples, they found higher levels of IL-17 in CD4+ and CD8+ cells in the bronchial mucosa, which correlated with frequent asthma exacerbations.[78]

Paucigranulocytic asthma is characterised by the absence of airway inflammation and the presence of neutrophils and eosinophils.[71] Patients with asthma who have this phenotype are associated more stable trajectories of asthma with less exacerbations, most likely due to the lack of inflammation.[77] Pauci-granulocytic asthma involves neural mediators of airway smooth muscles to account for the lack of inflammation, which gives rise to AHR, which has been seen in mice. [79] As such, there are no validated biomarkers associated with T2-low asthma. [71]

Sputum inflammatory endotyping.

Asthma has also been classified according to the presence of inflammation in induced sputum, with asthma classified according to the presence of eosinophils (eosinophilia >2-3%) or neutrophils (neutrophilia >61-76%).[80]

When there is the presence of both eosinophilia and neutrophilia, this is defined as “mixed” and; “paucigranulocytic” when the opposite occurs

where both eosinophil count and neutrophil count are within normal range.[80] The benefit of classifying asthma according to sputum is that this modality is much more accessible than that of a bronchial biopsy or obtaining BAL fluid. In patients not on corticosteroid therapy for asthma symptoms, sputum eosinophilia has been reported in 70-80%. Sputum eosinophilia has been reported in 50% of corticosteroid-treated asthmatics, irrespective of atopy status. [81,82]. Non-eosinophilic asthma is found in 25-55% of corticosteroid-naïve patients with asthma.[83]

Airway inflammation in clinical practice using sputum.

Airway inflammation is a key feature of asthma and, as such, is a key target for asthma therapies. It forms the basis of most guidelines in managing asthma and asthma exacerbations, emphasising ICS use and OCS for asthma exacerbations. Asthma is commonly associated with sputum eosinophilia.

Louis and colleagues demonstrated a strong relationship between sputum eosinophilia and asthma severity in a cross-sectional study of 74 asthmatics with various severities. Notably, the study also revealed sputum neutrophilia in those who remained symptomatic despite treatment with high doses of ICS. [84]

The utility of adopting an approach of utilising sputum inflammation in asthma exacerbations has been explored. Seventy-four patients with moderate to severe asthma were randomly allocated to management by BTS guidelines or sputum eosinophil count. Patients were followed up over 12 months during the study. If control was maintained for two months, an attempt to reduce corticosteroid treatment was made. Patients in the sputum management group have a reduced number of asthma exacerbations than in the BTS management group, as well as a reduced number of hospitalisations. The sputum eosinophil count was 63% lower in the sputum management group than in the BTS managed group. However, ICS/OCS doses did not differ between the two groups. The types of exacerbation observed were not examined.

Following this, Jayaram and colleagues used sputum induction to guide treatment. One hundred seventeen patients with asthma were randomised into either a clinical strategy group consisting of a symptom-based approach and spirometry or a sputum strategy group in which induced sputum was utilised with spirometry to guide ICS use. 79 exacerbations occurred in the clinical strategy group and 47 in the sputum strategy group. However, there was no influence on the number of non-eosinophilic exacerbations or the cumulative corticosteroid dose used during the trial. [85]

Viruses associated with asthma exacerbations.

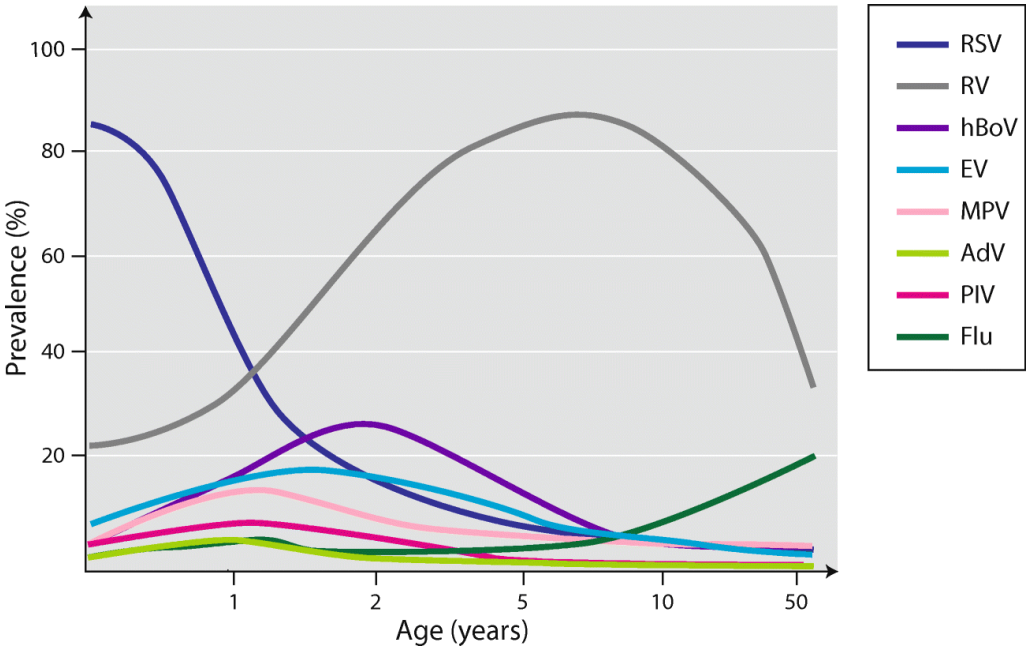


Figure 1. 6: Virus pathogens across the ages, which commonly cause asthma exacerbations.

RSV= Respiratory Syncytial Virus; RV= Rhinovirus; hBoV, human bocavirus; Flu= influenza virus; EV= Enteroviruses; MPV= metapneumovirus; PIV= Parainfluenza virus; AdV= Adenovirus.

Image obtained from Jarrti T et al. Role of viruses in asthma Seminars in Immunopathology 2020:42:61-74.

RNA viruses cause many asthma exacerbations. Human Rhinovirus (RV), is a significant cause of exacerbations. Viral infections have been identified in 50-80% of patients with asthma exacerbations. It is estimated that RV is the cause of 50-80% of asthma exacernations.[86] Other RNA viruses known to cause asthma exacerbations are Respiratory Syncytial Virus, Influenzas and Human Metapneumovirus.[87] One exception to this is Bocaviruses, which are DNA viruses and have been associated with asthma exacerbations, albeit less common.[87]

Corne and colleagues conducted one of the earlier studies that recognised the significance of the virus in asthma.[88] They performed a longitudinal cohort study on 76 cohabiting couples, one with and the other without asthma. They followed them up from autumn to winter in 1 year to assess the occurrence of viral infections in those with and without asthma. The advantage of this methodology is that the controls, in this case, are likely to be exposed to the same environmental factors and conditions. They obtained nasal lavage samples from participants every 2 weeks over this period.

Interestingly, the incidence of upper respiratory tract infections (URTIs) was not different between asthmatics (38/378 (10%)) or health controls (3/375 (9%)). However, what was shown was that the asthmatics experienced worse symptoms and slower viral clearance from the lower respiratory tract due to having a viral infection.[88].

This association has also been observed in epidemiological studies, highlighting that viruses in asthma exacerbations are associated with seasonality. Asthma exacerbations in school-age peak in autumn due to returning to school and the rise in prevalence in RV. A similar trend is observed in young children and adults. In one study, RVs were the most commonly detected in 65%. The next most common after this was coronaviruses (17%), followed by influenza and parainfluenza viruses (9%) and RSV (5%).[89] Similar findings have been demonstrated in adults with asthma exacerbation, with the prevalence of respiratory viruses is

more variable, ranging from 40% to 65%.[90] Given its high detection frequency, western research has focused on RV- induced Asthma Exacerbations. These epidemiological studies were possible due to the development of PCR technology for the detection of respiratory viruses

Rhinovirus (RV)

Rhinoviruses (RVs) are non-enveloped positive-strand RNA viruses.[91] They are classified as Rhinovirus A (RV-A) and B (RV-B). New RVs have been discovered, with the majority being strains of RV-C.[92] They are classified as A, B, or C according to the virus's capsid coding and non-coding regions.[93]

The transmembrane receptor protein for most Rhinoviruses A and B is ICAM-1(CD-54), and for rhinovirus-C, it is cadherin-related family member 3 (CDHR3).[92] ICAM-1 is upregulated in endothelial and epithelial sites of inflammation.

Mechanism of rhinovirus

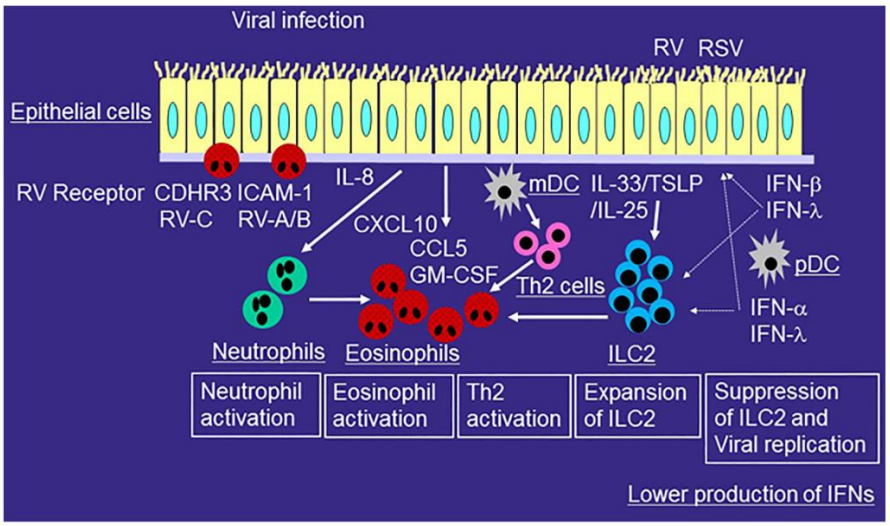


Figure 1. 7: Proposed mechanism of viral-induced asthma exacerbations.

Viruses enter the epithelial cells of airways by endocytosis. They are recognised by receptors PPR and TLR. This leads to an inflammatory cascade of T-cell differentiation, cytokine release and recruitment of inflammatory cells to the airways.

Image obtained from: Nakagome et al. Innate Immune Responses by Respiratory Viruses, Including Rhinovirus, During Asthma Exacerbation. Frontiers in Immunology 2022(13).

RV enters the airway epithelial cells and binds to its receptor. RV is recognised by pattern recognition receptors (PRRs). PRR causes the release of nuclear factor kappa-light-chain-enhancer of activated β-cells (NF-κB)[94]As a result, an inflammatory cascade ensues, with the release of IL-6, IL-8, and interferons (IFNs) as well as the T20-associated cytokines IL-33 and IL-25.[95] RV causes immunomodulation via the release of transforming growth factor (TGF) β, which can increase viral load. [96]

Respiratory Syncytial Virus (RSV)

Respiratory syncytial virus (RSV) is a single-stranded RNA virus. The most common strains are A and B, which are differentiated according to

the type of glycoprotein G attached.[97] RSV is known to cause bronchiolitis in infants.[98] In the immunocompromised, such as children and elderly patients, it can lead to severe pneumonia .[99] RSV is often commonly detected in asthma exacerbations studies involving children and as such RSV is more common than RV in preschool children who present to health care services with a wheeze.[100] RSV is thought to contribute to 8-18% of asthma exacerbations.[101]

RSV, until recently, followed a seasonal pattern of being prevalent in the winter months. However, since 2020, with COVID-19 and shielding policies that resulted from the pandemic, isolation resulted in lower levels of transmission, particularly in the winter of 2020. This affected children's immunity towards RSV. This resulted in a surge in the summer of 2021 of children with RSV in the UK and the northern hemisphere, according to Public Health England. [102]

Why do viruses lead to asthma exacerbations?

Why is it in some that respiratory viruses can lead to complications such as asthma exacerbation, and in some who are colonised with the same virus, are asymptomatic? This can be explained by the severity of the virus which is associated with 3 factors:

(1) Host deficiencies
(2) Viral capability
(3) Genomic susceptibility

Host deficiencies.

Intrinsic factors are also thought to contribute to susceptibility to asthma exacerbations caused by viruses, such as epithelial cells or airway remodelling itself, which is known to occur in those who experience multiple asthma exacerbations. Viruses infect the epithelial cells, affecting the airways' protective barrier function. Excessive mucus

production may also play a role in the development of asthma exacerbations.[103] and abnormalities in MU5AC in airway epithelial cells.[104] Immune mechanisms and how they increased the susceptibility to an asthma exacerbation are described later in this chapter.

Virus-capability

Some viruses are more pathogenic than others. It is unclear why RV is the most common cause of adult asthma exacerbations. [105] As previously explained, T2-associated inflammation is the most common phenotype seen in asthma, and RV contributes to this pathway through the release of key chemokines, as described above, but also through the release of IL-25, IL-33 and the cytokines IL-4, 5 and 13 from human bronchial epithelial cells.[106]RSV infection also activates T2 inflammation via CX3CR1, which leads to an allergic immune response with the release of T2-associated cytokines. [105]

Viruses may also exert different inflammatory responses. [107] Kato and colleagues explored asthma exacerbations in 267 subjects and performed PCR on nasal secretions. They looked at the cytokine profile associated with other viruses. RV was associated with raised IL-5 and eosinophil cationic protein (ECP); however, the IL-5 concentration in serum during an RSV infection was not increased, suggesting RSV utilises other pathways and not T2-biased pathways associated with an RV infection.[107]

There is a possibility that a more robust IFN response from viruses suppresses T2 inflammation, and conversely, a reduced inflammatory response by a virus upregulates T2 associated inflammation.[105] There are also intrinsic and structural differences in the airways of asthmatics compared to those without asthma, which could be a contributing factor.

Genomic susceptibility.

CDHR3 is a known receptor for Rhinovirus C and this receptor is expressed in the airway epithelium. A single nucleotide polymorphism (SNP) in CDHR3 is associated with a change in the base from G to A, which leads to worse outcomes in children who experience asthma exacerbation, such as admission to the hospital.[108]

Jannsen and colleagues identified 22 SNPs associated with RSV-associated asthma exacerbations, including the genes for vitamin D receptors (VDR). [109]

Immune dysregulation in asthma exacerbations.

Interferons

Some studies have found interferon synthesis is found to be deficient in asthma. It has been found from in-vitro from asthmatic patients after viral exposure that the epithelial cells will often have impaired production of important antiviral cytokines such as interferon α, β and λ. [110] Wark and colleagues demonstrated that cultured bronchial epithelial cells followed by rhinovirus inoculation in vitro, compared to non-asthmatics, produced lower levels of type 1 interferon and higher levels of rhinovirus replication.[111] This is evident not only in epithelial cells but also in pulmonary macrophages and circulating leukocytes, such as plasmacyte dendritic cells and monocytes. Pritchard and colleagues demonstrated that in peripheral blood mononuclear cells (PBMCs) of those with asthma compared to healthy controls after stimulation with rhinovirus and inoculation with rhinovirus for 24 hours, there was a significant reduction in IFN production.[110].

However, studies have shown that reduced type I and type III IFN production is not seen in all asthma patients. It is less evident in those

with mild asthma, raising the possibility that it occurs only in a subset of people with asthma.[112]

The phenotype of the asthma population sampled is also an important consideration. Simpson and colleagues explored interferon production in a subset of patients in the effect of azithromycin on asthma exacerbation and quality of life in adults with persistent uncontrolled asthma (AMAZES) study, which was a double-blind Randomised Control Study of low-dose azithromycin versus placebo.[113] They looked at 86 asthmatic patients and phenotyped them according to their inflammatory subtype. The group had poorly controlled asthma with a mean ACQ of 1.7 and a mean ICS dose of 1180 mg/day BDP equivalent. The mean age was 59.1(14.5) years. 76% demonstrated atopy. Those with predominately neutrophilic asthma had the most significant IFN deficiency, while those with eosinophilic asthma and those with the pause-granulocytic phenotype produced more interferon.

Dendritic cells (DC)

Dendritic Cells are antigen-presenting cells to lymphocytes and help to activate adaptive immunity. They help in the maturation of T-cells into Th2 or Th1 cells. They are present in airway epithelial cells and can present antigen that the airway may be exposed or can undergo phagocytosis in response to the antigen.[114]

Levels of DC's are greater in the airways of patients with asthma compared to controls and contribute to the exaggerated allergic immune response. Gill and colleagues identified that rhinovirus-stimulated purified blood plasmacytoid DCs to produce less IFN [115] than cells from healthy controls, suggesting a role of DC in the viral response.

T-cells

T-cells in asthmatic patients have also been identified to produce less interferon when exposed to viral infections. Johnston and colleagues examined T-cells from patients with asthma, obtained cells from bronchoscopy in patients before inoculation with a rhinovirus challenge, and performed bronchoscopy afterwards. [116]They found reduced induction of Th1 cytokines and the regulatory cytokine IL-10 and increased production of Th2 cytokines, often associated with asthma.

IgE Immunoglobulins

IgE has long been associated with asthma in epidemiological studies and is associated with type I hypersensitivity. Stimuli that promote the production of IgE are allergens, predominately, but also viruses and pollution. Dendritic cells, Th-2 cells, and ILC-2 are known to contribute to the release of IgE.[117]

Durrani and colleagues identified that children with allergic asthma had higher expression of FcεRIα on pDCs and mDCs from peripheral blood mononuclear cells. This inversely correlated with RV-stimulated IFN-α and INF-λ levels in asthmatic exacerbations, which occurred in those with higher IgE levels. [118]

The benefits of Omalizumab on asthma exacerbations have been established with a meta-analysis identifying the reduction of asthma exacerbation frequency (OR 0.52, 95% CI, 0.41-0.65).[119] On a molecular level, anti-IgE enhances the pDC antiviral response by reducing pDC surface receptor (FcεRIα) expression and restoring IFN-α production.[120] The Preventative Omalizumab or Step-up Therapy for Severe Fall Exacerbations (PROSE) study performed weekly nasal mucus samples on children with allergic asthma over 90 days. They found that 57% of exacerbations were attributed to rhinovirus, and those who were on

omalizumab had decreased duration of infection and reduced frequency of RV illnesses.[121]

Neutrophils and Eosinophils

Both neutrophils and eosinophilic inflammation may contribute to viral-induced asthma exacerbations. As described, type 2 inflammation influences viral-related exacerbations, but viral infection causes the recruitment of neutrophils to the airway inflammation via cytokine and chemokines such as IL-8(CXCL8) (Figure 1.7)

Johnston and colleagues explored the role of neutrophil extracellular traps (NET) formation in viral-induced asthma exacerbations.[122] NETs occur when neutrophils undergo NETosis, which releases NETs. These NETs contain dsDNA and histone and non-histone proteins, such as neutrophil elastase and myeloperoxidase, and have been found to increase type 2 immunity.[122] They found that dsDNA is released in rhinovirus-induced asthma exacerbations, which correlated with symptoms and type-2 cytokine release. [122] IL-8, a cytokine produced from neutrophils, increases the migration of eosinophils.

Neutrophilic inflammation has been observed during asthma exacerbations and in a proportion of those who have severe asthma. [123] The European Network for Understanding Mechanisms Of Severe Asthma (ENFUMOSA) found a more significant proportion of higher neutrophil counts in those with severe asthma than those with controlled asthma. [123]

It is known that eosinophilia can occur during asthma exacerbations.[124] [125] Respiratory viruses have been shown to increase the number of eosinophils in airway epithelium in those with allergic asthma exacerbations[105]. [126]

There is evidence that eosinophils possess antiviral properties, including the production of NO free radicals on viral entry to eosinophils, leading to antiviral effects on RSV and parainfluenza virus. [127]. Eosinophils have also been found to form eosinophilic extracellular traps, which consist of DNA, histones and granulins and reduce the migration of pathogens. However, overproduction of these traps has been found in those with allergic asthma.[128]

Innate Lymphoid Cells (ILCS)

ILCS has been described above as an important component in innate immunity and in the response to viruses. They cause the release of cytokines are central type 2 immunity, and are part of the inflammatory cascade in response to external stimuli such as viruses and allergens in airway epithelial cells.[129]

A landmark study by Jackson and colleagues identified the importance of ILC2. [130] After Rhinovirus inoculation of Bronchial epithelial cells,, they found the release of T2-associated cytokines IL4,5 and 13 that correlated with asthma exacerbation severity and that IL-33 levels correlated with viral load. They cultured T-cells and ILC2 with viral supernatant and found this resulted in the production of type 2 cytokines.[130]

Similarly, Dhariwal and colleagues identified an increased ILC2 to ILC1 ratios in BAL in patients with asthma compared to healthy controls after inoculation with rhinovirus.[131] This ratio correlated with the severity of exacerbation and the induction of type 2 cytokines in nasal fluid.

TSLP

TSLP has been considered important in type 2 inflammation through its influence on naïve T-cells and dendritic cells. In response to viral infections, TSLP is released and is thought to play a counter-regulatory

role in ILC-2.[129]. TSLP is also believed to contribute to steroid resistance found in asthma. [132]

IL-33

IL-33 binds to its cognate receptor (ST2) and activates the IL-1 receptor, activating innate and adaptive immunity.[133] Data from GWAS studies have shown that variance in IL-33 has been linked to an increased risk of developing asthma.[134135].

IL-33 is known to be associated with viral load during experimental rhinovirus infection in asthmatic patients. A study recruited 22 healthy donors and 14 with mild allergic asthma who, at baseline, had no recent respiratory infection. They exposed PMBCs to IL-33 and then subsequently cultured them with rhinovirus. In the people with asthma who were not in the healthy control group, they found an increase in IL-5 and IL-13 production.[136] This suggests that IL-33, in the context of HRV infection, may facilitate type 2 inflammation seen in asthma.

Werder and colleagues explored the role of anti-IL33 therapy in mice. They found that it reduced the magnitude of type 2 inflammation during rhinovirus-induced asthma exacerbations, boosted antiviral immunity, and decreased viral replication.[137]. In humans, phase 2 trials of Itepekimab, an anti-IL-33, have shown promise in improving lung function and symptoms in those with poor asthma control. [138]

IL-25

IL-25 is a crucial mediator of T2 inflammation observed in asthma. Beale and colleagues reported that in RV-induced asthma exacerbations, there was increased expression of IL-25 in cultured bronchial epithelial cells in asthmatics and increased viral load. RV-induced responses that are specific to exacerbation, such as type 2 cytokine production, reduced recruitment of neutrophils and eosinophils, and mucus production are reduced by blockade of the IL-25 receptor. [139]

The synergy between allergy and virus infections.

Asthma exacerbations occur during the pollen season in spring and autumn or exposure to an allergen. These tend to develop a viral infection superimposed, leading to a viral-induced exacerbation.

There are many hypotheses explaining why this may occur. One is the common predisposing factor of deficient innate immunity, which reduces IFN-α and INF-β, increasing vulnerability to viral infections and increased deposition of Th2 inflammation immunity to allergens and viruses. [140]This could be associated with abnormal regulation of immune function and potential loss of barrier function.

Another hypothesis is that allergens affect how the immune system responds to viruses. Airway inflammation and hyperresponsiveness could amplify the clinical effects of the virus. Studies show that children who have biological parents with asthma develop allergies at an earlier age and have recurrent viral infections.[141] Those that develop just allergy or virus infection alone do as well have viral infections, but the effect size is much greater when both are present.[142] Studies have shown that in those with an established diagnosis of asthma, the engagement of IgE receptors with immune cells inhibits their ability to produce cytokines, including interferon, in response to viral infections. [143]

The virus could also influence the host immune system's response to the allergens. Viral proteases could affect the Th2 response. Viruses could also influence airway growth and epithelial cell development. [144]

Prevention and treatment of viral-induced asthma exacerbations.

Anti-viral

Anti-viral medications have not been shown to provide benefits to those with asthma. Several drugs have been developed to treat rhinovirus infection, which has had minimal effect. Oseltamivir (Tamiflu) has been approved for the treatment of influenza, but its impact is modest, reducing illness by approximately a day and reducing illness severity.[145] Tamiflu therapy must be commenced early in the disease course to have any effect. There are currently no effective drugs to treat rhinovirus infections. mTORC inhibition has been proven to reduce the incidence of infections in healthy older people. mTORC inhibition has also been shown to prevent rhinovirus infections in people with asthma.[146]

Inhaled interferon

Trials assessing inhaled interferon therapy for asthma exacerbations have not identified therapeutic benefits. The effect of inhaled INF-β within 24 hours of developing a cold was evaluated [147]. The study did not reach the primary endpoint, as there was no overall improvement in asthma symptoms. However, there was a suggestion of some benefit in those with more severe disease as there was a clinically significant improvement in ACQ-6 score in those with asthma who inhaled IFN-β within the first 24 hours. Inhaled INF-β was associated with slightly higher morning peak flow than placebo, but the effect was much more significant in those with severe asthma. Viral load in the sputum was lower in those who had inhaled INF-β, but this was not statistically significant. Recently, a phase 2 RCT by McCrae and colleagues was performed in 121 patients with severe asthma defined as GINA 4/5, and they were randomised to 14 days of inhaled therapy or placebo. The trial drug did not reduce exacerbation frequency or reliever use or ACQ-6 scores[148]

Vaccinations

Influenza vaccination is modestly effective in preventing influenza, and it is recommended that people with asthma have it.[149] There have been attempts to develop a vaccination for rhinovirus; however, this is very difficult as there are several strains of rhinovirus.

Macrolides

Macrolides have been shown to reduce asthma exacerbation risk, as shown in the AMAZES study.[113] Macrolides are anti-inflammatory, and some data suggest they possess antiviral properties to prevent rhinovirus-associated infections. [150113]

Biologicals

Teach and colleagues studied in older children and teenagers the effect of omalizumab.[121] They administered omalizumab treatment before the season for rhinovirus to see if this affected autumn-associated asthma exacerbations. [151] The study demonstrated that asthma exacerbation was reduced in a subgroup of children. This was confined to those who experienced an asthma exacerbation during the study's run-in period. There was no effect on asthma exacerbation rate in those with stable asthma during the run-in period. In the follow-up aspect of the study, treatment in the Omalizumab group, compared to placebo, reduced the prevalence of laboratory-confirmed rhinovirus infections. A slight reduction in peak RV shedding was noted in those with Omalizumab compared to placebo. Benefits were seen in the average number of colds per subject, but no difference was seen in the average duration of colds.

Piñeros and colleagues explored the impact of mepolizumab on rhinovirus-induced asthma exacerbations. [120] In this Randomized Controlled Trial, they gave the anti-IL-5 to mild asthmatics who were steroid naïve patients and exposed them to rhinovirus two weeks later. They found that Mepolizumab could not reduce eosinophil activation, but

higher viral loads were seen in nasal swabs. This may reflect increased replication or decreased clearance. [152] The study's endpoint was lung function, and factors such as asthma severity may have contributed to the findings.

Why do inhaled corticosteroids prevent viral exacerbations?

The AMAZES study also demonstrated an inverse relationship between IFN production with viral inoculation and the dose of ICS. The greater the dose of ICS, the lower IFN-α after inoculating peripheral blood mononuclear cells with RV in vitro. In the multivariate analysis variables, sputum neutrophils were negatively associated with INF-a production, as was higher inhaled steroid dose, but interestingly, atopy was associated with greater INFα production. Although statistically significant, the correlation coefficient (R^2) was 0.26, suggesting other factors influenced the findings. Several mechanisms have been suggestive to explain the differences. In vivo studies have demonstrated that those with neutrophilic inflammation produce more IL1-β, which could inhibit interferon production.

Therefore, with these findings, the question remains: How could ICS be responsible for fewer viral-associated exacerbations? Studies that have looked at cells in the peripheral blood show it is difficult to see how ICS can affect these cells unless there is systemic absorption in those with higher doses of ICS. However, what is more likely is that ICS are a surrogate marker for asthma severity, with the increased severity of asthma being associated with higher doses of prescribed ICS, and the link with lower interferon production is more likely due to asthma severity.

Based on this reasoning, a subgroup of asthmatics who are prescribed a higher dose of ICS may be predisposed to viral infections. However, this needs to be explored in more detail. In vitro data suggests that if you treat cells with corticosteroids, the ability to inhibit interferon or other cytokines is dose-dependent, as we have learnt from COVID-19.[153,154]

Symptoms of asthma and asthma exacerbations.

The symptoms of asthma can vary amongst patients. The most characteristic feature is a wheeze on expiration. Other symptoms include cough, shortness of breath, shortness of breath on exertion and chest tightness. These symptoms can cause nocturnal awakening and limitations in activities of daily living.[155]

Several other respiratory conditions share similar symptoms. Therefore, it is imperative to elicit the context of the symptoms, such as triggers, timing, relieving factors, and response to therapeutic strategies, when establishing a diagnosis of asthma. [155]. The intensity of symptoms varies over time and can be brought on by seasonal changes.

Symptoms such as wheeze, cough, and shortness of breath have a low sensitivity and specificity to support a diagnosis of asthma, as they are shared among several respiratory conditions.[42] The predictive value of symptoms is increased if the symptoms are episodic. [156,157]. An auscultation finding of an expiratory wheeze increases the likelihood of asthma. [158]

When the symptoms of a person's asthma deteriorate from their usual day-to-day and their usual medications are not providing relief, it could be the mark of an impending asthma exacerbation.

Diagnosis of Asthma and Asthma Exacerbations

There is no gold-standard diagnostic test for asthma. The diagnosis of asthma is clinical and is currently based on identifying characteristic symptoms of asthma and examination findings through chest auscultation for an expiratory wheeze. However, examination findings can be normal; therefore no individual finding is central to the diagnosis of asthma. Evidence of airway obstruction reversibility is usually targeted to support a diagnosis of asthma.[155] Clinically, this is traditionally done by measuring PEFR in primary care. Sometimes, spirometry is utilised to

demonstrate airflow limitation or reversibility of FEV1 after the administration of bronchodilators such as salbutamol or advanced tests such as bronchial provocation tests. Patients are also assessed for the presence of airway inflammation, most commonly through FeNO and blood eosinophils.

As previously discussed, symptomology, inflammation and lung function can vary over time and at the point at which such reviews or tests are performed, the patient may not exhibit features of asthma. Therefore, a combination of tests is sometimes required, which are taken ideally at different times. Bronchial provocation tests are considered to elicit airway hyperresponsiveness. There have been studies that have demonstrated that asthma can be under [159] and over-diagnosed [160–162]. The former leaves a patient with symptoms that can have a broader impact. The latter leads to treatment that is not required, incurring costs for the patient and healthcare system as well as potential side effects from the medication prescribed.

Investigations:

PEFR

The PEFR is the best of three expiratory blows from total lung capacity (TLC). Further blows are encouraged if the two largest values are not within 40 L/min of each other. [163]. PEFR is measured by a peak flow meter, a device patients can take home. It is therefore ideal as a measurement of variability. Patients suspected of having a diagnosis of asthma are asked to keep a peak flow diary for two weeks and to take two readings; in the morning and at night to demonstrate diurnal variation in the form of variability in readings. The highest and lowest difference is taken and expressed as a percentage of the average PEF.[164,165] The challenges associated with this method are that it requires a patient to be compliant with taking readings, depends on the patient's breathing effort to be maximal, and requires good technique at using the device. The

upper limit of normal is 20% variability, with a value more excellent, increasing the likelihood of a diagnosis of asthma. [166].

Spirometry

Spirometry is the investigation of choice to demonstrate airflow obstruction. Normal spirometry, when a patient is symptomatic, does not exclude a diagnosis of asthma. The correlation of FEV1 to symptoms is poor. The FEV1/FVC changes with age therefore, levels below 70% may be normal and not indicative of airflow obstruction. A positive result from spirometry is defined as an improvement of FEV1 by 12% and 200ml after administering either a beta-agonist or a trial of corticosteroid therapy. An improvement of 400ml is strongly suggestive of asthma.

Direct Challenge Tests

Direct challenge tests are helpful for the assessment of airway hyperresponsiveness. An agent that is known to cause bronchoconstriction is inhaled, and the concentration of the agent, in conjunction with the change in FEV1, is taken over a set period of time. The most common agents used for this test are histamine or methacholine. The response is recorded as the provocative concentration (PC20). Linear interpolation of the log concentration is performed, and a positive test is regarded as a 20% fall in FEV1 with a concentration of agent of 8mg/ml or less.[167] This test is usually performed when no evidence of airflow obstruction is demonstrated by PEFR or spirometry. Other less commonly performed tests are indirect challenge tests with exercise or mannitol.

FeNO

Measuring the fraction of exhaled nitric oxide (FeNO) has become a core component in assessing airways disease. A high FeNO value is suggestive of Type 2 airway inflammation. GINA suggests that a FeNO of more than 20 ppb [168] may be indicative of Type 2 inflammation, whilst the ATS use a value of ≥ 25 ppb to be suggestive of type 2

inflammation.[169] FeNO can be affected by other variables, such as an increase in the presence of allergic rhinitis, sex(male), and the consumption of nitrates. Smoking is known to reduce FeNO levels. FeNO is independently associated with the risk of an asthma exacerbation, [170,171] and response to inhaled corticosteroids. [172] FeNO levels correlate well with the presence of eosinophilic airway inflammation and are useful for identifying type 2 inflammation, but a raised FeNO may not always be caused by asthma.[169] An improvement in FeNO is suggestive of a good response to ICS, similar to that found in sputum eosinophilia and therefore is used as biomarker to assess response to ICS therapy.[173]

Guidelines in asthma management:

The National Institute for Health and Care Excellence and British Thoracic Society have joint guidance on the diagnosis and management of asthma.[174]

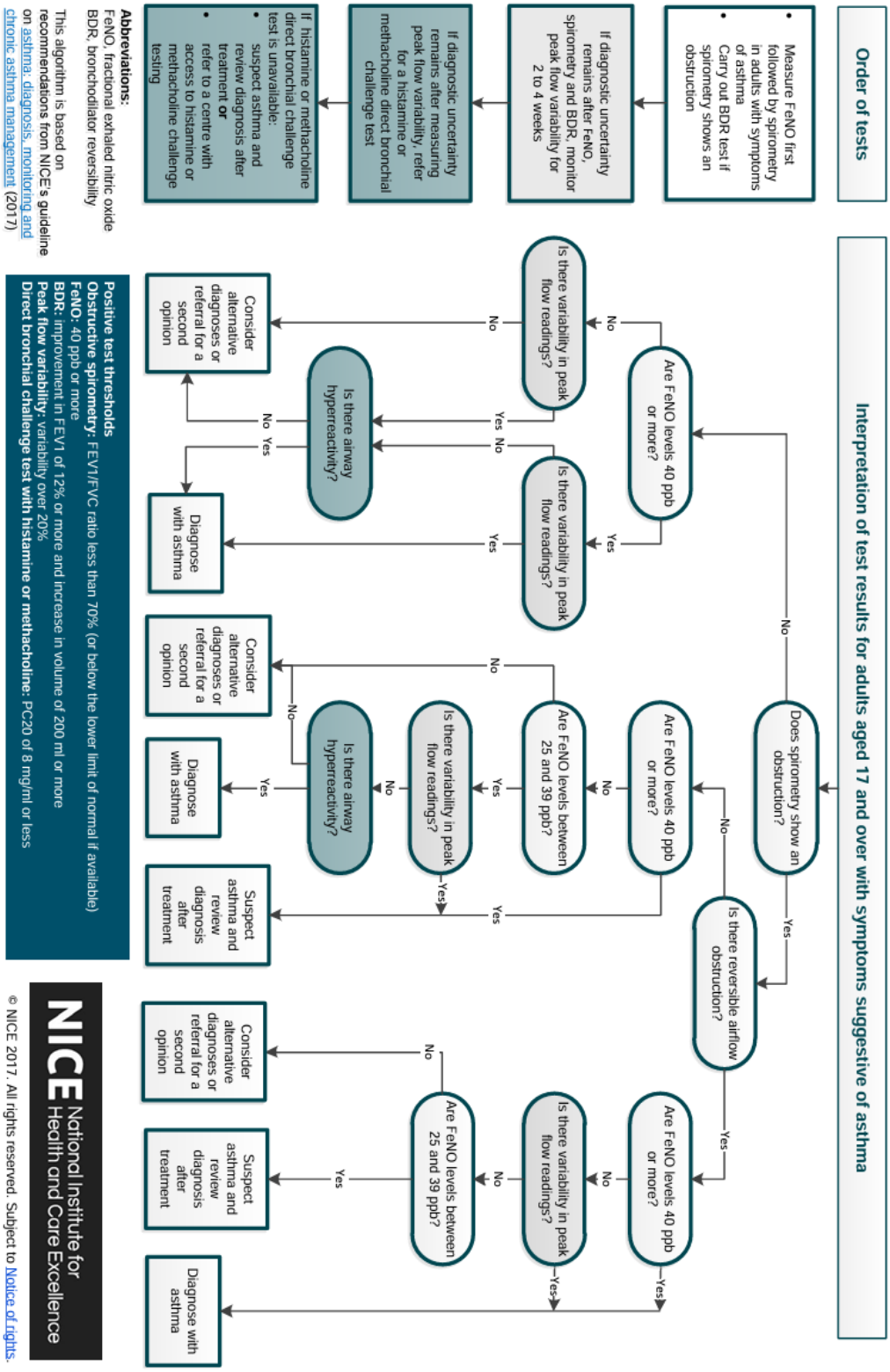


Figure 1. 8: NICE guidance on the diagnosis of asthma

Emphasis is placed on obtaining a detailed history from the patient, by a clinician with experience in asthma, to elicit symptoms of asthma as well

as a history of atopy. A normal value for the measurement of airflow obstruction does not exclude a diagnosis of asthma. If there remains, diagnostic uncertainty, then other tests, such as assessing for bronchodilator reversibility, are suggested.

Management of Asthma

Asthma Control

The focus of asthma management is to achieve reasonable asthma control. Asthma control has two components associated with it: symptom control and the prevention of adverse outcomes including asthma exacerbations, fixed airflow obstruction and side effects from prescribed medications. [175]There are validated tools available to assess asthma control, such as the Asthma Control Questionnaire (ACQ),[176] the Asthma Control Test (ACT) [177] and the "RCP-3Q's" which is a validated questionnaire produced by the Royal College of Physicians to gauge asthma control [178] Uncontrolled asthma is a risk factor for asthma exacerbations. However, achieving asthma control has been found to not always eliminate the risk of exacerbation.[179] [180,181] To further add to the complexity of asthma management, it has been shown that there can be discordance between symptoms and the risk of asthma exacerbations,[175] as well as discordance between symptoms and lung function, too. [182]

Risk management and factors for risk of future asthma exacerbations.

As discussed, a core component of asthma control is the prevention of future asthma exacerbations. There are known risks for future asthma exacerbations:

Prior history of asthma exacerbations.

A prior history of asthma exacerbations has been identified as a risk of future asthma exacerbations in several observational studies. [183] [184–186] The TENOR study group identified in an analysis of 2780 patients with

severe or difficult-to-treat asthma; the odds ratio after adjustment for demographics was 3.77 (95% CI 2.62,5.43).[186] Most studies have defined exacerbation on ERS/ATS definitions and therefore, have included hospitalisation, emergency department visits and a prescription of OCS [185] as a risk for future asthma exacerbations. The number of prior OCS courses is also a risk for future asthma exacerbations. [183]

Overuse of short-acting beta-agonists (SABA)

Overuse of SABA to aid in symptom control is commonly reported in studies. [187,188] Overuse of SABA is of concern as it does not address the underlying airway inflammation that can predispose to an asthma exacerbation.

Overuse of short-acting beta-agonist therapy is suggestive of poor asthma control. A Swedish population-based study identified that in a population of over 365,000 patients with asthma, 30% were reported to overuse SABA inhalers (defined as requiring more than two canisters over a 1-year period), with 21% collecting between 3 and 5 canisters a year. The number of canisters collected correlated with an increased risk of exacerbation. [189] Furthermore, the metrics use of salbutamol has been identified as a risk of future asthma exacerbations, with the number of days of salbutamol use and the number of actuations of salbutamol used over 24 hours. [190] A post-hoc analysis of this study explored inhaler use patterns prior to hospital attendance for severe asthma exacerbations. Excessive salbutamol use (median of 46 actuation) was identified with a delay in seeking medical care. Despite excessive SABA use, a pattern of no ICS use was also demonstrated. [191] Overuse of salbutamol can cause hypokalaemia [192] and cardiac instability.[193]

Excessive use of SABA inhalers (more than 11 canisters) has been associated with an increased risk of asthma-related mortality. [194,195]

Poor asthma control

Uncontrolled asthma has been shown in studies to be a predictor of future risk of asthma exacerbations. [196] [184] [197] With the level of poor control

correlating with an increased risk of an asthma exacerbation. [181] Asthma control has been shown to correlate with GINA-based level of asthma control. [198] The area under the receiver operating characteristic curve for the Asthma Control Test (ACT) score for predicting GINA control was 0.84 (95 CI 0.82-0.85).

Obesity

Obesity is a significant health problem with a rising prevalence, particularly in the Western world. The incidence of asthma is higher in obesity. A meta-analysis of over 300,000 asthmatics found the odds ratio of asthma in the overweight being 1.38 (95% CI 1.17-1.62) and obese being 1.92 (95% CI 1.43-2.59) [199]Obesity is defined as a Body Mass Index (BMI) of more than 30kg/m^2. Obese patients with asthma were found to have increased proinflammatory adipokines, such as leptin, which correlated with airway expression of leptin and adiponectin. [200] This also showed a significant correlation with airway reactivity. However, the study showed no correlation with airway inflammation. [200] It is known that airway epithelial cells have receptors for adipokines, suggesting they play a role in pathogenesis.[200] Obesity is a risk factor for poor control of asthma as well, with obese patients responding less well to ICS and ICS/long-acting beta-agonists (ICS/LABA) to achieve control and also as a factor for increased risk of future asthma exacerbations. [201] [202] . Obesity has been shown to modulate the effect of key signalling proteins for steroid responsiveness, such as mitogen-activated kinase phosphatase-1, through the pro-inflammatory state of obesity. [203] Obesity is noted to cause a reduction in lung function, in particular functional residual capacity and residual volume, which can have an impact on asthma control. [204]

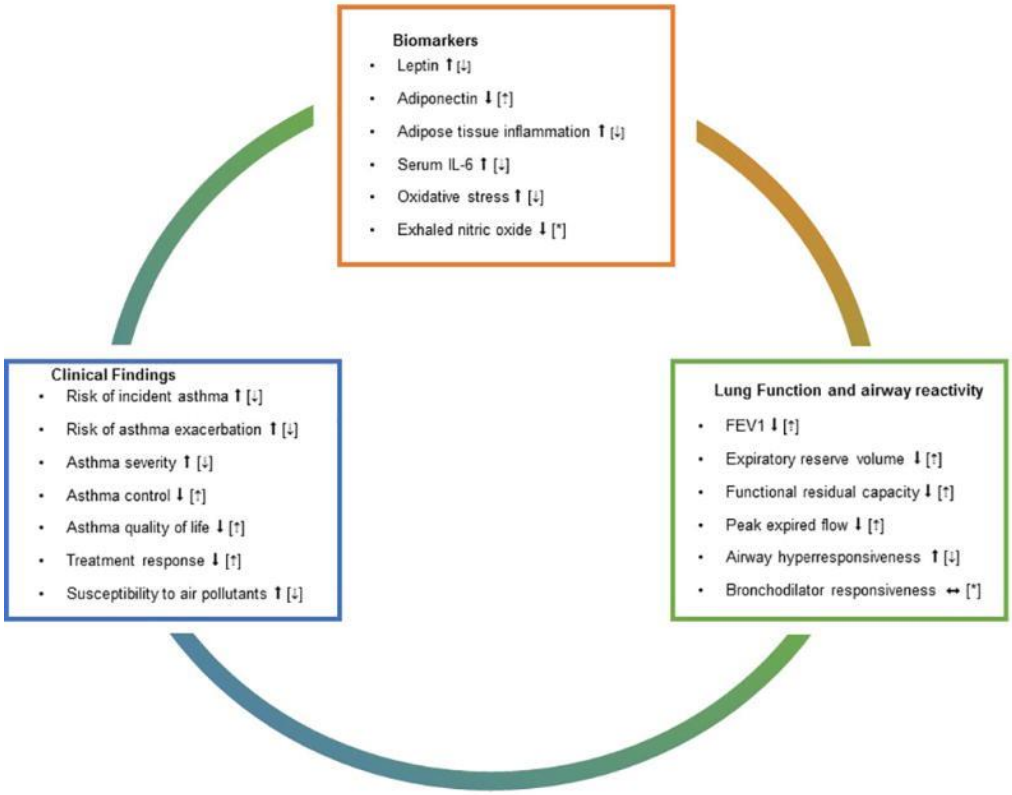


Figure 1. 9: The pathways by which obesity leads to poor outcomes in asthma

Bold arrows indicate the sequelae of obesity in asthma, whilst light arrows are indicative of improvements with a reduction in weight.

Image obtained from Peters et al.: Obesity and Asthma Journal of Allergy & Clinical Immunology 2018,141(4):1169-11179**.** [202]

Smoking

Patients with asthma who smoke have worse outcomes than those asthmatics who do not smoke, both in terms of morbidity and mortality.[205]. Between these populations, smokers are found to have worse asthma control and increased asthma exacerbation frequency.[206]This seems to strongly correlate with pack-years, with a packed year of more than >20 having worse clinical outcomes than a lower pack-year history OR 5.59 (95% CI 1.44, 21.67) to develop severe asthma. [207]. Smoking is also known to cause a reduction in lung function and contribute to obstructive

airways disease. Patients with asthma who smoke are also less sensitive to the benefits of ICS. [208]or OCS therapy[209].

Similar inflammatory changes occur in Chronic Obstructive Pulmonary Disease (COPD). Smoking leads to macrophage activation and neutrophil activation, which can lead to mucus hypersecretion. [210]

FeNO

Approximately 50% of patients with severe asthma demonstrate eosinophilic inflammation, as identified in real-life data from the Severe Asthma Registry Programme (SARP), which includes 1716 patients.[211] [212] As mentioned, FeNO is an established biomarker of type 2 inflammation caused by the cytokines IL-4 and IL-13. It is responsible for features such as AHR, goblet cell hyperplasia, and mucus hypersecretion.[213] A raised FeNO has been demonstrated to be associated with a greater risk of asthma exacerbation.[214] Notably, the greater the absolute FeNO values, the greater the risk of exacerbation, specifically in those with uncontrolled moderate to severe asthma.[215]

Adherence

It has been demonstrated that low adherence to inhaled medication leads to a greater risk of asthma exacerbation. [216] The most common methods to assess adherence are self-reporting and prescription pick-ups. The former has a risk of overreporting, whilst the latter suggests that a patient is collecting prescriptions, which does not necessarily mean they are taking their inhaled medication. Adherence to inhaled medicines for asthma has been reported widely as being poor. [217] [218] Several factors contribute to medication adherence, including the costs of the medication, the relevance of the medication to the disease, and side effects associated with taking the medication. A retrospective observational study explored whether an asthma exacerbation influenced adherence over 12 months.[219] They found that adherence improved after one exacerbation and was greater if there was more than one

exacerbation. Still, their method of assessing adherence was through a portion of days covered – which looks at the proportion of days a patient has access to their medication over a period of time,[220] obtained from healthcare system databases.

Management of Asthma Exacerbations

Management of asthma includes modifying risk factors such as allergy avoidance and comorbidities of asthma. Another significant component is medical therapies. Inhaled corticosteroids (beclomethasone, budesonide, ciclesonide, fluocinonide, fluticasone and mometasone are the mainstay in the treatment of asthma and have been shown to improve daily symptoms, quality of life, lung function, airway hyperresponsiveness as well as a reduction in the frequency and severity of asthma exacerbations. Studies have highlighted that those who benefit the most from ICS and OCS are those who express T2 inflammation in mild-moderate asthma. [69] [221] as well as in those with moderate-severe asthma. [222]

Treatable traits

In 2016, the “treatable traits” concept was introduced for asthma. [223] Treating the phenotypical observation or targeting of a causal pathway encompasses individually treating patients and addressing asthma's heterogeneity. This concept was further developed. [224] breaking the components of asthma care into pulmonary treatable traits, extra-pulmonary traits and behavioural/lifestyle traits.[224] This approach could potentially avoid unnecessary asthma medications, which could lead to patients experiencing unwanted side effects; such as in those patients who have been prescribed OCS as maintenance therapy for their asthma. They are at increased risk of complications such as infection, osteoporosis, fractures, cataracts and metabolic complications such as adrenal insufficiency and diabetes. [225] and an overall increased risk of mortality.[226]For example, patients who demonstrate poor evidence of

airway inflammation but clear evidence of airflow obstruction may benefit from LABA therapy. [13].

The benefits of using this approach in asthma management have been demonstrated in studies. The utility of treatable traits versus a standardised step-wise approach has been explored. A randomised control trial study examined the Asthma Quality of Life Questionnaire (AQLQ) as the only endpoint. Although a sample size of only 55, the study demonstrated a significant improvement in AQLQ score and asthma control. However, the arm of treatable traits did have a case manager and increased contact time compared to the standardised approach. [227]

Concerns from this approach are risks associated with focused care on targeting traits rather than providing holistic care for patients with “asthma syndrome”, i.e. not targeting components that may influence symptomology, such as Airway Hyperresponsiveness (AHR) with exclusively targeting airway inflammation.

When asthma becomes uncontrolled, there is a tendency to escalate inhaled therapies or add additional treatment options to gain control. Modifiable risk factors contributing to the loss of control, such as medication adherence and correct inhaler technique, are often not addressed [228] and smoking, which can affect the response to inhaled corticosteroids. [229]Clinicians are encouraged to adopt a phenotype-based approach to asthma care. Guidelines have changed to incorporate ICS use for those with mild asthma.

The benefits of utilising low dose-ICS have been proven to include a reduction in severe exacerbations, improved symptom control, and a reduction in lung function decline [20]. There has also been a change with the adoption of ICS plus a rapid onset LABA to be used on an as-needed basis as a rescue medication. This has reduced symptoms and exacerbation frequency even in those with no baseline features of T2-

inflammation [230] [231]. This has led to the inclusion in the GINA 2019 guidelines of inhaled corticosteroid-formoterol as an alternative regime to low-dose inhaled corticosteroid therapy in those with mild asthma. [231]. This would benefit patients with mild asthma who are less likely to take a daily ICS as a controller [231].

The benefits of escalating ICS therapies to high dose ICS are unclear in those with poorly controlled asthma and whether this contributes to the prevention of future asthma exacerbations. [232] [233] Pavord and colleagues looked at a large cohort of patients derived from electronic medical records. They found no difference in the mean time to an asthma exacerbation defined as a course of OCS in those who remained on medium-dose ICS compared to those who stepped up from medium-dose to high-dose ICS.[232] Those who were on a higher dose of ICS experienced a higher risk of an asthma exacerbation compared to those on medium-dose ICS. However, this could represent increased surveillance because of an escalation of therapy.

The BTS, NICE, and GINA guidelines all adopt a stepwise approach to asthma care. Asthma is used as an umbrella term, and changes in treatment are implemented primarily based on patient-reported symptoms rather than a biomarker-based approach such as elevated FeNO. [234] or the presence of sputum eosinophilia [235] which has been shown to reduce exacerbation frequency. GINA recommends that alternative inhaled therapies to be considered when high-dose ICS/LABA is being considered.

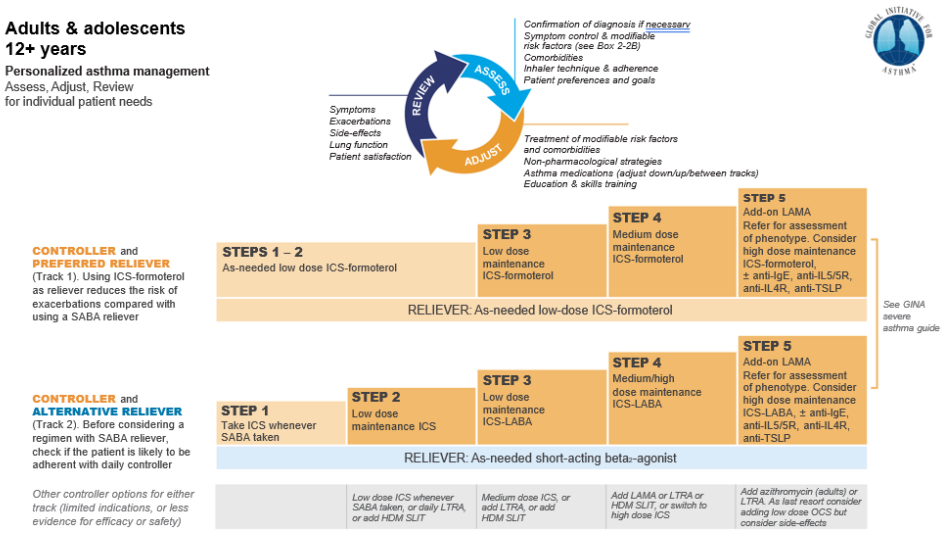


Figure 1. 10: The stepwise management of asthma from the Global Initiative for Asthma 2022.

ICS= inhaled corticosteroids, LABA= long-acting b-2 adrenergic bronchodilators. LAMA long-acting muscarinic antagonists (LAMA).

This will achieve adequate control for the majority of patients, as demonstrated by the Gaining Optimal Asthma Control study (GOAL). [236] However, there is a risk of escalating treatment without identifying another plausible explanation for symptoms. This can also potentially lead to patients being on inappropriately high doses of ICS and OCS, especially in those with low evidence of T2 inflammation.

A meta-analysis conducted by Beasley and colleagues demonstrated that after reviewing six studies of adolescents and adults with moderate to severe asthma, assessing the dose-response on important outcomes such as lung function and exacerbation; the benefit plateau.[237] A pooled analysis demonstrated that most of the therapeutic benefit of Fluticasone Propionate was achieved with a daily dose of 200µg/ day with reduced benefits at a higher dose. For exacerbations, the odds of withdrawal from the respective studies were no different, with 200µg versus ≥ 500µg being 1.27 (95%CI 0.78 to 2.07). However, there is a proportional increased risk of side effects with increasing doses. (Figure 1.9)

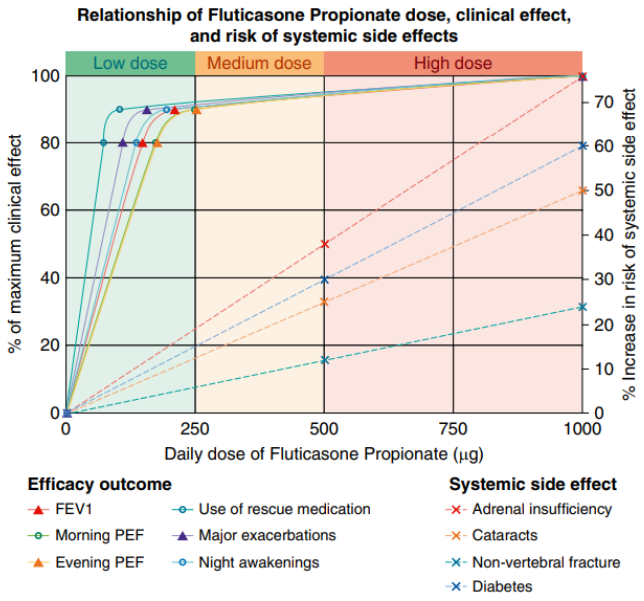


Figure 1. 11: Limitations of benefits from ICS therapy and associated adverse effects with increased dosage.

Image obtained from Beasley et al: Inhaled Corticosteroid Therapy In Adult Asthma. Time for a New Therapeutic Dose Terminology. Am J Respir Care Med 2019 15;199(12):1471-1477. [233]

Self-management of asthma exacerbations through ICS therapies

Recognising a developing exacerbation is the cornerstone of managing asthma exacerbations. Written personalised asthma action plans (PAAPs) are widely advocated to help support patients with this.[168] This is a written plan for patients when they develop worsening of their asthma symptoms and advice on escalating their inhaled controller therapies or reliever therapies and when to seek medical support for their asthma.

The escalation of as-needed low-dose ICS-formoterol has been shown to reduce the risk of severe exacerbations compared to SABA inhaled therapy alone in those with mild asthma.[238] Using ICS-LABA in a maintenance reliever regimen has been shown to reduce asthma exacerbation requiring OCS, and hospitalisation is of more significant benefit when started earlier on into an impending exacerbation. [222] [239]Quadrupling ICS has been shown to reduce the risk of requiring OCS for an asthma exacerbation [18]. For most asthma exacerbations, a short course of OCS is prescribed, i.e. 40mg for 5-7 days, to gain control of symptoms. The decision to prescribe a course of OCS is through a

combination of symptomology, failure of their usual medication to gain control of symptoms after escalation and a demonstration of reduced lung function, most commonly FEV1 or PEFR.

Management of asthma exacerbations in primary and secondary care

The British Thoracic Society and Scottish Intercollegiate Guidelines Network have published guidelines on the management of an acute asthma exacerbation; they have classified the severity of asthma exacerbations through a combination of symptoms, lung function in the form of Peak Expiratory Flow Rate (PEFR) and Arterial Blood Gas (ABG) parameters (Table 1.1)

<table>
<tr><td>Moderate acute asthma</td><td colspan="2">Increasing symptoms
PEF >50–75% best or predicted.
No features of acute severe asthma</td></tr>
<tr><td>Acute severe asthma</td><td colspan="2">Any one of:
- PEF 33–50% best or predicted.
- respiratory rate ≥25/min
- heart rate ≥110/min
- inability to complete sentences in one breath</td></tr>
<tr><td rowspan="8">Life-threatening asthma</td><td colspan="2">Any one of the following in a patient with severe asthma:</td></tr>
<tr><td>Clinical signs</td><td>Measurements</td></tr>
<tr><td>Altered conscious level</td><td>PEF <33% best or predicted</td></tr>
<tr><td>Exhaustion</td><td>SpO_2 <92%</td></tr>
<tr><td>Arrhythmia</td><td>PaO_2 <8 kPa</td></tr>
<tr><td>Hypotension</td><td>’normal’ $PaCO_2$ (4.6–6.0 kPa)</td></tr>
<tr><td>Cyanosis</td><td></td></tr>
<tr><td>Silent chest</td><td></td></tr>
<tr><td>Poor respiratory effort</td><td></td></tr>
<tr><td>Near-fatal asthma</td><td colspan="2">Raised $PaCO_2$ and/or requiring mechanical ventilationwith raised inflation pressures[555-557]</td></tr>
</table>

Table 1. 1 Level of severity of acute asthma exacerbations in adults. BTS/SIGN British guideline on the management of asthma. July 2019

Moderate exacerbations tend to be managed in primary care, while features of acute severe asthma require secondary care support. However, if there is no improvement after initial treatment in primary care, prompt referral to secondary care is needed.

Management of asthma exacerbations includes the use of bronchodilators, usually a SABA (most commonly as an inhaler in primary care) or nebulised SABA and short-acting anticholinergics (ipratropium) in secondary care, in addition to corticosteroid therapy and if required; prescribed oxygen therapy. Nebulised ipratropium is associated with an earlier recovery of FEV1 and FVC than nebulised salbutamol alone. [240] Antibiotics have not been advocated for prescribing alongside oral corticosteroid therapy unless there is a strong suspicion of a bacterial infection. [168]

Phenotyping Asthma

To encompass the heterogeneity of asthma, there have been several attempts to define asthma based on a combination of biological and clinical features. This is known as phenotyping. A phenotype is defined as:

"*The composite, observable characteristics of an organism, resulting from the interaction between its genetic make-up and environmental influences, which are relatively stable, but not invariable, with time. Phenotyping integrates biological and clinical features, ranging from molecular, cellular, morphological and functional to patient-orientated characteristics with the goal to improve therapy*" [17].

ERS/ATS guidelines severe asthma 2014.

The success of phenotyping asthma has been identified through clinical trials, such as the targeting of blood eosinophils with biological therapies benralizumab [241] and mepolizumab [242]in those with severe uncontrolled asthma . There is currently no consensus on classifying asthma phenotypes. Most phenotypes are developed now by integrating clinical, immunological, and physiological data as described below. First, it is important to consider the immunological basis and asthma-associated mechanisms that integrate to lead to the observed clinical phenotypes in studies. However, the clinical phenotypes (e.g., childhood-onset vs late-onset, allergic vs non-allergic) have poor correlation with a specific mechanistic pathway. As such, there is no unifying biological basis for phenotypes. [243]

Common T2-high clinical phenotypes

Early-onset allergic asthma

This typically starts during infancy and is triggered by allergens and other environmental stimuli such as viruses, cigarette smoking, and pollution that induce inflammation, leading to bronchoconstriction. Early onset allergic asthmatics have characteristics of atopy, including raised circulating allergen-specific IgE and comorbidities associated with allergy, including allergic rhinitis and atopic dermatitis. It is suggested there is a genetic predisposition to this. Kuo and colleagues explored asthma control and lung function according to allergic status. Those with no allergy, defined as a negative skin prick test (SPT), had worse lung function, demonstrated a greater extent of small airway disease measured by IOS, and scored higher in the ACQ. Interestingly, although statistically insignificant, compared to the allergic group, the non-allergic group were on a higher mean dose of ICS by 100 mcg.[244]

Late-onset non-allergic asthma

This occurs in adulthood and is characterised by a T2-high inflammatory and immune signature; however, unlike early allergic asthma, there is a lack of atopy present, resulting in low levels of allergen-specific IgE.[245] ILC2 is thought to contribute to the phenotype by increasing epithelial cell proliferation and promoting airway remodelling in response to tissue damage through the production of amphiregulin.[77] [246] Clinical characteristics include fixed airflow obstruction and steroid-resistant eosinophilia. Resistance to ICS has been suggested to be associated with ILC2. The memory T-cell CD45RO is expressed by ILC2, and has been shown to contribute to steroid resistance and sputum eosinophilia. [247]

Aspirin-exacerbated respiratory disease (AERD)

Asthma exacerbated by aspirin or asthma Aspirin-exacerbated respiratory disease (AERD) is associated with the development of respiratory symptoms after the consumption of aspirin and Nonsteroidal anti-inflammatory drugs (NSAIDs). It is associated with nasal polyps, aspirin sensitivity and asthma. However, asthma is not always present, hence the preferred term AERD.[248] It is associated with eosinophilia and occurs to cyclo-oxygenase-1 (COX-1 blockade). [249]

Common T2-low clinical phenotypes

Smoking associated asthma

From the literature, it is estimated that approximately 25% of asthmatics are reported to smoke. [250] An average decline of 58ml a year in FEV1 of smokers compared to 33ml in adult males was demonstrated by the Copenhagen City Heart Study.[251] Cigarette smoking causes oxidative stress by releasing reactive oxygen species (ROS) and reactive nitrogen

species (RNS) and suppressing antioxidant enzymes, causing cellular damage. Smoking also leads to the upregulation of neutrophils, macrophages and CD8+ T-cells[250] Cumulatively, this results in lung tissue damage and impaired epithelial barrier function, increasing susceptibility to bacterial infections.[77] Smoking is associated with steroid resistance. Pollution and emissions can also lead to the development of asthma.

Obesity-realted asthma

Obesity is frequently associated with neutrophilic asthma, later onset asthma and females. Approximately over 50% of asthmatics are obese. [252] Features include steroid resistance and AHR, which can improve with weight reduction. As described above, this is associated with a predominately T2-low phenotype.

Older onset asthma

Older onset asthma is usually defined as developing after age 65. In this population, it is associated with higher rates of morbidity and mortality, which has been attributed to under-diagnosis and, therefore, undertreatment.[253] Ageing is related to changes in lung physiology, including peripheral airway narrowing, reduced elastic recoil, and sarcopenia. The neutrophilic inflammation associated with this is elevated by Th1 and Th17 response. Comorbidities such as impaired motor skills and cognition, if present, can make the management of asthma in this subpopulation challenging.

Examples of phenotyping asthma in adults

Several cohort studies have attempted to encompass the heterogeneity of asthma through phenotyping, most often using cluster analysis, which can be an unbiased approach, depending on the methodology.

One of the first studies To look at asthma phenotypes was performed by Halder and colleagues. [254] This was a retrospective analysis of three data sets based on primary and secondary care, consisting of refractory asthmatics from secondary care and a clinical trials database. The most clinically relevant parameters were selected as per the judgment of investigators. Principal Component Analysis (PCA) was applied to reduce the number of variables. The outcome was 7 clusters being formed: three clusters in the primary care data set and four in the secondary care data set. However, of interest was that two clusters were identified that were common to both datasets: the “early onset-symptom-predominant group with minimal eosinophils” cluster , and the “late-onset, male predominant eosinophilic inflammation with few symptoms’ cluster. Whether the clusters identified remained after longitudinal follow-up is unknown. Selecting clinically relevant parameters, adds subjectivity to the analysis rather than a genuinely data-driven approach, but conversely adds clinical relevance.

Following this; Moore and colleagues conducted the Severe Asthma Research Programme (SARP) study. The study included 726 patients, using 34 variables based on individual judgment to generate 5 clusters, which were integrations of symptoms, medication use and lung function

Both studies identified groups with characteristics of females with adult-onset, non-atopic asthma, and a group of severe late-onset atopic asthma associated with fixed airflow obstruction Sputum count as a discriminator for phenotyping was not used in the SARP project, unlike the work performed by Haldar and colleagues

Patrawalla and colleagues[255] replicated the SARP methodology in an urban predominant asthma population in an attempt to recreate the 5 phenotypes identified in the SARP cohort.

A prospective study of adult-onset asthma in the Netherlands was conducted. [256] This involved 200 patients with asthma over the age of 18 were included, and variables that encompassed clinical physiological and inflammatory markers were included. Three clusters were identified, all with older-onset asthma. One cluster had airflow obstruction associated with eosinophilia despite high-dose ICS but a low symptom burden. The second cluster was predominantly female with a high symptom burden and no objective evidence of T2 inflammation, and the third was controlled asthma with no evidence of airflow obstruction or inflammation. This study therefore reinforced findings from the SARP and Haldar study.

Recent cluster analysis work includes the Mechanisms underlying the Genesis and evolution of asthma (MEGA) Cohort study[257] which adopted cluster analysis with detailed phenotyping on a prospective multicentre cohort of 512 patients with asthma. They identified four clusters using seven variables and had findings similar to those of SARP and Haldar et al.

All these pieces of work demonstrate that emergent phenotypes are consistently identified (Figure 1.10) in what seems to be a heterogeneous asthma population with distinct clinical and physiological biomarkers.

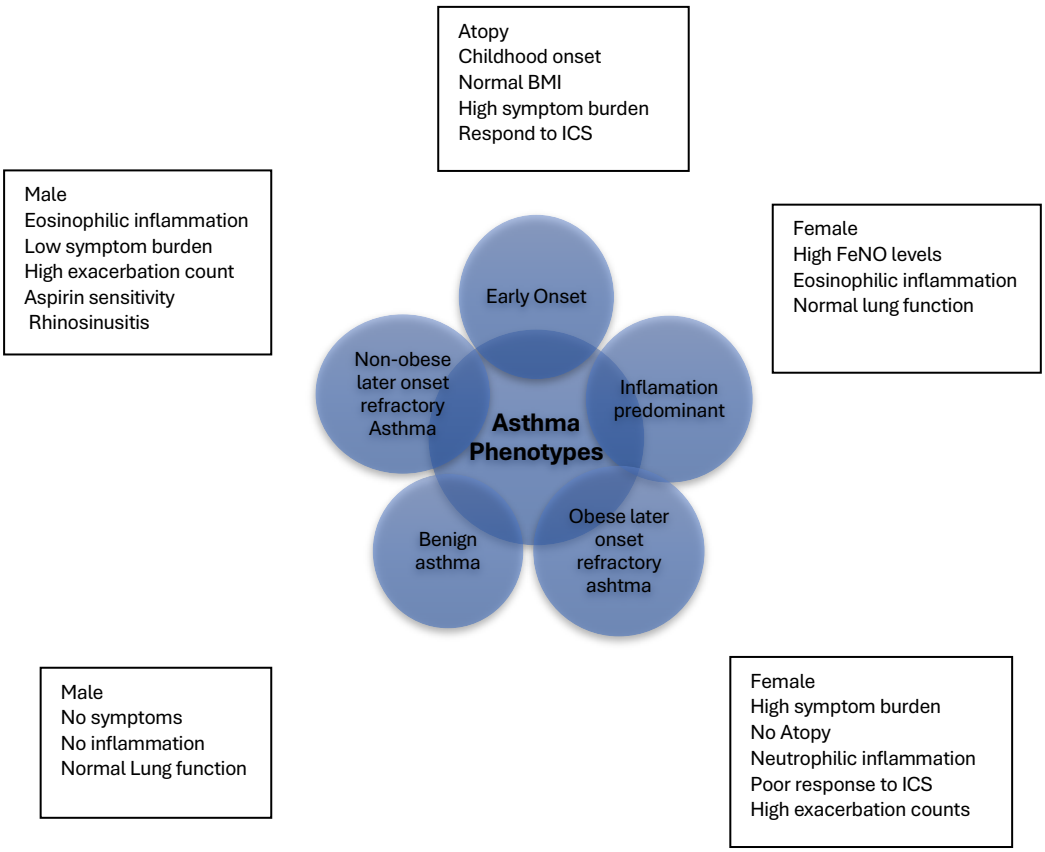


Figure 1. 12: Commonly identified asthma phenotypes observed through clustering-based methodologies.

These works also demonstrate that the sample population used for phenotyping asthma exacerbation is reflected in the clusters identified. It is also essential to have a detailed level of clinical information for the clusters to be meaningful. Integrating important physiological and inflammatory parameters is needed to add granularity to any identified phenotypes. Studies that have attempted to phenotype asthma exacerbations are explored within Chapter 2.

Author (Year)	Title	Population	Parameters considered	Outcome
Wenzel et al. (1999)[258]	Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiological and clinical characteristics	34 severe refractory corticosteroid-dependent asthmatics.	Endobronchial biopsy, lung function, allergy history and clinical variables	Two groups of eosinophil (-) and eosinophil (+). Eosinophil (+) had a thicker subbasement membrane and required intubation more.
Pavord et al. (1999)[259]	Non-eosinophilic corticosteroid unresponsive asthma	23 patients with asthma treated with SABA prn prior and then two months ICS	Spirometry, methacholine challenge, and induced sputum stratified according to the presence of eosinophils or not.	Improvement in lung function and bronchial hyperresponsiveness seen in the eosinophilic group suggestive of a greater improvement to ICS in those that demonstrated T2-inflammation
Green et al. (2002)[260]	Analysis of induced sputum in adults with asthma: identification of a subgroup with isolated neutrophilia and poor response to inhaled corticosteroids	250 adults with symptomatic asthma receiving step 1 to 3 BTS therapy compared to 34 controls	Lower airway inflammation assessed with induced sputum, lung function and clinical variables	Heterogeneity in induced sputum accounts. 60 patients had a nigh neutrophil count, older female and non-atopic that responded poorly to ICS.
ENFUMOSA study group (2003)[123]	The ENFUMOSA cross-sectional European multicentre study of the clinical phenotype of chronic severe asthma	163 with severe asthma compared with 158 with asthma controlled low dose ICS	Clinical history, questionnaire, clinical parameters, SPT, lung function, induced sputum, bood for differential cell count, FeNO	Female dominance of severe asthma, greater BMI. Markers of atopy showed an inverse correlation to asthma severity. Severe asthma had a higher ICS burden and worse lung function and neutrophilia in sputum.
Haldar et al (2008)[254]	Cluster Analysis and Clinical Asthma Phenotypes	3 independent asthma populations: 184 mild to	Clinical demographics, lung function, sputum cell counts,	5 clusters identified:

		moderate asthmatics managed in primary care, 187 refractory asthma in secondary care and 68 refractory asthmatics in a RCT	FeNO, ICS dosage, Juniper Asthma Control Score. 2-step cluster analysis. Ward's methods and K-means cluster analysis	Early symptom predominant, obese non-eosinophilic, benign asthma, early-onset atopic asthma and inflammation-predominant asthma
Author (Year)	**Title**	**Population**	**Parameters considered**	**Outcome**
Moore et al. (2010)[261]	Identification of Asthma Phenotypes using Cluster Analysis in the Severe Asthma Research Program	The Severe Asthma Research Program (SARP) cohort.	Cluster analysis using 34 variables on 726 asthmatics.	Five clusters were identified. Early-onset atopic asthma with normal lung function has medication requirements, and early-onset atopic asthma with normal lung function has greater medication requirements. Older, obese, late-onset, non-atopic asthma. 4th and 5th cluster severe airflow obstruction with bronchodilator responsiveness but differed in attainment of normal lung function, age of onset, atopy status.

Author (Year)	Title	Population	Parameters considered	Outcome
Boudier et al (2013)	Ten-year follow-up of cluster-based asthma phenotypes in adults. A pooled analysis of three cohorts	3320 asthmatics who participated in the European Community Respiratory Health Survey, the Swiss Cohort Study on Air Pollution and Lung and Heart Disease in Adults or the Epidemiological Study on Genetics and Environment of Asthma.	9 variables considered clinical and phenotypic characteristics twice, ten years apart. Asthma symptom score, questionnaire, asthma exacerbation history, treatment, allergic sensitisation, Total IgE, FEV1 and Bronchial hyperresponsiveness. Latent transition analysis (LTA) utilised.	Seven phenotypes were identified, differing in their asthma symptoms (low, moderate, high), allergic status, and pulmonary function. Phenotypes 10 years apart showed strong similarities.
Kim et al. (2013)[262]	Identification of asthma clusters in two independent Korean adult asthma cohorts	724 asthmatics from the Cohort for Reality and Evolution of Adult Asthma in Korea (COREA), and in 1843 subjects from another independent Korean asthma cohort of Soonchunhyang University Asthma Genome Research Centre (SCH)	Hierarchical cluster analysis was performed using Ward's method, followed by k-means cluster analysis.	4 clusters identified smoking asthma, severe obstructive asthma, early-onset atopic asthma, late-onset mild asthma. Clusters in COREA were replicated in SCH.

Author (Year)	Title	Population	Parameters considered	Outcome
Loza et al (2016)[263]	Validated and longitudinally stable asthma phenotypes based on cluster analysis of the ADPET study	One hundred fifty-six participants from ADPET and 82 participants from a subset of U-BIOPRED.	Fuzzy partition-around-medoid clustering. ADEPT was the training cohort and validated in the U-BIOPRED cohort	Four phenotypes were identified. 1)mild, good lung function early onset with T2-high low inflammation; 2)moderate-hyper-responsive, eosinophilic" phenotype, with moderate control, mild airflow obstruction and predominant type 2 inflammation 3) mixed severity, predominate fixed obstruction, non-eosinophillic and neutrophilic. 4) Severe uncontrolled, severe reversible obstruction, mixed granulocytic with moderate t2-inflammation. Reproduced in two subsets of U-BIOPRED Cohort.

Author (Year)	Title	Population	Parameters considered	Outcome
Ilmarinen et al (2017)[264]	Cluster Analysis on Longitudinal Data on Patients with Adult-Onset Asthma	Seinäjoki Adult Asthma Study - a 12-year follow-up study of 171 patients with new-onset adult asthma.	Lung function, asthma control test, Airways Questionnaire 20, clinical demographics including smoking history, FeNO, blood neutrophil and eosinophil levels, K-means cluster analysis was performed by using variables from baseline and follow-up visits.	Five clusters were identified. 1) Non-atopic males with a smoking history. 2)Older men, heavy smokers, reduced lung function and persistent obstruction. 3) Non-smoking females with reasonable control and function. 4) Obese symptomatic patients at baseline/follow-up. 5) Atopic early-onset asthma.
Jackson et al. (2021)[265]	Characterisation of patients with severe asthma in the UK Severe Asthma Registry in the biologic era.	2225 asthmatics attending 15 specialist severe asthma centres. Split into T2-high (N=992); FeNO >25 and blood eosinophil count>150 cells/µL and T2-low (N=210); FeNO <25 ppB and eosinophil count <150 cells/µL at registration.	Patient demographics, medical history, allergy testing, ACQ, EuroQoL Questionnaire, asthma medication, lung function, FeNO, Total IgE, differential white cell count.	T2-high were more likely to be male, older in age of symptom onset, older never-smokers, have nasal polyposis, worse lung function, and higher total IgE. T2-low had a higher BMI, depression/anxiety and OCS use, higher history of atopy.

Author (Year)	Title	Population	Parameters considered	Outcome
Han et al. (2021)[266]	Multidimensional Assessment of Asthma Identifies Clinically Relevant Phenotype Overlap: A cross-sectional study	522 stable asthmatics T2 was if 2 or more of the following were present: eosinophil count ≥0.14 $x10^9$ cells/L, IgE levels ≥ 100 IU/ml, or FeNO levels ≥30 ppb. Otherwise, it was classified as non-T2 asthma.	Clinical demographics, medications, atopy, asthma history, comorbidities, exacerbation history, sputum induction, differential white cell count, lung function, FeNO, ACQ, AQLQ. Ten phenotypes were identified based on this, e.g. allergic, early onset, obese and eosinophilic.	A Phenotype Overlap Score (POS) was generated. 383 had phenotype overlap. Patients with features of mixed T2 and non-T2 inflammation had elevated T2 inflammation biomarkers, worse asthma control, and were associated with severe exacerbations.
Khalfaoui et al (2022)	Airway remodelling rather than cellular infiltration characterises both type 2 cytokine biomarker-high and low severe asthma.	Stratified asthmatics into T2-high (N=17) despite high dose ICS FeNO≥45 ppb and blood eosinophils ≤ 0.2 $x10^9$/LT2intermediate (N=21), T2-low FeNO 30ppb and blood eosinophils 0.2$x10^9$/L (N=20). and T2intermediate (N=21) who had treatment adjusted against T2-biomarkers.	Bronchoscopy samples for immunohistochemistry, sputum for cytokine measurements. Full medical history, lung function testing, methacholine challenge, induced sputum	FeNO and blood eosinophil were increased in T2-high and highest sputum eosinophil. T2-low patients were more symptomatic. T2-intermediate and low had historical evidence of raised blood eosinophils. Tissue eosinophil neutrophil and mast cell count were similar across severe asthma phenotypes.
Matabuena et al (2023)[267]	Identification of Asthma Phenotypes in Spanish MEGA Cohort Using Cluster Analysis	Five hundred twelve patients with asthma from The Mechanisms Underlying the Genesis and Evolution of Asthma (MEGA) cohort.	Asthma history, ACT, mini AQLQ, the Sino-Nasal Outcome Test 22 (SNOT-22) and Hospital Anxiety and Depression Scale (HADS). BMI lung function.	Four clusters were identified: 1) adult-onset atopic patients with better lung function, lower BMI, reasonable asthma control 2) adolescent-

			Methacholine challenge, SPT, aeroallergen specific IgE. Differential white cell count from blood. Cluster analysis using kernel k-groups algorithm.	onset atopic asthma with normal lung function, low adherence to treatment and smokers 3) adult-onset severe non-atopic, overweight, poor lung function and control 4) Elderly onset, female, atopic, higher BMI preserved lung function with comorbidities and more likely to be smokers.

Table 1. 2 Summary of key studies that have used a phenotype-based approach to classify asthma

Chapter 2: Background and methodologies of the APEX study

Introduction

Exploring the literature for phenotyping asthma exacerbations has identified gaps in the research. The often-sporadic nature could explain the relative lack of published literature on the onset of asthma exacerbation, making it challenging to capture prospective data related to asthma exacerbations. As such, longitudinally assessing how asthma exacerbations influence asthma phenotypes has not been addressed to a high level of granularity in a prospective approach.

Phenotyping asthma exacerbations could allow for a shift from the "one treatment for all" approach to a more treatable trait approach by understanding the mechanisms involved in an exacerbation and potentially identify pathways for future therapeutic targets.

As highlighted in Chapter 1, airway inflammation is a crucial component to consider when phenotyping asthma. This approach should also extend to asthma exacerbations. The different triggers of inflammation in asthma lead to various clinical and physiological manifestations observed in clinical practice.

The Asthma Phenotyping Exacerbation (APEX) cohort study was developed to address these gaps. The aim is to phenotype an exacerbation using mixed-method approaches to understand the mechanisms associated with exacerbations.

One of the first studies that explored the heterogeneity of asthma exacerbations was performed by Turner and colleagues, who explored clinical characteristics of asthma exacerbations, with sputum eosinophilia and without, in 34 patients with symptoms suggestive of a mild asthma exacerbation, defined as symptoms of chest tightness, dyspnoea or

wheezing for two weeks without improvement and demonstration of a reduction in lung function by spirometry.[268] 16 of the 34 participants demonstrated no sputum eosinophilia (<4% eosinophil in the differential cell count) and this was associated with less evidence of reduced airflow obstruction and airway hyperresponsiveness compared to those with sputum eosinophilia (≥4%sputum eosinophils). Interestingly, in those patients with no evidence of T2-high inflammation at baseline based on sputum counts, there was no difference in median ICS dosage used as maintenance therapy for their asthma.

Similarly, Fahy and colleagues demonstrated the presence of neutrophilic inflammation in 18 adults with asthma exacerbation based on clinical assessment, at presentation to the emergency department.[269] 55% of those had evidence of sputum neutrophilia (75% of sputum cells), and 50% of those reported an infection as a trigger to their asthma exacerbation. However, this was self-reported and not confirmed through any analysis of sputum for evidence of bacteria or viruses. Only three patients demonstrated evidence of sputum eosinophilia (75% of sputum cells).

Serrano-Pariente and colleagues explored the heterogeneity of asthma exacerbations on 179 patients admitted with near-fatal asthma by conducting a prospective multicentre study, in 33 hospitals across regions in Spain, on those admitted who were above or equal to the age of 15.[270] They used a definition of near-fatal asthma exacerbation as an exacerbation accompanied by respiratory arrest, requiring mechanical ventilation or evidence of hypercapnia or respiratory acidosis. They chose 72 variables, which were then reduced to 44 for cluster analysis, selecting those the authors felt were clinically relevant. Demographic variables considered, included sex, age, GINA severity, a history of atopy, and asthma medications. Biomarkers considered were eosinophil count, presence of atopy via skin prick test and sensitisation to fungi. Factors related to their ITU admission, such as length of stay, were also incorporated. Three clusters were identified, with Cluster 1 being older

and predominately female with high ICS usage and worse lung function. Cluster 2 had a higher proportion of current smokers, and 50% used ICS inhalers, whilst Cluster 3 was predominately male and had low ICS usage. While this study did identify risk factors for near-fatal asthma, its use was limited due to the lack of inflammatory profiles and a high proportion of missing data.

Similarly, Seikya and colleagues examined the clinical characteristics of severe asthma exacerbations.[271] However, they collected data from completed questionnaires and did not use physiological parameters or biomarkers to assess the inflammatory status of asthma exacerbations. Using cluster analysis, they identified 5 clusters based on the clinical demographics and healthcare utilisation of 204 patients with severe or life-threatening asthma exacerbations, with an oxygen saturation of less than 90% on admission. One cluster was early onset asthma with high SABA use and a higher frequency of hospitalisation. As such, they didn't provide granularity into the different mechanisms of asthma exacerbations and relied on patients' perception of symptoms without objectivity. However, one could potentially identify targets to modify behaviours to prevent future exacerbations, such as education on SABA overuse, which has been associated with an increased risk of exacerbation, hospitalisation for asthma and mortality [272] and a switch to an ICS/LABA reliever approach when there is a deterioration in symptoms.

The inflammatory profile of exacerbations in patients with severe refractory eosinophilic asthma receiving Mepolizumab (MEX) study explored asthma exacerbation in asthmatic patients taking Mepolizumab.[214] An analysis of those who exacerbated whilst on Mepolizumab was compared to those who did not. In those who had an exacerbation in the study, there was evidence of a higher symptom burden and higher exacerbation rate in the year before study entry. In patients on biological therapies who exacerbated, heterogeneity in those asthma exacerbations was also identified. From the induced sputum of

these exacerbations, it was determined that there were both eosinophilic and non-eosinophilic exacerbations. Non-eosinophilic exacerbations were associated with infections and a raised CRP. In the exacerbations associated with the presence of a virus, there was no associated increase in sputum neutrophils. A paired sputum analysis of baseline to the first exacerbation experienced whilst on Mepolizumab identified that 71% had a preserved inflammatory phenotype.

More recently, McDowell and colleagues explored the heterogeneity of asthma exacerbations in the RASP-UK cohort.[273] They explored asthma exacerbations identified in a 48-week multicentre RCT comparing the use of a composite biomarker approach to steroid adjustment. Their definition of T2-low was a FeNO of less than or equal to 20 and a peripheral blood eosinophil count (PBE) of ≤ 150 cells/µL, and T2-high was defined as a FeNO greater than 20 or PE greater than 150 cells/µL.

They explored the exacerbation events, including asthma history, induced sputum, lung function, ACQ and AQLQ scores. As a secondary endpoint, they examined the differences between those who exacerbated the study and those who did not. In those who did experience an exacerbation, the question was raised about the difference between those who had T2-high versus T2-low-associated exacerbation. 390 exacerbation events occurred from 183 participants, of which 118 of those were captured. Those who experienced an asthma exacerbation were female, had high steroid usage, and, as a result of this, lower sputum, eosinophil counts via suppression of the T2-axis.

When comparing T2-high and T2-low asthma exacerbation events, there were no differences in mean lung function reduction (FEV1) or asthma control as measured by the ACQ score at exacerbation. During the exacerbation of T2-low patients, there was no rise in T2-biomarkers, FeNO or peripheral blood eosinophils.

Of those 118 exacerbations – 27% were phenotyped as T2-low and 73% as T2-high. T2-low had a more significant number of viruses detected on sputum than T2-high at exacerbation. From spontaneous sputum samples, the predominately inflammatory pattern was mixed, which contrasts with the findings from the MEX study, which was predominately dichotomous to the presence of eosinophils or the presence of neutrophils at exacerbation through immunomodulation via IL-5-associated pathways.[274]

These studies have revealed no consensus on the mechanisms associated with the inflammatory phenotypes of asthma exacerbations. In the studies above, exploring the phenotypes of asthma exacerbations was often a secondary endpoint. As such, the studies could not address this question despite being multicentre studies with large sample sizes.

What determines whether an exacerbation is T2-high or low could be alluded to by whether there is the presence of an infective agent; however, the mechanisms responsible for this are unclear. It seems exacerbations do not influence a change from the inflammatory phenotype at baseline, but the change in T2 status post-exacerbation has not been explored.

Author	Title	Population	Parameters Considered	Outcome
Turner et al[268] (1995)	Exacerbations of asthma without sputum eosinophilia	34 asthmatics with mild exacerbations (uncontrolled)	Sputum for differential cell count. (4% eosinophils on two occasions or >10% eosinophils once), lung function, methacholine challenge	Half of the sputum had no eosinophilia associated with less airway obstruction and less airway hyperresponsiveness.
Fahy et al[269] (1995)	Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation	18 adults with acute severe asthma and 12 adults with cystic fibrosis. Cellular and biochemical analysis.	Sputum cell counts. Sex, Atopy smoking status and lung function and inhaled medication usage.	Sputum eosinophilia was found in 3 samples, whilst neutrophilia was found in 7 samples. This correlated with those who self-reported a respiratory tract infection as the cause of exacerbation.
Pauwels et al (1997)[13]	Effect of inhaled formoterol and budesonide on exacerbations of asthma	852 patients assigned at random to 4 treatment groups	1 year follow up. exacerbation rate, symptoms, lung function recorded	The exacerbation rate for low-dose ICS/LABA was reduced by 26% and 49% with high-dose ICS/LABA. Mechanisms between symptoms and exacerbation are postulated to be distinct
M M Pizzichini et al[275] (1997)	Sputum in severe asthma exacerbations: kinetics of inflammatory indices after prednisolone treatment.	10 asthmatics who experienced a severe exacerbation	Symptoms, FEV, blood eosinophils and sputum day 1,2,3,7 and 21 days after treatment with OCS.	An eosinophilic response and increased fluid phase ECP, fibrinogen and IL-5 levels were identified. Heterogeneity in the degree of inflammation was identified with 1 neutrophilic response. 3 patients had low levels of ECP fibrinogen despite sputum eosinophilia.
Ortega et al[276] (2012)	Characterisation of asthma exacerbations in primary care using cluster analysis	2205 adults and 2435 children and adolescents with asthma	2 cross-sectional surveys consisting of clinical demographics and exacerbation history health care utilisation Supervised cluster analysis with recursive partitioning.	T clusters identified in adults. Predictors of exacerbation included hours worked, visiting an asthma specialist and excessive use of rescue medication. The rate ratio of one of the clusters associated with exacerbation had a female predominance, severe disease, atopy, and comorbidities associated with asthma and a lower asthma control score.

Author	Title	Population	Parameters Considered	Outcome
Serrano-Pariente et al [270](2015)	Identification and characterisation of near-fatal asthma phenotypes by cluster analysis	179 asthmatics admitted for an episode of near-fatal asthma admitted to 33 hospitals in Spain	40 variables, including Clinical demographics, General Health Questionnaire (GHQ) score, GINA severity of asthma, lung function, asthma history, atopy comorbidities, level of medication, eosinophil count, ICU stay information. Cluster analysis	3 clusters were identified: 1) older patients with clinical features and high medication burden, 2) worse outcomes of NFA admission, including requiring mechanical ventilation and respiratory arrest, and 3) younger with features of sensitisation and low medication levels on admission.
Seikya et al [271] (2016)	Severe or life-threatening asthma exacerbation patient heterogeneity identified by cluster analysis	Multicentre prospective study of 17 institutions across Japan.	Variables from patient and physician-orientated structured questionnaires include: Clinical demographics, comorbidities, symptom score, medication use, SpO_2 on admission, patient-perceived trigger of current exacerbation, demographics and comorbidities. K-medoids clustering with Gower's distance metric	5 clusters: 1) younger-onset asthma with severe symptoms at baseline, limitation of activities, greater OCS and SABA use and hospital utility. 2) elderly females, comorbidities with long duration of asthma 3)allergic asthma, history of atopy and features of atopy 4) Male with features of OCPD. 5) mild symptoms but history of hospitalisation
Ghebre et al [277] (2018)	Biological exacerbation clusters demonstrate asthma and chronic obstructive pulmonary disease overlap with distinct mediator and microbiome profile	32 asthmatics and 73 patients with COPD seen at the point of exacerbation.	Lung function, dyspnoea and cough VAS questionnaire, inflammatory mediators from sputum and serum, microbiome communities by RT-PCR of viruses and assessing bacterial load obtained from sputum samples.	3 exacerbation biological clusters. Cluster 1 was COPD predominant, cluster 2 mixed with asthma and COPD, and Cluster 3 was asthma predominately. Asthma and COPD had different clinical characteristics and inflammatory profiles but similar microbial ecology.

Author	Title	Population	Parameters Considered	Outcome
McDowell et al. [214] (2021)	The inflammatory profile of exacerbations in patients with severe refractory eosinophilic asthma receiving mepolizumab (the MEX study): a prospective observational study	145 participants, 18-80ys with severe eosinophilic asthma (GINA 4/5), receiving mepolizumab.	Peak flow, symptom diaries, CRP sputum eosinophil.	Eosinophilic and non-eosinophilic exacerbation events were predominately mutually exclusive.
McDowell et al. [273] (2022)	Exacerbation Profile and Risk Factors in a Type-2-Low-Enriched Severe Asthma Cohort	Secondary analysis from RCT. Patients phenotype at base to T2-low (N=71) and T2-High (N=230). Exacerbation events phenotype (N=118)	Lung function, ACQ-7, FeNO, Peripheral blood eosinophil, CRP. Sputum for differential cell count. PCR for viruses and Bacterial load measured from sputum. Bacterial load measured from sputum.	T2-low asthma exacerbations were no different from T2-high exacerbation events in lung function and ACQ-5 score. However, the T2-high cohort had a more significant increase in FeNO and PBE from study entry to the time of exacerbation than the T2-low cohort.

Table 2. 1: Summary of critical studies that have used a phenotype-based approach to explore asthma exacerbations.

Study population.

For this study, I, alongside the APEX team, recruited patients with asthma who have previously experienced an asthma exacerbation. Recruitment occurred from primary and secondary care to capture a wider population. We also recruited patients from our research register at the Nottingham Asthma Centre.

Two groups of asthmatic participants were recruited:

APEX-1

APEX-1 were participants with asthma who had an asthma exacerbation within less than or equal to 24 months of entering the study (initially this was set to an asthma exacerbation within 12 months prior to baseline study entry; however, it was felt, after consensus discussion, that the COVID-19 pandemic and shielding strategies, could have impacted on the exacerbation frequency participants may have experienced and this was therefore subsequently lengthened to 24 months).

APEX-2

Those with asthma exacerbation 24 to 60 months before study entry. APEX-2 was created as an important comparator group. Patient and public involvement was utilised to guide the study set up; a consensus was reached that those without recent exacerbation were also important to include to improve the generalisability of results generated from APEX. Although it was hypothesised that APEX-2 would provide fewer exacerbations than the APEX-1 cohort based upon the presence of the risk factor of a history of exacerbation alone, findings would be strengthened by comparing findings in APEX-1 to a group where exacerbations were not frequent.

Eligibility criteria

The full inclusion and exclusion criteria for the APEX study were set as follows:

Inclusion Criteria

- The ability to give fully informed written consent.
- Have a clinician diagnosis of asthma- this was self-reported by the patient and checked on their medical records after patient consent.
- Male or female aged ≥ 18 and ≤ 85 years of age.
- Participants were required to have had at least one asthma exacerbation requiring additional treatment in the 24 months prior to informed consent or 24-60 months prior to informed consent for APEX-2. This was defined as either:
    - Three or more consecutive days treatment with oral corticosteroids, for participants not on maintenance oral corticosteroids.

OR

    - At least a doubling of treatment with oral corticosteroids for 3 or more consecutive days, from a stable dose, for participants on maintenance therapy with oral corticosteroids.

These criteria were patient reported:

- On British Thoracic Society (BTS) step 1-5 treatment
- Current smokers were included, providing there was good evidence of underlying asthma (for example, a life-long history of asthma, > 12% FEV1 reversibility or sputum or blood eosinophilia).

- Non-English-speaking participants had to be proficient in their understanding of the English language, to be able to fully participate in the study.

Exclusion Criteria

- An exacerbation in the 4 weeks prior to the Baseline visit.
- The use of a biologic therapy, including Omalizumab, Mepolizumab or Benralizumab, at any time during the 3 months prior to informed consent.

  Participants who commenced biologic therapy prior to study entry were excluded. However, should they have commenced therapy whilst in the study they were allowed to continue.
- A history more in keeping with smoking related Chronic Obstructive Pulmonary Disease (COPD), (for example, where the participant had smoked > 20 pack years, without evidence of significant reversibility or eosinophilia).
- Other clinically significant respiratory diseases e.g Lung fibrosis or lung cancer.
- Use of regular high-dose maintenance systemic corticosteroids (for example, a dose of > 10mgs of Prednisolone daily)
- Any other clinically significant medical disease or uncontrolled concomitant disease, that was likely, in the opinion after clinical review, to impact on the ability to participate in the study or the study results.
- Pregnant women at the time of baseline entry to the study
- Participation in a study involving an investigational medicinal product at any time during the 3 months prior to informed consent.

Sample size calculation.

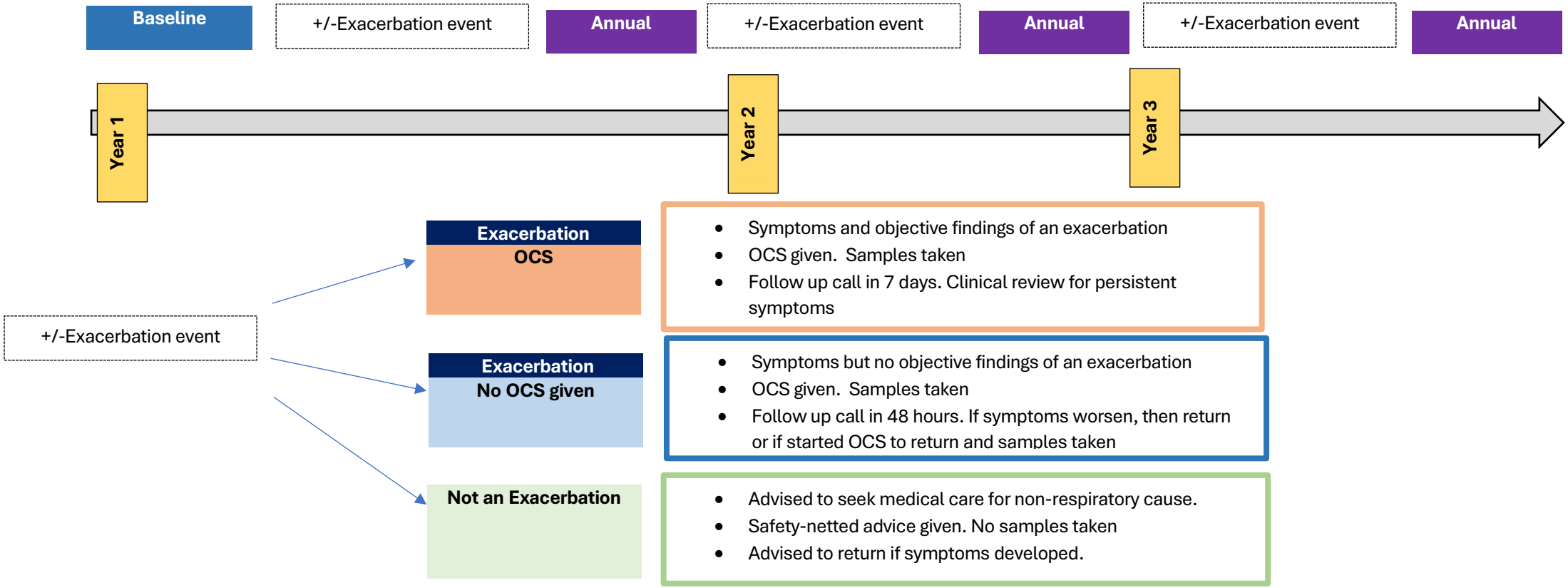
There is a paucity of data on the proportion of asthma exacerbations that are T2-high versus T2-low and results are inconsistent between studies. One of the aims of this study was to identify heterogeneity associated with asthma exacerbations, i.e. the proportion of asthma exacerbations that was T2-high and T2-low.

As such, the proportions of T2 high to T2 low asthma exacerbations were set to 50% respectively. This was with a confidence level of 95%, with a margin of error of 7%. A minimum sample size of 196 was required; hence the sample size for APEX-1 was set to 200 and any recruitment beyond 200 was considered acceptable.

APEX-2 was designed to be a comparator group, in particular for baseline features to the sister-cohort (APEX-1). A study suggested the mean exacerbation rate over a 12-month period to be 0.11/patient/year/[278]. We aimed to capture at least 50 asthma exacerbations over a 3 year period and therefore, a sample size of 100 would be adequate to make meaningful comparisons.

Study design

Figure 2. 1: APEX study design



Participants were in the study for a maximum period of 3 years. Annual visits occurred 1 year after baseline. If participants perceived their symptoms were at the point where they would usually seek help, they visited the Nottingham asthma centre and 1 of 3 outcomes were given.

Scheduled event.

Baseline visit

The study was an observational cohort study with a 3-year follow up period. Participants entered the study at baseline. To see patients at their "normal" in regard to their usual asthma control; we ensured patients had at least a 4-week period of not having an exacerbation before entering the study. Participants were not required to withhold any medications prior to study entry. Informed consent was obtained at the baseline visit prior to any data being collected or tests undertaken. The other events listed in Figure 2.1 will be described in detail in Chapter 4.

Methods

Recruitment strategy.

Recruitment into APEX was focused across the East Midlands North region as this was a single-centre study. GP practices in Nottingham were invited to participate in the research and act as a participant identification centre (PIC). Media, in the form of posters, were placed across health care centres and places of public interest to ensure recruitment into the study was across a varied setting. Patients were also recruited from secondary care, from acute admissions, outpatient clinics and through the asthma register held at our research facility at the Nottingham Asthma Centre based in Nottingham City Hospital NHS Foundation trust. Participants were provided with the study patient information sheet before recruitment began. (Appendix V)

Measurements:

The following measurements were taken during the study at the following study visits.

Visit	Baseline	Exacerbation	Telephone follow-up visit	Annual Visit
Time		**Day1 (+ 2 Days)**	**Day 7**	
Investigations				
Informed consent	**X**			
Medical History	**X**	**X**	**X**	**X**
Demographic Data	**X**			
Concomitant Medications	**X**	**X**	**X**	**X**
Inhaler Technique check	**X**	**X**		**X**
Height and Weight	**X**	**X**		**X**
Blood Pressure	**X**	**X**		**X**
ACQ (7) and MARS	**X**	**X**		**X**
Spirometry	**X**	**X**		**X**
Fractional Exhaled Nitric Oxide (FeNO)	**X**	**X**		**X**
Impulse Oscillometry (IOS)	**X**	**X**		**X**
Sputum (induced or spontaneous) for differential cell count, supernatant storage and microbiological assessment	**X**	**X**		**X**
Haematology (FBC, Blood Eosinophil and Neutrophil count)	**X**	**X**		**X**
Clinical Chemistry (C-Reactive Protein)	**X**	**X**		
Nasal Epithelium Samples for Genetic Analysis	**X**	**X**		
Skin prick test	**X**			
Total IgE	**X**			**X**

Table 2. 2 Measurements taken in the APEX study according to the schedule of visits.

Clinical History and Body composition.

A detailed asthma history, including previous hospital admissions and exacerbation frequency, was recorded on a participant's baseline visit. Relevant co-morbidities associated with asthma and their current asthma medications were also recorded. Additional information, such as participant perceived allergies and smoking status, was collected (Appendix I). Participant height (cm), weight (kg) and blood pressure (mm/Hg) were measured at baseline visits. Fluctuations in weight and blood pressure were captured during repeat measurements at exacerbation and annual visits and were entered for further spirometry or impulse Oscillometry measurements.

Asthma Control Questionnaire (ACQ)

Asthma control is defined as the extent to which the various manifestations of asthma have been reduced or removed by treatment.[4] There are two components to asthma control, as stated by Reddel et al. in the ATS/ERS task force in asthma control and asthma exacerbations. The first is "*patients' symptoms and their ability to perform their daily living activities*." The second component is "*the risk of future adverse events including loss of control described formerly, exacerbation risk and side effects associated with treatment and decline in lung function*". [4]
From the definition alone, it is clear that asthma control can vary with time and depends on the patient's perception of their symptoms, which could give rise to subjectivity. The second component has the potential for the introduction of bias, based on the physician's perception and thus the medication they prescribed to achieve control.

In order to minimise this, the Asthma Control Questionnaire (ACQ) was developed (Appendix II). [176] The ACQ was based on 91 asthma clinicians' responses to the importance of a set symptom used to assess asthma control. [176] The top 5 important symptoms were then developed into a questionnaire consisting of 7 equally weighted questions. 5 of the questions are based upon symptoms, 1 question on reliever medication

use over the past week and the final question is based upon recorded FEV_1% predicted. A score of 0 to 6 is given for the 7 questions. The mean score is then calculated. A higher mean score is suggestive of poorer control. From a 9 week observational study of patients with stable asthma, it was found to be sufficiently sensitive to detect changes in control, [176] with a change in score by 0.5 deemed an important clinical change in control. An ACQ7 score below 0.75 suggests well controlled asthma control whilst a score above 1.5 suggests poor control.

Medicines Adherence Report Scale (MARS)

Poor adherence to medication has been well described as a risk factor for poor asthma control. The Medicines Adherence Report Scale (MARS)© is a set of 5 questions, which assess patients' self-reported adherence to medication, both intentional and unintentional behaviours.[279,280] The questions were phrased in a manner that reduced the risk of appearing judgmental and therefore minimised the risk of over representation of adherence. The questionnaire is commonly adopted in clinical practice as it is easily implemented, inexpensive and can be used longitudinally to assess adherence over time.[280] A copy of the questionnaire is found in Appendix IV.

Venepuncture

Venepuncture was performed using the 23G blue BD® Vacutainer Safety Lok Blood Collection Set 7" Tubing. Full Blood Count (FBC) C-Reactive Protein (CRP) and IgE were sent to NHS laboratories at Nottingham University Hospital.

Nasal Epithelial samples

From previous studies performed at the Nottingham Asthma Centre, the Dent-O-Care® Interdental Brushes 4.0mm were used to obtain nasal epithelial samples. The brushes were handled with non-sterile gloves holding the shaft of the brush.

Nasal brushes were obtained from patients by inserting a nasal brush into the nasal passage along the floor of the nasal cavity to collect airway epithelium from the posterior aspect of the inferior turbinate. The brush rotated in a clockwise and anti-clockwise fashion repeatedly. Samples were immediately placed in 3ml of cold Lonza culture media (Lonza Bioscience, UK) and stored on ice for same-day processing. The brushes were processed by vortexing and scraping the nasal brushes to isolate the cells into the media. The cell suspension was centrifuged at 200g, and the nasal brush was discarded. The media supernatant was removed and the cell pellet was subsequently stored in a -80°C freezer for further analysis. This is similar to the previous workflow conducted in our study group.[281]

Skin Prick testing (SPT)

Skin Prick Testing (SPT) was measured to assess the presence of atopy in participants by detecting sensitivity to common allergens. The aeroallergens tested were:

- Felis domesticus
- Dog dander
- Penicillium notatum
- Aspergillus fumigatus
- Alternaria altenate
- Dermatophagoides pteronyssinus (House Dust mite)
- Cladosporium herbarium
- Tree pollen
- Grass pollen
- Normal saline (negative control)
- Histamine (positive control)

A drop of the aeroallergen was placed on the skin on the volar aspect of a participant’s forearm. A small lancet was then used to prick the skin's epidermis gently. Participants were observed following administration for 20 minutes. A positive SPT reaction was classified as the development of a wheal of at least 3mm in diameter in comparison to the negative

control. The longest diameter produced was measured. Participants were advised to withhold any antihistamine medication for 48 hours to ensure the best result. The Soluprick SQ kits© (ALK, Reading UK) were used for testing.

FeNO

Fractionated exhaled nitric oxide (FeNO), as described, is a noninvasive marker of inflammation in asthma. The NIOX VERO® (Morrisville, US) was used to measure this. Participants passively blew through the disposable mouthpiece, following the instructions on screen. They were also verbally coached. One measurement was taken.

Spirometry

The Easy on-PC spirometer® (Medizintechnik AGZurich, Switzerland) was used to measure the FEV1 and FVC. Initially, participants were screened for absolute and relative contraindications when performing spirometry. Calibration was performed before use. Participant information was inputted, including Study ID, date of birth, smoking history, height, weight, ethnicity and contraindications.

Patients performed a forced vital capacity manoeuvre after being coached on the correct technique to ensure they exhaled as hard and as fast as possible to obtain FVC. A nose peg was provided prior to the manoeuvre. Participants blew through a disposable spirette™ inserted into the Easy on-PC sensor. Over-reading of spirometry was performed manually, and at least three manoeuvres were performed. A maximum of eight were undertaken if required.

IOS methodology

Respiratory impedance was measured using the tremoflo® Ariwave Oscillometry device (Thorasys, Canada) according to the manufacturer's recommendation. The resistance load of the oscillometry device was

verified by using a mechanical test load prior to patient testing. This verifies the impedance load. The height, weight, gender, age, ethnicity and smoking status, including pack-years, were inputted prior to performing any tests.

The participant was instructed to wear a nosepiece and place it to seal the nasal passages fully. With the participant in a fully upright position, the participant was instructed to place both hands and compress the soft tissue of their cheeks to achieve an adequate seal. Participants were advised to relax their elbows and avoid compressing their chest by having their arms slightly outward.

With the use of examination gloves, the device was held to the participant's mouth with a single-use mouthpiece and they were advised to seal their lips to avoid any possible air leaks. The participant's face was held at a 15-degree angle from the horizontal plane to relieve any upper airway compression that could lead to elevated readings. Patients were advised to breathe normally throughout the test while holding their check with their palms and fingers to support the soft tissue of the jaw during measurements, which creates upper airway shunting and, therefore, loss of airflow motion to the upper airway walls. This is also why participants were required to wear a nose-peg for the manoeuvres. Patients were advised to keep their tongue below the mouthpiece and to avoid swallowing.

Once the participant was used to the device and able to demonstrate normal tidal breathing for at least 3 breaths, they were advised that they would experience a gentle oscillation and after that, the measurements were taken. Within the device, is a vibrating mesh, that generates soundwaves that are transmitted through the bronchial tree.

A minimum of three measurements were taken, with an interval between each measurement. A maximum of 8 attempts for adequate measurements was adopted. The aim is to impose the oscillations onto

the patient's normal tidal breath and thus allow the generation of respiratory impedance, as described below. The following measurements are taken with each manoeuvre:

Value	Definition	Cutoffs kPa/L/s / cmH_20/L [282]
R_5	Total resistance observed at 5Hz	>0.5 / >5.1
R20	Central resistance observed at 20Hz	-
R_{5-20}	Fall in Resistance 5Hz-20Hz indicative of peripheral resistance	>0.08 / 0.8
X_5	Area under reactance at 5Hz	-
A_X	Area of low-frequency reactance	>1.0 / 10
V_T	Tidal Volume.	-

Table 2. 3 Values obtained from the performance of impulse oscillometry (IOS) with definitions.

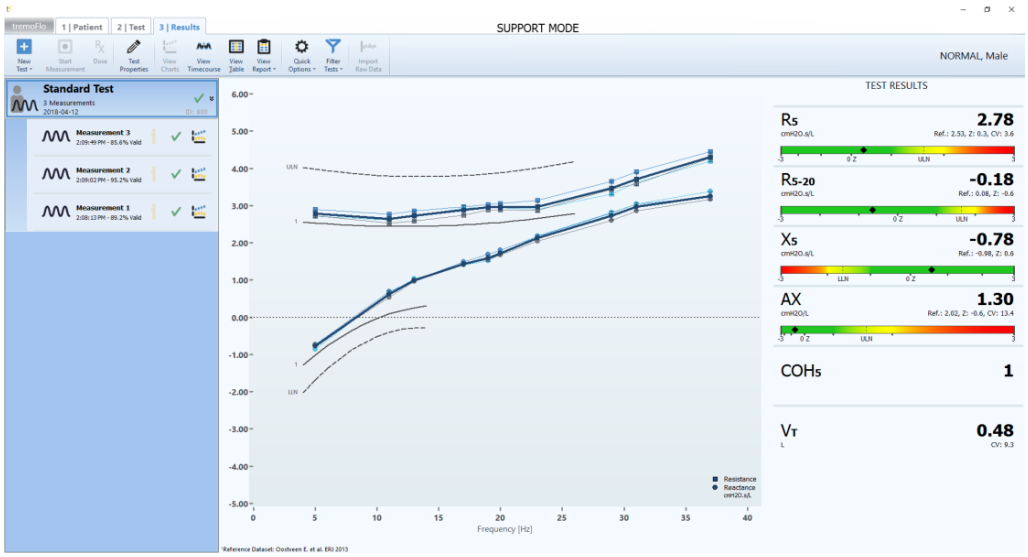


Figure 2. 2: Software Output obtained from performing IOS using the Thorasys device.

The software has built-in quality control for the measurements being recorded. Based on the reactance and resistance curves created, the software can assess whether a participant is coughing or if there is a significant air leak.

The manufacturer recommendations are a minimum of three measurements with a coefficient of variation of respiratory system

resistance of ≤ 10%, a coherence of more than >0.7 and above 70% for validity data points across all frequencies.

Spirometry and reversibility

Spirometry was performed using the Easy on-PC spirometer ®. The Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC), PEFR and FEV_1/FVC ratio were measured. The best of 3 acceptable manoeuvres was recorded. The joint guidelines from the American Thoracic Society (ATS) and European Respiratory Society (ERS) were adopted to perform spirometry.[283] The repeatability criteria demanded that the two most significant FEV_1 values were within 150ml of each other.[283] For participants who were not able to achieve this, their values were still recorded, as well as the grading of the session as per ERS/ATS guidelines. [283]

Reversibility was performed via the inhalation of 400mcg of salbutamol and repeat spirometry after 15 minutes. Reversibility was calculated as a percentage using the following equation, using the largest FEV1 measured:

$$\frac{\textbf{(Post Bronchodilator } FEV_1 \textbf{- Pre-Bronchodilator } FEV_1\textbf{)}}{\textbf{Pre bronchodilator } FEV_1} \quad \textbf{X100}$$

Sputum induction

Hypertonic saline was utilised to obtain sputum plugs from participants. Sputum was collected at all participant visits where possible unless the participant produced a sample spontaneously. The protocol described by Pavord and colleagues was adopted.[284] The protocol for inducing sputum is in Appendix III.

The induction process requires participants to inhale nebulised hypertonic saline starting at the percentage of 0.9% normal saline or 3% hypertonic depending on post bronchodilator FEV_1 and their percentage

predicted FEV_1. Participants inhaled the solution for 5 minutes and attempted to produce sputum using the techniques described in Appendix III. If the participant was unable to produce a sputum sample at that percentage and providing there was not a significant drop in their lung function from baseline due to bronchoconstriction caused by the hypertonic saline, participants had an increased saline concentration of 4% and then if no sample produced; 5% hypertonic saline utilised if lung function again permitted. All samples produced were processed in the lab within 2 hours of collection.

Sputum processing

Method: The method was based on the standard operating procedure (SOP) implemented for respiratory research conducted at the University of Nottingham. Thus, below is the methodology as described in the SOP. The protocol was created by Laura Matthews.

Sputum extraction

The sputum samples were processed within 2 hours of final expectoration. The initial part of the process involved isolation of sputum plugs. The selection and isolation of sputum plugs from saliva is an important factor in reducing squamous contamination and improving sample quality.[285] Isolated sputum plugs were cleared from the salivary fluid using curved forceps and transferred onto a petri dish lid. If any blood was present in the sample, that part of the sample was avoided and the presence of blood was documented. Using the forceps, the sample was collected into a single mass; aided by moving the mass around the lid in a circular motion to spread saliva around the lid but to keep the sputum in one mass.

The purified sputum sample was transferred into a pre-weighed 15ml falcon tube and re-weighed to determine the weight of the sputum isolated. The falcon tube was always placed on ice, where possible.

Sputum processing

The sputum plug was incubated with phosphate-buffered saline (PBS) (Sigma-Aldrich, Darmstadt, Germany). The amount of PBS was 8 volumes (ml) x sputum weight (g) of PBS (the volume required is the ratio of 8 ml PBS to 1g of selected sputum).

The sample was vortexed for 15 seconds and rocked on ice for 10 minutes. It was then centrifuged at 600G for 10 minutes at 4°C. Six volumes of the produced supernatant (6 volumes of PBS added in the previous step) were removed from the tube and placed into a new falcon tube. This was centrifuged at 1500g for 10 minutes at 4°C. The supernatant was split between cryovials' and stored at -80°C.

Two volumes of PBS (the original volume of PBS added initially) were added to the sputum sample. Four volumes of 0.2% Dithiothreitol (DTT) (Sigma-Aldrich, Darmstadt, Germany) were then added to the sputum sample (equal volumes to that of supernatant removed in the previous step). This ensured a final DTT concentration of 0.1%. · The sample was vortexed for 15 seconds and rocked on ice for 10 minutes. The sample was transferred into another pre-weighed 15ml falcon and re-weighed to determine the weight of the sample. 10µl of the sample was removed and mixed with 10µl Trypan blue and mixed well. 10µl of the cell suspension/Trypan blue solution was added to a haemocytometer chamber for the process of cell counts. Live leukocytes, dead leukocytes and Squamous were recorded according to morphology. The total number of cells in the sample and cell concentration were calculated to then calculate the total number of cells per gram of sputum.

The cell suspension was centrifuged at 600G for 10 minutes at 4°C. The resulting DTT supernatant was stored at -80. The cell pellet formed was resuspended in an appropriate volume of d-PBS to give a concentration of $5x10^5$ cells/ml.

Cytospin preparation ·

Two poly-lysine slides were utilised, and 75µl of the resuspended cell pellet was added to one slide and 150µl to the other. It was then spun in a Shandon cytospin at 450rpm for 6 minutes. The slides were then air-dried, and cell density was checked, and volume was added or diluted if necessary. Slide production was repeated as described above, and an additional slide was created with the optimised volume. Slides were fixed in methanol, and the initial slide stained with RappiDiff II. 1 slide was retained and stored for future use.

Disposal

Disposables (pipette tips, falcon and petri dishes) were left in 10 %Distell overnight before disposal in clinical waste. 10% Distell was poured into the sputum collection pot.

Chapter 3: Clinical and inflammatory characteristics of the APEX cohort at baseline

Aims

This chapter sets to describe the APEX-1 and APEX-2 cohorts at baseline and to phenotype the cohorts at baseline whilst addressing key aims, namely:

- To characterise the APEX cohorts at baseline with key clinical, demographic, inflammatory and physiological parameters.
- To identify the T2 status of the cohort at baseline according to being either T2-high or T2-low and if there are clinical differences between the two.
- To assess whether there are differences in the APEX cohort of those who underwent an exacerbation event in APEX versus those who did not, with the hypothesis that those who did undergo an exacerbation event had worse clinical measurements.

Data analysis

Analysis of cross-sectional data.

The data analysed was collected as described in Chapter 2 Table 2.2.

For comparisons between groups, paired or unpaired tests were performed. Unpaired t-tests were performed for unpaired analyses with normal distribution. If not normal in distribution, then the Mann-Whitney test was performed. If the samples were paired and had a normal distribution, then a paired t-test was performed. A Wilcoxon matched-pairs signed rank test was conducted appropriately if paired and not of normal distribution. The one-way analysis of variance (ANOVA) or test was used to compare three groups or more of normal distribution. If not normal distribution, then the Kruskal-Wallis test was conducted. For repeated measures, ANOVA was performed if there was a normal distribution, and if there was no normal distribution, then the Friedman test was performed. For categorical variables the Chi-squared test was performed unless stated otherwise.

In this thesis, parametric data is presented as mean(SD). Non-parametric data is presented as median, and the interquartile range (IQR) is presented with the 25th and 75th centile values stated. Initially, all data was assessed for normal distribution by plotting histograms, and subsequently, the data was transformed by calculating the natural log if required. If data could not be converted successfully, this would be presented as non-parametric data, as described above. A p-value of <0.05 was taken as the threshold of significance for all statistical analyses, unless stated otherwise. Statistical software packages used to perform calculations were Excel® (Microsoft, US), SPSS® (IBM, US), and Prism® (GraphPad, US)

The first exacerbation outcome, exacerbation requiring OCS=Primary OCS or exacerbation not requiring OCS=Primary No OCS of a participant was taken for analyses only, and subsequent exacerbation visits a participant may have had in the study were recorded as "**multiple exacerbation event**s." This was done to avoid overrepresenting a participant who may have exacerbated multiple times during the study.

Definitions of inflammatory phenotypes:

Based on a review of the existing literature described in Chapter 1, the following cut-offs were used to define inflammatory phenotypes:

Parameter	**T2-high**	**T2-low**
Sputum Eosinophilia	≥2%[260]	<2%
Sputum Neutrophilia	≥65%[260]	<65%
FeNO (ppb)	≥40[286]	<40
Blood Eosinophil (x10^9/L)	≥0.3[242]	<0.3

Table 3. 1: Classification of T2-high and low inflammation used in the APEX study.

The presence of a raised FeNO in this study was defined as greater than or equal to 40ppb, which is used as a cut-off by NIOX (Oxford, UK) to support a diagnosis of asthma. A cut off $0.3x10^9$ per L was used to define the presence of blood eosinophilia.

Results

Baseline Characteristics

305 participants were recruited into the APEX study, with 202 in APEX-1 and 103 in APEX-2 (Table 3.2). There were more females in APEX-1 (73.1% vs. 64.1%). Mean age was similar in both cohorts (50.8 vs. 49.6 years).

Parameter	APEX-1	APEX-2	p-value
Age (Yr)	50.78(14.78)	49.55(17.51)	0.52
R5 $cmH_20/L/s$ *	4.28(1.79)	4.04(1.63)	0.30
R20 $cmH_20/L/s$ *	3.35(1.07)	3.18(0.97)	0.29
R5-R20 $cmH_20/L/s$ *	0.98(1.08)	0.87(0.92)	0.88
AX cmH_20/L *	16.18(21.20)	14.28(15.15)	0.30
FeNO ppb **	19.50(13.00-32.75)	19.00(12.00-17.00)	0.58
FEV1pred (L)	3.02(0.66)	3.24(0.83)	0.02
FEV1 (L)	2.57(0.82)	2.74(0.87)	0.08
FVCpred (L)	3.77(0.81)	4.0(1.00)	0.02
FVC (L)	3.43(0.96)	3.70(1.06)	0.03
PEFR (L/min)	436.47(123.57)	471.41(119.72)	0.02
FEV1/FVC	74.28(11.19)	73.93(9.93)	0.54
FEV1% pred **	87.00(73.40-97.96)	88.56(70.51-97.67)	0.91
FVC% pred	91.01(15.21)	92.03(13.82)	0.57
Reverse (%) **	4.56(0.79-9.38)	3.45(0.67-7.49)	0.50
Neutrophils $(x10^9/L)$ **	4.22(3.33-5.38)	4.02(3.17-5.35)	0.19
Eosinophils $(x10^9/L)$	0.23(0.22)	0.19(0.13)	0.12
CRP $(x10^9/L)$ **	3.00(1.75-5.35)	2.00(1.00-5.00)	0.08
IgE (UI/ml) **	55.5(0.8-2.4)	69.50(22.55-235.00)	0.34
ACQ5**	1.60(1.05)	1.00(0.40-1.60)	<0.001
ACQ6 **	1.42(0.83-2.33)	0.83(0.33-1.50)	<0.001
ACQ7**	1.57(1.0-2.29)	1.00(0.57-1.57)	<0.001
MARS**	4.6(4.0-4.8)	4.6(3.68-4.80)	0.12
(+) SPT N(%)	115(57.5)	52(53.6)	0.53

Table 3. 2: Baseline characteristics of APEX cohorts.

Due to mechanical faults, IOS was not collected in 18 participants for APEX-1 and 5 for APEX-2. 2 participants could not perform an adequate test for FeNO in APEX-1 and 4 in APEX-2. 7 participants, a blood sample could not be obtained in APEX-1 and 1 in APEX-2. *=mean **= median(IQR). Bonferroni correction for multiple tests was undertaken.

Spirometry

The FEV1% predicted for APEX-1 and APEX-2 were similar at baseline (87% vs 88.6% p=0.91), as were measurements for FVC% predicted (91.01% vs 92.03% p=0.57). Normal FEV1/FVC ratios were observed in both cohorts. APEX-1 had a slightly lower mean FEV1 2.57 vs 2.74 L compared to APEX-2; however, this was not statistically different (p=0.08). Post Bronchodilator reversibility for APEX-1 and APEX-2 was similar, with a mean reversibility of 4.56% for APEX-1 and 3.45% for APEX-2 (p=0.50).

Impulse Oscillometry

No difference in total resistance was observed at 5Hz for APEX-1 and APEX-2. (4.28±1.79 vs 4.04± 1.63 cmH20/L/s p=0.30). The Central resistance observed at 20Hz for APEX-1 and APEX-2 was 3.35±1.07 vs 3.18±0.97 cmH20/L/s (p=0.29). Significant peripheral airway resistance was observed in both APEX-1 and APEX-2 cohorts; however, there was no difference between the two cohorts with mean R5R20 0.98±1.08 vs 0.87±0.92cmH20/L/s p=0.88. The area of low-frequency reactance was 16.18 ±21.20 vs 14.28±15.15 for APEX-1 and APEX-2, respectively, p=0.30.

Questionnaires

The ACQ5, ACQ6, and ACQ7 were recorded with ACQ5 recording symptoms, ACQ6 was added with reliever use, and ACQ7 combined pre-bronchodilator FEV1 with the two variables. There was a significant difference between ACQ7 scores, with APEX-1 having a greater median ACQ7 of 1.57 versus APEX-2 of 1.00 p=<0.001. However, when using the ACQ5 score, which is patient-reported only, both cohorts still scored above 1.0 for median ACQ5, suggesting the perception of poor control of asthma, but again, this was greater for APEX-1 than 2 (1.60 vs 1.00) p=<0.001. The adherence to asthma therapies of both cohorts was reported to be the same through the MARS score, with the median for APEX-1 and APEX-2 being 4.6 p=0.12

Atopy

Skin prick tests were performed to assess the presence of atopy within the two cohorts, and total IgE was collected. 57.5% of APEX-1 and 56.6% of APEX-2 had 1 or more positive skin prick tests, p=0.53. The Median Total IgE was 55.5UI.ml; in APEX-2, it was higher at 69.5 UI/ml, p=0.34.

Smoking status

	APEX-1 N(%)	APEX-2 N(%)	p-value
Smoking status Never smoker Ex-smoker Current smoker	 135 (66.8) 55 (27.2) 12 (5.9)	 72 (69.9) 29 (28.2) 2 (1.9)	 p=0.15
Mean Age of Onset, smoking	18.52	17.39	
Mean Pack-year	9.19	8.14	
E-cigarette use (N)	5	2	

Table 3. 3: Smoking status of the APEX cohort. Fisher's exact test assessed the difference in current smokers in APEX1 and 2.

Both cohorts had similar percentages of never-smokers and ex-smokers. However, APEX-1 had 5.9% current smokers and APEX-2 1.9% P=0.12. The mean age on onset was similar in both cohorts. Both had an average pack year of less than 10, reflecting the exclusion criteria to ensure patients with COPD or a significant pack year history were excluded.

Inflammatory cell composition of sputum.

Sputum Characteristic	APEX-1 N(%)	APEX-2 N(%)
Neutrophilic	27(60)	14(51.9)
Eosinophilic	3(6.7)	4(14.8)
Mixed	4(8.9)	0(0)
Paucigranulocytic	11(24.4)	9(33.3)

Table 3. 4: Baseline sputum characteristics of the APEX cohort.

At baseline, every participant attempted to produce a sputum sample. Of these, 22.2% of APEX-1 and 26.2% of APEX-2 produced samples that could successfully undergo sputum processing and cell counts.

27 (60%) of the APEX-1 samples were neutrophilic, compared to 14 (51.9%) of the APEX-2 samples. Four samples met the criteria for eosinophilia in APEX-2 and three samples in APEX-1. 24% of samples obtained in APEX-1 showed no evidence of inflammation, and in APEX-2, 33%. However, fewer samples were collected in APEX-2 than in APEX-1.

Biomarkers of T2 inflammation: FeNO and Blood Eosinophil

T2 inflammation	APEX-1 N(%)	APEX-2 N(%)
Raised FeNO	38/202(18.8)	16/103(15.5)
No raised FeNO	162/202(80.2)	87/103(84.5)
Raised Eosinophil	42/202(20.8)	19/103(18.5)
No Raised Eosinopil	153/202(75.7)	84/103(81.5)
Either raised FeNO OR Eosinophil OR Sputum Eosinophilia	63/202(31.2)	27/103(26.2)
Both raised FeNO AND Eosinophil	17/202(8.42)	9/103(8.74)
No raised FeNO and No raised eosinophil	135/202(66.8)	76/103(73.8)

Table 3. 5: Inflammatory phenotype of APEX cohorts according to the presence of T2 inflammation.

T2-high was defined as FeNO (≥40 ppb), Blood eosinophil (≥0.3 x10^9/L) or sputum eosinophilia (≥2% eosinophils).

Table 3.5 records the proportion of patients demonstrating the presence of T2-related inflammation for APEX-1 and APEX-2. Most of the APEX-1 and APEX-2 cohort at baseline did not present any evidence of T2 inflammation with a raised FeNO and a raised eosinophil, 66.8% vs 73.8% for APEX-1 and APEX-2, respectively.

31.2% of APEX-1 showed evidence of T2 inflammation, and 26.2% for APEX.2 The median FeNO for APEX-1 was 19.5 ppb for APEX-1 and 19 ppb for APEX-2. Median Eosinophil count for APEX-1 and APEX 2 was 0.16 vs 0.14 $x10^9$ per L, respectively.

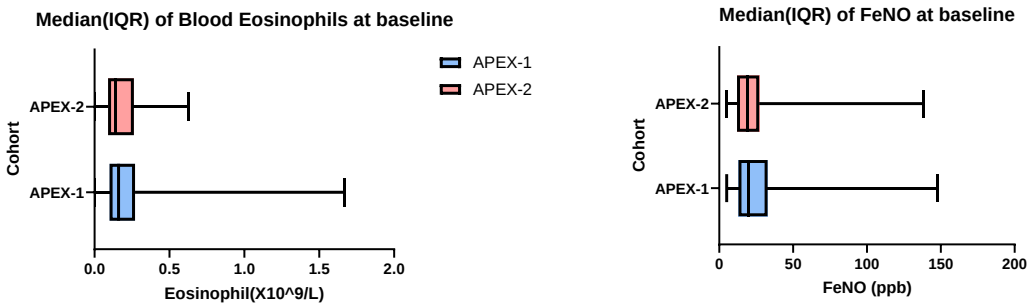


Figure 3. 1: Median and IQR for baseline FeNO and blood eosinophils for the APEX cohort.

Demographic characteristics of the APEX cohort, including comorbidities

	APEX-1 N(%)	APEX-2 N(%)	P value*
Female	149(73.8)	66(64.1)	0.08
BMI (kg/m^2)	30.3(7.93)	29.5(6.14)	0.28
Asthma	202(100)	103(100)	-
COPD	0(0)	0(0)	-
Arthritis	55(27.2)	23(22.3)	0.36
Depression	80(39.6)	31(30.1)	0.10
Eczema	76(37.6)	42(40.8)	0.59
GORD	89(44.1)	40(38.8)	0.38
Hypertension	35(17.3)	16(15.5)	0.69
Nasal Polyps	21(10.4)	8(7.8)	0.46
Rhinitis	53(26.2)	23(22.3)	0.46
Sleep apnoea	13(6.4)	6(5.8)	0.84
Lung Surgery	2(1)	1(1)	0.99
Age of Onset of Asthma N(SD)	21.5(17.8)	20.2(19.2)	0.25
Hospital admission for asthma	100(49.5)	36(35)	0.02
Hospital admission for asthma 12 months	26(12.9)	0(0)	<0.001
ITU admission for asthma	13(6.4)	3(2.9)	0.04
Intubation for admission	3(1.5)	1(1)	0.41
Unscheduled visit for asthma 12 months	165(81.7)	12(11.7)	<0.001
Exacerbations in 24 months/24-60months	3.31(3.98)	1.45(0.84)	<0.001
FH of asthma	115(56.9)	54(52.4)	0.45
Aspirin allergy	18(8.9)	2(1.9)	0.05
Caucasian	177(87.6)	92(89.3)	-
African	2(1)	1(1)	-
Asian	9(4.5)	6(5.8)	-
Black	3(1.5)	1(1)	-
Hispanic	3(1.5)	0(0)	-
Other	8(4)	3(2.9)	-

Table 3. 6: Demographic characteristics of APEX-1 and APEX-2.

A chi-squared test was performed to obtain p-values for categorical variables.

APEX-1 cohort had a more significant percentage of females in the cohort than males (73.8% vs 64.1%). However, this was not statistically different p=0.08. Body Mass Index (BMI) for both cohorts were similar

(30.3 for APEX-1 and 29.5 for APEX-2 p=0.28). There was no statistical difference in comorbidities between APEX-1 and APEX-2 (Table 3.6) for reflux (GORD), depression, eczema, hypertension, the presence of nasal polyps, allergic rhinitis or sleep apnoea. APEX-1 experienced statistically significant healthcare utilisation, with 49% of patients experiencing a hospital admission for their asthma versus 35% for APEX-2. Due to the recruitment strategy for APEX-2, participants had to have experienced an asthma exacerbation greater than two years prior to study entry and, as such, had no hospital admission in the 12 months prior to study entry; however, in APEX -1 just over 1 in 10 had a hospital admission for their asthma in the past 12 months. APEX-1 had 13 ITU visits related to asthma, in which three reported being intubated. 81.7% reported an unscheduled visit to their primary care physician, out-of-hours or A+E for their asthma, in comparison to APEX-2 at 11.7%. There was a statistical difference in the presence of an Aspirin allergy, with 8.9% reporting this in APEX-1 and just under 2% for APEX-2. There was no difference in ethnicities recruited into both cohorts, with the majority being Caucasian for both cohorts; 87.6% for APEX-1 and 89.3% for APEX-2

Baseline Inhaled medications

	APEX-1 N(%)	APEX-2 N(%)
SABA	183 (90.6)	90 (87.3)
Type of Inhaler Glucocorticoid Combination	 17 (8.4) 175 (86.6)	 17 (17.2) 82 (82.8)
Maintenance dose of inhaled corticosteroid ICS/ LABA/ICS Median ICS dose Budesonide Equivalent N(IQR) Low Dose (200-≤400 µg/day) Medium Dose (400-800 µg/day) High Dose (≥800 µg/day) MART therapy	 800(800-1600) 17 (8.9) 107 (55.7) 68 (35.4) 30(14.9)	 800(800-800) 19 (19.2) 57 (57.6) 23 (23.2) 14(13.6)
LAMA	22(10.9)	5(4.9)
LTRA	33(16.3)	11(10.7)
OCS	5(2.5)	0
Theophyllines	5(2.5)	0
Biological therapy	0	0

Table 3. 7: Asthma medications utilised by the APEX cohorts at study entry

SABA =Short-Acting Beta Agonist, ICS= Inhaled Corticosteroid LABA= Long-Acting Beta Agonist LAMA= Long-Acting Muscarinic Antagonist LTRA= Leukotriene Receptor Antagonist. Low, medium and high dose ICS based upon budesonide or equivalent doses. NICE Asthma Guidelines 2023 [287].

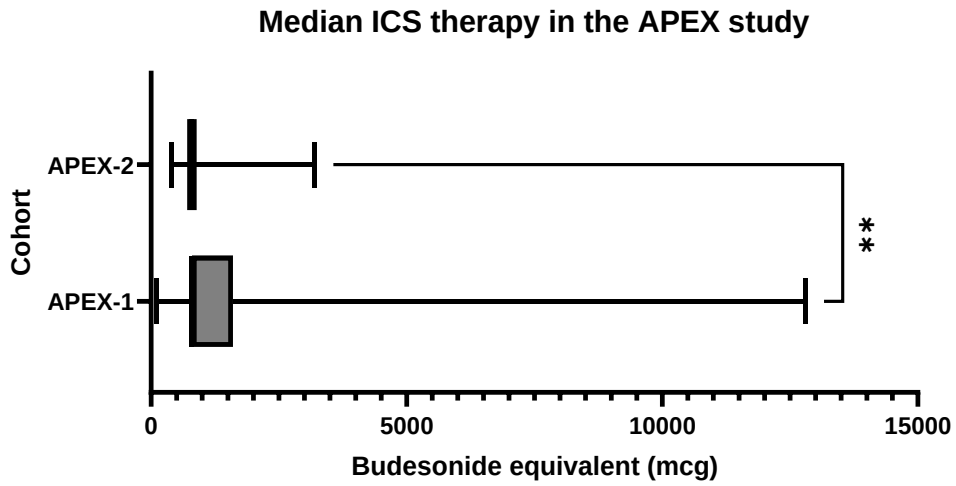


Figure 3. 2: Median ICS therapy in the APEX study stratified by cohort.

Asthma medication

A high proportion of APEX-1 and APEX-2 were using a form of short-acting beta-agonist (SABA) for their asthma as part of their daily care (90.6% vs 87.3%). 95% of APEX-1 and 96% of APEX-2 were on Inhaled corticosteroid therapy for their asthma. Over a third of APEX-1 were on a high-dose ICS to maintain asthma control versus APEX-2, where under a quarter of the cohort were on high ICS doses. Similar percentages of inhaled medium dose inhalers were present in APEX-1 and APEX-2 (55.7% vs 57.6% respectively). The median dose of Budesonide equivalent for APEX-1 was 800mcg (800-1600), and for APEX 2 was 800mcg (800-800). 30 (14.9%) of participants in APEX1- adopted MART therapy, and 14/103 (13.6%) in APEX-2.

The APEX-1 cohort had a higher medication burden than APEX-2. 10.9% were additionally on long-acting antimuscarinic antagonists (LAMA), and 16.3% of APEX-1 were on Leukotriene Receptor Antagonists (LTRA). Additionally, five patients were on maintenance steroids, and five were on theophyllines.

Comparing T2 status at baseline

	T2-High	T2-Low	p-value
(N)(%)	90(29.5)	215(70.5)	-
R5 cm$H_2$0/L/s**	3.99(2.97-5.24)	3.83(3.01-4.93)	0.51
R20 cm$H_2$0/L/s**	3.23(2.48-3.87)	3.16(2.61-3.82)	0.42
R5-R20 cm$H_2$0/L/s*	1.11(1.06)	0.84(1.01)	0.04
AX cm$H_2$0/L**	11.04(4.44-24.01)	6.50(3.22-19.35)	0.06
FeNO (ppb)**	46(30.50-69.00)	15(11.00-22.00)	<0.0001
Eosinophils (x10^9/ml)*	0.41(0.27(	0.14(0.07)	<0.0001
ACQ5*	1.20(0.80-2.20)	1.40(0.75-2.20)	0.60
ACQ6*	1.17(0.67-2.17)	1.17(0.67-2.17)	0.76
ACQ7*	1.43(0.86-2.14)	1.43(0.86-2.07)	0.32
FEV1%*	76.8(19.54)	87.86(16.90)	<0.0001
FVC%*	87.84(16.80)	92.86(13.57)	0.007
MARS**	4.6(3.80-4.80)	4.6(4.00-4.80)	0.61

Table 3. 8: T2 status of the APEX cohorts combined at baseline

*=mean and unpaired t-test **=median and Mann-Whitney test

Table 3.8 shows that just under 30% of the APEX cohort demonstrated evidence of T2 high inflammation at baseline through sputum eosinophilia, or blood eosinophilia or FeNO. At baseline, those with a T2-high status demonstrated greater peripheral resistance (1.11 vs 0.84 $cmH_20/L/s$. p=0.04, and worse lung function at baseline (FEV1% predicted 76.8% vs 87.9%). There was no statistical difference in ACQ5,ACQ6 or ACQ7 scores between T2-high and T2-low,

Exacerbator vs non-exacerbators at baseline

The difference between APEX-1 and APEX-2 was based on a self-reported exacerbation requiring a course of oral corticosteroids. This may be subject to recall bias. Therefore, to further strengthen the baseline analysis, a prospective review of those who had called the Nottingham Asthma Centre and underwent an exacerbation visit within the course of the APEX study (exacerbators) versus those who did not undertake an exacerbation visit in APEX (non-exacerbators) was undertaken. This analysis included all patients who visited regardless of whether a course of steroids was prescribed.

	Exacerbators	Non-Exacerbators	p-value
	N=86	N=219	
Age	52.58(14.15)	49.61(16.22)	0.78
R5 $cmH_20/L/s$*	4.15(1.66)	4.22(1.81)	0.84
R20 $cmH_20/L/s$*	3.28(0.97)	3.30(1.09)	0.99
R5-R20 $cmH_20/L/s$*	0.93(1.06)	0.87(0.97)	0.67
AX cmH_20/L*	15.83(20.94)	15.22(18.92)	0.92
FeNO*	35.77(30.11)	23.67(21.15)	**0.04**
FEV1pred (L)*	3.05(0.80)	3.10(0.74)	0.54
FVCpred (L)*	3.83(0.86)	3.85(0.89)	0.84
PEFR*	446.51(125.86)	449.27(121.72)	0.91
FEV1 post (L)*	2.98(1.01)	2.95(0.75)	0.87
FEV1/FVC*	71.99(11.27)	75.05(10.49)	**0.03**
FEV1% pred*	82.67(18.60)	85.42(18.25)	0.24
FVC% pred*	91.25(14.53)	91.38(14.87)	0.95
Reverse (%)*	7.57(8.12)	5.12(7.70)	0.14
Neutrophils ($x10^9$/L)	4.21(3.57-5.31)	4.12(3.24-5.50)	0.84
Eosinophils ($x10^9$/L)**	0.22(0.11-0.33)	0.14(0.10)	**0.01**
CRP ($x10^9$/L)**	3.00(1.00-5.00)	3.00(1.00-5.00)	0.37
IgE (UI/ml)**	65.2(29.25-273.5)	51.5(22.20-187.0)	**0.007**
ACQ-5**	1.20(0.80-2.20)	1.40(0.60-2.20)	0.39
ACQ-6**	1.17(0.67-2.17)	1.33(0.67-2.17)	0.40
ACQ-7**	1.43(0.86-2.14)	1.43(0.86-2.14)	0.83
MARS**	4.6(4.20-4.85)	4.4(3.80-4.80)	**0.01**

Table 3. 9: Key differences in clinical, physiological and biomarkers according to exacerbation status.

Bonferroni correction was performed for multiple testing of *mean(SD) **median(IQR).

Those who experienced an exacerbation event had evidence of T2 inflammation in both FeNO (35.8 vs 23.7 ppb p=0.04) and blood eosinophils (0.22 vs 0.14 =0.01). This is supported by findings in the

literature in that those who demonstrated T2 inflammation demonstrated more frequent courses of OCS for asthma. [288]They also demonstrated greater atopy with significantly raised IgE levels than those that did not have an exacerbation event: 65.2 vs 51.5 UI/ml. FEV1/FVC ratio was also reduced in those who experienced an exacerbation event (71.99% vs 75.05% p=0.03). Adherence to therapies for asthma was also greater in those that exacerbated (4.6 vs 4.4 p=0.01).

Discussion

This chapter aimed to identify the key differences at baseline between APEX-1 and APEX-2. Comparisons were also made according to the T2 status at baseline and a prospective comparison at baseline of those who exacerbated in APEX (Baseline Exacerbators) versus those who did not have an exacerbation event in APEX (Baseline Non Exacerbators).
APEX-1 was enriched in those with a recent asthma exacerbation compared to APEX-2. The APEX-1 cohort had features of those with severe asthma, such as high healthcare utilisation and a more significant medication burden. As highlighted in previous studies, the presence of T2 inflammation is a risk factor for the development of future asthma exacerbations. Despite over 30% of APEX-1 and just under 25% of APEX-2 being prescribed higher doses of inhaled corticosteroids, there was still evidence of T2-inflammation with over 30% of APEX-1 having either proof of a raised FeNO or raised blood eosinophil, and in APEX-2 which is based on an exacerbation not occurring within five years of study entry 26% of APEX-2 having either a raised FeNO or blood eosinophil. This is despite the similar adherence to medications for asthma in both APEX-1 and APEX-2.

The heterogeneity of both cohorts has been demonstrated through sputum profiling for cell counts. 60% of participants at baseline had neutrophilic predominant sputum at baseline in APEX-1 and 52% for APEX-2. The neutrophilic phenotype is well-established in asthma. It is characterised by being male predominant with older onset asthma and

typically responding poorly to inhaled corticosteroid therapy. They were on high ICS doses and, as such, had high symptom burden from the side effects of prolonged steroid use, such as GORD and hypertension. [289]

The presence of neutrophils in sputum is often considered a reflection of steroid therapies with neutrophilia post steroid therapy or after taking high dose ICS, and whilst this may be the reason for APEX-1 having a high sputum neutrophilia and APEX-2 having eosinophilia; steroids have also been shown to increase the survival of neutrophils. The neutrophilic phenotype has been seen in steroid naïve patients too[290] and the presence of neutrophils in high-dose ICS has not always been consistent. [291]Unfortunately, despite performing sputum induction on the cohort, only 22.3% of APEX-1 and 26.2% of APEX-2 were able to produce a sputum sample, which limits the interpretation of findings. However, producing sputum successfully in patients with asthma can be difficult.[292]

In APEX-1, the median ACQ-7 score at baseline was 1.57, and APEX-2 was 1.15, suggesting worse asthma control. This reflects, firstly, a higher average number of asthma exacerbations and, secondly, greater healthcare utilisation than that of APEX-2, who perceived their asthma to be better controlled. An interesting finding was that in both APEX-1 and APEX-2 the median ICS dose was the same. This could be interpreted in many ways. It could be firstly that they were both on adequate doses of ICS, or it could mean that given the fact that APEX-2 had not had a recent asthma exacerbation, the cohort might have been overprescribed high ICS dosages.

There was no difference in FEV1% predicted between APEX-1 and APEX-2 (median FEV1% predicted 87% vs 89%). An important consideration is a variation in FEV_1 during follow-up, which is 15% year-to-year in healthy individuals and more in patients with airway disease. [293] FEV_1 should, therefore, change by at least 12% before accounting for a meaningful change in lung function. There have been studies that have demonstrated that in a population admitted to a hospital for a physician-

based diagnosis of an asthma exacerbation – 83% had obstructive lung function. [294]

The presence of small airway disease was significant in both cohorts. Peripheral resistance indicative of small airway disease was above the normal threshold for both APEX-1 and APEX-2 and at baseline.

There were key differences when comparing those who went on to have an exacerbation event in the APEX study to those who did not. On average, those with an exacerbation event demonstrated more significant T2 inflammation in FeNO and blood eosinophils and more significant airflow obstruction. They also had a statistically greater adherence to their medication for asthma management. At baseline, there was no statistical difference between ACQ scores and measurements of small airway disease between exacerbators and non-exacerbators. These findings are similar to those of previous cohort studies, such as the SARP and U-BIOPRED.

A significant proportion of APEX-1 and APEX2 demonstrated T2-inflammation in blood but not on FeNO and vice versa. As previously described, there is a good correlation between blood eosinophils and exhaled FeNO, so whether this represents a different correlation or whether this could represent a phenotype overlap is unclear.

Another important point to consider is that we did not look at historical blood eosinophil count. Therefore, those labelled as T2-low may have a historical eosinophil count that would classify them as being T2-high. This highlights the difficulty in labelling T2-low, given the variability of biomarkers such as the blood eosinophil count and FeNO. However, those with T2-high inflammation at baseline had worse lung function as measured by FEV1% predicted and FVC% predicted, and peripheral resistance and it is known that those who experience multiple asthma exacerbations experience a more significant decline in lung function.[288]

I have explored key clinical and physiological markers of inflammation at baseline. To improve the generalisability of findings, a broader recruitment strategy was implemented from primary care and secondary care with no strict criteria for study entry. As such, the severity of asthma in the cohort differs from that of other cohort studies, such as SARP and U-BIOPRED. Both APEX-1 and APEX-2 were predominantly Caucasian, affecting the generalisability of these findings to other ethnicities.

To phenotype asthma exacerbations, obtaining a detailed understanding of the cohort's characteristics is essential to understand potential future risks in the development of asthma exacerbations, such as what would occur in a severe asthma clinic. To phenotype at the point of an asthma exacerbation, it is important to have a detailed understanding of the cohort's characteristics at baseline to put any findings found into context. The next chapter will extend our findings by comparing baseline findings to when an exacerbation events occurs.

Chapter 4: Phenotyping Asthma Exacerbations- Exacerbation Profiles

Aims

The APEX study was designed to collect inflammatory, clinical, and physiological parameters during an asthma exacerbation. Ideally, this would be before commencing steroid therapy or treatment that may influence any findings. This chapter focuses on understanding the impact of inflammatory phenotypes identified in Chapter 3 and exploring this through paired analyses as well as assessing the inflammatory stability of exacerbation events. My hypothesis is that T2 high-associated exacerbations in which OCS was required were associated with worse clinical outcomes than T2-low-associated exacerbations. The following aims were generated based on this.

- To first describe the natural history of an exacerbation. This will be through exploration of exacerbation frequency, the behaviours that occurred before exacerbation and symptoms at exacerbation.
- To identify the inflammatory, physiological and clinical changes during a suspected exacerbation.
- Whether a T2-high exacerbation event was associated with worsening lung function than T2-low exacerbation events

The following analyses will address these aims:

1. Paired analysis
    a. Baseline to exacerbation primary event where OCS prescribed. (Primary OCS)
    b. Baseline to exacerbation primary event where no OCS is prescribed. (Primary No OCS)
2. Paired analysis
    a. T2 status at Primary OCS exacerbation event to baseline.

b. T2 status at baseline to exacerbation event requiring OCS.

3. Comparison of Lung function at T2- High and Low exacerbation events
4. Assessment of phenotypical stability at the point of exacerbation

Method

Unscheduled events

Exacerbation visits

As described in Chapter 2, patients were invited to the Nottingham Asthma Centre, should they develop symptoms suggestive of an asthma exacerbation. They were advised to contact the research centre when they would typically seek medical attention for a deterioration in their asthma symptoms.

Participants were triaged first to ensure they were safe to enter the unit. This was based upon NICE guidance, and the questions posed to participants were to encompass the following themes:

- Unable to complete full sentences
- Evidence of cyanosis
- Features of altered consciousness or exhaustion

We aimed to bring participants to the centre as soon as they called, ideally on the same day. However, if patients had already commenced a course of oral corticosteroid therapy, they were invited to the centre if they had had less than three days' worth of corticosteroid therapy. If they had commenced OCS, this was recorded.

The same measurements that were taken at baseline were measured during the exacerbation visit. A real-world approach was undertaken to assess a possible asthma exacerbation, which relied on taking an asthma history, including a comparison of the patient's usual symptoms to current ones and looking for evidence of exacerbations via examination. All

exacerbation visits were overseen by myself, and the decision as to whether steroids were given or not was based on the history and examination findings. If there was some uncertainty to an exacerbation with history and examination findings alone then spirometry at baseline was compared to that seen in exacerbation. Although infrequently done, I acknowledge this could be a source of selection bias, but it was done to maintain patient safety. There were three outcomes from the visits:

- **No exacerbation**: On the assessment of the participant, there were no features at the time of review to suggest the participant was experiencing an asthma exacerbation. No measurements were obtained for these events, as described below in the “clinical measurement section.”
- **Exacerbation event not requiring OCS**: On the participant's assessment, there were features of an exacerbation (i.e. cough or shortness of breath), but objectively, there was no evidence of an asthma exacerbation (history more in keeping with an upper respiratory tract infection or lack of wheeze). Clinical measurements were taken of these participants. Participants were followed up in 48 hours with a telephone call to reassess their symptoms. If they were still symptomatic, their symptoms worsened, or they had started taking their corticosteroid therapy, they were invited to the trials unit for a repeat assessment to assess whether the event was an exacerbation. If symptoms had resolved, they were given safety advice and told to ring should their symptoms return.

  This was considered an important event to capture, as what defines an exacerbation is unclear. Clinically, this is a combination of symptoms, examination findings, and a lung function test, which is often in England, a PEFR. Blood eosinophils can sometimes be used in conjunction but are often unavailable before any therapies are initiated. These events have a high symptom burden that

results in patients seeking medical attention and receiving often inappropriate steroid therapy.

Therefore, understanding what occurs in such events is essential. Whether this differs from events where OCS are given will be answered when a multidimensional approach is undertaken to define this period of uncontrolled symptoms.

- **Exacerbation requiring OCS:** On assessment, the participant had symptoms and objective measurements to suggest they were experiencing an asthma exacerbation or they had already commenced a course of oral corticosteroid therapy. A prescription for oral corticosteroid therapy was provided to these patients for ten days. We aimed to contact participants 7 days after coming for their Asthma exacerbation event to assess their symptomology. Should they have persistent symptoms, they were then invited back to the Nottingham Asthma Centre for a clinical review. No study measurements were taken at this point. If needed, clinical care would be provided as appropriate, and their primary care physician would be informed of this to ensure continuity. After an OCS event, a written letter was sent to their primary care physician to allow an update of the participant's medical records, with permissions obtained from participants first to allow for this.

Results

Natural History of Exacerbation Events

Tables 4.1, 4.2 and Figure 4.1 show the exacerbation events that occurred during the APEX study. The total mean and median for all parameters are in Appendix VI. 305 patients entered the study, and 140 exacerbation events were observed. There were 122 exacerbation events in APEX-1 and 18 in APEX-2. APEX-1 had 40 unique exacerbation events in which steroids were prescribed and 27 events in which no steroids were prescribed. There were 42 multiple exacerbation

events in APEX-1, which involved 25 participants. APEX-2 had four exacerbation events in which steroids were prescribed and 14 unique exacerbation events where steroids were not required. Therefore, there were 44 first exacerbation events requiring OCS in APEX and 41 first exacerbation events where OCS was not needed. No multiple exacerbations were experienced by any participant in APEX-2. Four multiple exacerbations did not require OCS, and this was from 3 participants.

Frequency of asthma exacerbations

Figure 4.2 shows the frequency of exacerbation events according to seasonality. The most significant frequency of asthma exacerbations occurred in the autumn and winter, with 78 of them occurring in the Autumn and Winter of 2021 and 2022. The COVID-19 pandemic did affect the number of exacerbations that could be seen from the winter of 2019 until the summer of 2020 due to the UK government's shielding policies and lockdown strategies.

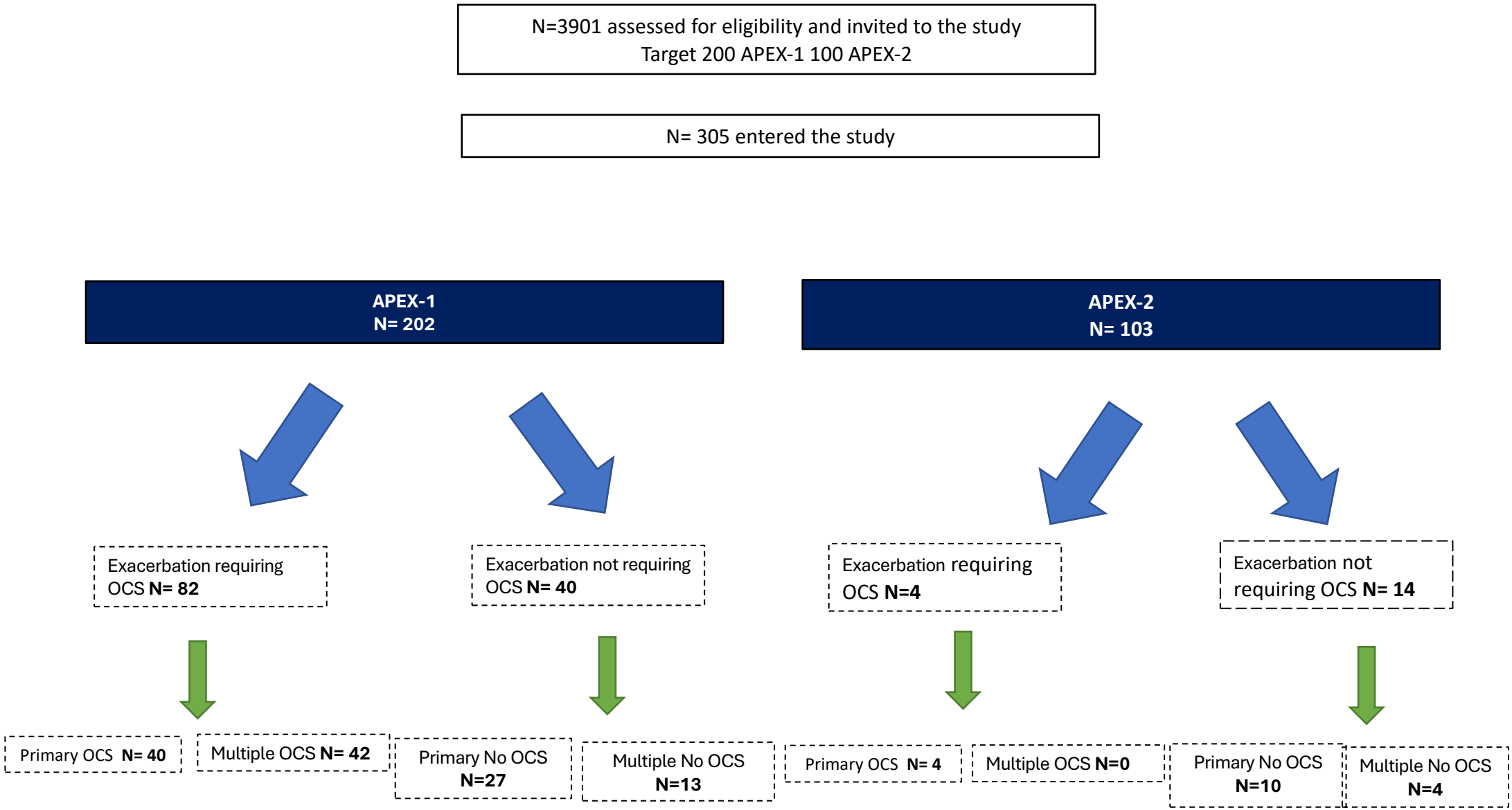


Figure 4. 1 Exacerbation events during study duration.

The first exacerbation a participant experienced was classified as the “first event.” “Multiple events were any further exacerbation a participant experienced.

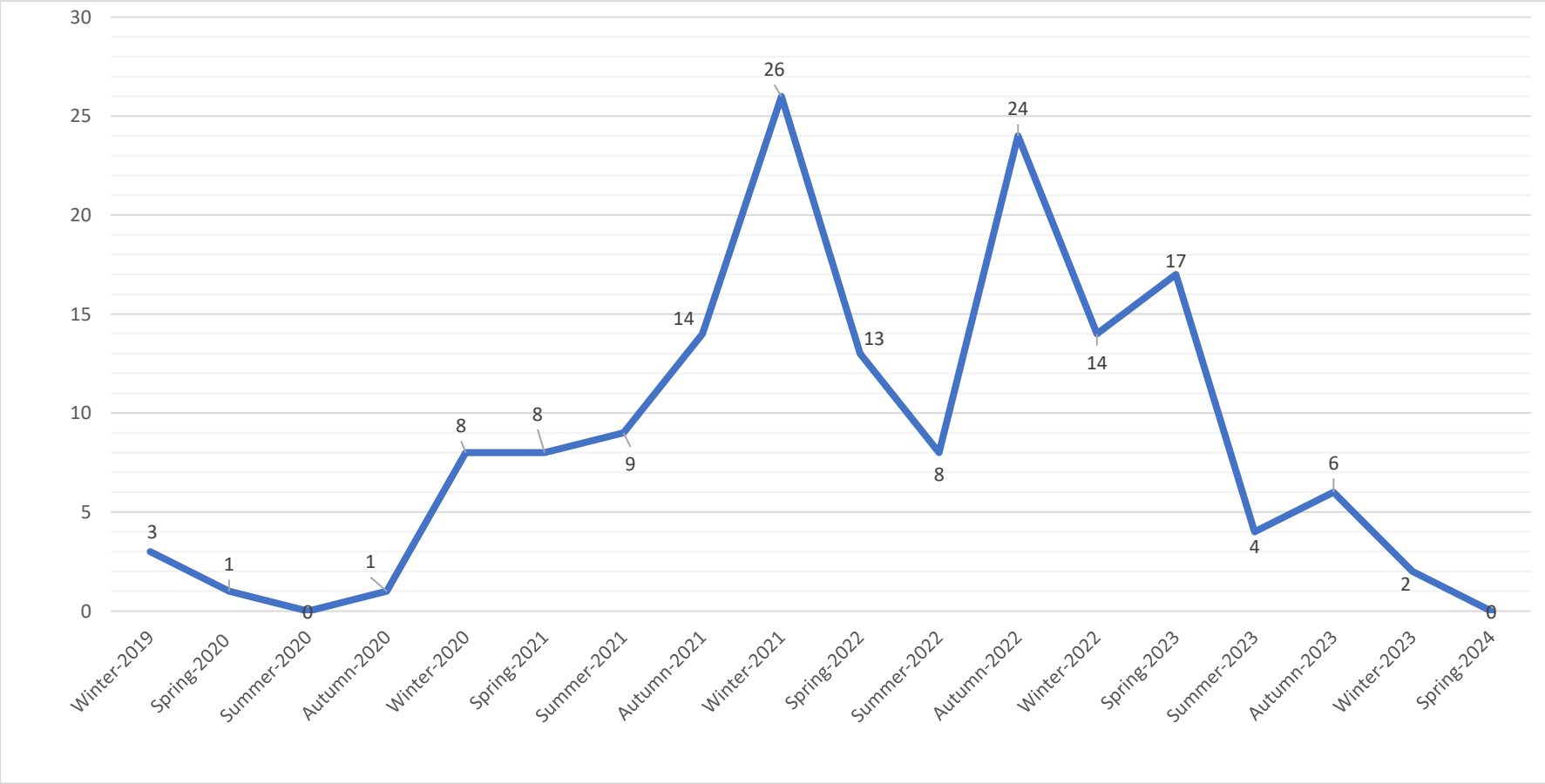


Figure 4. 2: Frequency of Exacerbation events during study duration according to season.

Study commenced during winter 2019 and due to COVID-19 the study was paused for a period of 9 months.

APEX-1	Value (N)
Total exacerbation events captured	125
Exacerbations requiring OCS	82
Exacerbations not requiring OCS	40
No evidence of exacerbation	3
Unique exacerbation requiring OCS †	40
Unique exacerbations not requiring OCS†	27
Multiple exacerbation requiring OCS	42
Number of participants contributing to a multiple exacerbation requiring OCS	25
Multiple exacerbations not requiring OCS†	13
Number of participants contributing to a multiple exacerbation not requiring OCS	11

Table 4. 1: Breakdown of APEX-1 exacerbations.

First exacerbation recorded was 21st January 2020. Last exacerbation recorded was 20th December 2023.
Exacerbation events:
Total number of exacerbation events recorded = 134
Missed number of exacerbation events recorded= 9
Exacerbation events captured= 125

APEX-2	Value (N)
Total exacerbation events captured	22
Exacerbations requiring OCS	4
Exacerbations not requiring OCS	14
No evidence of exacerbation	4
Unique exacerbation requiring OCS †	4
Unique exacerbations not requiring OCS†	10
Multiple exacerbation requiring OCS	-
Number of participants contributing to a multiple exacerbation requiring OCS	-
Multiple exacerbations not requiring OCS†	4
Number of participants contributing to a multiple exacerbation not requiring OCS	3

Table 4. 2: Breakdown of APEX-2 exacerbations.

The first exacerbation recorded was 1st October 2021. The last exacerbation recorded was 4th May 2023.

Exacerbation events:
Total number of exacerbation events recorded = 23
Missed number of exacerbation events recorded 1
Exacerbation events captured= 22

Self-management of asthma exacerbations by participants prior to study visit:

	APEX-1 N/N(%)	APEX-2 N/N(%)
Events captured	125	22
Escalation of treatment prior to the visit	94(75.2)	12(66.6)
Increased SABA	77(61.6)	2(11.1)
Increased ICS	0	9(50)
Increased ICS/LABA	50(40)	1(5.5)
Commenced antibiotics.	3(2.4)	0
Commenced OCS	7(5.6)	0
Mean number of days of symptoms prior to visit	9.7	9.2

Table 4. 3: Participants modification of their usual asthma medication before an exacerbation visit according to the cohort.

We captured what they did with their usual asthma care to understand the participants' behaviours when experiencing worsening asthma symptoms (Figure 4.3). The mean number of days participants would persist with their symptoms before seeking help with their symptoms was nine days. In APEX-1, 75% of participants altered their therapies, with 60% of patients preferring to escalate their SABA inhaler to help abort their symptoms. These values were 66% and 11.1%, respectively, in APEX-2. From Chapter 3, 8.4% of APEX-1 participants were on ICS alone for their asthma. None of these participants went on to have an exacerbation event in the APEX study. However, in APEX-2, 50% of participants escalated their ICS therapy. 40% of patients in APEX-1 escalated their ICS/LABA therapy to gain control of their asthma symptoms. All the participants in APEX-1 who exacerbated were on a form of ICS/LABA therapy. In APEX-2, 1 participant began to escalate their inhaled therapy to help with their symptoms. 7 participants in APEX-1 began a rescue pack of their OCS therapy prior to seeing the APEX team, and these were all prior to consultation with their primary care physician for advice.

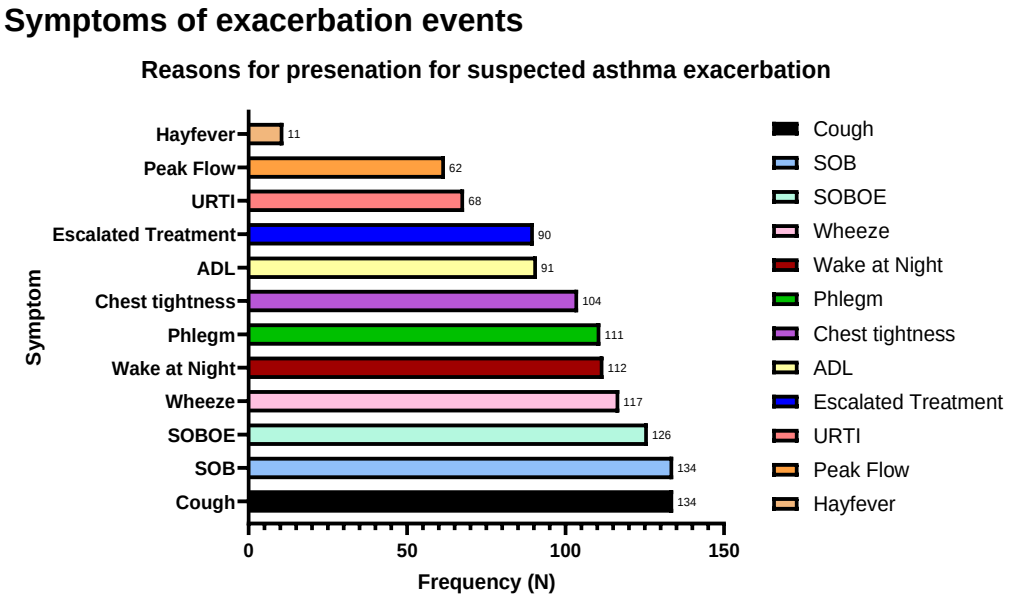
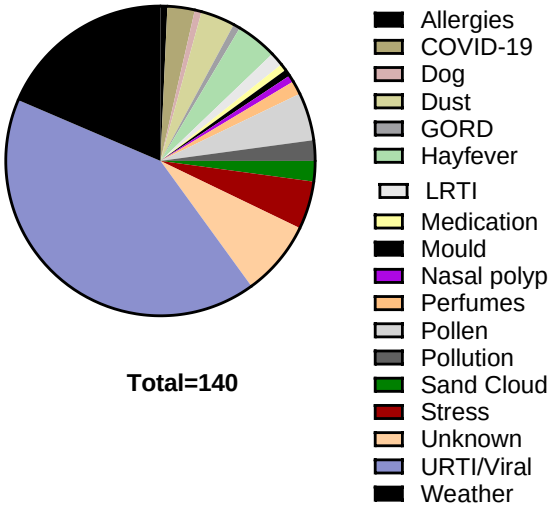


Figure 4. 3: Symptoms participants experienced at the presentation of an exacerbation visit in the APEX study.



Trigger	Frequency N(%)
Allergies	1(0.7)
COVID-19	4(2.9)
Dog	1(0.7)
Dust	5(3.5)
GORD	1(0.7)
Hay fever	6(4.3)
LRTI	2(1.4)
Medication	1(0.7)
Mould	1(0.7)
Nasal Polyps	1(0.7)
Perfumes	2(1.4)
Pollen	7(5.0)
Pollution	3(2.1)
Sand Cloud	3(2.1)
Stress	7(5.0)
Unknown	11(7.9)
URTI/Viral	58(41.4)
Weather	26(18.6)
Total	140/100

Figure 4. 4: Participants perceived the trigger to worsen asthma symptoms.

I recorded the participants' reasons for calling the study centre regardless of the cohort of origin. Participants were given 12 options to consider and could choose more than one reason for attendance. The top four symptoms' patients presented with were cough, shortness of breath, shortness of breath on exertion and wheeze. Nocturnal awakening followed. 68 participants described having coryzal symptoms that have resulted in worsening of their asthma, whilst 11 participants described hay fever symptoms. The perceived trigger to their worsening asthma symptoms was also asked. The participants could consider more than one aetiology. The most common triggers (Figure 4.4) that participants described as the cause of their symptoms were viral /upper respiratory tract infections (URTI) 41.4% and a change in weather 18.6%. Just under 8% of aetiologies for worsening asthma symptoms were unknown, according to the participants.

Follow up on exacerbation events.

	Number N/N(%)
Number of telephone follow-ups	124
Resolved symptoms	112(90.3)
Number with persistent symptoms. *Exacerbation event* OCS No OCS	 12/102(9.7) 6/12 6/12
Further OCS prescriptions *Exacerbation event* OCS Characteristic of event T2-High T2-Low	2/8 2 2 0
Antibiotics prescribed *Exacerbation event* OCS No OCS	2 2 0

Table 4. 4: Summary of telephone follow-up calls during the APEX study.

As part of the study, exacerbation events were followed by a 7-day follow-up call to assess symptoms as part of clinical care and to assess therapy's impact upon symptoms (Table 4.4).140 exacerbation events occurred in which we could contact 124 (88.5). Most participant's symptoms resolved after seven days (90.3%). Of the 12 participants, 6 of those were participants who were not initially prescribed steroids for their exacerbation. After clinical review, 2 of them were prescribed additional OCS, both of which were initially exacerbations that were provided with a course of OCS. Of the two prescribed further OCS, their initial exacerbation event was suggestive of T2-high inflammation. 4 participants went to their GP after their initial visit to the study, and 2 participants were given antibiotics by their GP, but their symptoms persisted despite this. No participant was prescribed a further course of OCS by their GP. No participant required hospital admission.

APEX Exacerbation analysis.

Exacerbation outcome nomenclature:

Events that occurred in the APEX-1 cohort and APEX-2 cohort were grouped according to the following outcomes:

- **Primary OCS:** First Exacerbation events where OCS was prescribed.
- **Primary No OCS:** First Exacerbation events where OCS was not prescribed.
- **Multiple OCS events:** Further exacerbation events where OCS was prescribed.
- **Multiple no OCS events:** Further exacerbation events where OCS was not prescribed.

Visits unrelated to asthma and therefore no treatment was provided are not included in any of the analyses.

1)Paired analysis of exacerbation events.

Table 4.5 describes the key features of exacerbation events according to a participant's first presentation to the research centre (primary) and secondly to whether a course of OCS therapy was prescribed for worsening asthma symptoms (OCS or No OCS). Of note is that the decision to prescribe OCS therapy was made before any samples were taken and was based on clinical findings. Full descriptive data of the parameters collected are in Chapter 2.

To analyse what occurs at the point of an exacerbation in which a course of steroids was prescribed, a paired analysis was performed comparing parameters to baseline. In relation to the statistical analyses, a paired t-test was carried out if values followed a normal distribution. If values did not follow a normal distribution, a Wilcoxon test was carried out. P-values were calculated for each test.

Variable	(Baseline vs Primary OCS)	p-value	(Baseline vs (Primary No OCS)	p-value
N	44	-	37	-
R5 cmH_20/L/s **	4.11(3.11-5.55) 5.83(4.07-7.04)	0.002	3.17(2.57-4.50) 3.87(2.83-4.50)	0.60
R20 cmH_20/L/s **	3.13(2.68-3.99) 3.85(2.9404.47)	0.23	3.00(2.39-3.64) 3.07(2.55-3.64)	0.34
R5-R20 cmH_20/L/s**	0.55(0.32-1.84) 2.15(1.00-2.84)	0.0002	0.45(0.13-0.97) 0.64(0.20-1.25)	0.38
AX cmH_20/L**	11.92(4.07-31.00) 39.93(11.7-62.56)	0.0001	5.59(2.71-11.55) 7.46(3.37-20.36)	0.14
FeNO (ppb)*	40.67(32.34) 51.51(56.62)	0.12	33.38(28.81) 35.35(34.31)	0.57
PEFR*	406(108.5) 338.50(104.5)	<0.0001	501.9(137.8) 485.32(124.54)	0.18
FEV1 % predicted*	78.52(20.71) 69.45(21.52)	<0.0001	84.88(14.35) 86.78(12.22)	0.47
FVC% predicted*	90.02(15.83) 81.71(18.94)	<0.0001	89.95(12.91) 93.32(11.68)	0.03
Neutrophils**	4.19(3.58-5.34) 4.76(3-34-6.44)	0.22	4.19(3.38-4.87) 4.54(3.56-5.58)	0.24
Eosinophils* ($x10^9$/L)	0.29(0.21) 0.36(0.31)	0.04	0.22(0.17) 0.26(0.27)	0.14
CRP ($x10^9$/L)**	3.00(1.00-5.75) 4.00(2.00-10.25)	0.004	2.5(1.00-5.00) 3.00(2.00-5.25)	0.24
MARS**	4.60(4.20-4.80) 4.60(4.05-5.00)	0.67	4.80(4.60-5.00) 4.80(4.80-5.00)	0.82
ACQ5**	1.20(0.80-1.80) 3.20(2.60-4.00)	<0.0001	1.60(0.80-2.45) 2.80(2.30-3.40)	<0.0001
ACQ6**	1.17(0.83-1.79) 3.17(2.54-3.96)	<0.0001	1.33(0.67-2.33) 2.83(2.33-3.33)	<0.0001
ACQ7**	1.43(0.86-2.07) 3.21(2.71-3.82)	<0.0001	1.43(0.82-2.21) 2.71(2.21-3.14)	<0.0001

Table 4. 5: Paired analysis from baseline to exacerbation according to exacerbation outcome for worsening asthma symptoms – OCS or No OCS.

*=Paired t-test mean(SD) presented. **= Wilcoxon test median(IQR) presented. Bonferroni correction for multiple testing applied.

Asthma control

Asthma control was worse in both these events regardless of whether the ACQ 5, 6, or 7 was measured, all with p=<0.0001. Adherence was consistent at baseline and exacerbation events, irrespective of whether

a course of steroids was provided. The median MARS-A being 4.60(4.05-5.00) for Primary OCS and 4.80(4.80-5.00) for Primary No OCS events.

Small airways disease

The presence of peripheral resistance suggestive of small airways disease was evident in Primary OCS but not in Primary No OCS (median R5-R20 2.15 $cmH_20/L/s$ p=0.0002 vs 0.64 $cmH_20/L/s$ p=0.38.) Reactance was greater at Primary OCS versus no OCS; median 39.93 p=0.0001 for those provided with OCS vs 7.46 $cmH_20/L/s$ p=0.14 for those without. In those events in which a course of steroids was not offered for exacerbation symptoms, there was no significant change in small airways disease parameters in any markers of small airways disease. There was no statistically significant difference seen in total airway resistance, central airway resistance, peripheral airway resistance or reactance to that observed at baseline through paired analysis (p=0.60, 0.34, 0.38, 0.14) in Primary No OCS events.

Inflammation

T2 inflammation was evident with a mean FeNO, which was more significant in those who required OCS than those who did not (51.51 vs 35.35 ppb). However, there was no statistical difference in paired analysis to baseline for both exacerbation events. Similarly, in Primary OCS events, there was a difference in blood eosinophils when compared to Primary No OCS (0.36 vs 0.26 x109/L). there was statistical difference when compared to respective baseline values for Primary OCS (0.29 to 0.36 x109/L p=0.04) but not for Primary No OCS (0.22 to 0.26 p=0.14).

The FeNO IQR for those who did receive a course of OCS was 21-50 ppb. The IQR for those who did not receive OCS was 15-33ppb. The breakdown of T2-associated inflammation observed in primary exacerbation events is illustrated in Table 4.6.

Exacerbation Sputum Characteristic	N(%)	Exacerbation requiring OCS N(%)	Exacerbation not requiring OCS N(%)
Neutrophilic	17(53.0)	10(52.6)	7(53.8)
Eosinophilic	11(34.4)	7(36.8)	4(30.8)
Mixed	2(6.3)	1(5.3)	1(7.7)
Paucigranulocytic	2(6.3)	1(5.3)	1(7.7)

Primary OCS	**N(%)**
High FeNO	15/44(34.1)
Low FeNO	29/44(65.9)
High Eosinophil Count	21/44(47.7)
Low Eosinophil Count	23/44(52.3)
High FeNO OR High Eosinophil OR Sputum Eosinophilia	25/44(56.8)
High FeNO AND High Eosinophil	1/44(25.0)
Low FeNO and Low Eosinophil	19/44(43.2)

Primary No OCS	N(%)
High FeNO	14/37(37.8)
Low FeNO	23/37(62.2)
High Eosinophil Count	9/37(24.3)
Low Eosinophil Count	28/37(75.7)
High FeNO OR High Eosinophil OR Sputum Eosinophilia	14/37(37.8)
High FeNO AND High Eosinophil or Sputum Eosinophilia	9/37(24.3)
Low FeNO and Low Eosinophil	23/37(62.2)

Table 4. 6: Inflammatory profile of primary exacerbation events in the APEX study

56.8% of exacerbations showed evidence of T2 inflammation with either a raised FeNO or a raised blood eosinophil. At the point of review, 43.2% of unique exacerbation events showed no evidence of T2 inflammation.

For exacerbations in which no OCS was prescribed, there was less evidence of T2 inflammation—37.8% of these events showed evidence of T2 inflammation, while 62.2% showed no evidence of any T2 inflammation. 19 sputum were collected for primary OCS and 13 for Primary No OCS. Over half of the sputum showed neutrophilia predominance in both events (52.65% for Primary OCS and 53.8% for Primary No OCS). 36.8% of sputum showed eosinophilia in Primary OCS and 30.8% in Primary No OCS. Again, however, the number of adequate samples collected was quite small.

Lung Function

Lung function differed between the two groups. In Primary OCS, those who received a course of steroids had worse lung function FEV1% predicted (69.5% p=<0.0001 vs 86.8% p=<0.47) with greater reversibility on average (12.69% vs 7.00% see Appendix V) compared to the baseline. FVC% predicted, and PEFR were statistically different for Primary OCS compared to baseline; however, there was no difference in PEFR for Primary No OCS.

Neutrophils and CRP

Measurements in inflammation not related to T2 inflammation – neutrophils and C-reactive protein (CRP) were measured at baseline and exacerbation. There was no significant change in neutrophil count for both Primary OCS and Primary No OCS events. However, CRP was statistically different, with it being raised at Primary OCS exacerbations compared to baseline (p=0.004). The clinical meaningfulness of this change in CRP is questionable.

Frequent Exacerbators – Exacerbations where multiple courses of OCS were prescribed. (Data in Appendix VI)

In the APEX study, 25 participants had multiple exacerbation events. These exacerbation events are cumulatively described in Appendix VI, which defines parameters for those with multiple exacerbations in which OCS was prescribed and those with multiple exacerbations in which a course of OCS was not prescribed. Briefly, those with multiple exacerbations had T2-inflammation similar to those with primary exacerbations seen in (Appendix VI) but were not to a greater value, with mean Primary OCS being 51.51 ppb and Primary multiple OCS being 50.24 ppb. Similarly, blood eosinophil was of similar values with mean eosinophil count for Primary OCS= 0.36 $x10^9$/L and Multiple Primary OCS= 0.38 $x10^9$/L. Those with multiple OCS exacerbations had a reduced FEV1% predicted of 70.7%, similar to primary OCS events.

There was small airways disease with R5-R20 of 1.47 $cmH_20/L/s$ and a reactance of 30.65 cmH_20/L. However, this was no greater than Primary OCS events

Frequent Exacerbators – Exacerbations where multiple courses of OCS were not prescribed. (Data in Appendix VI)

In the APEX study 14 participants contributed to 17 events were repeated events occurred in which a course of OCS was not prescribed. There was evidence of small airways disease in these events but less than that of exacerbations in which OCS. These events had no evidence of T2 inflammation with a mean FeNO of 25 ppb, which is less than the median of those events in which multiple OCS were prescribed 30 ppb. There was also evidence of no eosinophilia, with a mean eosinophil blood count of 0.25 $x10^9/L$ (SD=02.6). Lung function was preserved with a mean FEV1% predicted of 87% and demonstrated less reversibility than primary OCS (mean 12.7% vs 5.21%)

2A) Paired analysis of T2 status at baseline to an exacerbation requiring OCS.

	T2-High (Baseline) (Primary OCS)	p-value	T2-Low (Baseline) (Primary OCS)	p-value
N(%)	22(50)		22(50)	
FeNO (ppb)	61.81(33.48) 78.43(70.99)	0.25	19.52(8.41) 25.82(14.00)	0.05
Eosinophils ($x10^9$/L)	0.41(0.21) 0.55(0.32)	0.03	0.15(0.07) 0.25(0.12)	0.92
R5 cmH_20/L/s **	4.24(3.14-5.25) 5.71(4.21-6.31)	0.006	3.65(3.06-6.18) 5.81(3.62-7.36)	0.14
R20 cmH_20/L/s**	2.91(2.53-3.79) 3.63(2.77-4.17)	0.07	3.16(2.81-4.47) 3.91(2.96-4.73)	0.97
R5-R20 cmH_20/L/s**	0.96(0.35-1.85) 2.15(1.06-2.78)	0.008	0.53(0.30-1.77) 1.80(0.39-2.87)	0.01
AX cmH_20/L**	12.79(4.35-32.30) 40.41(14.-05-59.18)	0.0007	11.04(2.79-30.77) 32.25(4.90-64.24)	0.08
FEV1% predicted	73.11(20.56) 60.67(21.22)	<0.0001	83.94(19.85) 78.06(17.88)	0.004
FVC% predicted	87.88(17.18) 75.11(20.76)	<0.0001	92.16(14.43) 87.65(13.50)	0.02
ACQ5	1.26(0.96) 3.54(0.95)	<0.0001	1.41(0.79) 3.14(0.78)	<0.0001
ACQ6	1.19(0.88) 3.45(0.99)	<0.0001	1.36(0.75) 3.13(0.76)	<0.0001
ACQ7	1.45(0.80) 3.53(0.82)	<0.0001	1.47(0.72) 3.08(0.72)	<0.0001

Table 4. 7: Paired analysis of baseline to exacerbations requiring OCS according to T2 status at baseline.

*Mean (SD) **Median (IQR). Bonferroni correction for multiple testing applied.

There were 22/44(50%) participants that had a Primary OCS event in which there was at least one feature of T2 inflammation on study entry i.e at baseline. There were 22/44 (50%) participants with a Primary OCS event without evidence of T2 inflammation on study entry. There was no significant increase in FeNO from baseline T2-high to the point of primary OCS (mean FeNO 61.8 at baseline to 78.43 p=0.25); however, these participants at baseline were already T2-high. In T2-low at baseline, there was a significant difference at Primary OCS (19.52 to 25.82 ppb p=0.05),

but the absolute values are suggestive of a modest increase in T2-inflammation and below the threshold of what was considered “T2-high” in the APEX study.

For blood eosinophils, there was a significant change in T2-high at baseline but not for T2-low at baseline to the point of Primary OCS. Looking at the absolute values, the mean eosinophil count was 0.41 x109/L, and at exacerbation, it was 0.55 x109/L p=0.03. In T2-low eosinophil status at baseline, there was no significant increase in eosinophil count (0.15 x109/L to 0.25 x109/L p=0.92), and 0.25 is considered lower than what is classified as T2-high in this study.

Comparing T2-high and T2-low at baseline with respective Primary OCS, there was a significant increase in peripheral resistance measured for T2-high (median baseline 0.96 to 2.15 $cmH_20/L/s$ at exacerbation p=0.008) and T2-low (median baseline 0.53 to 1.80 p=0.01). The other spirometry values and all ACQ scores showed significant changes from baseline T2-high to Primary OCS and T2-low at baseline to Primary OCS.

Of note, in exacerbation events in which no course of OCS was prescribed, 12/37 at baseline had evidence of T2 inflammation, and 25/37 were associated with no evidence of T2 inflammation.

2B) Paired analysis of T2 status at exacerbation requiring OCS to baseline features

	T2-High (Baseline) (Exacerbation)	p-value	T2-Low (Baseline) (Exacerbation)	p-value
N(%)	25(56.8)		19(43.2)	
FeNO (ppb)*	54.50(36.21) 75.63(66.37)	0.05	25.45(18.13) 21.05(9.80)	0.31
Eosinophils* (x10^9/L)	0.38(0.23) 0.54(0.31)	0.003	0.17(0.10) 0.13(0.10)	0.20
R5 cmH$_2$0/L/s **	4.28(3.40-5.77) 5.52(4.06-6.86)	0.005	3.89(3.02-5.25) 5.53(3.29-7.19)	0.01
R20 cmH$_2$0/L/s**	3.54(2.68-3.99) 3.63(2.78-5.24)	0.36	3.44(2.82-4.03) 3.83(2.98-4.47)	0.29
R5-R20 cmH$_2$0/L/s**	0.87(0.34-2.05) 2.18(0.88-5.75)	0.001	0.53(0.29-1.13) 1.10(0.26-2.76)	0.01
AX cmH$_2$0/L**	12.43(5.27-33.04) 39.50(12.61-64.61)	0.0002	8.79(2.56-16.37) 26.35(2.50-50.98)	0.04
FEV1% *predicted	74.04(20.94) 64.40(22.47)	<0.0001	83.90(19.59) 75.91(18.29)	0.0053
FVC% *predicted	86.49(16.72) 76.68(20.84)	0.0004	94.26(13.93) 87.67(12.70)	0.0134
ACQ5*	1.23(0.92) 3.46(0.91)	<0.0001	1.45(0.81) 3.19(0.85)	<0.0001
ACQ6*	1.19(0.86) 3.38(0.93)	<0.0001	1.36(0.77) 3.18(0.84)	<0.0001
ACQ7*	1.45(0.81) 3.44(0.76)	<0.0001	1.48(0.69) 3.15(0.81)	<0.0001

Table 4. 8: Paired analysis of baseline to exacerbations requiring OCS according to T2 status at exacerbation.

*Mean(SD) **Median(IQR). Bonferroni correction for multiple testing applied.

25/44 exacerbations required OCS, and there was at least one feature of T2 inflammation at the point of Primary OCS. 19/44 exacerbations requiring OCS, had no evidence of T2 inflammation at the point Primary No OCS.

Table 4.8 illustrates that through paired analysis, stratified according to the presence of T2 inflammation at the point of exacerbation. At baseline, there was the presence of T2-high inflammation for FeNO and Blood eosinophil (54.50 ppb at baseline and 75.63 ppb at exacerbation, and 0.38 x10^9/L at baseline and 0.54 x10^9/L at exacerbation). This was statistically significant compared to the baseline values. This significance was not seen in the comparison to those who were T2-low at

exacerbation compared to their respective baseline values for FeNO and blood eosinophil count. There was a greater magnitude of difference in peripheral resistance from exacerbation to baseline in those with T2-high inflammation at exacerbation compared to those with T2-low at exacerbation (0.87 to 2.18 $cmH_20/L/s$ (p=0.001) vs 0.53 to 1.10 $cmH_20/L/s$(p=0.01). This was seen in Reactance (Ax), too, with a greater amount in T2-associated exacerbations (12.43 to 39.50 H_20/L p=0.0002) vs (8.79 to 26.35 H_20/L p=0.04). There was also a greater decline in lung function (FEV1% predicted) when compared to baseline in those with T2-high inflammation at exacerbation compared to T2-low inflammation (74.04 to 63.98 p=<0.0001 vs 83.90 to 75.84 p=<0.005).

In exacerbation events in which no course of OCS was prescribed, 14/37 events were associated with evidence of T2 inflammation, and 23/37 events were associated with no evidence of T2 inflammation.

3)Comparing physiological airway parameters according to the presence of T2 inflammation.

To explore the impact of T2 status upon exacerbations, a sub-analysis was performed to assess whether the presence of T2 inflammation (raised FeNO, raised blood eosinophil, or evidence of sputum eosinophilia) was associated with a greater degree of decline in lung function obtained from spirometry and Impulse Oscillometry.

	Primary OCS			**Primary no OCS**		
Parameter	T2 High	T2 Low	p value	T2 High	T2 Low	p value
N	25	19	-	12	25	-
FEV1%*	64.40(22.10)	75.91(18.79)	0.07	87.30(9.77)	86.53(13.42)	0.84
FVC%*	76.68(20.84)	87.67(12.70)	0.04	92.63(10.60)	93.65(12.37)	0.80
R5 $cmH_20/L/s$ **	5.52(4.06-6.86)	5.53(3.29-7.19)	0.78	4.12(2.70-5.07)	3.44(2.93-4.40)	0.76
R20 $cmH_20/L/s$**	3.63(2.78-5.24)	3.83(2.98-4.47)	0.69	3.18(2.38-3.50)	3.02(2.58-3.69)	0.69
R5-R20 $cmH_20/L/s$ **	2.18(0.88-5.75)	1.10(0.26-2.76)	0.35	0.88(0.29-1.54)	0.50(0.17-1.23)	0.36
AX cmH_20/L**	39.50(12.61-64.61)	26.35(2.50-50.98)	0.11	8.62(5.07-28.98)	6.99(2.95-14.70)	0.22

Table 4. 9: Lung function according to T2-status high or low at exacerbation in Primary OCS and Primary No OCS events.

*mean (SD) unpaired t-test **median(IQR) Mann-Whitney test.

There was no statistical difference in lung function measurements for the larger airways and smaller airways between T2-high associated exacerbations and T2-low associated exacerbations according to Primary OCS and Primary No OCS. However, the absolute values obtained for spirometry were worse in those with T2-high inflammation for primary OCS events. Total resistance (R5) (5.52 $cmH_20/L/s$ vs 5.53 $cmH_20/L/s$ p=0.78), central resistance (R20) (3.63 $cmH_20/L/s$ vs 3.83 $cmH_20/L/s$ p=0.69), and Peripheral Resistance (R5R20) (2.18 $cmH_20/L/s$ vs 1.10 $cmH_20/L/s$ p=0.35). Reactance (Ax) (29.50 cmH_20/L vs 26.35 cmH_20/L p=0.11) was not statistically different but again absolute values for all IOS measurements were greater in T2-high primary OCS events compared to Primary No OCS events. FVC was associated with a statistically significant difference between T2 high and T2 low. (76.68% vs 87.76% p=0.04) in primary OCS events, with FVC being lower in T2-high Primary OCS events.

In events where no OCS was prescribed, there was no statistical difference between T2-high associated exacerbations and T2-low associated exacerbations according to FEV1% predicted (87.3% vs 86.53% p=0.84), FVC% predicted (92.6% vs 93.7% p=0.80) total resistance (R5) (0.88 $cmH_20/L/s$ vs 0.50 $cmH_20/L/s$ p=0.76), central resistance (R20) (3.18 $cmH_20/L/s$ vs 3.02 $cmH_20/L/s$ p=0.69), peripheral resistance (0.88 $cmH_20/L/s$ vs 0.50 $cmH_20/L/s$ p=0.46) and reactance (Ax) (8.62 cmH_20/L vs 6.99 cmH_20/L p=0.22).

4)Assessment of inflammatory phenotype stability at exacerbation and for multiple exacerbations in the APEX study.

During the study, a participant may have experienced multiple exacerbation events. The stability of the inflammatory phenotypes in these events is essential. Firstly it is important to assess whether the inflammatory phenotype changes significantly from baseline to exacerbation. Secondly, it is of interest to identify if the inflammatory phenotype from the first exacerbation is similar to that observed in the second exacerbation.

All exacerbation events in which a course of OCS was prescribed were therefore included in this analysis ie (Primary OCS and Multiple OCS), and only those participants that at least had one annual visit in which the inflammatory phenotype was included to assess the longitudinal stability of the phenotype as a significant number of exacerbation events occurred after the first annual event for a participant. Only exacerbation events are considered in this chapter. The same criteria described above were used to distinguish T2 high inflammation from T2 low inflammation. A comparison of longitudinal stability with the addition of annual reviews will be discussed in Chapter 5.

Statistical Methodology

The McNemar test, a nonparametric test used to analyse binary data, was used for paired analyses. It is similar to Fisher's test, which is used

for unpaired data.[295]. For the McNemar test, it requires events to be mutually exclusive. The Chi-squared test is utilised for the test. If the Chi-square test has a p=<0.05, then the null hypothesis can be rejected and would suggest a change between two events, a change in T2-status. If the p-value exceeds this, we accept the null hypothesis, i.e. there is no significant change in T2-status, which indicates no difference between the two events. The formula used in this test is as follows. B and C represent the discordant pairs in a 2x2 contingency table.

$$X^2 = \frac{(b-c)^2}{(b+c)}$$

Results

Figure 4.10 illustrates a paired analysis of Baseline and exacerbation events. A total of 66 pairs were compared. There were 12 pairs in which, at baseline, their inflammatory status was T2-low, and at exacerbation, it became high. There were nine pairs in which at Baseline had a T2-high status and then at an exacerbation event was T2-low. The p-value obtained from performing McNemar was 0.66, suggesting no significant change in phenotype at exacerbation.

		Exacerbation-1	Exacerbation-1	McNemar (p-value)_
		High	**Low**	
Baseline	**High**	18	9	
Baseline	**Low**	12	27	
		Total	**66**	**0.66**

Table 4. 10: Assessing the stability of the inflammatory phenotype at baseline from an exacerbation event.

		Exacerbation-2	Exacerbation-2	McNemar (p-value)_
		High	**Low**	
Exacerbation-1	**High**	11	3	
Exacerbation-1	**Low**	3	12	
		Total	**29**	**1.00**

Table 4. 11: Assessing the stability of the inflammatory phenotype at a second exacerbation event from the first exacerbation event.

This was also the case for a participant who had a first exacerbation in the study and then went on to have a second exacerbation event. In the second exacerbation event, there were three pairs in which the first exacerbation was T2-high and then became T2-low. Conversely, there were three pairs in which the first exacerbation was T2-low and then became T2-high in the subsequent second exacerbation (Figure 4.11).

		Annual-1	Annual-1	McNemar (p-value)
		High	**Low**	
Baseline	**High**	21	11	
Baseline	**Low**	11	89	
		Total	**132**	**1.00**

Table 4. 12: Assessing the stability of the inflammatory phenotype from baseline to annual in participants with no exacerbation event.

To strengthen the validity of the findings, a paired analysis was performed on non-exacerbators by comparing the inflammatory phenotype observed at baseline to the phenotype at their first annual visit to the study. There were 132 pairs in this, with 89 pairs being t2-low at baseline and t2-low in their paired annual review. There were only 22 pairs in which the inflammatory phenotype changed- 11 going from a T2-low baseline to a high at their annual review and 11 pairs going from a T2-high at baseline to a T2-low in their annual review. There was no

significant change in phenotype from baseline to their first annual event, with a P value obtained of 1.00 (Figure 4.12)

Discussion

This chapter focused on changes that occurred at the point of exacerbation. First, a paired analysis was undertaken of changes that happened in an exacerbation event, Primary OCS or Primary no OCS, compared to baseline values. I hypothesised that T2-high-associated exacerbations were associated with worse clinical outcomes than T2-low-associated exacerbations. To answer this, analyses were created, incorporating a stratification of T2 status at baseline compared to exacerbation and, conversely, T2 status at exacerbation compared to baseline. Further analyses were conducted to assess if a T2-high status at primary OCS resulted in worse lung function than a T2-low associated primary OCS. Finally, the stability of exacerbation phenotypes was explored.

Exploring the natural History of asthma exacerbations.

The mean number of days participants persisted with their symptoms before seeking healthcare support was nine days. The presence of a dedicated number could have influenced this to help with their symptoms and hence be an underestimation of the number of days participants would usually persist with their symptoms; however, there are currently no studies to date that have explored this, and this could reflect normal behaviour. Despite evidence of the benefits of escalating ICS/LABA therapy to help abort exacerbation symptoms[18], only 40% of patients who were on ICS/LABA therapy undertook this, with 60% of exacerbation events adopting up-titration of their SABA inhaler.

The symptoms presented for a suspected asthma exacerbation event were similar to those identified in the literature: shortness of breath at rest and exertion, a worsening cough, and wheezing.

Most patients who were identified as requiring steroids had their symptoms resolved after seven days. This highlights the importance of

phenotyping patients who present with exacerbations to prescribe steroids based upon a combination of not just symptoms alone but objective measures of inflammation and airway physiology, too.

Exacerbation events in which NO OCS was provided and small airway disease.

Exacerbation events in which no OCS were given could represent several clinically relevant situations. They could be episodes of “moderate asthma exacerbations” where the symptoms resolved themselves with appropriate changes in their usual medication. However, what was evident in these events was that they, too, had inflammatory heterogeneity. As demonstrated in these episodes, there was a significant amount of T2 inflammation, 37.8% showing evidence of T2 inflammation. These episodes could also represent a period of uncontrolled asthma. What has been highlighted is the significance of IOS in distinguishing events. The markers of small airway disease R5, R20, R5R20 and Ax were not significantly different from the matched baseline in Primary No OCS, unlike exacerbation events in which OCS was prescribed. Primary OCS. Prospective studies with a larger sample size would be required to validate this finding and designed in such a way as to address this finding. What is of interest and needs further work is the utility of inhaled corticosteroids and their deposition in the smaller always during exacerbations. The potency of, for example, fluticasone is greater than prednisolone, yet oral corticosteroids are the cornerstone management for an asthma exacerbation.[296] Could it be that particle deposition is insufficient in the smaller airways or that oral steroids provide a systemic anti-inflammatory effect not seen with inhaled oral corticosteroids, which only have a local effect?

These events did not go on to have worsening symptoms after a follow-up call. What remains unclear and requires further research is what mechanisms lead to an episode of “uncontrolled”/mild exacerbation,

develop into an exacerbation event requiring steroids or even hospitalisation.

T2- status at exacerbation for Primary OCS influences the decline in lung function.

56.8% of Primary OCS events demonstrated T2-high inflammation, and 43.2% of exacerbations observed in the APEX study that required OCS were classified as having T2-low inflammation. Although sputum samples to identify phenotypes were limited by the number of samples obtained, in the samples collected, 52.6% demonstrated neutrophilia. The role of neutrophils has been illuded in Rhinovirus-provoked exacerbations and their formation of neutrophil extracellular traps.[297] This suggests the heterogeneity of inflammatory phenotypes in asthma exacerbations.

Chapter 6 in this thesis will explore the proportion of asthma exacerbation observed in APEX of viral aetiology. Of note is that patients in the APEX study perceived that 41% of exacerbations were brought on by a viral/ upper respiratory tract infection. Both T2-high and T2-low had a loss of lung function from paired baseline, although the magnitude of difference seemed to be greater in Primary OCS events that were T2-high than T2-low, proving my hypothesis correct. The T2-low primary OCS events had small airway disease with increased peripheral resistance (R520) but not reactance (Ax), whilst T2-high primary OCS events had a reduction in both R520 and Ax.

When analysis was performed on T2-inflammation changes from baseline to exacerbations according to T2-status at baseline, there was no change in FeNO and a modest increase in blood eosinophil change

for primary OCS. At baseline for FeNO, there was already significant T2-high inflammation at 61.8 ppb.

However, on the sub-analysis performed comparing baseline to exacerbation according to T2-status at the point of exacerbation, there was a significant increase in blood Eosinophils. Again, there was no significant change from baseline- but these participants already had a high mean FeNO-54.5 ppb.

What was consistent across all exacerbation events was the loss of asthma control, with high ACQ scores suggestive of poor asthma control during these events, regardless of whether a course of steroids was prescribed or not.

The modest degree of T2-high associated inflammation observed during these exacerbation events could be explained by the exacerbation events captured were not severe enough to require hospitalisation for nebulisation or oxygen therapy. Therefore, they may not be as “severe”, probably resulting in higher levels of FeNO and Blood Eosinophilia and greater loss of lung function.

Both T2-high and T2-low Primary OCS and Primary No OCS events are associated with similar declines in lung function.

Exacerbation events were observed during the APEX study, whether OCS was prescribed or not. An analysis was conducted according to the presence of T2 inflammation and the absence of T2 inflammation based on the combination of blood eosinophil and FeNO and evidence of sputum eosinophilia at exacerbation to assess if there was a difference in lung function loss according to T2-status. Both T2-high and Low exacerbation events for Primary OCS and Primary No OCS were associated with similar declines in lung function with no significant difference in most lung function parameters except FVC% predicted was worse in T2-high Primary OCS compared to T2-low Primary OCS. However, T2-high primary OCS did have worse absolute values for FEV1% predicted and R5, R20 and R520. An insufficient number of Primary OCS exacerbation events observed could explain the lack of

statistical power to differentiate. This is similar to findings observed from McDowell and Colleagues.[273]

Stability of inflammatory phenotypes

A review of the stability of the inflammatory phenotype through paired analyses was conducted: first, from baseline to exacerbation, and second, a comparison between a first exacerbation and a subsequent second exacerbation. Most participants who were T2-high at baseline went on to have a T2-high exacerbation, and conversely, a T2-low status at baseline was associated with T2-low at exacerbation. This suggests there is inflammatory stability at exacerbation from baseline.

From reviewing patients in a clinical setting and throughout the APEX study- a participant may have an exacerbation that requires a course of steroids and then, following this, an episode where there is a deterioration of symptoms sufficient for them to seek assistance from their primary care physician, but it may be deemed that a course of steroids is not required. The possibility that the episode that occurred later may influence the inflammatory profile of future exacerbations is a consideration, but for this analysis, only exacerbation events in which a course of OCS was prescribed were considered as this is the current definition on which asthma exacerbations are based. 68.2% of observed exacerbations had the same T2 status from baseline to exacerbation and 79.3% in the second exacerbation event compared to the first. McNemar p values were insignificant in both cases, suggesting phenotype stability from baseline to exacerbation. There were a limited number of pairs for second exacerbation events, so these findings must be interpreted cautiously. I was the only physician assessing the outcome of exacerbation, and thus, this could add confirmation bias to the findings; however, current clinical practice is the interpretation of usually a sole physician to assess symptomology and exam findings to assess for an exacerbation.

Conclusions

There is evidence of heterogeneity in asthma exacerbations. A significant proportion of exacerbations observed had no evidence of T2 inflammation. Small airways disease was present at exacerbation but there was a substantial change in R5, R20 and AX for exacerbations that require OCS from paired baseline versus exacerbations in which no OCS was prescribed. This is suggestive that IOS could be helpful in discrimination exacerbations that require OCS versus episodes of uncontrolled/mild asthma exacerbations. T2-high associated Primary OCS events had a more significant decline in lung function and inflammation compared to their paired baseline values than T2-low Primary No OCS events compared to baseline values.

There was no significant difference in inflammatory phenotype change from baseline to exacerbation and among participants who had multiple exacerbations; however, these findings must be interpreted with caution due to the small number of second exacerbations. This stability could allow a more targeted approach to therapeutic options for these patients. The strength lies in having baseline values for comparison, reinforcing the importance of phenotyping patients who experience asthma exacerbations during periods of “normal” asthma symptoms.

Chapter 5: The longitudinal stability of asthma exacerbation associated inflammatory profiles

Introduction

The longitudinal stability of asthma exacerbations has been largely unexplored, with no consensus on the stability of inflammatory phenotypes. It is known that asthma exacerbations are associated with a reduction in lung function.[21]. A large population-based study identified that asthma exacerbations were associated with a decrease in peak flow by 1.34 L/min per year.[21]Newby and colleagues determined that those with eosinophilia were associated with twice the loss of lung function over six years compared with those who did not demonstrate eosinophilia. However, the two groups had no difference in the frequency of asthma exacerbations. [298]

In a small sample population of 21 participants, Dente and colleagues identified after a follow-up period of 3 years that eosinophilia persisted in 87%. This sample was characterised as having difficult-to-treat uncontrolled asthma.[299] . Conversely, McGrath and colleagues identified that a non-eosinophilic group identified at baseline was persistent in patients treated with ICS after 29 weeks of treatment, but whether that

represented the effect of treatment is unclear, but they responded poorly to inhaled steroid therapy based on asthma control assessment. [265]

Al-Samri and colleagues explored the inflammatory stability of asthma by performing monthly sputum counts in patients with severe asthma and three monthly counts in patients with moderate asthma over one year. [300] They identified a stable phenotype in only a third of the 61 study participants. After one year in the moderate group, a sputum eosinophil count of less than 3% was present in 39%, greater than 3% in 32%, and a neutrophil count greater than 65% in 1%. In the severe asthma group, persistent eosinophilia of >3% was present in 32% and eosinophilia < 3% in 35% after one year of follow-up. The factors contributing to this were unclear.

The SARP III program performed annual sputum analysis for differential sputum counts in its cohort of 206 patients with severe asthma, involving seven research sites. A baseline sputum sample and at least two further sputum samples were required.[301] They organised the cohort into three groups based on the overall inflammatory type identified across all sputum samples obtained: a T2-high with evidence of sputum eosinophils ≥2%, a second group with sputum eosinophil of ≤2% and a third group in which the eosinophil count was variable (>2SDs from baseline). They found stability in the inflammatory profile of those in the eosinophil high and low group.

However, the studies above do not explore the impact of asthma exacerbations on the longitudinal stability of asthma phenotypes, whether the inflammatory profile changes from baseline at exacerbation and the influence this has on the overall inflammatory phenotype after an exacerbation. McDowell and colleagues explored this concept at the point of an exacerbation. [273] They were using data from a 48-week randomised controlled trial exploring the adjustment of steroids in a T2-low cohort of 301 patients. They found that those participants who exacerbated the inflammatory profile at baseline were not associated with the phenotype at exacerbation or in their second exacerbation.

The APEX study was designed to address this question directly in a cohort of well-phenotyped patients and longitudinally assess identified phenotypes at baseline.

Aim:

The aim is to assess the longitudinal impact of asthma exacerbations, focusing on the inflammatory stability of identified phenotypes. I hypothesise inflammatory phenotypes are stable annually after accounting for an exacerbation event, based on my findings from Chapter 4. The longitudinal stability will be explored by addressing the following aims.

1) Explore the inflammatory profile of the APEX study by identifying the T2 status of the cohort annually.

2) Longitudinally assess the trajectories of markers of airway inflammation and lung function in participants with an exacerbation event, with primary OCS and primary No OCS, to evaluate if there is a difference.

3) Whether a T2 high inflammatory phenotype at baseline versus a T2 low inflammatory phenotype at baseline have different trajectories in clinical physiological and inflammatory markers of airway disease

4) Assessing the stability of inflammatory phenotypes according to T2 status, having identified the T2 status at baseline, exacerbation and for an annual event.

Method

Participants were invited to return to the research site approximately a year after entering the study. At the visit, any changes in circumstances that may affect the participant's ability to continue in the study were addressed. The same investigations performed at baseline were carried out during the visits to ensure continuity of the data obtained for longitudinal assessment.

As recruitment was prospective, annual visits occurred at various times during the study's duration. As such, some participants may not have had an annual visit by the end of the study if they had been recruited late into it. I must mention that the pandemic and government restrictions during the study delayed some annual visits until participants felt it was appropriate to return to the research site and thus had an impact on the number of annual events captured.

The same criteria to distinguish T2-high inflammation from T2-low inflammation was used for this analysis as before.

Results

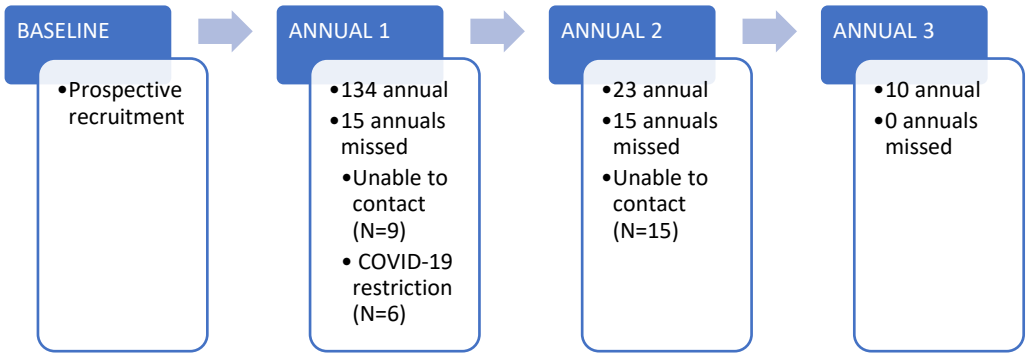
	APEX-1	**APEX-2**
Annual year 1 (N)	134	55
Annual year 2 (N)	23	8
Annual year 3 (N)	10	6

Table 5. 1: The number of participants that had annual visits in the APEX study.

Table 5.1 illustrates the number of consecutive annual visits per year that occurred in the APEX study. Figure 5.1 details the consecutive yearly

visits, including details on missed ones. One hundred thirty-four participants completed an annual visit in year 1 in APEX-1 and 55 in APEX-2. Fifteen participants could not have their first annual visit, nine were uncontactable, and six could not come in due to COVID-19 restrictions. 23 participants in APEX-1 and 8 participants in APEX-2 had a second yearly visit. Fifteen were unable to have a second annual visit as they were uncontactable. In total, 16 participants had a third consecutive annual visit: 10 in APEX-1 and 6 in APEX-2.

APEX-1 follow up



APEX-2 follow up

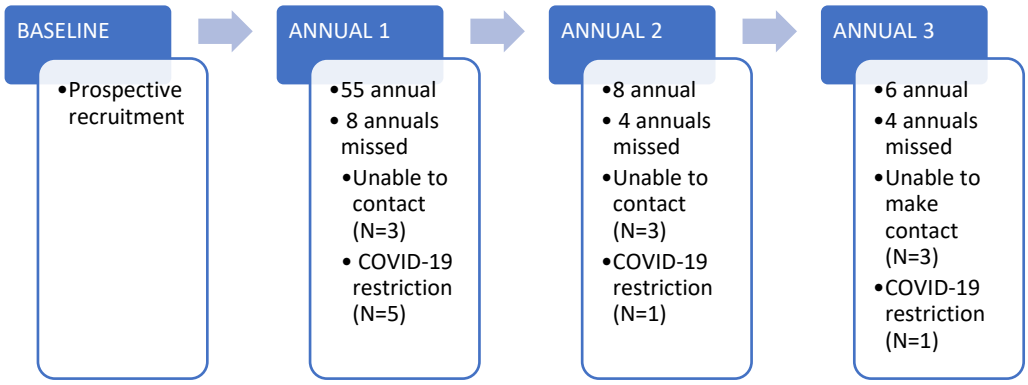


Figure 5. 1: Participants' flow charts for annual visits in the APEX study accounting for COVID-19. .

Longitudinal data assessing inflammatory phenotypes in the APEX study.

Annual 1

Annual review (Year 1)	Value No.(%)
Associated Raised FeNO [+]	51/189(27.0)
No raised FeNO [+]	137/189(73.0)
Associated Raised Eosinophil [*]	29/189(15.3)
No Raised Eosinophils [*]	157/189(84.7)
Associated with either raised FeNO OR Eosinophil OR Sputum Eosinophilia	55/189(29.1)
Associated with both raised FeNO AND Eosinophil	17/189(9.0)
No raised FeNO and No raised eosinophil	134/189(70.9)

[+]1 participant unable to perform the procedure to obtain FeNO
[*] 3 participants were unable to provide a venous blood sample.

Annual 2

Annual review (Year 2)	Value No.(%)
Associated Raised FeNO	5/31(16.1)
No raised FeNO	26/31(83.9)
Associated Raised Eosinophil*	5/31(16.1)
No Raised Eosinophils	24/31(77.4)
Associated with either raised FeNO OR Eosinophil OR Sputum Eosinophilia	8/31(25.8)
Associated with both raised FeNO AND Eosinophil	2/31(6.5)
No raised FeNO and No raised eosinophil	23/31(74.2)

*2 participants were unable to provide a venous blood sample

Annual 3

Annual review (Year 3)	Value No.(%)

Associated Raised FeNO	4/16(25.0)
No raised FeNO	12/16(75.0)
Associated Raised Eosinophil	4/16(25.0)
No Raised Eosinophils	12/16(75.0)
Associated with either raised FeNO OR Eosinophil OR Sputum Eosinophilia	5/16(31.2)
Associated with both raised FeNO AND Eosinophil	3/16(18.8)
No raised FeNO and No raised eosinophil	11/16(68.8)

Table 5. 2: Longitudinal inflammatory exacerbation profiles in the APEX studies APEX-1 and APEX-2 cohorts were combined.

Table 5.2 illustrates the proportions of T2 inflammation observed in consecutive annual reviews in both APEX-1 and APEX-2 combined. The predominant inflammatory profile observed in each successive year was a T2-low, with no evidence of raised FeNO or eosinophilia in the blood. In the annual reviews, current inhaled therapies for asthma were recorded. No de-escalation of ICS was recorded in APEX-1 or APEX-2 cohort on review of any medication changes that had occurred over the year since study entry.

On average, 71.3% of the APEX cohort had no evidence of T2 inflammation in their annual reviews over three years. As such, there were similar percentages of patients with T2 inflammation with either evidence of a raised eosinophil or FeNO. In the first annual review, this was 30%, followed by 26% in year 2 and 31% in year 3. Only a small percentage of the APEX cohort showed consistent levels of T2-high inflammation with both a raised FeNO and blood eosinophil. This was 9.0% in year 1, 6.5% in year 2.

Longitudinal trajectories of markers of airway inflammation and lung function about participants with an exacerbation event.

FeNO and Blood Eosinophils

To analyse T2 inflammation, FeNO and blood eosinophils were longitudinally reviewed according to exacerbation outcome. For FeNO and blood eosinophils, multiple testing of paired samples were performed, and distribution was transformed to normality; a one-way ANOVA was performed with Geisser-Greenhouse correction as equal variability of difference was not assumed a follow-up test of the mean rank of each event being compared with the mean rank of baseline. Tukey's multiple comparison test was performed with individual variances computed for each comparison. A mixed-effect model was applied for pairs with missing parameters. The output reported was adjusted p values for each comparison. Multiple t-tests with Bonferroni correction are illustrated to reduce Type 1 error. NS is indicative of no significance.

	Tukey's multiple comparisons test	(N)	Mean(SD)	Summary	Adjusted p-value
Primary OCS	Baseline vs. Exacerbation	37 37	0.29(0.22) 0.39(0.33)	*	0.01
	Baseline vs. Annual	37 32	0.29(0.22) 0.25(0.20)	ns	0.27
Primary No OCS	Baseline vs. Exacerbation	28 24	0.22(0.16) 0.23(0.20)	ns	0.73
	Baseline vs. Annual	28 25	0.22(0.16) 0.10(0.15)	ns	0.97

Table 5. 3: ANOVA for Blood Eosinophils ($x10^9$/L) for exacerbation events. Analyses used the first annual event only. ns=not significant

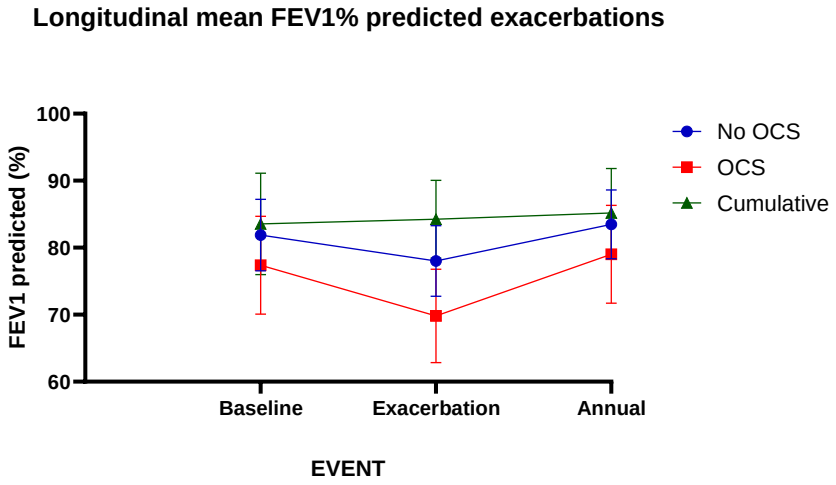
	Tukey's multiple comparisons test	(N)	Mean(SD)	Summary	Adjusted p-value
Primary OCS	Baseline vs. Exacerbation	37 37	42.8(36.1) 56.1(59.1)	*	0.04
	Baseline vs. Annual	37 37	42.8(36.1) 40.85(32.3)	ns	0.89
Primary No OCS	Baseline vs. Exacerbation	28 28	25.1(16.6) 26.8(20.7)	ns	0.83
	Baseline vs. Annual	28 26	25.1(16.6) 24.7(18.5)	ns	0.90

Table 5. 4: ANOVA for FeNO (ppb) for exacerbation events. Analyses used first annual event only. ns=not significant

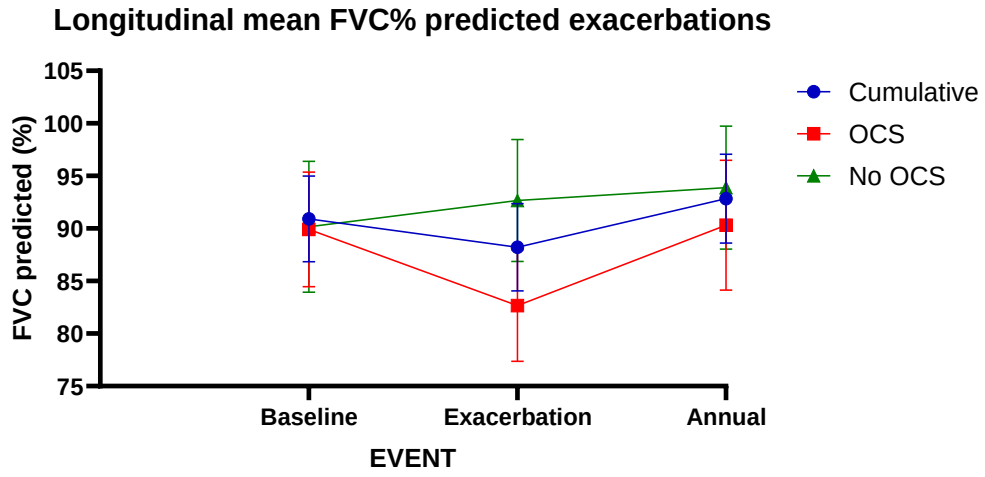
After a one-year follow-up, there was no significant change in FeNO or Blood eosinophils after a Primary OCS or Primary no OCS event from baseline to the first annual post-exacerbation event after adjustment for multiple testing in those exacerbation events (Table 5.3 and 5.4.)

Significant changes as expected were found in those exacerbation events required steroids in blood eosinophil count and FeNO. However, values returned to a similar level observed at study entry into APEX.

FEV1% and FVC% predicted.



	Tukey's multiple comparisons test	(N)	Mean (%)	Summary	Adjusted p-value
OCS	Baseline vs. Exacerbation	38 37	77.36 69.82	****	<0.0001
	Baseline vs. Annual	38 32	77.37 79.02	ns	0.52
No OCS	Baseline vs. Exacerbation	28 28	83.55 84.24	ns	0.89
	Baseline vs. Annual	26 26	83.55 85.17	ns	0.54



	Tukey's multiple comparisons test	(N)	Mean (%)	Summary	Adjusted p-value
OCS	Baseline vs. Exacerbation	38 37	89.91 82.66	****	<0.0001
	Baseline vs. Annual	38 32	89.91 90.32	ns	0.94
No OCS	Baseline vs. Exacerbation	28 28	90.91 88.20	*	0.03
	Baseline vs. Annual	26 26	90.91 92.84	ns	0.16

Figure 5. 2: Longitudinal assessment of lung function FEV1% and FVC% predicted in APEX study post exacerbation presented.

ANOVA results displayed between baseline and 1st year annual according to exacerbation outcome

Small airways Disease

Exacerbation where OCS is given.

	Tukey's multiple comparison test	(N)	Mean	Summary	Adjusted p-value
R5 $cmH_20/L/s$	Baseline vs. Exacerbation	28 30	4.69 5.70	**	0.0016
	Baseline vs. Annual	28 33	4.69 4.18	ns	0.13
R20 $cmH_20/L/s$	Baseline vs. Exacerbation	28 30	3.57 3.77	ns	0.2
	Baseline vs. Annual	28 33	3.57 3.33	ns	0.11
R520 $cmH_20/L/s$	Baseline vs. Exacerbation	28 30	1.12 1.94	***	0.0006
	Baseline vs. Annual	28 33	1.12 0.84	ns	0.33
AX cmH_20/L	Baseline vs. Exacerbation	28 30	21.38 45.86	***	0.0001
	Baseline vs. Annual	28 33	21.38 16.40	ns	0.56

Exacerbation where No OCS given.

	Tukey's multiple comparison test	(N)	Mean	Summary	Adjusted p-value
R5 $cmH_20/L/s$	Baseline vs. Exacerbation	22 21	3.74 3.64	ns	0.85
	Baseline vs. Annual	22 20	3.74 3.75	ns	1.00
R20 $cmH_20/L/s$	Baseline vs. Exacerbation	22 21	3.13 3.00	ns	0.58
	Baseline vs. Annual	22 20	3.13 2.93	ns	0.34
R520 $cmH_20/L/s$	Baseline vs. Exacerbation	22 21	0.62 0.64	ns	0.97
	Baseline vs. Annual	22 20	0.62 0.81	ns	0.16
AX cmH_20/L	Baseline vs. Exacerbation	22 21	9.71 14.05	ns	0.28
	Baseline vs. Annual	22 20	9.71 12.34	ns	0.61

Table 5. 5: Longitudinal assessment of IOS parameters to measure small airway disease in the APEX study. ANOVA was performed with adjusted p-values presented. First annual event used for analyses. ns=not significant.

There was no significant evidence of a longitudinal change in spirometry values and parameters of small airway disease, regardless of whether a course of OCS was prescribed or not for an exacerbation event. As seen in Chapter 4 from baseline to exacerbation there were significant changes in FEV1% predicted from baseline to exacerbation. Only in exacerbations where OCS was prescribed was a decline in peripheral resistance and reactance noted. (Table 5.5).

Longitudinal assessment according to T2 status in the APEX study in exacerbations requiring OCS.

In this section, the longitudinal profile of asthma exacerbations that required a course of OCS will be analysed according to whether, at baseline, a participant had evidence of T2-high inflammation or T2-low inflammation.

Parameter	Comparison	(N)	Mean	T2-High (adj. p-value)	(N)	Mean	T2-Low (adj. p-value)
R5 $cmH_20/L/s$	Baseline vs Exacerbation	16 17	4.74 5.75	0.01	14	4.98 6.04	0.05
	Baseline vs Annual	16 16	4.74 4.63	0.94	14	4.98 4.02	0.09
R20 $cmH_20/L/s$	Baseline vs Exacerbation	16 17	3.33 3.70	0.01	14	3.88 4.02	0.74
	Baseline vs Annual	16 17	3.33 3.44	0.62	14	3.88 3.32	0.03
R520 $cmH_20/L/s$	Baseline vs Exacerbation	16 17	1.23 2.12	0.003	14	1.15 2.07	0.01
	Baseline vs Annual	16 17	1.23 1.15	0.94	14	1.15 0.68	0.22
AX cmH_20/L	Baseline vs Exacerbation	16 17	23.0 46.7	0.001	14	23.8 48.9	0.02
	Baseline vs Annual	16 17	23.0 22.7	1.00	14	23.8 12.4	0.36
FEV1%	Baseline vs Exacerbation	19 19	71.2 58.9	0.0005	19 19	83.6 77.3	0.01
	Baseline vs Annual	19 18	71.2 68.7	0.51	19 17	83.6 86.1	0.44
FVC%	Baseline vs Exacerbation	19 19	87.7 75.1	0.0006	19 19	92.1 87.4	0.06
	Baseline vs Annual	19 18	87.7 84.2	0.21	19 17	92.1 94.3	0.47
ACQ-5	Baseline vs Exacerbation	19 19	1.27 3.56	<0.0001	19 19	1.45 3.15	<0.0001
	Baseline Annual	19 18	1.27 1.60	0.49	19 17	1.45 1.25	0.645
ACQ-6	Baseline Exacerbation	19 19	1.20 3.46	<0.0001	19 19	1.40 3.15	<0.0001
	Baseline Annual	19 18	1.20 1.48	0.568	19 17	1.40 1.19	0.5221
ACQ-7	Baseline Exacerbation	19 19	1.49 3.59	<0.0001	19 19	1.51 3.11	<0.0001
	Baseline Annual	19 18	1.49 1.77	0.49	19 17	1.51 1.32	<0.55
FeNO (ppb)	Baseline Exacerbation	18 18	58.3 76.7	0.02	18 19	22.4 27.8	0.32
	Baseline Annual	18 18	58.3 56.1	0.98	18 19	22.4 25.8	0.41
Eosinophil ($x10^9/L$)	Baseline Exacerbation	19 19	0.41 0.51	0.13	19 19	0.16 0.19	0.46
	Baseline Annual	19 16	0.41 0.34	0.34	19 16	0.16 0.14	0.47

Table 5. 6: Longitudinal assessment of markers of lung function and airway inflammation and asthma control in the APEX study according to the presence or absence of T2 inflammation at study entry in APEX.

Table 5.6 demonstrates ANOVA of key parameters collected at each study visit in APEX according to T2 inflammation. There was no significant difference across all parameters longitudinally in those who were T2-high or T2-low at the study baseline, who then went on to have an exacerbation event and then had an annual review.

Stability of phenotypes according to T2 status in the APEX study.

Identifying phenotype stability and the influence an exacerbation has upon this is important as this could potentially alter how we approach the management of patients with asthma. This has not been explored to this extent before. The McNemar test was performed on exacerbation OCS events, i.e., primary and multiple OCS, which had at least one annual review in the study to assess longitudinal stability. Each comparison (Figure 5.3 & Table 5.7-5.11) shows the most common inflammatory pair is T2-low inflammation, followed by T2-high inflammation. A mixed inflammatory pair where there was a T2-status in one event that differed from the other event i.e. T2-high at baseline and then T2-low on the annual review and vice versa. Mixed pairs were observed in all comparisons generated. However, the number of mixed pairs is statistically insignificant, as the non-significant p-values suggested. This indicates that the inflammatory profile from one event to another was stable, i.e., most T2-high at one event, i.e., the baseline will be a T2-high at exacerbation, and a T2-high at exacerbation would remain after a 1-year annual follow-up. This was evident over a two-year follow-up period.

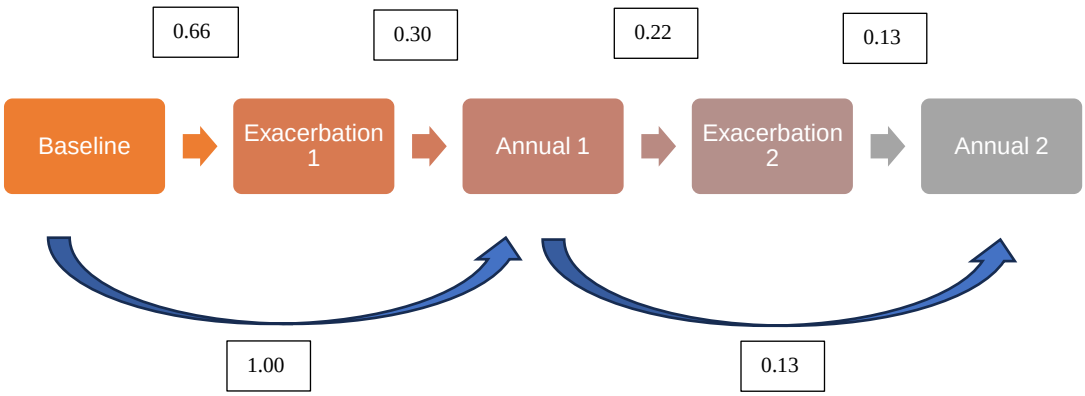


Figure 5. 3: Phenotypic stability was present in the APEX study over a two-year follow-up period.

McNemar p-values are presented, and the comparisons made to generate these p-values are denoted with a blue arrow.

		Annual-1	Annual-1	McNemar
		High	**Low**	
Baseline	**High**	17	7	
Baseline	**Low**	6	34	
		Total	**64**	**1.00**

Table 5. 7: Stability of phenotype according to T2-status from study entry to the first annual event in OCS events.

		Annual-1	Annual-1	McNemar
		High	**Low**	
Exacerbation-1	**High**	18	10	
Exacerbation-1	**Low**	5	28	
		Total	**61**	**0.30**

Table 5. 8: Stability of phenotype according to T2-status from first exacerbation event to first annual event

		Exacerbation-2	Exacerbation-2	McNemar
		High	**Low**	
Annual-1	**High**	8	1	
Annual-1	**Low**	5	15	
		Total	**29**	**0.22**

Table 5. 9: Stability of phenotype according to T2-status from the first annual review event in participants who went on to have a second exacerbation event.

		Annual-2	Annual-2	McNemar
		High	**Low**	
Exacerbation-2	**High**	2	0	
Exacerbation-2	**Low**	4	5	
		Total	**11**	**0.13**

Table 5. 10: Stability of phenotype according to T2-status from second exacerbation event in participants who went on to have a second annual review.

		Annual-2	Annual-2	McNemar
		High	**Low**	
Annual-1	**High**	1	0	
Annual-1	**Low**	4	11	
		Total	**16**	**0.13**

Table 5. 11: Stability of phenotype according to T2-status from the first annual review in participants who went on to have a second annual review.

Discussion

In this chapter, I set to identify the longitudinal changes that occurred in the APEX cohort, both of relevant clinical measurements and T2-status. I then went on to assess the longitudinal stability of identified phenotypes according to T2-status with the hypothesis of there being stability.

Longitudinal assessment of airway inflammation and Lung function according to T2-status

In the APEX study, I recorded clinically relevant measures of lung function and airway inflammation longitudinally. After one year of follow-up, there was no longitudinal difference in lung function parameters or markers of inflammation, FeNO, and blood eosinophil in those with a primary OCS and no primary OCS exacerbation event.

Also, as described in Chapter 3, patients were phenotyped according to having features of T2-high inflammation and T2-low inflammation. From baseline to the first annual review, there was no significant difference in all clinical parameters measured for primary OCS events. The lack of a substantial decrease or increase in T2 inflammation from an exacerbation event from baseline to an annual event post-exacerbation can explain this. A year after exacerbation may not be long enough to detect a meaningful change in the FEV1% predicted.[302]

Longitudinal inflammatory phenotypes

The APEX study was designed to assess the longitudinal stability of airway inflammation in asthma, revealing inconsistent findings as described in the literature review. Whether an asthma exacerbation influences the T2-inflammatory phenotype on follow-up has not been explored. The results of this study suggest that exacerbations do not affect the T2 status of a participant on follow-up, i.e. T2-high inflammation

at baseline is likely to lead to a T2-high inflammation after longitudinal follow-up post asthma exacerbation. After one year of follow-up, 20% did have a change in phenotype, with 6 undergoing an inflammatory change of T2-low to T2-high and seven changing from T2-high to T2-low. However, this was not statistically significant.

These findings must be interpreted with caution. The design of this study was observational, and therefore, no treatment modifications occurred after an exacerbation. As previously mentioned in the annual review, no changes were made by their usual inhaled medication for their asthma by their regular health care practitioner, and this may be due to the fact they were in the APEX study. As part of an asthma review post-exacerbation, you would expect a change in medication to prevent future exacerbations. As such, a change in medication after an exacerbation may influence the observed findings, with an escalation of inhaled ICS therapy that could affect the T2-axis. This stability has implications for potentially offering therapeutic strategies for those who remain, for example, T2-low; however, a patient must be phenotyped to elicit this information.

Of note, three participants in the APEX study commenced biological therapies but did not experience an asthma exacerbation after commencing treatment.

Those with an annual review were phenotyped to the same detail as at baseline, allowing for detailed characterisation of those who underwent an exacerbation event on follow-up. Limitations include the reduced number of follow-up events in years 2 and 3. This is partly due to the study design of prospective recruitment as the study ran and COVID-19, which resulted in delays in annual reviews. A limited number of exacerbation events also limit the generalisability of findings.

Conclusion

Key clinical parameters did not significantly change after an exacerbation event. There seems to be inflammatory stability from baseline and one year after an asthma exacerbation, but this stability must be interpreted cautiously.

Chapter 6: Viral detection and human genomic expression at exacerbation in nasal epithelial samples from APEX.

Introduction

As described in Chapter 1, the heterogeneity of asthma is influenced by gene-environment interactions, including within the airway epithelium. The link between early childhood infection and the subsequent development of asthma has been well established. [303]

This thesis has previously discussed the immunological basis of asthma exacerbations and viruses in the context of asthma exacerbations. However, the important mechanisms associated with asthma exacerbations remain poorly understood. This lack of understanding is partly due to the complex nature of the exposure that triggers an exacerbation, highlighting our research's significance.[304]

This work will identify the viral aetiology of asthma exacerbations and then perform RNA profiling of nasal epithelial cells through sequencing, with the aim of providing novel insights into the pathways associated with asthma exacerbations. Figure 6.3 provides an overview of the work undertaken in this thesis chapter.

Literature review of nasal transcriptomic work related to asthma exacerbations

A comprehensive literature search was conducted, focusing specifically on nasal transcriptomics and asthma. This search was then updated in January 2024, ensuring the inclusion of the most recent and relevant studies. The findings of this search are summarised in Table 6.1.

Initial studies that looked at gene expression in asthmatic patients compared asthmatic patients with non-asthmatic patients or allergic controls, making it difficult to make meaningful interpretations of the gene expressions during an exacerbation as it's not from asthmatic patients at their “baseline”. McErlean and colleagues explored transcriptional profiling of patients with asthma, allergies and controls. They performed three visits, the first during the peak onset of worsening symptoms on average two days into symptoms, a nasal brush during symptom resolution six days and finally one after which was defined as “baseline”, which was on average 89 days with a wide range from 29 to 297 days however the gene expression before the exacerbation event was unclear. They identified 22 genes uniquely associated with asthma exacerbation, including TMEM178, associated with lower airway obstruction, and TMEM16A, associated with mucus production and smooth muscle cell contraction in the airways.

However, some genes they identified were not associated with a specific mechanistic pathway or cell lineage, suggesting that unexplored mechanistic pathways may be at play.

The study revealed a significant finding: individuals experiencing asthma exacerbations express a reduced inflammatory response to viruses. This is evidenced by a reduction in genes associated with a viral immune response, such as CXCL11, CXCL9, and CCL2. Such a finding furthered our understanding of the immune response in asthma exacerbations.

During the recovery period when samples were taken, they noticed no statistically significant suppression in inflammation gene expression

previously associated with asthma exacerbation even after treatment with steroid therapy, but they identified genes related to modulation of immune response and mucosal repair being activated.

Wesolowksa-Andersen and colleagues in 2017 explored the effect of viruses on the airway epithelium in children using PolyA-selected whole transcriptome sequencing in 92 patients with asthma and compared that to 69 healthy controls as part of the Genes-Environment and Admixture in Latino Americans (GALA II) cohort. They identified that 21 children (9 asthmatic and 12 controls who were not experiencing asthma exacerbations) carried viruses in their nasal passages. They did not find a difference in gene expression in those with viruses when comparing patients with asthma and healthy controls. However, they identified that in those patients with virus and asthma exacerbation – there was a suppression of T2 inflammation. However, the cross-sectional nature makes it difficult to interpret as no samples were taken at a “baseline” event.

Altman and colleagues in 2019, conducted a study to characterise the nasal transcriptome in a cohort of 207 children aged 6-17. They used RNA sequencing of nasal epithelium samples obtained via nasal lavage and blood. The raw reads were normalised and corrected for batch effect. They then performed differential gene expression and weighted gene correlated network analysis to identify gene co-expression modules. They used STRING and Cytoscape to assess protein interaction between identified networks. The networks identified include SMAD3-related cell differentiation, eosinophil activation, extracellular matrix (ECM) production and epidermal growth factor receptor (EGFR) signalling. This rigorous methodology ensures the reliability and validity of the study's findings.

SMAD3 signalling is integral to developing asthma exacerbations. SMAD3 transduces signals from the activation of TGF-receptors from the release of TGF-β;[305] which is involved in tissue homeostasis and, as

mentioned in the introductory chapter at the start of the thesis, has been shown to increase during viral replication. They identified the upregulation of T2-high modules, including those responsible for the upregulation of IL-33 and dysregulation of type 1 interferons. They also determined that only a subset of genes was suppressed with steroids associated with mucus hypersecretion and type 2 inflammation. Signals not suppressed included those responsible for EGFR and ECM production. This infers possible heterogeneity of asthma exacerbations on a transcriptomic level. Similar findings were identified in the Mechanisms Underlying Asthma Exacerbations Prevented and Persistent with Immune Based Therapy (MUPPITS-1&2) trials to assess the effects of mepolizumab on asthma exacerbations. They identified that asthma exacerbations whilst on Mepolizumab, which is known to target T2-associated inflammation, caused downregulation of mast cell, eosinophil and IL-33 response genes but had no effect on TGF-β and SMAD3 signalling in 284 asthma exacerbations in which nasal lavage measurements were taken from children.

Recently, Phelan and colleagues compared the transcriptomic expression of frequent exacerbators in asthma, defined as requiring at least two hospital admissions for an asthma exacerbation, to those without frequent exacerbations to identify a molecular mechanism that drives multiple exacerbations in children.[306] They identified expression modules that involved neuronal pathways and were associated with repeated exacerbations, which led to neuronal remodelling.

Similarly, the U-BIOPRED researchers compared frequent exacerbators, defined as those who had more than or equal to 2 exacerbations in a year, to infrequent exacerbators, defined as those who had less than 2 exacerbations in a year, and a third group, described as “persistent frequent exacerbators,” who had exacerbated at least twice in the following year.[307] They identified genes differentially expressed between frequent and infrequent exacerbators. CEACAM5, was determined to be highly expressed in the bronchial epithelium of patients with persistent

frequent exacerbations. The power to detect a significant difference was limited due to loss to follow-up at exacerbation.

These studies highlight that multiple pathways contribute to the development of asthma exacerbations. Still, it is difficult to ascertain which of these pathways are causally related and how they lead to the development of asthma exacerbations.

To date, there have been no studies looking at nasal transcriptomic at the point of asthma exacerbations in an adult cohort, and there are very few studies that have longitudinally explored transcriptomic expression over multiple years.

Title	Author	Synopsis	Method	Outcome
Dissecting childhood asthma with nasal transcriptomics distinguishes sub-phenotypes of the disease 2014[308]	Poole et al.	Whole-transcriptome RNA sequencing was performed on nasal airway brushings from 10 asthma and ten controls. This was compared to bronchial and small airway transcriptome	RNA sequencing	90.2% overlap in expressed genes in nasal and bronchial transcriptomes. Cluster identified Th2-high and Th-2 low differentiated by 70 genes.
Asthmatics with exacerbation during acute respiratory illness exhibit unique transcriptional signatures within the nasal mucosa[309] 2015	McErlean et al.	Patients with asthma (N=44), allergies(N=7), and control (N=9)l were assessed at three stages: peak-onset, recovery, and a baseline visit afterwards.	microarray	Asthmatics with exacerbation exhibited a unique gene expression pattern associated with reduced immune response.
Transcriptome networks identify mechanisms of viral and non-viral asthma exacerbations in children[310] 2019	Altman et al.	Comparison of gene expression in viral-induced exacerbation (n=47), non-viral-induced exacerbation (n=107) and no exacerbation-associated events (n=107).	RNA sequencing	A set of "core exacerbation modules identified" independent of the presence of a virus. Associated with epithelial-associated SMAD3 upregulation and lymphocyte downregulation
Airway transcriptome networks identify susceptibility to frequent asthma exacerbations in children[306] 2023	Phelan et al.	Part of the Ohio Paediatric Asthma Repository (OPAR). Children age 5-19. Nasal brush samples were collected at the point of exacerbation. 49 frequent exacerbators compared to 83 non-frequent exacerbators.	RNA sequencing	Nine expression modules, consisting of 2342 genes, were identified. Frequent exacerbators expressed increased genes associated with the nervous system, and non-frequent exacerbators had higher expression of immunity, including natural killer cells.
Dual RNA-seq reveals viral infections in asthmatic children without respiratory illness, which are associated with changes in the airway transcriptome 2017[311]	Agata Wesolowska-Andersen et al	Detection of respiratory viruses in asthmatics (N=92) and control of children (N=69)	Whole transcriptome RNA sequencing	A large proportion of children without respiratory illness were carrying respiratory viruses that altered the nasal epithelial transcriptome.

Title	Author	Synopsis	Method	Outcome
Clinical and transcriptomic features of persistent exacerbation-prone severe asthma in U-BIOPRED cohort[307] 2022	Hoda et al.	Part of the U-BIOPRED consortium. Compared frequency exacerbators to non-frequent exacerbators	RNA sequencing	Those with frequent exacerbations had upregulated T1 and T2 inflammatory pathways compared to non-frequent exacerbators.
Associations between outdoor air pollutants and non-viral asthma exacerbations and airway inflammatory responses in children and adolescents living in urban areas in the USA: a retrospective secondary analysis. [312] 2023	Altman et al.	A retrospective analysis of the MUPPITS1 cohort of 208 children was performed and replicated in the ICATA cohort.	RNA sequencing	As measured by PM2.5 and O3 levels, poor air quality was associated with asthma exacerbations and reduced lung function, with altered gene expression in pathways related to mucus hypersecretion, barrier function, and T2-associated inflammatory pathways.
Epithelial associated Inflammatory Pathways Underlie Residual Asthma Exacerbations in Urban Children Treated with Mepolizumab Therapy[313] 2023	Patel et al.	MUPPITS-1 (n=106) and MUPPITS-2 (n=290) were randomised to Mepolizumab and placebo. Nasal lavage samples taken at exacerbation	RNA sequencing	Type-2 inflammation-associated pathways driven exacerbations experienced in children whilst on Mepolizumab therapy.

Table 6. 1: Studies that have assessed the nasal transcriptome in relation to asthma exacerbations.

Aim:

The APEX study has identified that a significant proportion of asthma exacerbations demonstrated no evidence of T2 inflammation, and this could be related to the presence of the virus at the point of exacerbation. Therefore, nasal brush samples were taken to subsequently test for the presence of a virus at the point of exacerbation.
The second aim of this work is to explore transcriptomic changes of participants who had an exacerbation event through analysing differential gene expression by performing RNA sequencing to illude to possible mechanisms associated with the exacerbations observed in the APEX study. The biological relevance of findings of gene expression will be placed into context using Ingenuity Pathway Analysis (IPA) (Qiagen ©)

Based upon clinical findings in APEX so far, I hypothesise that a viral positive exacerbation causes more significant deterioration in clinical parameters than a viral negative exacerbation. I also hypothesise that there is significant differential gene expression in Primary OCS events compared to Primary No OCS events. To address these hypotheses, the following questions will be addressed:

1. What proportion of asthma exacerbations observed during APEX had a respiratory virus present at the point of exacerbation.
2. In those with an exacerbation event, what proportion had a virus present at baseline?
3. In viral (+) exacerbations, is there a difference in important clinical parameters compared to those with viral (-) exacerbations?
4. At the point of exacerbation event (Primary OCS and Primary No OCS), what are the essential changes in genomic expression compared to baseline?
5. In those participants who went on to develop an exacerbation in APEX, is there a difference in the expression profile from those who did not exacerbate during their time in the study?

Methodology

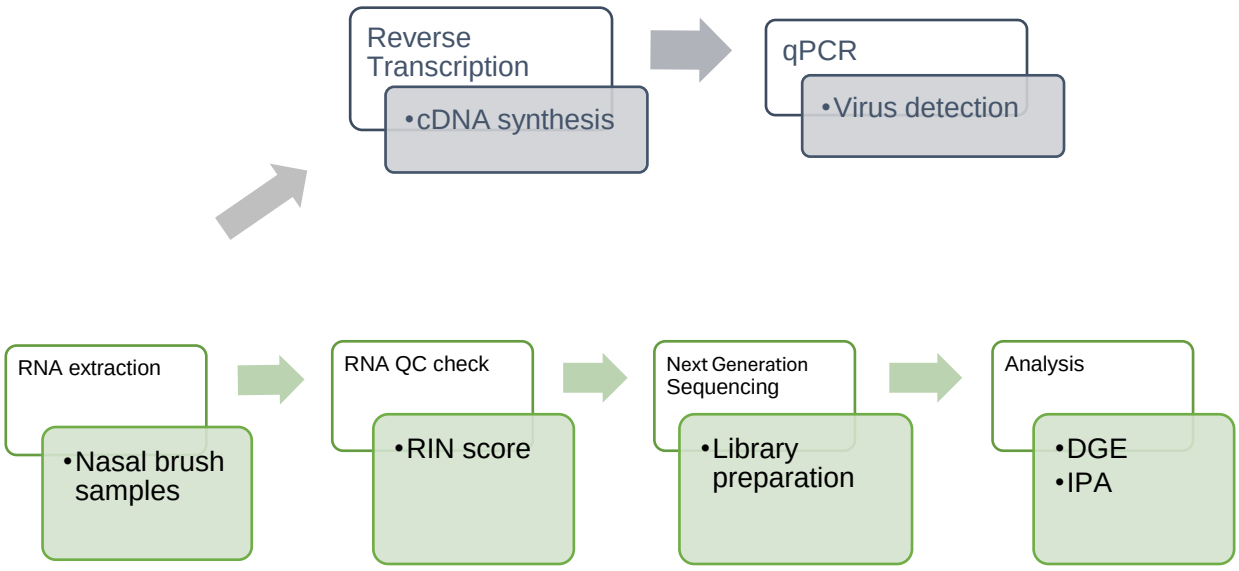


Figure 6. 1: Overview of analysis undertaken. Nasal epithelial cells were extracted from nasal brush samples.

Sample were used for cDNA synthesis and then Quantitative Polymerase Chain Reaction (qPCR) was performed to detect the presence of respiratory viruses. RNA was also sequenced for Differential Gene Expression (DGE) and Ingenuity Pathway Analysis (IPA)

Nasal Brush Sampling

Chapter 2 describes the sampling methodology for nasal brushes, which were used to collect nasal epithelial cells.

RNA extraction

Total RNA was extracted from nasal pellets using RNA/DNA purification Kits (Norgen Biotek Corp ®). The kit allows for the isolation and purification of total RNA and DNA from the sample. The method is based on spin column chromatography. Below is a description of the methodology as described by the manufacturer.

Preparation to perform extractions was implemented to achieve an RNase-free environment, including cleaning all equipment with industrial methylated spirits (IMS), using pipettes solely used for RNA extractions, using clean disposable gloves when handling all equipment and changing these frequently. All surfaces were cleaned using RNase decontamination solution RNaseZAP™ (SIGMA® life sciences), and all equipment needed for extraction was also decontaminated.
The manufacturer's methodology was followed for the extraction process. 300µL of Buffer SKP was added to the pellet. Cells were subsequently lysed by vortexing for at least 15 seconds, ensuring the pellet was dissolved entirely before moving on to the next steps. Using the DNA purification column placed in a collection tube, the sample was then pipetted into the column. 600µL of the lysate generated was put into the column. This was centrifuged at 5200 relative centrifugal force in units of g (RCF) for 2 minutes. This was then spun for an additional 1 minute at 14000 RCF. The flowthrough was placed temporarily in a box containing ice ready for RNA purification. The spin column and collection tube were then reassembled for the genomic wash stage to extract DNA from the sample.

500 µL of wash solution A was added to the column and centrifuged at 3500 RCF for 1 minute. The flowthrough was discarded. 500 µL of

solution A was added to the column and centrifuged at 3500 RCF for 1 minute. The flow-through was discarded. The column was spun at 14000 RCF for 2 minutes to dry the resin thoroughly, and the contents from the collection tube were discarded. To elute the RNA. The column was placed in a 1.7ml elution tube provided by the kit. 100 µL of Elution Buffer F was added and left in the column for four minutes. This was centrifuged initially at 200 RCF for 2 minutes and then 14000 RCF for 2 minutes. DNA was placed in the ice box. To bind the RNA to the RNA column, to every 100µL of flowthrough from the previous step, 60µL of 100% ethanol was added. Therefore, 180µL was added. This was mixed well by vortexing. The RNA purification column was placed in a provided collection tube. 600 of the flowthroughs mixed with ethanol was added to the column. This was centrifuged at 3500 RCF for 2 minutes and then 14000 RCF for 1 minute. The flowthrough was discarded. The spin column and the collection tube were reassembled.

400 µL of wash solution A was added to the column and centrifuged at 3500 RCF for 1 minute. The flowthrough was discarded, and the column was reassembled with the collection tube. 400µL of wash solution A was added to the column again and centrifuged at 3500 RCF for 1 minute; flow-through was discarded. A final volume of 400 µL was added to the spin column and centrifuged at 3500 RCF for a further minute. After the flow-through was discarded, the resin was dried by spinning the column for 2 minutes at 14000 RCF.

To elute the RNA, the column was placed in a 1.7ml elution tube 50µL of elution A solution was added, and it was allowed to bind for 4 minutes. The sample was centrifuged at 200 RCF for 2 minutes and then at 14000 RCF for an additional 2 minutes. Samples were labelled and stored at -80°C for subsequent cDNA synthesis and sequencing.

Assessment of total RNA, RNA concentration and A260/280 ratio

To assess the quality and quantity of RNA obtained from the samples, the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific®) was used. This microvolume spectrophotometer records the measured RNA concentration, total RNA content, and A260/280 ratio. Each Sample was measured in triplicate to ensure accuracy.

Assessment of RNA quality: RNA Integrity Score (RIN)

To assess the RNA integrity, the Agilent 2100 Bioanalyser System® (Agilent Technologies, Cheshire, UK) was used in combination with the Agilent RNA 6000 Nano Kit® (Agilent Technologies). The 28S/18S ribosomal RNA ratio is used to assess the quality of the total RNA from a sample and is produced via the cleavage of an RNA transcript. [314] A high ratio suggests that a large amount of intact RNA is present and has not undergone degradation. A ratio of > 2 is generally considered acceptable. [315]The RIN score is calculated based on the strength of the RNA peaks, which in eukaryotic cell samples are 28S and 18S. However, it has been widely recognised that RNA integrity numbers from nasal passage samples will be lower than expected due to the nature of the obtained sample and contamination present within the samples. [316] The RIN scores for the samples were performed externally by Oxford Genomics (Oxford, UK). The RIN scores of the samples are presented in Appendix VII.

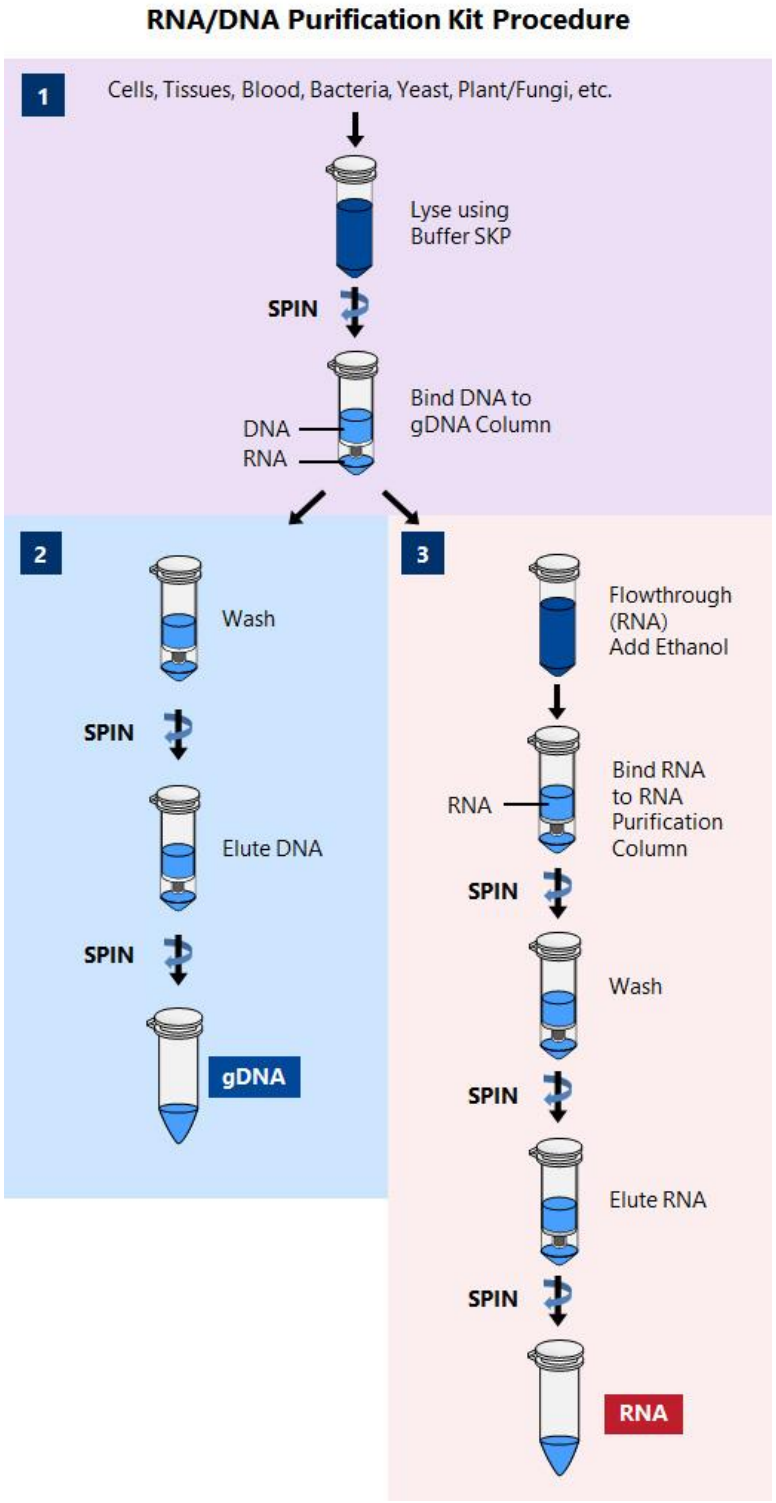


Figure 6. 1: RNA extraction process using the Norgen RNA/DNA purification kit. Image obtained from Norgen Biotek Corp® (Ontario, Canada)

cDNA synthesis

cDNA synthesis is essential to performing qPCR. CDNA synthesis involves reverse transcription, using reverse transcriptase to convert RNA into cDNA. [317]

The Superscript™ First-Strand Synthesis System for RT-PCR is optimised to synthesise first-strand cDNA from total RNA. The cDNA can then be used downstream for qPCR by amplification with appropriate primers by qPCR for the gene of interest. The first-strand cDNA synthesis reaction is catalysed by SuperScript™ II Reverse Transcriptase (RT).

The work area was prepared to ensure reduced contamination, including using IMS to clean all equipment, RNaseZAP™ (SIGMA® life sciences) application on equipment, pipettes utilised only for PCR, and regularly changing gloves. All Eppendorf's contained in the Superscript first-strand synthesis kit™ were kept on ice and centrifuged before use.

The reaction used 50ng of total RNA. The volume of the reaction was made up to 10µL using nuclease water. Nuclease-free water was water that had been deionised, filtered, autoclaved, and tested for contaminants, such as endonucleases, exonucleases, and RNases. Diethyl pyrocarbonate-treated water (DPEC) contains a chemical used to inhibit RNases.

The following components and quantities were used for making cDNA as per manufacturer protocol:

Component	The amount required for one reaction (µL)	X reactions
First Master mix		
dNTP	1	1 X Samples (RT+ & -) + 1
Random Hexamers (RH)	0.2	0.2 X Samples (RT+ & -) + 1
Second Master mix		
10X RT buffer	2	2 X samples (RT+ & -) +1
25mM $MgCl_2$	4	4 X samples (RT+ & -) +1
0.1M DTT	2	2 X samples (RT+ & -) +1
RNaseOUT™	1	1 X samples (RT+ & -) +1
RNase H	1	(For RT+ 1 µL DPEC H_20 for RT-)

A master mix of dNTP and RH was made depending on the number of reactions being performed. An additional amount for one further reaction was added to account for pipette loss. A Reverse transcriptase-negative (RT-) reaction mix was made for each sample of total RNA.

Samples were centrifuged briefly on the UNIVERSAL 320 benchtop centrifuge (Hettich Lab Technology®) at 1000 revolutions per minute (rpm). The required RNA and DPEC treated water to make the reaction to 10 µl was made. The mixture was first incubated at 65°C for 5 minutes. The Prime Thermal cycler (Techne®) was used for cDNA synthesis. In those 5 minutes, the second master mix was made using the quantities stated in the table. 9 µL of the master mix was added to each reaction mixture. This was centrifuged briefly at 1000rpm. It was then incubated at 25°C for 2 minutes. 1µl of SuperScript™ II was added to the samples (RT+) and 1µL of DPEC-treated water to RT-. The samples were then incubated at 25°C for 10 minutes. The temperature was then raised to 42 °C for 10 minutes. The reaction was then terminated at 70 °C for 15 minutes. Samples were then placed on ice. The samples were centrifuged briefly. 1µL of RNase H was added to the samples and incubated at 37 °C for 20 minutes before proceeding to qPCR. Samples were stored at -20°C.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

qPCR is a method that allows for high-sensitivity detection of viruses even with a small quantity of initial nucleic acids through amplification. The amount of DNA is measured after each PCR cycle through the emission of a fluorescent signal that is proportional to the amount of product (amplicons) made.[318]

The principals that make up each cycle in a real-time qPCR reaction are in three steps (Figure 6.4):

1) Denaturation. High-temperature are used to generate single-stranded DNA from double-stranded DNA structures in single-stranded.
2) Annealing: complementary primers hybridise to the strands of DNA. The temperature of this is based on the calculated melting temperature (T_m) of the primers (5°C below the T_m of the primer).
3) Extension: This is where DNA polymerase activity occurs, and the optimal temperature is set to achieve this

Components

TaqMan™ Gene Expression Master Mix

The TaqMan™ Gene Expression Master Mix contains all components required for qPCR, excluding the template and primers.

Pre-developed TaqMan™ Assay Reagents (PDAR)

PDARs are designed to detect and quantify specific genetic sequences using target and control assays in separate wells for this project. They are optimised to work with TaqMan® gene expression Master Mix.

Assay ID	Virus Detected	Reporter Dye	Quencher Dye
Vi99990016_po	Rhinovirus	FAM	MGB
Vi99990015_po	RSV	FAM	MGB
Vi99990011_po	Influenza A	FAM	MGB
Vi06439642_s1	HP1	FAM	MGB
Vi06439670_s1	HP3	FAM	MGB
Vi07922146_s1	MPV	FAM	MGB
Hs99999901_s1	(-) 18S rRNA housekeeper	FAM	MGB

Table 6. 2: TaqMan Assay ID and their respective reporter and quencher dyes used in the APEX study.

A select panel of viruses was chosen based on the epidemiological surveillance data collected by the government for influenza and respiratory viruses in the year 2019-2020. This is undertaken by the Public Health England's National Infection Service.[319] For that year, there were 192 outbreaks of viral-related illness with Rhinovirus, RSV, Influenza A, Human Parainfluenza and Metapneumovirus. These PDARS (Table 6.2) were commercially brought from Thermofisher Scientific©. The company does not disclose primers and probes associated with these PDARs.

Taq Man Probe.

Taqman probes (Figure 6.2) are joined to two other molecules- at the 5' prime end, a fluorescent molecule known as a reporter (R) that "reports" signals as more product is generated, and at the 3' prime end, it is attached to a molecule known as the "quencher" (Q); this "quenches" the fluorescent signal from the reporter in certain circumstances, such as an intact probe.[320]

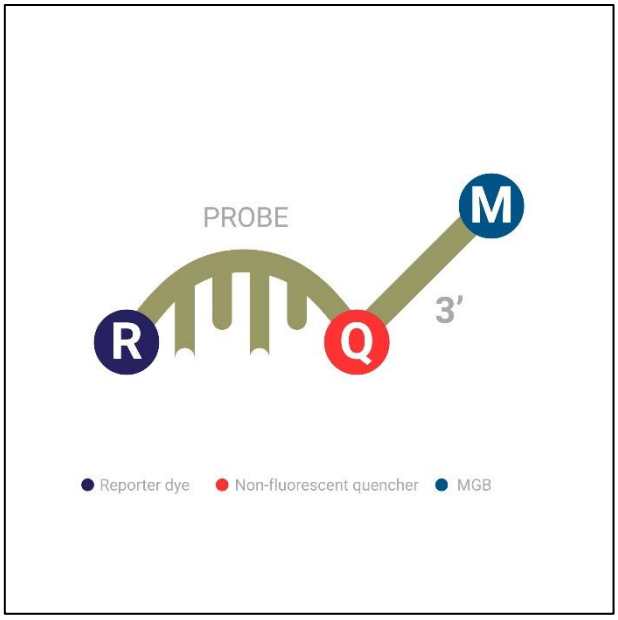


Figure 6. 2: The structure of a TaqMan Probe. Image obtained from Thermofisher UK.

The Minor Groove Binder (MGB) dye is a short probe. [321] The MGB is attached to the quencher itself, which is nonfluorescent

If the reporter and quencher are separated during the reaction and light strikes the response, the reporter fluoresces, producing a signal that the PCR machine can detect. Each time a new PCR amplicon is created, the aim is for the reporter and quencher to split, and thereby, the fluorescent will proportionally increase with the quantity of product made.

Housekeeper genes

Housekeepers are utilised to normalise for the differences in the quantity of cDNA synthesised in each well and the quality of the product to reduce errors. [322,323] A good gene should be expressed well in the cells under investigation. The expression should remain the same regardless of a disease state. However, housekeeping genes are not perfect.[324] Typically, the used housekeeping genes have been found to show varying expression levels [325].

After reviewing the literature, I chose the housekeeper gene. Three candidate housekeeper genes were considered: the 18s ribosomal RNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and beta-actin (ACTB). I found that GAPDH and ACTB are commonly used in respiratory studies.[326] However, they were found to have a variable expression in a metanalysis.[327] The 18s housekeeper gene was chosen because it has been identified as reliable in human bronchial epithelial cell (HBEC) studies.[328] I tested the house-keeper gene on HBEC cells infected with RV-18 before analysing any APEX nasal brush samples, and it was found to be stable.

Methodology for qPCR

The following reaction mixes were made:
RT+: cDNA made with reverse transcriptase, Master Mix, DPEC-treated water and PDAR.
RT-: cDNA made without reverse transcriptase, Master Mix, DPEC-treated water and PDAR.
Non-template control (NTC): sample containing DPEC-treated water, PDAR and Master Mix

Component	Value (µL)
cDNA	1
Master Mix	10
H20	8 (4 for master mix and 4 for cDNA)
PDAR	1
Total	**20**



Figure 6. 3: 96-well plate setup for qPCR of cDNA from nasal brush samples.

2 samples were used per plate. Each row had 1 PDAR per well. Row H contained non-template control (NTC). PDAR used were Rhinovirus, Respiratory Syncytial Virus (RSV), Human Parainfluenza Virus 1 (HP 1), Human Parainfluenza Virus 3 (HP 3), Human Metapneumovirus (HMPV), 18S ribosomal RNA (18s). Reporter dyes were FAM (blue) and ROX was used as a reference dye (orange).

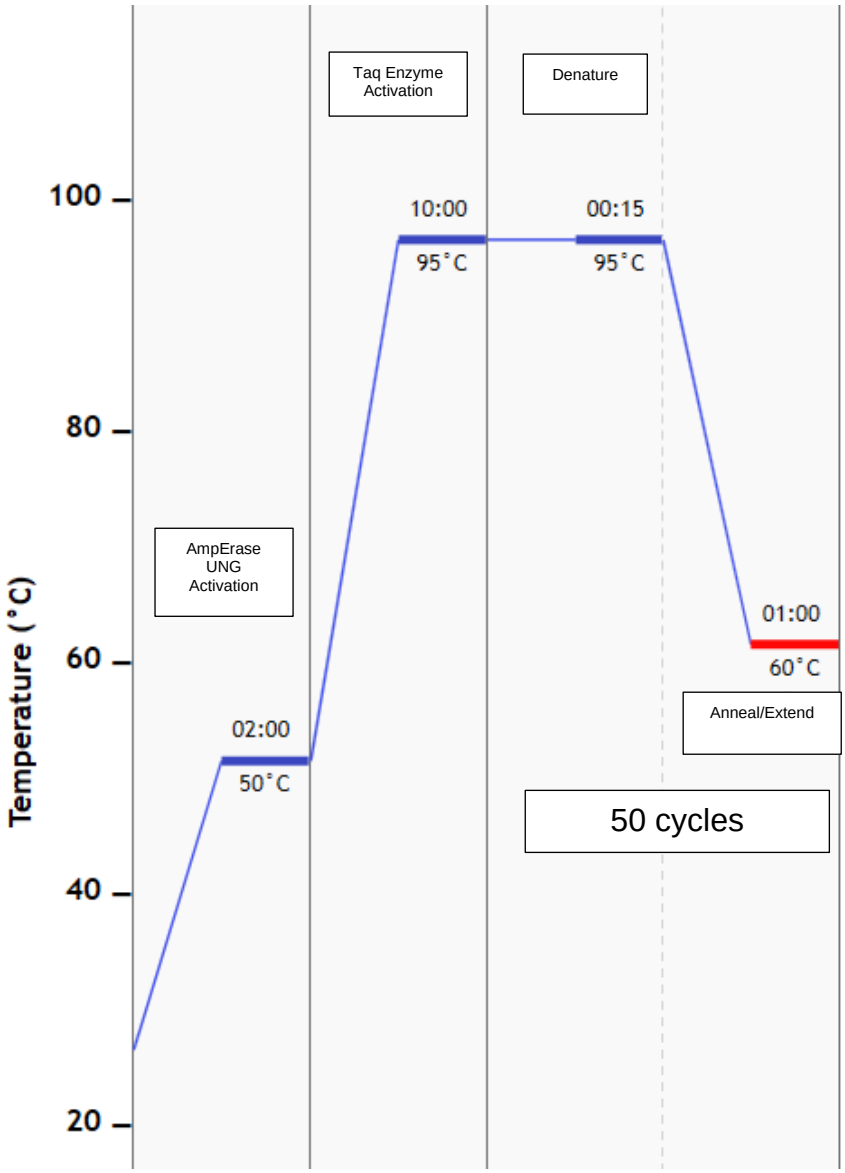


Figure 6. 4: PCR cycle settings with the temperature and duration of each step of the PCR process.

Statistical analyses

50 cycles were chosen for PCR; this was chosen due to the relatively low quantities of RNA as well as contaminants due to the nature of the samples. The cycle threshold (Ct) ranged from 32-38. The housekeeper gene 18s had a Ct on average of 22. The delta Ct SD was set at 0.25, and the triplicates were considered reliable if they were less than 0.25. If 2 out of the triplicates amplified, this was considered acceptable. For this analysis, the presence of a virus or not and the type of virus present was

considered the most clinically relevant outcome to establish rather than the quantity of cDNA present.

RNA sequencing methodology and analysis pathway

RNA sequencing helps us understand diseases such as asthma and can identify potential biomarkers; it also allows us to measure gene expression of disease in different states, such as at the point of an asthma exacerbation. The transcriptome is the collection of RNA within a cell that has been transcribed from genomic DNA. In humans, the transcriptome represents 85% of the human genome. Of that, only 3% of the genome encodes protein-coding genes messenger RNA (mRNA). The rest consists of non-coding and regulatory RNA, ribosomal RNA and transfer RNA. [329] The RNA analysis pipeline was based on previous work undertaken by our study group.[330,331]

Due to the low-quality RIN scores obtained from Oxford Genomics (mean RIN 3.11 SD(1.32), median 3.0 (IQR=2.25-3.65) (Appendix VII)) partly because of the nature of the sample itself containing contaminants, a ribodepletion approach was undertaken for RNA enrichment. The minimum concentration of RNA needed for sequencing was 10ng.

Ribodepletion

As mentioned, due to the low-quality RIN scores, ribodepletion was adopted. Ribodepletion involves directly removing rRNA from the total RNA sample by hybridising the sample with DNA oligonucleotides that capture rRNA and remove it from the sample.[332]

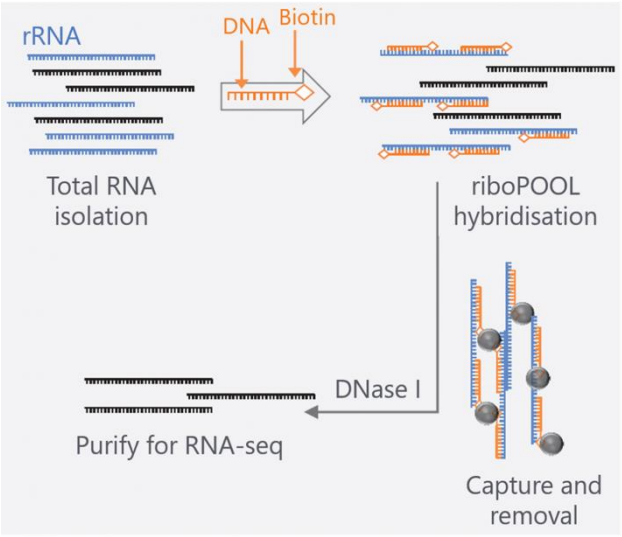


FIgure 6. 5: The process of Ribopletion of rRNA from samples.

In the process of ribodepletion the olionulceotide probes in this case "riboPOOL" will bind to human ribosomal RNA and are removed through magnetic beads. Image obtained from Cambridge Bioscience (Cambridge, UK)

This results in a read distribution that allows exonic, intronic, and intergenic regions to be sequenced. This method, as opposed to other enrichment pathways such as mRNA capture, allows the sequencing of lower-quality samples and provides better 5' coverage than mRNA seq.

Methodology

Oxford Genomics performed sequencing using Illumina© (San Diego, US), which uses a sequence-by-synthesis approach. In this approach, a sequence is generated by adding bases to create base pairs.[333] The methodology for sequencing is detailed as previously described.[334]

Paired-end sequencing

Paired-ended sequencing was adopted because this methodology allows differential expression detection for lower-expressed genes and improves mapping for novel isoform discovery. There is also better mapping across exon-exon junctions.[335]

Sequencing Depth

Reading or sequencing depth is important to consider when sequencing. The read depth is the number of reads for a nucleotide, and the average read depth is the average depth across the region sequenced. [336] For whole genome sequencing, an average read depth of 5-15M is considered sufficient. In a sample of RNA, most of the RNA will be highly expressed genes. If the read depth is not enough, then the majority of the read will be of the highly expressed genes rather than detecting differential expression of lower expressed genes.[336] As a result of ribodeption methodology, over-sequencing is required to explore the exonic content in detail to get adequate coverage. Hence, 40M was chosen for this work. The number of replicates was considered to allow sufficient detection of lower-expressed genes, considering the sequencing depth chosen. Coverage is an important consideration. Coverage represents the proportion of mapped reads to the number of bases of the reference genome. The aim is to have adequate coverage so that there are no missing areas across the target DNA. [337]

Results

Viral pathogen in nasal brush samples of exacerbation events detected by q-PCR.

305 participants were enrolled into the APEX studies. However, for this proportion of work, samples up to May 2023 were utilised. qPCR was performed on 108 exacerbation samples at exacerbation and 64 matched baseline samples. Example qPCR curves obtained for each virus detected, and non-template controls are in Appendix VIII.

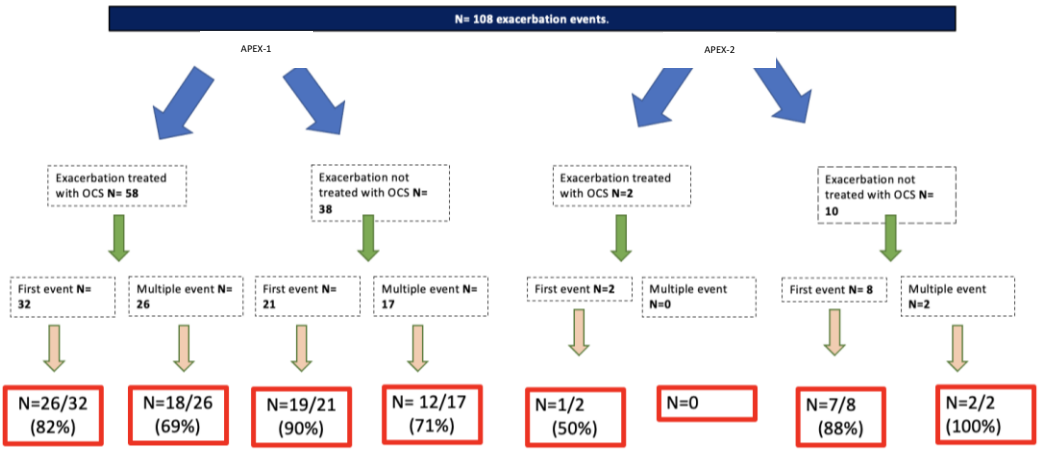


Figure 6. 6: Flow chart illustrating number of nasal brush samples positive for the presence of a virus detected by q-PCR

There was a total of 92 viruses detected in those 108 exacerbation samples, with seven samples testing positive for two viruses (Figure 6.7). Those seven samples came from 7 individual participants. There were 64 participants who contributed to these exacerbation events. The figure below illustrates the frequency of the virus detected according to the exacerbation event. It also demonstrates the number of viruses detected according to event and APEX cohort with the number of samples positive. The maximum number of multiple viruses detected in a sample were 2.

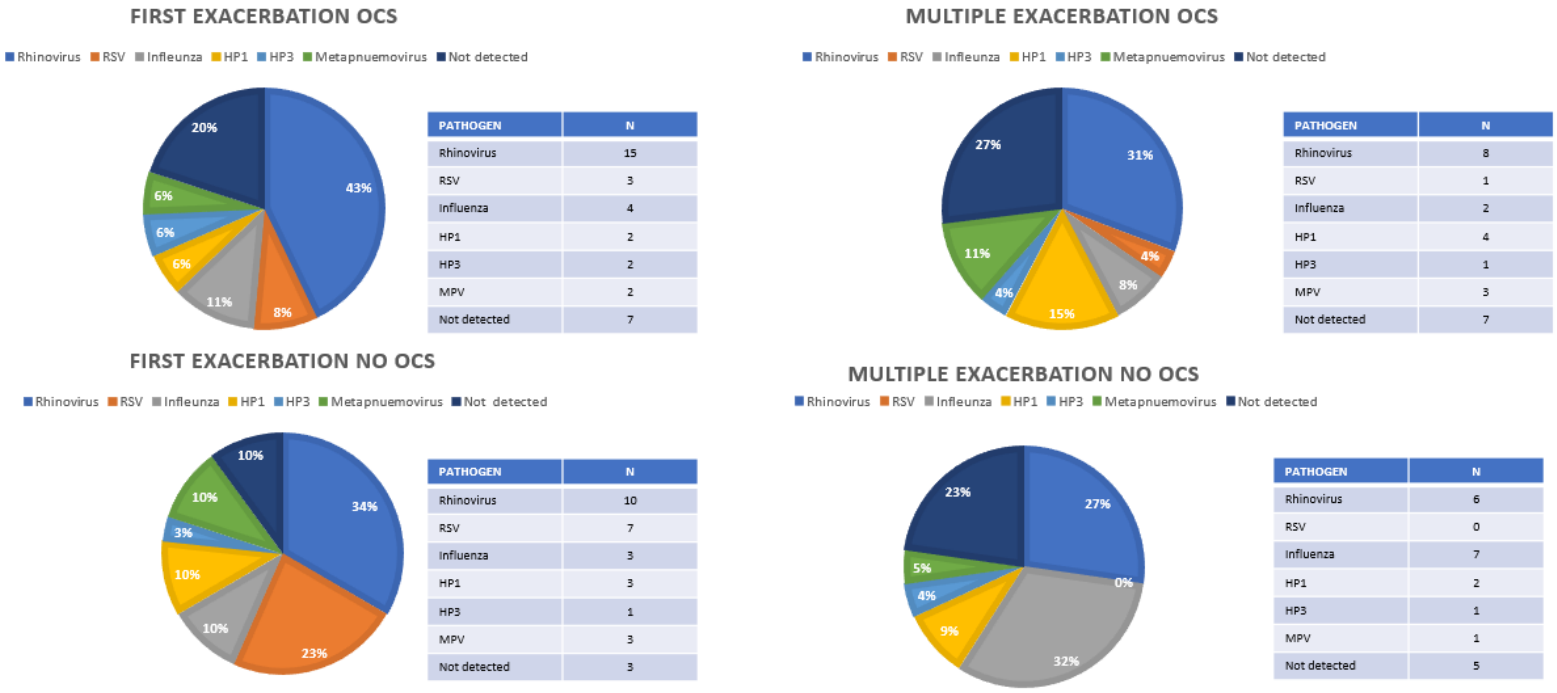


Figure 6. 7: Viral pathogen presence in primary and multiple events in the APEX study.

This was from the panel of 6 PDARS of common respiratory viruses in 2019-2020

	APEX-1	APEX-2	Total
Primary OCS	28 (2 samples 2 viruses)	2 (1 sample 2 viruses)	30
Primary No OCS	20 (1 sample 2 viruses)	7	27
Multiple OCS	18	0	18
Multiple No OCS	15 (3 samples 2 viruses)	2	17
			92

As Figure 6.7 illustrates, 80% of primary OCS had a virus present (28/34 samples, with 2 of those samples having two viruses present). There were 20% of Primary OCS events where no virus was detected based on six respiratory viruses, with the highest incidence occurring between 2019 and 2020. I acknowledge there may be viruses in the samples that were not tested for. The most common virus present was Rhinovirus (43%). This was followed by 11% for Influenza A and 8% for RSV. In Primary No OCS events, 90% were associated with a viral pathogen being detected (27/30). In 10% (3/30), no viral pathogen was detected. The most common pathogen detected was again Rhinovirus. However, 23% of these exacerbations were associated with RSV. Similar percentages of Influenza were present (10% vs 11%).

26 exacerbation events were multiple OCS events. Over a third of these events were associated with Rhinovirus as the primary pathogen; only 10% had no virus detected. In those exacerbation events, in which no OCS was provided (multiple No OCS), over a third were associated with Influenza, and 27% were related to rhinovirus. No RSV was detected in these samples. A Fisher Exact test was performed to assess whether there was a significant difference between pathogens present in primary events (Primary OCS vs Primary No OCS). There was no statistical difference between the type of viral pathogen present at primary exacerbation events between OCS and no OCS events, as shown in Table 6.3.

Pathogen	OCS (N)	No-OCS (N)	p-value
Rhinovirus (+)	15	10	
Rhinovirus (-)	20	20	0.46
RSV (+)	3	7	
RSV (-)	32	23	0.17
Influenza (+)	4	3	
Influenza (-)	32	27	1.00
HP1 (+)	2	3	
HP1 (-)	33	27	0.66
HP3 (+)	2	1	
HP3 (-)	33	29	1.00
MPV (+)	2	3	
MPV(-)	33	27	0.66

Table 6. 3: Fisher exact tests exploring viral pathogen between primary OCS and no OCS events in the APEX study.

There was no significant difference in the type of virus present according to whether a course of steroids (Primary OCS) was required or not (Primary No OCS)

To assess whether the presence of a viral (+) exacerbation event was associated with worse clinical parameters than a viral (-) exacerbation, an analysis of Virus (+) and Virus (-) in primary events, i.e. regardless of whether a course of steroids was provided or not (Table 6.4). A focus on critical clinical markers of asthma was adopted for this analysis. Bonferroni correction was added for multiple testing.

There was no significant difference in FEV1% predicted, with FEV1% predicted being greater in those events with a virus present, with mean FEV1% being 77.87% versus 68.44%. Markers of T2-inflammation, namely FeNO and peripheral blood Eosinophil Count, showed no difference with median FeNO in viral (+) exacerbation events at 25 ppb and viral (-) at 32 ppb. Similar eosinophil counts were also measured in viral (+) and viral (-) exacerbation events (0.19 vs 0.20 $x10^9/L$) Ax. However, on measurements of small airway disease, peripheral resistance was significantly greater in those exacerbation events where viruses were present at an exacerbation event than when a virus was not detected. (1.30 $cmH_20/L/s$ (0.64-2.45) vs 0.20 $cmH_20/L/s$ (0.05-2.14)) p=0.03. There was no statistical difference for reactance, although the absolute values were much greater in exacerbation events where a virus was detected. No difference in Asthma control or inflammation measured by CRP was found.

Parameter	Virus (+)	Virus (-)	p-value
N	55	10	-
FEV1%*	77.87(19.57)	68.44(21.11)	0.17
FeNO (ppb)**	25.00(15.75-37.00)	32.00(26.50-58.00)	0.08
Eosinophil ($x10^9$/L)**	0.19(0.08-0.43)	0.20(0.09-0.50)	0.83
R5R20 (cmH_20/L/s)**	1.30(0.64-2.45)	0.20(0.05-2.14)	0.03
Ax (cmH_20/L)**	21.62(7.50-49.06)	6.29(5.30-39.92)	0.19
ACQ-5**	3.40(2.40-3.80)	2.90(2.35-3.30)	0.29
CRP** ($x10^9$/L)	5.00(2.00-14.25)	4.002.50-7.50)	0.93

Table 6. 4: Comparing key parameters primary exacerbation events in the APEX study according to the presence or absence of a virus.

*= Mean(SD) unpaired t-test **=Median(IQR) Mann-Whtiney test.

Viral pathogens detected by q-PCR on Baseline Samples in APEX.

As mentioned, 64 participants contributed to the 108 exacerbation events. Of these, 44 participants had one exacerbation event. 20 multiple exacerbation events occurred that contributed to the exacerbation samples, as illustrated by the table below:

Exacerbation Events	Number of Participants
1	44
2	9
3	5
4	5
5	1
Total	64

Six viruses were detected in 5 participants, with 1 participant having Rhinovirus and Influenza A present. All those who tested positive at baseline for a virus had multiple exacerbation events in the APEX study.

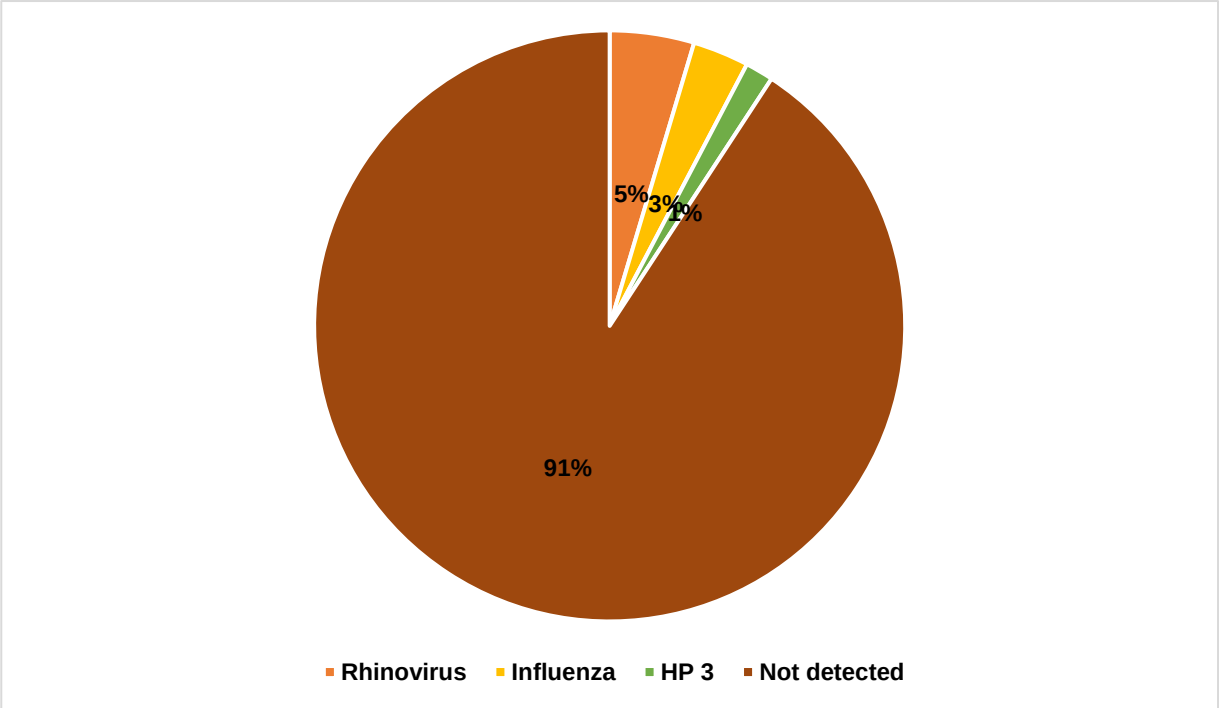


Figure 6. 8: PCR was performed on baseline samples that went on to have an exacerbation event in APEX.

In those who had a positive sample at baseline (N=5).

Differential gene expression analysis of exacerbation events in APEX.

206 nasal epithelial samples underwent RNA sequencing. The sample and presence of a virus at the primary exacerbation event are presented below (Table 6.5). Only primary events were considered for these analyses.

RNA Sample	N	Virus Present N(%)
Baseline	**126**	-
Baseline that did exacerbate	53	5(9.4)
Baseline that did not exacerbate	73	0
Exacerbation	**69**	-
Primary OCS	25	19(76)
Primary No OCS	24	20(83)
Total:	**195**	
Samples used in analysis:	**175**	

Table 6. 5: Nasal epithelial samples that underwent RNA-sequencing and Viral pathogen present at qPCR utilising PDARs from 6 common respiratory viruses.

Of note, 11 first-year annual samples were sequenced but not used for the analyses.Twenty multiple exacerbation event samples were not used in the analysis. Four patients had an exacerbation event, but a sample was not collected on their first exacerbation, but a sample was collected on multiple events. They were removed from the paired analyses.

The purpose of this analysis is to identify differential gene expression from baseline to exacerbation event, and as such, the following analyses were undertaken:

- Baseline participants that underwent an exacerbation event (exacerbators) versus those that did not experience an exacerbation event in the APEX study (non-exacerbators)
- Paired analysis of baseline versus primary OCS
- Paired analysis of baseline versus primary No OCS.

Chapter 3 described the cut-offs for T2-high and low inflammation. For the analyses, adjustments were made for BMI, gender, and ICS usage. For differential gene expression, Cut-offs were applied to make meaningful interpretations of the DEG fold change (FC) with a log fold change of -2 to +2. FC alteration can impact data interpretation, but using a more prominent fold change does not necessarily make interpretations more biologically relevant.[338] The most common fold change adopted from a literature review is between 1 and 2.

Mapped reads:

Table 6.6 describes the mapping output from the 206 samples that underwent sequencing. The mean uniquely mapped reads were 75%. This is because all RNA is being sequenced, not just human RNA, which means RNA due to other pathogens associated with asthma exacerbations could also have been sequenced. The mean read length was 292.

	Uniquely mapped reads %	Average input read Length	Average Mapped Length	Mismatch rate per base	unmapped reads-other %	unmapped too short %
(N)	206	206	206	206	206	206
Range	92.23	111.0	268.2	0.60	0.62	94.34
Mean	75.13	292.1	290.5	0.32	0.13	21.92
SD	25.07	10.42	27.53	0.06	0.09	25.72

Table 6. 6: Quality assessment of Mapped Reads after RNA sequencing.

The mean mismatch rate was 0.32%. On review of the unmapped reads, 21.92% of reads were not mapped as their length was too short for mapping, which suggests some RNA degradation in the analysis process and reflects the RIN scores obtained and attrition.

352 samples were sent to Oxford genomics. There were 37 samples in which RIN scores were unavailable, leaving 315 potential samples. For the aims of this work, focusing on a paired analysis of transcriptomic profiles from baseline to exacerbations which did or did not require steroids, 208 samples were analysed. 2 samples could not be sequenced due to quality, leaving 206 samples for potential analysis. (Table 6.5)

Analysis Pathway



Figure 6. 9: Analysis pathway used for RNA sequencing with the software packages used to perform the analyses.

Concatenation of data.

Initially, the sequencing data obtained due to the large quantity of data was first concatenated to combine the data. This generated two fastq.gz files- one forward and one reverse. The zcat command was used to combine multiple lanes for sequencing. Commands used for this process were generated by the High-Performance Computer team (Alen's Script© University of Nottingham, UK) and are detailed in Appendix IX.

Read Alignment

Mapping of raw data to the human reference genome was performed, and the Spliced Transcripts Alignment to a Reference (STAR) software was used to map reads to the human genome (STAR version 2.7.11b). The human reference genome was obtained from Ensembl genome browser 112 (EMBL's European Bioinformatics Institute, Cambridge, UK). The file used was Homo sapiens.GRCh37.87.gtf.gz.

The method of alignment chosen was true alignment, in which the reads are directly mapped to the reference genome, allowing for splice junctions across exons.[339] mRNA is spliced out, and exons joined together, and as such, short reads will cross exon-exon junctions that do not directly map back to the reference. As such, an aligner was required

to recognise this, and as such, complete alignment was performed to discover any isoforms using the STAR pipeline (Appendix IX).[339]

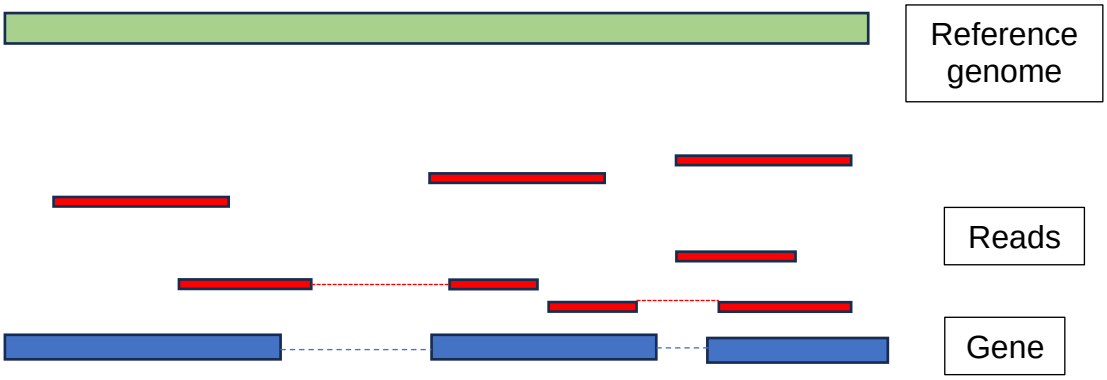


Figure 6. 10: Splice-aware alignment pipelines are critical as mRNA reads can cross exon-exon junctions.

It is expected that 70-90% of RNA-seq reads obtained should map to the human genome with loss due to unannotated transcripts or reads falling onto exons whose transcripts match an isoform of that particular gene.[340]

Adaptor sequences were removed for downstream analysis via Trimmomatic (GPL V2, USADELLAB), which performs read trimming and filtering.[341] . Both STAR and Trimmomatic are embedded within the Sankemake© package. The code used for this process is in Appendix IX.

edgeR normalisation and Differential expression testing

The data is first organised into a count matrix file for edgeR to process into an all_counts.text for further analysis in RStudio (2023.09.0® Build 463). The necessary packages were downloaded via R to run essential analyses and produce necessary plots. The count's matrix file was reviewed to ensure all samples with labels were present. Gene IDs were added to the Ensembl identifier using the maps' function and the Annotation Dbi package from Bioconductor. Normalisation and

differential gene expression were performed under the direct supervision of Dr Robert Hall.

All genes not transcribed in the samples were removed from the analysis. (Figure 6.11). Lowly expressed genes were also filtered out as they were not considered biologically relevant. Ten read counts or more were kept in.

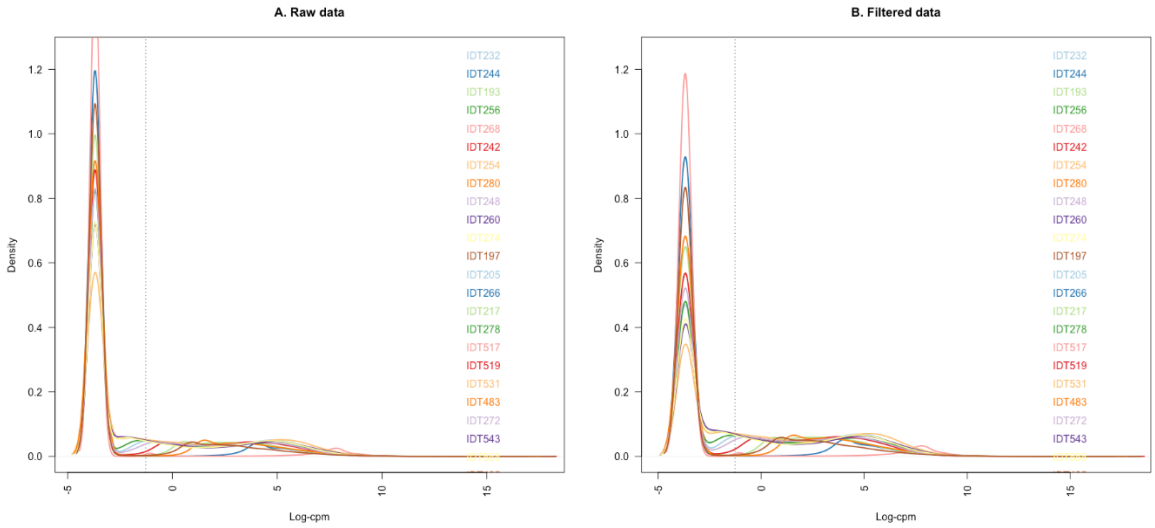


Figure 6. 11: The density of log-CPM values for pre-filtered “raw data” (left). Post-filtered data (right).

The left plot shows a significant number of genes that are not expressed or have a low degree of expression. The right plot shows after filtering these out. The Vertical dashed line represents the log-CPM threshold used in the filtering step.

Normalisation of the RNAseq data was performed using two Bioconductor packages, edgeR[342] and limma.[343] The principle of normalisation involves scaling each sample according to the respective read counts for each gene and dividing this by the total read counts for the sample. Normalisation removes bias introduced from different library sizes that may lead to the overrepresentation of a particular sample.

First, raw counts were transformed into counts per million (CPM). Raw counts were scaled, as those libraries were sequenced to a greater depth, which means they would result in a higher raw read count and not necessarily greater gene expression.

The next step is to determine the value of 75% of the scaled data, which is equal to or smaller than it, and then calculate the total average of the 75% centiles. Dimensionality reduction on expression data for outliers and batch effects is performed to ensure that the expression distribution is similar across the samples. Data was normalised using the trimmed means of M-values (TMM) using the calcNormFactors function in edgeR.

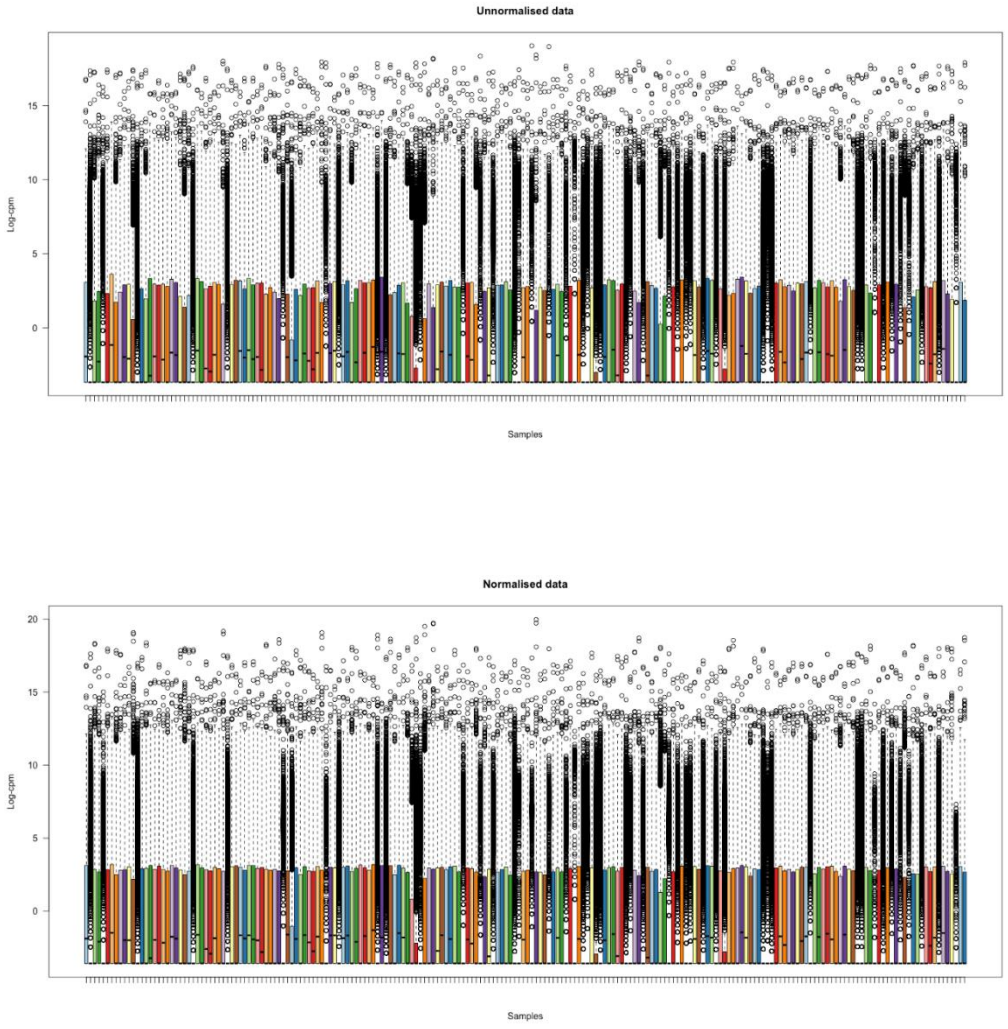


Figure 6. 12: Box plots of log-CPM values showing expression distributions for each sample's unnormalized data (top) and normalised data (bottom).

Two Bioconductor packages, edgeR and Limma, were used to perform normalisation to remove biases from the sequenced data using the TMM

method. Several samples had to undergo normalisation, which is visually challenging to interpret (Figure 6.11); however, on review after normalisation, the data became more evenly spread.

Unsupervised clustering.

This is important to assess how samples relate to each other and to assess for variance. Principal component analysis reduces the complexity of expression data to demonstrate relationships on two axes.[344,345]

PCA also allows for the assessment of clusters, which suggests that the replicates within each condition are more similar to each other than they are to different groups. Therefore, it is an essential tool for considering outliers, which could skew the results obtained. To do this, the plotMDS function was performed using Limma.

The first dimension or principal component reflects the fold change that best separates it, is the most significant cause of variation between the samples and further dimensions and is orthogonal to the one before.

PCA was performed to see if there were generally any clusters that formed from the RNA seq data. PCAs were performed according to:

- APEX event: Baseline, Exacerbation and Annual
- Study ID: if participants clustered together
- Study Cohort: APEX-1 or APEX-2

On review of the PCA plots, there was no significant clustering that occurred, suggesting considerable heterogeneity of the samples that underwent sequencing.

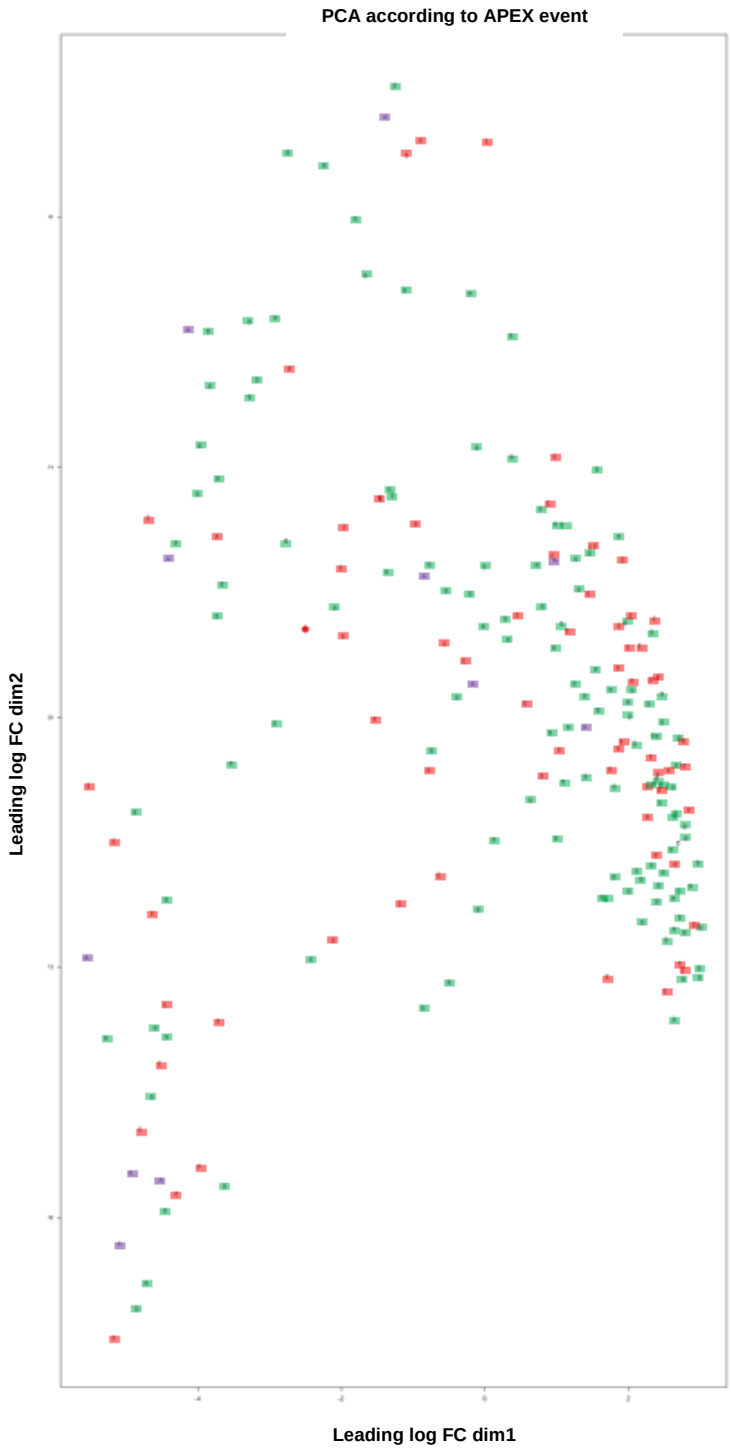


Figure 6. 13: PCA of samples according to APEX events:

Baseline =Green **Exacerbation event=Red** **Annual=Purple**

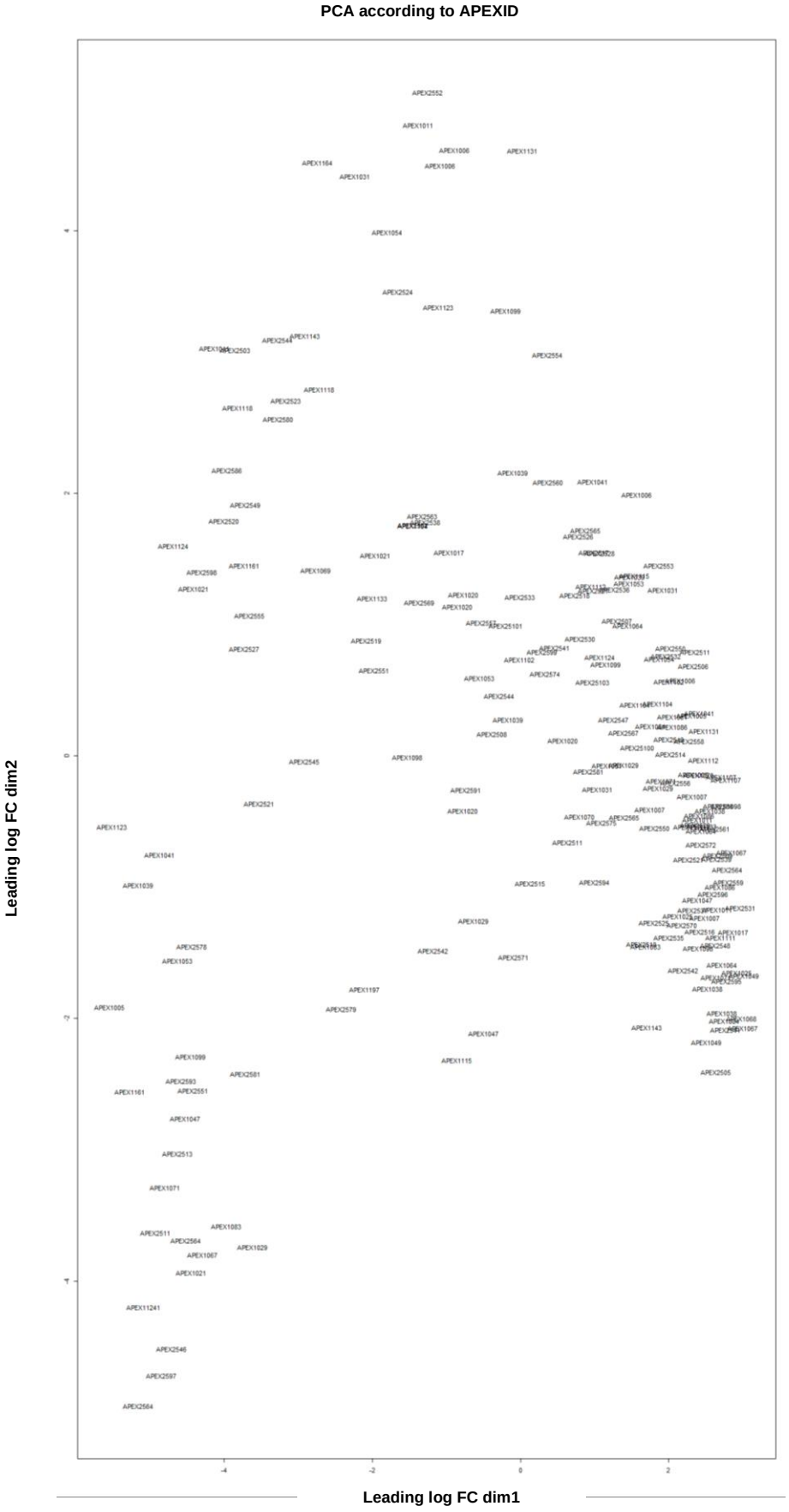


Figure 6. 13: PCA of samples according to participant.

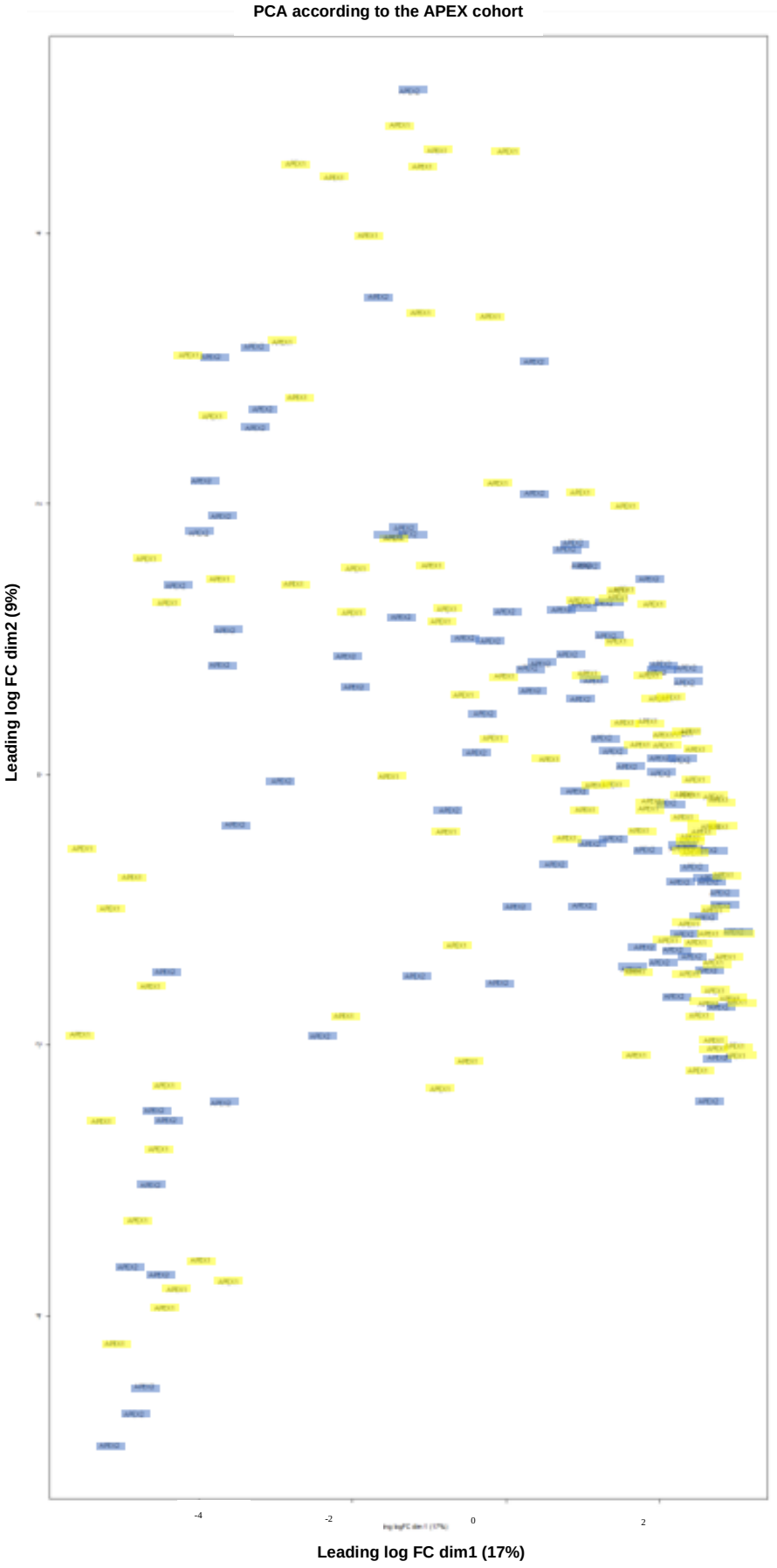


Figure 6. 14: PCA of samples according to study cohort (APEX-1 & APEX-2)

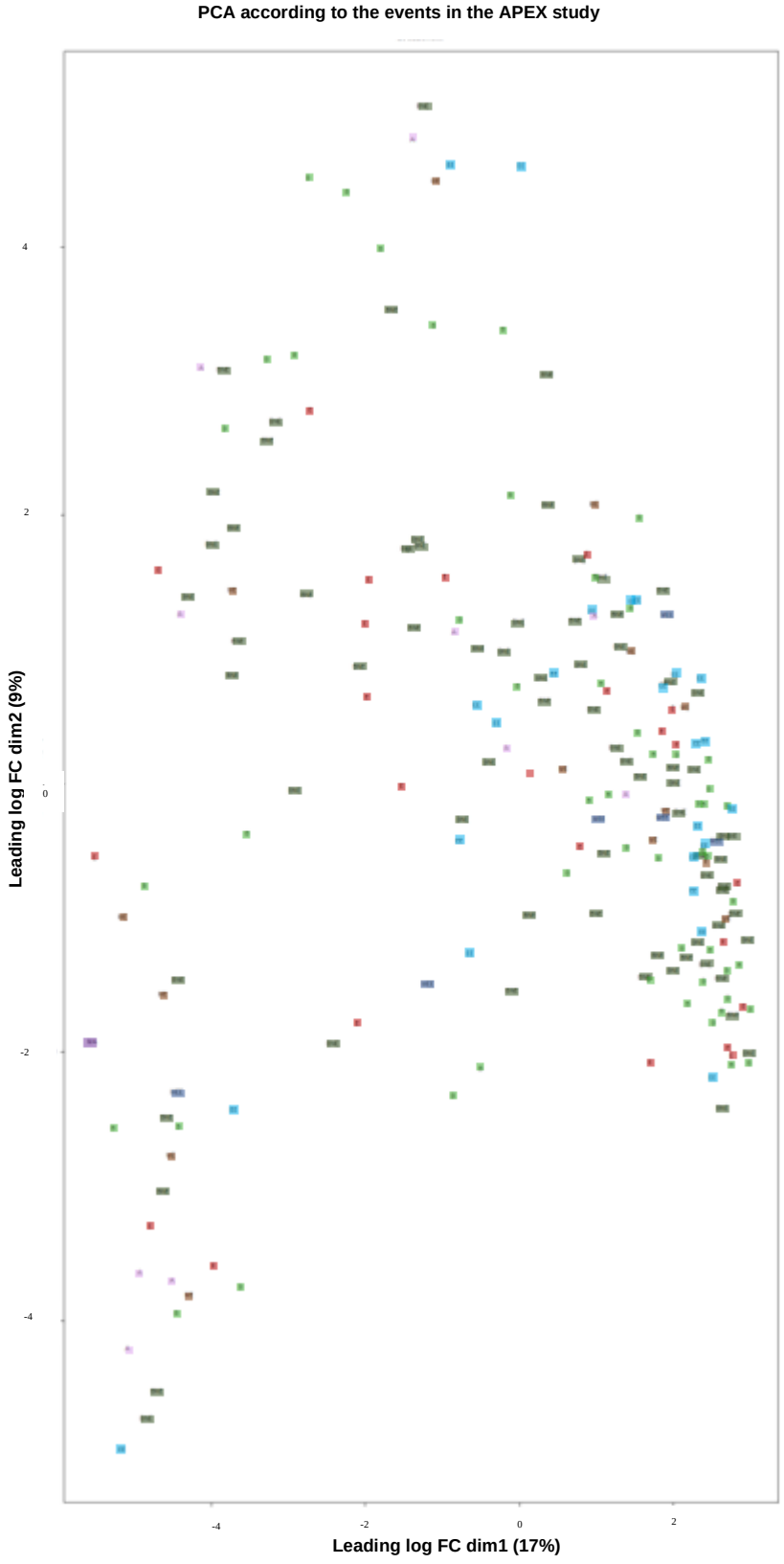


Figure 6. 15: PCA of nasal brush samples according to events: Baseline that exacerbated, Baseline that did not exacerbate, Primary OCS, Primary No OCS, Multiple OCS, Multiple No OCS and Annual.

The next step is performing linear modelling in log-CPM values, which is essential. The mean-variance of log counts generates a particular weight to account for variations in precision for measurements, as count size may vary from sample to sample for the same gene. This is inputted into a Bayes analysis pipeline[346] The function is performed using the voom function with the code used in Appendix IX.

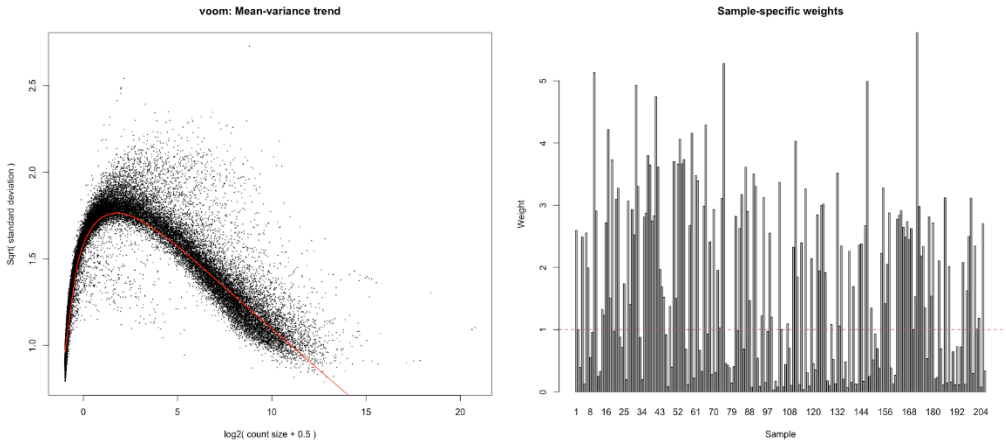


Figure 6. 14: Voom Plot (left) with the quality weighted approach.

The sample's weight is plotted on the bar chart (right). The left voom plot is from residual variance left after linear modelling and square rooted and plotted against the mean log2 transformed with an offset of 2 representing a gene's mean expression.

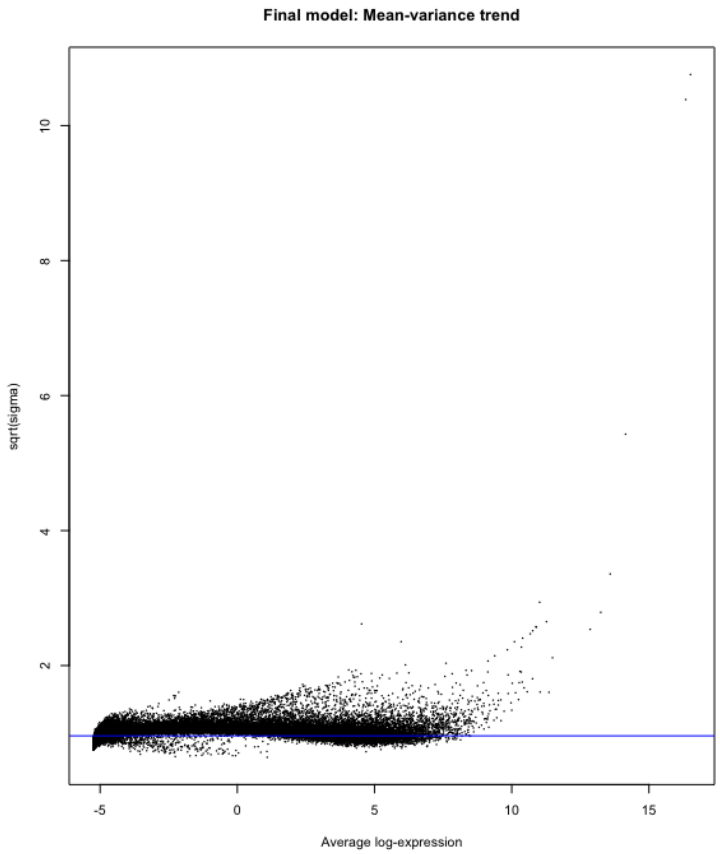


Figure 6. 15: Final Voom plot post transformation demonstrates the residual standard deviation against log-CPM values.

The blue line represents the average log2 residual standard deviation.

As illustrated by the bar chart, there was evidence of sample-level variation, so the “voomWithQualityWeights” function was used to incorporate sample-level weights with the abundance-dependent weights, as seen in Figure 6.14.

Differential expression testing aims to identify whether there is a significant difference in the counts or expression between baseline and exacerbation events within one gene or transcript or whether the differential expression is determined by chance.

In differential gene expression, I am testing for differential expression for each gene, and as such, testing across thousands of genes requires correction for multiple comparisons. Many genes could result in difficulty in meaningful interpretations. One of the most common methodologies for testing this is the False Discovery Rate (FDR). The FDR aims to keep

the sample false positive rate below a threshold usually set to 5%. This provides an FDR-corrected p-value known as the q-value.

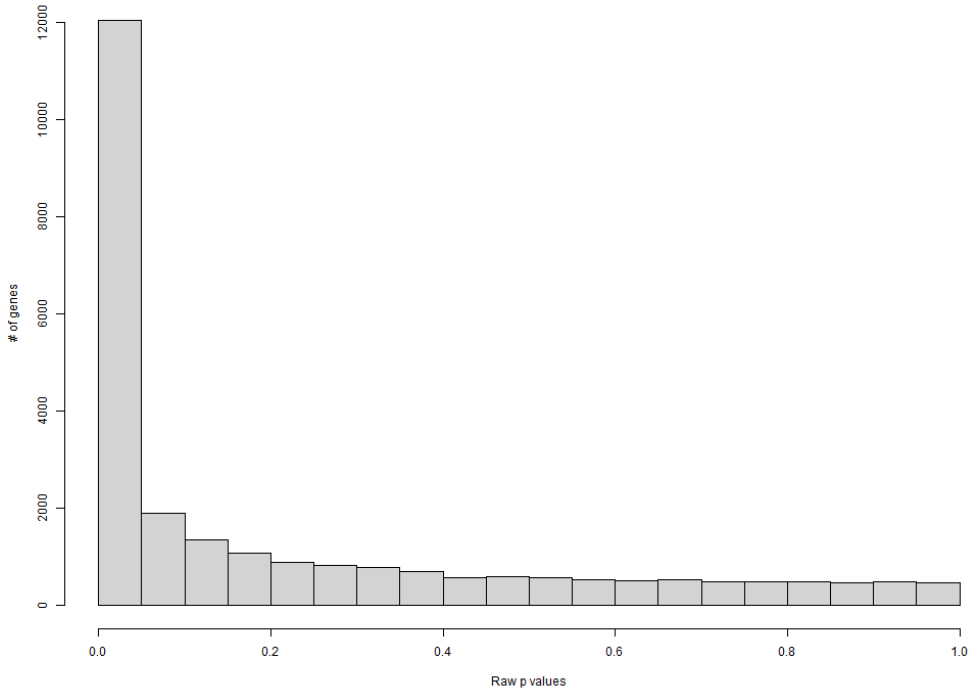


Figure 6. 16: Density distribution of raw p-values for the RNA sequence analysis.

Raw p-values are displayed on the x-axis with the number of genes within a specific p-value. q-values are generated from p-values with an FDR set to 5%

Functional Profiling

Functional profiling puts the findings into a relevant biological and clinical context. Functional profiling is a process by which the list of genes changes under different conditions or disease states and identifies pathways, transcription networks, or regulators that are enriched beyond what you would expect to have occurred by chance. Ingenuity Pathway Analysis (IPA) was performed (©QIAGEN). The IPA process generates p-values for molecules expressed by the genes and whether they are associated with a particular pathway or function. A Fisher's exact test is utilised for this. A p-value of ≤ 0.05 is suggestive of a non-random association.

For all analyses used in IPA, a Benjamini-Hochberg correction was added for multiple testing to reduce any findings' false positive discovery rate. Canonical Pathway Enrichment was used to input the Gene IDs, fold changes, and q-values; the output is enriched pathways, an associated q-value is generated, and a z-score. Z scores predict activation or inhibition where a z-score ≥2 is suggestive of activation and a z-score ≤2 is suggestive of inhibition, which will identify whether a pathway is upregulated or downregulated in an asthma exacerbation. The ratio output informs the ratio of genes inputted to the ratio of genes involved in the pathway. The downstream effects analysis is similar to the canonical enriched pathways but is according to the disease type and biological process that may occur based on the sequenced data.

Paired analysis of Primary OCS to baseline

Demographics

The first analysis performed was a paired analysis of exacerbation events in which a course of steroids was given for a suspected exacerbation (Primary OCS). This was compared to parameters at study entry in APEX.

Table 6.7 illustrates key clinical parameters. The median age was 62.2, and 76% of Primary OCS were female. There was no difference in T2-status at baseline to Primary OCS in either FeNO count 22.0 ppb at baseline vs 28.0 ppb at primary OCS p=0.83, and blood eosinophil count 0.23 $x10^9$/L vs 0.30 $x10^9$/L at primary OCS p=0.38. This could reflect the sample size. As expected, there was a significant difference in Primary OCS in decline in lung function measured as the FEV1% predicted and ACQ5 score with loss of asthma control in both events

	Baseline	**Primary OCS**	**p-value**
	N=25	N=25	
Female N(%)	19(76)	-	-
Age Yr(IQR)*	62.2(45.7-69.3)	-	-
FeNO (ppb) **	22.0(9.0-41.0)	28.0(15.5-43)	0.83
Eosinophil ($x10^9$/L) **	0.23(0.11-0.34)	0.30(0.09-0.50)	0.38
FEV1% pred (SD) *	77.2(20.4)	65.6(21.0)	<0.0001
ACQ5 score**	1(0.8-2.0)	3.2(2.4-3.8)	<0.0001

*=Paired t-test mean(SD) presented. **= Wilcoxon test median(IQR) presented. Bonferroni correction for multiple testing applied.

Table 6. 7: Paired analysis between baseline and Primary OCS whose nasal brush samples underwent RNA-sequencing

PCA plot

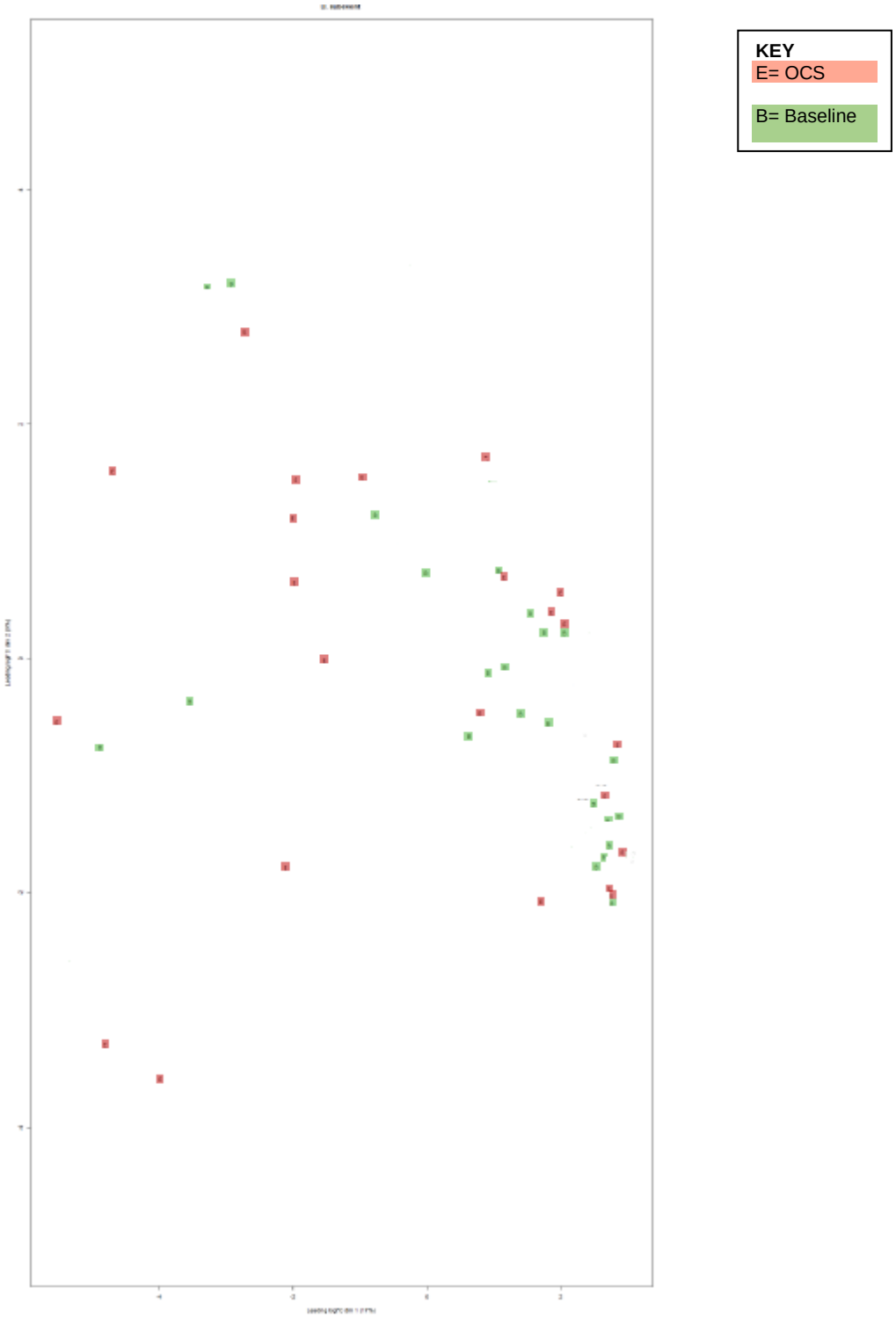


Figure 6. 17: PCA according to Primary OCS and Baseline

Significant differential gene expression in asthma exacerbations requiring OCS.

Comparing Primary OCS to baseline, 4962 genes were downregulated, and 436 were upregulated. Figure 6.22 illustrates the volcano plot of differentially expressed genes. Gender, age, and ICS dose were covariant inputted and adjusted for in the analysis. Due to many differentially expressed genes, an adjusted p-value of $1x10^{-5}$ was set.

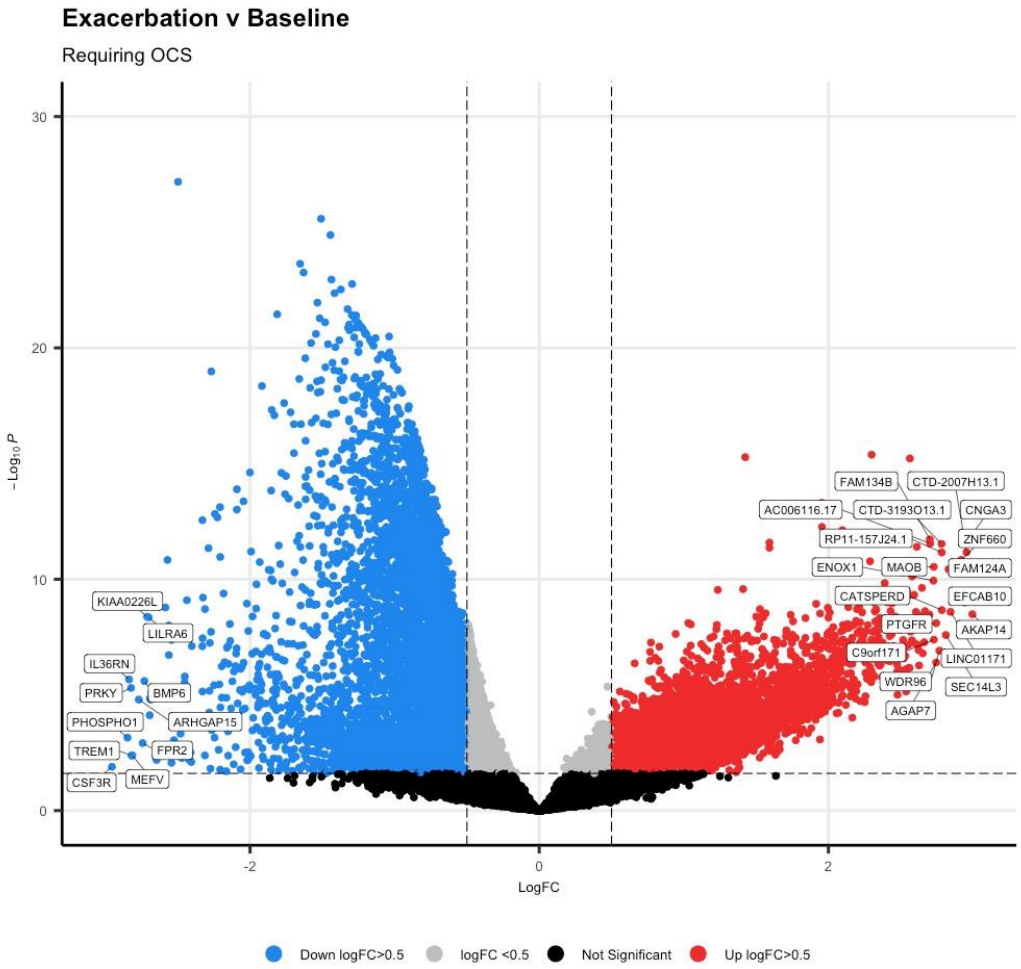


Figure 6. 18: Volcano plot illustrating paired Primary OCS (N=25) vs Baseline (N25) differential gene expression.

The log fold change of individual genes are on the x-axis. The negative log of the genes p-value to the base 10 is on the y-axis. The red represents the upregulation of genes compared to the baseline. The blue represents downregulation. Key genes that are upregulated or downregulated are labelled. Genes that did not cross the FDR of 0.05 are coloured in black.

All nasal brush samples were taken before any course of steroids was provided to the participant for their asthma exacerbation.

On review of the most significant fold change of DEG's obtained from the volcano plot identified and in conjunction with a review of the literature, some genes are novel in being associated with asthma exacerbations, such as **CNGA3** (cyclic nucleotide-gated channel subunit alpha 3) and **CTD-2007H13.1.** **ZNF660** has been associated with wheezing and airway obstruction in preschool children presenting with recurrent wheezing after a viral infection obtained from leucocyte transcriptomes.[347]FAM134B is related to endoplasmic reticulum phagy, which is required for protein clearance. Its significance in asthma is unknown.[348] **KIAA0226L** is an unexplored gene that was downregulated at the point of an asthma exacerbation. **PHOSPHO1** has been identified in a GWAS study of moderate to severe asthma.[349] The receptor of IL36, whose expression was downregulated. IL-36 antagonists have been identified to reduce inflammation in asthma from in-vitro studies. [350]

The heatmap (Figure 6.19) illustrates the gene expression of the top 500 genes upregulated and downregulated grouped to baseline and Primary OCS. It shows that genes associated with exacerbation are upregulated, but compared to the paired baseline, these genes are downregulated.

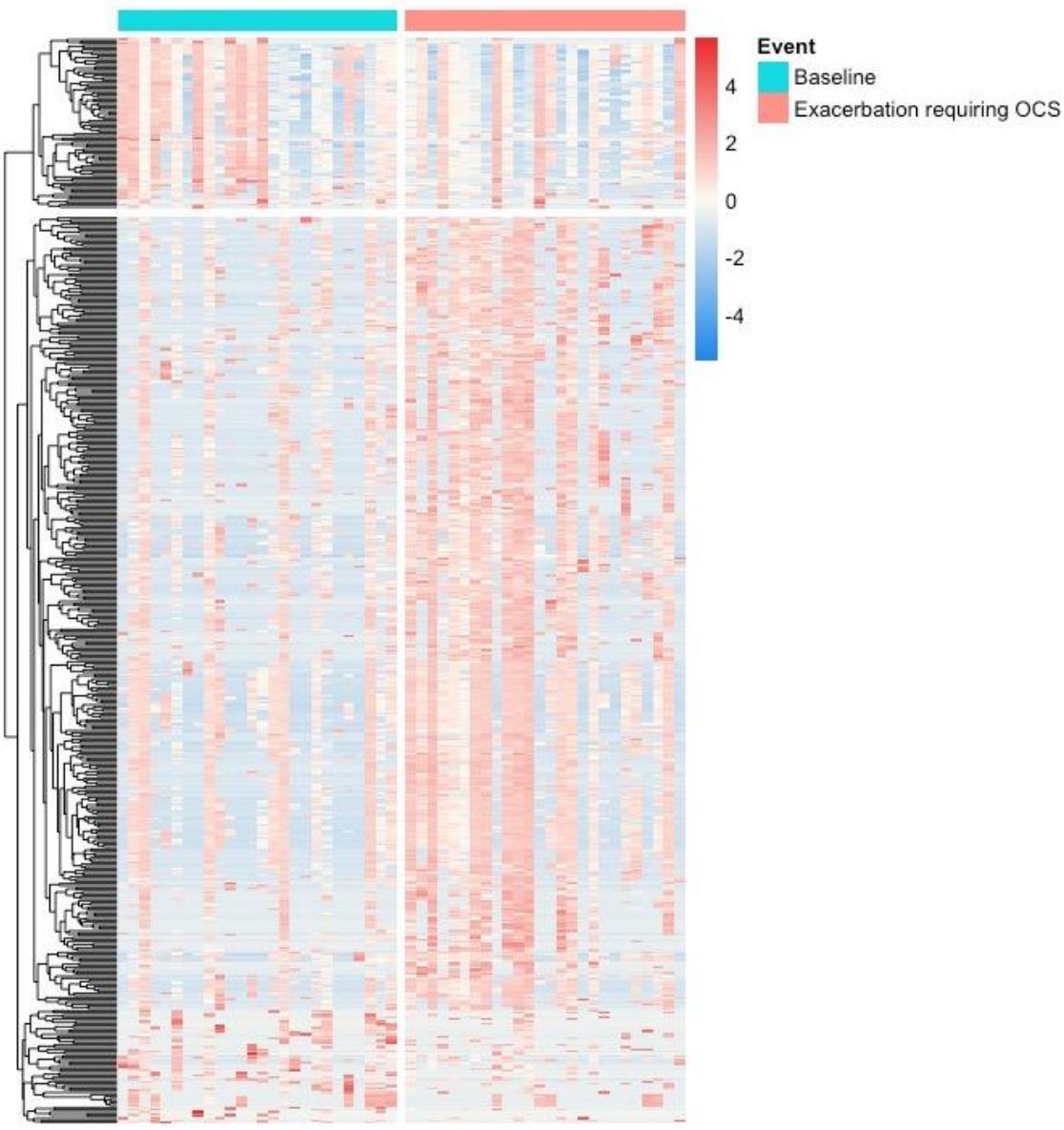


Figure 6. 19: Heatmap of differential gene expression in Primary OCS vs Baseline shows upregulation of genes at exacerbations requiring OCS.

Top 500 DEG determined using Log_2 FC and FDR p<0.05.

The top 10 highly expressed and lowly expressed genes identified by IPA are shown in Table 6.8 and 6.9, and their relation to asthma is described. After reviewing the literature, I found that most of these molecules have

been identified in microRNA studies, and their direct role in asthma exacerbations is unclear. **CWH43** was identified in a study which identified differentially methylated genomic regions in rhinovirus-induced wheezing and asthma in children after whole blood RNA sequencing. [351] Downregulated genes were **IL36A** and **CXCR1**, which are associated with neutrophilic inflammation.

Gene	Protein name	Fold Change	Known Relation to asthma	Chromosome Location	Protein Function/Significance
CWH43	Cell Wall Biogenesis 43 C-Terminal Homolog	3.799	Y	4p11	The methylated genomic region associated with rhinovirus-induced wheeze + asthma[352]
DCHS2	Dachsous Cadherin-related 2	3.563	Y	4q31.3	Upregulated by degranulated products from eosinophils pre-activated with IL-3[353]
BICC1	Bicaudal C Homolog family RNA Binding Protein 1	3.466	Y	10q21.1	An intergenic variant associated with adult-onset asthma[354]
FAM66D	Family with sequence similarity 66 Member D	3.466	Y	8p23.1	Found to be upregulated in mild asthma in children with inv-8p23.1[355]
CLU1	Clusterin 1	3.388	Y	18p11.32	Clusterin expression levels associated with increased reactive oxidative stress in patients with asthma[356]
TTC29	The tetratricopeptide repeat domain 29	3.355	Y	4q31.22	Negatively correlated asthma-associated gene with miR-199b-5p[357]
PDE7B	Phosphodiesterase 7B	3.349	Y	6q23.3	Associated with reduced cAMP in epithelial cells of allergic asthma[358]
OR7E47P	Olfactory Receptor Family 7 Subfamily E Member 47 pseudogene	3.289	Y	12q13.13	DEG upregulated at baseline in patients with asthma. Expressed in CpG site cg22681114[281]
TMEM212	Transmembrane Protein 212	3.184	Y	3q26.31	Negatively correlated asthma-associated gene with miR-199b-5p[357]
DNAAF6	Dynein Axonemal Assembly Factor 6	3.164	Y	Xq22.3	Associated with defective airway cilia and Primary Ciliary Dyskinesia[359]

Table 6. 8: Top 10 genes that are highly expressed in Primary COS and their respective fold change.

Gene	Protein name	Fold change	Known relation to asthma	Chromosome Location	Protein Function/Significance
IL36A	IL36	-3.385	Y	2q14.1	Associated with upstream amplification of neutrophilic lung inflammation[350]
RUBCNL	Rubicon Autophagy Enhancer / Pacer Protein	-2.711	Y	13q14.13	Found to be downregulated in sensitised children with allergic airways disease[360]
LILRA6	Leukocyte Immunoglobulin-Like Receptor A6	-2.702	Y	19q13.42	Associated with immune repose in asthma in the analysis of gene ontology analysis in asthmatics[361]
SLC25A18	Solute Carrier Family 25 Member 18	-2.585	Y	22q11.21	Mitochondrion-related gene signature in neutrophilic asthma[362]
RPL21P122	Ribosomal Protein L21 Pseudogene 122	-2.571	N	17p12	-
CLDN18	Claudin 18	-2.563	Y	21q21.3	Claudin deficiency associated with epithelial barrier dysfunction in asthma[363]
CXCR1	CXC motif chemokine receptor	-2.562	Y	2q35	CXCR1 involved in peripheral neutrophil migration in asthma[364]
ADGRE3	Adhesion G protein-coupled receptor 3	-2.544	Y	19p13.12	Downregulated in asthma associated with chronic rhinosinusitis with nasal polyps[365]
MRPS15P1	Mitochondrial Ribosomal Protein S15 Pseudogene 1	-2.498	N	15q24.1	-
Cl6orff95-DT	Chromosome 16 open reading frame 95	-2.437	N	16q24.2	-

Table 6. 9: Top 10 genes that are highly expressed in Primary COS and their respective fold change

Upstream Regulators

To contextualise the gene expression changes seen in Primary OCS events, the IPA Upstream Regulator© analysis was undertaken (Figure 6.21). The analysis is based on the Ingenuity® Knowledge Base, which examines the transcriptional regulator and its target gene. The software identifies the present targets of transcriptional regulators.

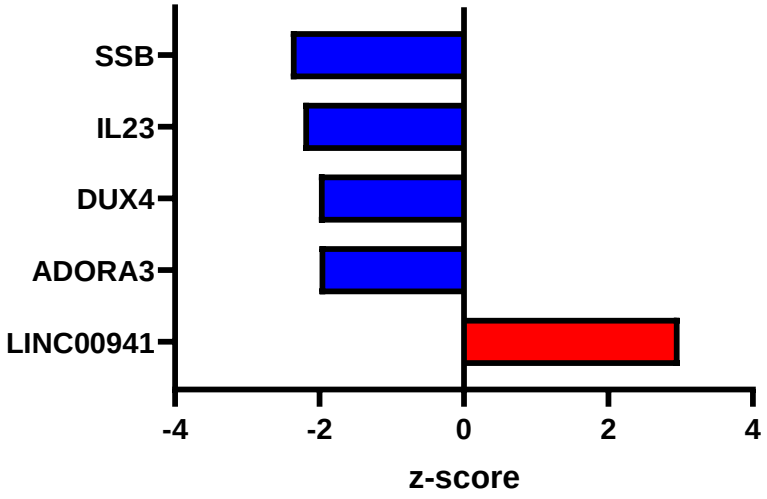


FIgure 6. 20: Upstream regulators identified in Primary OCS, identified by Ingenuity Knowledge Base.

Red = predicted activation state to be activated. Blue=predicted activation state to be inhibited. Absolute Z-scores ≥+/- 2.0 with a P-value >1x10^{-4} were used as a cut-off for significance.

LINC00941 is a long noncoding RNA associated with the development of fibrosis via the upregulation of fibroblasts and myofibroblasts, which produces an increased extracellular matrix commonly seen in pulmonary fibrosis.[366] . Upstream regulators predicted to have inhibitory effects include the single-stranded DNA binding protein (**SSB**), which prevents the degradation of single-stranded DNA from degrading. The gene has also been negatively correlated with the microRNA miR-199N-5p in microRNA studies of bronchial biopsies from patients with asthma. [357] **IL-23** is associated with type 2 airway inflammation and has been a target in phase 2 studies of Risankizumab as a potential biological target for severe asthma.[367] This is suggestive of other pathways apart from T2-inflamamtion being upregulated.

Canonical pathways

Pathway analysis using IPA revealed seven canonical pathways: 3 are upregulated, and 4 are downregulated (Table 6.10 and Figure 6.22). The expected activity of the gene is denoted by (↑=activated) or (↓=inhibited).

Module	Genes	Fold change & p-value	Synopsis of function
Cellular stress & injury	CELA3B↑ HNF1-α↓ MMP27↑ PAX2↓ PPP3R2↑	0.636, 1.86E-06 -0.818, 8.70E-07 0.482, 1.14E-06 -1.100, 2.82E-07 0.581, 4.37E-07	**Chaperone Mediated Autophagy Signalling Pathway** CELA3B CpG site associated with DNA methylation in asthma[368] MMP27 regulates airway remodelling[369]
Intracellular + secondary messenger signalling	NPY2R↑ TRAJ2↓ TRAV5↓ TRGJ↓	0.763, 1.11E-10 -0498, 6.05E-0.6 -0.781, 2.47E-08 -0.483, 7.10E-0.6	**G Protein Signalling mediated by Tubby** Coined "Tubby" as this protein is upregulated in delayed onset obesity in mice. [370]Tubby protein act as a transcription factor and couple to G-protein activity. Found to be upregulated in severe asthma[371] Upregulation of T cell receptor alpha in murine model of asthma [372]
Transcriptional regulation	LTB↑ NFGR↑ TNFSF-12↑ TNFSF-18↑ TRAJ2↑	2.101, 4.79E-.06 2.569, 1.99E-0.7 -2.569, 1.99E-07 -2.336, 1.39E-07 0.498, 6.05E-06	**NFKBIE signalling pathway** NF-kB transcription factor upregulated in asthma.[373] Lymphotoxin Beta drives airway smooth muscle hyperresponsiveness and airway remodelling.[374] Nerve Growth Factor produced by smooth muscle cells, endothelial cells and immune cells in asthma exacerbations[375]
Cellular growth, proliferation and development	ADGRE3↓ ADGRG4↓ CXCR1↑ CCR9↑ APLNR↓	-2.544, 6.23E-07 -1.011, 5.84E-0.8 2.562, 2.13E-06 0.748, 2.49E-0.6 -1.524, 1,49E07	**FAK signalling** Upregulation of G protein-coupled receptor by inhaled therapies leads to bronchodilatation.[376] FAK regulates airway smooth muscle signalling.[377] CCR9 is expressed in inflammation and has a vital role in eosinophil and lymphocyte recruitment[378]
Humoral immune response	COL12A1↑ COL13A1↓ COL4A5↑ HLA-DQB2↑	2.422, 1.77E-07 -1.242, 4.45E-06 1.444, 5.16E-06 2.577, 6.50E07	**IL-4 signalling** COL41 associated with airway remodelling[379]
Pathogen induced signalling	CSF2↑ DEFB118↑ DEFB123↑ INF-γ↑ IL-10↓ IL-26↑	1.026 1.02E-07 0.488 2.63E-0.6 0.499 5.05E-0.6 0.925 6.97E-07 -1.802 1.57E-10 0.651 3.61E-06	**TH17 Activation pathway** Human β-Defensin 2 mutations associated with asthma in children[380] Il-10 inversely correlates with the severity of asthma.[381] IL-26 upregulated in uncontrolled asthma[382]
Cellular immune response	CD28↓ HLA-C↓ TRAJ2↓ HLA-DQB2↑ PLD5↑	-1.569 3.51E-07 -0.773 3.11E-06 -0.498 6.05E-06 2.577 6.50E-07 0.996 5.60E-.9	**CTLA-4 signalling in cytotoxic T-lymphocytes pathway** CD28 blocking found in steroid-resistant asthma[383] 4 SNP's in the CTLA-4 gene were associated with asthma, atopy and reduced lung function[384] DQB2 expression correlated with reduced lung function[385] PDL5 downregulates NPSR1 involved in pathogenesis of asthma[386]

Table 6. 10: Pathways involved and the identified genes and their expected activity (upregulated=↑ or downregulated=↓) in Primary OCS.

Fold change and p-values stated as well as description of the pathway which the genes contribute to.

Reviewing the canonical pathway analysis for exacerbations that required OCS revealed pathways associated with T1 and T2 inflammation and airway remodelling. The Chaperone-Mediated Autophagy Signalling Pathway involves genes responsible for normal airway functioning, such as **MMP27**, a matrix metalloproteinase involved in the degradation of proteins in the extracellular matrix. This is thought to be upregulated and has been identified to contribute to the emphysematous changes observed in COPD. This has been unexplored in asthma.[369] The Tubby pathway, which is associated with maturity-onset obesity in mice, is, and its activity is upregulated in severe asthma. However, the relevance of the genes in this pathway is unexplored in the context of asthma.

The Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon (**NFKBIE**) pathway was upregulated. Genes involved in smooth muscle cell airway remodelling include Nuclear factor-κB (**NF-κB),** which is upregulated in murine airway smooth muscle cells.[373] The **FAK** signalling pathway is associated with airway bronchodilation and genes **ADGRE3** and **ADGRG4** are downregulated. Edwards and colleagues highlighted the importance of NF- κB in asthma and COPD.[387] OCS therapy has been shown to down-regulate NF- κB activity[388]Chemokines associated with neutrophilic inflammation, such as CXCR1, were activated. CCR9 has been identified in murine models and was found to be upregulated after OVA administration and gene knockout. Still, it had no influence on IL-4 and IL-5 cytokines, suggesting a role of both neutrophilic and eosinophilic inflammation.[378] Another pathway involved in Primary OCS was the Th-17 pathway, which is associated with CD4+ T-cells associated with T2-low asthma that are less responsive to steroids.[389] **IL-10** is associated with Th17 pathways.

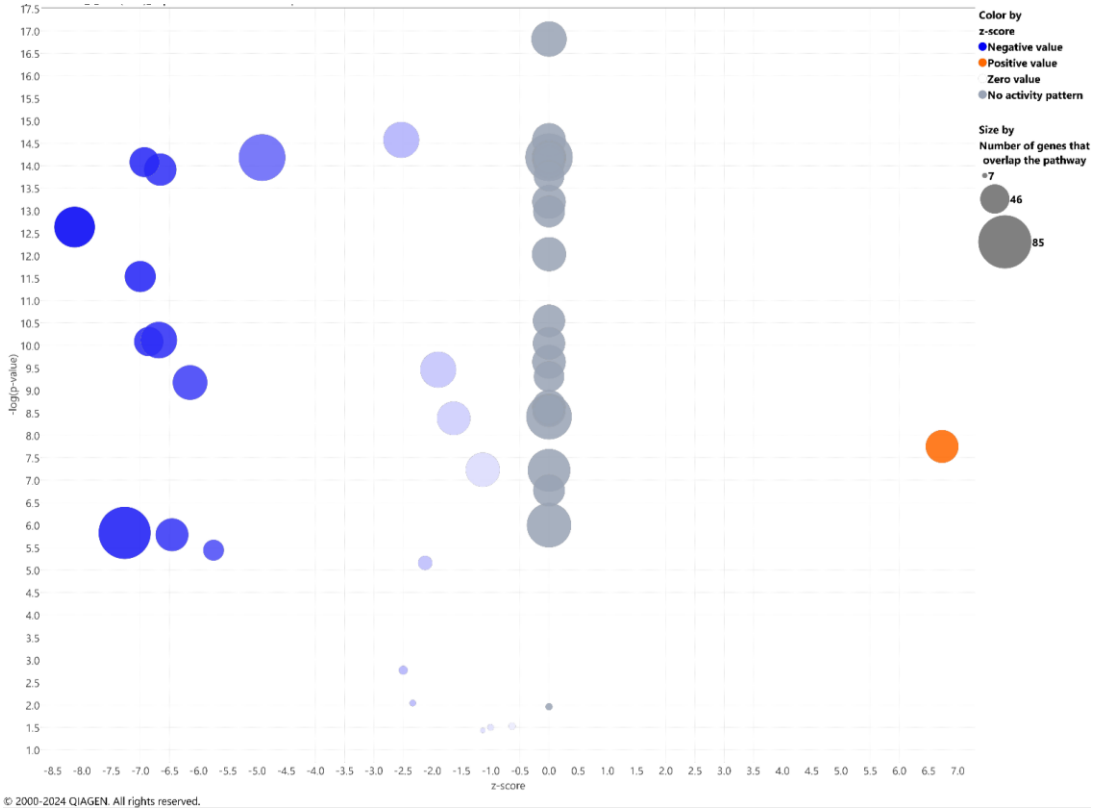


Figure 6. 21: Bubble plot of downstream functional pathway association in relation to Primary OCS.

Pathways with z-scores on the x axis and -log p-values on the Y-axis and in log format due to the wide range of p-values. The bubble's size indicates the number of genes involved in the pathway. The details of those bubbles are illustrated further in Figure 6.25. Adjusted p-value of 0.05 was used for all data

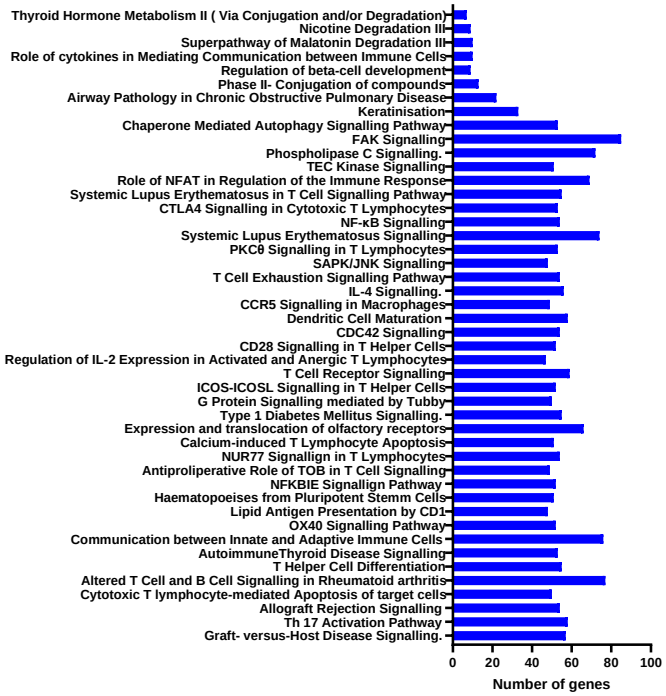


Figure 6. 22: Pathways associated with Primary OCS-associated gene expression in the APEX study.

The number of genes identified in that pathway is illustrated. An adjusted p-value of 0.05 was used for all data.

Figure 6.21 and 6.22 illustrates the genes associated with a particular pathway. Pathways with the highest number of genes included pathways involving innate and adaptive immunity, T & B-cells in Rheumatoid arthritis, CDC42 which is a helper T-cell pathway found to be over-expressed in children with asthma who were obese[390]. The pathways with high amounts of genes can be related to the pathogenesis seen in asthma.

Disease and Function analysis

Based on the RNA sequence data, an analysis of biological functions or disease processes expected to increase, or decrease was conducted. Z scores were used for this analysis to assess increased or decreased activity, and z-scores greater than or equal to +/—2 were only included. Overall, there is an increase in the activity of inflammatory cells. A decrease in macrophage activity was noted. There was also a decrease in the secretion of endogenous steroid hormones. This would explain some of the common features seen in asthma exacerbations.

Category	Increased or Decreased Activity according to z-score	Activity
Haematological System Development	Increased	↑Formation of leucocytes ↑Formation of APC. ↑Formation of haematopoietic progenitor cells.
Cellular Growth and Proliferation	Increased	↑Formation of leucocytes ↑Formation of APC ↑Formation of eosinophils ↑Stimulation of lymphatic cells
Cellular development	Increased	↑Colony formation of leucocytes ↑Colony formation of phagocytes ↓ Formation of macrophages
Tissue Development	Increased	↑Colony formation of leucocytes ↑Colony formation of phagocytes ↓Development of macrophages
Cell signalling	Decreased	↓Concentration of cAMP
Lipid Metabolism	Decreased	↓Secretion of hormones ↓Decreased secretion of steroids ↓Decreased conjugation of 12-hydroxyeicosetetranenoic acid
Immune cell trafficking	Decreased	Priming of leukocytes.

Table 6. 11: Disease and Function IPA analysis in Primary OCS.

Key categories associated with a z-score of +/-2 included. The activity according to Z-score is highlighted, and some of the key features of each category are described, stating increased or increased activity.

Paired analysis of Primary No OCS to Baseline

Demographics

The next analysis performed is a paired analysis of exacerbation events in which a course of steroids was not required for a suspected exacerbation (Primary No OCS). This was compared to parameters at baseline study entry in APEX.

The table below illustrates key clinical parameters. The median age was 48.7, and 70.8% (N=17) of Primary No OCS were female. There was no significant difference in T2-status at baseline to Primary No OCS in either FeNO or blood eosinophil count (23.5 ppb at baseline vs 27.0 ppb at primary No OCS for FeNO p=0.52 and blood eosinophils, 0.17 $x10^9$/L vs 0.18 $x10^9$/L at primary OCS p=0.05). This, again, could reflect the sample size. No significant difference at Primary No OCS was noted in lung function measured as the FEV1% predicted. However, there was an increase in ACQ5 score with loss of asthma control.

	Baseline	**Primary No OCS**	**p-value**
	N=24	N=24	
Female N(%)	17(70.8)		
Age Yr(IQR)**	48.7(14.6-61.2)		
FeNO (ppb) **	23.5(13.5-40.5)	27.0(15.0-33.5)	0.52
Eosinophil ($x10^9$/L) **	0.17(0.08-0.31)	0.18(0.11-0.27)	0.05
FEV1% pred (SD) *	88.1(16.5)	85.63(15.5)	0.80
ACQ5 score(IQR)**	1.40(0.80-2.35)	2.80(2.25-3.60)	<0.0001

*=Paired t-test mean(SD) presented. **= Wilcoxon test median(IQR) presented. Bonferroni correction for multiple testing applied.

Table 6. 12: Paired analysis between baseline and Primary No OCS whose nasal brush samples underwent RNA-sequencing.

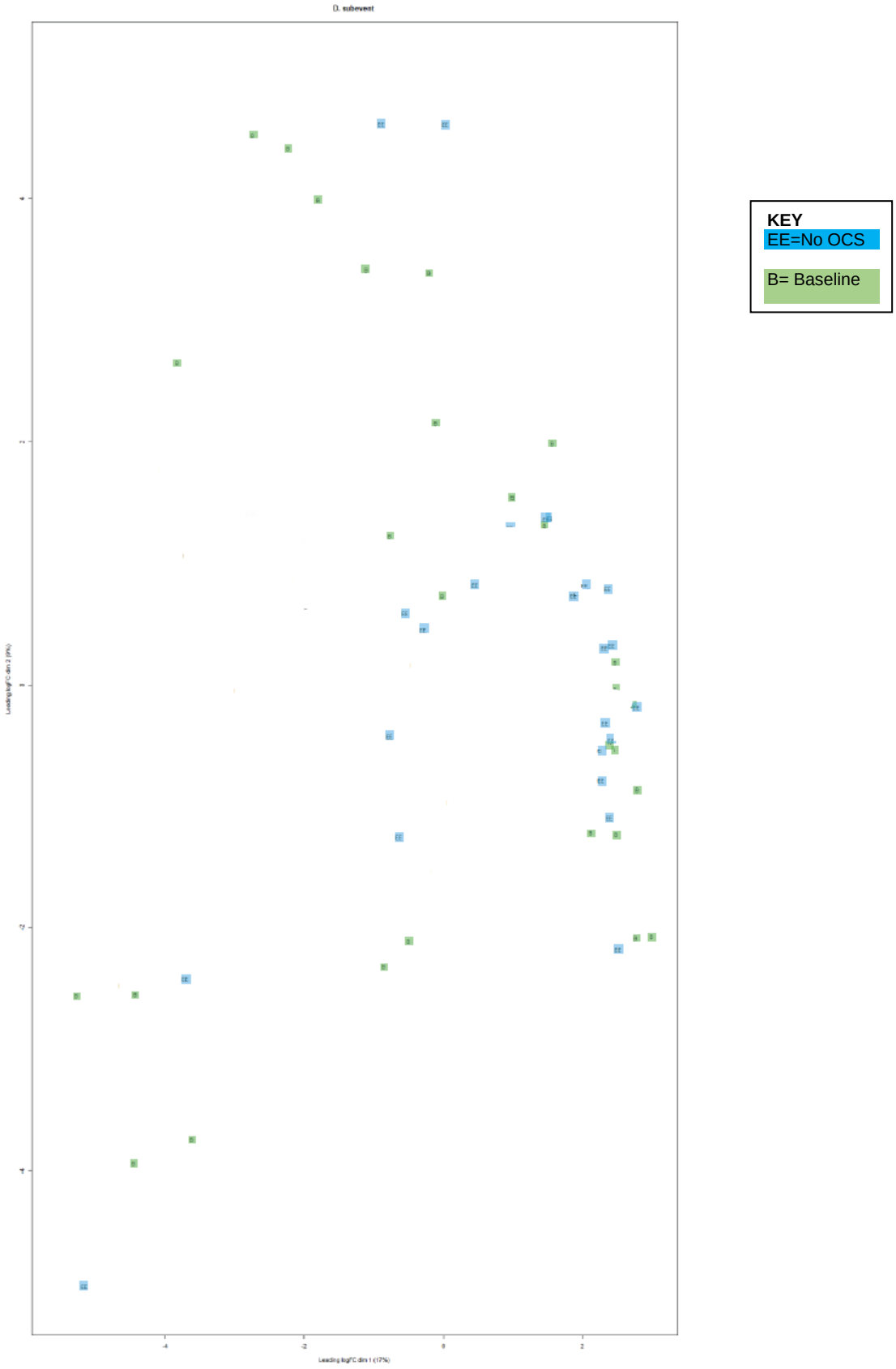


Figure 6. 23: PCA according to Primary OCS and Baseline. No clusters were identified

Significant downregulation of genes in differential gene expression in asthma exacerbations not requiring OCS.

Comparing Primary No OCS to baseline, 1553 genes were downregulated, and 2271 were upregulated. Using a p-value of $1x10^{-3}$, Figure 6.25 illustrates the volcano plot of differentially expressed genes. Gender, age, and ICS dose were covariant inputted and adjusted for in the analysis.

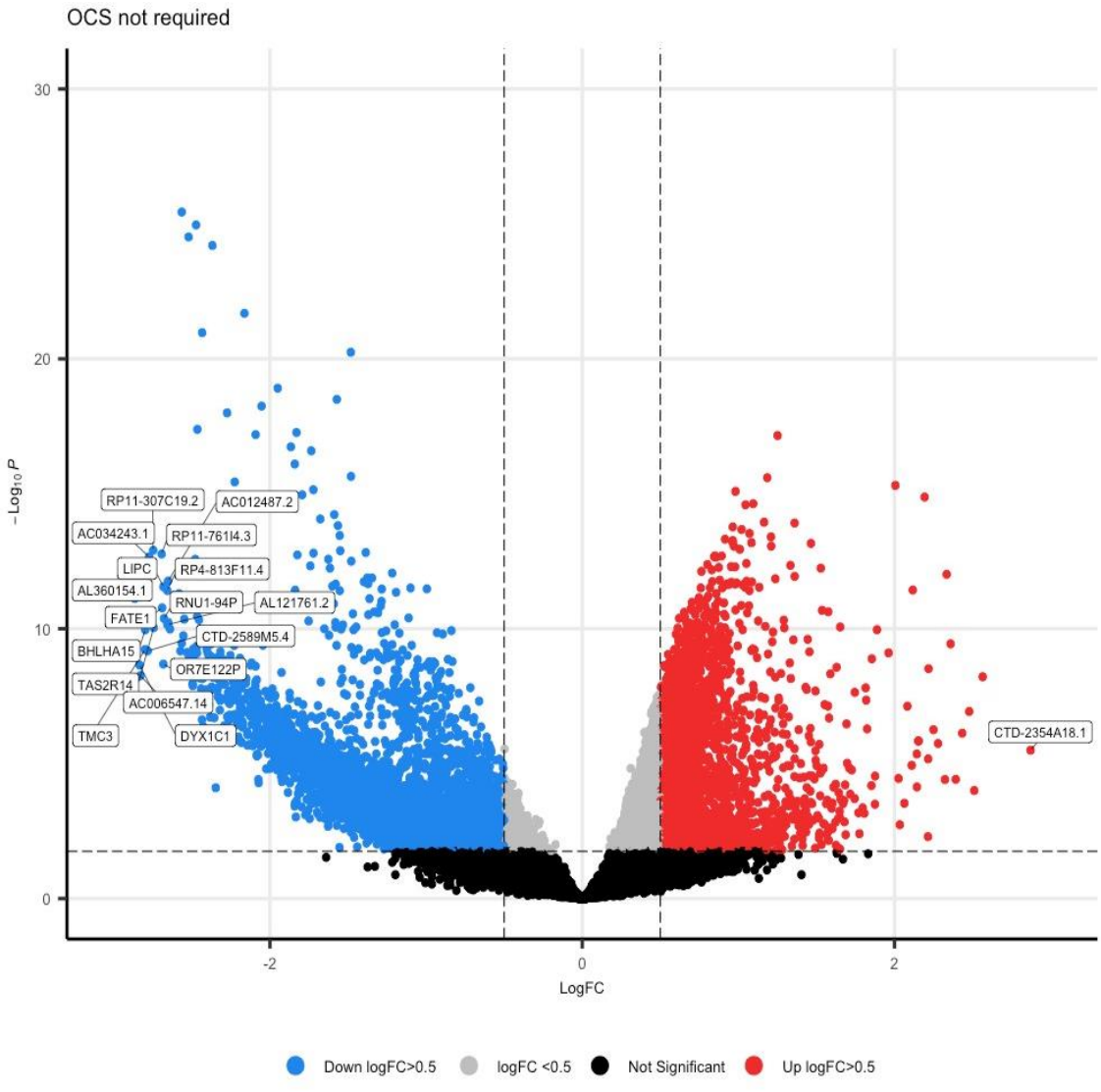


Figure 6. 24: Volcano plot illustrating paired Primary No OCS (N=24) vs Baseline (N=24) differential gene expression.

The log fold change of individual genes is on the x-axis. The negative log of the gene's p-value to the base 10 is on the y-axis. The red represents gene upregulation compared to the baseline, and the blue represents downregulation. Key genes that are upregulated or downregulated are labelled. Genes that did not cross the FDR of 0.05 are coloured in black.

The volcano plot visualised shows a significant number of downregulated genes. **CTD-2345A18.1** is of unknown significance in the context of asthma. **BHLHA15** is associated with mucus secretory cell function after performing RNA sequencing of 681 nasal epithelium samples in children [391]**FATE1** has been demonstrated to be upregulated in chronic rhinosinusitis with nasal polyps. Through single-cell transcriptomics, TMC3 has been associated with developing broncho-constriction and dilation, directly affecting end-expiratory lung volumes.[392]. The upregulated gene CTD-2354A18.1, is a non-coding RNA novel in asthma.

The heat map (Figure 6.25) illustrates the top 500 expressed genes grouped according to the baseline study entry into APEX and the Primary No OCS event. At the Primary No OCS event, these genes seem to be downregulated, and is upregulated at the paired baseline.

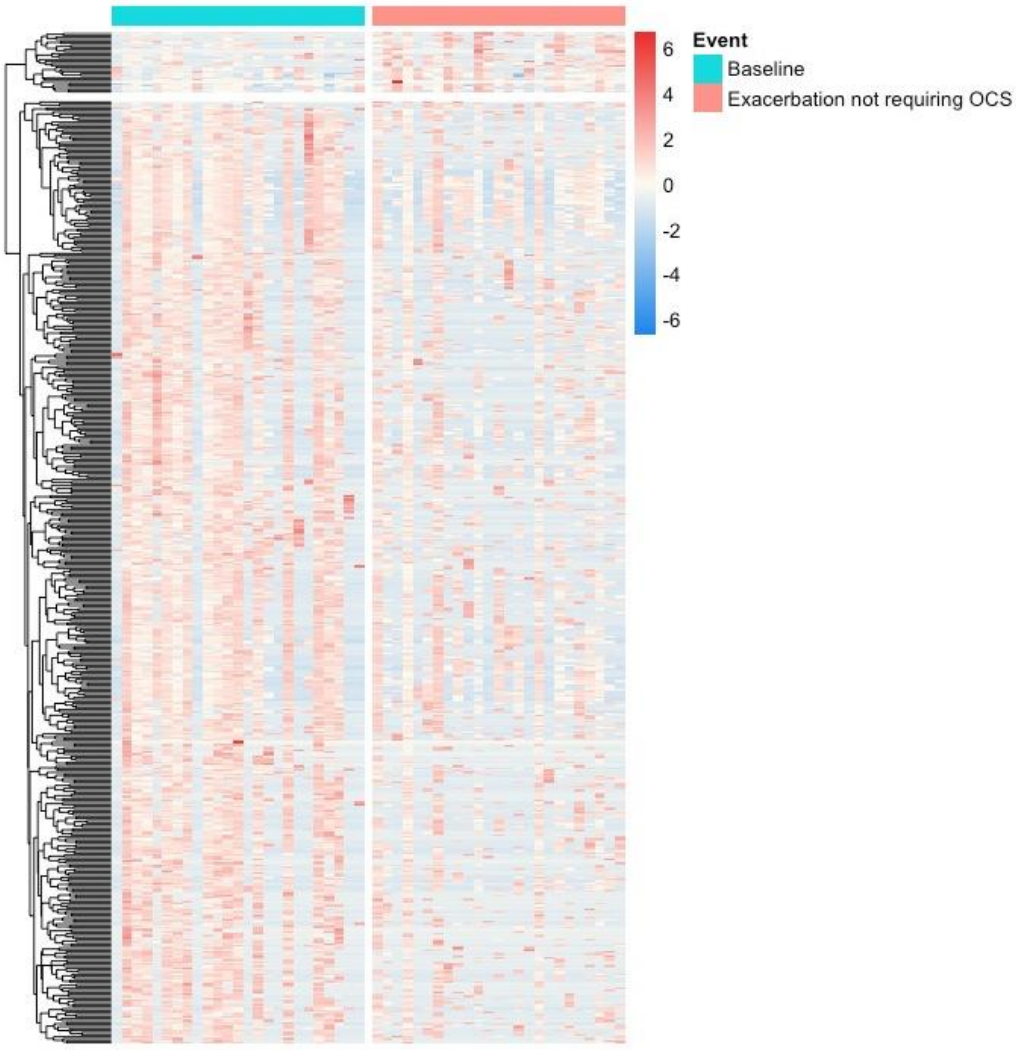


Figure 6. 25: Heatmap of differential gene expression in Primary No OCS vs Baseline.

Top 500 DEG determined using Log_2 FC and FDR p<0.05.

Gene	Protein name	Fold Change	Known Relation to asthma	Chromosome Location	Protein Function/Significance
LINC02582	Long Intergenic Non-Coding RNA 2582	2.871	Y	18q22.3	MicroRNA 200 targets LIN0258, which is related to asthma severity[393]
CNTN1	Contactin 1	2.565	Y	12q12	Found to promote monocyte-derived dendritic cell-derived T-cell response in asthma [394]
KLK7	Kallikrein-related peptidase 7 (KLK7)	2.511	Y	19q13.41	Lympho-epithelial Kazal-type-related inhibitor (LEKTI) inihibits KLK7 which offers protection of epithelium against allergens[395]
NPR3	Natriuretic Peptide Receptor 3	2.478	Y	5p.13.3	Associated with Bronchial hyperresponsiveness[396]
LGI3	Leucine-rich glioma inactivated 3	2.433	N	8p21.3	-
IL36RN	Interleukin-36 receptor antagonist	2.392	Y	2q14.1	Levels down-regulated in asthmatics and reduces IL-36 inflammation [350]
IL36A	IL36	2.359	Y	2q14.1	Associated with upstream amplification of neutrophilic lung inflammation[397]
SPRR2C	Small proline-rich protein (SPRR) family	2.334	Y	1q21.3	Associated with IL-13 inflammation in epithelial cells in asthmatics[398]
PLA2G2F	Phospholipase A_2	2.323	Y	1p36.12	Associated with the pathogenesis of asthma and allergic disease and features of AHR[399]
RPTN	Reptin	2.279	Y	1q21.3	SNP identified in GWAS study as having a role in asthma[400]

Table 6. 13: Summary of top 10 genes that were upregulated at exacerbation events where OCS was not required versus baseline

A review of the top 10 molecules upregulated and downregulated during exacerbation events in which OCS were not required revealed an insight into the mechanistic pathways associated with this (Table 6.13-6.14). The most upregulated molecule was **LINC02582**, a non-coding RNA associated with microRNA 200c, which correlated with worsening asthma severity. There was upregulation of genes, which are found in T1-associated inflammatory pathways such as CNTN1, and SPRR2C, associated with IL-13 inflammation, which is related to T2-inflammatory pathways. Two genes associated with bronchial hyperresponsiveness

were upregulated too - **PLA2G2F** and **NPR3**. Another upregulated gene was **IL36A**, which was associated with inflammation and is attributed to neutrophilic inflammation**.**

Table 6.14 below shows the top-downregulated genes in exacerbations in which OCS was not required. CDKL4 is a gene involved in the mTOR signalling pathway. As identified in Chapter 1, the mTOR pathway has gained interest in preventing the effects of rhinovirus infection. It has been found to be upregulated in the airways of patients with asthma and is involved in goblet cell upregulation and airway metaplasia. [401]

Gene	Protein name	Fold change	Known Relation to asthma	Chromosome Location	Protein Function/Significance
C2orf72	Chromosome 2 Open Reading Frame 72	-3.890	Y	2q37.1	SNP downregulated in persistent asthma in childhood asthma[402]
CHMP5P1	CHMP5 Pseudogene 1	-3.093	N	Xp11.3	-
CDKL4	Cyclin Dependent Kinase Like 4	-3.047	Y	2p22.1	Regulates mTOR signalling, which is responsible for metaplasia of airway epithelium in asthmatic airways [401]
LRRIQ3	Leucine Rich Repeats and IQ containing 3	-3.036	N	1p31.1	Found to be downregulated in GWAS studies of allergic rhinitis[403]
RN7SKP24	RN7SK pseudogene 4	-3.025	N	9p13.3	-
KRT39	Keratin 39	-3.018	Y	17q21.2	SNP rs2107212 associated with obesity only in asthmatics[404]
GSTM1	Glutathione S-transferase mu 1	-3.009	Y	1p13.3	Null genotype is associated with severe adult-onset genotype. [405]
DNAAF4	Dynein Axonemal Assembly Factor 4	-2.836	N	15q21.3	Point mutations in PIHD3 affect the DNAAF4-HSP90 complex associated with primary ciliary dyskinesia[406]
CCDC188	Coiled-coil domain-containing protein	-2.830	N	22q11.21	-
TMC3	Transmembrane Channel Like 3	-2.800	Y	15q25.1	Expressed by lung-innervating vagal ganglia reduced expression associated with bronchoconstriction [407]

Table 6. 14: Summary of top 10 genes that were downregulated at exacerbation events where OCS was not required versus baseline

Upstream regulators

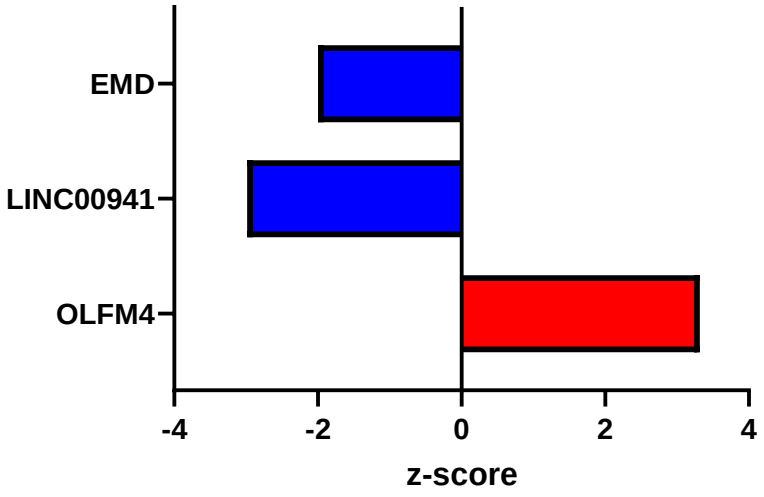


Figure 6. 26: Upstream regulators identified in Primary No OCS, identified by Ingenuity Knowledge Base.

Red = predicted activation state to be activated. Blue=predicted activation state to be inhibited. Absolute Z-scores ≥ +/-2.0 with a P-value >$1x10^{-4}$ were used as a cut-off for significance.

Three upstream regulators met the z-score threshold of ≥+/-2.0. **Neutrophils produce OLFM4 (Olfactomedin 4)**, and serum levels were found to be raised in 35 uncontrolled asthmatics and correlated with sputum neutrophil count.[408] **EMD** (emerin), found in the nucleus of neutrophils', is an upstream regulator whose activity is inhibited.[409]Of interest, **LINC00941**, which was an activated upstream regulator in the paired Primary OCS analysis, is now downregulated in exacerbations which did not require OCS.

Canonical pathways analysis of Primary No OCS.

Core modules identified through IPA include those associated with "signal transduction" and "homeostasis" and upregulation of "Developmental Biology." Table 6.17 and Figure 2.7 detail the key genes related to the canonical pathways. The expected activity of the gene is denoted by (↑=activated) or (↓=inhibited).

Module	Genes	Fold change & p-value	Synopsis of function
Signal transduction	ADCY2↓ ADCY5↓ ADRA2A↓ AGT↓ ANXA1	-1.319, 7.80-05 -1.522, 3.77E-04 -2.240, 1.97E-06 -1.388, 9.81E-04 0.518, 4.59E-04	**G alpha (i) signalling events** Adenylate cyclase 2 is upregulated after exposure to NO_2 in asthmatics in a GWAS study of childhood asthma.[410] Alpha-2A-receptor (ADRA2A) is upregulated in GWAS on bronchial biopsies of asthma compared to control. Angiotensinogen AGT gene enhances bronchoconstriction and peptides that accumulate in airways. [411]
Haemostasis	ACTA1↑ ADCY10↑ ADCY5↑ ADGRB2↓	1.143, 8.26E-07 2.040, 1.50E-05 1.522, 3.77E-04 -1.569, 2.44E-04	**Cellular effects of sildenafil** ACTA1gene upregulated in elderly patients with asthma after sputum transcriptomic analysis.[412] SNP variant of ADVY9 associated with severe asthma and correlates with reduced BDR[413]. ADBR2- Beta-2 Adrenergic receptor.
Developmental Biology	CASP14↑ EVPL↑ JUP↑ KLK12↑ KRT12↑	0.690, 2.90E-05 0.611, 1.31E-04 0.3989, 2.00E-04 1.487, 4.45E-04 0.576, 3.06E-04	**Keratinisation** CASP14 was identified as a regulatory gene for cell apoptosis and upregulated in a GWAS study of severe asthma[349]

Table 6. 15: Pathways involved in the identified genes and their activity in Primary No OCS (upregulated=↑ or downregulated=↓).

Genes associated with this pathway were related to nitric oxide inflammation. One pathway was shared with Sildenafil, which is known to enhance NO-mediated vasodilatation.[414] Genes associated with this pathway were **ACTA** variants. There was downregulation of **AGT,** which enhances bronchoconstriction in aspirin-intolerant asthma. AGT produces Angiotensin II, which is a vasoconstrictor of smooth muscles[411] The adenyl cyclase (**ADCY**) gene is involved in bronchodilatation and immunomodulation and was reduced in Primary No OCS. [413]

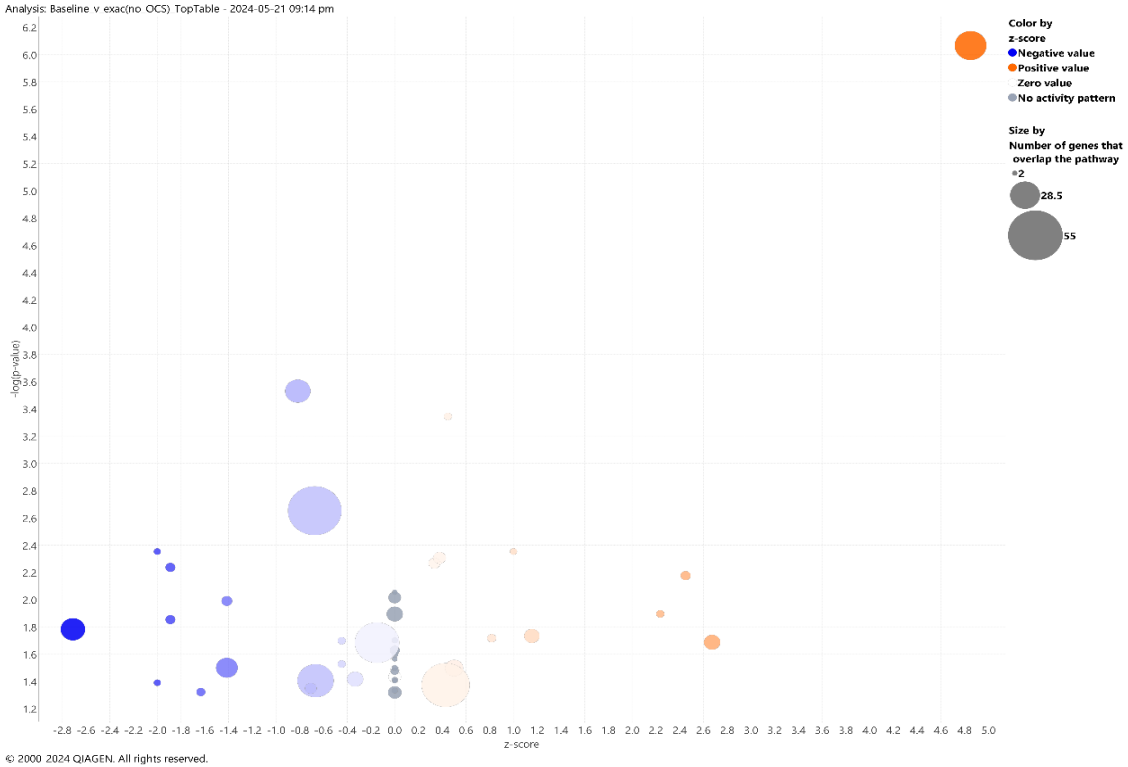


Figure 6. 27: Bubble plot of downstream functional pathway association about Baseline Exacerbators.

Pathways with z-scores on the x axis and -log p-values on the Y-axis and in log format due to the wide range of p-values. The bubble's size indicates the number of genes involved in the pathway. Adjusted p-value of 0.05 was used for all data. Details of the bubbles and gene quantities are in Figure 6.28.

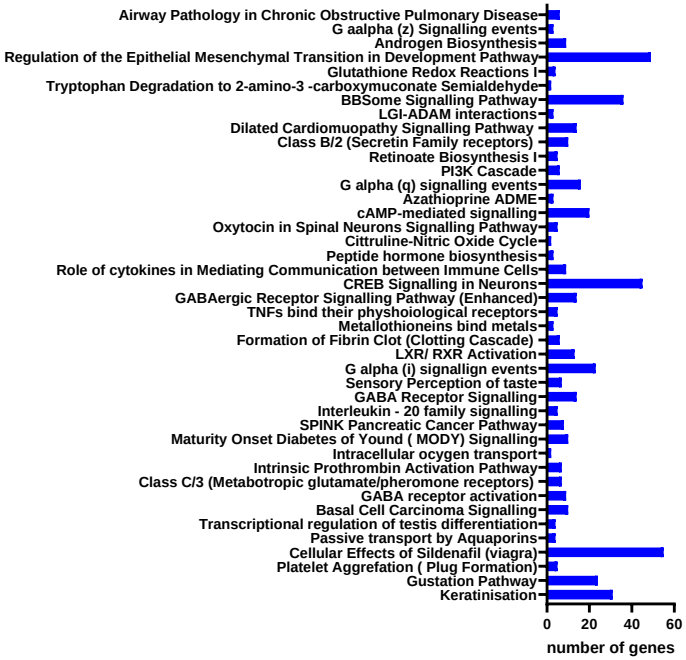


Figure 6. 28: Pathways associated with Primary No OCS-associated gene expression in the APEX study.

The number of genes identified in that pathway is illustrated. An adjusted p-value of 0.05 was used for all data.

Figure 6.28 illustrates the frequency of genes associated with pathways. The pathways with the most genes included the inflammatory pathway associated with sildenafil, the CREB signalling pathway and a pathway associated with the regulation of mesenchymal cells.

Disease and Function analysis

Based on the RNA sequence data, an analysis of biological functions or disease processes expected to increase or decrease was conducted. Z scores were used for this analysis to assess increased or decreased activity, and z-scores greater than or equal to +/—2 were only included.

Category	Increased or Decreased Activity according to z-score	Activity
Small Molecule Biochemistry	Increased	↑metabolism of terpenoid, cAMP, neurotransmitter release, cyclic nucleotides ↓transport of amino acids.
Molecular transport	Increased	↑neurotransmitter release, cyclic nucleotides, cAMP ↓ calcium, transport of amino acids
Nucleic Acid Metabolism	Increased	↑cyclic nucelotides, cAMP
Cell signalling	Increased	↑cAMP ↓calcium
Cellular Function and Maintenance	Decreased	↓reorganisation of actin cytoskeleton, calcium
Tissue Development	Decreased	↓stromal cells, extracellular matrix

Table 6. 16: Disease and Function IPA analysis in Primary OCS.

Key categories associated with a z-score of +/-2 included. The activity according to Z-score is highlighted, and some of the key features of each category are described, stating increased or decreased activity.

This analysis has identified increased terpenoid activity, a terpene derivative that contains oxygen molecules. Studies have identified it as reducing inflammation associated with IL-1, TNF-α, and NF-κB cytokines.[415] There was also increased activity of cAMP involved in molecular messengers of physiological pathways. In Primary No OCS events, pathways associated with airway remodelling were reduced,

such as activation of stromal cells and genes related to extracellular matrix formation.

Commonly Expressed genes between Primary OCS and Primary No OCS.

The Venn diagram (Figure 6.30) reveals that Primary OCS and Primary No OCS events shared 10,016 common genes, suggesting common mechanisms driving both pathways. However, significantly more genes were uniquely expressed in Primary OCS events than in Primary No OCS events.

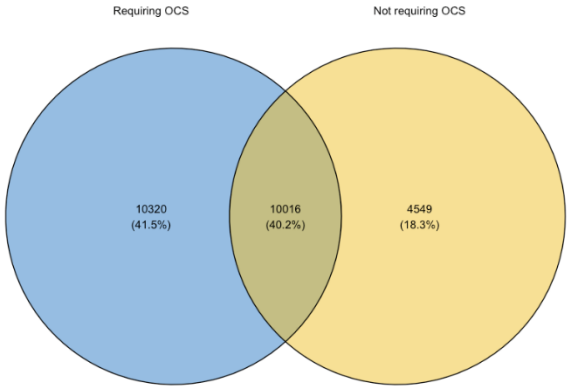


Figure 6. 29: Venn diagram illustrating 10016 genes commonly expressed between those exacerbation events requiring steroids versus those not requiring steroids in the APEX study.

Exploring this further, Venn diagrams were created for downregulated genes in Primary OCS versus Primary No OCS (Figure 6.30) and a separate Venn diagram for upregulated genes in Primary OCS versus No OCS (Figure 6.31). 782 common genes were downregulated in Primary OCS vs Primary No OCS, and 134 common genes were upregulated. There were more genes downregulated in Primary OCS and more genes upregulated in Primary No OCS. The relevance of these genes in the context of asthma should be considered with these findings.

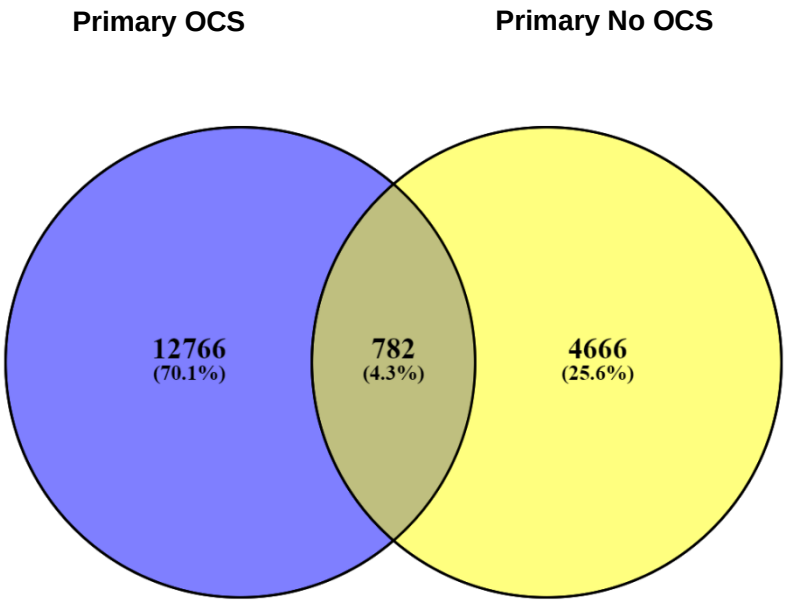


Figure 6. 30: Venn Diagram illustrating downregulated genes in Primary OCS and Primary No OCS events in the APEX study.

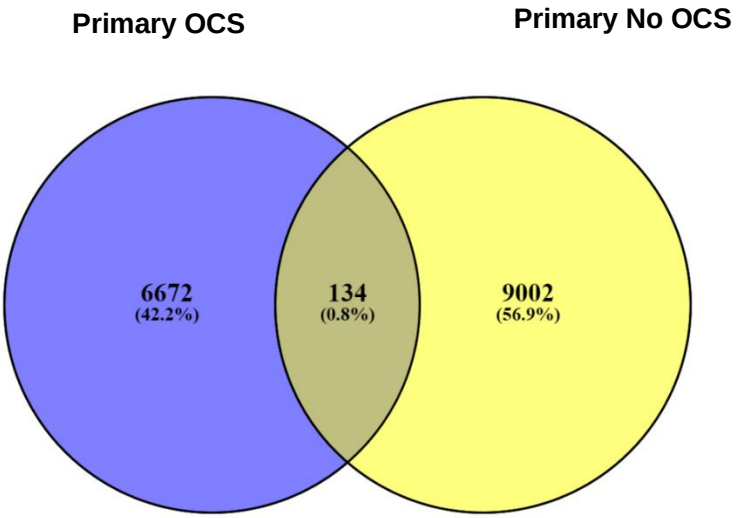


Figure 6. 31: Venn Diagram illustrating upregulated genes in Primary OCS and Primary No OCS events in the APEX study.

Exacerbators versus non-exacerbators at baseline

I set out to identify whether there was a difference in differential gene expression among those who went on to have an exacerbation event that required and did not require steroids in the APEX study. This final analysis assesses if there were differences at baseline. The table below illustrates key demographics of those who had an exacerbation event in APEX, regardless of whether it was a primary OCS event or a primary no OCS event i.e. Baseline Exacerbators (BE). A comparison was made to assess the difference to those that did not, i.e. Baseline Non-Exacerbators (BNE).

Demographics

	Baseline Exacerbators (BE)	**Baseline Non-Exacerbators (BNE)**	**p-value**
	N=53	N=74	
Female N(%)	38(71.7)	47(63.5)	0.72
Age Yr(IQR)**	51.7(44.0-68.4)	47.4(34.3-63.6)	0.04
BMI*	30.1(7.1)	29.0(5.6)	0.89
Depression N(%)	25(33.8)	23(31.1)	0.06
Reflux N(%)	24(32.4)	27(36.5)	0.31
Eczema N(%)	16(21.6)	29(39.2)	0.30
Nasal Polyps N(%)	6(8.1)	7(9.5)	0.73
Rhinitis N(%)	18(24.3)	19(25.7)	0.31
FeNO (ppb) **	25(13-41)	18 (12-27)	0.03
Eosinophil ($x10^9$/L) **	0.22(0.09-0.33)	0.14(0.10-0.25)	0.12
FEV1% pred (SD) *	81.7(18.6)	85.7(17.7)	0.24

Table 6. 17: Key parameters at baseline between exacerbators and non-exacerbations whose nasal brush samples underwent RNA-sequencing.

*=Paired t-test mean(SD) presented. **= Wilcoxon test median(IQR) presented. Bonferroni correction for multiple testing applied.

In the samples that underwent sequencing, at baseline, those with an exacerbation event were significantly older (51.7 vs 47.4 p=0.04). The two groups had no difference in the frequency of comorbidities commonly associated with asthma. However, at baseline, there was a higher FeNO

(25 vs 18 ppb p=0.13) in those with an exacerbation event. Lung function was similar, with FEV1% predicted to be 81.7% in BE and 85.7 in BNE.

Principal component analysis (PCA) was performed on BNE and BE samples. No clustering occurred at the study entry.

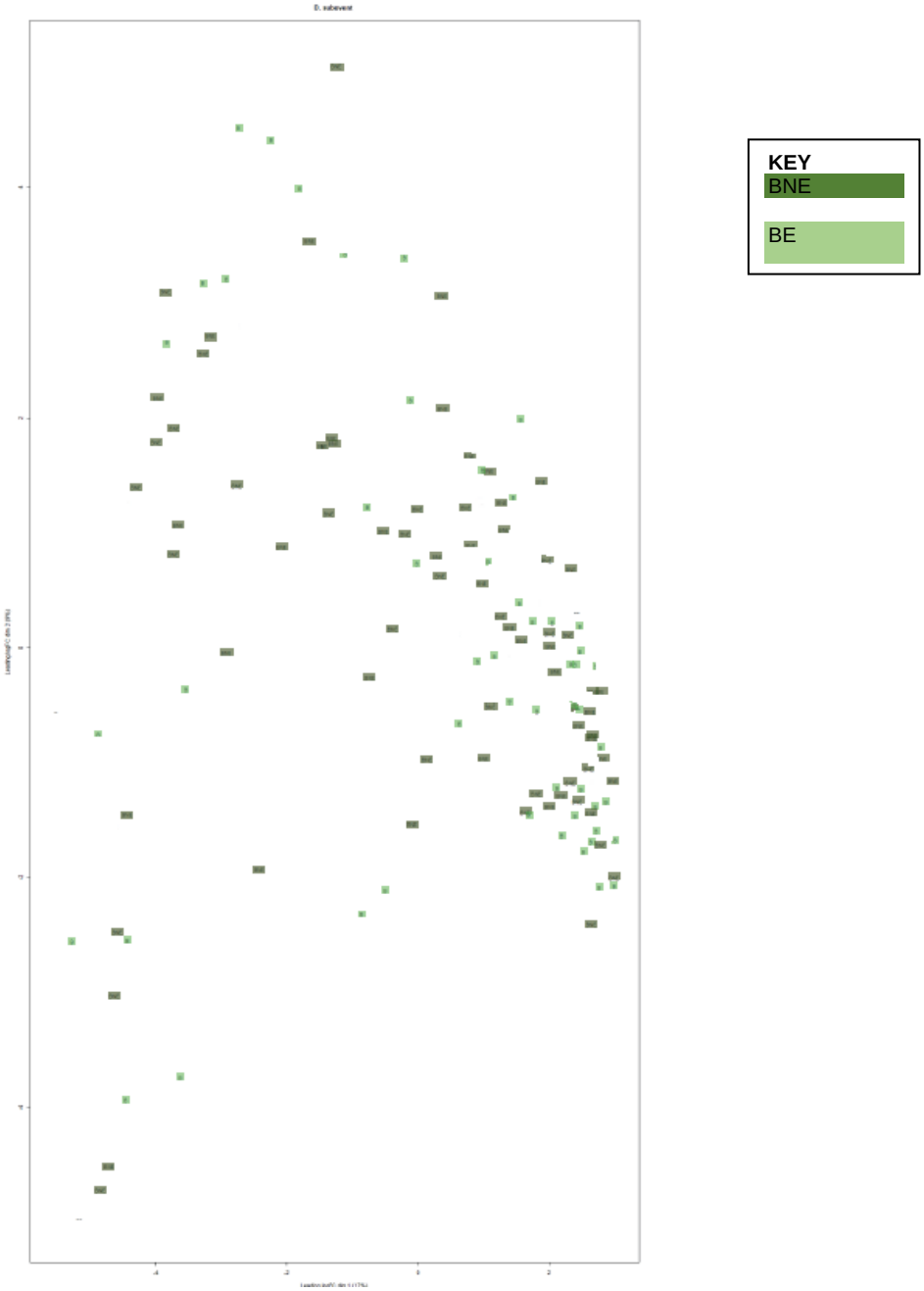


Figure 6. 32: PCA shows no significant clustering of participants at the study entry.

Significant differential gene expression between exacerbators and non-exacerbators

At baseline, 1348 genes were downregulated, and 1755 were upregulated, comparing BE to BNE. Figure 6.19 illustrates the volcano plot of differentially expressed genes. Gender, age, and ICS dose were covariant inputted and adjusted for in the analysis.

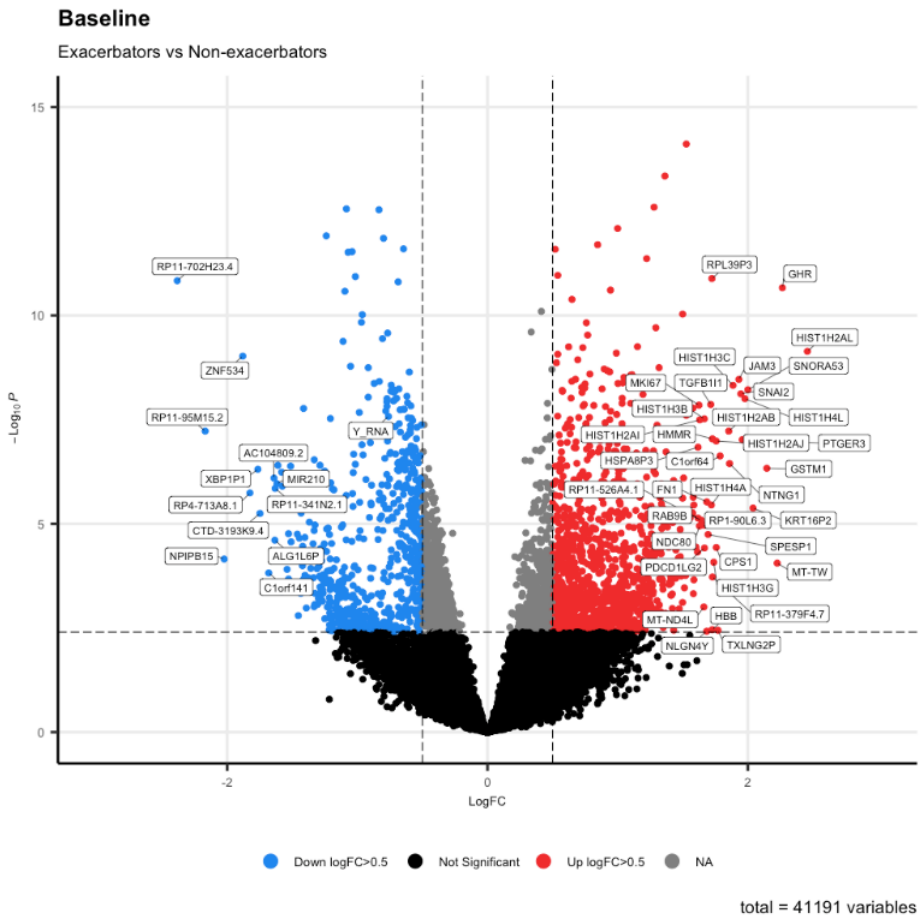


Figure 6. 33: Volcano plot illustrating differential expression analysis of nasal transcriptome at baseline.

The log fold change of individual genes are on the x-axis. The negative log of the genes p-value to the base 10 is on the y-axis. The red represents the upregulation of genes compared to the baseline. The blue represents downregulation. P-value was set to <0.05. Key genes that are upregulated or downregulated are labelled. Genes that did not cross the FDR of 0.05 are coloured in black.

On review of the greatest fold change and significant p-values produced in the volcano plot, in those who underwent an exacerbation event (BE), there was upregulation of genes associated with inflammation in asthma, such as **PTGER3**, which is a Prostaglandin E2 involved in T1 and T2 inflammation. [416] **RPL39P3,** which is associated with IL6 signalling, is thought to be associated with "high metabolic activity" epithelial cells in asthma.[417] **GHR** (Growth Hormone receptor) has been established as being suppressed in the presence of a high dose of steroids. The upregulation of this gene may suggest unsuppressed inflammation in those who experienced an exacerbation event or inadequate ICS dosages. **ZNF534** has been known to be downregulated in males associated with atopy-associated asthma. **RP11-70H23.4** is of unknown significance in the context of asthma.

Figure 6.34 illustrates a heatmap of the top 500 differentially expressed genes grouped into "exacerbated" (BE) and "Did Not Exacerbate" (BNE) categories. In the BE category, genes are upregulated, and in BNE, genes are downregulated, albeit not to the degree seen during the exacerbation event analyses.



Figure 6.34: Heatmap of gene expression in exacerbators vs non-exacerbators in the APEX study.

Top 100 DEG determined using Log_2 FC and FDR p<0.05.

The top 10 upregulated and down-regulated genes based on fold change in Exacerbators (BE) and Non-Exacerbators (BE) in the APEX study identify genes associated with severe asthma

The top 10 genes that were upregulated and downregulated in the IPA analysis are displayed in Tables 6.18 & 6.19. Molecules that were upregulated have been identified in studies researching severe asthma and risk factors, such as **MT-TW** associated with the obese asthma phenotype-RNA in the allergic asthma phenotype, and **KRT17P2**, which is upregulated in severe asthma and **SNORA53**, which has been associated with increased methylation and associated with asthma exacerbations. The sequelae of asthma exacerbations include a loss of lung function, and **H4C13** was expressed, which is associated with fixed airflow obstruction observed in COPD.

Some genes seemed to represent a response to injury, such as the upregulation of **GSTM1**, which has been identified as a molecule that protects against reactive oxidative species in the epithelial cells. There was upregulation of **H2AC16**, which studies have shown to be reduced in RV-inoculated epithelial cells of patients with asthma, which may reflect a response to previous infection. Conversely, there was a downregulation of **NPIBP15**, which is associated with the expression of transcription factors in response to virus infection in patients with asthma. Down-regulated genes included **C1orf141**, a non-coding microRNA that is upregulated in patients with asthma versus controls after adjustment for smoking and inhaled corticosteroids. This negatively correlates with microRNA miR-1999b-5p, associated with cilium assembly and organisation. **ALG1L6P** is downregulated in the presence of IL-6 inflammation and is also downregulated in this analysis.

Gene	Protein name	Fold Change	Known relation to asthma	Chromosome Location	Protein Function/Significance
H2AC16	H2A clustered histone 16	2.457	Y	Chromosome 6p22.1	H2A is a core histone in chromatin which is reduced in response to RV infections in patients with asthma[418]
GHR	Growth Hormone Receptor	2.265	Y	Chromosome 5p13.1-p12	Known to be suppressed in asthmatics with high dose ICS[419]
MT-TW	tRNA-Trp	2.225	Y	Chromosome 59-10	Known to be upregulated in obese asthmatics.[420]
GSTM1	Glutathione Peroxidase	2.146	Y	Chromosome 1p13.3	Known to be upregulated in airway epithelial cells of asthmatic. Protects against reactive oxidative species[421].
KRT17P2	Keratin 17 pseudogene 2	2.040	Y	Chromosome 17p11.2	Associated with severe asthma[422]
SNORA53	Small nucleolar RNA, H/ACA box 53	2.000	Y	Chromosome 12q23.1	eQTL for rs2253681, which is significantly associated with both DNA methylation and severe asthma exacerbations[423]
H4C13	H4 clustered histone 13	1.977	N	Chromosome 6p22.1	Associated with fixed airflow obstruction COPD phenotype[424]
Y-RNA	Cytoplasmic sncRNA	1.970	N	-	Small non-coding RNA involved in epigenetic regulation, over expressed in allergic patients[425]
PTGER3	Prostaglandin E receptor 3	1.958	Y	Chromosome 1p31.1	G-protein coupled receptor whose genetic variant is associated with severe asthma [416]
SANI2	Snail family transcriptional repressor 2	1.946	Y	Chromosome 8q11.21	Associated with airway remodelling via Epithelial-Mesenchymal Transition.[426]

Table 6. 18: Top 10 genes that are highly expressed in BE and their respective fold change.

Gene	Protein name	Fold Change	Known relation to asthma	Chromosome Location	Protein Function/Significance
RP11_702H234	Unknown	-2.384	-	Chromosome 15q25.2	Rp11 702h234 antisense
PTPN1PP3	PTPN11	-2.169	Y	Chromosome 6q23.3	In-vivo studies suggestive of increased recruitment of leucocytes and T2-high associated cytokines[427]
NPIPB15	Nuclear pore complex interacting protein	-2.023	Y	Chromosome 16q23.1	Reduced levels found in viral replication associated with reduced immune transcription factors against viruses[428]
ZNF534	KRBO3, zinc finger protein 534	-1.880	Y	Chromosome 19q13.41	demonstrated a significant sex interaction for atopy with opposite direction of effects between females and males.[429]
RP4_713A81	Rp4 713a81 sense intronic	-1.826	-	-	-
XBP1P1	X-box binding protein 1 pseudogene 1	-1.765	Y	Chromosome 5q22.2	Associated with upregulation of T2-reled cytokins via "T2-polarisation" [430]
CTD_3193K94	ctd 3193k94	-1.748	-	-	-
C1orf141	Chromosome 1 open reading frame 141	-1.681	Y	Chromosome 1p31.3	Found to be downregulated in microRNA in bronchial biopsies in patients with asthma[357]
AC1048092	AC1048092 antisense	-1.640	-	Chromosome 2q241.8	-
ALG1L6P	ALG1 like 6,	-1.635	Y	Chromosome 3p12.3	Found to be downregulated in IL-6-associated inflammation in patients with asthma[417]

Table 6. 19: Top 10 genes that are lowly expressed in BE and their respective fold change.

Upstream Regulators

On review of the molecular drivers with the highest z-scores, Transforming growth factor-β (**TGFB1**) has been associated with airway remodelling in asthma and, as identified in Chapter 1, is upregulated with viral infections. Epithelial cells, macrophages and eosinophils produce it.[431] Bromodomain-containing protein 4 (**BRD4**) has been identified as being involved in the immune pathway of T2-high asthma and upregulates the expression of IL-9.[432] **DDX3X** is involved in antiviral activity and promotes type I interferon production[433]

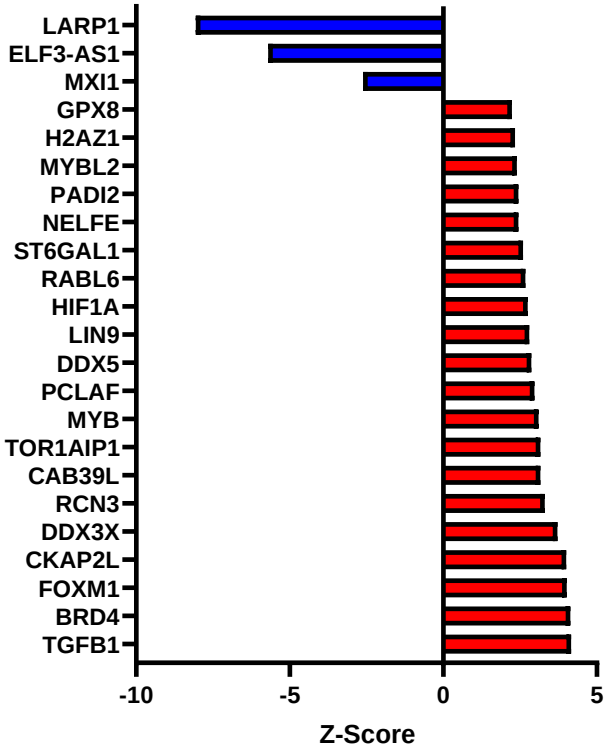


Figure 6. 34: Upstream regulators identified in exacerbators at baseline identified by Ingenuity Knowledge Base.

Red= predicted activation state to be activated. Blue=predicted activation state to be inhibited. Absolute Z-scores ≥ +/-2.0 with a P-value >$1x10^{-4}$ were used as a cut-off for significance.

Of the 2 proposed inhibited activity states, **LARP1** is novel in its association with asthma and acts as an RNA-binding protein.[434] **MXI1**

has been identified as a gene responsible for mast cell activation in COPD associated with eosinophilia[435]

Canonical Pathways analysis identifies genes associated with increased cellular activity in baseline exacerbators.

Canonical analysis utilising IPA allowed further study of these genes and the identification of pathways associated with those genes that underlie a predisposition to asthma exacerbation to be identified in Baseline Exacerbators in the APEX study. Figure 6.35 illustrates a bubble plot downstream function pathway analysis. The number of genes associated with the most upregulated pathways is illustrated in Figure 6.36. The bubbles with the most significant z-scores and p-values are then described in Table 6.20. Five modules were strongly upregulated. These modules included “cellular response to stimuli”, “increased metabolism”, “protein metabolism”, “response to stress and injury” and “metabolism of RNA”. A downregulated module included reduced “cellular immune response.” Exploration of these modules identified pathways which are biologically relevant to asthma exacerbation and upregulation of genes that would be expected to manifest as clinical features of asthma exacerbation, such as **ADCY9,** which is responsible for producing secondary messenger cyclic AMP (**cAMP**). Regulator T-cells produce this. This was found to be upregulated in asthma and associated with reduced bronchial hyperresponsiveness .[413] **NNMT** expression has been upregulated in the muscles of people with COPD. It is thought to improve myogenesis and protect against oxidative damage.[436] The relevance of this gene in the context of asthma is unexplored. The **EEF2** gene codes for a translation elongation factor for protein synthesis. [437]Pinilla and colleagues identified that EEF2 inactivation activates the ribosomal stress-associated kinases ZAKα and NLRP1 phosphorylation, which results in proteasome degradation of cultured human epithelial cells. [438]

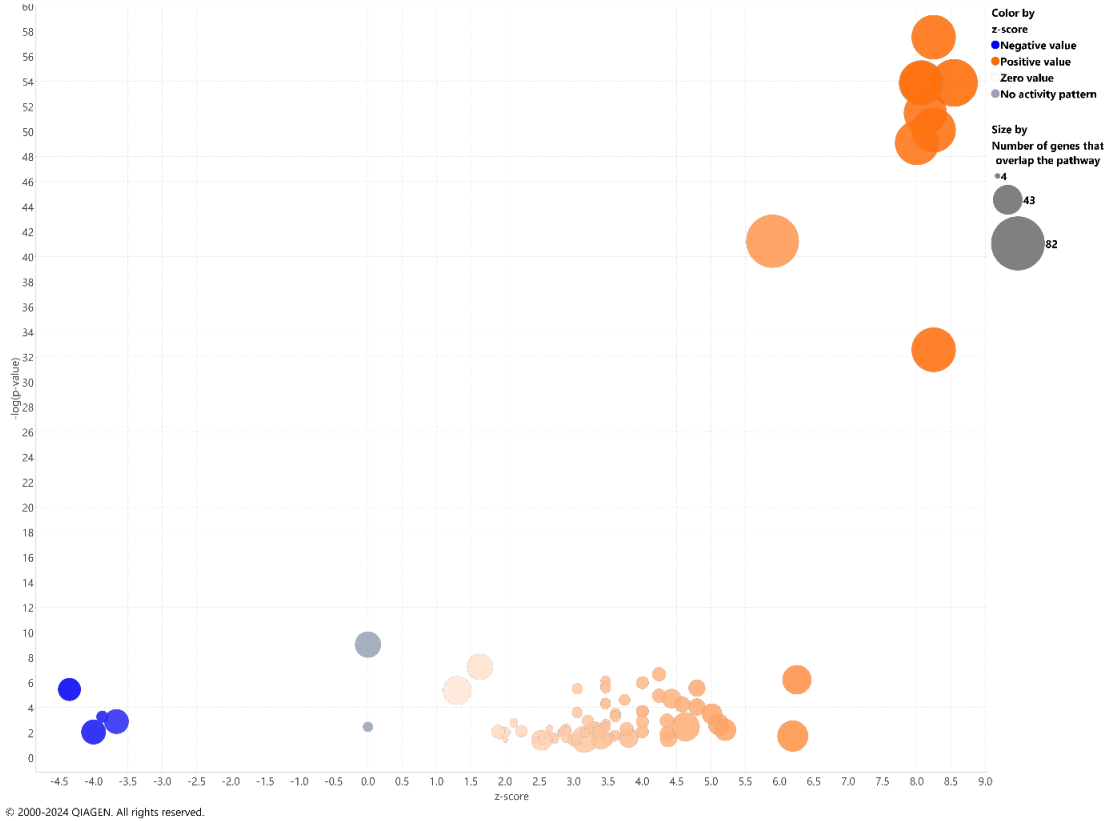


Figure 6. 35: Bubble plot of downstream functional pathway association about Baseline Exacerbators.

Pathways with z-scores on the x-axis and -log p-values on the Y-axis are in log format due to the wide range of p-values. The bubble's size indicates the number of genes involved in the pathway. The details of those bubbles are illustrated further in Figure 6.24. An adjusted p-value of 0.05 was used for all data.

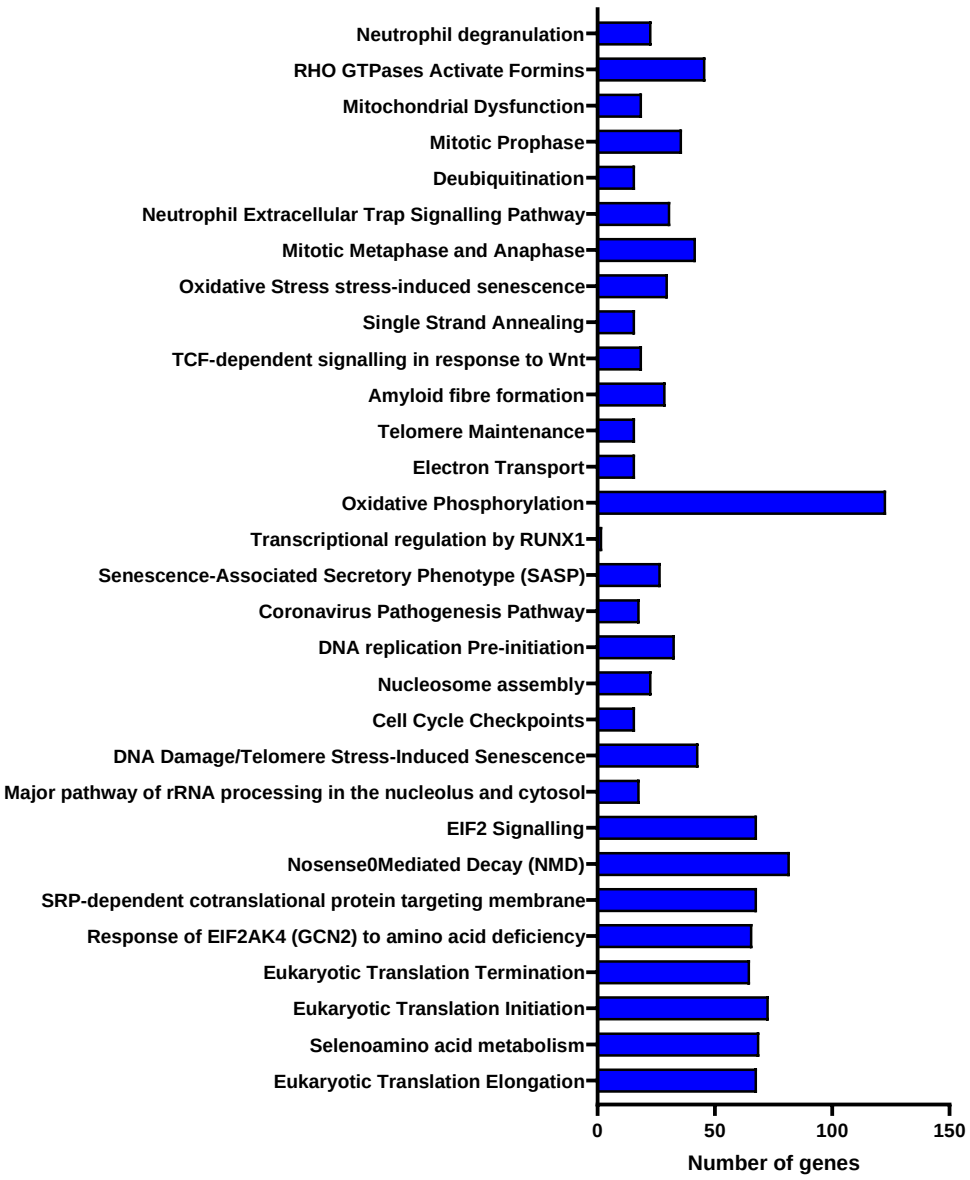


Figure 6. 36: Pathways associated with Exacerbator-associated gene expression in the APEX study. An adjusted p-value of 0.05 was used for all data.

Module	Genes	Fold change & p-value	Synopsis of function
Metabolism	ADCY9↑ CTH↓ INMT↑ NNMT↑ RPL3↑	0.424, 1.64E-02 -1.149, 2.37E-02 0.900, 2.73E-05 1.176, 9.53E-03 0.310, 2.18E-03	**Selenoamino acid metabolism** Adenylyl cyclase type 9 enzyme produces cAMP and is associated with severe asthma and reduced BDR response.[413] Cystathionine-lyase (CTH) inhibition upregulates tryptophan metabolism, which is involved in allergic inflammation in murine models. [439] Nicotinamide N-methyl Transferase is upregulated in the skeletal muscle of those with COPD.[436] Ribosomal Protein S3 gene (RPL3) downregulation in murine models leads to reduced features of asthma → AHR and cytokine release[440]
Metabolism of Protein	EEF2↑ EEF1A1↑ EEF1B2↑ RPL3↑ RPL4↑	0.238, 2.88E-0.3 0.414, 1.40E-07 0.327, 1.44E-02 0.310, 2.18E-03 0.389, 2.09E-05	**Eukaryotic Translation Elongation** EEF-2 upregulation attributed to epithelial barrier disruption[438] Ribosomal Protein L-3 (RPL3) gene upregulated during viral-induced exacerbations [441]
Cellular response to stimuli	ATF4↑ RPL3↑ RPL4↑ RPL6↑ RPL10↑	0.302, 8.20E-03 0.319, 2.18E-03 0.484, 2.09E-05 0.340, 1.82E-03 0.403, 3.77E-05	**Response of EIF2AK4 (GCN2) to amino acid deficiency** EIF2AK4 is a kinase that phosphorylates the eukaryotic translation initiation factor -2 (EIF2) in inflammation.[442] The ATF4 gene is upregulated in response to cellular stress in the airway epithelium.[443]
Metabolism of RNA	RPL8↑ RPL10↑ RPL11↑ RPL12↑ RPL14↑	0.360, 3.07E-04 0.403, 3.77E-05 0.473, 7.07E-03 0.429, 6.20E-04	**Nonsense-Mediate Decay (NMD)** Unexplored in the pathogenesis of asthma
Cellular Stress and Injury	AFT4↓ CCND1↑ EIF1↑ EIF2A↓ EIF3E	-0.302, 8.20E-03 0.320, 2.58E-02 0.279, 3.69E-03 -0.5518,.22E-03 0.390 3,.70E-02	**EIF2 signalling** This pathway is responsible for protein synthesis regulation. Reduced AFT4 response leads to an increase in TH17 response.[444] Cyclin D1 is associated with obese asthma phenotype in murine models.[445] EIF2A is downregulated in IL-6-associated airway inflammation in human cultured cells in asthma. [446]
Immune response	ATF4↑ CCND1↓ EEF1A1↓ HIFI1A↑ IL6↑	0.302, 8.20E-03 -0.320, 2.58E-02 -0.414, 1.40E-07 0.394, 1.58E-02 0.742, 3.35E-3	**Coronavirus Pathogenesis Pathway** AT4 is elevated in lung tissue murine models after OVA stimulation leading to ER stress and cytokine release[447] Raised IL-6 seen in asthma patients with reduced lung function.[448]

Table 6. 20: Pathways involved, the identified genes, and their expected activity (upregulated=↑ or downregulated=↓) in Baseline Exacerbators.

Fold change and p-values are stated, and a description of the pathway to which the genes contribute to is provided.

Disease and Functions

Increased cell activity and inflammatory upregulation in Baseline Exacerbators at study entry

IPA analysis of “Disease and Functions” using IPA has identified an upregulation of activity (Table 6.21) associated with the recruitment of cells such as epithelial cells and leucocytes. There is also an increase in metabolic activity within cells. This is again when participants entered the study and thus were not experiencing exacerbation symptoms.

Category	Increased or Decreased Activity according to z-score	Activity
Protein synthesis	Increased	↑protein synthesis and metabolism, ↓translation of mRNA
Gene expression	Increased	↑ Binding of DNA ↓translation of mRNA
Connective Tissue Development and Function	Increased	↑connective tissue cells, keratinocytes, fibroblasts
Cellular Movement	Increased	↑Cell movement of epithelial cells ↑Cell movement of leukocyte lines
Cell cycle	Increased	↑Binding of DNA endogenous promoter ↑Binding of DNA ↑G1/S phase transition ↑Interphase ↑Mitosis
Cell Growth and Proliferation	Increased	↑Proliferation of connective tissue cells ↑Proliferation of vascular cells ↑Proliferation of blood cells ↑Proliferation of lymphocytes ↑Proliferation of immune cells ↑Proliferation of epithelial cells
Development	Increased	↑Development of fibroblasts

Table 6. 21: Disease and Function IPA analysis in Baseline Exacerbators. Key categories associated with a z-score of +/-2 included.

The activity according to Z-score is highlighted, and some of the key features of each category are described, stating increased or increased activity.

Discussion

This thesis chapter initially aimed to identify the proportion of asthma exacerbations with a viral pathogen present and assess if this presence was clinically associated with different changes in clinical parameters used to evaluate asthma. I then made comparisons to exacerbation events where no virus was detected. I then explored the gene expression associated with exacerbation events through paired analyses to identify possible mechanisms related to asthma exacerbations.

A significant finding is that 80% of Primary OCS events had a virus present, with 43% of those cases showing the presence of Rhinovirus. This aligns with the existing literature, indicating that most asthma exacerbations have a viral aetiology. Notably, our study detected a pathogen in 80% of samples, a detection rate surpassing most studies' average rate, typically ranging from 41 to 55%.[449,450]Studies have suggested that the detection rate of pathogens is greater in NPA samples than in sputum.[451] A viral infection could have been present in 20% of asthma exacerbations where no viral pathogen was detected, but we only tested for six respiratory viruses. I must also mention that the study ran through the COVID-19 pandemic and limits generalisability due to shielding policies influencing findings.

It would be interesting to explore what proportion of those had a bacterial pathogen, but also, in those exacerbations with a viral pathogen detected, how many of those had an additional bacterial infection? We have discussed the interaction between allergy exposure and virus infection. We have not explored allergic triggers that could contribute to the aetiology. I have not explored viral loads in this portion of the work, which could suggest that viral pathogens may be present at baseline. However, qPCR was performed on the participants at baseline to address this by assessing if the findings observed at the exacerbation reflected the participants at baseline. Five participants had a virus present at baseline, with 1 participant having two viral pathogens present- rhinovirus

and Influenza A. Of interest, the five participants who had a pathogen at baseline had multiple asthma exacerbations.

The subsequent analysis aimed to determine whether a viral-positive exacerbation was associated with different worse clinical parameters than viral-negative exacerbation. My hypothesis was proven wrong. The only discernible difference was that a viral-positive asthma exacerbation was linked to greater peripheral resistance than seen with a viral-negative asthma exacerbation. This is a novel finding, as no previous study has explored the relationship between viruses and small airways disease. It has been established in earlier research that a viral-positive asthma exacerbation is associated with a more severe decline in lung function than a non-viral-associated asthma exacerbation.

RNA was isolated from nasal brush samples and sequenced to identify meaningful changes in gene expression in a cohort of well-phenotype patients who have exacerbated symptoms and to identify possible mechanisms contributing to the pathogenesis of asthma exacerbations. The IPA analysis allows for the placement of biological context to meaningful interpretations.

The clinical chapters in this thesis have revealed heterogeneity associated with asthma exacerbations, which is also evident on a genomic level. Primary OCS events differed from Primary No OCS which was seen in differential nasal gene expression which is what was postulated in my hypothesis. Many DEGs were identified, and as such, it is challenging to underpin a specific pathway associated with asthma exacerbations, which can allude to some possible mechanisms. Upstream analysis of Primary OCS found downregulation of IL-23, which is known to cause Th17 activation and neutrophil recruitment in inflammation.[389] There was upregulation of Linc00941, which is associated with airway remodelling.

I performed canonical analysis of Primary OCS and Primary No OCS pathways. Some genes were novel in asthma exacerbation such as those associated with G protein signalling. Pathways associated with airway remodelling were dominant in the Primary OCS, and the Primary No OCS events had pathways related to airway inflammation. Pathways found in Primary OCS were the **FAK signalling pathway.** FAK signalling has been associated with airway smooth muscle activity. This correlates with findings from Chapter 4, which identified significant difference in lung function from baseline to Primary OCS and not in Primary No OCS. These pathways were not seen in the Primary No OCS canonical pathway analysis. Pathways of T1 and T2-associated inflammation were upregulated in Primary OCS events such as genes associated with Th17 and IL-4 signalling.

Studies exploring nasal gene expression in asthma exacerbation have identified important genes associated with T2-associated inflammation through canonical pathway analysis, such as CEAACAM5, an IL-13-regulated epithelial gene, and Carboxypeptidase A3, a mast cell protease.[379] IL-33 and TSLP are upregulated in the nasal epithelium of patients with asthma who are at an increased risk of exacerbation due to T2-related cytokines.[391] The Mucin MUC5AC is increased in airway epithelial cells and associated with airway hyperresponsiveness and mucus plugs.[104]

I then explored any difference between exacerbators and exacerbators at baseline. Upstream analysis identified several genes associated with airway remodelling. This is interesting, as nasal brush samples collected for these events were when patients were “stable” and not experiencing symptoms suggestive of exacerbation. This suggests that even during periods of stability in patients who experience worsening, genes are activated and associated with airway remodelling and inflammation.

Identifying mechanisms associated with asthma exacerbation development requires minimal biases that could influence any findings.

One of the main strengths is integrating detailed clinical information, which has supported the genomic analyses and allowed clinically relevant comparisons. A strength of the approach adopted is a real-world approach, at the point at which the patient would usually visit their healthcare professional for worsening asthma symptoms, adding clinical relevancy to the analysis. Also, samples were collected regardless of whether a course of steroids was given or not to allow meaningful interpretations rather than only collecting samples based on the clinician's interpretation of whether an asthma exacerbation was occurring. The samples were collected prospectively before OCS use, which may have influenced the findings. The upper airway was sampled as a surrogate for the lower respiratory tract. Performing bronchoscopies to obtain lower airway samples in symptomatic patients would be challenging. Whilst differential gene expression of upper airway sampling and lower airway sampling is similar, there are differences in inflammatory molecules between upper and lower airways, such as interferon responses. [452]

The findings of this chapter suggest that T1 and T2 inflammatory pathways are essential mechanisms involved in asthma exacerbations. Identifying the aetiology of the captured exacerbations, which were viral predominant, supports such findings.

There are several methods to assess differential gene expression. Williams and colleagues identified significant heterogeneity in the performance of RNA-Seq workflows to identify differently expressed genes. They highlighted the differences as "due to differences in recall", "the proportion of reference-identified genes identified by an adopted workflow", and "precision of which is the proportion of genes identified by the workflow".[453] The pipeline used for analysis is consistent with what our study group uses.[281,331] Assessing the proteomic expression level with the exact comparisons would strengthen this study's findings.

Limitations include the limited number of Primary OCS witnessed in the study. The next step would be to analyse the Multiple OCS samples to see if similar pathways and genes are expressed in these nasal epithelium samples and annual samples to validate the findings observed.

Conclusions

Most asthma exacerbations observed in the APEX study had viral aetiology. Evidence shows that T1 and T2 inflammatory pathways are essential in asthma exacerbations. Pathways associated with lung function appear predominately in Primary OCS events compared to Primary No OCS events in which inflammatory pathways are observed. The next step would be to assess if these genes are present in annual events. It would also be interesting to ascertain what proteins are observed from proteomic analysis of nasal brush samples.

Chapter 7: Discussion and Conclusions

This thesis aimed to phenotype a cohort of participants with asthma at baseline and to compare these phenotypes with those observed in an exacerbation event. These exacerbation events were then characterised according to the presence or absence of a respiratory virus using a select choice of viruses. Phenotyping was undertaken using clinically relevant measurements and exploration on a transcriptomic level through RNA sequencing of nasal brush samples to understand possible mechanisms that allude to the clinical findings. I then longitudinally followed up with these participants to see whether phenotypic stability was present after an asthma exacerbation.

Due to the sporadic nature of asthma exacerbations, this study was designed to capture these events at the point of an asthma exacerbation over a three-year period, which, as previous research has shown, can be challenging. The influence of an asthma exacerbation on the longitudinal stability of an inflammatory phenotype has not been explored so far in research.

Clinical findings at baseline.

This thesis has incorporated several analyses, beginning with the recruitment and subsequent analysis of over 300 participants at study entry into APEX, as shown in Chapter 3. At study entry, there were crucial differences in APEX-1 and APEX-2, which differed in time of a prior history of asthma exacerbation, with APEX-1 having a recent exacerbation (within two years of study entry) and APEX-2 (within 2-5 years of study entry).

APEX-1 had evidence of poorer asthma control and a reduced FEV1 but also more significant evidence of small airway disease with higher absolute value for peripheral and central resistance. At baseline, there was heterogeneity in inflammation, with a third of APEX-1 demonstrating evidence of T2 inflammation in APEX-2. This was just over 26%.

Although this was a “snapshot” of inflammation at one point, both APEX-1&2 showed evidence of T2 inflammation despite ICS therapy, which is known to suppress this pathway. APEX-2, a cohort of patients without a recent exacerbation, still demonstrated evidence of unsuppressed T2 inflammation. We chose to exclude those patients who were on biological therapies as the immunomodulation by biologics may impact the phenotypes observed, and Biologics reduce the risk of future asthma exacerbations[454]However, these patients have severe asthma, which may reflect the modest changes we saw in demographic differences between APEX-1, such as co-morbidities.

When organising the APEX cohorts according to T2-high and T2-Low, those who demonstrated a T2-high inflammatory phenotype at baseline had worse FEV1% predicted (76.8%(19.54) vs 87.9%(16.9)) and a greater degree of peripheral airways resistance, which has been found in those who have T2-associated inflammation.[214] Those who went on to have an exacerbation event in the APEX study (regardless of whether a course of steroids was prescribed or not) demonstrated evidence of T2 inflammation at baseline. These findings are comparable to those seen in the U-BIOPRED cohort, which had features of T2-high airway inflammation and poor symptom control.[455] Although not observed at baseline in APEX, women are more likely to have increased severity of asthma.[456]

Exacerbation Profiles

Participants were encouraged to call the APEX team for worsening asthma symptoms. All of the exacerbation events captured were before OCS use, with 7 participants contacting to query whether to start a rescue

pack of steroids. As expected, APEX-1 had more exacerbation events than APEX-2. 44 primary OCS events were captured, and 42 multiple OCS events were from 25 participants. This is similar to findings from a database study of 51,462 patients with asthma in which half went on to have a further asthma exacerbation.[457]

As expected, APEX-1 had more exacerbation events than APEX-2, with a history of exacerbation being a strong predictor for future asthma exacerbations. The modest number of exacerbation events captured could be explained by study design. The study was a single-centre, and recruitment was prospective, limiting the potential number of exacerbations, although recruitment was aimed across the east midlands north. This could limit the generalisability of findings. We recorded the natural history of a participant's exacerbation event, which provided insight into behaviours. ICS therapy was escalated before calling the APEX study, which is what is advised to abort asthma exacerbations.[18] However, in those that escalated therapy – they were on ICS/LABA. It demonstrates that for those who had an exacerbation event, the MART therapy approach could have been underutilised, with 77 (61.6%) of those experiencing an exacerbation event opting to increase their inhaled SABA; which, when utilised alone, is a risk for severe asthma exacerbations as demonstrated in the SABINA study.[458] I acknowledge that the increased ICS use before the study visit could be a potential limitation, but the study's design was real-world. As such, it is expected that a participant will attempt to abort an impending exacerbation by an appropriate escalation of inhaled therapies.

A paired analysis of Primary OCS and No OCS to the respective baseline revealed the importance of small airways disease (Figure 7.1). In those who had an exacerbation, there was only a difference in peripheral resistance and reactance in those who required a course of OCS for their symptoms. Kuo and colleagues demonstrate that IOS BDR was associated with poor asthma control.[459] However, it’s a novel finding that

IOS has the potential to distinguish between exacerbation events in which steroids are required



Figure 7.1: Key findings from the work undertaken in this thesis of phenotypes observed in asthma exacerbations.

and is worth exploring in a study explicitly designed to address this question. In Primary OCS exacerbation events, 57% of exacerbation events had evidence of T2 inflammation with either a raised FeNO or raised Eosinophil count. 43.2% had no evidence of T2 inflammation. This suggests the heterogeneity of inflammatory phenotypes in asthma exacerbations. The importance of deconstructing the concept of asthma and providing treatment using a biomarker/trait approach has been advocated in a Lancet Commission article.[460]

I explored exacerbation events further with the T2 status at respective exacerbation events (Primary OCS and No OCS) and whether there was a difference in measures of lung function and small airway disease. There was no difference according to T2 status regardless of whether a course was prescribed, suggesting that T2-high and low exacerbations are both associated with a physiological loss of large airway and small airway function. This is similar to findings found by Chan and colleagues, who demonstrated that in those with moderate to severe asthma, there was

no difference in FEV1% predicted between T2-high and T2-low asthma– there was, however, worse FEF_{25-75}% in T2-high asthma (OR=.2.75(1.01-7.46)).[288]

A limitation, I acknowledge, is the limited number of sputum samples, which limits phenotypical interpretation on a cellular level. This again limits the generalisability of findings. The APEX study assessed the observed phenotypes' longitudinal stability and the characteristics that comprise the phenotypes observed from a participant's baseline. The design of the study was an observational longitudinal study with prospective recruiting. As such, some participants may have been recruited in the second year of the research and have one annual review, or participants may have been recruited in the third year of the study and may not have had an annual event. This is reflected in the difference in the number of observed annual events between year one, year 2 and in year 3 of both cohorts. The proportions of participants that had evidence of T2 -high inflammation in the APEX study was 29.1% in year 1, 25.8% in year 2 and 31.2% in year 3, with 70.9% in year 1, 74.2% in year 2 and 68.8% in year 3 having T2-low asthma. Annual reviews were undertaken at a period of “stability” where they did not require escalated ICS or ICS/LABA therapy. I acknowledge these findings must be interpreted with caution owing to the reduced number of participants having a year 2 and 3 annual event.

The APEX study occurred during the COVID-19 pandemic. This may have influenced the observed number of asthma exacerbations, with a change in participant behaviours due to shielding policies. It has been shown that the was a reduction in asthma exacerbations for 18 months after the first lockdown.[461] I also acknowledge that COVID-19 significantly delayed a participant's annual review events. As such, most analyses performed in this thesis were focused on one-year post-study entry.

A series of variance analyses were performed to assess changes in clinical measurements that comprise the identified phenotypes.

The first analysis was conducted to assess for changes according to a Primary OCS versus a Primary No OCS event. The second analysis focused on a Primary OCS event and was stratified according to T2 status at baseline. After one year, there was no difference in airway physiology by spirometry measurements, small airway disease through IOS measurements or airway inflammation by measuring FeNO and blood eosinophils. T2 status at baseline did not result in a significant change in characteristics that comprised the phenotypes after one year of follow-up, such as asthma control, lung function and markers of small airway disease. I acknowledge that only analysing 1-year of follow-up and the limited sample size of these analyses may not be sufficient to detect a meaningful change in lung function. It is well established that asthma exacerbation is associated with a loss of lung function.

The longitudinal stability of the phenotypes identified was stable over a 2-year follow-up period according to T2-Inflammation observed at baseline. This finding is variable in other studies examining phenotypic stability in adults. The Airway Disease Endotyping for Personalized Therapeutics (ADEPT) study assessed the stability of 158 patients with asthma, across a range of severities, in 5 visits over 12 months.[263] They found group stability in FEV1/FVC ratio and FeNO but variation in ACQ and sputum inflammatory phenotype. The BIOAIR study group, similarly, after a one-year follow-up of 169 patients across a range of asthma severities, found that 48.6% of the cohort changed clusters according to inflammation. Still, lung function measurements were stable.[462] A strength of our approach is that an exacerbation was accounted for, which has been addressed as a possible confounding factor in previous studies. However, we could not obtain sputum samples to assess inflammatory phenotype stability on a cellular level. Also, in my research, clinical measurements were recorded cross-sectionally one year from study entry only and as such would not detect changes associated with fluctuations according to seasonality or allergen exposure.

80% (28/35) of the Primary OCS events witnessed in the APEX study were viral positive and based on a panel of 6 common respiratory viruses. As already discussed, this is higher than the number of studies exploring viruses in asthma exacerbations. In the 20% where no virus was detected, it could be that a virus was present but was not detected by our select panel. Also, the COVID-19 pandemic may have had an influence, but I would expect the presence of a virus to be less during this time. Mapping the RNA sequencing data to the viral genome could answer this question. A virus-associated exacerbation showed significant peripheral airway resistance compared to asthma exacerbations where no virus was detected.

Transcriptomic analysis of nasal brush samples.

The genomic work in this thesis supports the clinical findings in the clinical research. Following an analysis of exacerbators and non-exacerbators in the APEX study, paired analyses were undertaken between Primary OCS and No OCS events compared to baseline. PCA analysis did not identify any clusters suggestive of the participants' heterogeneity. All analyses revealed significant differential gene expression. In Primary OCS events, genes were upregulated compared to baseline; conversely, in Primary No OCS, genes were downregulated. There were 10016 (42%) common overlapping genes between Primary OCS and Primary No OCS.

Upstream regulator analysis identified **LINC00941** activated in Primary OCS, and its activity was inhibited in Primary No OCS, a gene associated with airway remodelling. Exploration of canonical pathways further identified pathways related to airway modelling in Primary OCS but not in Primary No OCS. Pathway analysis also identified pathways associated with T1 and T2 inflammation, such as IL-4 signalling and TH17. Genes related to these pathways were expected to have upregulated activity, suggesting heterogeneity in inflammatory pathways. Other nasal transcriptomic studies, carried out in children, found

canonical pathways associated with ciliary function and also identified T1 and T2-associated pathways.[463]

Of particular interest was the upstream analysis between BNE and BE. At baseline, when participants were meant to have “controlled asthma,” genes associated with airway inflammation and airway remodelling, such as BRD4 and TGFB1, were activated in BE in performing upstream analysis.

The small sample size of Primary OCS events limits the generalisability of such findings. The study design was observational; therefore, the differential gene expression analysis, upregulated and downregulated molecules and mechanistic pathways identified by IPA cannot be attributed to a purely viral-associated asthma exacerbation. Pollutants, bacteria or allergens could have contributed to the findings. An in-vivo approach to identifying an isolated cause of an asthma exacerbation would be challenging and there may be multiple aetiologies at play for a single exacerbation event. This thesis does not account for seasonality as a contributing factor to asthma exacerbation but could contribute to the findings.

Future work

Hypothesis-free approach to asthma exacerbations

Exacerbation events were organised into whether a course of steroids was provided or not. This was based on the physician's interpretation of history and examination findings, and some of the outcomes of this thesis are based on this. It is interesting to identify if a hypothesis-free approach can phenotype exacerbation events, according to the integration of key, clinically relevant measurements and history findings. I am working with a health data scientist, Dr Figueredo, using APEX baseline and exacerbation data, to see if we can phenotype the data into clusters using this hypothesis-free approach, to generate unique groups. Integration of RNA-sequencing data would enrich the analysis.

Discovery of microbiome associated with asthma exacerbations

Through qPCR, I identified the proportion of asthma exacerbations with a virus present, which is the most common cause. However, there are many other causes of asthma exacerbations. It would be interesting to identify what proportion of exacerbations in APEX had other viruses, bacterial or fungal pathogens associated with them. Mapping the RNA sequences to these genes could be a way to explore this.

The importance of the lung microbiome through nasal sampling and how it influences asthma exacerbations during and after an exacerbation has been explored in children,[464] but it is unexplored, among adults. It would be interesting to know how the microbial flora alters from baseline to an exacerbation event after an annual review. It is also essential to identify the critical interactions between the host and microbia colonising the nasal epithelium and whether a course of steroids influences this.

Post exacerbation changes

In this thesis, I have explored the longitudinal stability of an asthma exacerbation. On a transcriptomic level, I have explored primarily the changes that occur at exacerbation and compared this from baseline. The longitudinal transcriptomic changes that occur after an exacerbation and how this compares to baseline at study entry, would further strengthen the assessment of the stability of asthma exacerbation phenotypes. Assessing the immediate transcriptomic changes that occur post-exacerbation and how this compares to that seen at baseline with the aim of seeing whether expression modules alter, is of interest and is currently underway in APEX-3, exploring asthma exacerbations in a hospital setting. RNA sequencing could help identify whether there is resolution of transcriptomic changes seen at the point of exacerbation or not.

Inflammatory profiling using protein expression in the APEX study.

Adding proteomic work could provide a further depth to phenotyping asthma exacerbations. We have phenotyped exacerbations using an inflammatory approach and identified differences between T2-high and low exacerbations. Proteomics could offer biomarkers for "eosinophilic asthma exacerbations" or "neutrophilic asthma exacerbations". The U-BIOPRED study group identified 13 serum proteins associated with eosinophilic asthma and 12 serum proteins associated with neutrophilic asthma by examining 756 sputum and serum samples.[465] It would be interesting to see if this is replicated in the APEX cohort at baseline and exacerbation.

Conclusions

It is essential to recognise the heterogeneity associated with asthma exacerbations. Small airway disease as a contributing factor to asthma exacerbations is an important consideration. It should be considered as part of clinical practice, especially in cases of severe asthma or for those who have experienced multiple asthma exacerbations. IOS appears to be sensitive to distinguish between an exacerbation event and uncontrolled asthma, providing an existing measurement is known. Both T2-high and T2-low asthma exacerbations appear to have similar physiological changes in lung function and measurements of smaller airways.

The phenotypes identified from baseline were stable after longitudinal follow-up over two years, with asthma exacerbations being factored in. The mechanisms associated with exacerbations and potential pathways according to T2 status have been alluded to.

Phenotyping exacerbations not only allows the potential for judicious prescribing of corticosteroids for uncontrolled asthma but also has the potential to deconstruct asthma exacerbations into traits which could be targeted.

Chapter 8-References

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Appendices

Appendix I Asthma history obtained at baseline visits.

ASTHMA HISTORY

YEAR OF DIAGNOSIS: ____ (YYYY) (If not know to obtain from GP records)

Previous hospital admissions for asthma : YES ☐ NO ☐ If YES; number of admissions ☐

Number of hospital admissions in past 12 months ☐

ITU admissions YES ☐ NO ☐

Number of unscheduled visits to A & E / OOH / GP in past 12 months ☐

Occupation ..... If not working:

Asthma related ill health ☐

Other cause ☐

Family history of asthma YES ☐ NO ☐

Allergy Cat ☐ Dog ☐ Grass ☐ Pollen ☐ Tree ☐ House dust mite ☐ Other: .....

SMOKING HISTORY

Smoking status: Current ☐ Ex Smoker ☐ Never Smoker ☐

If **Current**: Age of onset ☐☐ Number a day ☐☐ Years smoked ☐☐ Pack-Year ☐☐

If **Ex smoker**: Age of onset ☐☐ Number a day ☐ Years smoked ☐☐ Pack-Year ☐☐

MEDICATION ALLERGY

DRUG	REACTION

ASTHMA MEDICATIONS:

Property	Brand Name	Strength	Dose: Please state Puffs for inhalers (mg) for tablets/neb	Frequency	Compliancy
SABA ☐					Yes ☐ No ☐
ICS ☐					Yes ☐ No ☐
LABA/ICS ☐					Yes ☐ No ☐
Montelukast ☐					Yes ☐ No ☐
LAMA ☐					Yes ☐ No ☐
Theophylline ☐					Yes ☐ No ☐
Nebuliser ☐					Yes ☐ No ☐
OCS ☐					Yes ☐ No ☐

Appendix II Asthma Control Questionnaire

Asthma Control Questionnaire

Please answer Questions 1–6.
Circle the number of the response that best describes how you have been during the past week.

1. On average, during the past week, how often were you **woken by your asthma** during the night?
   - **0** Never
   - **1** Hardly ever
   - **2** A few times
   - **3** Several times
   - **4** Many times
   - **5** A great many times
   - **6** Unable to sleep because of asthma

2. On average, during the past week, how **bad were your asthma symptoms when you woke up** in the morning?
   - **0** No symptoms
   - **1** Very mild symptoms
   - **2** Mild symptoms
   - **3** Moderate symptoms
   - **4** Quite severe symptoms
   - **5** Severe symptoms
   - **6** Very severe symptoms

3. In general, during the past week, how **limited were you in your activities** because of your asthma?
   - **0** Not limited at all
   - **1** Very slightly limited
   - **2** Slightly limited
   - **3** Moderately limited
   - **4** Very limited
   - **5** Extremely limited
   - **6** Totally limited

4. In general, during the past week, how much **shortness of breath** did you experience because of your asthma?
   - **0** None
   - **1** A very little
   - **2** A little
   - **3** A moderate amount
   - **4** Quite a lot
   - **5** A great deal
   - **6** A very great deal

5. In general, during the past week, how much of the time did you **wheeze**?
   - **0** Not at all
   - **1** Hardly any of the time
   - **2** A little of the time
   - **3** A moderate amount of the time
   - **4** A lot of the time
   - **5** Most of the time
   - **6** All the time

6. On average, during the past week, how many **puffs of short-acting bronchodilator** have you used each day?
   - **0** None
   - **1** 1–2 puffs most days
   - **2** 3–4 puffs most days
   - **3** 5–8 puffs most days
   - **4** 9–12 puffs most days
   - **5** 13–16 puffs most days
   - **6** More than 16 puffs most days

To be completed by a member of the clinic staff:

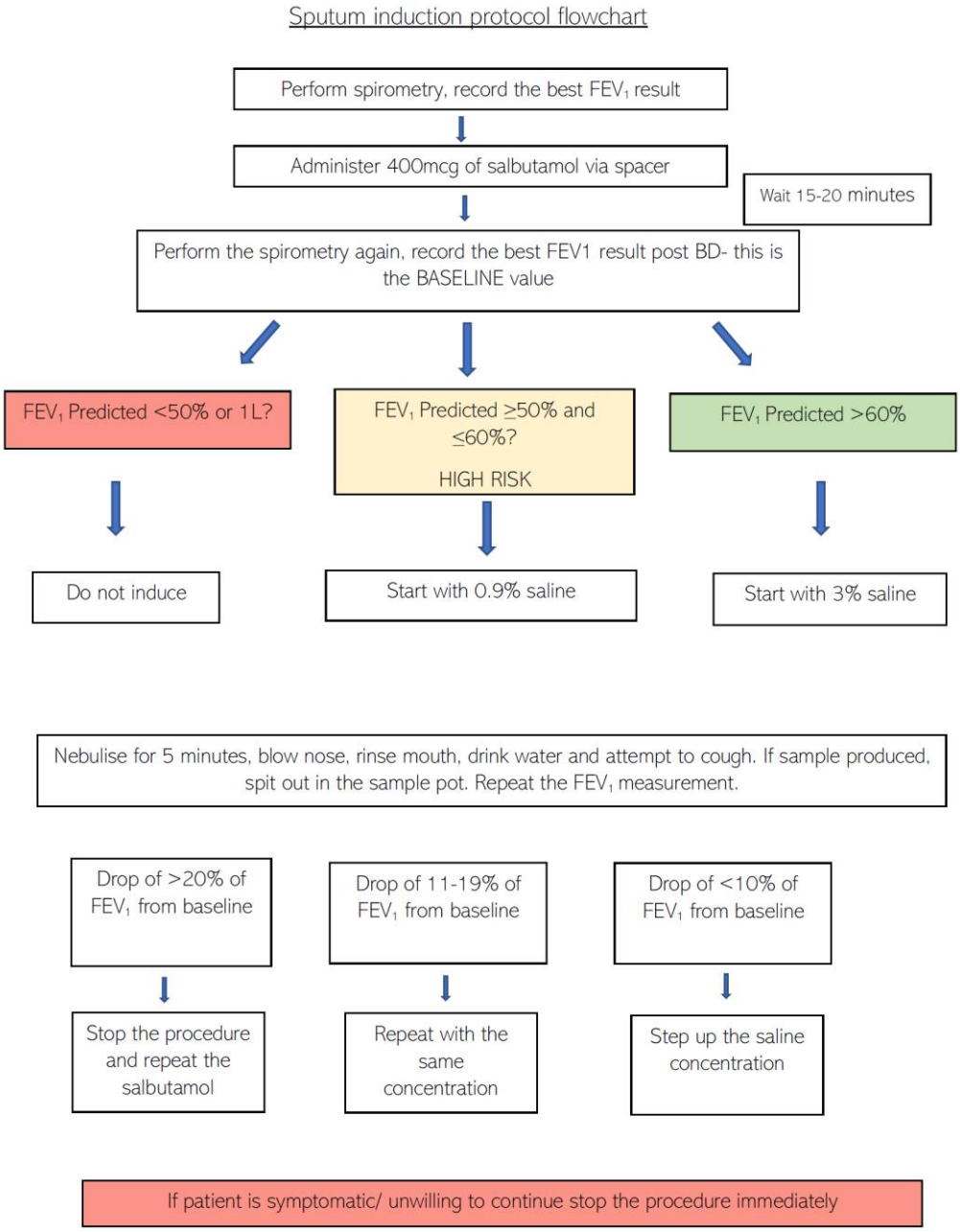
7. FEV_1 prebronchodilator:
   - **0** > 95% predicted
   - **1** 95–90%

   FEV_1% predicted:
   - **2** 89–80%
   - **3** 79–70%
   - **4** 69–60%
   - **5** 59–50%
   - **6** < 50% predicted

(Record actual values on the dotted lines and score the FEV_1% in the next column)

Juniper EF, O'Byrne PM, Guyatt GH, et al. *Eur Respir J.* 1999;14:902-907.

Appendix III Sputum induction protocol flow chart



Appendix IV Medicines Adherence Report Scale (MARS)

Patient Number **Visit** **Date**

Medicines Adherence Report Scale (MARS)

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- Many people find a way of using their medicines which suits them
- This may differ from the instructions on the label or form what their doctor has said
- We would like to as you a few questions about how you sue your medicines

Here are some ways in which people have said they use their medicines. For each of the statements, please tick the box which best applies to you:

Your own way of using your medicines	Always	Often	Sometimes	Rarely	Never
	1	2	3	4	5
I forget to take them					
I alter the dose					
I stop taking them for a while					
I decide to miss out a dose					
I take less than instructed					

Appendix V Patient information sheet APEX

The APEX Study
To better understand asthma exacerbations

Study information leaflet
Everything you need to know about this pioneering asthma study.



Chief Investigator: Prof Tim Harrison
Researchers: Dr Matthew Martin, Dr Ashish Pradhan & Prof Tricia McKeever

Background to the study

Asthma is a common lung condition affecting millions of people across the globe. With your help, we can try to understand more about the triggers of asthma exacerbations and how to prevent the early symptoms of asthma exacerbations from developing into a serious event. It is the Nottingham Asthma Centre's number one priority to turn this research into potentially lifesaving treatments for the future.

Join our research study

We would like to invite you to take part in this vital study.

- Before you decide, we would like you to understand why the research that is being done is so important and what it would involve for you.
- Please be assured that one of our team will go through this information sheet with you and answer any questions you may have.
- Talk to others about the study if you wish and please ask us if there is anything that is not clear, or if you would like more information.



A big hello from the APEX research team based in Nottingham!

What is the purpose of the study?

1. Key facts about asthma exacerbations

- Asthma exacerbations lead to difficulty in breathing, which can be very frightening. Some can be treated at home with extra puffs of inhalers, but some require steroid tablets and very severe exacerbations can require hospital treatment.

2. The reason for doing this research

- Lots of things can cause an asthma attack and we believe different triggers may lead to different types of exacerbations. If we can confirm this, it may be possible to find new ways to treat asthma exacerbations and reduce the use of steroid tablets which may have undesirable side effects.

3. The aims of this study

- We want to study a large group of people with asthma of any severity.
- We will perform some tests when your asthma is stable and then try and repeat them if you have an attack.

- WE WILL NOT CHANGE ANY OF YOUR CURRENT TREATMENT AND WE ARE NOT TESTING ANY NEW TREATMENTS ON YOU IN THIS STUDY.

4. This will

- Help us to understand more about what causes asthma exacerbations and whether people have different types of exacerbations.

Why have I been invited?

You are being invited to take part because we believe you have asthma and you have had an asthma attack in the last two years.

Do I have to take part?

It is completely up to you to decide whether you want to take part and your decision will not affect your care in any way. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form.

If you do decide to take part, you will be joining ground-breaking research into understanding asthma exacerbations and the information we learn from your participation could help us to discover new, potentially lifesaving treatments for asthma patients in the future. It may also help you to better understand and cope with your asthma. Please be assured that you would be free to withdraw at any time and without giving a reason. This would not affect your legal rights.

What happens if I join the study?

1. General information

- If you decide you would like to take part in this study, we will ask you to attend for several study visits and to have tests and procedures performed.

- The total length of the study is four years and we will be recruiting participants for three years. You will take part for up to three years or up to when the study ends, whichever is the soonest.

- All the visits will take place at the Nottingham Asthma Centre, which is part of the Nottingham Respiratory Research Unit, located at Nottingham City Hospital. We will try to arrange the visits at a time and day that is convenient for you and free parking is available at the unit. You should take all your medications as usual, including your inhalers, on the day of each visit and during your involvement in the study.

An overview of the study

If you agree to take part in the study, we will ask you to:

- Attend an initial visit (Baseline) which will last about 2.5 hours.
- Attend a visit at the end of each year you are in the study (Annual Visit), which will last about 2 hours.
- If, during the study, your asthma gets worse or you start to develop an asthma attack, we would like to try and arrange another visit (attack visit) as soon as possible, to assess how bad the attack is and provide you with any additional treatment we think you need. Hopefully this may avoid you having to make an appointment with your GP.
- With your consent, the research team will also send you a monthly reminder by text, from study specific mobile phones, asking you to contact them if you feel your asthma symptoms are worsening.
- Finally, we would also like to keep in touch with you every 3 months to provide you with study updates.

Activity	Baseline visit	Annual visit	Asthma Attack visit
Informed consent	x		
Medical history and medications	x	x	x
Inhaler technique check	x	x	x
Height/weight	x	x	x
Blood pressure	x	x	x
Questionnaires	x	x	x
Lung function tests	x	x	x
Sputum test	x	x	x
Blood tests	x	x	x
Urine tests	x	x	x
Nasal samples and throat swab	x	x	x
Skin prick (allergy) test	x		
Device training	x		

The visit table below summarises the study and the procedures which are involved at each visit.

The asthma attack visit

If you feel that your asthma is getting worse and you would normally ask a healthcare professional (for example, your doctor, nurse or pharmacist) for help, we will ask you to contact the research team. If possible, we will arrange for you to come to the Clinical Trials Unit for an assessment by a unit NHS doctor, at Nottingham City Hospital.

- This assessment will last about 40 minutes and is shaded in grey on the visit table above.
- After the assessment, there will then be 3 possible outcomes:

1. **The doctor thinks that you are definitely having an asthma attack**.

   You will be asked if you can complete Stage 2 of the visit immediately (shaded in blue in the table above) and afterwards, you will be given the appropriate treatment. This will be dispensed by the Nottingham City Hospital Out-Patients Pharmacy on the same day and we will contact you on Day 7 to see how you are.

2. **The doctor thinks that you may be in the early stages of an asthma attack or have a possible viral illness.**

You will be asked if you can complete Stage 2 of the visit immediately. You will be given a prescription for the appropriate treatment, which can be dispensed by any community pharmacy, in case you get worse over the next few days. We will ask you to contact us if you have any concerns or if you feel you need to start the attack treatment. You may be asked if you can come back to the unit to repeat Stage 2. If however, you do not get worse, we will contact you on Day 7 to check how you are.

3. **The doctor does not think you are having an asthma attack**.

You will not need to complete Stage 2 of the visit. We will ask you to contact us if you have any concerns or you feel your symptoms are worsening. If this happens, we may ask you if you can attend the Unit for a repeat assessment. If we do not hear from you, we will contact you on Day 7.

Whatever the outcome of the Asthma Attack Visit, we hope that you will remain in the study because we want to see if people with more than one attack, have the same or different types of exacerbations. You will be invited to your next annual review and we will ask you to contact us again, the next time you feel your asthma is getting worse.

IMPORTANT

If you feel you require urgent assessment by a healthcare professional, you should follow standard out-of-hours clinical practice, to ensure your safety. If you have an asthma attack and receive treatment elsewhere, then please let us know as soon as you are able to.

Please note that you will be able to contact us during office hours, which are Monday to Friday, from 9am until 5pm.

Tests and procedures

A member of the research team will be with you while you are doing any of the tests described below. We will ensure you are given full instructions on how to perform them. If you have any questions about any of these tests then please do not hesitate to ask. Any of the tests or procedures will be stopped immediately if you find them too unpleasant or develop significant side effects.

Medical History and Medications

At each visit, we will review your medical history (including respiratory and non-respiratory history, smoking and allergies) with you. We will also ask you about any treatments you are taking or have taken for asthma or other conditions.

Respiratory Questionnaires

You will be asked to complete 2 short questionnaires. These look at how asthma affects your life, what medications you use and how you use them.

Lung Function Tests

Spirometry

This is a blowing test, which is used to measure your lung function (the size of your lungs and how quickly you can empty them). Your height and weight will be recorded and then you will be asked to take a seat. You will remain seated for the test. You will be asked to take a deep breath in and then blow out as hard and fast as you can, into a mouthpiece until your lungs are empty. You may be asked to put a clip on your nose to make sure that no air escapes from your nose.

Occasionally some people can feel light-headed or faint whilst performing spirometry. It may also cause you to cough or make your chest feel tight.

Exhaled Nitric Oxide (FeNO)

Nitric Oxide is a gas present in everyone's breath. This is a blowing test which measures the amount of inflammation in the airways (breathing tubes), by measuring the concentration of exhaled nitric oxide. It involves breathing into a mouthpiece, which is connected to an analyser, for a few seconds.

Impulse Oscillometry (IOS)

This test is used to measure lung movement during normal breathing. You will be asked to breathe normally, though a mouthpiece into a tube, during which time measurements are made. This will be repeated 3 times.

Sputum Samples

Sputum is the mucus substance that is produced from the lungs and airways (breathing tubes) when a person coughs or spits. We will ask you to provide a sputum sample at every visit you attend by inhaling a salty solution through a machine called a nebuliser. This creates an aerosol of the salty water, which often helps people to produce a sputum sample. This process is usually well tolerated, however, you will experience a salty taste in your mouth and may feel nauseous or

have a sore throat. The salty solution may also irritate the airways, causing chest tightness, wheeze, cough and shortness of breath, so you will be monitored closely by trained staff, before, during and after the test, as described on the next page. If, for any reason, it is not possible to use the nebuliser, we will ask you to cough some sputum into a pot

- We will measure your lung function on a spirometer.
- You will be given a dose of Salbutamol (blue inhaler), using an inhaler and spacer, to open up your airways. We will let this work for 15-20 minutes. This can sometimes make you feel a little shaky and increase your heart rate.
- We will re-measure your lung function.
- We will ask you to inhale the salty solution for 5 minutes. We may ask you to do this up to 3 times at each visit.
- After each inhalation we will ask you to blow your nose and rinse out your mouth with some water. You will then be asked to cough to see if you can produce a sputum sample.
- We will check your lung function, using the spirometer, after each inhalation period and ask you to report any symptoms you may have. Further Salbutamol can be given for relief if necessary.

Skin Prick Test

This test is used to assess how allergic you are to up to 10 different substances, such as grass or pollen, which will be dissolved in liquid to make a solution. A small drop of the solution is placed on the forearm, which is scratched slightly with a needle, to see whether a reaction such as a red itchy bump occurs.
A skin prick test may cause mild discomfort from the scratch of the needle. In extremely rare cases, you may have a whole body allergic reaction that might cause a rash, swelling of the tongue or skin, shortness of breath or a fall in blood pressure. Emergency care is always available if this rare reaction occurs.
This test will usually be performed once during the study, at the Baseline Visit. However, if this is not possible for any reason, we can do this at any subsequent visit.

Blood Tests

You will be asked to provide up to 50mls (about 10 teaspoons) of blood at each visit. This will include samples for routine clinical screening and for future research into asthma and lung diseases.
Taking blood may cause pain where the needle is inserted and there is a small risk of bruising and infection at the needle site. Some people experience dizziness or fainting during a blood test, so it will be done with you sitting down.

Urine Sample

We will ask you to provide a urine sample of approximately 20mls. This will be stored in a freezer and we will look at inflammation molecules related to asthma.

Nasal Samples

To detect viruses and inflammation, we will take a sample of secretions from your nose. Firstly, we will insert a small piece of paper up one nostril to abosrob any mucus. Following this we will then squirt salty water into your nose and then immediately draw the fluid back into a syringe.

If you agree, we will also take cell samples from your nose for genetic analysis. These will be obtained by using a small brush. This will be optional and you will be asked to consent specifically to this.

Throat Swab

This will also be taken to detect viruses. A sterile cotton swab will be rubbed across the back of your throat for a few seconds. This may make you gag slightly, but it should not be painful.

Study Devices

Albus Home RD (Passive Bedside Monitoring Device)

Albus Home RD is an automated non-contact monitoring device that is plugged into the mains and placed by your bedside at home. Each night it monitors symptoms and signs relevant to asthma using different sensors. A wireless motion sensor (similar to those used in automatic lights) monitors breathing rate, a microphone records sounds of coughing and an environmental sensor measures temperature and humidity. The microphone captures all room sounds, but an automatic computer program identifies any recorded speech and scrambles it so it cannot be heard or identified by the research team. The audio recording function of the device can also be turned off or paused at any time.

The device uses minimal electricity (<1p per day) and is safe for long-term home use. All data is stored on a secure USB stick which we will occasionally ask you to bring with you to study visits for the data to be downloaded. If you agree, the device may be connected to your home wireless internet (WiFi) to allow the company (Albus Health) to check that the device is working properly, but the company would have no access to the data stored on the device. If you do have home internet or do not wish it to be used, pre-paid internet dongles, which connect to the device, can be provided.

If you use this device during the study, training on its use will be provided. Any technical support you require, will be provided by the research team. The devices will need to be returned to the research team at the end of your involvement with the study.

Expenses and payment

Unfortunately you will not be paid to take part in this study, but we can cover any travelling expenses or arrange a taxi if necessary. Please note that the costs of any prescribed treatment arising from attendance at study visits, will be met by yourself, according to your existing circumstances. For example, you may usually pay for prescriptions, have a pre-payment certificate or qualify for free prescriptions.

What are the benefits of taking part?

We cannot promise the study will help you, but we hope the information we obtain from this study will help us to understand more about the nature of asthma exacerbations, which could lead to the development of more targeted treatments. We also hope that you may gain a better personal understanding of your asthma and asthma exacerbations and that by seeing us we can avoid you having to make an appointment with your GP.

What are the risks?

As with all tests and procedures, some people experience side effects, some of which are detailed in the “Tests and Procedures” section. However, we believe the risks of this study are very small.

What happens when the study ends?

The study will end 4 years after the first participant is entered. The day-to-day care of your asthma will remain with your GP throughout your involvement in the study and with your permission, we will provide them with updates as detailed on Page 12.

What happens if there’s a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers, who will do their best to answer your questions. The researchers' contact details are given at the end of this information sheet. If you remain unhappy and wish to complain formally, you can do this by contacting the Patients Advice and Liaison Service (PALS) office.

PALS Telephone: 0800 1830204 (free from a landline) or 0115 9249924 Extension 65412.

Email: pals@nuh.nhs.uk

Post: NUH NHS Trust, c/o PALS, Freepost, NEA 14614, Nottingham NG7 1BR or via www.pals.nhs.uk

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence, then you may have grounds for a legal action for compensation against the University of Nottingham, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in the study be kept confidential?

We will follow ethical and legal practice and all information about you will be handled in confidence.
If you join the study, we will use information collected from you (and your medical records) during the course of the research. This information will be kept strictly confidential, stored in a secure and locked office, and on a password protected database at the University of Nottingham. Under UK Data Protection laws the University is the data controller (legally responsible for the data security) and the Chief Investigator of this study (named in this information sheet) is the Data Custodian (manages access to the data). This means we are responsible for looking after your information and using it properly. Your rights to access, change or move your information are limited, as we need to manage your information in specific ways to comply with certain laws and for the research to be reliable and accurate. To safeguard your rights, we will use the minimum personally identifiable information possible.

You can find out more about how we use your information and to read our privacy notice at:

https://www.nottingham.ac.uk/utilities/privacy.aspx.

The data collected for the study will be looked at and stored by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people from regulatory organisations to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

Information about you which leaves the research site will have your name and address removed and a unique code will be used, so that you cannot be recognised from it. However, sometimes we need to ensure that we can recognise you, to link the research data with your medical records, so in these instances, we will need to know your name and date of birth.

If you use the Albus Home RD bedside monitoring device during the study, to monitor the signs and symptoms of asthma, your speech will be scrambled by a computer so it will not be understood. All other data (breathing rate, coughing, room temperature and humidity will be transferred from a secure USB stick to Albus Health via secure servers, for analysis. All the data will be anonymised and identity code numbers, rather than your personal details will be used.

Your contact information will be kept by the University of Nottingham for one year after the end of the study, so that we are able to contact you about the findings of the study and possible follow up studies (unless you advise us that you do not wish to be contacted). This information will be kept separately from the research data and only those who need to, will have access to it. If you consent to the collection of your home and work addresses with postcodes, for the analysis of your exposure to pollen and air pollutants, this will be kept for two years after the end of the study. All other data (research data), will be kept securely for seven years. After this time, your data will be disposed of securely. During this time all precautions will be taken by all those involved, to maintain your confidentiality. Only members of the research team given permission by the data custodian will have access to your personal data.

In accordance with the University of Nottingham's, the Government's and our funder's policies, we may share our research data with researchers in other universities and organisations, including those in other countries, for research in health and social care. Sharing research data is important to allow peer scrutiny, re-use (and therefore avoiding duplication of research) and to understand the bigger picture in particular areas of research. Data sharing in this way will be anonymised (so that you cannot be identified).

Although what you say during the study visits is confidential, should you disclose anything to us which we feel puts you or anyone else at any risk, we may feel it necessary to report this to the appropriate persons.

What happens if I don't want to carry on with the study?

Your participation is voluntary and you are free to withdraw at any time, without giving any reason, and without your legal or medical treatment rights being affected. If you withdraw, we will no longer collect any information about you or from you, but we will keep the information about you that we have already obtained, as we are not allowed to tamper with study records and this information may have already been used in some analyses and may still be used in the final study analyses. To safeguard your rights, we will use the minimum personally identifiable information possible.

What involvement will my General Practitioner (GP) have?

With your permission, your GP will be notified that you are taking part in this study.
If you attend the Asthma Attack visit, we will send a letter to your GP, informing them of the outcome of the visit. We will also, with your permission, notify you and your GP of any findings that are of clinical concern. This will be discussed with you in the first instance. Please note that the results of the genetic analysis will not be given to your GP.

What happens to the samples I give?

We would also like to seek your consent, so that samples may be stored and used in possible future research (please indicate you agree to this on the consent form). These samples will be stored with a code unique to you and securely at the University of Nottingham, under the University's Human Tissue Research Licence (No. 12265).

Some of these future studies may be carried out by researchers other the current team of Professor Tim Harrison and Dr Matthew Martin, who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised and you will not be identified in any way. If you do not agree to this, any remaining samples will be disposed of in accordance with the Human Tissue Authority's codes of practice.

Will any genetic tests be done?

Yes. The samples we collect using the nasal brushes and whole blood will undergo genetic analysis. Please note that this part of the study is optional. If you agree to these samples being collected and analysed, you will be asked to sign an optional clause on the consent form. Please note that the results of this analysis will not be given to you.

What happens to the results of the research study?

We anticipate that the results of this study will be published in scientific journals and will be presented at scientific meetings and conferences. Copies of these can be sent to you on request. We may also publish a summary of the research on relevant websites and social media and in our newsletters. You will not be identified in any reports or publications as all data used is anonymous.

Who is organising and funding of research?

This research is being organised by the University of Nottingham and is being funded by AstraZeneca.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been checked and approved by the Health and Social Care Research Ethics Committee A (HSC REC A).

Who can I contact for more information?

For further information please contact the APEX Team
Email Karen.Shaw@nottingham.ac.uk
Rob.Needham@nottingham.ac.uk

Telephone 07967327345 or 07970224153
We look forward to hearing from you.
The Nottingham Asthma Centre Team.

Appendix VI Description of Exacerbation Events according to outcome.

	Exacerbation OCS Mean(SD)	Exacerbation OCS Median(IQR)	Exacerbation No OCS Mean(SD)	Exacerbation No OCS Median(IQR)
ACQ-5 Score	3.39(0.89)	3.20(2.60-4.00)	2.90(0.79)	2.80(2.30-3.40)
ACQ-6 Score	3.33(0.89)	3.17(2.54-3.96)	2.83(0.76)	2.83(2.33-3.33)
ACQ-7 Score	3.34(0.78)	3.21(2.71-3.82)	2.67(0.68)	2.71(2.21-3.14)
MARS-A Score	4.39(0.79)	4.60(4.05-5.00)	4.62(0.56)	4.80(4.80-5.00)
R5 cmH_20/L/s	5.77(2.19)	5.83(4.07-7.04)	3.84(1.40)	3.87(2.83-4.50)
R20 cmH_20/L/s	3.72(1.00)	3.85(2.94-4.47)	3.06(0.86)	3.07(2.55-3.64)
R5-R20 cmH_20/L/s	2.04(1.51)	2.15(1.00-2.84)	0.78(0.84)	0.64(0.20-1.25)
AX cmH_20/L	45.02(37.93)	39.93(11.17-62.56)	14.76(21.05)	7.46(3.37-20.36)
FeNO (ppb)	51.51(56.62)	29.00(21.00-50.00)	35.35(34.31)	25(15.00-35.50)
FEV1 Predicted (L)	2.85(0.68)	2.81(2.35-3.21)	3.26(0.70)	3.23(2.76-3.68)
FEV1 (L)	1.98(0.81)	1.81(1.47-2.43)	2.85(0.78)	2.80(2.35-3.30)
FVC Predicted (L)	3.61(0.85)	3.54(3.00-4.04)	4.08(0.87)	4.05(3.46-4.60)
FVC (L)	2.94(0.98)	2.86(2.37-3.39)	3.82(0.99)	3.78(3.08-4.40)
PEFR L/min	338.50(104.46)	328.00(273.25-398.75)	485.32(124.54)	481.00(386.00-568.00)
FEV1 (Post bronchodilator) (L)	2.31(0.90)	2.17(1.64-2.89)	3.03(0.79)	3.16(2.48-3.41)
FEV1/FVC Ratio	67.08(13.03)	68.74(57.78-75.51)	74.73(9.57)	77.23(68.83-82.36)
FEV1% Predicted	69.45(21.52)	68.54(57.13-84.86)	86.78(12.22)	89.38(82.60-95.03)
FVC%Predicted	81.71(18.94)	83.62(72.32-94.28)	93.32(11.68)	94.05(86.166-101.65)
Reversibility (%)	12.69(11.50)	8.22(4.97-18.93)	7.00(6.57)	5.46(2.90-8.96)
Sputum Neutrophil Count (%)	77.58(34.45)	93.00(56.50-99.13)	79.78(21.97)	87.75(64.13-98.25)
Sputum Eosinophil Count (%)	15.91(27.38)	0.50(0.13-32.63)	6.78(18.68)	0.00(0.00-2.00)
Eosinophils	0.36(0.31)	0.28(0.11-0.50)	0.26(0.27)	0.18(0.11-0.28)
CRP (x10^9/L)	8.86(11.78)	4.00(2.00-10.25)	4.79(7.85)	3.00(2.00-5.25)

Table 8.1: Descriptive features of unique exacerbation events

	Multiple OCS Mean(SD)	Multiple OCS Median(IQR)	Multiple No OCS Mean(SD)	Multiple No OCS Median(IQR)
ACQ-5 Score	3.68(0.62)	3.70(3.20-4.20)	2.70(0.60)	2.80(2.20-3.20)
ACQ-6 Score	3.53(0.61)	3.33(3.00-4.17)	2.50(0.63)	2.50(1.83-2.92)
ACQ-7 Score	3.52(0.66)	3.71(2.86-4.14)	2.47(0.56)	2.71(1.86-2.86)
MARS-A Score	4.72(0.27)	4.80(4.60-5.00)	4.62(0.40)	4.60(4.50-5.00)
R5 cmH_20/L/s	4.78(2.10)	4.66(2.99-5.91)	4.32(1.71)	3.82(2.83-6.31)
R20 cmH_20/L/s	3.31(1.00)	3.18(2.56-4.01)	3.15(0.83)	3.11(2.53-3.79)
R5-R20 cmH_20/L/s	1.47(1.40)	.87(0.41-2.37)	1.17(1.06)	0.77(0.28-2.14)
AX cmH_20/L	30.65(37.00)	12.59(5.40-45.24)	27.39(31.60)	12.74(4.42-30.75)
FeNO (ppb)	50.24(48.45)	34.00(16.00-59.50)	40.59(31.44)	43.00(17.00-53.50)
FEV1 Predicted (L)	2.96(0.63)	3.08(2.59-3.41)	2.90(0.67)	2.77(2.30-3.48)
FEV1 (L)	2.12(0.84)	1.99(1.47-2.87)	2.44(0.90)	2.17(1.78-2.98)
FVC Predicted (L)	3.74(0.81)	3.84(3.18-4.35)	3.70(0.85)	3.46(2.89-4.46)
FVC (L)	3.21(1.02)	3.06(2.31-3.77)	3.61(1.19)	3.12(2.79-4.58)
PEFR L/min	391.71(137.05)	382.00(310.50-470.50)	474.00(150.94)	438.00(378.50-521.50)
FEV1 (Post bronchodilator) (L)	2.14(0.86)	2.15(1.59-2.87)	2.42(0.91)	2.17(1.78-2.69)
FEV1/FVC Ratio	65.21(13.98)	66.82(55.20-74.65)	68.03(11.28)	69.60(63.38-74.58)
FEV1% Predicted	70.71(20.83)	75.02(52.20-85.95)	82.81(15.55)	84.05(73.97-89.13)
FVC%Predicted	84.97(14.64)	83.72(72.15-95.30)	96.06(13.59)	97.09(83.63-107.33)
Reversibility (%)	8.99(11.38)	9.35(0.00-13.38)	7.30(9.49)	5.21(0.74-10.57)
Sputum Neutrophil Count (%)	42.96(36.79)	24.85(12.25-81.94)	53.56(29.43)	43.37(33.00-84.31)
Sputum Eosinophil Count (%)	25.77(35.20)	1.76(0.00-65.88)	9.06(12.51)	4.38(0.75-22.06)
Eosinophils	0.38(0.32)	0.31(0.11-0.58)	0.34(0.20)	0.39(0.21-0.49)
CRP (x10^9/L)	7.31(3.00)	3.00(1.00-10.00)	12.35(31.41)	2.00(1.00-6.00)

Table 8.2: Descriptive features of multiple exacerbation events. Mean(SD) and Median (25%-75% IQR)

Appendix VII RIN scores from Oxford Genomics.

Sample Conc.	QC Conc (ng/uL)	Total mass (ng)	Vol (ul)	Volume (ul)	**Input Mass for prep (ng)**	RIN Values	Tape Conc. ng/ul
5	**5.90**	**176.92**	30	**30**	176.92	3.5	7.4
5	**1.80**	**44.43**	30	**24.7**	44.43	3.8	2.22
5	**3.79**	84.2	30	**22.2**	84.2	2.1	2.3
5	**1.01**	5.1	30	**22**	5.071	4.3	0.7
5	**5.46**	**136.44**	30	**25**	136.44	5.2	6.37
5	**3.43**	**92.57**	30	**27**	92.57	1.9	8.3
5	**1.38**	6.9	30	**25**	6.9	3.3	3.8
5	**1.22**	**30.38**	30	**25**	30.38	4	1.47
4.8	**6.26**	**169.08**	30	**27**	169.08	5.3	6.57
5	**0.69**	3.5	30	**25**	3.5	2.9	0.9
5	**2.21**	**58.54**	30	**27**	58.54	3.1	7.82
5	**2.56**	**76.23**	30	**29.8**	76.23	3	4.26
5	**0.82**	4.1	30	**28.6**	4.1	3.4	0.9
5	**2.13**	10.6	30	**25**	10.6	4.9	2.5
5	**10.50**	**262.46**	30	**25**	262.46	5.1	7.37
5	**10.05**	**271.47**	30	**27**	271.47	3.2	53.1
5	**0.88**	22.9	30	**26**	22.9		0.2
5	**2.50**	12.5	30	**25**	12.5	3.1	5.9
5	**0.99**	**24.71**	30	**25**	24.71	3.7	1.06
5	**5.38**	**145.14**	30	**27**	145.14	3.5	17.5
5	**0.80**	20.9	30	**26**	20.9		0.3
5	**14.45**	**361.16**	30	**25**	361.16	4.80	22.8
5	**1.46**	38.0	30	**26**	38.0	3.4	0.5
5	**2.48**	**66.94**	30	**27**	66.94	4.3	4.4
5	**1.33**	6.6	30	**25**	6.6	2.8	3.6
5	**2.44**	12.2	30	**25**	12.2	5.2	3.4
5	**1.26**	**33.90**	30	**27**	33.90	1.1	2.44
5	**2.40**	**60.05**	30	**25**	60.05	2.6	4.73
5	**3.44**	89.4	30	**26**	89.4	6.4	3.5
5	**1.20**	29.9	30	**24.9**	29.9	3.6	1.0
5	**2.54**	**68.71**	30	**27**	68.71	1.6	7.22
5	**8.66**	**226.76**	30	**26.2**	226.76	2.7	13.9
5	**0.62**	3.1	30	**25**	3.1		0.1
5	**1.33**	**33.13**	30	**25**	33.13	4.5	1.9
5	**0.86**	4.3	30	**25**	4.3	1.8	0.6
5	**21.52**	**516.58**	30	**24**	516.58	3	14.7
5	**2.05**	53.4	30	**26**	53.4	5.2	4.4

5	1.55	41.88	30	27	41.88	2.7	2.1
5	5.64	28.2	30	25	28.2	3.4	13.7
5	0.71	17.81	30	25	17.81	3.6	0.751
5	0.86	22.3	30	26	22.3	1	0.8
5	1.35	58.29	30	43	58.29	1.9	2.2
5	11.10	55.5	30	25	55.5	3	33.6
5	2.40	60.03	30	25	60.03	5.1	3.88
5	4.57	98.65	30	22	98.65	1.9	2.5
5	12.90	64.5	30	27	64.5	3	50.0
5	3.18	75.29	30	23.7	75.29	5.8	3.78
5	0.48	11.6	30	24	11.6		0.2
5	4.84	149.16	30	30.8	149.16	4.2	4.57
5	2.26	60.96	30	27	60.96	4.6	6.0
5	0.86	4.3	30	25	4.3	3	2.0
5	0.91	4.6	30	25	4.6	1.5	1.3
5	1.17	29.30	30	25	29.30	4.1	1.55
5	2.95	79.54	30	27	79.54	3.4	2.77
5	0.98	26.99	30	28	26.99	2.5	3.02
5	2.88	74.20	30	25.8	74.20	3.7	2.46
5	0.68	3.4	30	25	3.4	1.2	0.5
5	1.28	31.94	30	25	31.94	3.6	1.2
2.9	0.63	17.12	30	27	17.12		0.2
5	2.51	12.5	30	25	12.5	3.8	2.4
5	0.87	22.6	30	26	22.6		0.4
5	1.08	5.4	30	25	5.4	1.8	2.0
5	4.24	106.11	30	25	106.11	2.4	5.34
5	3.24	87.39	30	27	87.39	1.7	5.6
5	0.50	13.1	30	26	13.1		0.1
5	1.48	40.08	30	27	40.08	2.3	2.3
5	0.97	25.7	30	26.4	25.7	2.4	0.4
5	1.54	38.61	30	25	38.61	3.2	1.05
5	2.17	10.8	30	25	10.8	2.7	5.0
5	1.51	7.5	30	25	7.5	6.8	1.9
5	2.38	64.13	30	27	64.13	1.4	4.91
5	2.94	76.5	30	26	76.5	2.8	3.2
5	1.23	32.68	30	26.6	32.68	2.6	1.46
5	1.65	43.0	30	26	43.0	3.2	0.9
5	12.23	330.24	30	27	330.24	3.9	173
5	9.36	299.39	30	32	299.39	2.3	20.1
5	0.86	4.3	30	25	4.3		0.3
5	1.70	42.39	30	25	42.39	1.7	3.66
5	0.98	25.5	30	26	25.5	3.3	0.8
5	2.02	54.67	30	27	54.67	2.1	2.7

5	1.05	5.2	30	25	5.2		0.3
5	1.71	46.19	30	27	46.19	3.3	7.4
5	17.97	89.8	30	25	89.8		9.6
5	2.12	55.1	30	26	55.1	1.4	2.3
5	0.90	22.49	30	25	22.49	3.2	0.916
5	3.00	81.10	30	27	81.10	1.1	7.5
5	0.78	3.9	30	25	3.9	3.4	0.4
5	3.07	76.77	30	25	76.77	2.3	3.86
4.4	1.32	52.88	30	40	52.88	1.6	0.8
5	0.85	20.5	30	24	20.5	1.8	0.5
5	1.41	7.0	30	29	7.0	4.6	2.1
5	3.20	78.64	30	24.6	78.64	3.4	3.76
5	0.86	21.40	30	25	21.40	3	0.787
5	2.52	68.02	30	27	68.02	2.9	5.3
5	27.08	135.4	30	25	135.4	2.9	23.4
5	6.23	31.2	30	25	31.2	3.3	34.6
5	1.85	46.15	30	25	46.15	4.6	2.02
5	2.39	64.42	30	27	64.42	3.1	2.98
5	3.11	87.15	30	28	87.15	3	10.8
5	0.62	3.1	30	25	3.1	3.3	0.5
5	5.52	167.26	30	30.3	167.26	3.6	5.65
5	3.10	77.41	30	25	77.41	1.2	3.44
5	1.02	5.1	30	25	5.1	2	0.5
5	3.04	82.16	30	27	82.16	2.6	2.8
5	0.66	17.1	30	26	17.1		0.2
5	4.58	114.43	30	25	114.43	4.5	5.28
5	2.80	14.0	30	25	14.0	4.8	5.2
5	2.17	58.50	30	27	58.50	1	2.6
5	0.45	11.6	30	26	11.6		0.1
5	53.92	1455.72	30	27	1455.72	3.4	13.7
5	4.39	109.70	30	25	109.70	3.1	5.66
5	1.57	7.8	30	25	7.8	2.7	3.8
5	0.68	17.6	30	26	17.6		0.2
5	11.24	303.52	30	27	303.52	3.1	26.4
5	0.75	18.63	30	25	18.63	3.3	0.755
5	0.43	11.3	30	26	11.3	2.8	0.6
5	0.94	4.7	30	25	4.7		0.4
5	0.74	19.2	30	26	19.2	2.9	0.6
5	0.58	24.45	30	43	24.45	3	1.4
5	6.22	155.59	30	25	155.59	3.1	7.81
5	0.49	2.4	30	25	2.4		0.0
5	1.27	6.3	30	25	6.3	2.1	0.9
5	1.35	33.79	30	25	33.79	2.5	0.758

5	0.72	18.6	30	26	18.6	2.9	0.6
5	2.99	80.73	30	27	80.73	1.2	2.6
5	6.51	175.79	30	27	175.79	4.5	7.6
5	1.19	5.9	30	26.5	5.9	7	1.5
5	0.85	22.2	30	26	22.2	2.8	0.6
5	2.63	65.85	30	25	65.85	4.1	2.96
5	5.21	140.70	30	27	140.70	6	5.8
5	18.69	93.5	30	25	93.5	2.4	20.4
5	3.69	92.26	30	25	92.26	3.3	4.13
5	15.48	464.52	30	30	464.52	3.8	64.3
5	3.35	83.77	30	25	83.77	3.4	4.62
5	3.68	95.8	30	26	95.8	3.1	1.5
5	2.75	13.7	30	25.5	13.7	2.8	9.6
5	1.07	32.86	30	30.8	32.86	3.1	1.12
3	1.82	49.15	30	27	49.15	2.6	2.5
5	1.52	7.6	30	25	7.6	2.7	2.6
5	5.39	134.67	30	25	134.67	2.5	6.07
5	2.39	63.92	30	27	63.92	2.3	4.73
5	6.43	32.1	30	25	32.1	3.2	12.7
5	1.14	34.32	30	30	34.32	2.9	1.74
5	0.62	3.1	30	25	3.1	3.3	0.5
5	6.62	200.61	30	30.3	200.61	2.6	9.82
5	1.79	44.85	30	25	44.85	3.1	1.94
5	1.99	53.77	30	27	53.77	1.9	1.3
5	3.32	16.6	30	25	16.6	7.3	4.7
5	0.67	14.7	30	21.9	14.7		0.2
5	4.59	114.64	30	25	114.64	4.7	5.15
5	1.70	44.1	30	26	44.1	1.2	1.0
5	10.65	53.2	30	25	53.2	3.9	75.7
1.7	1.49	47.60	30	32	47.60	1.3	1.2
5	2.95	79.78	30	27	79.78	1.1	4.81
5	1.16	30.2	30	26	30.2	6.5	0.6
5	1.99	10.0	30	25.3	10.0	2.1	2.2
5	4.25	106.25	30	25	106.25	2.4	0.918
5	1.25	33.87	30	27	33.87	2.8	0.972
5	0.66	17.1	30	26	17.1	1.2	0.6
5	7.91	39.6	30	25	39.6	3.3	14.9
5	1.24	30.88	30	25	30.88	2.8	0.934
5	0.95	4.8	30	25	4.8	1.6	0.6
5	1.26	34.08	30	27	34.08	2.6	1.9
5	0.94	23.41	30	25	23.41	3.4	0.854
5	0.45	11.7	30	26	11.7		0.3
5	4.80	120.11	30	25	120.11	3.7	4.91

5	0.64	3.2	30	25	3.2		0.2
5	2.26	58.7	30	26	58.7	1.3	1.7
5	2.82	76.26	30	27	76.26	1.2	4.3
5	4.47	120.82	30	27	120.82	4.8	7.6
5	0.65	3.2	30	25	3.2		0.3
5	4.46	115.64	30	25.9	115.64	1.4	3.7
5	1.02	22.8	30	22.2	22.8	1.5	0.5
5	3.50	87.43	30	25	87.43	4.9	3.17
5	0.84	22.69	30	27	22.69	2.7	1.2
5	1.39	6.9	30	25	6.9	2.1	1.4
5	2.52	63.03	30	25	63.03	3.8	4.12
5	1.61	50.06	30	31	50.06	3.4	1.8
5	1.16	30.1	30	26	30.1	2.2	0.5
5	4.17	20.9	30	25	20.9	3.3	10.3
5	4.29	107.24	30	25	107.24	5.4	16.4
5	3.04	82.05	30	27	82.05	2	5.2
5	0.59	2.9	30	30.2	2.9	1	0.7
5	1.19	32.23	30	27	32.23	2.3	3.32
5	0.49	2.5	30	25	2.5	1.5	0.5
5	2.12	53.03	30	25	53.03		
5	1.79	48.76	30	27	48.76	2.4	6.44
5	2.78	71.64	30	25.8	71.64	2.6	2.2
5	0.64	3.2	30	25	3.2	1	4.2
5	1.26	34.01	30	27	34.01	3	1.2
5	3.11	15.6	30	27.4	15.6	3.1	4.3
5	1.27	31.80	30	25	31.80	2.8	1.29
5	0.49	11.8	30	24	11.8		0.2
5	4.08	101.97	30	25	101.97	1.2	4.71
5	3.23	16.1	30	25	16.1	3.8	9.2
5	0.59	15.4	30	26	15.4		0.1
5	2.97	80.20	30	27	80.20	1.4	5.1
5	0.90	24.33	30	27	24.33	1.1	1.02
5	3.11	15.5	30	25	15.5	3.2	6.7
5	2.65	68.8	30	26	68.8	2.9	2.2
5	4.25	106.35	30	25	106.35	1	5.39
5	3.97	101.16	30	26	101.16	2.8	7.51
5	0.59	13.2	30	22.5	13.2	3.2	0.8
5	0.66	16.60	30	25	16.60	3.3	0.737
5	0.75	3.7	30	25	3.7		0.2
5	1.19	5.9	30	25	5.9	1.3	1.2
5	0.92	24.78	30	27	24.78	1.5	1.78
5	2.96	74.12	30	25	74.12	3.3	2.99
5	0.55	14.4	30	26	14.4	3	0.4

5	3.83	103.43	30	27	103.43	4	9.0
5	2.64	68.7	30	26	68.7	1.4	2.0
5	6.76	169.07	30	25	169.07	6	7.3
5	0.73	3.6	30	25	3.6		0.4
5	2.99	74.76	30	25	74.76	4.2	4.43
5	2.67	71.96	30	27	71.96	3.7	6.6
5	0.62	3.1	30	25	3.1		0.3
5	1.19	44.5	30	37.5	44.5	2.8	1.0
5	2.83	71.82	30	25.4	71.82	2.7	2.66
5	1.15	30.99	30	27	30.99	3.9	2.8
5	0.54	2.7	30	26.4	2.7	1	0.7
5	2.62	65.44	30	25	65.44	6	3.26
5	0.92	28.51	30	31	28.51	2.1	0.9
5	1.99	9.9	30	25	9.9	3.3	4.2
5	0.65	17.0	30	26	17.0		0.1
5	3.06	94.17	30	30.8	94.17	3.2	2.97
5	0.94	25.34	30	27	25.34	2.9	1.7
5	1.02	5.1	30	25	5.1	2.2	1.0
5	0.66	3.3	30	25	3.3	2.8	1.8
5	1.09	29.43	30	27	29.43	4.8	5.65
5	2.72	68.02	30	25	68.02	3.4	2.13
5	46.47	1254.76	30	27	1254.76	3.4	30.4
5	18.59	464.67	30	25	464.67	3.20	17.5
5	0.94	4.7	30	4.5	4.7	1.7	0.9
5	3.17	15.9	30	25	15.9	3.5	3.7
5	0.62	16.83	30	27	16.83		0.2
5	6.11	152.78	30	25	152.78	4.7	5.13
5	1.02	26.5	30	26	26.5		0.4
5	2.12	50.9	30	24	50.9	2.1	1.3
5	432.45	2162.3	30	24	2162.3	3.1	29.2
5	1.28	31.13	30	24.4	31.13	3	1.76
5	1.71	46.11	30	27	46.11	1.7	3.5
5	2.63	13.1	30	25	13.1	3.3	4.6
5	4.21	105.33	30	25	105.33	2.7	3.96
5	1.11	29.0	30	26	29.0	2.6	1.2
5	0.82	22.01	30	27	22.01	2.7	1.62
5	0.77	20.0	30	26	20.0	2.5	1.0
5	7.56	204.13	30	27	204.13	3.4	15.7
5	11.61	290.35	30	25	290.35	1.4	9.2
5	0.60	3.0	30	25	3.0		0.1
5	9.70	242.60	30	25	242.60	2.8	10.4
5	1.21	31.5	30	26	31.5	4.1	1.2
5	1.03	5.2	30	25	5.2	1.8	0.6

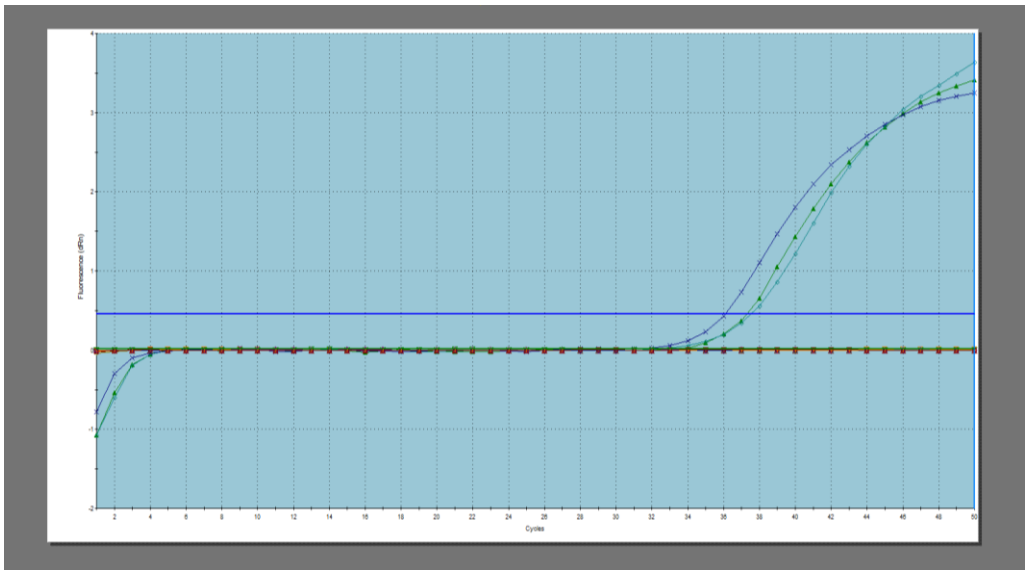
5	6.03	162.74	30	27	162.74	1.5	9.47
5	2.00	53.93	30	27	53.93	2.8	4.5
5	2.91	75.6	30	26	75.6	2.3	2.8
5	3.00	74.90	30	25	74.90	3.4	3.04
5	1.66	8.3	30	25	8.3	4.5	1.4
5	2.43	65.67	30	27	65.67	3.3	13.2
5	0.62	16.1	30	26.2	16.1	1.8	0.7
5	0.69	3.4	30	25	3.4		0.3
5	4.57	114.15	30	25	114.15	3.8	7.13
5	2.21	59.77	30	27	59.77	2.8	6.6
5	5.25	131.19	30	25	131.19	3.9	8.6
5	5.31	26.5	30	25	26.5	4	38.9
5	4.89	131.00	30	27	131.00	3.6	3.9
5	2.31	57.69	30	25	57.69	1.1	2.36
5	2.28	11.4	30	25	11.4	5.4	1.7
5	1.05	27.2	30	26	27.2		0.4
5	1.98	49.49	30	25	49.49	3	2.5
5	3.63	97.91	30	27	97.91	4	5.2
5	14.61	73.1	30	25	73.1	3.3	64.3
5	1.27	34.38	30	27	34.38	4.3	4.85
5	1.20	29.93	30	25	29.93	3.4	1.46
5	0.58	2.9	30	25	2.9	3.2	0.6
5	1.42	7.1	30	25	7.1	7	1.0
5	2.64	66.08	30	25	66.08	4.1	2.12
5	1.26	34.14	30	27	34.14	5.6	1.61
5	1.88	9.4	30	25	9.4	1.3	1.7
3.6	2.58	69.68	30	27	69.68	2.5	1.7
5	7.78	198.30	30	25.5	198.30	3.4	20.8
5	0.45	11.7	30	26	11.7		0.1
5	1.15	5.8	30	25	5.8	1.1	2.4
5	5.17	129.33	30	25	129.33	5.4	2.75
5	2.30	62.05	30	27	62.05	2.5	1.6
5	0.60	15.6	30	26	15.6		0.1
5	1.35	6.7	30	25	6.7	1	1.0
5	7.03	175.64	30	25	175.64	2.9	5.74
5	0.56	14.7	30	26	14.7	1.9	0.5
5	2.53	71.21	30	28	71.21	4	4.11
5	6.49	175.24	30	27	175.24	3.1	7.63
5	4.57	114.20	30	25	114.20	2.7	7.62
5	0.79	4.0	30	25	4.0	1.9	0.5
5	0.81	21.0	30	26	21.0	2.2	0.8
5	2.50	62.42	30	25	62.42	5.2	2.34
5	0.91	4.6	30	25	4.6	1.9	0.4

5	0.96	25.97	30	27	25.97	2.6	1.24
5	1.13	29.4	30	26	29.4	1.5	1.0
5	2.74	74.02	30	27	74.02	3.2	4.3
5	1.24	6.2	30	25	6.2	2.3	1.2
5	2.21	55.16	30	25	55.16	5.7	2.14
5	0.62	16.0	30	26	16.0	3.3	0.9
5	6.62	178.68	30	27	178.68	1.3	8.0
5	1.30	32.61	30	25	32.61	2.1	1.64
5	2.66	13.3	30	25	13.3	6.3	2.4
5	3.30	86.37	30	26	86.37	3.6	4.9
5	1.33	33.24	30	25	33.24	3.5	1.31
5	0.68	3.4	30	25	3.4	3.1	1.2
5	2.91	73.89	30	25.4	73.89	6.2	3.57
5	1.66	47.39	30	29	47.39	2.4	0.9
5	1.21	6.0	30	28.8	6.0	1.8	0.7
5	0.76	19.7	30	26	19.7		0.2
5	16.51	417.82	30	25.3	417.82	2.90	16.6
2.9	1.55	88.45	30	57	88.45	2.9	0.9
5	0.66	3.3	30	25	3.3	3.3	0.5
5	5.64	152.38	30	27	152.38	4.9	17.2
5	3.27	81.66	30	25	81.66	5.6	1.76
5	1.61	8.0	30	25	8.0	2.1	0.8
5	2.22	60.04	30	27	60.04	4.4	4.36
5	2.08	51.99	30	25	51.99	2.7	1.29
5	0.77	3.8	30	25	3.8		0.2
5	3.58	17.9	30	25	17.9	8.3	5.2
5	13.31	359.39	30	27	359.39		10.8
5	1.60	39.96	30	25	39.96	6.6	1.34
5	0.68	17.6	30	26	17.6		0.3
5	3.64	91.00	30	25	91.00	3.9	3.36
5	1.01	26.3	30	26	26.3	5	0.5
4.3	1.35	36.49	30	27	36.49	3.3	0.7
5	1.15	5.8	30	25	5.8	1.4	0.7
5	3.06	82.71	30	27	82.71	1.1	2.72
5	0.77	19.15	30	25	19.15	3.5	0.798
5	1.83	47.6	30	26	47.6	1.5	1.6
5	3.18	15.9	30	25	15.9	4.9	4.5
5	56.70	1417.48	30	25	1417.48	3.20	39.2
5	1.24	6.2	30	24	6.2	2.3	0.4
5	2.69	72.67	30	27	72.67	3.3	3.55
5	0.52	13.6	30	26	13.6	2.6	0.5
5	5.11	141.00	30	27.6	141.00	5.5	7.64
5	0.90	4.5	30	25	4.5	2	0.8

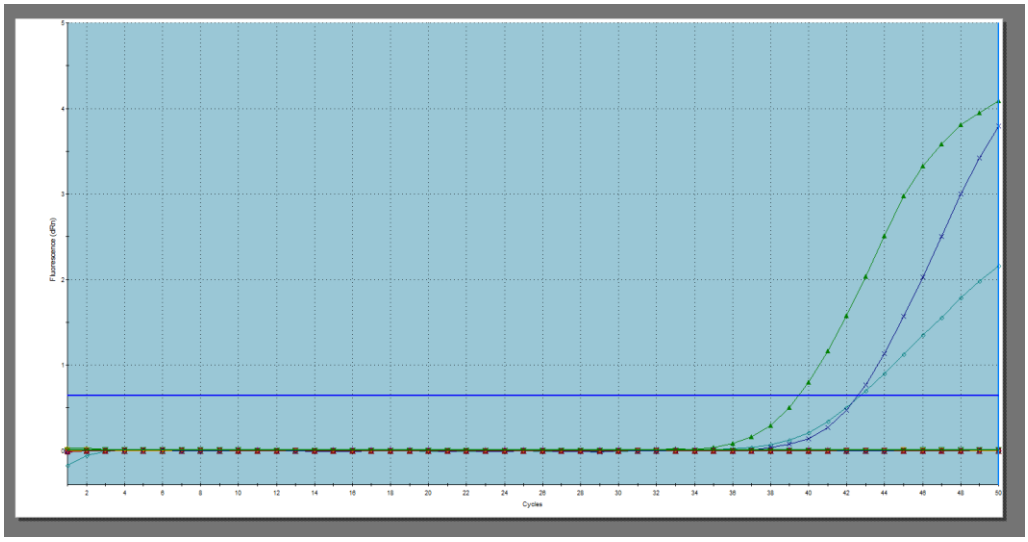
5	**2.99**	**80.73**	30	**27**	80.73	3.1	3.89
5	**0.58**	15.1	30	**26**	15.1	1.8	0.8
5	**2.28**	**59.32**	30	**26**	59.32	5.3	4.1
5	**2.00**	10.0	30	**25**	10.0	1.3	0.9
5	**1.92**	**48.00**	30	**25**	48.00	4.5	1.51
5	**0.87**	22.7	30	**26**	22.7	3.1	0.9
5	**0.73**	**18.21**	30	**25**	18.21	3.6	0.541
3.1	**0.67**	**30.87**	30	**46**	30.87	1	0.7
5	**6.16**	30.8	30	**25**	30.8	3.4	14.0
5	**1.09**	**29.54**	30	**27**	29.54	2.4	1.4
5	**21.96**	**549.05**	30	**25**	549.05	3.6	80
5	**6.40**	32.0	30	**25**	32.0	2.8	26.1

Appendix VIII – Amplification Plots of QPCR in the APEX study.

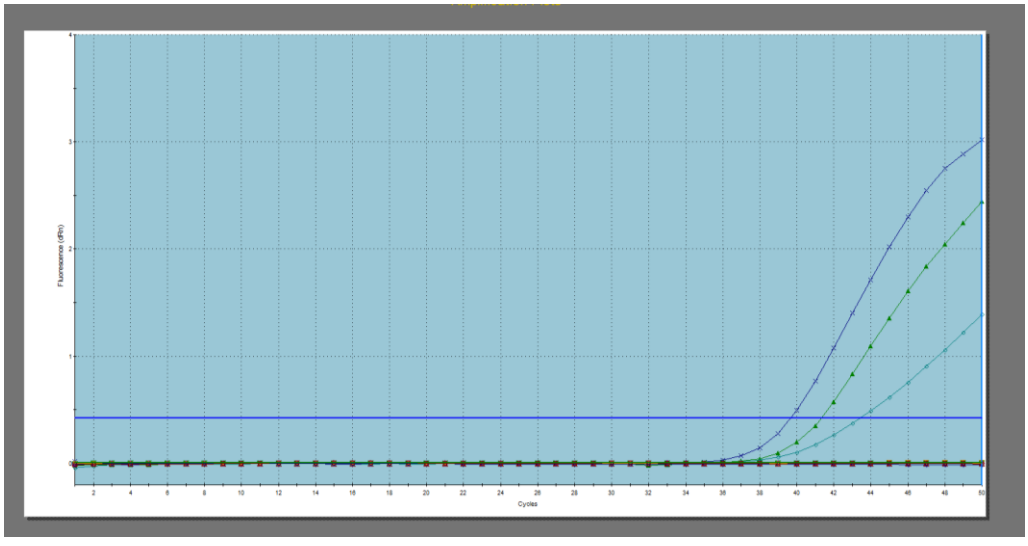
Amplification curves for each PDAR. Each PDAR was performed in triplicate. Amplification plot also illustrates NTC in triplicate.



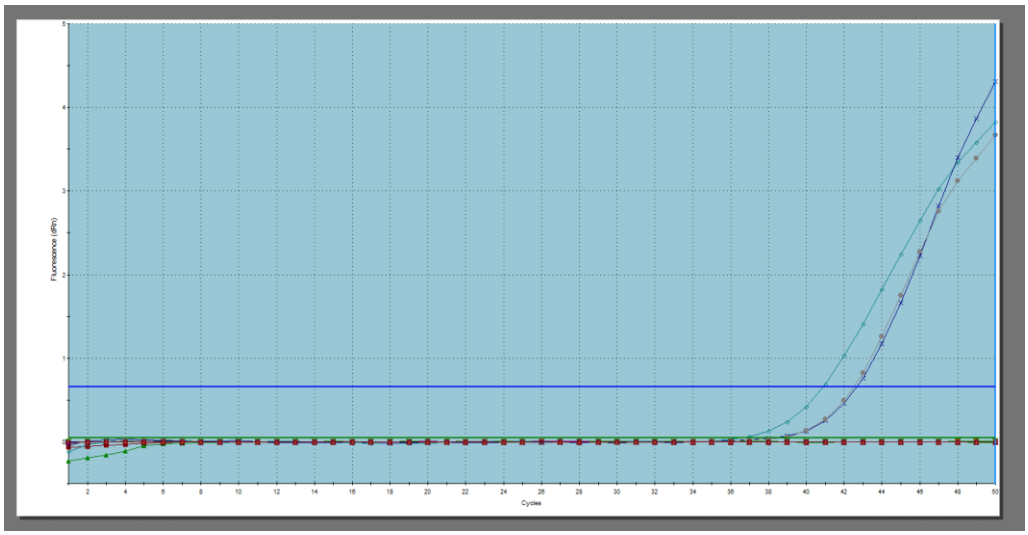
Rhinovirus



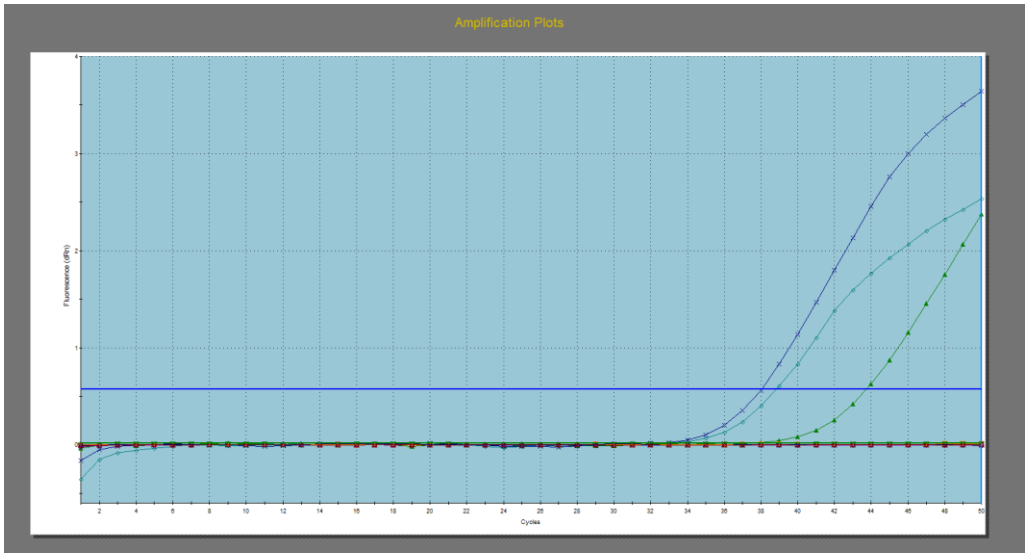
RSV



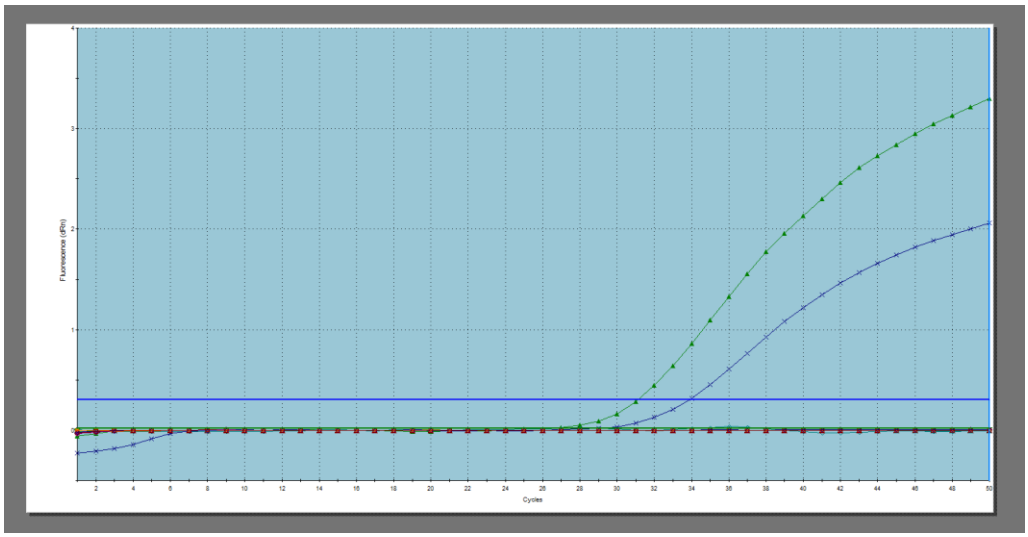
Influenza



Human Parainfluenza 1



Human Parainfluenza 3



Metapneumovirus

Appendix IX– R code used for RNA sequencing of transcriptomes.

Due to the large quantity of concatenated data and to organise this snake file were used and generated using the Snakemake command:

```
zcat file1_R1.fastq.gz file2_R1.fastq.gz
file3_R1.fastq.gz | gzip > R1.fastq.gz
```

```
rule zcat:
       input:
              "{sample}L1.fastq.gz",
              "{sample}L2.fastq.gz"
       output:
              "{sample}.fq.gz"
       shell:
              "zcat {input} | gzip>{output}"
```

Snakemake© (snake make team) is a workflow management system that allows data to be organised within a framework and is reproducible.

Both STAR and Trimmomatic are embedded within the Sankemake© package. The following piece of code was used:

```
configfile: "config.yaml"

rule all:
  input: config['GRC_dir']+"/Genome"

rule star_genome:
  input:
    fa=config['fa'],
    gtf=config['gtf']
  output:
    expand(config['GRC_dir']+"/{name}",
name=config['GRC_names'])
  params:
    out_dir=config['GRC_dir'],
    ram_limit=config['star_genome_ram_limit']
  threads: 28
  shell:
    "STAR "
    "--runThreadN {threads} "
    "--runMode genomeGenerate "
    "--genomeDir {params.out_dir} "
```

```
    "--genomeFastaFiles {input.fa} "
    "--sjdbGTFfile {input.gtf} "
    "--sjdbOverhang 101 "
    "--limitGenomeGenerateRAM {params.ram_limit}"



rule trim:
  input:
    "{input_base}_1.fastq.gz",
    "{input_base}_2.fastq.gz"
  output:
    "{input_base}_trimmed_1.fq.gz",
    "{input_base}_trimmed_1np.fq.gz",
    "{input_base}_trimmed_2.fq.gz",
    "{input_base}_trimmed_2np.fq.gz"
  params:
    trimming_lib=config['trim_fa']
  threads: 28
  shell:
    "trimmomatic PE "
    "-threads {threads} "
    "-phred33 "
    "-trimlog {wildcards.input_base}.trimlog "
    "{input} "
    "{output} "
    "ILLUMINACLIP:{params.trimming_lib}:2:30:10 "
    "LEADING:3 "
    "TRAILING:3 "
    "SLIDINGWINDOW:4:15 "
    "MINLEN:36 "



rule star_mapping:
  input:
    fq1="{input_base}_trimmed_1.fq.gz",
    fq2="{input_base}_trimmed_2.fq.gz",
    gen=config['GRC_dir']+"/Genome"
  output:
    "{input_base}Aligned.sortedByCoord.out.bam",
    "{input_base}Log.final.out",
    "{input_base}Log.out",
    "{input_base}Log.progress.out",
    "{input_base}ReadsPerGene.out.tab",
    "{input_base}SJ.out.tab"
  params:
    genome_dir=config['GRC_dir']
  threads: 28
```

```
  shell:
    "STAR "
    "--runThreadN {threads} "
    "--genomeDir {params.genome_dir} "
    "--readFilesIn {input.fq1} {input.fq2} "
    "--outFileNamePrefix {wildcards.input_base} "
    "--outSAMstrandField intronMotif "
    "--readFilesCommand zcat "
    "--outSAMmapqUnique 50 "
    "--outSAMtype BAM SortedByCoordinate "
    "--outSAMattrRGline ID:{wildcards.input_base}-RNAseq
SM:{wildcards.input_base} LB:{wildcards.input_base}-
3RNAseq PL:Illumina "
    "--quantMode GeneCounts
```

```
#!/usr/bin/bash
#SBATCH --time=72:00:00
#SBATCH --job-name=apexconcat
#SBATCH --mem=100000
#SBATCH --nodes=1
#SBATCH --ntasks-per-node=28

module load anaconda-uon/3
source
/gpfs01/software/miniconda/miniconda3/etc/profile.d/conda
.sh
conda activate /gpfs01/software/conda-extra/respmed

cd /gpfs01/home/msxap20/apex
```

The following codes were used for scaling of raw counts:

```
```{r}
cpm <- cpm(DGEL)
lcpm <- cpm(DGEL, log=TRUE)
```
```

The following codes were used for Voom transformation:

```
```{r}
par(mfrow=c(1,2))
v <- voom(DGEL, design, plot = TRUE)
corfit <- duplicateCorrelation(v,design=design, block= block)
corfit
v2 <- voom(DGEL, design, block = block, correlation =
corfit$consensus
fit <- lmFit(v2, design, block = block, correlation =
```
```

```
corfit$consensus)
fit <- contrasts.fit(fit, contr.matrix)
efit <- eBayes(fit)
plotSA(efit, main="Final model: Mean-variance trend")
```