NON-INFLAMMATORY MECHANISMS IN ASTHMA

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Hajar Ali

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Abstract

Until relatively recently asthma was considered to be an allergic disease characterised by airway inflammation. However, emerging evidence suggests that approximately 50% of asthmatics have no overt signs of inflammation. The pathophysiological mechanisms underlying disease in this group - non-eosinophilic asthma (NEA) - remain poorly understood.

In 130 young-adults with generally well-controlled asthma and 79 non-asthmatics (aged 14-21 years), various non-invasive approaches were used to assess both inflammatory and non-inflammatory mechanisms in eosinophilic asthma (EA) and NEA. Studies focussed on: (i) neural mechanisms (including autonomic nervous system (ANS) activity, sensory nerve activity, and levels of neural mediators in induced sputum); and (ii) airway remodelling (measuring induced sputum levels of airway remodelling mediators), in different asthma phenotypes, whilst also taking into account clinical and inflammatory characteristics. In addition, the response to short acting β-agonist (SABA) and short acting muscarinic-antagonist (SAMA) treatment was compared between asthma phenotypes.

Differences in some aspects of neural regulation were observed between EA, NEA, and non-asthmatics. In particular, airway sensory nerve reactivity was enhanced in NEA compared with non-asthmatics ($p<0.05$). Sensory nerve reactivity was also higher in NEA compared to EA, but this did not reach statistical significance ($p=0.07$). There was no evidence of an imbalance in ANS activity when comparing asthmatics and non-asthmatics, or EA and NEA. Increased levels of nociceptin were found in asthmatics and EA compared with non-asthmatics ($P<0.05$); nociceptin was positively associated with sputum eosinophils. Several inflammatory and remodelling mediators (including IL-1β, ECP, periostin, and VEGF-A) were elevated in EA but not NEA. There was no evidence of a differential response to SAMA between EA and NEA; however, a small subgroup of asthmatics responded better to SAMA.

In conclusion, the studies described in this thesis suggest that sensory nerve reactivity may play an important role in the pathophysiology of NEA, but not EA, and may potentially represent a novel phenotype-specific treatable trait. Autonomic dysregulation does not appear to play a role in well-controlled asthma or specific asthma phenotypes. Findings suggest a potential involvement of nociceptin in asthma pathology, particularly in relation to airway eosinophilia. Finally, the identification of a small group of asthmatics who responded better to SAMA than SABA challenges current asthma treatment guidelines and suggest a need for a more personalised treatment approach. Further investigation of non-inflammatory mechanisms is warranted to improve understanding of other mechanisms underlying different asthma phenotypes, which will contribute to the identification of more specific treatable traits.

"The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes."

- *Ibn Sina (Avicenna)*

“Doubt is the beginning of wisdom; it leads us to question, explore, and seek deeper understanding.”

- *Abu Rayhan al-Biruni (Al-Biruni)*

Author's declaration

This thesis was produced according to Massey University's "Thesis with publication" requirements. That is, it is based on research that is published, under review or submitted for publication. Chapters 3, 4, 5 and 6 have been written as individual research papers, and the first three of these chapters were written in the style of the journal to which it was submitted. Consequently, there is some repetition (particularly in the methods, and study participants characteristics sections), and there are minor stylistic differences between chapters.

The submitted manuscripts include other authors who provided expertise and contributed to the writing of the papers, including my PhD supervisors and in some cases, collaborators in different institutes in New Zealand, Australia, and the United Kingdom. However, for each chapter, my input was greatest, as reflected by being first author on these papers. I was the lead project coordinator for the studies described, involved in developing patient information sheets and consent forms, written questionnaires and organising the ethics application with supervision from my supervisors prior to the conduct of these studies. Upon commencement of the study, I was involved in organising recruitment of the study participants, work coordination, sample collection and processing, data collection, cleaning, and analysis, as well as preparation of the manuscripts.

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you, and I dedicate this thesis to each and every one of you, who will always be my greatest source of happiness.

Abbreviations

ACh:	Acetylcholine
ACQ:	Asthma control questionnaire
AHR:	Airway hyperresponsiveness/hyperreactivity
ANS:	Autonomic nervous system
APC:	Antigen-presenting cells
ASM:	Airway smooth muscle
ATS:	American Thoracic Society
BAL:	Bronchoalveolar lavage
BDNF:	Brain-derived neurotrophic factor
BDR:	Bronchodilator reversibility / response
BHR:	Bronchial hyperreactivity/hyperresponsiveness
Blood EOS-high	A high blood eosinophil count
BTS:	British Thoracic Society
C2:	Concentration of tussive agent inducing at least 2 coughs
C5:	Concentration of tussive agent inducing at least 5 coughs
CGRP:	Calcitonin gene related peptide
COPD:	Chronic obstructive pulmonary disease

DAMP: Damage-associated molecular pattern

DCC: Differential cell count

DTE: Dithioerythritol

DTT: Dithiothreitol

EA: Eosinophilic asthma

EBUS: Endobronchial ultrasound

ECG: Electrocardiogram

ECM: Extracellular matrix

ECP: Eosinophil cationic protein

ECRHS: European Community Respiratory Health Study

EDN: Eosinophil-derived neurotoxin

EEG: Electroencephalogram

EGF: Epithelial growth factor

ELISA: Enzyme-linked Immunosorbent Assay

FcεR: High-affinity immunoglobulin E receptor

FENO: Fraction of exhaled nitric oxide

FEV_1: Forced expiratory volume in 1 second

FEV_1/FVC: Ratio of forced expiratory volume in 1 second to forced vital capacity

fMRI: Functional magnetic resonance imaging

FVC:	Forced vital capacity
GINA:	The Global Initiative for Asthma
GM-CSF:	Granulocyte Macrophage Colony Stimulating Factor
GM:	Geometric means
HDM:	House dust mite
HF:	High frequency
HRCT:	High resolution computed topography
HRV:	Heart rate variability
Hz:	Hertz (cycles/minute)
IB:	Ipratropium bromide
ICS:	Inhaled corticosteroid
IFN-γ:	Interferon gamma
IgE:	Immunoglobulin E
IL:	Interleukin
ILC:	Innate lymphoid cell
iNOS:	Inducible nitric oxide synthase
IQR:	Interquartile range
ISAAC:	International Study of Asthma and Allergies in Childhood
LABA:	Long acting β-agonist

LAMA:	Long-acting muscarinic antagonist
LF:	Low frequency
LF/HF Ratio:	Ratio between the power of Low Frequency and High Frequency bands
LOD	limit of detection
MBP:	Major basic protein
MCP:	Monocyte chemoattractant protein
MGA:	Mixed granulocytic asthma
MHC:	Major histocompatibility complex
MIP:	Macrophage inflammatory protein
MMP:	Matrix metalloproteases
MPO:	Myeloperoxidase
NA:	Neutrophilic asthma
NAEPP:	National Asthma Education and Prevention Program
NANC:	Non-adrenergic-non-cholinergic nervous system
NE:	Neutrophil elastase
NEA:	Non-eosinophilic Asthma
NGF:	Nerve growth factor
NKA:	Neurokinin A
NO:	Nitric oxide

PAMP: Pathogen-associated molecular pattern

PEF: Peak expiratory flow

PGA: Paucigranulocytic asthma

PNS: Parasympathetic nervous system

PRR: Pathogen recognition receptor

RANTES: Regulated on activation, normal T cell-expressed and secreted protein

RAR: Rapidly adapting receptors

RMSSD: Square root of the mean squared differences of successive RR intervals

ROS: Reactive oxygen species

RR: Inter-beat

SABA: Short acting β-agonist

SAR: Slowly adapting receptors

SD: Standard deviation

SDRR: Standard deviation of the RR intervals

SNS: Sympathetic nervous system

SP: Substance P

SPT: Skin prick test

TCC: Total cell count

TGF-β: Transforming growth factor beta

TH:	T helper cell
TIMP:	Tissue inhibitor of metalloproteinases
TLR:	Toll-like receptor
TNF:	Tumour necrosis factor
TP:	Total power
TRP:	Transient receptor potential
$TRPV_1$:	Transient receptor potential vanilloid 1
TSLP:	Thymic stromal lymphopoietin
VEGF:	Vascular endothelial growth factor
VIP:	Vasoactive intestinal peptide
VLF:	Very low frequency
WHO:	World Health Organisation

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Chapter 1 Introduction

Asthma is one of the most common non-communicable diseases and is responsible for considerable morbidity and healthcare costs globally.[1] Over 300 million people have asthma worldwide[2] and it is estimated that within the next five years a further 100 million may be affected.[3] In New Zealand, asthma affects up to 20% of the population[4] with an estimated cost of over $1 billion per annum.[5] A considerable amount of research has been conducted over recent decades,[6,7] resulting in some advances in treatment, clinical management, and understanding of the pathophysiology of asthma.[8] However, this has not resulted in a cure or primary prevention for asthma; and a large proportion of patients (30-50%) continue to have symptoms with standard therapy.[9] One reason for the lack of progress may, at least in part, be due to asthma having been considered a single disease entity,[9] despite an early recognition that it consists of different phenotypes.[10,11]

Until relatively recently, asthma was considered predominantly an allergic disease[12] characterised by eosinophilic inflammation.[13] However, it is increasingly considered a heterogeneous disease involving either separate (but partially overlapping) diseases with similar symptomatology or different clinical subtypes with distinct underlying mechanisms.[14,15] Whilst various approaches have been proposed to categorise or phenotype asthma, two broad pathophysiological categories, variously described as allergic and non-allergic (based largely on skin prick test (SPT) positivity or serum immunoglobulin E (IgE)),[2] TH_2-high and TH_2-low (based on the presence/absence of TH_2-inflammation or levels of blood or sputum eosinophils, fractional exhaled nitric oxide (FeNO) or confirmed allergy),[16] or eosinophilic (EA) and non-eosinophilic asthma (NEA; using induced sputum)[17] are commonly used in research and clinical studies.[15,18] As greater resolution is required for improving the understanding of asthma pathology and optimising personalised treatment,[19]

further stratifications may be warranted. Consistent with this, four inflammatory subtypes of asthma have been proposed on the basis of induced sputum assessment: EA, mixed granulocytic asthma (MGA), neutrophilic asthma (NA) and paucigranulocytic asthma (PGA)(with the latter two phenotypes often combined into a NEA category).[20] Studies using these approaches suggest that inflammation may not be a universal feature of asthma[21] and that 50% of asthma cases, identified as NEA, occur in the absence of allergy, or eosinophilic/neutrophilic airway inflammation.[22]

The precise characteristics and biological mechanisms underlying NEA, particularly PGA, are not fully understood,[17] but there is some evidence that the autonomic nervous system (ANS) and airway nerves[23] may play a role, and that airway remodelling and structural changes may also potentially be involved.[24] However, it is unclear whether these alternative pathways occur in the presence or absence of airway inflammation.

The notion that neural mechanisms play a role in asthma pathogenesis is not new;[25] prior to the mainstream acceptance of the inflammatory paradigm,[26] asthma was often considered a neural disorder, commonly triggered by emotional stress.[27] It was hypothesised that an imbalance between bronchodilating and bronchoconstricting influences of the ANS or parasympathetic dysfunction led to increased airway hyperresponsiveness (AHR) and bronchospasm.[28] More recent studies have also shown that sensory nerves may play a critical role in asthma pathophysiology.[29,30] However, in general, studies examining the role of neural mechanisms have been relatively rare, and findings have been inconsistent.

The relationship between airway neural regulation and inflammation is also unclear. Animal models suggest that proteins released by immune cells can directly affect sensory nerves [31,32] and potentially cause bronchoconstriction by activating the parasympathetic cholinergic reflex.[33] Conversely, release of neuropeptides by sensory nerves[27] or airway parasympathetic

nerves[34] can affect leukocyte migration and function. However, there is a relative paucity of clinical data, and, as result, the pathophysiologic relevance of neuro-immune crosstalk to asthma pathology remains inconclusive.[35] It is also unclear whether asthma symptoms can occur as a direct result of neural dysregulation without airway inflammation, or whether these mechanisms are "merely" an intermediate stage between airway inflammation and symptoms. Moreover, whether neural dysregulation is associated with specific inflammatory phenotypes, or clinical features of asthma, remains unidentified.

Asthma is often accompanied by a range of structural changes collectively known as airway remodelling[24] which (alongside inflammation) is considered a major cause of chronic airway obstruction and progressive lung function loss.[36] The pathogenesis of remodelling is incompletely understood, but for a long time it was believed to be aberrant repair process largely resulting from chronic inflammation.[24] However, there is increasing evidence that remodelling can develop both in parallel with,[37] and in the absence of,[38,39] airway inflammation. Despite its clear importance, airway remodelling has received far less attention than inflammation in asthma, due to both the understandable focus on inflammation in many asthmatics, and the lack of non-invasive methods available to assess remodelling.[38] Consequently, the role of remodelling in different asthma inflammatory phenotypes, particularly in the absence of inflammation, remains unknown. Moreover, it is unclear whether remodelling processes overlap or are associated with the neural-associated mechanisms described above.

Taken together, it is increasingly clear that asthma is probably not solely an inflammatory disorder, with the underlying causes of "non-inflammatory asthma" (PGA) remaining unknown. Given that conventional asthma therapies that target predominantly eosinophilic airway inflammation are not fully effective for many asthma patients,[40] there is a clear need to improve understanding of underlying mechanisms as this will facilitate the development of

improved treatment options,[41] potentially targeting phenotype-specific "treatable traits". For example, if neural mechanisms and/or airway remodelling play a key role in (non-inflammatory) asthma then specific agents targeting airway nerves (i.e. anticholinergics as suggested previously)[42] or certain aspects of airway remodelling may provide additional options for asthma treatment, particularly for asthmatics for whom current treatment options are not fully effective.

The research described in this thesis aimed to improve understanding of the role of neural mechanisms and remodelling in different asthma phenotypes. It also assessed differential responses to treatment in different asthma phenotypes. The work is based on an observational study of 130 asthmatic and 79 non-asthmatic young adults (aged 14-21 years) which involved: a respiratory questionnaire, induced sputum and AHR testing, lung function, FeNO, and SPT; additionally, in the majority of this group, blood collection, heart rate variability (HRV) analysis, and assessment of differential treatment response were also assessed (see Appendix 1 for detailed information). In a sub-population (39 asthmatics and 21 non-asthmatics) capsaicin challenges (to assess airway sensory nerve reactivity) were conducted.

For each results chapter, there is a specific focus on: autonomic nervous activity; airway sensory nerve reactivity; airway inflammatory, neural and remodelling mediators; or treatment response.

Aims

The aims of this thesis were to assess:

I. Differences in neural regulation, as determined by airway sensory nerve reactivity (using capsaicin challenge), autonomic nervous activity (using HRV analysis) and measurement of neurogenic mediators in induced sputum, in EA, NEA and non-asthmatics.
II. The association between markers of neural regulation and clinical and inflammatory characteristics.
III. Differences in sputum mediators associated with inflammation and airway remodelling in EA, NEA, and non-asthmatics.
IV. The association between mediators implicated in airway remodelling and inflammatory characteristics in EA, NEA and non-asthmatics.
V. Differential response to anticholinergics and short acting β-agonist treatment in EA and NEA.

The structure of this thesis is as follows:

Chapter 1- General introduction

This chapter provides a brief rationale for the studies described in this thesis. This is followed by the aims and structure.

Chapter 2- Literature review

This chapter provides a review of the relevant asthma literature and background for the research. Asthma is defined and the associated characteristics, epidemiology, and pathophysiology, as well as methods to assess pathophysiology, are described. The concepts

of asthma phenotypes is introduced and conventional as well as novel mechanisms involved in asthma pathogenesis are described.

Chapter 3- Enhanced airway sensory nerve reactivity in non-eosinophilic asthma (Aims I, II and III)

This chapter presents the results of capsaicin challenges conducted to assess sensory nerve reactivity in EA, NEA, and non-asthmatics. Associations with EA and NEA phenotypes and clinical characteristics of asthma are reported.

Chapter 4- Heart rate variability as a marker of autonomic nervous system activity in eosinophilic and non-eosinophilic asthma (Aims I, II and III)

This chapter describes the results of HRV assessments (conducted as a proxy indicator of ANS activity) in EA, NEA, and non-asthmatics. Associations with EA and NEA and clinical characteristics of asthma are examined.

Chapter 5- Assessment of inflammatory, neural and remodelling mediators in sputum of eosinophilic and non-eosinophilic asthma (Aim I, II, III, IV, V and VI)

This chapter describes the results of sputum inflammatory, neural, and remodelling mediator analyses in EA, NEA, and non-asthmatics. Correlations among mediators and inflammatory cells are described stratified by asthma phenotype.

Chapter 6- Bronchodilator response in eosinophilic and non-eosinophilic asthma (Aim VII)

This chapter compares bronchodilator responses in EA and NEA treated with either anticholinergic or short acting β-agonists and examines if any other characteristics are associated with treatment response.

Chapter 7- General discussion and conclusions

This chapter summarises the main findings and makes comparisons with previous studies. The relevance of the findings, strengths and limitations of the studies, and recommendations for future research and conclusions are provided.

Chapter 2 Literature Review

The scientific literature describing asthma is vast, complex, and rapidly changing. Therefore, a complete and comprehensive review is outside the scope of this thesis. Instead, the focus here will be to provide context for the subsequent chapters of this thesis, specifically describing:

1. The general characteristics of asthma. These include the definition and assessment of asthma, epidemiology, risk factors and management.
2. The pathophysiological processes that lead to asthma manifestations and heterogeneity.
3. Methodologies for assessing underlying pathophysiology, with a particular focus on the specific methods used in subsequent chapters.
4. Phenotyping asthma on the basis of pathophysiology.

2.1 Asthma: An overview

2.1.1 Definitions

The word asthma has its origins in the Greek noun Άσθμα, derived from the word aazein, meaning 'to pant' or 'short of breath'. The earliest known description of asthma was provided by the renowned Greek clinician Aretaeus of Cappadocia in the 1st century B.C.:[26]

"Heaviness of the chest; sluggishness to one's accustomed work and to every other exertion; difficulty of breathing in running or on a steep road; they (the patients are hoarse and troubled with cough; flatulence and extraordinary evacuations in the hypochondriac region; restlessness; heat at night small and imperceptible; nose sharp and ready for respiration."

More than two millennia later, some of these features are still recognisable in contemporary definitions.

The World Health Organisation (WHO) defines asthma as:

"…a long-term condition affecting children and adults. The air passages in the lungs become narrow due to inflammation and tightening of the muscles around the small airways. This causes asthma symptoms such as cough, wheeze, shortness of breath and chest tightness. These symptoms are intermittent and are often worse at night or during exercise. Other common triggers can make asthma symptoms worse. Triggers vary from person to person, but can include viral infections (colds), dust, smoke, fumes, changes in the weather, grass and tree pollen, animal fur and feathers, strong soaps and perfume."[1]

The Global Initiative for Asthma (GINA) defines asthma as:

"… a heterogeneous disease, usually characterized 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."[2]

Thus, current definitions generally describe four main features: symptoms (the most common being cough, shortness of breath, wheezing or whistling and chest tightness),[2] (variable) airway obstruction, hyperresponsiveness, and inflammation, which together form the basis of the pathological, physiological, and clinical features of asthma.

2.1.2 Epidemiology

Whilst asthma has been recognised for at least 2000 years, its prevalence has increased significantly in the last 50 years.[43] It is currently estimated that more than 300 million people worldwide have asthma,[7] and one in eight adults and one in seven children are currently affected in New Zealand.[5] If current trends continue, it is estimated that by 2025 a further 100 million worldwide may be affected.[3] Although this increase has been observed globally, there appears to be marked geographical variation. The highest prevalence of asthma symptoms is seen in English-speaking Western countries, and New Zealand has amongst the highest rates in the world.[44,45] Between 2000 and 2003, the International Study of Asthma and Allergies in Childhood (ISAAC) Phase 3 Survey, a large-scale international collaboration on asthma and atopic disease surveying over 700,000 children, showed that high-income countries had higher prevalence of asthma symptoms. It also suggested that low- and middle-income countries generally had higher rates of severe asthma and asthma mortality.[46] Asthma prevalence may have peaked or even began to decline in high income countries, whilst it continues to rise in low- and middle-income countries.[45] Although the reasons for the

disparity in prevalence between higher- and lower- income countries are not clear, it has been proposed that adoption of a western lifestyle and urbanisation may play a role.[47]

Patterns in incidence and prevalence also differ between children and adults. Although asthma often begins in childhood, symptoms can occur at any time throughout life and whilst asthma incidence and prevalence are higher in children, morbidity and mortality are higher in adults.[7] Also, incidence and prevalence differ by sex throughout life.[48] Pre-pubertal boys are more likely to have asthma and are twice as likely as girls to be hospitalised for exacerbation,[49] but this trend reverses during adolescence.[50] In adults, asthma prevalence is higher in women, and they also have an elevated risk of hospitalisation during and after puberty.[51] The reasons for these differences are not entirely understood but may be related to smaller airway size and higher prevalence of atopic sensitisation (described in section 2.1.3.3) observed in boys and hormonal changes in females.[51]

The social and economic costs of asthma are substantial in both high and low/middle income countries[43,52] and the overall cost to the New Zealand economy is estimated to be around 1 billion per annum.[5] The cost-burden is mostly due to the direct costs from treatment and hospitalisation[52] but there are also significant (indirect costs) associated with lost time from work and school. Asthma also significantly impacts on quality of life through impairment of social, emotional, physical, and occupational activity.[53]

2.1.3 Assessment

There is currently no single test or established "gold standard" for the clinical diagnosis or identification of asthma. However, practical guidelines for clinical diagnosis and management, in both children and adults, are available from international respiratory associations, such as GINA[2], the American Thoracic Society (ATS)[54] or the British Thoracic

Society (BTS).[55] The current general consensus is that clinical diagnosis should be based predominantly on evidence of recurrent respiratory symptoms and variable expiratory airway obstruction.[2]

2.1.3.1 Symptoms

The primary symptoms are dyspnea, wheeze, chest tightness and cough. These symptoms can occur alone or in combination and can vary over time and in intensity.[2] They often occur at night or early in the morning and may be triggered or worsened with exercise, infection, or exposure to allergenic or non-allergenic (irritant) exposures.[2,56]

When assessing and defining asthma in primary care settings or in large population-based studies where access to objective testing is limited, a common approach is to use medical history and symptom-based questionnaires.[57] The most commonly used symptom questionnaire in adults is that developed for the European Community Respiratory Health Study (ECRHS).[58] Questions primarily relate to respiratory symptoms and medication-use during the previous 12 months. A similar questionnaire has been developed for use in children for the ISAAC.[59]

However, whilst useful due to their low costs and convenience,[60,61] and whilst generally correlating with objective testing (see below), defining asthma on the basis of symptoms alone has some limitations. Firstly, “asthma symptoms” are not exclusive to asthma and may be present in other airway diseases including chronic obstructive pulmonary disease (COPD), dysfunctional breathing, and vocal cord dysfunction.[62] For example, “wheeze” is a hallmark of asthma but is also reported in other airway diseases including COPD[63] and cardiac disease (“cardiac asthma”).[64] Secondly, asthma is an extremely variable condition and the variability in presence and severity of symptoms at the time of assessment can lead to both under and

over-diagnosis and affect disease prevalence estimates. For example, a review assessing 5 studies including 21,530 participants reported 54% underdiagnosis and 34% overdiagnosis in primary health care units.[65] Finally, there may also be potential problems with patient recall and individual differences in symptom perception; therefore, reliance on self-reported symptoms alone may potentially result in misclassification.

2.1.3.2 Airway obstruction/airflow limitation

Variable airway obstruction is often described as a key physiological manifestation of asthma.[2] Airway obstruction is often assessed using spirometry, either alone, or in combination with tests to measure AHR to certain triggers or pre- and post-treatment with rapid-acting bronchodilators or inhaled corticosteroids (ICS).[2]

Spirometry

Spirometry is a simple, reproducible procedure commonly performed to assess lung function and involves the measurement of volume and airflow over time during exhalation following a full inhalation.[66] The most commonly used spirometric parameters are: Forced Expiratory Volume in one second (FEV_1; the amount of exhaled air within the first second of forced deep exhalation); Forced Vital Capacity (FVC; the total volume of air exhaled in a forced exhalation), and FEV_1/FVC ratio.[67] These parameters are typically expressed as absolute flow/volume, or as the percentage of the predicted values using data derived from average values in healthy individuals of similar age, gender, ethnicity, and height.[67] Decreased FEV_1 (<80 % predicted) and FEV_1/FVC (<70 %) are generally considered indicative of airway obstruction in adults.[68]

Peak Expiratory Flow (PEF, or 'peak flow') is another lung function parameter that is commonly used, providing a measure of peak expiratory flow after a full inspiration.[2] PEF

diurnal variation of >20% or an increase in PEF of ≥15% after bronchodilator administration is suggested to be supportive of an asthma diagnosis in some guidelines.[2] As noted above, the difference in pre- and post-bronchodilator spirometry may also be used. This allows identification of reversible airway obstruction often seen in asthmatics (bronchodilator reversibility/responsiveness: BDR).[69] BDR is often defined as the increase in FEV_1 of >12% and >200 mL from baseline after administration of a Short acting β-agonist (SABA; i.e. 200-400 mcg salbutamol or equivalent).[70] However, the validity of these cut-offs has been questioned in paediatric populations in particular; many children with asthma have baseline FEV_1 within the normal reference ranges and any increase in FEV_1 after bronchodilator administration is often limited when compared with adults.[70] Instead, use of BDR cut-off values of 8% and 9% have been proposed in young people.[71,72]

Lung function assessments are useful in asthma diagnosis, but they are often effort-dependent and have some degree of insensitivity, especially in individuals with stable asthma.[73] In addition, they may not correlate directly with asthma symptoms.[74,75] For example, while airflow obstruction is often associated with asthma, as mentioned above many asthmatics may present with normal lung function at assessment,[56] or display a degree of irreversible airflow obstruction or reversibility that may not be present at the time of assessment.[76] This is particularly true for patients who experience symptoms predominantly during viral infections or who have well controlled symptoms due to medication.[77] One study demonstrating that lung function alone may not be adequate to define asthma was conducted on 333 adults with doctor-diagnosed asthma, of whom only 21% had airflow obstruction (defined as a ratio of FEV1/FVC <70%).[78] When spirometry does not indicate airflow obstruction, but subjects have a clinical history of symptoms, bronchoprovocation challenge testing may be helpful to assess airway/bronchial hyperreactivity (BHR).[77]

AHR

AHR (BHR) is present in many (but not all) asthmatics[79] and is defined as an excessive airway narrowing (bronchoconstrictive response) to a range of stimuli, e.g. allergens, methacholine, cold, or smoke.[80] AHR is conventionally measured using a histamine, methacholine, or hypertonic saline bronchial challenge and generally reported in terms of dose or volume of the challenging agent producing a 15-20% fall in FEV_1.[2] As with airflow obstruction, AHR may not necessarily be present at the time of assessment and may only be detected with specific stimuli i.e. a positive response to one stimuli does not necessarily identify a response to other stimuli, and vice versa.[81] AHR can also be observed in non-asthmatics or during viral infection[82,83] and may have a poor overall correlation with clinically diagnosed asthma.[84] Moreover, AHR testing is generally only undertaken in specialist centres and as such is not routinely used for asthma diagnosis in primary care[85] or in epidemiological research.

2.1.3.3 Other objective tests used for asthma assessment

Allergy tests

Asthma is often associated with "atopy" and "allergy"[86] (discussed in more detail in sections 2.2.1.1 and 2.4.1.1) and the presence of atopy often increases the probability that a patient with respiratory symptoms has asthma.[2] In a clinical or research setting, atopy is usually determined by means of a skin-prick test or a blood test with specific or serum IgE measurements.[87] However, the presence of a positive skin test or increased serum IgE does not necessarily mean that asthma is caused by allergen exposure.[86]

FeNO

Increased nitric oxide (NO; or FeNO) has often been associated with asthma (discussed in more detail in section 2.3.1.1). In a clinical or research setting, FeNO levels can be detected in exhaled breath.[88]

2.1.4 Management

This section will not comprehensively discuss all aspects of asthma treatment and management (the reader is referred to the review by Papi *et al* [89] for this). Instead, a brief overview is provided to introduce the most commonly used therapies. The concept that that different types of asthma (or "phenotypes") may have differential treatment response is further explored in section 2.4.1.3.

Current asthma management guidelines are based on a stepwise approach with treatment progressively increased (stepped-up) to achieve symptom control and minimise future exacerbation risk, with the option to step-down treatment after a period of prolonged control.[90] Composite measures e.g. Asthma Control Questionnaire (ACQ),[91] Asthma Control Test,[92] and National Asthma Education and Prevention Program (NAEPP) classifications[93] have been developed and validated[91,93,94] to assess change in control for this purpose.[2] Initial treatment generally depends on symptom severity, lung function, and modifiable risk factors, whereas ongoing treatment decisions are based on an individualised cycle of assessment, treatment adjustment, and review of the response.[2]

Conventional pharmacological treatments predominantly target either airway smooth muscle (ASM) tone or inflammation,[95] and are generally divided into reliever and maintenance (also known as controller and preventer) or add-on therapy. Reliever/controller medications

mediate the rapid relief of acute asthma symptoms, whereas maintenance/preventer medications generally need to be taken regularly on a long-term basis to be effective.[89]

2.1.4.1 Reliever medication

β_2-agonists are one of the oldest classes of medicines used in this setting. Their main airway action is through engagement of G-protein-coupled β-receptors at the surface of smooth-muscle cells to induce muscle relaxation and subsequent bronchodilatation.[96] β_2-agonists are subdivided into SABA and long-acting β_2 agonists (LABA), depending on their onset and duration of action. SABA generally have an onset of action of under 5 minutes and are effective for 3-4 hours, LABA, may exhibit comparable onset of action to SABAs but remain active for 12-24 hours.[2]

Other reliever bronchodilators less commonly used include short and long-acting muscarinic antagonists (SAMA/LAMA).[97] SAMAs, like SABAs, are used in the acute management of symptoms, whereas LAMA, like LABA, are considered as an option for maintenance therapy.[42]

2.1.4.2 Controller medication

ICS are the cornerstone of maintenance therapy and are mainly used to suppress airway inflammation by inhibiting the release of airway pro-inflammatory cytokines and, recruitment of immune cells, and inducing anti-inflammatory gene expression.[98] Their main action in the airways is through binding to cytosolic glucocorticoid receptors that are present on nearly all cell types.[99] Both initiation and daily treatment with ICS have been shown to decrease eosinophil numbers in the airways,[98,100,101] attenuate AHR,[102] improve lung function and asthma control, and reduce exacerbation frequency.[103]

2.1.4.3 Add-on treatments

When symptoms remain uncontrolled with low-to-moderate doses of ICS, the recommended step-up option in the GINA management guidelines is the addition of other agents e.g. LABA or LAMA.[104,105] This can result in improved lung function, symptom control, and reduced need for reliever therapy compared with patients receiving higher ICS dose.[106,107] Recently, add-on maintenance medication in the form of targeted biological therapies has been introduced for groups with severe eosinophilic inflammation (discussed in more detail in 2.4.1.3). In particular, monoclonal antibodies targeting IgE, interleukin (IL)-4, IL-5, and IL-13 have been shown to improve asthma symptom control and reduce exacerbation in some groups.[108-110]

2.1.5 Aetiology

As with management, for the purposes of this thesis it is not feasible to comprehensively discuss all risk and protective factors, but the reader is directed to reviews by Stern *et al*[111] and Beasley *et al*,[112] for further information. Briefly, there is no single cause of asthma, and several factors may contribute.[112] Asthma aetiology depends on the interplay between endogenous/ host factors[113] and exogenous/ environmental factors.[111] Identified risk factors include atopy and allergy,[114,115] (discussed in section 2.2.1.1) family history,[116,117] certain childhood respiratory infections,[118,119] psychological/ emotional stress,[120,121] gender,[111] paracetamol or antibiotic use,[122,123] vitamin D deficiency,[124,125] indoor and outdoor pollution,[126-128] occupational exposures,[129,130] some bacterial exposures,[131,132] obesity,[126,133] diet,[134,135] and several other exposures (e.g. pesticides, gases, mould).[136] Given the wide range of risk factors and environmental exposures identified, it is likely that, within a given population, the underlying mechanisms causing asthma symptoms are different, and different exposures/risk factors may contribute to different subtypes of asthma.

2.1.6 Heterogeneity

As described in Chapter 1, asthma has been recognised as heterogenous in nature for decades.[9] To date, various clinical and statistical approaches have been utilised in both clinical and research settings to classify this heterogeneity, dividing asthma into different subgroups with similar characteristics (Table 2.1).[9,19] For example, in the early twentieth century, one approach was to classify asthma on the basis of atopy status, distinguishing allergic (extrinsic) asthma (associated with sensitisation to allergens and other allergic diseases) and non-allergic (intrinsic) asthma (described in section 2.4.1.1). Other approaches include stratification on the basis of characteristics such as severity,[2] control status,[2] age of onset,[137] treatment response,[138] or trigger.[15] However, these approaches are largely descriptive, overlapping, and rely on a single aspect or dimension of the disease, limiting their usefulness.

A recently developed approach to asthma classification has been the use of statistical methods to integrate large amounts of data into a single analysis and combine multiple characteristics to identify subgroups of asthma patients.[139] Approaches include cluster and latent class analysis,[140,141] principal component analysis,[142] and exploratory factor analysis.[143] However, such methods are still in the relatively early stages of development and have some limitations, including the effect of subjective selection of different variable sets used and inconsistencies across different population demographics.[144] Moreover, they often fail to provide pathophysiological insights, in large part due to the relative lack of pathophysiological data included in studies using these approaches; for example, inflammatory and biomarker data reflecting underlying disease processes are often not included.[145,146] The need to characterise asthma on a pathophysiological rather than only a clinical basis has long been recognised[37] and is primarily driven by the need for improved

(and personalised) treatment options.[9] Whilst multiple types (or phenotypes) of asthma have been described, based on both clinical features and pathophysiological characteristics, for the purposes of this thesis "phenotype" is used to identify subtypes of asthma in terms of underlying pathology/pathophysiology (the term "endotype" is also used by many researchers).[147,148] Two broad (and often overlapping) divisions of asthma on the basis of pathophysiology are commonly used in research and clinical studies: allergic, or TH_2-high or EA, and non-allergic, TH_2-low or NEA.[148] Before further discussing these pathophysiological phenotypes of asthma in more detail (in section 2.4), some background about the various underlying pathophysiological processes involved and the methods by which they are assessed will be provided.

Table 2.1. Approaches to classification

Clinical phenotypes	• Severity (Intermittent, mild persistent, moderate persistent, severe persistent) • Control (well controlled, partly controlled, uncontrolled) • Treatment response • Bronchodilator reversibility • AHR • Intrinsic, extrinsic • Atopic, non-atopic • Unsupervised clusters (using statistical clustering approaches)
Trigger-related phenotypes	• Aspirin exacerbated respiratory disease • Exercise/cold air-induced • Environmental/occupational allergen • Emotional stress
Demographic phenotypes	• Age of onset • Obesity • Sex
Pathophysiological phenotypes	• Inflammatory phenotypes (eosinophilic, neutrophilic, mixed granulocytic, paucigranulocytic) • TH_2-high or TH_2-low

2.2 Pathophysiology

The pathophysiology of asthma is complex and involves a diverse range of underlying mechanisms and pathways. The reader is directed to recent reviews[8,34,149] for a comprehensive overview. For the purpose of this thesis, this section focuses on the underlying mechanisms, cells, and mediators involved in allergic/TH_2-mediated inflammation and non-TH_2-mediated inflammation, as well as alternative pathways such as airway remodelling and neural mechanisms, with the latter in particular being discussed in greater detail (as this is the focus of subsequent chapters).

2.2.1 Inflammation

2.2.1.1 TH_2 inflammation

Allergy and Atopy

The terms 'atopy' and 'allergy' are often used interchangeably; however, they refer to two different, but related, concepts. Atopy is defined as a tendency to become sensitised and produce allergen-specific IgE against common environmental allergens such as house dust mite (HDM), animal proteins pollens, or fungi.[115] It involves an exaggerated IgE-mediated immune response, known as type I immediate hypersensitivity (i.e. allergic) reaction after allergen exposure.[150] Allergy is an exaggerated immune response to an allergen resulting in symptoms (unlike atopy, which does not necessarily lead to symptoms), regardless of the specific underlying mechanism. As described in section 2.1.3.3, atopy and allergy are associated with asthma in both children and adults.[115,151,152] However, atopy and allergy are not always present in asthmatics[86] and the proportion of asthma attributable to atopy is approximately 50%.[153] In allergic (IgE-mediated) asthma, after sensitisation has occurred

and, upon re-encountering allergens, symptoms are associated with two phases: the early and late effector phase.[154]

Although it is still unclear what initiates sensitisation upon initial exposure to an allergen,[155] sensitisation involves active allergen uptake by antigen-presenting cells (APCs).[156] Allergen then undergoes proteolytic processing and epitopes are loaded onto major histocompatibility complex (MHC) class II molecules. After APC migration to regional lymph nodes, this is presented at the cell surface[157] to naive T lymphocytes, resulting in the selective expansion and activation of allergen-specific TH_2 cells.[158] These allergen-specific TH_2 cells then secrete TH_2 cytokines, e.g. IL-4, IL-5, IL-9, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Under the influence of some of these cytokines, B cells then undergo immunoglobulin class switching from IgG to IgE-producing cells and differentiate into plasma cells.[159] Once IgE antibodies specific to an allergen have been produced, an individual is deemed "sensitised" to that allergen.[155]

When an individual is subsequently re-exposed, allergen-specific IgE binds to allergen, and mast cells (often located in tissues with close contact with the external environment, such as skin and airways)[155] are activated upon cross-linking of FcεRI-bound IgE on their surface.[160] This results in the release of numerous mediators (e.g. histamine, cysteinyl leukotrienes, prostaglandins, and a range of cytokines and enzymes described in Table 2.3). In the airways, this initiates a plethora of physiological changes such as ASM constriction, vascular leakage, and mucus hypersecretion.[161] Many affected individuals experience the associated "early-phase" allergy symptoms (such as wheezing, cough or breathlessness) within an hour of allergen exposure.

The subsequent late allergic reaction phase generally develops 2-9 hours after allergen exposure.[162] In allergic asthma, this phase is characterised by recruitment, activation, and

migration of numerous inflammatory cells, particularly neutrophils and eosinophils into the lamina propria, epithelium, and the airway lumen.[155] These cells then augment and prolong the inflammatory response through the release of various mediators including lymphokines, immunomodulatory and proinflammatory cytokines, chemokines, growth factors, and eicosanoid lipid mediators.[155,163] Once established, a repetitive cycle of inflammation can become chronic even in the absence of sustained allergen exposure,[164] and both acute and chronic inflammatory responses are accompanied by airway structural changes, obstruction, and AHR.[165,166] Figure 2.1 provides an overview of the allergic immune response.

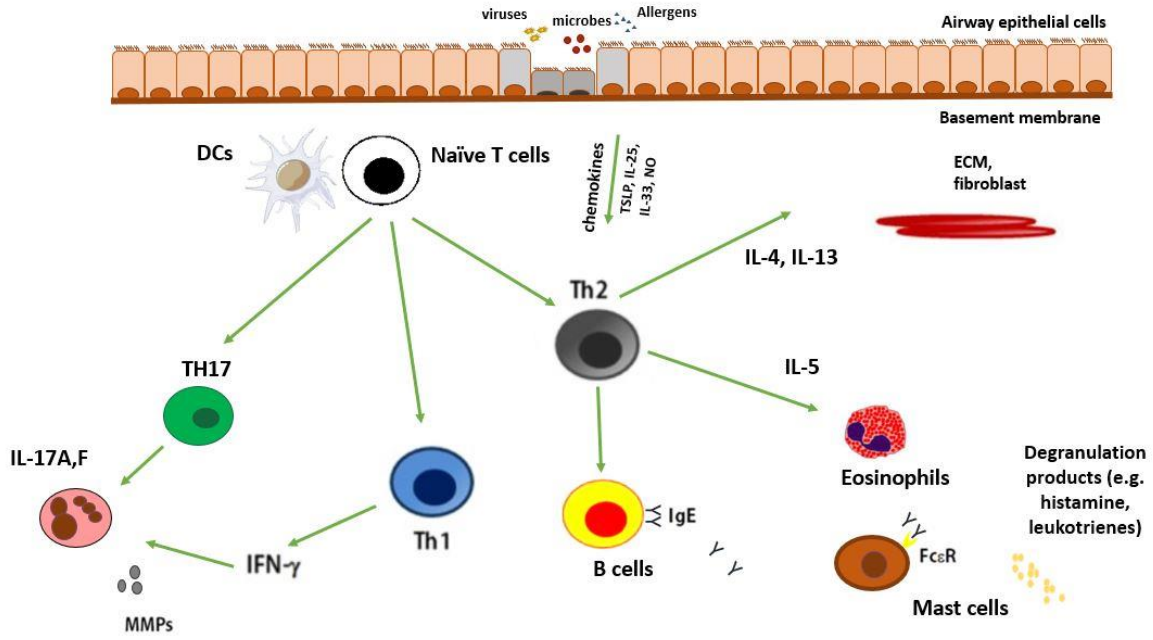

Figure 2.1. Airway allergic immune response.

Although numerous cell types from both innate and adaptive immune systems (and their mediators) are implicated in the inflammatory processes described above (and seen in Figure 2.1), eosinophils are the most characteristic effector cells associated with allergic asthma pathology (see below). An overview of other inflammatory cells and mediators involved in asthma are discussed in the next section and summarised in Tables 2.2 and 2.3, respectively.

Eosinophils

Eosinophils are granulocytic leukocytes often associated with parasitic infections as well as IgE-mediated allergy and asthma.[167] Eosinophil development and maturation occurs in the bone marrow upon exposure of myeloid precursors to IL-3, IL-5, and GM-CSF.[168] After leaving the bone marrow, they are recruited into the airways and sites of inflammation by chemokines such as eotaxin and RANTES (regulated on activation, normal T cell-expressed and secreted protein) as well as members of the macrophage inflammatory protein (MIP) and monocyte chemoattractant protein (MCP) families.[169] Once in the airways, activated eosinophils can degranulate and release numerous mediators, including major basic protein (MBP), eosinophil cationic protein (ECP), reactive oxygen species (ROS), GM-CSF, and lipid mediators.[170] These induce epithelial damage, AHR, and airflow limitation (through bronchoconstriction, mucosal edema, and mucus hypersecretion,[171] leading to asthma symptoms.[172,173] Eosinophils also produce a vast array of cytokines, chemokines and growth factors, including TH_2 cytokines (e.g. IL-4, IL-5, IL-9, IL-13, and IL-25), TH_1 cytokines (IL-12 and interferon (IFN)-γ), proinflammatory cytokines (tumour necrosis factor (TNF)-α, IL-1, IL-6, and IL-8), and immunomodulatory cytokines (e.g. transforming growth factor (TGF)-β and IL-10).[174] The immunomodulatory cytokines maintain and prolong inflammatory responses and induce ASM cell proliferation through a direct cell-contact mediated release of cysteinyl leukotrienes.[166]

Increasing evidence suggests that TH_2-mediated eosinophilic inflammation may also be triggered by allergens through non-allergic pathways.[175] A number of allergens, including HDM and moulds, are able to stimulate the airway epithelium through toll-like receptors (TLRs) and other damage-associated molecular pattern (DAMP) receptors.[176] This leads to release of IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) in the absence of specific

IgE antibodies.[177] These cytokines can lead to the activation of submucosal type two innate lymphoid cells (ILC2) resulting in the release of TH_2 cytokines IL-4, IL-5, IL-9, and IL-13.[164]

Table 2.2. Inflammatory cells and their role in asthma

Name	Mode of action	Role
Neutrophils	• First responder against pathogens • Phagocytosis • Degranulation • Release of mediators	• May play a role in lack of response to corticosteroid treatment[178] • Release ROS, myeloperoxidase (MPO) and neutrophil elastase (NE)[179] • Associated with more severe airflow obstruction, lower lung function and thicker airway wall[180]
Monocytes/ Macrophages	• Phagocytosis • Antigen presentation to T cells	• Release ROS, TNF-α, IL-1, IL-6, IL-8, and IL-12[181]
Mast cells	• Key mediators of type I hypersensitivity • Degranulation • Release of mediators	• Bronchoconstriction, vasodilation, mucus secretion upon degranulation[181] • Chemotaxis of inflammatory cells and development of AHR as well as release of TNF-α, TGF-β, IL-4, IL-5, and IL-13[181]
Basophils	• Degranulation • Release of mediators • IgE mediated allergic reaction	• Role in asthma is the least well defined of all inflammatory cells[182] • Implicated in severe inflammation, bronchoconstriction and worsening of asthma symptoms[183] • Release histamine, IL-4, IL-5, and IL-13[184]
Dendritic cells	• Antigen presentation to T cells • Allergic sensitisation	• Initiate and perpetuate T cell response in asthma[184] • Increase or decrease inflammation depending on subset[184] • Increased numbers found in blood and sputum of asthma patients after allergen challenge[184]
B lymphocytes	• Antibody production	• Allergic reaction[185] • Development of AHR and smooth muscle contraction[8]
T lymphocytes (CD4+ cells)	• Orchestrate immune response against pathogens	• Allergic reaction, eosinophilia, development of AHR and mucus secretion[181] • Increase or decrease inflammation depending on subset (e.g. TH_1 and TH_2 cells mediate inflammation, T-regulatory cells suppress persistent inflammation and TH_{17} cells induce neutrophilia and steroid insensitivity)[181]

Table 2.3. Inflammatory mediators

Cytokine	Source	Airway levels in asthma	Mode of action
Lymphocytes and T-cell regulatory cytokine			
IL-4	TH_2 cells, mast cells, eosinophils	↑	↑ IgE production and number of TH_2 cells[163]
IL-5	TH_2 cells, mast cells, eosinophils	↑	↑ number of eosinophils[163]
IL-9	TH_2, mast cells, eosinophils, neutrophils	↑	↑ number of mast cells[163]
IL-12	Macrophages, T and B cells, dendritic cells	↓	↑ number of TH_1 cells[186]
IL-13	Epithelial cells, fibroblasts, eosinophils, TH cells, ASM cells	↑	↑ IgE production and induce remodelling[163]
IL-17	TH_{17} cells	↑	↑ number of neutrophils (indirectly)[186]
IL-18	Macrophages	↓	↑ IFN-y release[187]
IL-25	TH_2 cells, mast cells	↑	↑ number of TH_2 cells[188]
IFN-γ	TH_1 cells, NK cells	↓	↓ number of TH_2 cells, IgE production[187]
Proinflammatory cytokines			
IL-1β	Macrophages, epithelium, neutrophils	↑	↑ number of eosinophils, mast cells and dendritic cells[163]
IL-6	Macrophages, T and B cells, eosinophils, epithelial cells, mast cells	↑	↑ number of TH_2 cells[189]
TNF-α	Macrophages, epithelial cells	↑	↑ number of TH_2 cells, AHR[190]
TSLP, IL-25, IL-33	Epithelial cells, stromal cells, mast cells, basophils	↑	↑ number of TH_2 cells[191]
Chemokines			
MCP family	Macrophages, dendritic cells, T lymphocytes, epithelial cells	↑	↑ number of monocytes, chemoattracting [163]
Eotaxin	Smooth muscle cells, macrophages, eosinophils, T cells	↑	↑ migration of eosinophils, lymphocytes, basophils[163]
IL-8	Eosinophils, neutrophils, macrophages, T lymphocytes	↑	↑ number of TH_1 cells, chemoattractant of neutrophils, downregulation of IgE production[192]
RANTES	CD8+ T cells, epithelial cells, fibroblasts	↑	↑ number of TH_2 cells[163]
Anti-inflammatory cytokines			
IL-10	T cells, mast cells, macrophages	↑	↓ number of TH_2 cells[193]

2.2.1.2 Non-TH_2 inflammation

Although "classical" TH_2-mediated inflammation is important in many asthmatics, a subset of asthma cases have an underlying pathology that is clearly different.[13] This has led to an increased interest in non-TH_2 inflammatory pathways.[194] Although, non-TH_2 inflammatory pathways are less well characterised, accumulating evidence often suggests involvement of innate immunity and associated neutrophil dominant inflammation.[17] The neutrophilic asthma phenotype associated with this pattern of inflammation will be discussed in greater detail in section 2.4.1.3.

A thorough review of innate immunity is beyond the scope of this review (the reader is referred to Marshall *et al,*[195] for more information). Briefly, innate immunity acts as the first line of defence against pathogens and is particularly important in the lungs since it facilitates the elimination of pathogens prior to the onset of adaptive immune response.[196] Innate immune responses are triggered by the detection of pathogens or irritants (e.g. bacterial endotoxins, particulate air pollution, ozone, viruses)- or pathogen-associated molecular pattern (PAMP)/DAMPs by a limited number of receptors deemed pathogen recognition receptors (PRRs), such as TLRs.[194] The TLRs are a highly conserved family of homologous signalling receptors expressed on the surface of most cells, including inflammatory, epithelial, and ASM cells.[193] Upon PAMP/DAMP recognition, PRRs present at the cell surface or intracellularly - signal to trigger proinflammatory and antimicrobial responses.[197] TLR activation can induce a shift towards activation of TH_1 and TH_{17} responses, leading to generation of a wide range of mediators including IL-2, IL-12, IFN-γ, TNF-α, IL-1β and IL-8.

The neutrophil is a primary cell type involved in the innate immune response. Neutrophils are short-lived leukocytes[198] that act as first responders against bacterial or fungal infections[199]

and/or irritant exposures.[200] They can sometimes be found in large numbers in the airways and play a key role in phagocytosis and some subtypes of asthma.[201] Neutrophils produce and release a wide range of mediators that are implicated in asthma pathogenesis. For example, mediators such as ROS, MPO, NE, cathepsin G[202] and neutrophil derived enzymes such as matrix metalloproteases (MMPs),[203] which are critical for microbial killing and tissue repair, are also associated with airway inflammation, remodelling and AHR in asthma.[204] Some neutrophil products such as IL-1β and IL-8 also act as neutrophil chemoattractant, and neutrophil activation can lead to further neutrophil influx and subsequent prolonged inflammation.[192] In the lungs, respiratory exposure to various microbial components such as endotoxins can lead to asthma-like symptoms and trigger neutrophilic inflammation.[194] Indeed, severe asthmatics with increased airways neutrophil counts show increased levels of cytokines responsible for TH_1 (IFN-γ, IL-12, TNF-α)[205] and TH_{17} differentiation and expression.[206] Initiation of TH_1 and TH_{17} responses may also suppress TH_2 responses (described in section 2.2.1.1).[207]

2.2.2 Airway remodelling

Alongside airway inflammation, airway remodelling is often observed in asthma pathology.[36] It is characterised by structural alterations such as epithelial shedding, sub-basement membrane thickening, sub-epithelial fibrosis, ASM hypertrophy and hyperplasia, blood vessel proliferation and dilation, and mucous gland hyperplasia and hyper-secretion.[208] Some of these changes have been associated with a progressive loss of lung function,[209] increased airflow obstruction, and AHR in asthma[210] that is not prevented by or fully reversible by current therapy.[211]

The underlying mechanisms driving airway remodelling are largely unclear and likely to involve a complex interaction between biochemical mediators, cellular processes, and genetic and environmental factors.[212] It is also widely believed that remodelling is associated with chronic inflammation[213] and numerous mediators released during the inflammatory process such as cytokines, growth factors, and endothelins have been implicated in different aspects of airway remodelling.[36] For example, cytokines associated with both TH_2-high and TH_2-low inflammation such as IL-1β and IL-6 have the capability to enhance smooth muscle proliferation,[214] and IL-13,[215] TGF-β,[216] and nerve growth factor (NGF)-β[217] have also been shown to induce fibrosis. Similarly, a number of other mediators, including epidermal growth factor,[218] fibroblast growth factor, vascular endothelial growth factors (VEGF),[219] NE,[220] endothelin,[221] and tissue inhibitor of metalloproteinases-1 (TIMP-1)[222] are associated with increased vascularity, dysregulated extracellular matrix (ECM) deposition, and smooth muscle cell proliferation.[219] An overview of the cells and mediators involved in remodelling is shown in Figure 2.2.

Despite this, the relationship between inflammation and remodelling is not fully understood. Studies in childhood asthma have shown that airway remodelling can be observed from a

very early age,[223] suggesting that remodelling may occur in parallel with, rather than as a consequence of inflammation. Furthermore, there is accumulating evidence that airway remodelling can occur in the absence of inflammation[39,224] and may even occur as a result of repeated bronchoconstriction.[225] For example, Grainge *et al* demonstrated that bronchoconstriction (induced by either allergen or methacholine) led to TGF-β activation of and downstream remodelling changes.[38]

It is also possible that some aspects of the remodelling process are dependent on airway inflammation while others are not. For example, a recent study of 80 asthmatics found that specific features of remodelling such as inner and outer airway wall thickening may be associated with airway inflammation, whereas thickening of the ASM layer and basement membrane may occur in the absence of inflammation.[226] Taken together, this implies that airway inflammation, mechanical forces, and/or as yet unidentified stimuli can all induce airway remodelling, and that specific aspects of remodelling may be distinguished by the roles those stimuli play.

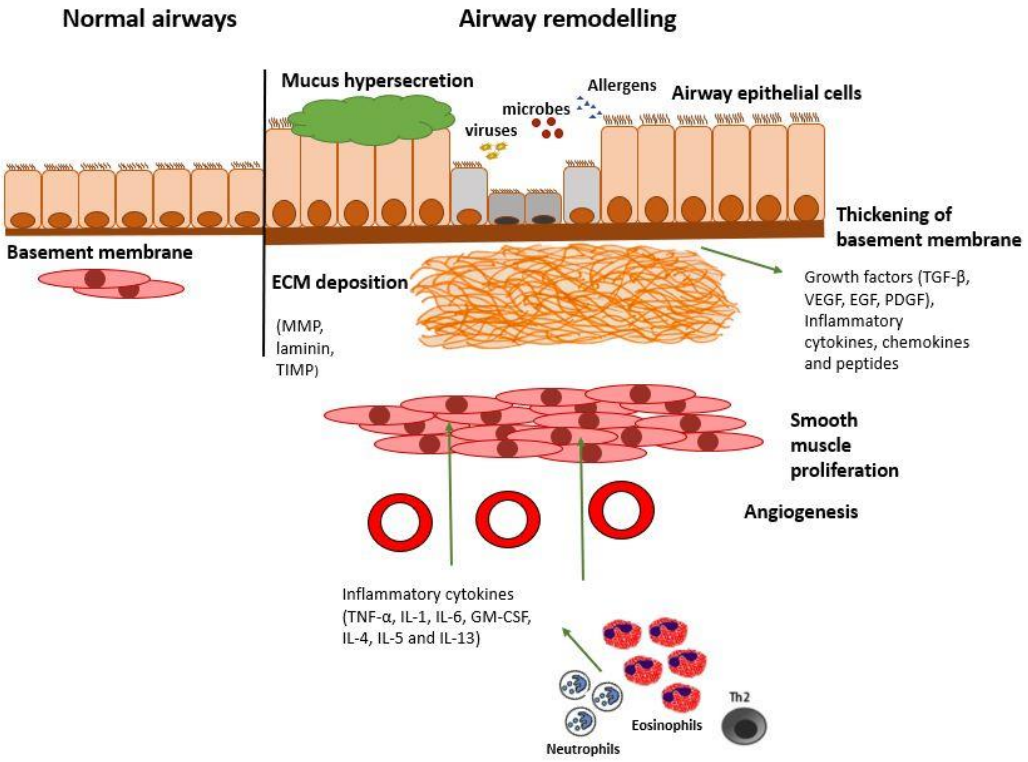

Figure 2.2. Airway remodelling processes.

2.2.3 Neural involvement

Over recent decades, a limited (but growing) body of evidence has accumulated suggesting that various components of the ANS may be involved in the development and pathology of asthma.[227] This is feasible as the ANS and airway innervation play a major contribution in regulating ASM tone and calibre.[228]

2.2.3.1 Airway innervation

Human airways are innervated by a network of autonomic nerves that are divided into motor (efferent) and sensory (afferent) neurons.[229] Airway nerves regulate many aspects of airway function including breathing patterns, airway muscle tone, cough reflex, and pain transmission.[28] These are all dependent on nerve activation/ transmission to the central nervous system, and result in sensory perception, motor function, or both.[230]

Efferent innervation

The airway efferent nerves are divided into the: (i) parasympathetic cholinergic; (ii) sympathetic adrenergic; and (iii) non-adrenergic-non-cholinergic nervous system (NANC) nerves.[231] These function through different pathways and act as complementary but oppositional systems.[232] Selectively stimulating these various pathways can either constrict or dilate[229] (e.g. either due to an increase in cholinergic activity or a decrease in nitrergic activity).[34]

i) **Parasympathetic cholinergic nerves:** These make up most innervation in human airways, and project to the airways via the vagus nerve.[233] The parasympathetic nervous system (PNS) primarily transmits via acetylcholine (ACh) which acts on two types of receptors, the muscarinic and nicotinic cholinergic receptors,[234]

expressed on smooth muscle cells,[230] submucosal glands, fibroblasts, epithelial cells, and inflammatory cells.[235] The PNS can induce either bronchoconstriction or bronchodilatation when activated or inhibited, respectively, and also regulates stimulation of respiratory mucus glands.[236]

ii) **Sympathetic adrenergic nerves:** These use the catecholamines epinephrine and norepinephrine as their neurotransmitter.[237] In humans, sympathetic innervation of ASM is sparse or non-existent and plays little role in regulating airway calibre.[229] However, β-adrenergic receptors are abundantly expressed on ASM and their activation, by circulating epinephrine released from sympathetic nerves, may result in bronchodilation.[34]

iii) **NANC nerves:** These oppose cholinergic activity and are the main neural bronchodilator pathways in human airways.[229] The major neurotransmitters for NANC nerves are vasoactive intestinal peptide (VIP) and NO.[238] Apart from acting on ASM, they may be involved in mucus secretion and vascular dilation.[239] NANC nerves are anatomically different to parasympathetic nerves.[240]

Afferent innervation

Afferent airway innervation occurs predominately via the vagus nerve.[34] Sensory nerves terminate at all levels of airways (including the bronchi) below and within the airway epithelium, smooth muscle, glands, and autonomic ganglia, and play a key role in regulating breathing patterns and inducing cough reflex.[241] Sensory nerve fibres can be classified based on their primary function and associated receptors. These include the unmyelinated C fibres and stretch-sensitive myelinated A fibres.[242]

i) **C-fibres:** Approximately two thirds of sensory afferents are non-myelinated C-fibre neurons with sensory terminals located between or underneath airway

epithelial cells.[243] They are sensitive to chemical stimuli, such as inflammatory mediators (e.g. histamine, bradykinin, and prostaglandins)[244] or inhaled environmental irritants (e.g. allergens, tobacco smoke, hot air, citric acid, or capsaicin)[241] and are activated by G-protein coupled receptors[245] and Transient Receptor Potential (TRP) channels.[246]

ii) **A-fibres:** These are classified as mechanoreceptors and conduct action potentials at a relatively high speed.[241] They are classified into three groups; thinly myelinated Aδ-fibres, which are predominantly nociceptive; or myelinated Aβ-fibres, which are cutaneous mechanoreceptors that can be slowly (SAR) or rapidly adapting receptors (RARs).[34] Both SARs and RARs are important for as they are involved in detecting changes in lung, volume (i.e. lung inflation and tidal volume), airway calibre, bronchoconstriction and airway oedema.[247] In particular, SARs are localised to smooth muscle and are activated by changes in the airway wall tone as well as neurotransmitters and mediators such as ACh and histamine, respectively,[247] resulting in cough and altered regulation of ASM tone.[27]

Upon activation of these sensory afferents, responses are mediated by central reflex pathways and/or local or axon reflexes.[241] The former involves transmission of action potentials to the brainstem, influencing the activity of autonomic or somatic efferent nerves.[248] This leads to subsequent release of ACh and activation of muscarinic receptors on the ASM cells (as discussed above), and triggers cough reflex or bronchoconstriction.[249] Axon reflexes are elicited by a stimulus that excites sensory neurons but does not require central input and involves peripheral axon terminals release of neuropeptides (see below).[250] This can produce potent local effects such as bronchoconstriction, extravasation, and inflammation.[251]

2.2.3.2 Efferent innervation in asthma

Efferent nerves may undergo changes in asthma that result in increased neural bronchoconstriction.[23] These changes are exhibited as: i) enhanced parasympathetic response; ii) diminished adrenergic and NANC responses; or iii) enhanced reflex bronchoconstriction.[228].

Evidence for enhanced parasympathetic response in "asthma" comes largely from experimental animal models of allergic airway disease. This includes the increased release of ACh in allergen-exposed IgE-immune mice,[252] increased bronchodilation in antigen-challenged guinea pigs upon vagotomy,[253] and inhibition of AHR with anticholinergic agents in antigen-challenged mice.[254] In humans, anticholinergic drugs such as atropine and ipratropium bromide (IB) diminish both bronchoconstriction and AHR in asthmatics through their antagonistic effect on muscarinic receptors,[255,256] indicating the possibility of enhanced parasympathetic response in asthma. However, directly assessing an enhanced parasympathetic response in asthma in humans is challenging due to the lack of non-invasive methods to measure ANS activity. To overcome this limitation, a number of studies have used proxy indicators such as analysis of heart rate variability (HRV; discussed in greater detail in section 2.3.3), or autonomic control tests to measure PNS activity in adults[257-259] and in children[260,261] with asthma. Some of these studies report increased parasympathetic activity, although findings have been inconsistent.

The decrease in bronchodilation induced by NANC and SNS adrenergic nerves, which can result in exaggeration of parasympathetic bronchoconstriction[262] could potentially be due to intrinsic defects in NANC nerves, altered expression of NANC receptors, or increased breakdown of the NANC neurotransmitters.[263] For example, oxygen free radicals from inflammatory cells break down NO[247] and enzymatic degradation of VIP by mast cells may

be enhanced in severe asthma.[264] The decrease in SNS activity may be due to reduced epinephrine release in asthmatics. In particular, studies have found that epinephrine secretion is reduced during acute asthma attacks in adults[265] and during exercise in asthmatic children.[266]

Finally, there may be increased cholinergic reflex bronchoconstriction due to increased sensitivity of sensory receptors in the airways[241] (discussed below).

Efferent innervation-inflammation crosstalk

Although it is often assumed that inflammation is the main cause of AHR in asthma.[267] increased parasympathetic activity may induce AHR in the absence of inflammation.[268] In support of this, it has been shown that asthmatics exposed to stressful situations exhibit increased vagal activity and show a greater degree of AHR in response to methacholine,[269-271] that are not associated with inflammatory markers such as FeNO.[270] It is also possible that inflammation and/or efferent nerve-mediated mechanisms may interact. For example, muscarinic signalling results in recruitment and migration of inflammatory cells and release of proinflammatory cytokines and chemokines, helping establish and prolong the immune response.[272] Alternatively, epithelial and inflammatory cells in the airways release ACh that may interact with muscarinic receptors that are expressed on fibroblasts, epithelial cells, and inflammatory cells as well as ASM cells.[273] This suggests non-neuronal ACh may mimic the classical cholinergic-driven bronchoconstriction effect described above.[235] However, the functional relevance of non-neuronal ACh in the asthmatic airways is yet to be established.

2.2.3.3 Afferent innervation in asthma

While sensory nerve activation serves as a protective mechanism, under certain pathological states, sensory nerves can develop hypersensitivity and exaggerated reflex responses that become deleterious to normal airway function,[274] and alterations in the activation, structure, or function of these nerves may contribute towards AHR, inflammation, and airway remodelling.[241]

Increasing evidence suggests that sensory nerves may be involved in asthma.[275] Epithelial damage resulting from chronic inflammation may expose afferent sensory nerve endings in the subepithelial layer to the airway lumen,[276] leading to increased stimulation. Furthermore, allergic inflammation and associated mediators can stimulate sensory nerve fibres directly,[241] which at least in in some animal models induces acute hypersensitivity of C fibres to several stimuli, including capsaicin.[277] As with efferent nerves, afferent nerve activity cannot currently be measured directly in humans. However, clinical evidence from studies using tussive agents for cough challenge testing (discussed in greater detail in section 2.3.3)[278] suggests that some asthmatics have increased sensory nerve sensitisation.[279]

Sensory nerve sensitisation in asthma may occur as described below:

i) **Increased or altered expression of cough receptors:**

Increased TRP vanilloid 1 ($V_{1)}$ expression,[280] density,[241] and $TRPV_1$ activation[281] have been reported in severe asthmatics and a role for TRP channel activation has been suggested in a rodent model of allergic asthma.[282]

ii) **Neurogenic inflammation through an axonal reflex:**

Activation and sensitisation of the sensory C-fibres may lead to release of neuropeptides such as neurokinin A (NKA), substance P (SP), VIP, and calcitonin gene related peptide (CGRP).[283] These agents are able to modify nerve function,

stimulate production of neuronal growth factors, and interact with immune cells and pathways.[275]

Neurogenic inflammation through axon reflex has been demonstrated in rodents,[284] but to date there is little clinical evidence. However, some studies have shown that airway neuropeptide levels increase upon exposure to allergens and during asthma exacerbation.[285,286] SP concentrations in induced sputum have also been shown to inversely correlate with airflow obstruction,[287] although findings have not been consistent.[288] VIP concentration levels have been reported to be significantly lower during asthma exacerbations[289] and a complete absence of VIP-immunoreactive nerves has been reported in post-mortem preparations of asthmatic lungs,[289] suggesting reduced bronchodilatory function.

iii) Sensory afferents influencing motor efferent activity:

Increased stimulation of sensory nerves by a variety of stimuli, such as inhaled methacholine,[290] histamine,[241] cold air,[291] allergens,[292] and exercise,[293] can potentiate the release of ACh from parasympathetic nerves to activate muscarinic receptors on ASM and trigger contraction and airway narrowing.[233]

Afferent-inflammation crosstalk

The relative rapidity of sensory nerve activation, and the non-immunological nature of some exposures (such as cold/ hot air or pH changes) suggest that in some cases pathways involving sensory nerves may be important in asthma even without any demonstrable airway inflammation,[14] but this has yet to be confirmed.

In addition, although neuropeptides have often been assumed to be of neural origin because of the abundance in the number of SP and NKA nerve fibres in asthmatic airways,[263] many of these “neuropeptides” can also be produced by inflammatory cells,[294-296] and play a role in

inflammatory processes.[297] Therefore, despite the (limited) evidence of involvement of neurogenic/neuropeptide-mediated mechanisms in asthma, it still remains unclear whether these mechanisms occur prior to or alongside airway inflammation or can exert their role in the absence of inflammation.[298]

2.3 Assessment of pathophysiology

To date, conventional methods assessing airway pathophysiology in asthma have been largely confined to assessment of airway inflammation, which, unsurprisingly, provide little insight into non-inflammatory mechanisms. In the last few decades, several methods have become available for the non-invasive assessment of alternative mechanisms, which will hopefully facilitate an improved understanding of their role in asthma pathophysiology. This section will review approaches for assessing airway inflammation, neural pathways, and airway remodelling in asthma, with a particular focus on methods employed in the subsequent chapters of this thesis.

2.3.1 Inflammation

There are a number of methods available to assess inflammation and airway pathology, both invasive (e.g. bronchoscopy; collecting bronchial washings, biopsy, and/or bronchoalveolar lavage (BAL))[299] and non-invasive (e.g. induced sputum, exhaled breath condensate, FeNO measurement).[300] The advantages and disadvantages of the different methods are summarised in Table 2.4. As FeNO measurement and induced sputum have been used throughout the studies described in this thesis, only they are described in detail.

2.3.1.1 FeNO

Increased levels of nitric oxide in exhaled breath of asthma patients were first reported in the early 1990s[301] and has since been found consistently through dozens of studies in different settings.[22,302-304] Nitric oxide is produced endogenously from the amino acid L-arginine by nitric oxide synthase,[305] which occurs as either constitutive or inducible (iNOS) isoforms in

epithelial cells and various inflammatory cells in the airways.[88] The iNOS form is typically produced in response to airway inflammation and in host defence against infection.[306]

NO can be detected in exhaled breath, through the bronchial tree, or in the lung parenchyma[88] and its measurement has been increasingly recognised as a convenient and cost-effective means of non-invasively assessing airway inflammation. Standardised guidelines and recommendations for clinical use and reference values for FeNO in normal healthy populations have been published,[307] and FeNO measurement is generally consistent and reproducible in adults and children.[308,309]

In asthma patients, high FeNO levels are often associated with eosinophilic inflammation, AHR, and disease severity.[310,311] FeNO levels decrease after initiating oral[312] or inhaled steroid treatment,[313] leading to recommendations that FeNO measurements should be used in predicting and documenting ICS response,[314] adherence monitoring [315,316] and as a diagnostic tool in ICS-naïve patients.[317] However, studies tailoring asthma treatment based on FeNO levels[318-320] have been inconclusive. For example, a meta-analysis of 1010 patients, found that whilst tailoring the ICS dose based on FeNO levels led to reduction of daily dose, it did not affect asthma exacerbations or lead to better asthma control.[321]

2.3.1.2 Induced Sputum

Sputum induction is a well characterised and validated procedure[322] used to examine inflammatory cells and biomarkers from the lower airways in the assessment of respiratory disorders.[323] It involves inhalation of an aerosolised solution to encourage sputum expectoration.[324] Various agents such as distilled water, normal saline, glucose solution, and surfactant agents (e.g. tyloxapril) have been used[325] but hypertonic saline remains the most commonly used, due to relatively high success rate and good safety profile.[326] It generally

involves inhalation of 4.5% hypertonic saline for increasing time intervals (to 10-20 minutes), or inhalation of increasing concentrations of hypertonic saline for the same period.[324]

For regular cytological assessment, either the whole sputum[327] or plug selection technique[328] are used for subsequent processing of the resulting expectorate. Either way, the expectorate is generally solubilised with reducing agents (e.g. dithiothreitol (DTT) or dithioerythritol (DTE)) that disrupt covalent disulfide bonds in mucin,[328] as mucus interferes with downstream analysis.[324] Generally, the resulting cell suspension is then filtered and centrifuged and a total cell count (TCC) and assessment of cell viability (using trypan blue exclusion) is performed.[329] Cytospins are then prepared, and differential cell counts (DCC) are performed on the resulting slides that have been stained appropriately (e.g. May-Grunwald Giemsa).[328] Various criteria and cut-offs have been used for determination of sample quality, often based on cell viability and/or squamous cell contamination,[327] as poor-quality samples (with low viability, high squamous cell contamination, or significant saliva contamination[330] are difficult to assess and are reproducible.[331]

Induced sputum is regularly used for the measurement of absolute and relative leukocyte numbers, as well as soluble mediators levels in the supernatant produced during processing.[326,332] Studies identifying reference values for leukocyte populations in healthy and diseased populations[327,333] show that, in general, the most common leukocyte population found in induced sputum of healthy individuals is the macrophage,[333] although neutrophils can be found in high numbers in some, particularly older, individuals.[334]A small population of lymphocytes (less than 5%) is generally observed. As discussed in section 2.2.1, eosinophils are found in increased numbers in many asthmatics.

Induced sputum has several advantages over other techniques used to assess airway pathophysiology. It can be performed in in children and adults, with a reported success rate of

~70-100%,[335] with minimal discomfort to the patient. However, it is technically challenging, laborious, and difficult to conduct in large population-based studies,[328] and unlike biopsy, only allows assessment of cells and mediators in the airway lumen.[336] The procedure itself may induce changes in inflammatory cell populations, particularly increases in neutrophils and eosinophils, within a 24–48-hour period,[337] suggesting that repeated visits within a short period of time may lead to artefactual findings. The presence of proteases within saliva or the use of DTT may result in reduced mediator detection.[338] Finally, some studies report considerable interindividual and intraindividual variability in the quality, quantity and viability of sputum produced; and in a proportion of cases, sputum cannot be successfully induced at all.[339]

Table 2.4. Methods used to assess airway inflammation

Method	Advantages	Disadvantages
Bronchoscopy	• Direct airway assessment • Provides information on structural changes • Downstream *in vitro* analysis	• Invasive • Rescue medication/procedures needed • Difficult to repeat in patients • Time consuming • BAL fluid relates to only one segment of lung/can be blood contaminated/diluted by saline • Laborious processing methods/results are not available immediately
Induced sputum	• Semi-invasive and safe • Validated tool • No expensive equipment required • Molecular and cellular biomarkers • Allows treatment guidance • Allows study of larger patient populations than e.g. bronchoscopy	• Risk of bronchoconstriction • Rescue medication/ procedures needed • Time consuming • Success rate around 80% • Cannot be conducted in a primary care setting • Procedure itself may induce changes in airway/lab results • Laborious processing methods/results are not available immediately • Little use for assessing airway structure and subject to dilution by saline
FeNO/ exhaled nitric oxide	• Non-invasive • Validated tool • Immediate results • Can be conducted repeatedly • Safe even in severe disease • May be collected across all ages • Allows study of large patient population	• Flow-dependent • Nasal sourced contamination is possible • Only one mediator is detected • Sensitive to range of factors including steroid administration
Exhaled air/ exhaled breath condensate/volatile organic compounds	• Non-invasive • Validated tool • Can be conducted repeatedly • May be collected across all ages • Allows study of large patient • population	• Method still under evaluation • Laborious processing methods/results are not available immediately • Low reproducibility of exhaled biomarkers • Upper airways/salivary possible contamination • Soluble markers subject to dilution
Nasal Lavage	• Non-invasive • Inexpensive • Molecular and cellular biomarkers • Allows study of large patient population	• Variable sample quality • Laborious processing methods/results are not available immediately • Upper airway may not be representative of lower airways • Little use for assessing airway structure and can be diluted by saline

2.3.2 Airway remodelling

Structural changes in the airways are generally evaluated through direct examination of airway tissues, indirect assessment (using tissue fluids), or through radiological assessment.[340] Direct airway tissue assessment is often used and can be conducted on specimens obtained surgically, post-mortem, or through bronchial bronchoscopy.[341] Histological analyses of airway tissue samples allow the study of the entire airway structure and identification of specific pathological changes such as sub-basement membrane thickening, subepithelial fibrosis or loss of epithelial integrity.[341] However, these sampling approaches are extremely invasive, costly, and impractical to conduct in population-based studies. As an alternative, some non-invasive techniques have been developed.

One approach is to measure mediators associated with airway remodelling and ECM degradation in body fluids such as induced sputum (discussed in section 2.3.1.2), BAL fluid, blood, urine, or saliva (reviewed in Manso *et al*).[340] However, a limitation of measuring mediators is that many of them may not be specific to airway remodelling and are often related to airway inflammation.[36]

Alternatively, imaging technologies such as such as high-resolution computed tomography (HRCT) or endobronchial ultrasound (EBUS)[36] also allow non-invasive assessment of airway remodelling. HRCT scanning uses X-rays to produce high resolution images of the airway lumen and wall dimensions.[342] However, it exposes the patient to radiation. EBUS was initially used to evaluate bronchial wall tumour infiltration,[341] but studies conducted over the last twenty years have demonstrated that EBUS can also be used to visualise and distinguish three to five layers of bronchial wall.[343] Unlike HRCT, EBUS is not associated with radiation exposure; however, it requires bronchoscopy.[344]

2.3.3 Neural pathways

2.3.3.1 Efferent nerves

Various approaches have been used to monitor efferent nerve activity.[345] These are often based on evaluation of cardiovascular reflexes triggered by performing specific provocative manoeuvres. For example, stimuli that raise blood pressure, such as isometric exercise,[346] cold pressor test,[347] or mental arithmetic,[345] as assessed through blood pressure measurements, reflect mainly SNS activity.[345] Conversely, changes in heart rate immediately after orthostatic testing, Valsalva manoeuvre[348] or during deep breathing[347] reflect PNS activity.[349] Other tests, such as using radiolabelled noradrenaline tracers[350] and measuring catecholamine levels in biological fluids[266] have also been used to evaluate ANS activity.

Given the complexity of the ANS there is no single test that accurately reflects its function. Traditionally, a battery of tests have been used to assess autonomic dysregulation, with the Ewing battery (including Valsalva manoeuvre, response to deep breathing, orthostatic testing, and sustained hand grip) being widely used.[351] More recently, techniques such as HRV measurement or microneurography have been introduced.[352] As HRV analysis used in this thesis to assess efferent nerve activity, only this is discussed in greater detail.

HRV analysis

HRV is a non-invasive indicator of cardiac autonomic function, based on measuring the variations in time-intervals between consecutive heart beats at rest.[353] It is generally calculated from the R-wave-to-R-wave on an electrocardiogram (ECG) or by a validated HR monitor.[352] HRV analysis is classified into two categories on the basis of data recording time;

short-term HRV which is typically calculated over 5 min, and long-term HRV which is calculated over a nominal 24-hour period.[352]

There are different approaches to HRV evaluation. The most common are the use of time or frequency domain methods.[352] Time domain analyses characterise R-R interval variation, whereas frequency domain analysis describes the frequency at which the length of the R-R interval changes.[354] Both methods provide data representing the activity and balance of the different branches of the ANS, and are strongly correlated.[355] The specific HRV parameters are described in Table 2.5. Time domain methods are commonly used for long-term HRV,[356] and frequency domain analysis is most commonly used for short-term HRV.

Although HRV analysis has gained popularity as a simple, non-invasive tool, it has some limitations. In particular, it is directly influenced by several methodological, environmental and physiological factors. For example, duration (e.g. short-term versus long-term), the instrument used (heart rate monitor versus ECG), or data selection and/or processing (e.g. time vs frequency domain) may all impact HRV findings.[357] Sex,[358] age,[359] blood pressure,[360] pubertal status,[361] body size,[362] and environmental factors like social stresses, loud noises, and exposure to pollution[363] can also influence HRV findings.

Table 2.5. HRV parameters

	Variable	Description	Indicative of/use:[352]
Frequency-domain	VLF	Very low frequency (power spectrum range between 0.0033-0.04 Hz)	It is not entirely clear what VLF represents, but it is presumed to reflect thermoregulation and vasomotor activity[352]
	LF	Low frequency (power spectrum range between 0.04-0.15 Hz)	SNS activity in long-term recordings PNS activity when respiration rate is lower than 9 per minute or during deep breathing
	HF	High frequency (power spectrum range between 0.15-0.4 Hz)	PNS activity
	LF/HF Ratio	Ratio between the power of LF and HF frequency bands	SNS/PNS balance Higher values reflects SNS/lower values indicate PNS dominance
	Normalised LF	Ratio between absolute LF and difference between total power/VLF (expressed as percentage)	Minimises the effect of changes in VLF power Emphasises changes in SNS
	Normalised HF	Ratio between absolute value of HF and difference between total power and VLF (expressed as percentage)	Minimises the effect of changes in VLF power Emphasises changes in PNS
Time-domain	RMSSD	Root mean square of successive RR interval differences.	PNS activity
	SDRR	Standard deviation of RR intervals	SNS and vagal activation

Use of HRV assessment in asthma

To date, relatively few studies have conducted HRV analysis in asthma. These studies have been predominantly conducted in adults,[257,364] or severe asthmatics,[260,365] with relatively few studies in young adults or children with mild-to-moderate asthma.[366-368] To date, results have been mixed, with asthma associated with increased PNS activity (as reflected by increased HF or RMSSD) in some studies,[260,364,365] but not others.[369,370] For example, in a study of children (49 asthma/ 58 non-asthma), resting HF was significantly higher in asthmatics compared to aged-matched controls.[367] Similar findings were reported in a study of 30 asthmatic adults.[364] In contrast, in a study of 19 asthmatic and ten non-asthmatics, both

symptomatic and acute asthmatics had significantly lower LF when compared with the controls, but HF (PNS activity) was not increased in asthma.[257]

Some studies have reported an association between increased PNS activity and features such as asthma severity[365] or AHR.[371] For example, in one study, asthmatics had high HF and low LF compared with the non-asthmatics, with the highest values being observed in severe asthmatics.[372] Similar results were obtained in a study of 94 children with severe asthma.[366] Increased SNS activity (increased LF) has been associated with improved asthma control[365] and β-agonist use.[373,374]

Currently, there are no clinical studies specifically assessing the association between airway inflammatory markers and HRV in asthma. However, reports suggest an inverse relationship between HRV indices, especially PNS-associated parameters, and markers of systemic inflammation (including white blood cell count) in healthy subjects,[375] in cardiovascular diseases [376] and in inflammatory conditions such as sepsis.[377] In a recent meta-analysis of 51 clinical studies examining HRV and inflammatory markers, most data suggested a negative correlation between both sympathetic (LF) and parasympathetic (HF) markers and inflammatory markers such as IL-1, IL-6, C-reactive protein, IFN-γ and TNF-α.[378]

2.3.3.2 Afferent nerves

Assessment of airway afferent nerve activity (also termed sensory nerve reactivity)[275] has often been conducted indirectly using a variety of approaches. These include electrophysiologic studies of neuronal cell bodies/isolated tissue or in anesthetised animals,[379] confocal bronchoscopy,[241] cough provocation challenge,[379] or measuring mediators/ peptides implicated in neurogenic inflammation.[35] Of these, the two used in this thesis are cough provocation challenge (discussed below) and sputum mediator measurement.

Sensory nerve reactivity

Cough provocation challenge is a relatively non-invasive, repeatable, and safe procedure[380] that has been standardised and validated.[278] There are different approaches,[381] but it generally involves inhalation of increasing concentrations of tussive agent via a nebuliser.[278] Various agents such as capsaicin, citric acid, tartaric acid, prostaglandin, and bradykinin[278] have been used, but capsaicin is widely used because of high success rate, potency, and specificity[281] (it activates $TRPV_1$ on sensory c-fibres).[382]

The challenge outcome is based on the number of coughs elicited in the period after inhalation,[383] with endpoints generally being either the dose eliciting 2 and 5 coughs (C2 and C5),[380] or maximum cough response and half-maximal cough response based on non-linear mixed-effect procedure.[381] These endpoints have been found to be adequate for identifying sensitivity to tussive agents,[384-386] and to be reproducible. For example, capsaicin response is 90-100% reproducible in both the short (14 days) and long-term (~ 6 months).[383]

The safety of inhaled capsaicin in cough challenge testing has been assessed in a review of 122 studies.[387] No serious adverse events were reported, although 9 of the 4,833 subjects experienced transient reduction in lung function, and transient throat irritation was common.

Use of sensory nerve reactivity assessment in asthma

There are relatively few studies directly comparing sensory nerve reactivity in asthmatics and non-asthmatics and have generally been conducted in severe asthmatics,[388,389] or adults,[281] with few studies in young adults or children.[390,391] To date, findings have been equivocal. While some studies show that asthmatics exhibit increased sensory reactivity to inhaled capsaicin,[279,388,392] or citric acid,[281] others demonstrate no difference in response to capsaicin,[281,384,393-396] citric acid,[397,398] or tartaric acid.[399,400] More recently, in a study

comparing capsaicin, citric acid, and prostaglandin challenges in healthy controls, healthy smokers, COPD, asthma, and chronic cough, no significant differences in C5 capsaicin and prostaglandin between healthy volunteers and patients with asthma were observed. However, there was a significant difference in C5 to citric acid, suggesting differences in expression or cough receptor sensitivity to citric acid.[281]

The association between capsaicin sensitivity and clinical characteristics such as asthma severity or AHR is also unclear. Most studies conducted in severe asthmatics suggest that this group may be more sensitive to capsaicin.[401-403] For example, in a study of 122 severe asthma patients, heightened capsaicin sensitivity was associated with asthma severity and frequent exacerbations, particularly in patients without atopy.[389] However, there is some limited evidence that stable or mild asthmatics may also exhibit enhanced sensitivity. For example, Satia *et al* found heightened capsaicin-evoked cough responses in 97 patients with stable asthma compared with 47 non-asthmatics;[279] this was also more apparent in non-atopic asthmatics.

There are very few studies exploring the association of airway inflammation with sensory nerve reactivity in asthma, but those available do not show a strong association. For example, in one study assessing the relationship between capsaicin sensitivity, airway inflammation and disease control in 157 asthmatics, capsaicin sensitivity was associated with asthma control but unrelated to inflammatory cells or markers such as FeNO and IgE.[389] Similarly, Minoguchi *et al* found no change in capsaicin response despite observing a significant increase in airway eosinophilia after challenging mild atopic asthmatics with inhaled HDM allergen.[404]

2.4 Pathophysiological phenotyping

2.4.1 Inflammation

2.4.1.1 Allergy or atopy

An early approach to asthma classification was stratifying into extrinsic and intrinsic asthma.[147] Extrinsic asthma was believed to be associated with an early onset and allergen sensitisation, while intrinsic asthma was associated with late onset, absence of atopy and female sex.[10,11] However, whilst initially considered distinct forms of asthma, a series of small clinical studies comparing intrinsic/extrinsic and atopic/non-atopic asthma found that they overlapped in both clinical presentation and in underlying inflammatory characteristics (including airway levels of IL-4 and IL-5 mRNA), and, on this basis, suggested that the majority of asthma was likely to be TH_2-mediated.[405,406] Subsequently, the assumption that asthma should be classified on the basis of atopy/allergy (allergic vs non-allergic asthma), and the relevance of this classification, has been increasingly challenged.[15] In particular, it is now clear that atopy can occur in the absence of allergic airway inflammation,[22] or that airway inflammation can occur in non-atopics.[407]

Given the potential problems with classification on the basis of atopy or clinical characteristics (as discussed in section 2.1.6), and the availability of methods for assessing airway pathophysiology, there is increasing emphasis placed upon phenotyping asthma on the basis of airway immunopathology.

To date, two broad categories of pathophysiological phenotypes are commonly used: TH_2-high and TH_2-low (on the basis of molecular signatures), or EA and NEA, based on cytological assessment of inflammatory cell populations in induced sputum.[147]

2.4.1.2 Molecular phenotypes

The application of high-throughput -omics approaches has been used to define molecular subsets of asthma.[147] An example of this approach involved gene-expression profiling of biopsy-derived airway epithelial cells, identifying a group of genes (including periostin (POSTN)) that were specifically induced in asthma, and directly regulated by IL-13 *in vitro*.[408] In a further study, this gene signature was used as a surrogate marker of the IL-13 inflammatory pathway to determine gene expression variability. Subgroups with high and low levels of IL-13 induced genes were found, indicating differential involvement of TH_2-like pathways, despite similar symptoms.[409] The TH_2-high profile was associated with increased markers such as airway eosinophils, mast cells, IgE levels, AHR, and ICS responsiveness.[409] It also appeared to be associated with a sputum gene expression signature comprising of 6 biomarkers.[410] Since these studies, various other omics-based approaches have been used to further profile asthma subsets and, in general, these provide further evidence for TH_2-based endotypes.[411,412]

2.4.1.3 Sputum inflammatory phenotypes

For more than a century, there have been reports of increased airway eosinophils in asthma,[413] and this is now considered a hallmark of allergic asthma;[9] often termed EA.[22] Despite previous suggestions that EA made up most asthma cases,[405,406] towards the end of the 20th century, an increasing number of studies identified subgroups of patients[414] with symptomatic disease, frequent β-agonist use, and AHR, but normal levels of sputum eosinophils.[322,415] EA only accounts for about 50% of all asthma cases,[22,416,417] ranging from 30% to 70%.[418,419] Studies using bronchial biopsy[224,420] or BAL[180] have also identified a proportion of asthma cases with little evidence of eosinophilic airway inflammation,[224,414,421]

even in severe asthmatics[315] or during exacerbation.[415] This is referred to as NEA and accounts for the remainder of all asthma cases.[13] Subsequently, a landmark study by Simpson *et al* suggested that asthma could be further categorised into four inflammatory subtypes on the basis of airway eosinophils and neutrophils, including the previously described EA phenotype divided into EA (≥1.01% sputum eosinophils and <61% neutrophils) and MGA (≥1.01% sputum eosinophils and ≥61% neutrophils) and the NEA phenotype divided into NA (≥61% sputum neutrophils and ≤1.01% sputum eosinophils) and PGA (≤1.01% sputum eosinophils and ≤61% neutrophils)[20] (Figure 2.3).

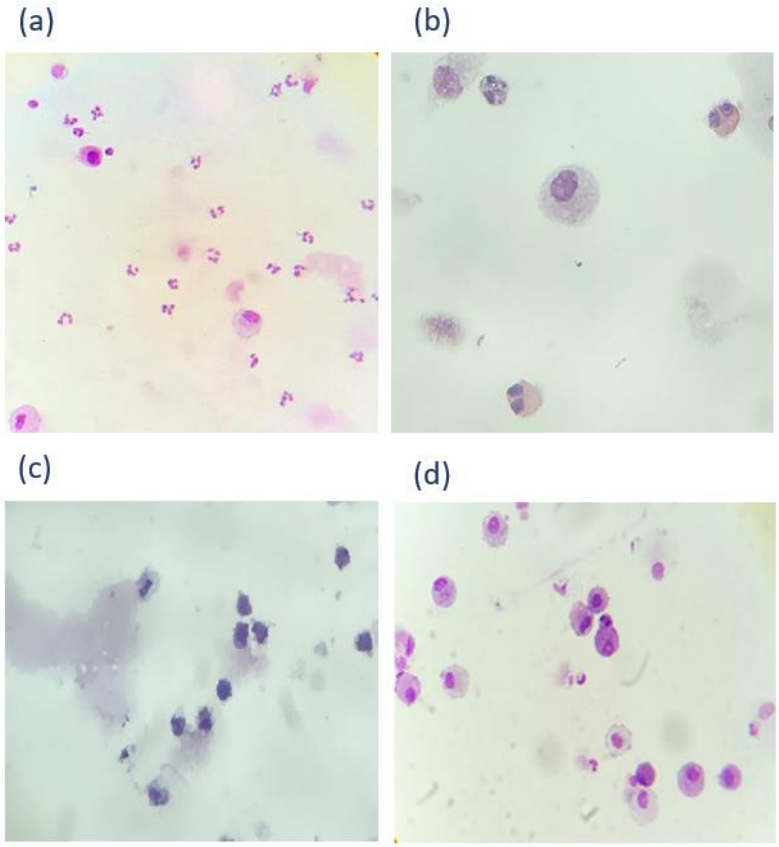

Figure 2.3. Sputum cytospins showing four inflammatory phenotypes of asthma: (a) neutrophilic asthma; (b) eosinophilic asthma; (c) mixed granulocytic asthma; (d) paucigranulocytic asthma.

Eosinophilic asthma

EA is the most well-characterised inflammatory phenotype.[422,423] Studies based upon DCC of slides produced using induced sputum generally classify EA using eosinophil cut-offs of greater than 1%[20,424]- 3%[425,426]. EA has been associated with increased asthma severity,[427] airway obstruction, AHR,[428] and exacerbation frequency.[429] EA also appears to be more responsive to ICS treatment when compared with NEA[430] in both adults[414,431] and children.[432,433]

EA is associated with increased expression of TH_2 cytokines IL-4, IL-5, and IL-13, in both sputum protein[434] and mRNA.[435] Other clinically useful biomarkers, such as increased FeNO[436] and serum and sputum periostin[437] are associated with EA and have been shown to correlate with sputum eosinophil counts.[438] Similarly, sputum levels of galectin 10,[439] ECP, and eosinophil-derived neurotoxin (EDN) are all increased in EA.[440]

Mixed granulocytic asthma

MGA makes up approximately 3%-8% of all asthma patients.[20,441-443] Although not well characterised, it is believed that MGA represents a combination of the pathology observed in both EA and NA i.e. involvement of both TH_2 and innate inflammation.[20,444] At least one study has shown that asthmatics with MGA have more severe airflow obstruction, more frequent exacerbations and daily symptoms, and increased health care utilisation compared to those with either EA or NA.[445] Unsurprisingly, MGA is associated with increased levels of airway biomarkers related to both eosinophils and neutrophils, including brain-derived neurotrophic factor (BDNF), IL-1β,[445] IL-8 and IFN-γ-inducible protein 10 kD,[446] and IL-6. The latter finding has led to suggestions that therapies targeting the proinflammatory IL-6 pathway may be beneficial in MGA.[447]

Non-eosinophilic asthma

Although NEA represents a substantial proportion of all asthmatics, less is understood about its clinical features and underlying mechanisms compared with EA.[448] NEA can be observed in all grades of severity, including mild-to-moderate,[417] severe refractory[443] and steroid resistant asthma.[414]

Whilst atopy may be present, NEA seems to be associated with a relatively TH_2-low inflammatory milieu.[17] In support of this, an *ex vivo* assessment of sputum cell cytokine production found that NEA was associated with less IL-4 and more TNF-α production than EA.[189] In at least a proportion, (see below) innate-mediated, neutrophil driven inflammation is important, with evidence of increased airway neutrophilia and neutrophil-associated cytokines[13] (discussed in greater detail in section 2.2.1.2). However, neutrophilic inflammation is not always present in NEA. In particular, there is no evidence of either neutrophilic or eosinophilic airway inflammation in over 30% of adults with asthma;[20] this is even more frequent amongst adolescents.[22] As described above, this has led to the stratification of NEA into NA and PGA.[20]

Neutrophilic asthma

NA is generally associated with airway neutrophilia and the absence of eosinophils and thought to involve the innate immune system[194] (described in section 2.2.1.2). It represents approximately 10-20% of adult asthma patients.[20,443] It is variously characterised by sputum neutrophil cutoffs ranging from 40% to 76%.[425] NA is more frequent in older patients[140] than children and adolescents (in some studies NA is undetectable in these populations),[22,182] obese women,[449] smokers,[450] in more severe disease[451,452] and corticosteroid-treated patients.[179] Regarding the latter, increased sputum neutrophil count may, at least in part, be driven by treatment, as ICS inhibit neutrophil apoptosis, and, in some settings, contribute to

neutrophil activation.[101] However, NA has been observed in steroid-naïve asthmatic individuals[417,422] as well as asthmatic individuals who have had steroids withdrawn.[101]

In NA, neutrophil percentage has been reported to inversely correlate with lung function,[453] air trapping,[454] airway wall thickness,[455] and increased levels of NE, MMPs and ROS,[456] but not with airway AHR, when compared with other asthma phenotypes.[428] In addition, in at least some cases, NA is associated with exposures such as bacterial endotoxins, particulate air pollution, and ozone,[13] as well as smoking and occupational exposures.[457] Recent studies assessing the airway microbiome have also reported reduced diversity and evenness of detectable airway microbiota in NA, with greater abundance of certain taxa such as *Haemophilus* and *Moraxella* in NA patients.[458,459]

Paucigranulocytic asthma

PGA is often characterised by less severe asthma, less atopy, and fewer exacerbations compared with EA and NA.[224] PGA is also associated with significantly better lung function than the other asthma phenotypes;[425] in a recent study of 240 asthmatics, PGA had mean (SD) FEV_1 of 81.9% (20.4) predicted compared with EA (FEV_1, 74.2% (19.8) predicted), MGA (FEV_1, 69.7% (18.2) predicted), or NA (FEV_1, 72.2% (20.2) predicted).[443] PGA represents between 31% and 47% of asthma cases[20,22,443,460] in both adults[431] and children.[224] As expected for a phenotype defined as having normal levels of eosinophils and neutrophils, PGA is characterised by low levels of eosinophil- and neutrophil-related biomarkers.[461]

The immunopathology of PGA remains poorly understood.[462] It has been proposed that the lack of granulocytic inflammation may be associated with airway remodelling after previous rather than current inflammation,[463] or may involve neural mechanisms; which may or may not be independent of airways inflammation or remodelling (discussed in section 2.2.3).

Stability of inflammatory phenotypes

Studies investigating the longitudinal stability of inflammatory phenotypes in both children[442,464] and adults[180,422] have yielded equivocal results. Some studies report stable phenotypes in mild to severe asthma followed-up over 6 months,[422] 12 months,[180] 2 years[465] and 5 years.[20,466] In contrast, other studies find considerable variation over time, with approximately half[467] to two thirds[468] of inflammatory phenotypes in severe asthmatics changing over a 12-month period. Some reports suggest that this is even higher; in 128 asthma patients assessed more than once, D'Silva *et al* found that only 23% maintained the same inflammatory phenotype.[469]

There are many potential influences on inflammatory phenotype stability, including medications, exacerbations, allergen exposure, air pollution, and viral infections.[442,470] Possibly the most studied influence in this context is that of medication (i.e. ICS use), with studies initiating ICS use leading to reduced sputum eosinophils over a 12-month period,[100,101,465] with the reverse observed with ICS withdrawal[471] or dose decrease.[472] As discussed previously, ICS use is also associated with increased neutrophil levels.[422] Asthma exacerbation can also be associated with temporal changes in airway eosinophilia (as occurs with environmental allergen exposure,[111] and respiratory viral infections be associated with temporarily increased sputum neutrophil levels).[473]

2.4.2 Other approaches to pathophysiological phenotyping

As noted above, pathophysiological phenotyping of asthma is increasingly common, and many of the approaches have included clinical and inflammatory characteristics. However, whilst the evidence summarised in sections 2.2.2 and 2.2.3 point towards the potential remodelling and neural pathways, to the author's knowledge, the assessment and identification of phenotypes on this basis has not yet been formally conducted. Despite this, the concept of phenotyping on the basis of remodelling and neural pathways has been acknowledged[40,474] with preliminary work identifying remodelling phenotypes using HRCT scans in adult asthmatics,[475] or assessing neural responses following allergen or irritant exposure using functional magnetic resonance imaging (fMRI)[476] or cough response to inhaled stimuli (discussed in section 2.3.3). However, these studies are still in the relatively early stages and to the author's knowledge there remains no formal classification system or phenotyping approach using these approaches.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Hajar Ali
Name/title of Primary Supervisor:	Prof. Jeroen Douwes
Name of Research Output and full reference:	
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and	
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Supervised and contributed to data collection, laboratory sample processing, all statistical analyses of the collected data and prepared the manuscript.	
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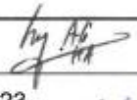
Candidate's Signature:	
Date:	31-07-23
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Date:	31/7/23

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Chapter 3 Enhanced airway sensory nerve reactivity in non-eosinophilic asthma

Hajar Ali, Collin Brooks, Julian Crane, Richard Beasley, Stephen Holgate, Peter Gibson, Philip Pattemore, Yu-Chieh Tzeng, Thorsten Stanley, Neil Pearce, Jeroen Douwes

Background: Neural mechanisms may play an important role in NEA. This study compared airway sensory nerve reactivity, using capsaicin challenge, in EA, NEA and non-asthmatics.

Methods: Thirty-eight asthmatics and nineteen non-asthmatics (aged 14-21 years) underwent combined hypertonic saline challenge/sputum induction, FeNO, atopy, and spirometry tests, followed by capsaicin challenge. EA and NEA were defined using a sputum eosinophil cut-point of 2.5%. AHR was defined as a ≥15% drop in FEV_1 during saline challenge. Sensory nerve reactivity was defined as the lowest capsaicin concentration that evoked 5 (C5) coughs.

Results: NEA (n=20) had heightened capsaicin sensitivity (lower C5) compared to non-asthmatics (n=19) (GM C5: 58.3μM, 95% confidence interval 24.1-141.5 vs 193.6μM, 82.2-456.0; $p<0.05$). NEA tended to also have greater capsaicin sensitivity than EA, with the difference in capsaicin sensitivity between NEA and EA being of similar magnitude (58.3μM, 24.1-141.5 vs 191.0μM, 70.9-514.0) to that observed between NEA and non-asthmatics; however, this did not reach statistical significance ($p=0.07$). FEV_1 was significantly reduced from baseline following capsaicin inhalation in both asthmatics and non-asthmatics but no differences were found between subgroups. No associations with capsaicin sensitivity and atopy, sputum eosinophils, blood eosinophils, asthma control, or treatment were observed.

Conclusion: NEA, but not EA, showed enhanced capsaicin sensitivity compared with non-asthmatics. Sensory nerve reactivity may therefore play an important role in the pathophysiology of NEA.

BMJ Open Respiratory Research 2021;8; with minor amendments

3.1 Introduction

Asthma is generally associated with TH_2-mediated, allergic airway inflammation.[12] However, some studies show that <50% of asthma cases are attributable to airway eosinophilia,[13] and that ~50% have no overt signs of either eosinophilic or neutrophilic inflammation.[22] In the absence of inflammation, the mechanisms underlying NEA remain unclear but it is plausible that neural pathways may be involved.[23] Whilst this notion is not new,[477] there is increasing contemporary literature supporting a role for neural involvement: some studies suggest that altered autonomic regulation, involving vagal tone and reduced sympathetic tone, may be important,[23] whilst others have suggested that sensory nerve activation may play a key role in asthma pathogenesis.[275,285]

To date, evidence of altered airway sensory nerve reactivity in asthma, often measured using capsaicin challenge to induce cough by specifically targeting the transient receptor potential $TRPV_1$ channel on sensory C-fibres, is equivocal.[275] One study observed an increase in capsaicin sensitivity amongst adult asthmatics,[388] whilst others found no difference between asthmatics and non-asthmatics.[395,478] Studies measuring associations between capsaicin response and inflammatory biomarkers including atopy,[279,479] soluble mediators,[393,480,481] fractional FeNO,[279,392] sputum[404,481,482] and blood[279,389] eosinophil percentages, have also shown mixed results. We hypothesise that these inconsistencies may be due to inflammatory asthma phenotypes expressing differential sensory nerve reactivity.

No studies have examined capsaicin responses across inflammatory asthma phenotypes assessed using induced sputum, which is considered representative of "actual" airway pathophysiology.[483] This study compared sensory nerve reactivity between young asthmatics and non-asthmatics and across different asthma inflammatory phenotypes, and examined

associations between sensory nerve reactivity and clinical, demographic, and inflammatory characteristics.

3.2 Methods

3.2.1 Study population

Participants (14-21 years), recruited from Wellington, New Zealand (either from a birth cohort study[484] or through separate community-based recruitment), completed a respiratory questionnaire based on the ISAAC Phase II survey.[485] The ISAAC study assessed the prevalence of respiratory symptoms in nearly 2 million children and adolescents in >100 countries; the survey is available at http://isaac.auckland.ac.nz/). Asthma was defined as wheezing/whistling in the chest and/or asthma medication use in the last 12 months. Non-asthmatics reported no asthma symptoms, no other respiratory conditions or asthma medication use. Informed consent was obtained from participants/parents, and the study was approved by the Northern B Health and Disability Ethics Committee (15/NTB/2).

3.2.2 Clinical assessments

Participants took part in a maximum of three assessments (the first involving all tests described below except capsaicin challenge) (Figure 3.1). To confirm inflammatory phenotype stability, asthmatics underwent another sputum induction 3-6 months later. Capsaicin challenge was conducted at a final assessment (2nd visit for non-asthmatics, 3rd for asthmatics) for a proportion of non-smoking participants identified as either EA, NEA, or non-asthmatics (recruitment was random within each subgroup). Capsaicin challenge was conducted 6-12 months after the final sputum induction. Asthma control was assessed using ACQ7.[91] Participants with respiratory infection within 1 month of assessment returned when symptom-free and those with FEV_1% predicted <75% were excluded. Prior to testing, asthma medication and antihistamines were withheld for ≥12 and ≥24 hours, respectively.

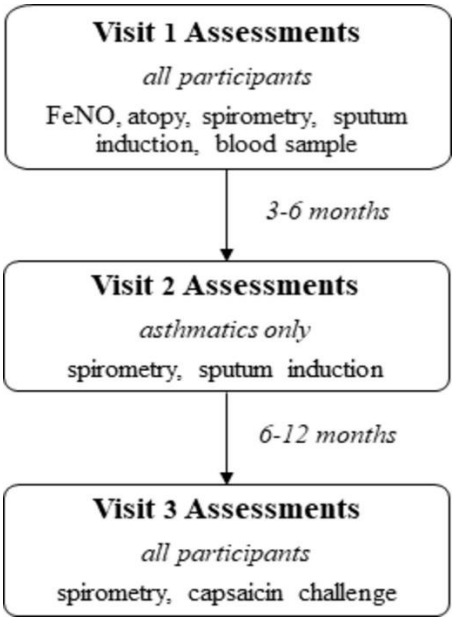

Figure 3.1. Timeline of clinical assessments.

3.2.2.1 Spirometry and FeNO

Spirometry and FeNO was measured using an Easyone spirometer (NDD Medizintechnik AG, Zurich, Switzerland) or Hypair FeNO analyser (Medisoft, Sorinnes, Belgium) as described previously.[22,486]

3.2.2.2 Atopy

Skin prick tests were conducted using a panel of aeroallergens as described previously: HDM, tree mix, grass mix, cat and dog dander, *Alternaria tenuis* and *Penicillium* mix (Stallergenes Greer, Sydney, Australia). Atopy was determined by presence of at least one weal ≥ 3mm.[22]

3.2.2.3 Blood eosinophils

Blood was collected using BD-vacutainers (BD, Auckland, New Zealand) for a complete blood count. A high blood eosinophil count (blood EOS-high) was defined as ≥250 eosinophils/mm^3.[487]

3.2.2.4 Combined hypertonic saline challenge and sputum induction

Hypertonic saline challenge/sputum induction was conducted as described previously.[488] Briefly, aerosolised hypertonic saline (4.5% w/v) was produced using an ultrasonic nebuliser (DeVilbiss Ultraneb 2000, Langen, Germany) and administered orally through a mouthpiece (Hans-Rudolph Inc, Kansas City, USA) for increasing intervals from 0.5-4 minutes, to a total of 16 minutes. Spirometry was conducted between intervals, and salbutamol was administered if FEV_1 dropped to ≤75%-predicted. During the procedure, the number of coughs was not counted. Participants were subsequently encouraged to produce sputum in a sterile plastic container. Sputum plugs were dispersed using DTT (Sputasol, Oxoid Ltd, Basingstoke, Hampshire, England). The suspension was filtered through a 60µm filter (Millipore, County Cork, Ireland) and TCC and viability performed. Following centrifugation, supernatant was aspirated and stored at -80ºC and the resulting cell suspension was used to prepare cytospin slides stained using a Diff-Quik® fixative/stain set (Dade Behring, Deerfield, IL). A DCC of 400 non-squamous cells was made using light microscopy. Samples were considered to be adequate for analysis if they had a squamous cell contamination <30% and >400 total non-squamous cells on one slide. EA was identified as ≥2.5% eosinophils at any visit and NEA as <2.5% eosinophils at both visits. AHR was defined as a ≥15% drop in FEV_1 from baseline.[488]

3.2.2.5 Capsaicin challenge

Capsaicin challenge was conducted as described previously[479] with minor modifications. Capsaicin (Sigma-Aldrich, Castle Hill, Australia) was solubilised in ethanol/Tween 80. Participants inhaled single breaths of aerosolised capsaicin solution in doubling concentrations (0.98 to 500μM) from a jet fnebuliser (model 646, DeVilbiss, Langen, Germany) controlled by a KoKo dosimeter (nSpire Health Inc, Louisville, CO, USA). One-minute intervals were maintained between different concentrations. The lowest concentration eliciting 2 (C2) and 5 (C5) coughs during a 30-second interval between each concentration was manually recorded by a nurse. The procedure was terminated if/when the C5 threshold was reached. If C2 or C5 was not reached, a value of 1000μM was assigned for analysis. Lung function was measured before and after capsaicin challenge.

3.2.3 Power and statistical analysis

The primary aim of this study was to compare capsaicin response in asthmatics and non-asthmatics, and EA and NEA. Based on power calculations conducted prior to commencing the study, which assumed a differences in concentration of capsaicin to elicit 2 coughs of 53.6 (19.0) μmol/l in asthmatics and 116.0 μmol/l (SD 58.1) in non-asthmatics,[489] we determined that 20 participants in each subgroup (non-asthmatics, EA and NEA) would be sufficient (>99% power) to detect statistically significant differences between asthma phenotypes, or between either asthma phenotype and non-asthmatics.

Analyses were performed using STATA version 11.0 (STATA Corp, College Station, TX, USA) and GraphPad Prism 7.0 (Graphpad Software Inc, La Jolla, CA, USA). C2 and C5 values were expressed as geometric means (GM) with 95% confidence interval (CI), and C5 used as the primary outcome.[479] Mann-Whitney *U* tests, unpaired t-tests, or Chi-square tests

were used as appropriate. Linear regression was conducted using log-transformed C5. Regression coefficients were exponentiated and presented as relative differences i.e. ratios (per unit increase for continuous variables and compared to the reference category for categorical variables). Ratios of >1 represent reduced capsaicin sensitivity whereas ratios of <1 represent heightened sensitivity. If significant associations were found, sensitivity analyses (excluding subgroups with or without specific characteristics) were conducted to assess robustness of findings.

3.3 Results

3.3.1 Population characteristics

Thirty-nine asthmatics and 21 non-asthmatics were recruited (12 asthmatics and 20 non-asthmatics from the previous birth cohort study[484] and 27 asthmatics and 1 non-asthmatic through community-based recruitment). One asthmatic and two non-asthmatics were excluded due their FEV_1 being ≤75% predicted, leaving 38 asthmatics and 19 non-asthmatics. Asthmatics were slightly younger but no differences in sex, ethnicity, or FeNO were observed (Table 3.1). Prevalence of atopy, AHR, and sputum eosinophil percentages were higher in asthmatics. Of the asthmatics, 18% were classified as uncontrolled, 26.3% as partly controlled and 55.4% as well-controlled. Participants recruited from the community were slightly younger than participants from the birth cohort (mean age: 18 vs 21 years), but all other baseline characteristics were comparable (data not shown).

Table 3.1. Population characteristics

	Non-asthma (N=19)	Asthma (N=38)	Eosinophilic asthma (N=18)	Non-eosinophilic asthma (N=20)
Age	21.0 (2.0)	19.0 (2.0) **	18.3 (2.0)	19.3 (2.0)
Males- n (%)	6 (32.0 %)	14 (37.0 %)	6 (33.0 %)	8 (40.0 %)
Height (cm)	170.0 (8.3)	167.4 (9.0)	165.0 (8.1)	168.7 (9.5)
Weight (Kg)	67.0 (12.6)	67.4 (15.4)	62.2 (12.1)	72.2 (17.0)
Ethnicity				
European-NZ (%)	18 (94.7 %)	30 (78.9 %)	14 (77.8 %)	16 (80.0 %)
Non-European-NZ (%)	1 (5.3 %)	8 (21.1 %)	4 (22.2 %)	4 (20 %)
Passive smoking[a]	2 (10.5 %)	3 (8.0 %)	1 (6.0%)	2 (10%)
Asthma medication[a]				
No asthma medication- n (%)		8 (21.1%)	3 (17.0 %)	5 (25.0 %)
ICS alone- n (%)		6 (15.7%)	4 (22.2 %)	2 (10.0 %)
β-agonist alone- n (%)		7 (18.4%)	2 (11.1 %)	5 (25.0 %)
ICS & β-agonist - n (%)		17 (44.8%)	9 (50.0 %)	8 (40.0 %)
Sleep disturbance due to cough[a]	0 (0.0 %)	14 (36.8 %) **	7 (39.0 %)	7 (35 %)
Dry cough at night[b]	0 (0.0 %)	13 (34.0 %) **	7 (39.0 %)	6 (30.0 %)
ACQ7 score		0.8 (0.3-1.3)	1.4 (0.7-1.7) ††	0.6 (0.2-0.9)
FeNO (ppb)	41.5 (38.1)	66.6 (76.1)	82.3 (75.2) †	53.0 (76.0)
Atopy[c]- n (%)	10 (53 %)	32 (84.2 %) *	17 (94.4 %)	15 (75.0 %)
Airway hyperreactivity[d]- n (%)	0 (0.0 %)	15 (39.5 %) **	11 (61.1 %) ††	4 (20.0 %)
Sputum eosinophils %	0.0 (0.0-0.3)	2.2 (0.0-10.7) **	12.0 (9.0-40) ††	0.0 (0.0-0.8)
Sputum neutrophils %	13.0 (7.0-33.0)	8.3 (4.3-24.0)	7.8 (5.0-24.0)	8.5 (4.1-24.4)
Blood eosinophils (mm^3)	200 (100-300)	500 (200-800) **	600 (500-900) ††	200 (100-400)

Means (standard deviation), median (IQR) or frequency (%), Mann-Whitney test and Chi-square tests were used as appropriate. * $p<0.05$; ** $p<0.01$ asthmatics versus the reference population, † $p<0.05$; †† $p<0.01$ non-eosinophilic versus eosinophilic asthmatics.

[a] In the past 12 months

[b] In the past 12 months without cold or respiratory infection

[c] Positive SPT against one or more common allergens

[d] ≥15% drop in FEV_1 from baseline following hypertonic saline challenge

SPT, skin prick test; FeNO, fractional exhaled nitric oxide

Eosinophilic asthma defined as ≥2.5% sputum eosinophils

3.3.2 Inflammatory phenotypes

Fifty-three percent (n=20) of asthmatics were NEA at both visit 1 and 2, with the remaining 47% (n=18) EA. EA were more likely to be atopic, and have AHR, higher FeNO, and more poorly controlled asthma than NEA (Table 3.1). There were no differences in nocturnal cough symptoms (Table 3.1). Neutrophilic asthma or mixed granulocytic asthma[20] were not detected, and sputum neutrophil levels were not significantly different between groups (Table 3.1).

3.3.3 Capsaicin response and inflammatory phenotypes

Capsaicin response did not differ between asthmatics and non-asthmatics (Figure 3.2A and 3.2B) and was not associated with recruitment source (data not shown). However, NEA had significantly greater capsaicin sensitivity than non-asthmatics (GM 58.3μM, 95% CI 24.1-141.5 vs 193.6μM, 82.2-456.0; Figure 3.2B). NEA tended to also have greater capsaicin sensitivity than EA, with the difference in capsaicin sensitivity between NEA and EA being of similar magnitude (58.3μM, 24.1-141.5 vs 191.0μM, 70.9-514.0) to that observed between NEA and non-asthmatics; however, this did not reach statistical significance (p=0.07). Using sputum eosinophil cut-offs of either 1%[20] or 3%[414] to define EA and NEA did not affect these findings (data not shown). Results for C2 showed no differences between groups (Figure 3.2A). When excluding participants with elevated blood eosinophil levels (to avoid potential NEA phenotype misclassification), capsaicin sensitivity remained higher in NEA (C5 72.9μM, 14.2-374.9) than non-asthmatics (170.0μM, 71.7-403.0), but findings were no longer statistically significant. When subjects were stratified by atopy (Supplementary Figure S3.1) or blood eosinophils (Supplementary Figure S3.2) rather than EA/NEA, we found no significant differences in C2 or C5 between groups.

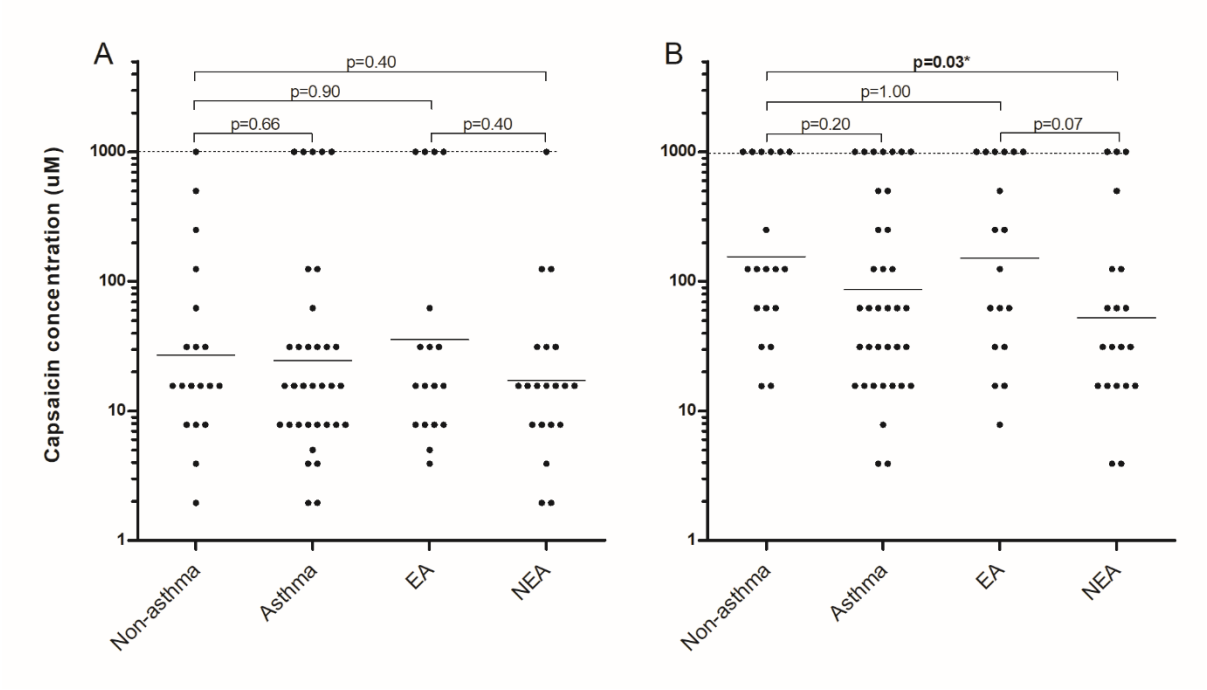

Figure 3.2. Concentrations (μM) of capsaicin eliciting (A) 2 coughs (C2) or (B) 5 coughs (C5) in participants with and without asthma, and eosinophilic asthma (EA) and non-eosinophilic asthma (NEA). Dashed lines at 1000 μM represent values assigned to those participants who did not achieve C2 or C5 during testing. Solid line represents geometric mean. Mann-Whitney test was used. * $p<0.05$

3.3.4 Capsaicin response and demographic/clinical characteristics

In asthmatics and non-asthmatics, no associations were found between capsaicin sensitivity and demographic parameters, asthma control, lung function, inflammatory markers, treatment or AHR (Table 3.2). In EA, capsaicin sensitivity was significantly lower for Europeans (n=14) compared to non-Europeans (n=4; Table 3.2). Capsaicin sensitivity was also significantly lower for those with (n=11) compared to without AHR (n=7; ratio=7.94, $p<0.05$). In NEA, C5 was inversely associated with FeNO (ratio=0.99 per unit increase, $p<0.05$) and positively associated with FEV_1/FVC% predicted (ratio=1.12 per unit increase, $p<0.05$; Table 3.2).

Table 3.2. Associations between demographic and clinical characteristics and capsaicin response (C5)

	Relative difference (ratio) in capsaicin concentration to elicit 5 coughs[#]			
	Non-asthma (N=19)	**Asthma (N=38)**	**Eosinophilic asthma (N=18)**	**Non-eosinophilic asthma (N=20)**
	Ratio (95%CI)	Ratio (95%CI)	Ratio (95%CI)	Ratio (95%CI)
Continuous variables				
Age (years)	1.10 [0.70,1.72]	0.89 [0.70,1.09]	0.79 [0.51,1.25]	1.26 [0.80,1.98]
FEV_1% predicted	0.99 [0.91,1.09]	1.02 [0.98,1.07]	1.07 [0.98,1.17]	1.02 [0.94,1.12]
FVC% predicted	0.96 [0.83,1.09]	1.00 [0.91,1.09]	1.05 [0.96,1.15]	0.96 [0.87,1.05]
FEV_1/FVC% predicted	1.10 [0.92,1.31]	1.07 [0.98,1.17]	1.07 [0.94,1.23]	1.12* [1.03,1.23]
FeNO (ppb)	0.99 [0.97,1.01]	1.00 [0.99,1.01]	1.01 [1.00,1.02]	0.99* [0.98,1.00]
Sputum eosinophil %	0.91 [0.76,1.09]	1.02 [0.98,1.07]	0.99 [0.95,1.04]	0.63 [0.26,1.56]
Sputum neutrophil %	1.00 [0.94-1.10]	0.96 [0.93,1.00]	0.96 [0.91,1.01]	0.98 [0.93,1.02]
Blood eosinophil/mm^3	0.99 [0.99,1.00]	1.01 [0.99,1.00]	1.00 [0.99,1.01]	0.99 [0.99,1.00]
ACQ7 score	-	1.74 [0.71,2.13]	1.59 [0.41,6.14]	0.40 [0.04,3.8]
Dichotomous variables				
Female (vs male)	0.81 [0.13,4.94]	1.59 [0.41,6.14]	3.98 [0.66,24.21]	0.63 [0.10, 3.84]
Ethnicity (Eur vs non-Eur)	3.23 [0.53,19.68]	2.04 [0.53,7.91]	10.23* [1.68,62.23]	0.48 [0.12,1.85]
Dry cough at night (yes vs no)	-	0.32 [0.08,1.22]	0.14 [0.02,0.91]	0.9 [0.14,5.81]
Sleep disturbance due to cough (yes vs no)	-	1.27 [0.32,4.98]	1.70 [0.19,14.53]	0.80 [0.15,4.26]
AHR (yes vs no)	-	2.51 [0.65,9.73]	7.94* [1.31,48.31]	0.20 [0.03,1.21]
Atopy (yes vs no)	1.26 [0.21,7.66]	0.63 [0.10,3.84]	0.40 [0.01,23.12]	0.40 [0.07,2.42]
Treated (yes vs no)	-	0.33 [0.07, 1.72]	0.23 [0.01, 4.21]	0.25 [0.04, 1.60]
ICS use (yes vs no)	-	0.81 [0.23, 2.88]	1.14 [0.17, 7.64]	0.41 [0.08, 2.21]
β-agonist use (yes vs no)	-	1.02 [0.28, 3.80]	1.14 [0.17, 7.64]	1.02 [0.20,5.70]

[#] As analyses were conducted on-log transformed C5 values, regression coefficients are shown as relative (ratios) rather than absolute differences (per unit increase in case of continuous variables and compared to the reference category in case of categorical variables); Ratios of >1 represent reduced capsaicin sensitivity whereas ratios of <1 represent heightened sensitivity. * $p<0.05$

3.3.5 Sensitivity analyses

Post-hoc sensitivity analyses were conducted for characteristics independently associated with capsaicin response. Limiting analysis to asthmatics with AHR, we found that capsaicin sensitivity was significantly greater in NEA (15.6µM, 2.6-95.0) than non-asthmatics (193.6µM, 82.2-456.2) and EA (441.0µM, 127.0-1533.0; Figure 3.3A). Excluding non-Europeans (n=9) showed significantly increased capsaicin sensitivity in NEA (50.3µM, 18.0-139.0) compared with non-asthmatics (206.0µM, 84-507), and EA (320µM, 104-989; Figure 3.3B). To clarify the potential role of treatment status, we conducted further sensitivity analysis including only asthmatics who used either ICS or β-agonists (excluding n=5 NEA and n=3 EA). This also showed statistically significant ($p<0.05$) enhanced capsaicin sensitivity in NEA (39.4µM, 16.9-91.4) compared with non-asthmatics (155.5µM, 76.2-317.6) and EA (150.4µM, 58.4-387.1; Figure 3.3C). We also conducted sensitivity analysis based on lung function, FeNO (excluding NEA with elevated FeNO levels to address the issue of potential phenotype misclassification), ICS-use alone, and gender (Supplementary results, supplementary Figure S3.3 & S3.4). These did not have an appreciable effect on the main findings (although in some cases results were no longer statistically significant).

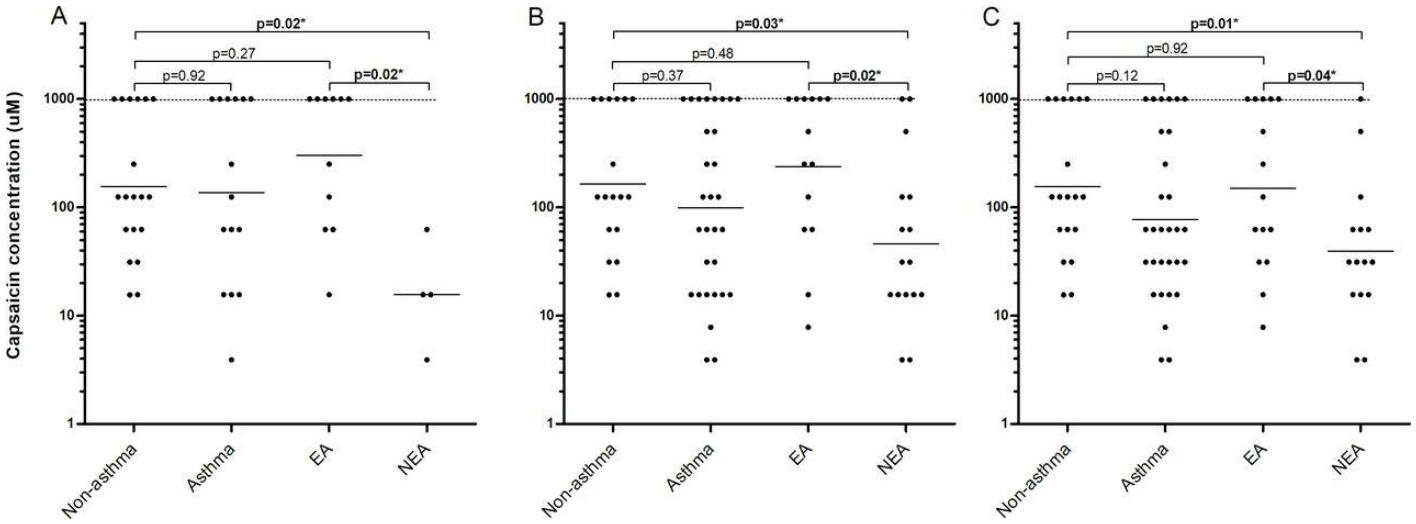

Figure 3.3. Concentration (μM) of capsaicin eliciting 5 (C5) coughs in participants with AHR (A), Europeans only (B), and in participants using ICS or β-agonist medication (C). Dashed lines at 1000 μM represent values assigned to those participants who did not achieve C2 or C5 during testing. Solid line represents geometric mean. Mann-Whitney test was used. * $p<0.05$

3.3.6 Capsaicin challenge and spirometry

FEV_1%-predicted and FVC%-predicted were significantly reduced following capsaicin challenge in asthmatics and non-asthmatics. However, this was not different between subgroups, including EA and NEA (Table 3.3).

Table 3.3. Changes in lung function following capsaicin challenge

	Non-asthma (N=19)	Asthma (N=38)	Eosinophilic asthma (N=18)	Non-eosinophilic asthma (N=20)
FEV_1 % predicted	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
Baseline	97.6 (7.9)	96.0(10.7)	91.5 (11.1) †	99.6 (9.0)
Post	95.0 (8.0)	92.2 (11.2)	88.4 (11.1) †	96.0 (10.3)
Δ	-2.7 (3.6) #	-3.7 (-2.7) #	-3.1 (2.7) #	-4.3 (2.7) #
FVC % predicted				
Baseline	97.7 (7.0)	100.0 (11.0)	98.0 (11.4)	102.0 (9.6)
Post	95.3 (7.5)	95.5 (10.9)	94.0 (11.2)	97.0 (11.0)
Δ	-2.4 (2.5) #	-3.3 (5.1) #	-4.0 (4.2) #	-4.6 (6.0) #
FEV_1/FVC % predicted				
Baseline	101.2 (4.4) *	97.3 (7.7)	94.9 (7.4) †	99.5 (7.4)
Post	100.0 (5.0)	97.0 (7.4)	94.5 (7.3) †	99.3 (7.0)
Δ	-1.4 (3.0)	-0.3 (4.4)	-0.4 (3.1)	-0.2 (5.3)

Data presented as mean (SD). t-test: * $p<0.05$; ** $p<0.01$ asthmatics versus the reference population; † $p<0.05$, †† $p<0.01$ non-eosinophilic versus eosinophilic asthmatics; # $p<0.01$ baseline versus post capsaicin challenge.

3.4 Discussion

This study found enhanced airway sensory nerve reactivity in NEA compared with non-asthmatics, whilst no difference between EA and non-asthmatics was found, suggesting that sensory nerve reactivity may play a role in the pathophysiology of NEA but not EA. No associations between capsaicin sensitivity and atopy, sputum eosinophils, blood eosinophils, asthma control, or treatment were observed. However, AHR was associated with reduced capsaicin sensitivity in EA, whilst FENO was associated with increased capsaicin sensitivity in NEA.

Although our findings are consistent with some previous reports showing no difference in capsaicin response between asthmatics and non-asthmatics,[395,478] other studies found a heightened capsaicin response in asthma.[279,388] These inconsistencies may be due to demographic and methodical differences, or alternatively, as suggested here, airway sensory nerve reactivity may be specific to inflammatory phenotypes, with differences masked for comparisons with general asthma.

To our knowledge, a direct relationship between sensory nerve reactivity and NEA has not previously been shown. However, recent studies have suggested an association with non-atopic asthma,[275] which, like NEA, may be driven by non-TH_2 mechanisms.[13] For example, one study reported that capsaicin-induced cough was more pronounced in non-atopic asthmatics compared to atopic asthmatics or non-asthmatics.[279] Another study suggested that heightened capsaicin sensitivity is associated with poor asthma control/severity in non-atopic asthmatics.[389] However, data are equivocal and a study in non-asthmatics found no association with atopy,[479] suggesting that atopy does not reliably predict capsaicin response. In agreement, we observed no differences between non-atopic and atopic asthmatics, or between atopic or non-atopic individuals in general. However, our study was not powered to

examine capsaicin response in non-atopics, who made up a small proportion (16%) of asthmatics, as is typical in New Zealand.[22]

Few studies have assessed associations between airway inflammation and sensory nerve reactivity; these yielded inconsistent results, possibly due to asthmatic airway inflammation heterogeneity. Three studies showed no association between capsaicin response and sputum eosinophilia;[404,481,482] however, in these studies capsaicin response was assessed in allergic asthmatics or following allergen challenge, which likely excluded individuals with TH_2-low inflammation and/or NEA. Other studies used FeNO[279,392] or blood eosinophils[279,389]as indicators of TH_2-mediated airway inflammation, and again, results varied.[392,474] In the present study, we used multiple TH_2-indicators; both systemic (atopy, blood eosinophils) and airway-specific (FeNO, sputum eosinophils), but an increased capsaicin response was observed only in NEA. Capsaicin sensitivity was also associated with FeNO in NEA, but this association (a 1ppb FeNO increase was associated with 1% greater capsaicin sensitivity) was small, and unlikely to be of clinical significance. The reasons for the mixed findings between studies are unclear, but it is possible that, whilst elevated FeNO and blood eosinophils are markers of TH_2 inflammation, they may not be specific enough to accurately identify airway inflammatory patterns, and in particular, NEA (in our study 75% of NEA were atopic). This is supported by previous data showing that blood eosinophils and FeNO levels do not accurately predict sputum eosinophil percentages.[483]

The causes of enhanced sensory nerve reactivity in NEA are unknown. However, viruses and irritants, identified as potential triggers of asthma,[275] and NEA in particular,[13] may play a role. These may result in sensory nerve $TRPV_1$ channel activation or increased expression, leading to increased cough response, even in the absence of other pathophysiology, such AHR (as observed in the EA group in this study), or inflammation.[275] Similar

hyperresponsive capsaicin-sensitive phenotypic changes have been reported in vasomotor rhinitis, despite no evidence of nasal mucosal inflammation.[490] Alternatively, increased capsaicin response may be due to alterations in the afferent pathways or neuronal networks upstream of initial $TRPV_1$ activation.[275]

Although we found no statistically significant associations with characteristics previously associated with capsaicin sensitivity such as age,[481] gender,[279] asthma control,[389]or treatment,[388] we observed an association with ethnicity in EA. There are few studies examining associations between either sensory nerve reactivity or inflammatory phenotypes and ethnicity, and of the former, no association has been found.[491] As our finding was based on very small numbers, it may be due to chance.

Consistent with other studies,[388,389] baseline lung function was not associated with capsaicin response. However, following capsaicin challenge, FEV_1%-predicted and FVC%-predicted were slightly decreased across all groups with no differences between subgroups. This is in agreement with previous studies showing that capsaicin does not cause clinically significant bronchoconstriction in asthmatics.[279] Our results suggest that whilst capsaicin produces an increased tussive response in NEA, it is not associated with clinically significant AHR in this (or any other) group.

The observation that increased sensory nerve reactivity is associated with NEA may have significant implications. As reported previously, NEA makes up >50% of asthma[22] and is less responsive to ICS,[422] the mainstay drug in asthma management. There is therefore a substantial and unmet need in the therapeutic management of this group. If sensory nerve reactivity plays a role in the pathology underlying NEA and is therefore a potential treatable trait,[19] then accurately identifying individuals with increased airway sensory reactivity, and developing specific therapeutic approaches targeting this, will be important. Of particular

interest, recent reports suggest that anticholinergics (which are effective in some but not all asthma)[492] may markedly reduce airway reactivity to a variety of stimuli including capsaicin.[23] Tiotropium bromide reduces both cough and cough-reflex sensitivity in asthma refractory to ICS/LABA.[402] Alternatively, P2X3 antagonists (which have shown promise in the treatment of refractory chronic cough) may be of benefit.[493] However, it is currently unclear whether these will be effective in NEA, which was not associated with nocturnal cough symptoms in this study. It is also possible that capsaicin treatment itself may be beneficial in sensory nerve hyperreactivity in NEA, as has been shown in vasomotor rhinitis.[494] Finally, in addition to results being relevant to treatment, our findings suggest that capsaicin challenge, in conjunction with other methods such as sputum induction, AHR, FeNO, and atopy testing, may be a useful tool to differentiate between asthma phenotypes, and provide important clues regarding causal (non-allergenic) exposures.

This study has limitations. Firstly, the number of participants, particularly when stratified by phenotype, were relatively small. Although power calculations, based on limited observations from other studies, suggested sufficient power (see methods), differences observed in our study were somewhat smaller than we had assumed and power to detect differences between groups was therefore reduced. This may explain why there was a significant difference between NEA and non-asthmatics, and a similar difference between NEA and EA that did not reach statistical significance, involving slightly smaller numbers. In addition to reduced power, this study involved multiple comparisons, which may have contributed to some chance findings. However, for our main aim (to assess whether capsaicin responses are different across asthma inflammatory phenotypes, and non-asthmatics) and focusing on the primary outcome (C5), we found 7 (43.8%) statistically significant ($p<0.05$) findings across 16 comparisons (Figures 3.2B/3.3A-C), which is considerably more than expected based on chance alone (0.8; 5%). Therefore, based on the fact that results were highly consistent across

multiple sensitivity analyses, we believe that these results are unlikely a chance finding. For Table 3.2, which summarises the results of our secondary aim (to examine associations between sensory nerve activity and clinical, demographic, and inflammatory characteristics in asthmatics) we had fewer statistically significant findings (4 out of 54 (7.4%) compared to 2.7 (5%) expected for comparisons in asthmatics). Therefore, those associations are more likely explained by chance and should therefore be interpreted with a degree of caution.

Secondly, asthmatics were generally well-controlled and identified using an epidemiological definition and not on the basis of objective tests (such as BDR and/or AHR). Therefore, some misclassification may have occurred, particularly for NEA, in which asthma symptoms are often present in the absence of objective measures (such as AHR).[495] However, we consider that any bias introduced as result will be minimal as this approach, used in previous studies,[57,304,496] generally compares well with clinical diagnoses,[57]and has been shown to be better than some objective measures.[496] Indeed, there are several issues with objective testing for confirmation of asthma diagnosis in a community based setting, particularly given the inherently variable nature of asthma, and that most asthmatics are not treatment naïve (>60% in the current study were using ICS at the time of assessment). This (amongst other reasons) has led to recommendations that asthma be considered on the basis of symptoms rather than pathophysiology.[9] In this study, of the 38 participants who we defined as asthmatics, 34 had their asthma diagnosed by a doctor (as indicated from the questionnaire); of the four subjects that were identified as asthmatic with no doctor diagnosis of asthma, three had used ICS in the past 14 days. Therefore, only one subject was defined based on respiratory symptoms alone. Excluding this person from the analyses did not materially change the results, although p-values increased marginally (data not shown). Also, the main study findings were similar when applying a more stringent definition of asthma, i.e. restricting analysis to only

asthmatics who used ICS or β-agonists, or with AHR; suggesting that associations observed are robust and unlikely to be due to asthma misclassification.

Thirdly, due to the cross-sectional nature of the study, capsaicin challenge was not repeated and reproducibility of capsaicin response in inflammatory phenotypes remains unstudied. However, a high degree of reproducibility of capsaicin response has been documented previously.[383] Fourthly, there is a possibility that at least some of the NEA cases may be EA in which ICS suppressed airway eosinophilia.[422] However, *post hoc* analysis, excluding the 4 NEA participants who used ICS in the last 14 days, did not have an appreciable effect (although results were no longer statistically significant). Fifthly, information regarding cough symptoms and medication use was collected on the basis of participant self-report using the ISAAC questionnaire. As such, data regarding ICS dose or asthma treatment step were unavailable, and although we have data regarding nocturnal cough symptoms, no information on daytime cough frequency was collected. We were therefore unable to determine if capsaicin response was associated with daytime or overall cough frequency. Finally, it has been suggested that that a non-linear fix-modelling procedure may be more appropriate than fixed C2/C5 endpoints.[279] However, in this study, capsaicin challenge was terminated upon reaching C5 (to avoid further participant discomfort). Hence, non-linear fix-modelling was not feasible.

In conclusion, our study shows that sensory nerve reactivity may play an important role in the pathophysiology of mild-to-moderate NEA in young adults. Although it is not yet clear if this is relevant in older groups or more severe asthma, we suggest that sensory nerve reactivity may represent a novel therapeutic target in NEA, a group in which current asthma medications have previously been shown to be less effective.[422]

3.5 Supplementary material

After conducting sensitivity analyses examining the association with atopy (Supplementary Figure S3.1) and blood eosinophils (Supplementary Figure S3.2), further sensitivity analyses were conducted examining capsaicin response in only participants with FEV_1% predicted <95% or excluding participants with high FeNO in NEA. Similar results were found between NEA and non-asthma (Supplementary Figure S3.3A and S3.3B). To exclude the possibility that NEA was in fact EA with ICS-suppressed eosinophilia, we conducted an analysis excluding all NEA who received ICS in the last 14 days (n=4). This did not have an appreciable effect on the main findings (although results were no longer statistically significant; Supplementary Figure S3.3C). Finally, as females have previously been shown to have enhanced capsaicin sensitivity, we also conducted a sensitivity analysis excluding males (n=20). This analysis showed significantly increased sensitivity in NEA (41.7 µM, 13.2-131.7) compared with EA (222.7µM, 81.3-610.5); a borderline statistically significant difference was also found comparing NEA with non-asthmatics (146.6µM, 57.3-375.2; Supplementary Figure S3.4A and S3.4B).

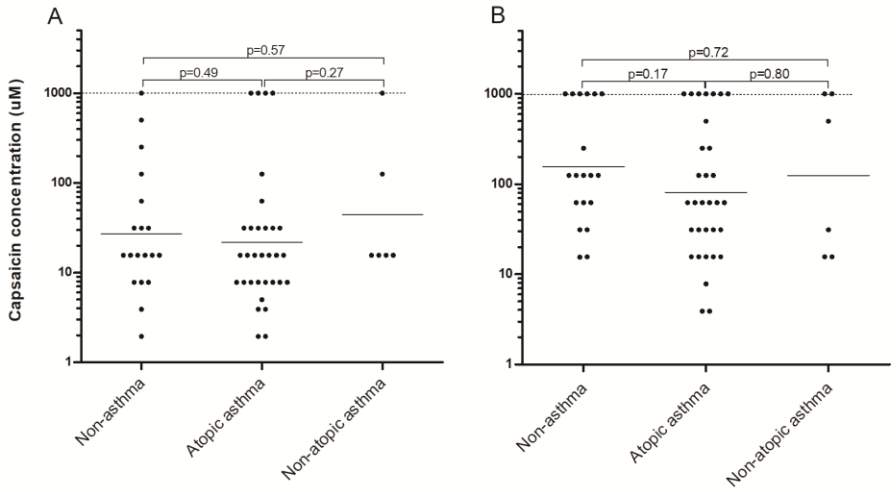

Figure S3.1. Concentrations (μM) of capsaicin eliciting (A) 2 coughs (C2) or (B) 5 coughs (C5) in non-asthmatics and asthmatics with and without atopy. Dashed lines at 1000 μM represent values assigned to those participants who did not achieve C2 or C5 during testing. Solid line represents geometric mean. Mann-Whitney test was used.

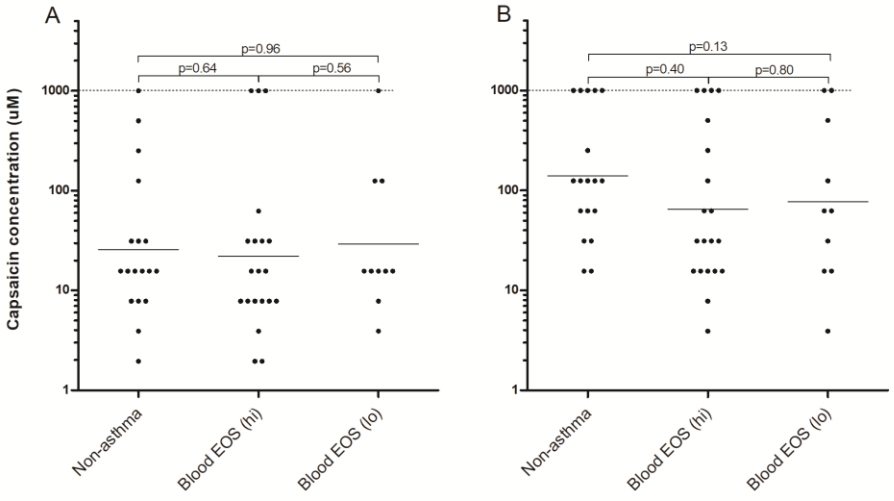

Figure S3.2. Concentrations (μM) of capsaicin eliciting (A) 2 coughs (C2) or (B) 5 coughs (C5) in participants with and without asthma, and asthmatics identified as blood EOS-high and blood EOS-low. Dashed lines at 1000 μM represent values assigned to those participants who did not achieve C2 or C5 during testing. Solid line represents geometric mean. Mann-Whitney test was used.

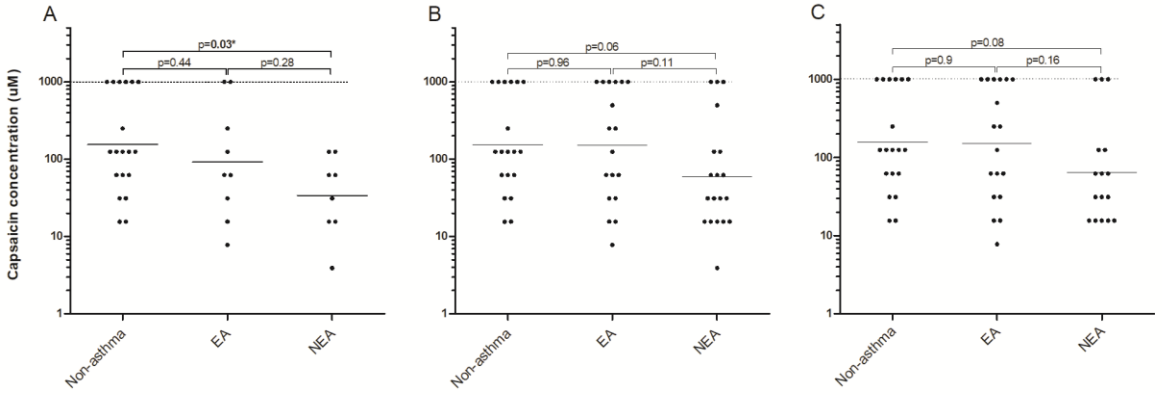

Figure S3.3. Concentrations (μM) of capsaicin eliciting 5 coughs (C5) in participants with airflow limitation (FEV_1% predicted <95%) (A), excluding participants with high FeNO (based on the 90th percentile of FeNO levels in non-asthmatics) of in NEA (B) and in NEA participants who did not use ICS in the last 14 days (C). Dashed lines at 1000 μM represent values assigned to those participants who did not achieve C2 or C5 during testing. Solid line represents geometric mean. Mann-Whitney test was used. * p<0.05

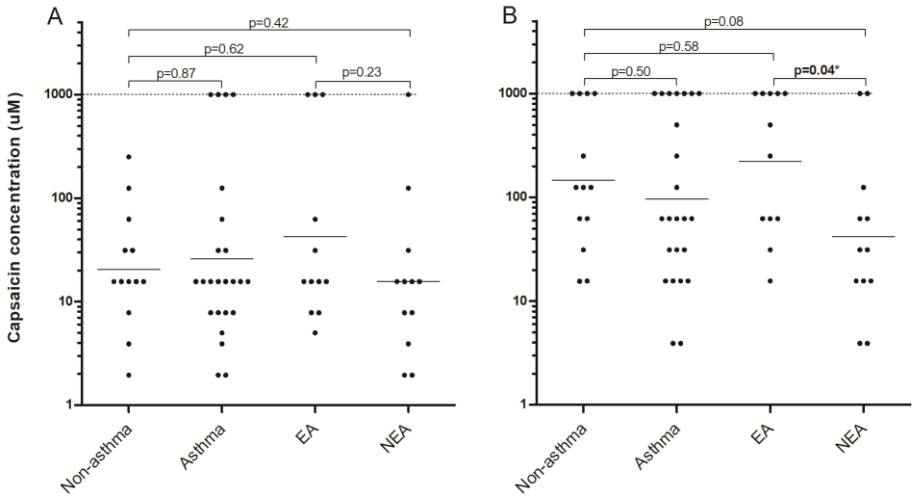

Figure S3.4. Concentrations (μM) of capsaicin eliciting (A) 2 coughs (C2) or (B) 5 coughs (C5) in female participants with and without asthma, and asthma stratified into eosinophilic asthma (EA) and non-eosinophilic asthma (NEA). Dashed lines at 1000 μM represent values assigned to those participants who did not achieve C2 or C5 during testing. Solid line represents geometric mean. Mann-Whitney test was used. * p<0.05

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Hajar Ali
Name/title of Primary Supervisor:	Prof. Jeroen Douwes
Name of Research Output and full reference:	
Heart rate variability as a marker of autonomic nervous system activity in young people with eosinophilic and non-eosinophilic asthma.	
In which Chapter is the Manuscript /Published work:	4
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	90%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Supervised and contributed to data collection, laboratory sample processing, all statistical analyses of the collected data and prepared the manuscript.	
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Chapter 4 Heart rate variability as a marker of autonomic nervous system activity in young people with eosinophilic and non-eosinophilic asthma

Hajar Ali, Collin Brooks, Yu-Chieh Tzeng, Julian Crane, Richard Beasley, Peter Gibson, Philip Pattemore, Thorsten Stanley, Neil Pearce, Jeroen Douwes

Background: An imbalance in ANS activity may play a role in asthma, but it is unclear whether this is associated with specific pathophysiology. This study assessed ANS activity by measuring HRV in EA and NEA and people without asthma.

Methods: HRV, combined hypertonic saline challenge/sputum induction, FeNO, SPT to measure atopy, and spirometry tests were conducted in teenagers and young adults (14-21 years) with (n=96) and without (n=72) generally well-controlled asthma. HRV parameters associated with sympathetic and parasympathetic ANS branches were analysed. EA and NEA were defined using a 2.5% sputum eosinophil cut-point. AHR was defined as ≥15% reduction in FEV_1 following saline challenge.

Results: HRV parameters did not differ between asthmatics and non-asthmatics or EA and NEA. They were also not associated with markers of inflammation, lung function or atopy. However, increased absolute low frequency ($LF\mu s^2$; representing increased SNS activity) was found in asthmatics who used β-agonist medication compared to those who did not (median: 1611, IQR: 892-3036 vs 754, 565-1592; $p<0.05$) and increased normalised low frequency (nu) was found in those with AHR compared to without AHR (64, 48-71 vs 53, 43-66; $p<0.05$).

Conclusion: ANS activity (as measured using HRV analysis) is not associated with pathophysiology or inflammatory phenotype in young asthmatics with generally well-controlled asthma. However, enhanced SNS activity can be detected in asthmatics with AHR or who use β-agonist medication.

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4.1 Introduction

Asthma is commonly characterised by eosinophilic,[12] or neutrophilic airway inflammation,[451] but there is increasing evidence that inflammation is not detectable in a large proportion of cases.[416] Furthermore, asthma therapies directed towards reducing airway inflammation (such as ICS) are not effective in controlling symptoms in some people.[422] This has led to an increased interest in non-inflammatory mechanisms, such as neural pathways in asthma.[23]

The ANS plays a critical role in regulation of ASM tone,[23] and historically it has been suggested that ANS dysregulation may be important in asthma.[228] If true, this may provide an alternative avenue for intervention,[228] particularly in asthmatics with little evidence of airway inflammation or for whom current medication is ineffective. Whereas direct assessment of autonomic activity is difficult, it can be assessed indirectly through analysis of HRV. HRV data is commonly evaluated using frequency domain analyses.[497] This assigns the distribution of periodicities in HR fluctuation into frequency bands, including HF and LF, considered to reflect PNS (principally vagus nerve activity) and SNS modulation, respectively. The LF/HF ratio is considered to reflect the balance between the two.[352]

To date, relatively few studies have conducted HRV analysis in asthma and the results have been mixed. Increased PNS activity in asthma (i.e. increased HF) was found in some studies[260,364,365] but not others.[369,370] Furthermore, some studies have reported an association between increased PNS activity and poor asthma control[365] and AHR,[371] while increased SNS activity (increased LF) was associated with improved control[365] and β-agonist use.[498] However, most studies have been conducted in adults or people with severe asthma, with few studies in young adults or children with mild-to-moderate asthma. Additionally, previous HRV studies have not considered the heterogeneity underlying different asthma pathologies or inflammatory phenotypes e.g. EA and NEA.[13] This may have contributed to some of the

mixed results reported previously. In particular, we have previously shown that NEA but not EA exhibit heightened sensory nerve reactivity.[499] Other neural pathways might therefore also be important in the pathology of this phenotype.

We hypothesised that an ANS imbalance is involved in NEA, for which there is little evidence of airway inflammation, and the pathophysiological basis is largely unknown.[13] The aim of the study therefore was to assess ANS activity in asthma by measuring HRV in EA and NEA in young (14-21 years) people with and without asthma. The reason for choosing this specific age-group was because sputum induction and airway hyperreactivity testing is difficult in young children and previous studies have shown that pubertal status significantly affects HRV measurements.[361] We also examined the associations between HRV parameters and clinical and inflammatory characteristics.

4.2 Methods

4.2.1 Study population

We recruited 96 asthmatic and 72 non-asthmatic participants aged 14–21 years from Wellington, New Zealand, either from a previous birth cohort study[484] (29 with and 70 without asthma) or through separate community-based recruitment (67 with and 2 without asthma). All participants completed a respiratory symptom questionnaire based on the ISAAC Phase II survey.[485] Asthma was defined on the basis of a positive response to: 'have you had wheezing or whistling in the chest in the past 12 months?', and/or 'have you taken asthma medication in the past 12 months'. β-agonist use was defined as any short or long acting β-agonist use, either in the last 12 months or the last 7 days; ICS use was defined as any ICS use in the last 12 months. Participants without asthma reported no respiratory symptoms or asthma medication use. Informed consent was obtained from all participants and their parents, and the study was approved by the Northern B Health and Disability Ethics Committee (15/NTB/2).

4.2.2 Clinical assessments

Participants underwent the clinical assessments described below. Asthma control status was based on ACQ7, with a value of ≤ 0.75 representing controlled asthma, 0.76–1.49 representing partially-controlled asthma, and ≥ 1.5 representing uncontrolled asthma.[91] Participants with symptoms resembling a respiratory infection within 1 month of assessment returned when symptom-free and those with FEV_1%-predicted <75% were excluded. Prior to testing, all asthma medication and antihistamines were withheld for at least 12 and 24 hours, respectively.

4.2.2.1 HRV measurement

HRV parameters were measured using a computerized ECG data acquisition device with 16 analogue input channels sampled at 1000 Hz (PL3516 PowerLab 16/35, ADInstruments Pty Ltd. New South Wales, Australia). Measurements were conducted with participants seated and motionless; they were asked to breathe naturally and avoid talking during recording. Following a 2-minute stabilisation period, R-R intervals (between two consecutive R waves) were recorded for 10 minutes. Computation of frequency-domain parameters and R–R interval filtering of artefacts/ectopic beats were performed using LabChart Software (v. 8.1.13, ADInstruments Pty Ltd. New South Wales, Australia). Parameters used included total power (TP), HF power (0.15-0.40 Hz), LF power (0.04-0.15 Hz), and LF to HF ratio (LF/HF). The power density of LF and HF parameters was calculated and expressed in absolute (μs^2) and normalised units (nu) to account for total power and very low frequency (VLF) band (0.0033–0.04 Hz) using the following equations: "(LF/TP-VLF) x 100" and "(HF/TP-VLF) x 100", respectively. HF (nu) was not reported as it can be determined from LF (nu) using the equation "(mean (HF nu) =100 – mean (LF nu))".[352]

4.2.2.2 Atopy

Skin prick tests were conducted using a panel of aeroallergens:[486] HDM, tree mix, grass mix, cat and dog dander, *Alternaria tenuis* and *Penicillium mix* (Stallergenes Greer, Sydney, Australia). Atopy was determined by the presence of at least one weal ≥ 3mm.

4.2.2.3 Spirometry and FeNO

Spirometry and FeNO were measured using an Easyone spirometer (NDD Medizintechnik AG, Zurich, Switzerland) and Hypair FeNO analyser (Medisoft, Sorinnes, Belgium) as described previously.[22,486]

4.2.2.4 Combined hypertonic saline challenge and sputum induction

Combined hypertonic saline challenge/sputum induction was conducted as described previously.[488] Aerosolised hypertonic saline (4.5%w/v) was produced using an ultrasonic nebuliser (DeVilbiss Ultraneb 2000, Langen, Germany) and administered orally through a mouthpiece (Hans-Rudolph Inc, Kansas City, USA) for increasing intervals from 0.5-4 minutes to a total of 16 minutes. Spirometry was conducted between intervals, and salbutamol was administered if FEV_1 dropped to ≤75%-predicted. Participants were subsequently encouraged to produce sputum into a sterile plastic container. The resulting cell suspension was used to prepare cytospin slides stained using a Diff-Quik® fixative and stain set (Dade Behring, Deerfield, IL). Using light microscopy, EA was identified as ≥2.5% eosinophils and NEA as <2.5% eosinophils. AHR was defined as a reduction of ≥15% in FEV_1 from baseline.[488]

4.2.2.5 Blood eosinophils

Blood was collected using BD-vacutainers (BD, Auckland, New Zealand) and a complete blood count was obtained.

4.2.3 Statistical analysis

Data analyses were performed using STATA version 11.0 (STATA Corp, College Station, TX, USA) and Prism 5 (Graphpad Software Inc, La Jolla, CA, USA). Data are expressed as mean/standard deviation (SD), median/IQR, or frequency (percentage) as appropriate. Mann-Whitney *U* tests or unpaired t-tests were used as appropriate to assess differences between groups. Chi-square tests were used to assess differences between groups for dichotomous data. Comparisons were made between people with and without asthma, and those with EA and NEA. Absolute and normalised HRV indices were used as the primary outcome variables.

Linear regression analyses (either unadjusted or adjusted for age, sex and ethnicity) were used to assess associations between demographic/clinical factors and normalised LF (nu), LF/HF ratio and LF (μs^2) and HF (μs^2) in asthma. Prior to regression, LF (μs^2) and HF (μs^2) values were log-transformed as data were not normally distributed. Regression outcomes were reported as regression coefficient for (non-log-transformed) LF (nu) and LF/HF ratio data, and a relative difference (i.e. ratios per unit increase for continuous variables; compared to reference for categorical variables) for (log-transformed) LF (μs^2) and HF (μs^2) data. To assess the robustness of our findings, which relied on an asthma definition solely based on symptoms, we conducted sensitivity analyses including only asthmatics who also had AHR. Further stratified analyses were also conducted as appropriate.

4.3 Results

4.3.1 Population characteristics

Three people with and nine without asthma were excluded due to either poor quality or no sputum sample; 93 participants with asthma and 63 without asthma were therefore included in analyses. Participants with asthma were slightly younger than those without asthma but there were no differences in sex, ethnicity, or lung function (Table 4.1). As expected, atopy and AHR were more prevalent, and sputum eosinophil percentages higher, in asthma. Among those with asthma, 18% were classified as uncontrolled, 30% as partially controlled and 52% as well-controlled. Of the asthmatics, 68 were using β-agonists, with 17 taking both short and long acting β-agonists, 7 were exclusively using long acting β-agonists, and 44 were exclusively using short acting β-agonists.

Table 4.1. Population characteristics

	Non-asthma (N=63)	Asthma (N=93)	Eosinophilic asthma (N=41)	Non-eosinophilic asthma (N=52)
Age	20.2 (1.1)	18.1 (2.0) **	17.9 (2.0)	18.4 (2.0)
Males- n (%)	23 (37%)	43 (46.2%)	21 (51.0 %)	22 (42.0 %)
Height (cm)	170.1 (9.2)	169.0 (9.0)	169.0 (9.0)	169.0 (9.0)
Weight (Kg)	66.3 (14.8)	66.6 (16.2)	64.9 (16.3)	68.1 (16.1)
Ethnicity				
European- n (%)	57 (90.5%)	70 (75.3%)	31 (75.6%)	39 (75.0%)
Non-European- n (%)	6 (9.5 %)	23 (24.7%)	10 (24.4%)	13 (25.0%)
Airway hyperreactivity[b]- n (%)	3 (4.7 %)	40 (43.0 %) **	22 (54.0 %) ††	18 (35.0 %)
FEV_1% predicted	101.27 (12.0)	99.5 (14.4)	97.3 (14.3)	101.2 (14.4)
FVC% predicted	100.4 (9.9)	102.3 (12.9)	101.1 (13.0)	103.2 (13.0)
FEV_1/FVC% predicted	100.8 (7.8)	97.1 (8.0)	95.9 (7.4)	98.0 (8.1)
ACQ7 score		1.0 (0 .68)	1.3 (0.7) ††	0.8 (0 .6)
FeNO (ppb)	28.2 (19.4)	63.0 (69.3) **	92.3 (76.4) ††	39.9 (53.4)
Atopy[a]- n (%)	24 (38.1%)	77 (83.0%) **	37 (90.2%)	40 (77.0%)
β-agonist use last 12 months n (%)		68 (73.1%)	36 (88.0%) ††	32 (61.5%)
β-agonist use last 7 days n (%)		49 (53.0%)	28 (68.3%)	21 (40.4%)
ICS use n (%)		44 (47.0%)	22 (53.6%)	22 (42.3%)
Sputum eosinophils %	0.0 (0.0-0.0)	2.0 (0.0-8.0) **	9.2 (5.0-18.0) ††	0.0 (0.0-1.0)
Sputum neutrophils %	20.0 (7.3-35.0)	11.1 (5.0-21.4) **	8.2 (5.0-19.6)	12.6 (5.0-25.6)
Blood eosinophils (mm^3)	100 (100-200)	400 (200-600) **	500 (400-800) ††	250 (100-500)

T-test or Mann-Whitney test and Chi-square tests were used. Data are presented as mean (SD), or number (percentages) as appropriate. * P<0.05; ** P<0.01 asthmatics versus the reference population, † P<0.05; †† P<0.01 non-eosinophilic versus eosinophilic asthmatics.

[a] Positive SPT against one or more common allergens.

[b] ≥15% drop in FEV1 from baseline following hypertonic saline challenge.

SPT, skin prick test, FENO, Fractional exhaled nitric oxide, ICS, inhaled corticosteroid includes monotherapy and combination therapy in the last 12 months.

Eosinophilic asthma defined as ≥2.5% sputum eosinophils.

4.3.2 Inflammatory phenotypes

Forty-four percent (n=41) of participants with asthma were classified as having EA and 56% (n=52) as NEA. Compared to NEA, those with EA were more likely to be atopic, have AHR, and have higher FeNO and ACQ7 scores (Table 4.1). Neutrophilic or mixed granulocytic asthma[20] were not detected, and sputum neutrophil levels were higher in people without asthma compared to those with asthma.

4.3.3 HRV parameters and inflammation

There were no differences in absolute or normalised HRV parameters between participants with and without asthma, or between those with EA and NEA (Table 4.2). Results remained similar when analyses were restricted to asthmatics with AHR (supplementary Table S4.1). There were also no significant associations observed between HRV parameters and inflammatory markers including sputum eosinophils and neutrophils, blood eosinophils, FeNO, or atopy in linear regression analyses, either unadjusted (Supplementary Table S4.2) or adjusted for age, sex and ethnicity (Table 4.3).

Table 4.2. HRV parameters

	Non-asthma (N=63)	**Asthma (N=93)**	**Eosinophilic asthma (N=41)**	**Non-eosinophilic asthma (N=52)**
Total power (TP)	4554 (2845-8455)	4635 (2337-8380)	4635 (2242-10100)	4558 (2490.5-7053.5)
Low Frequency (μs^2)	1389 (877.8-2347)	1364 (729.7-2723)	1386 (738.2-3322)	1324.5 (690.3-2495.5)
High Frequency (μs^2)	1008 (468.8-2704)	1097 (509.2-2242)	1067 (408.8-2922)	1142 (527.1-1837)
LF (nu)	60.28 % (44.01-67.90)	54.10 % (43.70-66.86)	58.40 % (47.80-68.80)	53.30 (43.60-64.10)
LF/HF % ratio	1.51 (0.78-2.12)	1.20 (0.77-2.02)	1.41 (0.91-2.21)	1.14 (0.77-1.80)

T-test or Mann-Whitney test were used. Data are presented as median (IQR), or percentages, as appropriate.

4.3.4 HRV parameters and clinical characteristics

No associations were observed between lung function parameters and HRV indices, in either unadjusted (Supplementary Table S4.2) or adjusted (Table 4.3) regression analyses. However, participants with AHR had higher LF (nu) (median 63.7, IQR 48.4-71.0 vs 53.2, 43.3-65.5; Regression Coefficient 9.8, 95% CI 3.7-16.0; $p<0.05$)) and LF/HF ratio (1.8, 0.9-2.4 vs 1.1, 0.7-1.9; ratio 0.7, 0.1-1.2; $p<0.05$) compared to those without AHR, and asthmatics who used β-agonists had higher LF (μs^2) (1611.0, 892.0-3036.0 vs 753.7, 565.2-1592.0; ratio=1.9, 95% CI 1.3-2.7; $p<0.05$) compared to those who did not (Table 4.3). Borderline significant ($p<0.1$) positive associations were found between absolute LF (μs^2) and β-agonist use in the last 7 days (ratio=1.39, 95% CI 0.9-2.0) or ACQ7 score (ratio=1.31, 95% CI 0.8-2.2; Table 4.3). No association was found with ICS use and as none of the participants used IB, the association between IB use and HRV could not be assessed.

As β-agonist use and AHR were each associated with HRV parameters, we attempted to further clarify the nature of these associations by comparing HRV data in asthmatics with: AHR and β-agonist use (Group A; n=33); AHR and no β-agonist use (Group B; n=7); no AHR and β-agonist use (Group C; n=35); and no AHR and no β-agonist use (Group D; n=18). Group A had higher normalised LF (nu) and LF/HF ratio compared to groups C and D (i.e. those without AHR; Figure 4.1A and 4.1B). Groups A and C had higher absolute LF (μs^2) compared to group D (Figure 4.1C). No differences in absolute HF (μs^2) were observed across groups (Figure 4.1D). As β-agonist use has been shown to have a short-term effect on HRV parameters,[500] we repeated analyses using β-agonist use in the last 7 days; this showed similar results (Supplementary Figure S4.1).

Table 4.3. Association of HRV parameters with clinical characteristics in asthmatics (adjusted for age, sex and ethnicity).

	LF (nu)	LF/HF ratio	Log LF (μs^2)	Log HF (μs^2)
	Regression coefficient [95% CI]		Relative difference, or ratio [95% CI]	
Atopy (n=77, yes vs no)	0.913 [-8.014,9.836]	-0.120 [-0.898,0.657]	0.835 [0.507,1.378]	0.815 [0.418,1.59]
AHR (n=40, yes vs no)	9.849 [3.688,16.010] **	0.660 [0.110,1.209] *	1.211 [0.842,1.741]	0.79 [0.485,1.286]
β-agonist use[a] (n=68, yes vs no)	1.552 [-5.712,8.815]	0.266 [-0.365,0.897]	1.863 [1.264,2.734] **	1.714 [1.005,2.922]
β-agonist use[b] (n=49, yes vs no)	1.031 [-5.475,7.538]	0.120 [-0.446,0.687]	1.398 [0.976,2.001] †	1.331 [0.820,2.160]
ICS use (n=44, yes vs no)	1.276 [6.869,9.420]	0.039 [-0.670,0.750]	0.997 [0.632,1.572]	0.935 [0.508,1.719]
FeNO (ppb)	0.017 [-0.029,0.063]	-0.000 [-0.004,0.004]	1.00 [0.9975,1.003]	1.00 [0.997,1.003]
FEV_1% pred	0.088 [-0.148,0.324]	0.008 [-0.012,0.029]	0.997 [0.984,1.010]	0.993 [0.98,1.006]
FVC% pred	0.013 [-0.254,0.279]	0.002 [-0.021,0.025]	1.000 [0.985,1.015]	0.999 [0.981,1.018]
Sputum eosinophils %	-0.014 [-0.319,0.290]	-0.004 [-0.031,0.023]	1.011 [0.992,1.029]	1.012 [0.989,1.035]
Sputum neutrophils %	-0.065 [-0.274,0.143]	-0.004 [-0.023,0.132]	1.006 [1.001,1.011]	1.003 [1.003,1.016]
Blood eosinophils %	-2.704 [-13.134,7.724]	-0.314 [-1.20,0.572]	1.194 [0.626,2.277]	1.361 [0.604,3.069]
ACQ7	4.147 [-1.499,9.792]	0.145 [-0.296,0.586]	1.305 [0.986,1.727] †	1.159 [0.793,1.693]

Data presented as regression coefficient and 95% confidence limit for LF (nu) and LF/HF ratio and as ratios (per unit increase in case of continuous variables and compared to the reference category in case of categorical variables (yes/no)) for log transformed LF (μs^2) and HF (μs^2). † P<0.1, * P<0.05; ** P<0.01

[a] β-agonist use in the last 12 months

[b] β-agonist use in the last 7 days

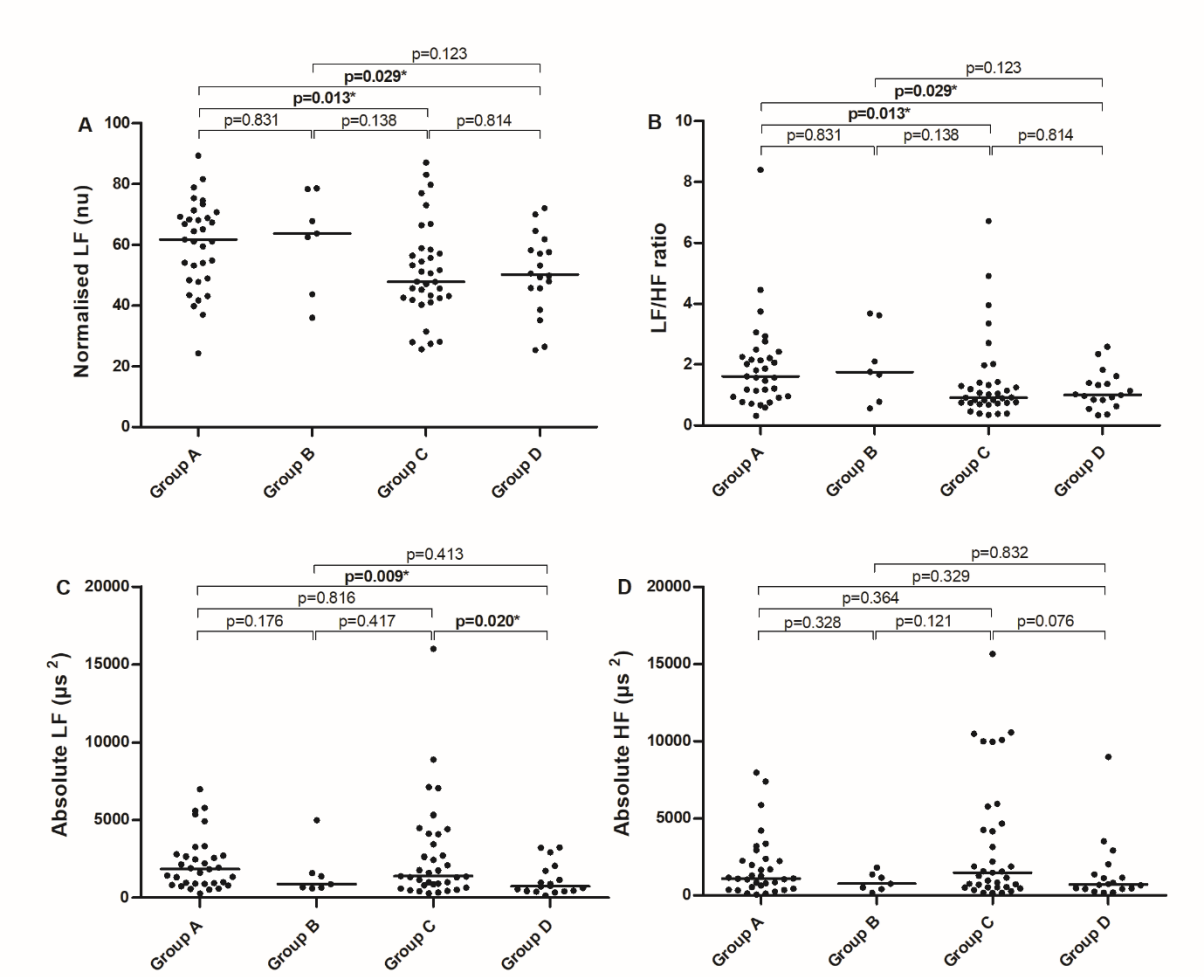

Figure 4.1. Normalised LF (nu) (A), LF/HF ratio (B), Absolute LF (μs^2) (A) and absolute HF (μs^2) (B) in asthmatics in (Group A) AHR and β-agonist use in the last 12 months; (Group B) AHR and no β-agonist use in the last 12 months; (Group C) no AHR and β-agonist use in the last 12 months; and (Group D) no AHR and no β-agonist use in the last 12 months. Solid line represents median. Mann-Whitney test was used. * $p<0.05$

4.4 Discussion

This study found no evidence of an imbalance or difference in ANS activity (as measured by HRV analysis) between people with and without asthma or between EA and NEA. However, increased absolute and normalised LF (representing increased SNS activity) was found in asthmatic participants who used β-agonist medication or had AHR. Differences in autonomic activity may therefore be associated with some clinical characteristics (i.e. β-agonist treatment and AHR) but appear independent of inflammatory pathology or phenotype.

Although increased HF (representing PNS predominance) in asthma has been reported,[365] we found no evidence of autonomic imbalance between people with and without asthma, which is consistent with previous findings.[370] We also found no significant associations between HRV indices and demographic characteristics that have previously been reported, such as age,[501] gender,[358] ethnicity,[501] body mass index[358] or with baseline FEV_1.[365] It is possible that this is due to differences in the populations studied. In particular, we recruited young participants from the general population with relatively well-controlled asthma, whereas most previous studies have assessed either older[369] or pre-pubertal populations[260] or more severe asthma in a tertiary setting.[260,364,365] Alternatively, mixed findings between studies may be due to methodological differences in HRV measurement (e.g. short-term vs long-term measurements), hampering valid comparisons between studies.[352] Finally, as speculated (see section 5.1), mixed results may be due to the heterogeneity underlying different asthma pathologies or inflammatory phenotypes. However, when inflammatory phenotypes were considered (to our knowledge this is the first study to do so), we found no association; likewise, no associations were found with the inflammatory markers studied. This suggests that mixed results are unlikely to be related to asthma phenotypes. It also suggests that, at least in younger people with well-controlled asthma, there is no evidence that autonomic

regulation, as assessed by using HRV analysis, is associated with airway inflammation. However, other neural pathways (not assessed in this study) may still play a role; indeed, we have previously shown that heightened sensory nerve reactivity may be involved, particularly in NEA.[499]

Previous studies have reported that increased HF was associated with poor asthma control[365] or severity.[260] In the present study, a borderline significant positive association was found between absolute LF and ACQ7. However, when conducting multivariate regression analyses adjusting for β-agonist medication, which was associated with both ACQ7 and LF (Table 4.3), the association with ACQ7 disappeared (data not shown), suggesting that the association was confounded by β-agonist use. The observation that β-agonist use was associated with higher absolute LF is consistent with two clinical studies showing a shift towards increased LF (SNS dominance) following β-agonist administration. In particular, Jartti *et al* reported that salbutamol administration within two hours of,[502] or two weeks preceding[498] HRV analysis was associated with decreased PNS and increased SNS activity in asthma. Although the underlying mechanism is not entirely clear, it is possible that β-agonists binding β_2-adrenoceptors in cardiac efferent SNS sites or peripheral vasculature may directly stimulate SNS activity.[230]

Relatively few studies are available assessing the association between HRV parameters and AHR in asthma, but those that did reported increased PNS activity.[371,503] One previous study of 53 people with untreated asthma[371] found that normalised HF was significantly higher in asthmatic subjects with AHR compared to those without, suggesting increased PNS activity. In contrast, our data showed a positive association between AHR and normalised LF and LF/HF ratio, suggesting increased SNS activity. However, most asthmatic participants with AHR in our study were undergoing β-agonist treatment, and it is possible that the effect of

the latter (see above) may have masked any associations with AHR. In an attempt to clarify this, we conducted a stratified analysis grouping on the basis of β-agonist use and/or AHR in asthma. This showed that normalised LF was reduced in those without AHR and absolute LF was reduced in subjects who did not use β-agonists. As absolute and normalised LF showed contrasting findings, we speculate that the association we observed between normalised LF and AHR may possibly be due to the process of data normalisation. Similar discrepancies between normalised and absolute HRV indices in asthma have previously been reported.[364]

While the present study did not find evidence of autonomic imbalance in asthma (or between asthma phenotypes), this may be because HRV analysis is not the most appropriate tool for evaluating autonomic airway regulation. Although widely accepted as a surrogate measure of autonomic function,[504] HRV is at best a proxy, and does not directly evaluate autonomic respiratory control.[352] Furthermore, it is unclear whether some HRV frequency bands are truly representative of distinct ANS components.[504] In particular, there has been debate about interpretation of the LF component, which is considered by some as solely a marker of sympathetic control,[505] while others have suggested that it is a marker of both sympathetic and parasympathetic control.[506] This is in part due to evidence suggesting that absolute LF values are determined by baroreflexes mediated by both PNS and SNS; therefore, LF may effectively reflect both PNS *and* SNS activity.[365]

Ultimately, the complexity of the ANS is such that there is currently no single "gold standard" test to accurately assess respiratory autonomic activity. To avoid further ambiguous or equivocal results when attempting to characterise ANS activity in asthma, we suggest that using a battery of tests, rather than relying on one single test may provide clearer results. An example could be the Ewing test battery; this consists of five tests assessing different aspects of ANS control and is often used in the diagnosis of diabetic neuropathy.[351]

This study has some limitations. Firstly, although almost all participants in the asthma group reported a doctor's diagnosis and/or recent symptoms, we did not use objective tests (such as BDR or AHR) to confirm diagnosis. It is possible that some misclassification may have occurred. However, we consider that any bias introduced as a result will be minimal as this approach, also used in many other studies,[57,304,496] compares well with clinical diagnoses[57] and has been shown to be better than some objective measures.[496] Secondly, as mentioned above, those with asthma in the present study were young with well-controlled asthma. It is currently unclear how generalisable these findings are to other age groups, or in more severe or uncontrolled asthma. Thirdly, this was a cross-sectional study, and therefore only HRV data representing a single timepoint are available. While studies in coronary artery disease have found that HRV is relatively stable over time,[507] it remains unclear if this is the case in asthma, which (as discussed above) is highly variable. Fourthly, as our study was conducted in a community rather than tertiary setting, we did not ask participants to abstain from LABA for long periods of time due to safety concerns. Finally, breathing frequency (which has been shown to affect HRV analysis)[508] was not recorded in this study. However, to minimise any potential effect, participants were advised to breathe normally during HRV measurement.

In conclusion, our study suggests that autonomic imbalance (as measured using HRV analysis) is not associated with pathophysiology or inflammation in asthma, or with any inflammatory phenotype, such as NEA, in young people with generally well-controlled asthma. However, altered ANS activity can be detected in asthmatic subjects with AHR or using β-agonist medication. Further studies using a more comprehensive battery of tests may be required to adequately evaluate autonomic activity in asthma.

4.5 Supplementary material

Table S4.1. HRV parameters in non-asthmatics and asthmatics with AHR

	Non-asthma (N=63)	**Asthma (N=40)**	**Eosinophilic asthma (N=22)**	**Non-eosinophilic asthma (N=18)**
Total power (TP)	4554 (2845-8455)	4186.5 (2289.5-8376.5)	4064 (2213-9265)	4623.5 (2644-8373)
Low Frequency (μs^2)	1389 (877.8-2347)	1513 (863.3-2688.5)	1478 (738.2-3269)	1669.5 (980.8-2653)
High Frequency (μs^2)	1008 (468.8-2704)	1082 (478.2-2096)	1045 (357.5-2373)	1216.5 (635.4-1686)
LF (nu)	60.28 % (44.01-67.90)	63.1 % (48.7-70)	61.8 % (48.4-71.4)	64.1 % (54.1-69.3)
LF/HF % ratio	1.51 (0.78-2.12)	1.7 (0.9-2.3)	1.6 (0.9-2.5)	1.8 (1.2-2.3)

T-test or Mann-Whitney test were used. Data are presented as median (IQR), or percentages, as appropriate.

Table S4.2. Association of HRV parameters with clinical characteristics in asthmatics (unadjusted).

	LF (nu)	LF/HF ratio	Log LF (μs^2)	Log HF (μs^2)
	Regression coefficient [95% CI]		Relative difference, or ratio [95% CI]	
Atopy (n=77, yes vs no)	-1.326 [-9.737,7.084]	-0.229 [-0.952,0.494]	0.902 [0.551,1.474]	0.964 [0.505,1.838]
AHR (n=40, yes vs no)	9.423 [3.316,15.530] **	0.619 [0.082,1.157] *	1.191 [0.577,1.221]	0.793 [0.487,1.290]
β-agonist use[a] (n=68, yes vs no)	2.031 [-5.119,9.182]	0.274 [-0.340,0.889]	1.811 [1.245,2.634] **	1.637 [0.950,2.807] †
β-agonist use[b] (n=49, yes vs no)	1.627 [-4.724,7.979]	0.130 [-0.413,0.681]	1.268 [0.876,1.835]	1.178 [0.723,1.917]
ICS use (n=44, yes vs no)	0.249 [-7.47,7.979]	0.033 [-0.698,0.633]	0.841 [0.536,1.321]	0.826 [0.459,1.485]
FeNO (ppb)	0.016 [-0.030,0.062]	-0.00007 [-0.004,0.004]	1.001 [0.998,1.003]	1.000 [0.997,1.003]
FEV_1% pred	0.07 [-0.152,0.292]	0.008 [-0.012,0.027]	0.993 [0.981,1.005]	0.989 [0.972,1.005]
FVC% pred	-0.010 [-0.2590,0.238]	0.002 [-0.019,0.023]	0.995 [0.982,1.009]	0.995 [0.978,1.014]
Sputum eosinophils %	-0.040 [-0.346,0.248]	-0.005 [-0.031,0.023]	1.012 [0.998,1.025]	1.016 [0.994,1.039]
Sputum neutrophils %	-0.081 [-0.286,0.125]	-0.005 [-0.023,0.012]	1.007 [1.002,1.012]	1.011 [1.004,1.018]
Blood eosinophils %	-2.253 [-12.108,7.602]	-0.322 [-1.15,0.510]	1.291 [0.693,2.407]	1.445 [0.665,3.141]
ACQ7	3.424 [-1.323, 8.172]	0.161 [-0.250,0.573]	1.155 [0.877,1.5222]	0.999 [0.693,1.440]

Data presented as regression coefficient and 95% confidence limit for LF (nu) and LF/HF ratio and as ratios (per unit increase in case of continuous variables and compared to the reference category in case of categorical variables (yes/no)) for log transformed LF (μs^2) and HF (μs^2). † P<0.1, * P<0.05; ** P<0.01

[a] β-agonist use in the last 12 months

[b] β-agonist use in the last 7 days

HRV parameters and β-agonist use in the last 7 days in asthmatics.

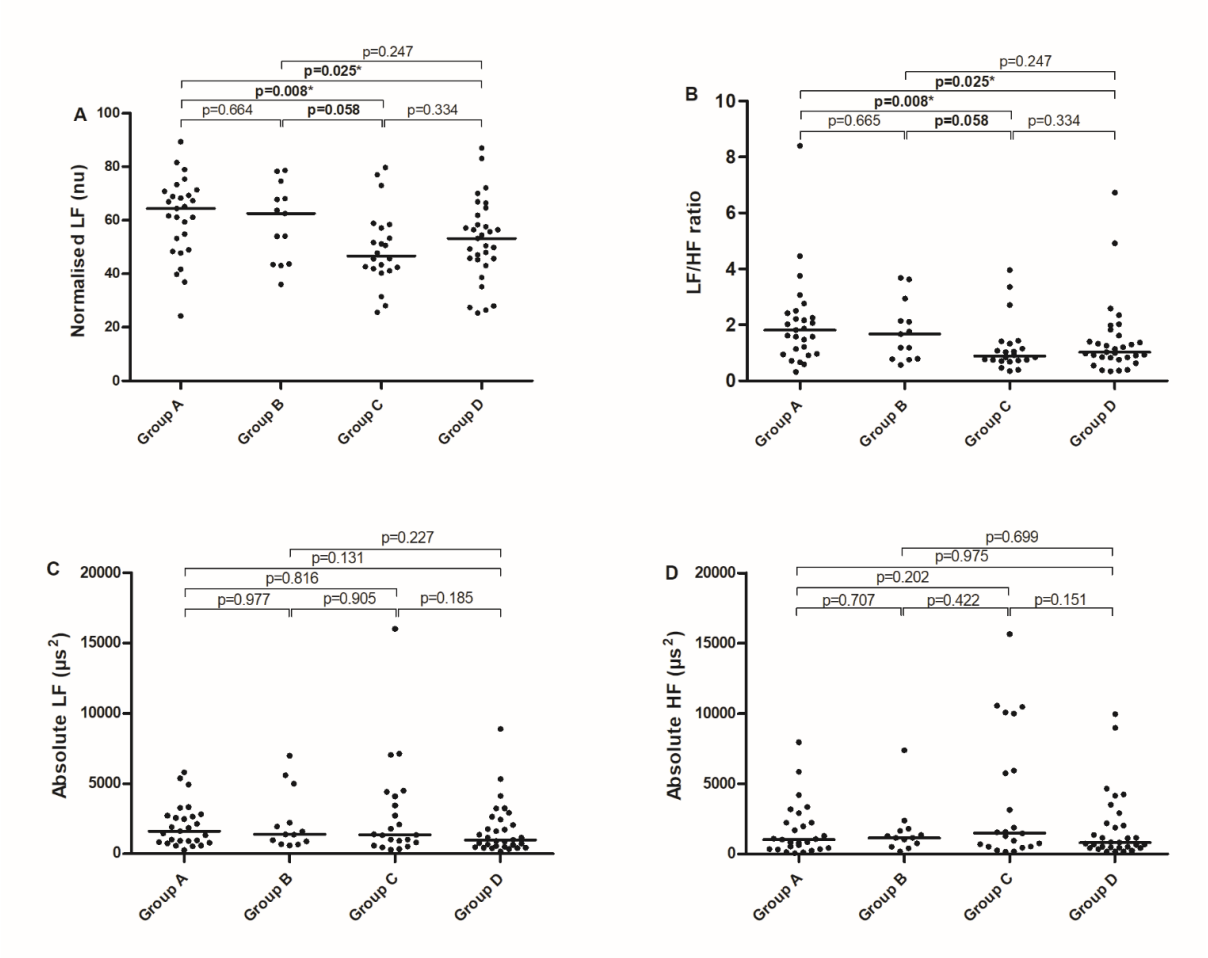

Figure S4.1. Normalised LF (nu) (A), LF/HF ratio (B), Absolute LF (μs^2) (A) and absolute HF (μs^2) (B) in asthmatics in (Group A) AHR and β-agonist use in the last 7 days; (Group B) AHR and no β-agonist use in the last 7 days; (Group C) no AHR and β-agonist use in the last 7 days; and (Group D) no AHR and no β-agonist use in the last 7 days. Solid line represents median. Mann-Whitney test was used. * $p<0.05$

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Hajar Ali
Name/title of Primary Supervisor:	Prof. Jeroen Douwes
Name of Research Output and full reference:	
Sputum inflammatory, neural, and remodeling mediators in eosinophilic and non-eosinophilic asthma.	
In which Chapter is the Manuscript /Published work:	5
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	90 %
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Supervised and contributed to data collection, laboratory sample processing, all statistical analyses of the collected data and prepared the manuscript.	
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Primary Supervisor's Signature:	
Date:	31/7/23

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

Chapter 5 Sputum inflammatory, neural, and remodelling mediators in eosinophilic and non-eosinophilic asthma

Hajar Ali, Jeroen Douwes, Jeroen Burmanje, Prachee Gokhale, Julian Crane, Philip Pattemore, Thorsten Stanley, Jacqueline Keenan, Collin Brooks

Background: Neural and remodelling mechanisms may play a role in asthma, particularly NEA. To assess sputum mediators associated with neural, remodelling, and inflammatory mechanisms in EA, NEA, and non-asthmatics.

Methods: 111 participants with and 62 without asthma (14-21 years) underwent sputum induction, FeNO, atopy, and spirometry tests. Twenty-four mediators were measured in sputum using ELISA or bead array. EA (n=52) and NEA (n=59) were defined using a sputum eosinophil cut-point of ≥2.5%.

Results: Elevated levels of nociceptin (median: 39.1 vs 22.4 ng/mL, p=0.03), periostin (33.8 vs 9.4 ng/mL, p=0.01), and ECP; (220.1 vs 83.7 ng/mL, p=0.03) were found in asthmatics compared with non-asthmatics. Nociceptin was elevated in EA (54.8 vs 22.4 ng/mL, p=0.02) compared with non-asthmatics. EA had higher levels of inflammatory (ECP: 495.5 vs 100.3 ng/mL, p≤0.01; IL-1β: 285.3 vs 209.3 pg/mL, p=0.03; histamine: 5805.0 vs 3172.5 pg/mL, p=<0.01) and remodelling (VEGF-A); 3.3 vs 2.5 ng/mL, p=0.03; periostin: 47.7 vs 22.1 ng/mL, p=0.04) mediators than NEA. Whilst macrophages were associated with neural mediators e.g. NKA (r=0.27, p=0.01) and nociceptin (r=0.30, p=0.02), granulocytes were associated with inflammatory/remodelling mediators; e.g. ECP and VEGF-A correlated with neutrophils (r=0.53 & r=0.33 respectively, p=<0.01) and eosinophils (r=0.53 & r=0.29 respectively, p≤0.01).

Conclusion: Elevated levels of nociceptin and inflammatory/remodelling markers were found in EA, but no evidence for neural and remodelling pathways was found in NEA. Neural and remodelling mechanisms appear to coexist with inflammation.

Annals of allergy, asthma & immunology 2023;130(6):776–783; with minor amendments

5.1 Introduction

Asthma is a heterogenous disease[509] involving several clinical phenotypes and pathophysiological processes.[510] To date, the focus in asthma pathophysiology has largely been on TH_2-mediated airway inflammation.[416] However, studies have found little evidence of airway inflammation in approximately 50% of asthma cases,[22] and there is increasing recognition that alternative pathways including neural mechanisms[511] and airway remodelling[24] may be important.

Induced sputum is often used to assess airway inflammation,[7] allowing the cellular profile-based characterisation of inflammatory phenotypes; i.e. EA, NEA,[13] or EA, NA, PGA and MGA.[20] Measuring soluble mediators allows further investigation of airway pathophysiology,[326] with most studies focusing on inflammatory proteins. For example, TH_2-associated mediators such as ECP[512] or EDN[513] are increased in EA; conversely, neutrophil-associated mediators such as NE and IL-8 are increased in NA.[514]

Studies measuring levels of sputum mediators associated with neural mechanisms and remodelling are limited, with some evidence of increased levels of tachykinins and neurotrophins (e.g. SP, NKA),[285,287] BDNF[515] and NGF[516] during exacerbations or in severe asthma. Similarly, increased levels of remodelling mediators such as TGF-β,[517] VEGF[518] and TIMP-1[519] have been reported in severe asthma. Although some (such as MMP-9 in NA)[520] have been shown to be associated with specific phenotypes, it remains unclear whether neural and remodelling mediators are associated with specific inflammatory phenotypes or occur in the absence of airway inflammation. Furthermore, as most previous studies have been conducted in severe asthma, adults, or during exacerbations, little is known about these mediators in young people with well-controlled asthma.

We hypothesised that neural mechanisms and remodelling may be particularly important in NEA, where there is little evidence of airway inflammation.[22] The aims of this study were therefore to measure sputum levels of mediators associated with airway inflammation, neural mechanisms, and remodelling, in young people with and without asthma, and to compare EA with NEA. Furthermore, as it is unclear whether neural or remodelling activity is associated with inflammation, we also assessed the interrelationships among specific mediators, and between mediators and specific sputum inflammatory cell populations.

5.2 Methods

5.2.1 Study population

Participants (14-21 years), recruited from Wellington or Christchurch, New Zealand (either from a birth cohort study[484] or through separate community-based recruitment), completed a respiratory questionnaire based on the ISAAC Phase II survey.[485] Asthma was defined as wheezing/whistling in the chest and/or asthma medication-use in the last 12 months. Non-asthmatics reported no asthma symptoms, other respiratory conditions, or asthma medication use. Informed consent was obtained from participants/parents. The study was approved by the Northern B Health and Disability Ethics Committee (15/NTB/2).

5.2.2 Clinical assessments

Participants underwent the clinical tests described below. Asthma control was assessed using the ACQ7.[91] Participants with respiratory infections within 1 month of assessment returned when symptom-free, and those with unrelated health conditions, or FEV_1 % predicted <75% were excluded. Prior to testing, asthma medication and antihistamines were withheld for ≥12 and ≥24 hours, respectively.

5.2.2.1 Spirometry and FeNO

Lung function and FeNO were measured using an Easyone spirometer (NDD Medizintechnik AG, Zurich, Switzerland) or Hypair FeNO analyser (Medisoft, Sorinnes, Belgium) respectively, as described previously.[307,486,521,522]

5.2.2.2 Atopy

Skin prick tests were conducted using a panel of aeroallergens as described previously.[22] Atopy was determined by presence of at least one weal ≥ 3mm after subtraction of the negative control (saline).

5.2.2.3 Combined hypertonic saline challenge and sputum induction

Hypertonic saline challenge/sputum induction was conducted as described previously.[488] Aerosolised hypertonic saline (4.5% w/v) was produced using an ultrasonic nebuliser (DeVilbiss Ultraneb 2000, Langen, Germany) and administered through a mouthpiece (Hans-Rudolph Inc, Kansas City, USA) for increasing intervals from 0.5-4 minutes to a total of 16 minutes. The procedure was stopped before 16 minutes if FEV_1 dropped more than 15% or if FEV_1 dropped to ≤75%-predicted. The minimum total inhalation time was 7 minutes. Spirometry was conducted between intervals, and salbutamol administered if FEV_1 dropped to ≤75%-predicted. Participants were encouraged to produce sputum into a sterile plastic container. Sputum plugs were dispersed using DTT (Sputasol, Oxoid Ltd, Basingstoke, Hampshire, England), filtered, and resuspended. Following centrifugation, supernatant was aspirated and stored at -80ºC. The cell suspension was used to prepare cytospin slides using Diff-Quik® (Dade Behring, Deerfield, IL). A differential count of 400 non-squamous cells was made using light microscopy. Samples were considered adequate quality if there was <80% squamous cell contamination and >400 non-squamous cells.[29] EA was identified as ≥2.5% eosinophils; NEA as <2.5% eosinophils. AHR was defined as a ≥15% FEV_1 drop from baseline.[488]

5.2.2.4 Mediator assessment

Sputum supernatants were assayed for mediators identified as being primarily involved in either inflammatory, neural, or remodelling processes (based on previous studies).[509-513] Mediators involved in multiple pathways (e.g IL-13) were assigned to a single group based on cellular origin/as appropriate, based on previous literature. Twenty-four mediators (8 inflammatory; 7 neural; 9 remodelling) were measured (Table 5.1). Assays (Table 5.1) were conducted according to manufacturer's instructions using either the Luminex MagPix (Luminex Corp., Texas, USA) or Enzyme-Linked Immunosorbent Assay (ELISA) using a TS800 microplate reader (BioTech®, VT, USA). To determine DTT effect, initial experiments were conducted spiking standards with and without DTT (data not shown); samples were diluted to minimise any DTT effect. Mediator concentrations were expressed as pg or ng/mL sputum.

Table 5.1. Sputum mediators

Group	Mediator	Limit of detection (pg/mL)	Non-asthma Frequency (%)	Asthma Frequency (%)	EA Frequency (%)	NEA Frequency (%)
Inflammatory	Interleukin-1β (IL-1β)[a]	1.6	58/58 (100)	106/106 (100)	50/50 (100)	56/56 (100)
	Interleukin-6 (IL-6)[a]	8.1	58/58 (100)	106/106 (100)	50/50 (100)	56/56 (100)
	Interleukin-8 (IL-8)[a]	2.3	58/58 (100)	106/106 (100)	50/50 (100)	56/56 (100)
	Interleukin-13 (IL-13)[a]	2.7	58/58 (100)	106/106 (100)	50/50 (100)	56/56 (100)
	Neutrophil elastase (NE)[a]	6.2	35/35 (100)	61/61 (100)	33/33 (100)	28/28 (100)
	Interferon y (IFN-γ)[a]	14	1/35 (3)	3/61 (5)	0/33 (0)	3/28 (11)
	Eosinophil Cationic Protein (ECP)[b]	125	34/34 (100)	57/57 (100)	31/31 (100)	26/26 (100)
	Histamine[c]	30	28/35 (80)	55/61 (90.2)	33/33 (100)	22/28 (77)
Neural	Acetylcholine[c]	3.0	35/35 (100)	61/61 (100)	33/33 (100)	28/28 (100)
	Neurokinin-A (NKA)[d]	0.1	100/103 (97)	48/50 (96)	48/49 (98)	52/54 (96)
	Substance P[c]	8.0	9/39 (23.1)	25/84 (30)	9/37 (24.3)	16/47 (34)
	Calcitonin gene related protein (CGRP)[e]	12.4	22/32 (69)	34/63 (54)	17/33 (52)	17/30 (57)
	Nociceptin[e]	180	35/35 (100)	61/61 (100)	33/33 (100)	28/28 (100)
	Nerve growth factor-β (NGF-β)[a]	7.4	58/58 (100)	106/106 (100)	50/50 (100)	56/56 (100)
	Brain-derived neurotrophic factor (BDNF)[a]	1.7	3/58 (5.2)	2/106 (2.0)	0/50 (0)	2/56 (3.6)
Remodelling	Angiopoietin[a]	32.0	2/58 (3.4)	2/106 (2.0)	1/50 (2.0)	1/56 (1.7)
	Matrix metalloproteinase-1 (MMP-1)[a]	8.5	58/58 (100)	106/106 (100)	50/50 (100)	56/56 (100)
	Matrix metalloproteinase-9 (MMP-9)[a]	0.9	58/58 (100)	106/106 (100)	50/50 (100)	56/56 (100)
	Tissue inhibitor of metalloproteinase-1 (TIMP-1)[a]	44.0	58/58 (100)	106/106 (100)	50/50 (100)	56/56 (100)
	Vascular endothelial growth factor (VEGF-A)[a]	5.4	58/58 (100)	106/106 (100)	50/50 (100)	56/56 (100)
	Osteopontin[a]	12.0	1/58 (2)	3/106 (3)	3/50 (6)	0/56 (0)
	Periostin[f]	120	32/35 (91.0)	55/61 (90)	30/33 (91)	25/28 (89)
	Transforming growth factor-β (TGF-β)[f]	2.5	13/35 (37)	25/61 (41)	16/33 (49)	9/28 (32)
	Elastin[g]	3.1	35/35 (100)	61/16 (100)	33/33 (100)	28/28 (100)

[a] Procartaplex panel; Invitrogen, CA, USA
[b] MBL International, MA, USA
[c] Abcam, Cambridge, UK
[d] RayBiotech, Norcross, GA, USA
[e] Creative Diagnostics, NY, USA
[f] Milliplex panel; Merck Millipore, Darmstadt, Germany
[g] CUSABIO, Wuhan, China

5.2.3 Statistical analysis

STATA version 11.0 (STATA Corp, College Station, TX, USA) and GraphPad Prism 7.0 (Graphpad Software Inc, La Jolla, CA, USA) were used. Data were expressed as mean/SD, median/IQR, or frequency (percentage) as appropriate. Mann-Whitney U tests, unpaired t-tests, or Chi-square tests were used as appropriate to assess differences between groups. Mediator concentrations were analysed as continuous data; those below the limit of detection (LOD) were assigned a concentration 2/3 of LOD.[523] Associations were assessed using Spearman rank-correlation; correlation n values correspond to Table 5.1 and correlation strength was reported as previously.[524] To assess the robustness of the asthma definition, and the effect of asthma treatment, some analyses were restricted to only asthmatics with AHR, or using ICS/any asthma medication in the last 12 months. The use of a more stringent sputum quality criterion (squamous contamination of <30% and >400 total non-squamous cells)[512] was also assessed.

5.3 Results

5.3.1 Population characteristics

One hundred-and-thirty participants with and 79 without asthma underwent assessments. Of these, 19 participants with and 17 without asthma were excluded due to poor quality/no sputum sample, leaving 111 asthmatic and 62 non-asthmatic participants (Table 5.2). Due to limited supernatant volume availability, some mediators were not measured in all participants (Table 5.1). Asthmatic participants were younger and had increased prevalence of atopy and AHR compared with non-asthmatics ($p<0.05$; Table 5.2). Forty six percent (n=52) of asthmatics were classified as EA and 53% (n=59) NEA. EA was associated with increased FeNO and AHR, and higher ACQ7 scores (all $p<0.05$) compared with NEA. No participants had NA or MGA,[20] and sputum neutrophil levels were not significantly different between EA and NEA (Table 5.2).

Table 5.2. Population characteristics

	Non-asthma (N=62)	Asthma (N=111)	Eosinophilic asthma (N=52)	Non-eosinophilic asthma (N=59)
Age	21.8 (1.2)	19.8 (1.8) **	19.6 (1.7)	20 (1.8)
Males- n (%)	23 (37.1%)	49 (44.1%)	25 (48.1 %)	24 (41.0 %)
Height (cm)	170.1 (9.9)	169.0 (8.6)	169.2 (8.8)	169.0 (8.5)
Weight (Kg)	66.0 (14.8)	67.4 (16.1)	67.3 (16.9)	67.6 (15.4)
Ethnicity				
European- n (%)	55 (89.0%)	90 (81.1%)	42 (81.0%)	48 (81.4%)
Non-European- n (%)	7 (11.0) %)	21 (18.9%)	10 (19.0 %)	11 (18.6%)
FEV_1% predicted	102.2 (12.0)	99.5 (14.4)	97.3 (14.3)	101.2 (14.4)
FVC% predicted	100.4 (9.9)	102.3 (12.9)	101.1 (13.0)	103.2 (13.0)
FEV_1/FVC% predicted	101.4 (7.6)	97.1 (8.0)	95.9 (7.4)	98.0 (8.1)
No asthma medication- n (%)		27 (24.3%)	7 (13.5%)	20 (34.0%)
β-agonist use- n (%)		82 (74.0%)	44 (85.0%)	38 (64.4%) *
ICS use- n (%)		52 (47.0%)	27 (52.0%)	25 (42.4%)
ACQ7 score		1.0 (0 .7)	1.1 (0.8)	0.8 (0 .6) **
Airway hyperreactivity- n (%)	4 (6.4 %)	44 (39.0 %) **	25 (48.1%)	19 (32.2%)
FeNO (ppb)	32.3 (31.1)	61.2 (69.0) **	86.3 (78.0)	39.0 (51.5) **
Atopy- n (%)	26 (43.0%)	92 (83.0%) **	45 (86.5%)	47 (80.0%)
TCC/mL x 10^6	2.5 (1.6-4.2)	1.4 (0.5-3.0) **	2.0 (0.5-3.7)	1.3 (0.5-3.0)
Sputum eosinophils %	0 (0-0)	2.2 (0-7.6)	8.2 (4.6-15.5)	0 (0-1.2) **
Total sputum eosinophils x10^4 mL	0.0 (00.00)	2.0 (0-11.0) **	12.3 (0.05-27.3)	0.0 (0.0-1.3) **
Sputum neutrophils %	20.0 (6.5-35.0)	9.0 (4.3-20.3)	8.3 (4.7-20.3)	9.4 (4.0-20.3)
Total sputum neutrophils x10^4 mL	37.2 (12.0-122.0)	9.3 (2.1-40.0) **	10.0 (4.0-40.2)	8.2 (1.4-40.0)
Sputum macrophages %	79.1 (64.0-92.0)	83.0 (68.1-91.3)	78.0 (60.0-86.0)	87.5 (78.3-94.3)
Total sputum macrophages x10^4 mL	202.2 (123.4-268.1)	96.4 (33.0-240.1) *	105 (33.0-252.0)	86.0 (43.0-233.2)
Sputum lymphocytes %	0.3 (0.0-0.8)	0.0 (0.0-0.7)	0.1 (0.0-0.7)	0.0 (0.0-0.5)
Total sputum lymphocytes x10^4 mL	0.5 (0-1.6)	0.0 (0.0-1.0) *	0.0 (0.0-1.2)	0.0 (0.0-0.6)
Sputum epithelial cells %	0.0 (0.0-0.3)	0.0 (0.0-0.5)	0.0 (0.0-0.3)	0.0 (0.0-0.7)
Total sputum epithelial cells x10^4 mL	0.0 (0.0-0.2)	0.0 (0.0-0.3)	0.0 (0.0-0.2)	0.0 (0.0-0.4)

Data presented as means (SD), median (IQR) or frequency (%). Mann-Whitney tests/Chi-square tests used as appropriate. * P<0.05; ** P<0.01 participants with versus without asthma and non-eosinophilic versus eosinophilic asthma. SPT, skin prick test; FeNO, Fractional exhaled nitric oxide; ICS, inhaled corticosteroid; TCC/mL= total non-squamous cells per mL sputum x10^6

5.3.2 Detectability of mediators

The majority of mediators were above the LOD (Table 5.1). However, TGF-β and SP were detected in 28% and 40% of samples, respectively, while BDNF, angiopoietin, osteopontin, and IFN-γ were detectable in <5% of the samples (Table 5.1). Of these, there were no significant differences in detection frequency between asthma and non-asthma, and EA and NEA (Table 5.1). Mediators detectable in <5% of samples were excluded from subsequent analyses.

5.3.3 Mediator levels

Levels of inflammatory mediators were generally comparable between asthma and non-asthma, except for increased ECP (median: 220.1 ng/mL, IQR: 83.7-839.4 vs 104.5 ng/mL, 44.3-253.8; $p<0.05$), and reduced IL-6 in asthma versus non-asthma (223 pg/mL, 112.1-598.0 vs 333.9 pg/mL, 189.9-790.2; $p< 0.05$; Table 5.3). Inflammatory mediator levels were also comparable between EA and NEA. However, ECP (495.9 ng/mL, 167.7-1232.1 vs 100.3 ng/mL, 49.1-250.4), histamine (5805 pg/mL, 4005-8235 vs 3172.5 pg/mL, 1327.5-4626), and IL-1β (285.3 pg/mL; 194.2-538.7 vs 209.3 pg/mL, 102.2-433.1; all $p< 0.05$) were elevated in EA (Table 5.3).

Of the neural mediators, no differences were observed between asthma and non-asthma, or EA and NEA, except for nociceptin, which was increased in asthmatics (39.1 ng/mL, 16.1-96.8 vs 22.4 ng/mL, 12.6-41.4), and in EA compared with non-asthmatics (54.8 ng/mL, 16.8-129.3 vs 22.4 ng/mL, 12.6-41.4); both $p<0.05$; Figure 5.1). Similarly, there were no significant differences in remodelling mediators between asthma/non-asthma, except for periostin, which was significantly higher in asthmatics (33.8 ng/mL, 7.7-68.4 vs 9.4 ng/mL, 4.1-34.7 $p<0.05$; Table 5.3). When comparing EA with NEA, higher levels of VEGF-A (3.3 ng/mL, 1.6-5.4; vs 2.5 ng/mL 0.6-4.2; $p<0.05$) and periostin (47.7, ng/mL 17.5-97.7 vs 22.1 ng/mL, 5.0-44.3; $p<0.05$; Table 5.3) were detected in EA.

Using a more stringent sputum quality criterion (Supplementary Table S5.1) or restricting the analyses to asthmatics who used ICS (Supplementary Table S5.4), those with AHR (Supplementary Table S5.5), or those using asthma medication in the last 12 months showed comparable results (data not shown). However, due to smaller group numbers, some results were no longer statistically significant.

Table 5.3. Mediator levels

	Non-asthma	Asthma	Eosinophilic asthma	Non-eosinophilic asthma
IL-1β	254 (150.3-765.2)	242.6 (151-469.8)	285.3 (194.2-538.7)	209.3 (102.2-433.1) *
IL-6	333.9 (189.9-790.2)	223 (112.1-598) *	273.8 (122.2-598)	183.1 (75.4-580.5)
IL-8	3631.3 (2281.1-7229.3)	3295.1 (1708.9-5311.8)	3995.6 (2376-5631.5)	2842.8 (1498.3-4446.7)
IL-13	22.9 (16.6-40.5)	30.1 (18.9-46.8)	29.3 (20.5-54.5)	30.1 (18.9-42.3)
ECP α	104.5 (44.3-253.8)	220.1 (83.7-839.4) *	495.9 (167.7-1232.1)	100.3 (49.1-250.4) *
Histamine	4140 (810-7200)	4320 (2790-6975)	5805 (4005-8235)	3172.5 (1327.5-4626) **
NE α	31.0 (16.0-59.0)	25.0 (15.0-59.0)	33.0 (16.0-58.0)	21.5 (13.5-60.5)
Acetylcholine	2248.6 (1983.5-2716.9)	2350.5 (2056.1-2685.4)	2323.7 (2041.7-2878.4)	2389.4 (2083.9-2575.6)
NKA	134.2 (52.9-451.4)	113.1 (48.6-332.5)	113.1 (46.9-445.5)	118.9 (53.8-276.6)
CGRP	931.2 (262-13830.8)	436.5 (262-7566.3)	395.9 (262-8905)	457 (262-4421.3)
Nociceptin α	22.4 (13.0-41.4)	39.1 (16.1-97.0) *	54.8 (16.8-129.3)	27.8 (15.8-77.4)
NGF-β	110.0 (32.4-327.6)	91.8 (32.4-324.9)	89.6 (47.3-327.6)	91.8 (26.5-281.8)
Substance P	21.3 (21.3-21.3)	21.3 (21.3-37.2)	21.3 (21.3-21.3)	21.3 (21.3-56.0)
MMP-1α	0.1 (0-0.2)	0.1 (0-0.2)	0.1 (0-0.2)	0.1 (0-0.1)
MMP-9α	7.4 (2.7-15.1)	4.8 (2.9-9.9)	5.6 (3.5-9.3)	4.1 (2.6-10.4)
TIMP-1α	392.2 (201.5-826.1)	363.8 (129.2-665.1)	460.6 (138.4-687)	286.8 (106-615.4)
VEGF-Aα	2.9 (1.6-5.2)	2.9 (1.2-5.2)	3.3 (1.6-5.4)	2.5 (0.6-4.2) *
Elastinα	13.5 (7.7-21.5)	14.6 (6.9-23.1)	15.7 (9.2-24.5)	9.6 (6.1-22.1)
Periostinα	9.4 (4.1-34.7)	33.8 (7.7-68.4) **	47.7 (17.5-97.7)	22.1 (5-44.3) *
TGF-β	45.3 (45.3-215.1)	45.3 (45.3-136.8)	45.3 (45.3-180.5)	45.3 (45.3-116.1)

All units are pg/mL except α denoting ng/mL, data are presented as median (IQR). Mann-Whitney tests were used. * P<0.05; ** P<0.01 participants with versus without asthma, and non-eosinophilic versus eosinophilic asthma.

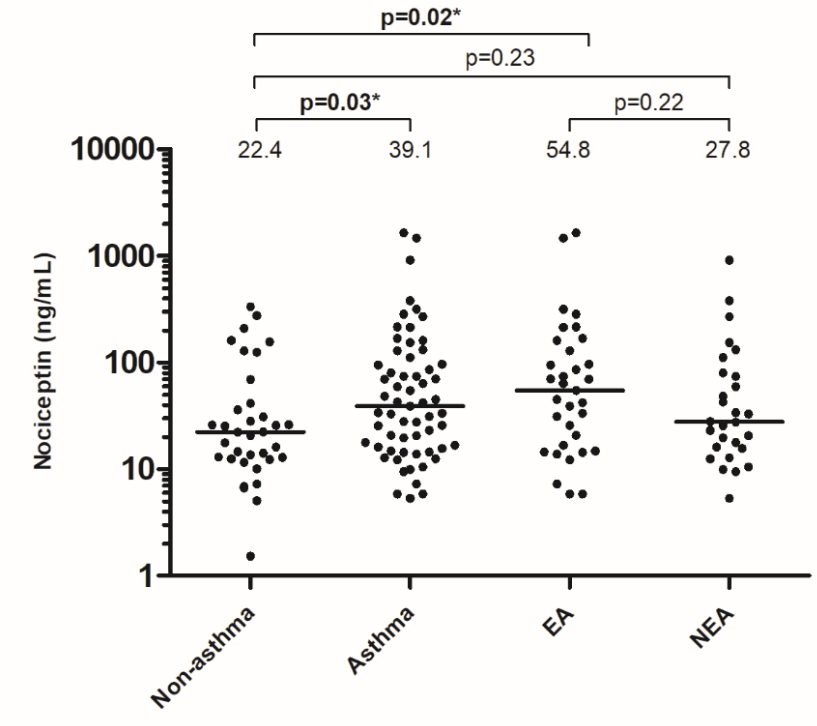

Figure 5.1. Nociceptin levels (ng/mL) in participants with and without asthma, and non-eosinophilic and eosinophilic asthma.Mann-Whitney test was used. * p<0.05. Median data values are expressed at the top of each group.

5.3.4 Correlations between mediators and sputum cells

Correlations between mediators and sputum inflammatory cells in asthmatics are shown in Table 4. Overall, we observed many significant positive correlations (r=0.2-0.6; p=0.01-0.05) between a range of mediators and macrophages, neutrophils, and eosinophils. In asthma, macrophages were significantly correlated with 13/20 (65%), and neutrophils and eosinophils were each significantly correlated with 12/20 (60%) of mediators (Table 5.4). Significant correlations were predominantly found with inflammatory and remodelling mediators, rather than neural mediators. Some of these correlations were moderately strong ($r \geq 0.5$): e.g. between ECP and neutrophils (r=0.53, $p<0.05$) and eosinophils (r=0.53, $p<0.05$), and elastin and macrophages (r=0.50, $p<0.05$). While there was little evidence of correlation between neutrophils and eosinophils and neural mediators, macrophages were significantly ($p<0.05$) correlated with 3/6 (50%) of neural mediators; in particular, NKA (r=0.27), nociceptin (r=0.30), and NGF-β (r=0.19). Similar patterns were observed in non-asthmatics (Supplementary Table S5.2) and when asthma was stratified into EA/NEA (Table 5.4).

Table 5.4. Correlations between mediators and sputum leukocyte populations

	Total Macrophages			Total Epithelial cells			Total Lymphocytes			Total Neutrophils			Total Eosinophils		
	Asthma	**EA**	**NEA**	**Asthma**	**EA**	**NEA**	**Asthma**	**EA**	**NEA**	**Asthma**	**EA**	**NEA**	**Asthma**	**EA**	**NEA**
Correlation coefficient (r)															
IL-1β	0.24*	0.22	0.25*	0.01	-0.05	0.07	0.11	0.01	0.17	0.25**	0.23	0.26*	0.25*	0.16	0.13
IL-6	0.40**	0.35*	0.41**	-0.04	0.14	0.05	0.08	-0.02	0.16	0.37**	0.33**	0.39**	0.33*	0.46**	0.32*
IL-8	0.48**	0.43**	0.53**	0.03	0.01	0.06	0.17	0.09	0.24	0.44**	0.38**	0.49**	0.34**	0.43**	0.23
IL-13	-0.04	0.04	-0.12	-0.09	0.00	-0.18	-0.05	0.02	-0.12	0.04	0.19	-0.09	0.11	0.12	0.10
ECP$^{\alpha}$	0.36**	0.36**	0.26	0.12	-0.25	0.01	0.04	0.00	0.18	0.53**	0.51**	0.55**	0.53**	0.56**	0.02
Histamine	-0.10	-0.13	0.30	-0.23	-0.08	-0.36	-0.15	0.10	-0.37*	0.03	0.03	-0.07	0.45**	0.08	0.23
NE$^{\alpha}$	-0.07	-0.13	-0.05	0.26*	0.34	0.26	0.35**	0.34**	0.32*	-0.11	-0.17	-0.09	-0.04	-0.02	-0.42*
Acetylcholine	-0.02	-0.29	0.28	-0.04	-0.06	-0.05	-0.15	0.13	0.02	-0.05	-0.34*	0.29	-0.09	-0.28	0.01
NKA	0.27**	0.33*	0.18	0.07	0.08	0.09	0.01	-0.02	0.05	0.10	0.07	0.15	0.12	0.25	0.11
CGRP	-0.10	-0.12	-0.05	-0.11	-0.03	-0.24	-0.10	-0.19	0.01	-0.19	-0.21	-0.11	-0.09	-0.22	0.10
Nociceptin$^{\alpha}$	0.30*	0.40*	0.16	0.02	-0.03	0.11	0.08	0.03	0.13	0.33**	0.47**	0.20	0.23	0.41*	-0.19
NGF-β	0.20*	0.32*	0.07	-0.09	-0.09	-0.07	0.15	0.11	0.18	0.18	0.28	0.10	0.18	0.44*	-0.01
Substance P	-0.14	-0.14	-0.13	0.06	-0.05	0.13	-0.01	0.22	-0.15	-0.02	0.13	-0.10	0.23*	-0.04	-0.33*
MMP-1$^{\alpha}$	0.38*	0.23	0.50**	-0.05	-0.22	0.10	0.10	0.11	0.11	0.33**	0.23	0.39**	0.23*	0.26	0.22
MMP-9$^{\alpha}$	0.41*	0.34*	0.44**	0.01	0.14	-0.07	0.15	-0.02	0.26	0.32**	0.42**	0.38**	0.20*	0.09	0.17
TIMP-1$^{\alpha}$	0.44**	0.29*	0.56**	-0.16	-0.24	-0.08	0.15	0.12	0.21	0.44**	0.32*	0.52**	0.35**	0.52**	0.22
VEGF-A$^{\alpha}$	0.38**	0.22	0.47**	-0.07	-0.17	0.02	0.12	0.08	0.16	0.33**	0.22	0.38**	0.29**	0.31*	0.17
Elastin$^{\alpha}$	0.50**	0.55**	0.41*	-0.02	-0.15	0.22	-0.07	-0.19	0.16	0.46**	0.39*	0.54**	0.26*	0.41**	0.02
Periostin$^{\alpha}$	0.42**	0.34*	0.52**	0.14	-0.03	0.36*	0.01	-0.01	0.15	0.32*	0.24*	0.47*	0.38*	0.36**	0.14
TGF-β	0.02	-0.14	0.21	-0.09	-0.09	-0.09	0.19	0.24	0.16	0.283*	0.31	0.28	0.20	0.16	0.14

All units are pg/mL except $^{\alpha}$ denoting ng/mL. Data are presented as Spearman's correlation coefficient. * P<0.05; ** P<0.01.

5.3.5 Correlations between mediators

Of all correlations between individual mediators in asthmatics (Table 5.5), 35% (73/210) reached statistical significance. The strongest correlations were observed between IL-8 and IL-1β ($r=0.70$, $p<0.05$) and IL-8 and MMP-9 ($r=0.63$, $p<0.05$), with moderate correlations observed between remodelling mediators or between remodelling and inflammatory mediators; e.g. IL-8 correlated with TIMP-1 and VEGF-A ($r=0.61$ and $r=0.55$, respectively; both $p<0.05$) and ECP correlated with elastin and periostin ($r=0.60$ and $r=0.58$, respectively; both $p<0.05$). Correlations between neural mediators were generally weaker, and there were no moderate correlations between neural mediators and other mediators, except for nociceptin, which was associated with ECP and periostin ($r=0.54$ and $r=0.52$, respectively; both $p<0.05$). Similar patterns were observed when asthma was stratified into EA/NEA (Supplementary Table S5.3).

Table 5.5 Correlations between mediators in participants with asthma

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
	Correlation coefficient (r)																			
1. IL-1β	1																			
2. IL-6	0.54*	1																		
3. IL-8	0.70*	0.52*	1																	
4. IL-13	0.41*	0.23*	0.16	1																
5. ECP$^{\alpha}$	0.14	0.32*	0.32*	-0.16	1															
6. Histamine	0.06	0.03	0.22	0.07	0.28*	1.00														
7. NE$^{\alpha}$	-0.11	-0.09	-0.14	-0.29*	0.01	-0.21	1													
8.Acetylcholine	-0.05	-0.14	0.09	-0.14	-0.01	0.14	0.06	1												
9. NKA	0.08	0.28*	0.24*	-0.37*	0.04	0.11	0.10	0.04	1											
10. CGRP	0.25*	-0.17	0.09	0.40*	-0.18	0.13	-0.19	-0.13	-0.37*	1										
11. Nociceptin$^{\alpha}$	-0.14	0.26*	0.07	-0.18	0.54*	0.21	0.05	0.01	0.46*	-0.42*	1									
12. NGF-β	0.37*	0.47*	0.26*	0.41*	0.04	-0.19	0.03	-0.27*	-0.02	0.17	0.09	1								
13. Substance P	-0.20	-0.35*	-0.20	0.12	0.07	-0.01	0.01	0.2	-0.38*	-0.01	-0.09	-0.29*	1							
14. MMP-1$^{\alpha}$	0.45*	0.64*	0.53*	-0.02	0.38*	-0.12	-0.11	-0.14	0.28*	-0.24	0.34*	0.14	-0.29*	1						
15. MMP-9$^{\alpha}$	0.54*	0.17	0.63*	0.15	0.2	0.11	-0.08	0.03	0.12	0.29*	-0.02	0.07	-0.16	0.33*	1					
16. TIMP-1$^{\alpha}$	0.46*	0.52*	0.61*	0.11	0.30*	-0.17	-0.10	-0.08	0.09	0.03	0.14	0.32*	-0.17	0.57*	0.46*	1				
17. VEGF-A$^{\alpha}$	0.35*	0.55*	0.55*	-0.08	0.27*	-0.02	-0.07	-0.15	0.27*	-0.17	0.27*	0.10	-0.39*	0.69*	0.39*	0.65*	1			
18. Elastin$^{\alpha}$	0.10	0.38*	0.36*	-0.31*	0.60*	0.16	-0.06	-0.02	0.32*	-0.24	0.46*	0.06	-0.14	0.50*	0.18	0.30*	0.41*	1		
19. Periostin$^{\alpha}$	-0.04	0.23	0.29*	-0.23	0.58*	0.32*	0.07	-0.10	0.40*	-0.16	0.52*	0.05	-0.16	0.35*	0.23	0.19	0.30*	0.60*	1	
20. TGF-β	0.08	0.15	0.18	-0.01	0.17	0.21	-0.14	-0.01	0.02	-0.17	0.33*	0.09	-0.23	0.05	0.19	0.15	0.21	-0.13	0.0	1

All units are pg/mL except $^{\alpha}$ denoting ng/mL. Data are presented as Spearman's correlation coefficient. * P<0.05.

5.4 Discussion

In this study we found increased airway levels of nociceptin, periostin, and ECP in asthma, and IL-1β, histamine, periostin, VEGF-A, and ECP in EA compared with NEA. Levels of all other inflammatory, neural, and remodelling mediators did not differ between asthmatic and non-asthmatic participants, or between EA and NEA. We found no evidence that neural or remodelling mediators were increased in NEA. However, many significant associations were observed between mediators representing inflammatory, neural, and remodelling pathways and between airway inflammatory cells and neural/remodelling mediators, suggesting that neural and remodelling processes are likely to occur alongside, rather than in the absence of inflammation.

Intriguingly, despite similar levels of most neural mediators between asthmatics and non-asthmatics, levels of nociceptin were elevated in asthma. Significantly higher levels of nociceptin were also found in EA compared with non-asthmatics (Figure 5.1; $p<0.05$). Nociceptin is an endogenous opioid-like peptide that acts through the nociceptin receptor.[525] In guinea pigs, administration of *exogenous* nociceptin inhibits bronchoconstriction[526] and capsaicin-induced cough;[527] however, little is known about its function in humans. Whilst there is evidence that nociceptin receptor expression may be altered in asthma,[528] to our knowledge only one previous study has assessed sputum nociceptin levels in humans. In a study of 85 older (>50 years) asthmatics, Singh *et al*[529] found elevated nociceptin levels in severe asthmatics compared with non-asthmatics; nociceptin was also positively associated with sputum eosinophils but not with lung function. We also found no association between nociceptin and lung function, AHR, or clinical parameters in asthmatics (data not shown). Taken together, this suggests a role for nociceptin in asthma (particularly EA) in older and younger populations, and across the spectrum of asthma severity. We speculate that increased

endogenous nociceptin production may ameliorate bronchoconstriction or cough in asthma; however, this remains unclear. If confirmed in other studies, the nociceptin/nociceptin receptor axis may present a novel therapeutic avenue, as suggested previously.[529]

The lack of difference observed with other neural mediators may be because most asthmatics had well-controlled asthma and increased neurogenic activity (previously associated with severe asthma/exacerbations)[285,287,515] may not have been present. This may, in part, reflect the transient and highly localised nature of neuropeptide signalling in the airways,[530] which potentially make it difficult to detect some sputum mediators. Another possibility is that these mediators may not play a role in asthma and/or NEA. This does not, however, preclude a role for other mediators or neural mechanisms (either not assessed, or unable to be assessed using sputum) even in well-controlled asthma. Indeed, using capsaicin challenge testing, we have previously shown that heightened sensory nerve reactivity may be important, particularly in NEA.[499]

Of the remodelling mediators, we found increased periostin and VEGF-A levels in asthma, and in EA versus NEA. This is consistent with previous studies showing that periostin is associated with TH_2-mediated inflammation[437] and increased eosinophil recruitment/degranulation,[531] whilst VEGF-A is associated with eosinophilic inflammation.[518] The lack of difference in other remodelling mediators is likely due to the absence of severe asthma or NA in our study. In particular, increased MMP-1, MMP-9, [520] TIMP-1[519] and TGF-β[517] were reported in severe asthma, and associated with NA and elevated levels of IL-8.[520]

We found no evidence of increased levels of remodelling mediators in NEA, suggesting that remodelling may not be important in this group. However, whilst remodelling may not be active at the time of assessment, previous infections, or inflammation[24] may have resulted in remodelling-related abnormalities that may not be detectable using sputum. For example,

increased ASM thickness has been reported in PGA in the absence of inflammation[226] and can currently only be assessed using bronchial biopsies and imaging techniques.[340]

Consistent with previous studies,[532,533] we found that remodelling mediators correlated with inflammatory mediators and inflammatory cell populations (especially macrophages, neutrophils, and eosinophils). This supports previous data showing that remodelling and inflammation are strongly associated in asthma.[24] It also suggests that airway leukocytes, such as macrophages and neutrophils (which can produce MMPs, TIMP-1,[534] TGF-β,[535] both *in vitro* and *in vivo*), produce these mediators in the airways. Interestingly, we also found that macrophages were associated with NKA, nociceptin, and NGF-β, suggesting that they may potentially be a source of these mediators, as reported previously.[298,536] However, with the exception of nociceptin, levels of these mediators were not increased in either phenotype, and the association patterns observed between inflammatory cells and mediators did not differ between EA and NEA, suggesting that this was not specific to phenotypes.

Of the inflammatory mediators, we found that ECP was increased in asthma compared with non-asthma, and levels of some mediators associated with TH_1 and TH_2 inflammation (including IL-1β, ECP, and histamine) were increased in EA compared with NEA. This is consistent with previous studies showing elevated levels of inflammatory markers (TH_1 and TH_2) associated with EA.[22,512] Previous studies have suggested that neutrophil-associated markers (i.e. IL-8) are increased in NEA, particularly in NA, which has been reported to make up 20-30% of NEA.[204,460] However, whilst we found increased neutrophils in non-asthmatics compared with asthmatics (statistically significant only when presented as total neutrophil numbers), no evidence of increased neutrophils or neutrophil-associated mediators was found in NEA, and no participants were identified as NA. Some other studies have also shown higher sputum neutrophil levels in non-asthmatics,[22] but results have not always been

consistent. The reasons for increased sputum neutrophil levels in non-asthmatics observed in this study are unclear.

There were several limitations. Firstly, 47% of asthmatics were using ICS, which may affect sputum cellular composition and mediator profiles.[537] However, when restricting analyses to those using ICS, results remained similar (with minor variation; Supplementary Table S5.4). Secondly, as lung function is often unaffected in young patients with asthma, particularly those undergoing treatment,[22] and repeated lung function testing or use of other objective measures to define asthma was not possible, some misclassification may have occurred. However, we consider that any bias introduced as a result is minimal given that this approach has been used in previous studies[57,496] and compares well with clinical asthma definitions.[57] Also, the main findings were similar when applying a more stringent asthma definition, i.e. restricting analysis to only those using ICS (Supplementary Table S5.4) or any asthma medication in the last 12 months (data not shown), or with AHR (Supplementary Table S5.5), suggesting that findings are unlikely to be due to misclassification. Thirdly, asthmatics were generally well-controlled young adults, recruited from the general population rather than a tertiary setting, making comparisons with previous studies (many of which assessed severe/uncontrolled asthma in adults) difficult. Fourthly, as our study was conducted in a community rather than tertiary setting, we did not ask participants to abstain from LABA and antihistamines for long periods of time due to safety concerns. Finally, mediator detection may be adversely affected by sputum sample quality and dilutions.[338] To test the effect of sample quality and dilution, additional analyses were conducted using a more stringent quality criteria, or with mediator concentrations normalised to sputum leukocyte numbers/ml. This showed similar results with only minor variations; in particular, p-values generally reduced resulting in a few more statistically significant findings. This suggests that sample quality (Supplementary Table S5.1) and dilution (Appendix 2) resulted in only a slight bias.

In conclusion, although we found increased sputum levels of nociceptin in asthma and IL-1β, histamine, periostin, VEGF-A, and ECP in EA, there was no evidence of increased neural and remodelling mediators in NEA. However, we did find that inflammatory cells/mediators were often associated with neural and remodelling mediators, suggesting that these mechanisms may coexist with inflammation. Our study suggests a role for nociceptin, particularly in EA, which is intriguing and warrants further study. More generally, further studies using alternative approaches, or assessing asthma during exacerbation/poor control, may be required to comprehensively assess neural and remodelling pathways in asthma.

5.5 Supplementary material

Supplementary Table S5.1. Mediator levels in participants with high sputum sample quality

	Non-asthma	Asthma	Eosinophilic asthma	Non-eosinophilic asthma
IL-1β	273.8 (132.3-781.0)	276.8 (156.6-453.6)	367.1 (242.6-567.0)	187.7 (111.2-293.9) **
IL-6	406.1 (249.3-801.4)	252.9 (162.0-603.0)	273.8 (199.4-603.0)	251.1 (136.8-731.7)
IL-8	3680.0 (2307.0-7687.0)	3466.0 (2078.0-4583.0)	3956.8 (3081.6-5612.8)	3003.5 (1984.1-4447.0)
IL-13	24.1 (13.3-43.9)	30.2 (18.9-54.5)	33.3 (22.1-54.9)	27.7 (18.9-35.2)
ECP$^{\alpha}$	178.8 (41.4-325.0)	256.5 (87.2-1107.7) *	881.1 (222.6-1382.6)	101.4 (49.1-256.5) **
Histamine	4298.0 (992.3-7346)	4320.0 (2610.0-7335.0)	6120.0 (4905.0-10192.5)	2880.0 (30.0-3645.0) **
NE$^{\alpha}$	27 (16.0-55.0)	33.0 (16.0-62.0)	28.5 (16.5-42.5)	33.0 (15.0-63.0)
Acetylcholine	2247.0 (1974.0-2720.0)	2241.0 (2055.0-2502)	2187.0 (1894.0-2452.0)	2294.0 (2083.0-2546.0)
NKA	140.5 (52.2-467.0)	223.9 (53.8-629.2)	229.8 (34.2-693.3)	202.9 (89.57-357.1)
CGRP	694.3 (262.0-13039.0)	624.8 (262.3-14216.0)	5447 (262.0-16234.0)	327.5 (262.0-2378.0)
Nociceptin$^{\alpha}$	23.9 (13.4-83.4)	42.7 (19.7-76.0) *	45.3 (21.0-95.0)	33.0 (16.1-74.2)
NGF-β	96.3 (28.0-327.6)	149.4 (32.4-327.6)	184.4 (66.7-327.6)	91.8 (32.4-281.9)
Substance P	21.3 (21.3-21.3)	21.3 (21.3-37.2)	21.3 (21.3-21.3)	21.3 (21.3-56.0)
MMP-1$^{\alpha}$	0.08 (0.01-0.19)	0.09 (0.03-0.2)	0.09 (0.04-0.2)	0.07 (0.1-0.1)
MMP-9$^{\alpha}$	8.1 (2.7-15.1)	5.5 (2.7-9.8)	5.7 (3.7-11.9)	3.9 (2.6-8.3)
TIMP-1$^{\alpha}$	442.2 (209.2-868.2)	407.1 (184.3-665.3)	535.2 (285.1-753.6)	330.7 (164.8-596.2)
VEGF-A$^{\alpha}$	2.9 (1.6-5.6)	3.4 (1.8-5.2)	3.5 (1.9-7.1)	3.4 (1.5-4.5)
Elastin$^{\alpha}$	15.5 (8.5-25.2)	19.0 (8.9-25.0)	18.8 (12.5-31.5)	15.3 (6.9-24.4)
Periostin$^{\alpha}$	9.9 (4.05-34.6)	41.8 (20.2-92.3) *	50.2 (35.3-107.7)	33.7 (7.6-60.3)
TGF-β	45.3 (45.3-215.1)	45.3 (45.3-136.8)	45.3 (45.3-180.5)	45.3 (45.3-116.1)

All units are pg/mL except α denoting ng/mL. Data are presented as median (IQR). Mann-Whitney tests were used. * P<0.05; ** P<0.01 participants with versus without asthma, and non-eosinophilic versus eosinophilic asthma.

Supplementary Table S5.2. Correlations between mediators and sputum cell populations in non-asthmatic participants

	Non-asthmatics				
	Total Macrophages	**Total Epithelial cells**	**Total Lymphocytes**	**Total Neutrophils**	**Total Eosinophils**
	Correlation coefficient (r)				
IL-1β	0.20	-0.13	0.06	0.45**	0.11
IL-6	0.30*	-0.16	0.05	0.57**	0.23
IL-8	0.24	-0.07	-0.05	0.47**	0.09
IL-13	-0.02	0.12	0.11	0.18	0.22
ECP$^{\alpha}$	0.16	-0.40*	-0.22	0.50**	0.26
Histamine	-0.26	-0.17	0.33*	0.01	0.21
NE$^{\alpha}$	-0.31	-0.08	0.27	-0.38*	-0.07
Acetylcholine	0.21	0.01	-0.05	-0.11	0.03
NKA	0.31*	-0.16	-0.01	0.08	0.15
CGRP	-0.27	0.07	0.21	-0.23	-0.06
Nociceptin$^{\alpha}$	0.40*	-0.28	0.03	0.45**	0.28
NGF-β	-0.06	-0.06	0.16	0.11	0.13
Substance P	0.01	0.23	-0.15	0.11	0.03
MMP-1$^{\alpha}$	0.24	-0.33	0.07	0.51**	0.17
MMP-9$^{\alpha}$	0.22	-0.05	-0.06	0.42**	0.03
TIMP-1$^{\alpha}$	0.10	-0.03	0.19	0.47**	0.08
VEGF-A$^{\alpha}$	0.46**	-0.19	0.03	0.55**	0.11
Elastin$^{\alpha}$	0.39**	-0.44**	-0.15	0.43**	0.43**
Periostin$^{\alpha}$	0.33	-0.34*	-0.14	0.34*	0.42*
TGF-β	0.25	-0.13	0.24	0.21	0.27

All units are pg/mL except $^{\alpha}$ denoting ng/mL. Data are presented as Spearman's correlation coefficient. * P<0.05; ** p<0.01.

Supplementary Table S5.3. Correlations between mediators in EA (above the diagonal line) and NEA (below the diagonal line)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1. IL-1β	**1**	0.46*	0.68*	0.16	0.05	-0.08	-0.16	-0.19	0.22	0.26	-0.29	0.23	-0.32	0.37*	0.41*	0.36*	0.24	0.1	-0.08	-0.01
2. IL-6	0.55*	**1**	0.40*	0.19	0.22	0.02	-0.13	-0.30	0.42*	-0.13	0.35*	0.44*	-0.40*	0.54*	-0.07	0.41*	0.51*	0.33	0.26	0.11
3. IL-8	0.64*	0.54*	**1**	0.05	0.09	0.17	-0.07	-0.01	0.39*	-0.05	0.08	0.28*	-0.29	0.43*	0.46*	0.57*	0.41*	0.28	0.22	0.07
4. IL-13	0.60*	0.25	0.22	**1**	-0.04	-0.09	0.19	-0.08	-0.39*	0.32	-0.08	0.40	0.20	-0.22	0.04	0.08	-0.28*	-0.22	0.00	-0.07
5. ECP$^{\alpha}$	0.1	0.36	0.41*	-0.23	**1**	0.08	0.02	-0.16	-0.14	0.16	-0.27	-0.09	0.03	-0.24	0.03	-0.12	-0.17	-0.05	-0.03	0.02
6. Histamine$^{\alpha}$	0.01	-0.05	0.09	0.3	0.19	**1**	-0.08	0.16	0.27	0.00	0.41*	-0.15	0.07	0.02	0.15	-0.04	-0.03	0.19	0.38*	0.24
7. NE	-0.06	-0.12	-0.23	-0.37*	0.04	-0.43*	1	-0.1	0.01	0.02	-0.12	-0.00	0.11	-0.17	0.01	0.01	0.07	-0.22	0.04	-0.13
8.Acetylcholine	0.12	0.08	0.19	-0.21	0.26	-0.06	0.3	**1**	-0.01	-0.11	-0.03	-0.24	0.10	-0.26	-0.16	-0.16	-0.20	-0.18	-0.19	-0.06
9. NKA	-0.07	0.18	0.06	-0.38*	-0.09	-0.14	0.34	0.1	**1**	-0.43*	0.53*	0.09	-0.41*	0.47*	0.20	0.17	0.48*	0.45*	0.36*	0.10
10. CGRP	0.23	-0.23	0.16	0.47*	-0.2	0.16	-0.46*	-0.19	-0.31	**1**	-0.52*	0.24	0.01	-0.36*	0.29	-0.02	-0.29	-0.05	-0.08	-0.38*
11.Nociceptin$^{\alpha}$	-0.03	0.24	0.04	-0.25	0.45*	-0.27	0.25	0.15	0.34	-0.3	**1**	-0.02	0.04	0.44*	-0.05	0.06	0.31	0.48*	0.67*	0.22
12. NGF-β	0.49*	0.46*	0.21	0.41*	0.14	-0.09	0.07	-0.24	-0.15	0.1	0.25	**1**	-0.08	0.04	-0.08	0.33*	0.06	0.05	0.19	-0.06
13. Substance P	-0.08	-0.32*	-0.1	0.05	0.02	0.03	-0.08	0.26	-0.36*	-0.06	-0.22	-0.44*	**1**	-0.26	-0.13	-0.24	-0.38*	-0.23	0.04	-0.13
14. MMP-1$^{\alpha}$	0.49*	0.70*	0.58*	0.16	0.29	-0.24	-0.07	0.04	0.06	-0.03	0.23	0.24	-0.26	**1**	0.14	0.44*	0.69*	0.58*	0.39*	0.07
15. MMP-9$^{\alpha}$	0.62*	0.29*	0.73*	0.24	0.22	0.03	-0.2	0.3	0.02	0.37*	-0.03	0.15	-0.19	0.48*	**1**	0.23	0.22	0.2	0.29	0.07
16. TIMP-1$^{\alpha}$	0.53*	0.55*	0.63*	0.13	0.27	-0.12	-0.35	0.05	0.03	0.21	0.25	0.26	-0.10	0.69*	0.65*	**1**	0.54*	0.27	0.14	-0.02
17. VEGF-A$^{\alpha}$	0.38*	0.55*	0.62*	0.09	0.27	-0.17	-0.26	-0.09	0.05	0.03	0.20	0.11	-0.34*	0.67*	0.53*	0.74*	**1**	0.36*	0.12	0.17
18. Elastin$^{\alpha}$	0.1	0.42*	0.42*	-0.41*	0.72*	-0.05	0.17	0.16	0.14	-0.49*	0.37	0.11	-0.08	0.45*	0.11	0.33	0.48*	**1**	0.52*	-0.29
19. Periostin$^{\alpha}$	-0.04	0.19	0.42*	-0.43*	0.39*	-0.03	0.09	0.12	0.40*	-0.20	0.24	-0.16	-0.33	0.34	0.22	0.33	0.49*	0.65*	**1**	0.05
20. TGF-β	0.1	0.25	0.23	0.15	0.23	0.02	0.20	0.10	-0.13	0.12	0.45*	0.26	-0.28	0.03	0.29	0.33	0.24	0.0	-0.05	**1**

All units are pg/mL except α denoting ng/mL. Data are presented as Spearman's correlation coefficient. * P<0.05.

Supplementary Table S5.4. Mediator levels in participants with asthma who used ICS and non-asthmatics

	Non-asthma	Asthma	Eosinophilic asthma	Non-eosinophilic asthma
IL-1β	254 (150.3-765.2)	224.6 (122.9-469.8)	242.6 (156.6-469.8)	193.5 (92-457.4)
IL-6	333.9 (189.9-790.2)	251.1 (72.5-603)	291.6 (112.1-446.4)	178.7 (38.3-681)
IL-8	3631.3 (2281.1-7229.3)	3110 (1529.1-4560.3)	3110 (1490.4-5904.9)	3092.7 (1558.6-4332.8)
IL-13	22.9 (16.6-40.5)	30.2 (18.9-52.2)	30.2 (20.5-54.5)	30.2 (18.9-43.7)
ECP$^{\alpha}$	104.5 (44.3-253.8)	309.5 (72.5-1167.9) *	738.6 (222.7-1232.1)	192.9 (56.3-362.5) *
Histamine	4140 (810-7200)	4603.5 (2835-6615)	5670 (2961-18135)	3285 (2790-5175)
NE$^{\alpha}$	31.0 (16.0-59.0)	17.5 (12.0-46.0)	18 (7-38)	16 (14-76)
Acetylcholine	2248.6 (1983.5-2716.9)	2471.1 (2097.7-2830.6)	2481.7 (2310.3-2903.8)	2471.1 (2050.4-2704.2)
NKA	134.2 (52.9-451.4)	133.9 (46.9-315.3)	113.1 (39.8-445.5)	133.9 (53.8-276.6)
CGRP	931.2 (262-13830.8)	262 (262-3944.7)	329 (262-5274.1)	262 (262-2515.9)
Nociceptin$^{\alpha}$	22.4 (12.6-41.4)	50.1 (16.2-142) *	54.8 (16.8-161.2)	42.8 (15.7-111.5)
NGF-β	110.0 (32.4-327.6)	82.4 (18-295.7)	87.3 (32.4-327.6)	46.1 (12-119.9)
Substance P	21.3 (21.3-21.3)	21.3 (21.3-54.1)	21.3 (21.3-28.6)	21.3 (21.3-85.7)
MMP-1$^{\alpha}$	0.1 (0-0.2)	0.1 (0-0.2)	0.1 (0-0.3)	0.1 (0-0.1)
MMP-9$^{\alpha}$	7.4 (2.7-15.1)	3.9 (1.6-9.3)	4.4 (1.6-6.8)	3.6 (1.8-11.3)
TIMP-1$^{\alpha}$	392.2 (201.5-826.1)	350.6 (101.7-644.7)	375 (104.8-687)	274.6 (81.9-586.6)
VEGF-A$^{\alpha}$	2.9 (1.6-5.2)	2.5 (1.1-5.2)	2.9 (1.1-6.9)	1.8 (0.5-4.1)
Elastin$^{\alpha}$	24.5 (15.7-)	13.4 (7.7-27.9)	16.9 (9.2-31.8)	10.4 (7.3-18.9)
Periostin$^{\alpha}$	9.4 (4.1-34.7)	25.2 (6.5-56) *	40.1 (17.6-92.3)	17.6 (5.9-32.4)
TGF-β	45.3 (45.3-215.1)	45.3 (45.3-158.6)	45.3 (45.3-180.5)	45.3 (45.3-45.3)

All units are pg/mL except α denoting ng/mL. Data are presented as median (IQR). Mann-Whitney tests were used. * P<0.05; ** P<0.01 participants with versus without asthma, and non-eosinophilic versus eosinophilic asthma.

Supplementary Table S5.5. Mediator levels in participants with asthma who had AHR and non-asthmatics

	Non-asthma	Asthma	Eosinophilic asthma	Non-eosinophilic asthma
IL-1β	254 (150.3-765.2)	242.6 (156.6-433.4)	285.3 (171.9-453.6)	231.5 (102.2-433.4)
IL-6	333.9 (189.9-790.2)	252.9 (112.1-603)	252.9 (112.1-603)	215.8 (87.8-1118.3)
IL-8	3631.3 (2281.1-7229.3)	3465.9 (1800.5-5311.8)	3929.4 (1800.5-5631.5)	3003.5 (1467.5-5311.8)
IL-13	22.9 (16.6-40.5)	30.2 (20.5-55.4)	38.3 (24.3-84.6)	30.2 (18.9-45)
ECP$^{\alpha}$	104.5 (44.3-253.8)	267.8 (148.4-685.9) **	528.8 (223.4-1169.9)	221.7 (102.4-309.5)
Histamine	4140 (810-7200)	5175 (2880-7380)	5737.5 (4680-8235)	3555 (2790-5175) *
NE$^{\alpha}$	31.0 (16.0-59.0)	16.0 (13.0-77.0)	20.5 (13-77)	16 (15-33)
Acetylcholine	2248.6 (1983.5-2716.9)	2294.3 (2041.7-2471.1)	2131.6 (1894-2685.4)	2401.4 (2294.3-2471.1)
NKA	134.2 (52.9-451.4)	89.6 (28.6-233.4)	64.9 (22.4-246.2)	133.2 (48.6-231.5)
CGRP	931.2 (262-13830.8)	1496.7 (262-16352.5)	1425.6 (262-14851.6)	1496.7 (262-17853.4)
Nociceptin$^{\alpha}$	22.4 (12.6-41.4)	45.3 (15.7-96.8) *	50.1 (16.8-161.2)	23.2 (15.7-74.2)
NGF-β	110.0 (32.4-327.6)	139.1 (32.4-327.6)	135.2 (32.4-327.6)	144.2 (12-327.6)
Substance P	21.3 (21.3-21.3)	21.3 (21.3-59.9)	21.3 (21.3-59.9)	21.3 (21.3-70.8)
MMP-1$^{\alpha}$	0.1 (0-0.2)	0.1 (0-0.2)	0.1 (0-0.1)	0.1 (0-0.3)
MMP-9$^{\alpha}$	7.4 (2.7-15.1)	4.9 (2.9-12)	5.1 (3.2-20.7)	4.4 (1.4-12)
TIMP-1$^{\alpha}$	392.2 (201.5-826.1)	502.5 (281.3-757.9)	535.2 (356.8-1000)	433 (138.1-719.8)
VEGF-A$^{\alpha}$	2.9 (1.6-5.2)	2.9 (1.5-5.2)	2.9 (1.5-5.4)	2.8 (1.4-5)
Elastin$^{\alpha}$	24.5 (15.7-)	15.7 (10.4-23.1)	16.3 (11.8-23.1)	15.3 (8.9-22.9)
Periostin$^{\alpha}$	9.4 (4.1-34.7)	38.7 (17.6-157.5) **	45.9 (17.6-176.4)	36 (13.5-134.6)
TGF-β	45.3 (45.3-215.1)	45.3 (45.3-116.1)	80.7 (45.3-116.1)	45.3 (45.3-116.1)

All units are pg/mL except α denoting ng/mL. Data are presented as median (IQR). Mann-Whitney tests were used. * P<0.05; ** P<0.01 participants with versus without asthma, and non-eosinophilic versus eosinophilic asthma.

Chapter 6 Bronchodilator response in eosinophilic and non-eosinophilic asthma

Hajar Ali, Collin Brooks, Sanne Muller, Rachelle Gabriels, Julian Crane, Philip Pattemore, Thorsten Stanley, Jeroen Douwes

Background: Although asthma phenotypes may have different underlying pathologies and response to ICS, it is unclear whether they have differential responses to bronchodilators. This study assessed the response to SAMA (IB) and SABA in EA and NEA.

Methods: 83 young adults (14-21 years) with asthma underwent combined hypertonic saline challenge/sputum induction, FeNO measurement, SPT, spirometry, and BDR tests with 40 μg IB and 200 μg salbutamol. Positive BDR was defined as a ≥8% increase from post-saline challenge FEV_1. EA and NEA were defined using a 2.5% sputum eosinophil cut-point.

Results: In all asthmatics, BDR was significantly increased with salbutamol compared to IB ($P<0.05$). Overall, 61% (n=54) responded to both treatments, 8% (n=7) responded to IB alone, 9% (n=8) responded to salbutamol alone, and 17% (n=14) responded to neither treatment. When stratified into EA (n=37) and NEA (n=46), BDR was significantly higher in EA than NEA with salbutamol (18.3% (14.2, 28.5%) vs 14.0% (5.7, 20.1%); $P<0.05$).

Conclusion: Salbutamol is the most effective bronchodilator in young people with generally-well controlled asthma. EA was associated with a greater response to either bronchodilator. The finding of a group responding to IB but not salbutamol suggests that the current "one size fits all" treatment approach in guidelines may not adequately address the needs of all asthmatics.

In preparation for submission

6.1 Introduction

In the early twentieth century, anticholinergics were considered a first-line asthma treatment.[538] However, in subsequent decades they have been largely superseded by adrenergic agonists, which are generally more effective and well-tolerated.[539] Studies comparing short acting anticholinergic/antimuscarinic (SAMA) agents, such as IB, with SABAs; currently routinely used as reliever medication), have also shown that SABAs generally provide greater bronchodilation overall.[540] Despite this, some subgroups, such as older patients,[541] or those with nocturnal,[542] intrinsic or non-atopic asthma,[543] may respond better to anticholinergics. Additionally, the need for more effective treatments for poorly controlled or treatment-refractory asthma has led to renewed interest in the use of anticholinergics,[544] and recent GINA guidelines[2] suggest the use of tiotropium bromide (a LAMA) for severe and uncontrolled asthma.[545]

In the last few decades, it has become increasingly clear that there is considerable pathophysiological heterogeneity underlying asthma. In particular, studies using induced sputum to assess airway inflammation often find that, alongside the well-characterised TH_2-mediated EA phenotype, NEA makes up ~50% of all asthmatics[417] and is largely charactered by an absence of airway inflammation.[22] It has been suggested that neural pathways may play a role in NEA,[499] and that anticholinergics may target airway narrowing associated with increased afferent nerve or vagal activity.[23] However, previous studies have rarely considered this heterogeneity when assessing BDR; in particular, no previous studies have compared the response to SAMA and SABA in different asthma inflammatory phenotypes. This may be important as NEA appears less responsive to another mainstay of asthma treatment, ICS.[422] Furthermore, previous studies have been primarily conducted in adults or people with severe asthma, with few studies in young adults or children with generally well-controlled asthma.

The primary aim of this study was to assess whether there was a differential effect in EA and NEA of SAMA and SABA treatment on BDR in young people (aged 14-21 years) with relatively well-controlled asthma.

6.2 Methods

6.2.1 Study population

One hundred and eighteen participants with asthma (14-21 years) were recruited in Wellington, New Zealand, either from a birth cohort study (n=49)[484] or through separate community-based recruitment (n=69). All completed a respiratory health questionnaire based on the ISAAC Phase II survey.[485] Asthma was defined on the basis of a positive response to: 'have you had wheezing or whistling in the chest in the past 12 months?', and/or 'have you taken asthma medication in the past 12 months'. Informed consent was obtained from participants/parents, and the study was approved by the Northern B Health and Disability Ethics Committee (15/NTB/2).

6.2.2 Clinical assessments

Participants undertook two assessments (the second assessment was conducted 3-6 months after the first) involving the tests described below. Asthma control was assessed using the ACQ7.[91] Participants with respiratory infection <1 month prior to the assessment returned when symptom-free, and, for safety reasons, those with FEV_1% predicted <75% were excluded. Prior to testing, asthma medication and antihistamines were withheld for ≥12 and ≥24 hours, respectively.

6.2.2.1 Spirometry and FeNO

Spirometry and FeNO were measured using an Easyone spirometer (NDD Medizintechnik AG, Zurich, Switzerland) and Hypair FeNO analyser (Medisoft, Sorinnes, Belgium) as described previously.[22,486]

6.2.2.2 Atopy

Skin prick tests were conducted (during the first assessment only) using a panel of aeroallergens: HDM, tree mix, grass mix, cat and dog dander, *Alternaria tenuis* and *Penicillium* mix (Stallergenes Greer, Sydney, Australia), and atopy was determined by presence of at least one weal ≥ 3mm after subtraction of the negative control (saline). Histamine (1%) was used as positive control.[22]

6.2.2.3 Combined hypertonic saline challenge and sputum induction

Hypertonic saline challenge/sputum induction was conducted as described previously.[488] Briefly, aerosolised hypertonic saline (4.5% w/v) was produced using an ultrasonic nebuliser (DeVilbiss Ultraneb 2000, Langen, Germany) and administered orally through a mouthpiece (Hans-Rudolph Inc, Kansas City, USA) for increasing intervals from 0.5-4 minutes to a total of 16 minutes. Spirometry was conducted between intervals, and bronchodilator (salbutamol at visit 1 and IB at visit 2) was administered as described below. Participants were subsequently encouraged to produce sputum, which was processed according to a well-characterised protocol, and the resulting cell suspension was used to prepare cytospin slides stained using a Diff-Quik® fixative/stain set (Dade Behring, Deerfield, IL). Using light microscopy, EA was identified as ≥2.5% eosinophils at any visit and NEA as <2.5% eosinophils at both visits. AHR was defined as a ≥15% drop in FEV_1 from baseline.[488]

6.2.2.4 BDR

To determine BDR, participants received 200 μg salbutamol (Ventolin, GlaxoSmithKline) at visit 1, and 40 μg IB (Atrovent, Boehringer Ingelheim) at visit 2, using a pre-primed volumatic spacer. At both visits, the appropriate bronchodilator was administered immediately after completing the combined hypertonic saline challenge/sputum induction, or if required, following a reduction in FEV_1 of ≤75%-predicted or >15% of baseline. Post-bronchodilator spirometry was performed 10 minutes after salbutamol administration (visit 1) and 20 minutes after IB administration (visit 2).[546]

6.2.3 Statistical analysis

Analyses were performed using STATA version 11.0 (STATA Corp, College Station, TX, USA) and GraphPad Prism 7.0 (Graphpad Software Inc, La Jolla, CA, USA). Mann-Whitney U tests, unpaired t-tests, Chi-square tests, or Kruskal Wallis tests were used as appropriate. BDR was calculated using the following equation: BDR % change= $\frac{Post-bronchodilator\ FEV_1 - Pre-bronchodilator\ FEV_1}{Pre-bronchodilator\ FEV_1}\ x\ 100\%$. BDR was assessed using two approaches: (1) comparing baseline FEV_1 (prior to the combined hypertonic saline challenge and sputum induction test) with post-bronchodilator FEV_1[547]; and (2) comparing post-challenge FEV_1 (the last FEV_1 obtained prior to treatment with salbutamol or IB) with post-bronchodilator FEV_1, thus representing saline challenge-induced bronchoconstriction reversibility (Appendix Figure 3.1).[548] BDR was compared between EA and NEA for salbutamol and IB separately, using both continuous (i.e. increase in FEV_1 (L)) and dichotomous measures (i.e. ≤8% versus >8% increase in FEV_1 (L).[71] To account for the differential response to saline challenge, we conducted further sensitivity analysis including only asthmatics with AHR (as defined above).

6.3 Results

6.3.1 Population characteristics

Thirty-five participants were excluded due to either poor quality/no sputum sample or incomplete bronchodilator data. Of the remaining 83 participants, 20% were classified as uncontrolled, 31% as partially controlled and 48% as well-controlled (Table 6.1). When classified based on sputum differential counts, 45% (n=37) had EA and 55% (n=46) had NEA. None were classified as neutrophilic or mixed granulocytic asthma.[20] EA had significantly reduced lung function, higher FeNO and poorer ACQ7 scores (all $p<0.05$; Table 6.1).

Table 6.1. Population characteristics

	Asthma (N=83)	**Eosinophilic asthma (N=37)**	**Non-eosinophilic asthma (N=46)**
Age	19.7 (1.8)	19.5 (1.7)	19.8 (1.7)
Males- n (%)	40 (48.2%)	18 (48.7%)	22 (47.8%)
Height (cm)	169.8 (8.7)	169.5 (9.3)	170.1 (8.2)
Weight (Kg)	67.1 (13.3)	66.1 (13.0)	67.8 (13.5)
Ethnicity			
European- n (%)	71 (85.5%)	31 (84.0%)	40 (87.0%)
Non-European- n (%)	12 (14.5%)	6 (16.2%)	6 (13.0%)
FEV_1% predicted	98.6 (14.7)	95.9 (15.2) †	100.7 (14.1)
FVC% predicted	101.2 (13.5)	100.7 (13.5)	101.5 (13.6)
FEV_1/FVC% predicted	97.3 (7.6)	95.2 (7.1) *	99.0 (7.6)
β-agonist use- n (%)	61 (73.5%)	31 (83.8%)	30 (65.2%)
ICS use- n (%)	44 (53.0%)	23 (50.0%)	21 (57.0%)
ACQ7 score	0.9 (0.7)	1.3 (0.8) **	0.65 (0.5)
Airway hyperreactivity[a]- n (%)	32 (39.0%)	17 (46.0%)	15 (32.6%)
FeNO (ppb)	62.9 (64.1)	81.8 (66.8) **	47 (58)
Atopy[b]- n (%)	68 (81.9%)	32 (86.5%)	36 (78.3)
TCC/mL x 10^6	2.2 (1.6-2.8)	2.8 (1.8-3.8)	1.6 (1.0-2.2)
Sputum eosinophils %	6.9 (4.4-9.4)	14.4 (10.0-18.9) **	0.6 (0.3-0.8)
Sputum neutrophils %	14.4 (11.0-17.8)	12.5 (8.2-16.7)	16.0 (10.8-21.2)

Data presented as mean (SD), median (IQR) or frequency (%), Mann-Whitney test and chi-square tests were used as appropriate. † $p<0.1$; * $P<0.05$; ** $P<0.01$ non-eosinophilic versus eosinophilic asthmatics.

[a] ≥15% drop in FEV_1 from baseline following hypertonic saline challenge

[b] Positive SPT against one or more common allergens

SPT, skin prick test; FeNO, fractional exhaled nitric oxide

Eosinophilic asthma defined as ≥2.5% sputum eosinophils

6.3.2 BDR

For all asthmatics, median baseline BDR was 0.9% (IQR -2.3-3.2%) and 0.4% (-4.0-3.2%) following salbutamol and IB administration respectively, whereas post-challenge BDR was 16.6% (6.9-25.0%) and 12.0% (7.1-22.0%). Using both approaches, BDR was significantly higher for salbutamol (P<0.05; Figure 6.1A). When analyses were restricted to asthmatics with AHR (n=32), a similar pattern was observed, although post-challenge BDR was elevated with both salbutamol (28.2%, 18.3-38.6%) and IB (16.9%, 10.4-24.9%; p<0.05; Figure 6.1B).

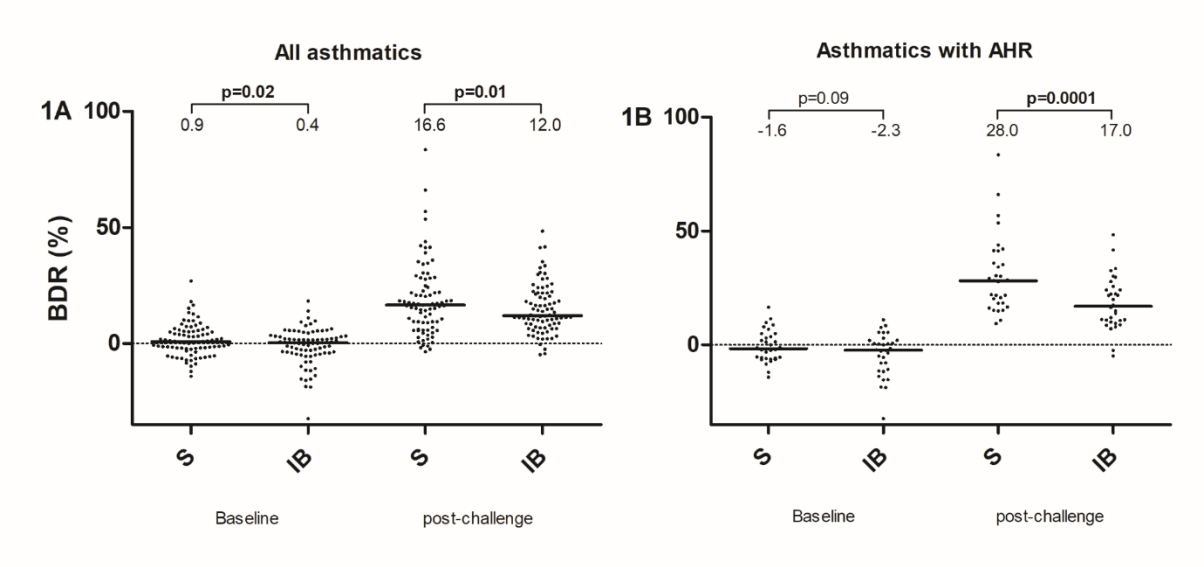

Figure 6.1. Bronchodilator response (percentage of change; BDR %): (A) baseline and post-challenge in all asthmatics (n=83); (B) baseline and post-challenge in asthmatics with AHR (n=32), following treatment with salbutamol (S) or ipratropium bromide (IB). Median data values are expressed at the top of each group.

6.3.3 BDR and inflammatory phenotypes

When stratified into EA and NEA, baseline (Figure 6.2A) and post-challenge BDR (Figure 6.2B) were similar to those observed in asthmatics overall in both cases. However, although baseline BDR was not significantly different between EA and NEA for either medication, post-challenge BDR was significantly higher in EA than NEA with salbutamol (18.3%, 14.2-

28.5% vs 14.0%, 5.7-20.1%); p<0.05; Figure 6.2B). There were no differences in BDR between EA with NEA when analyses were restricted to asthmatics with AHR regardless of bronchodilator or BDR definition used (Figure 6.2C, 6.2D).

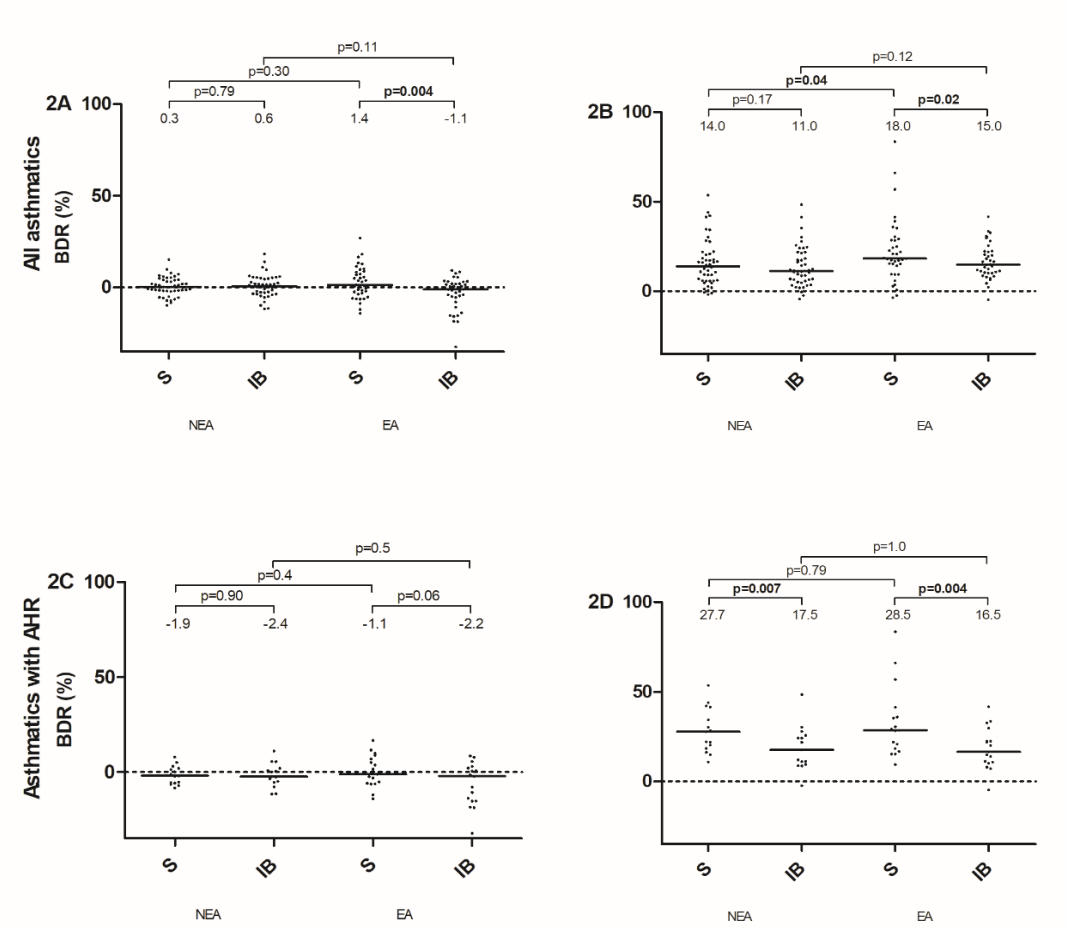

Figure 6.2. Bronchodilator response (percentage of change; BDR %): (A) baseline, (B) post-challenge in eosinophilic (EA) and non-eosinophilic (NEA) asthmatics; or (C) baseline; (D) post-challenge only in EA and NEA asthmatics with AHR, following treatment with salbutamol (S) or ipratropium bromide (IB). Median data values are expressed at the top of each group.

6.3.4 BDR prevalence

Using a >8% increase from baseline to define BDR, the majority of asthmatics (80%, n=67) were classified as non-responders to either treatment (Table 6.2). However, when using a >8% increase from post-challenge to define BDR, 61% (n=54) of asthmatics responded to both treatments, 8% (n=7) responded to IB alone, 9% (n=8) responded to salbutamol alone,

and 17% (n=14) responded to neither treatment (Table 6.2). When restricting analyses to asthmatics with AHR, similar response patterns were observed for baseline BDR. However, when assessing post-challenge BDR, all participants with AHR responded, of which 88% (n=28) responded to both treatments and 13% (n=4) responded to salbutamol alone; none of the participants responded to IB alone (Table 6.2).

When comparing baseline BDR in EA and NEA (Table 6.2), EA were more likely to have a positive response to any treatment (30% (n=11) vs 11% (n=5); $p<0.05$) and respond to salbutamol alone than NEA (24% (n=9) vs 2% (n=1); $p<0.05$). When comparing post-challenge BDR in EA and NEA, no statistically significant differences in the prevalence of response to salbutamol or IB alone were found, although EA were more likely to respond to both treatments than NEA (97% (n=36) vs 72% (n=33); $p<0.05$). When analyses were restricted to EA/NEA with AHR, a similar pattern was observed when defining BDR as FEV_1 >8% from baseline or post-challenge (i.e. the majority responded to both and the remainder to salbutamol alone), regardless of EA/NEA phenotype (Table 6.2).

Using 9%[72] or 12%[545] cut-off points to define BDR resulted in a similar pattern of results, although lower BDR prevalence was observed (data not shown).

Table 6.2. Prevalence of bronchodilator response (>8% FEV_1) in asthmatics (all and AHR only), EA, and NEA

	All asthmatics			Asthmatics with AHR		
	Asthma	**Eosinophilic asthma**	**Non-eosinophilic asthma**	**Asthma**	**Eosinophilic asthma**	**Non-eosinophilic asthma**
	(n=83)	**(N=37)**	**(N=46)**	**(n=32)**	**(n=17)**	**(N=15)**
>8% increase from baseline						
Both responders (SIB +/+), n (%)	2 (2.4%)	1 (2.7%)	1 (2.2%)	0 (0%)	0 (0%)	0 (0%)
Only S responders (SIB +/-), n (%)	10 (12.0%)	9 (24.3 %) *	1 (2.2%)	4 (12.5%)	4 (23.5 %) *	0 (0%)
Only IB responders (SIB -/+), n (%)	4 (4.8%)	1 (2.7%)	3 (6.5%)	2 (6.3%)	1 (6.0%)	1 (7.0%)
Non-responders (SIB -/-), n (%)	67 (80.7%)	26 (70.3 %) *	41 (89.1%)	26 (81.3%)	12 (71.0 %)	14 (93.3%)
>8% increase post-challenge						
Both responders (SIB +/+), n (%)	54 (65.0%)	26 (70.3 %)	28 (61.0%)	28 (87.5%)	14 (82.4 %)	14 (93.3%)
Only S responders (SIB +/-), n (%)	8 (9.6%)	5 (13.5%)	3 (6.5%)	4 (12.5%)	3 (17.6%)	1 (6.7%)
Only IB responders (SIB -/+), n (%)	7 (8.4%)	5 (13.5%)	2 (4.4%)	0 (0%)	0 (0%)	0 (0%)
Non-responders (SIB -/-), n (%)	14 (17.0%)	1 (2.7%) *	13 (28.3%)	0 (0%)	0 (0%)	0 (0%)

Data presented as frequency (%). * $p<0.05$ non-eosinophilic versus eosinophilic asthmatics. S, salbutamol; IB, ipratropium bromide

6.4 Discussion

In the present study, we found that salbutamol was generally the most effective bronchodilator in young asthmatics with relatively well-controlled asthma. The magnitude of response to both bronchodilators was greater in EA compared to NEA, although this was statistically significant only for salbutamol. There was no indication that IB was more effective in NEA; however, a small group of asthmatics responded to IB alone.

Our finding that salbutamol is more effective than IB in asthma is consistent with previous studies in different populations; for example, participants with mild to moderate airway obstruction,[549] persistent allergy,[541] young adult asthmatics,[540] and children with asthma.[550] Our study therefore provides further support for the preferential use of SABA rather than SAMA as reliever medication in asthma in general, as is currently advocated in international guidelines.[545]

The magnitude of response to both bronchodilators was greater in EA compared to NEA, although statistically significant only for salbutamol. This could suggest that BDR is associated with eosinophilic inflammation. However, EA had lower baseline lung function parameters (FEV_1, FEV_1/FVC), poorer asthma control, and were more likely to have AHR than NEA, which is consistent with previous studies in adolescents.[22] We therefore consider that the greater BDR observed in EA may simply be because they have more baseline airflow limitation and/or more poorly controlled asthma. Indeed, lower pre-bronchodilator lung function and poor asthma control have previously both been associated with greater BDR in asthmatics.[551] In an attempt to address this, we conducted further sensitivity analyses including only asthmatics with AHR i.e. those who had significantly reduced lung function during the hypertonic saline challenge, which included 17 EA (46%) and 15 NEA (33%).

This analysis showed no difference in BDR or prevalence of response between EA and NEA, regardless of the bronchodilator used. In addition, all asthmatics exhibited a positive response to both treatments or salbutamol alone when post-challenge FEV_1 was considered, supporting our belief that greater BDR is associated with poor asthma control and lower baseline lung function rather than specific inflammatory pathology.

Whilst we found no evidence of greater BDR with IB in NEA, we found a small group of asthmatics (8.4%, n=7 when examining post-challenge BDR) who responded to IB but not salbutamol. This group had relatively low ACQ7 scores (0.3 vs 1.0; $p<0.05$) and showed no evidence of AHR (0%, n=0 vs 52%, n=28; $p<0.05$), but were otherwise similar to other asthmatics. Some previous studies have also reported a subgroup of asthmatics responding to IB alone, with evidence this is associated with nocturnal,[542] non-atopic or intrinsic asthma.[543] In the current study we were unable to assess these characteristics due to either questionnaire limitations (nocturnal asthma) or limited participant number (only 18.1% were non-atopic). Nevertheless, our data (alongside previous studies) suggests that there may be a small group of well-controlled asthmatics (with no evidence of AHR) that are responsive to IB alone. However, as this response was observed only in a small group of participants, it is possible that it may be due to chance. To clarify and further characterise this group, we suggest that a larger study is needed involving multiple assessments over time.

BDR is conventionally assessed as change in FEV_1 from baseline after bronchodilator administration,[547] with a ≥12% increase often defined as clinically significant.[545] However, both approaches are based on studies with mostly adults conducted in tertiary settings[552,553] and may be too stringent in paediatric or adolescent asthma, in which baseline FEV_1 is less likely to be affected than older populations.[71] In agreement, we found that clinically significant baseline BDR was difficult to detect in majority of young asthmatics; 81% of

asthmatics were classified as non-responders using these definitions in the current study. To address this, we used two alternative approaches to identify BDR. Firstly we used a challenge-rescue model (using hypertonic saline) measuring BDR following a bronchial challenge-induced bronchoconstriction, proposed previously as a means to maximise BDR.[548] Secondly, we used a cut-off of 8% increase in FEV_1 to define BDR, as this has been shown to provide better sensitivity than the 12% cut-off to detect asthma in young people[71] Using this combination, we demonstrated BDR in the majority (83%) of asthmatics, suggesting that this approach may be more appropriate in this population of young asthmatics than strict adherence to the ≥12% cut-off from baseline to define BDR.

Our study has limitations. Firstly, asthmatics were identified using an epidemiological definition rather than objective tests, and therefore some misclassification may have occurred. However, this commonly used approach[57,304,554] generally compares well with clinical diagnoses.[57] Furthermore, although not using the conventional approach (see above), 83% of asthmatics identified using the epidemiological definition in the present study had BDR post-challenge. The remaining 17% may have been misclassified as has been shown previously,[555] or as asthma is a highly variable condition,[545] had little evidence of airway flow limitation at the time of assessment. Secondly, asthmatics in the present study were young and often had relatively well-controlled asthma. It is unclear how generalisable our findings are to other age groups, or those with severe or uncontrolled asthma. Thirdly, as our study was conducted in a community rather than tertiary setting, we did not ask participants to abstain from LABA for long periods of time due to safety concerns. Fourthly, BDR was only assessed at two-time points; once for salbutamol, once for IB. As asthma is characterised by considerable temporal variation in airflow limitation,[545] it is likely that BDR will also fluctuate. Studies assessing post-challenge BDR at multiple timepoints and in treatment-naïve patients are required to expand on the present findings. Finally, BDR to salbutamol was

consistently evaluated at visit 1 and IB at visit 2, which might introduce a potential bias. However, since the time intervals between assessments were several months apart, it is unlikely that this would significantly impact the results.

In conclusion, in this study of generally well-controlled young asthmatics we found that EA were more likely to respond to both bronchodilators (likely due to lower lung function at baseline), but there was no evidence of a differential response in NEA; in particular, IB was not more effective in NEA. Furthermore, although salbutamol was the most effective bronchodilator overall, a small group of asthmatics did not respond to salbutamol and responded to IB alone. This suggests that current guidelines and approach to the use of reliever medication may not be addressing the needs of a subgroup of asthmatics.

Chapter 7 General Discussion

7.1 Introduction

This thesis describes several studies utilising different non-invasive approaches to assess aspects of neural pathways and remodelling in the airways. They were designed to provide greater understanding of the role of: (i) neural mechanisms, particularly autonomic regulation, sensory nerve activity, and neural mediators; and (ii) remodelling, particularly levels of airway remodelling biomarkers. The work described in this thesis also assessed whether markers of neural and remodelling pathways were associated with inflammation. In addition, the response to SABA and SAMA treatments were compared between asthma phenotypes. Results were generally presented separately for EA, NEA, and non-asthmatics to assess whether features associated with neural mechanisms, remodelling and/or inflammation differed across these groups. This research adds to the relatively small body of research assessing non-inflammatory mechanisms in asthma. To the author's knowledge, no similar studies have previously been conducted in New Zealand, and only a limited number of studies have been conducted internationally.

This chapter summarises the main findings and discusses results of particular interest. This is followed by a discussion of the overall implications, the strengths and limitations, suggestions for future research, and overall conclusions.

7.1.1 The main findings are as follows:

- Approximately 50% of asthmatics were characterised as NEA (chapters 3, 4, 5 and 6).
- Airway sensory nerve reactivity was enhanced in NEA compared with non-asthmatics (Chapter 3).
- There was no evidence of an imbalance in ANS activity in asthma or in EA and NEA (Chapter 4).
- With the exception of nociceptin, there was little evidence of increased airway levels of neural mediators in asthma or in EA and NEA (Chapter 5). However, increased levels of some inflammatory and remodelling markers were found in asthma and EA, but not NEA (Chapter 5).
- A subgroup of asthmatics responded to IB but not salbutamol (Chapter 6).

7.2 Discussion and implications of main findings

7.2.1 NEA is a common phenotype

This research supports previous findings[22,460] showing that approximately 50% of young asthmatics in the general population have NEA. Additionally, those with NEA were predominantly PGA. No clear evidence of neutrophilic inflammation was found; in particular, none of the asthmatics had ≥61% neutrophils during assessment. Once again, this is consistent with previous studies in young asthmatics in New Zealand.[22,101,556] Whilst higher rates of NA have been reported in children in low-income countries (e.g. in Uganda, where it may be associated with infections or environmental exposures),[460] at least in New Zealand, neutrophilic inflammation may be less important in young adults with asthma compared with older asthmatics, who may have NA due to smoking and occupational exposures.[557]

It is possible that there may have been some misclassification, with some EA cases incorrectly classified as NEA due to ICS treatment, which is known to suppress airway eosinophilia.[422] However, given that only 42% of the NEA group had used ICS in the previous two weeks, and there was no difference in sputum eosinophil levels between those who had used ICS and those who had not, this is unlikely to have significantly affected the results i.e. if misclassification as a result of ICS-use had occurred, this would most likely be small. The commonly used sputum eosinophil cut-off of 2.5%[488] may have also resulted in misclassification. In particular, low-grade eosinophil-mediated inflammation (with associated lower levels of sputum eosinophils) may be important in NEA as has previously been suggested.[431] In support of this, in the current study, a *post hoc* analysis found that NEA had significantly higher sputum eosinophil percentages than non-asthmatics (median: 0.0, IQR: 0.0-1.24 vs 0.0, 0.0-0.0; $p<0.01$). However, detailed assessment of cytospin slides found that

52% of NEA had no evidence of any sputum eosinophils, indicating that "low-grade" eosinophilia would explain at most 48% of all NEA cases. It is therefore possible that NEA may be a heterogenous group consisting of asthmatics who present with a less evident form of EA and those without any detectable signs of inflammation. If true, the actual proportion of NEA may be lower than 50%. Despite some uncertainty about the exact percentage of asthmatics who classify as NEA, it is clear that a considerable proportion of asthmatics in the general population may not conform to the TH_2-mediated inflammatory paradigm, and therefore may not be optimally treated with therapeutic interventions solely targeting (eosinophilic) inflammation.[414] The high prevalence of cases identified as NEA highlights the need for further investigations of this phenotype to better understand its underlying pathology, with the aim of developing effective prevention strategies or more targeted interventions.

7.2.2 Sensory nerve reactivity is increased in NEA

One of the most important and intriguing findings of this research is that young people with NEA had enhanced sensory nerve reactivity when compared with non-asthmatics; sensory nerve reactivity was also higher compared to EA, but this did not quite reach statistical significance ($p=0.07$; Figure 3.2). While this is the first report to show a relationship between sensory nerve reactivity and NEA, recent studies have suggested an association with non-atopic,[279,389] and occupational asthma[558] which, like NEA, are likely to be driven by predominantly non-TH_2 mechanisms. This is important as it points towards an alternative pathology, potentially involving neural pathways.

Currently, the causes of enhanced sensory nerve reactivity, both in general and in NEA specifically, are not fully understood. As discussed in section 2.2.3.1, it has been suggested

that hereditary (intrinsic) sensory neuropathy,[559] along with airway remodelling (resulting from previous airway insults or chronic inflammation) may lead to-long lasting peripheral and central sensitisation of the cough pathways.[241] In the context of NEA, any of these factors could be responsible, although the absence of detectable airway inflammation suggests that chronic inflammation is unlikely to play a major role. Whether enhanced sensory nerve reactivity observed in NEA is due to central or peripheral sensitisation of sensory nerves, or previous airway insults, is unknown. Additionally, as only the response to capsaicin was assessed, it is unclear as to whether those with NEA also have enhanced sensory reactivity to other stimuli. As described in Chapter 3, participants did not report increased prevalence of nocturnal tussive symptoms, suggesting that this may be a $TRPV_1$-specific response, and not representative of "cough variant" asthma.[560]

The finding of increased sensory nerve reactivity in NEA highlights an identifiable and measurable component of airway disease that may have important implications. Firstly, if symptoms in NEA are specifically related to sensory reactivity, it identifies a novel non-inflammatory pathology, which may, at least partially, explain why NEA is less responsive to ICS.[422] Secondly, capsaicin sensitivity could be used to identify a potential treatable trait,[561] allowing the development of a more tailored approach to treatment. Thirdly, if the source or causes of increased sensory nerve reactivity observed in NEA could be identified, this may contribute to the development of prevention strategies to reducing causal exposures.

If proven useful, capsaicin challenge or sensory nerve reactivity testing could also become part of routine asthma assessments in specialised respiratory clinics to better identify those who may benefit from alternative treatment options. Further, it may be an important research tool that could allow more advanced asthma phenotyping, which would be particularly useful for population-based studies assessing the primary causes of asthma.

7.2.3 There is no evidence for altered autonomic activity in asthma

No evidence of an imbalance or difference in ANS activity was found when comparing asthmatics and non-asthmatics, or EA and NEA. Although this finding was consistent with some previous studies showing no difference in autonomic activity,[369,370] others have reported increased PNS activity.[260,364] As discussed in greater detail in Chapter 4, these inconsistencies may be attributed to differences in the populations studied. In particular, in contrast to the present study, most previous studies have assessed HRV in either older,[369] or pre-pubertal populations[260] or more severe asthma in a tertiary setting.[364,365] Additionally, methodological differences in HRV measurement and interpretations complicate comparisons between studies. Factors such as test conditions (e.g. resting versus post-exercise HRV measurements), changes in body position and the use of different software may also lead to differences in results.[562] Moreover, there are currently no validated tools developed specifically to assess ANS activity in the airways[345] and HRV analysis may therefore not be sufficiently sensitive/specific for assessing autonomic airway regulation. Ultimately, it is also possible that autonomic dysfunction does not play a critical role in asthma or in specific asthma phenotypes, or at least not in asthma in young people, particularly those with well-controlled asthma.

7.2.4 With the exception of nociceptin, there was little evidence of a role for neural mediators in asthma

Increased levels of sputum nociceptin were found in asthma and EA, when compared with non-asthmatics. As described in Chapter 5, nociceptin is not often studied and most studies examining the role and effect of nociceptin in the airways have been conducted in animal models.[526,563] Although studies have reported the importance of nociceptin in pathological

states such as neuropathic and inflammatory pain, and Parkinson's disease, as well as in anxiety and drug addiction,[564] there is relatively little known about the clinical function of nociceptin in asthma pathology. Whilst there is some evidence that nociceptin receptor expression may be altered in asthma,[528] to the author's knowledge only one previous study has reported increased sputum nociceptin levels in 55 adults (>50 years of age) with severe asthma.[529] The finding of increased airway nociceptin levels in young subjects with well-controlled asthma, suggests that nociceptin may be involved in asthma across a broader spectrum of severity.

Although it is suggested that nociceptin may be of neural origin (as grouped in Chapter 5 based on previous literature,[565] several studies suggest that different leukocyte populations can express the N/OFQ receptor and are capable of producing and secreting nociceptin.[296,529,566] There are also reports suggesting that nociceptin has immunomodulatory functions, such as leukocyte migration, lymphocyte proliferation, and cytokine production.[567,568] Thus, given the significant associations observed between sputum nociceptin levels and granulocytes in the present study and also reported previously by Singh *et al*,[529] sputum nociceptin may originate from inflammatory cells. Alternatively, as inflammatory cells often co-localise with airway nerves and inflammatory mediators are known to induce production of sensory neuropeptides,[241] it is also possible that nociceptin may be produced by sensory nerves as a result of inflammation involving these cells.

Despite enhanced sensory nerve reactivity observed in NEA (Chapter 3), no evidence of increased levels of any of the neural mediators studied was found in this group. This suggests that neurogenic inflammation is unlikely to play a role in this phenotype, at least in young asthmatics with well-controlled asthma. The reasons why enhanced sensory reactivity was detected in NEA without evidence of neurogenic mediator involvement is unclear. It is possible that enhanced sensory nerve reactivity in NEA is mediated by central reflex

pathways that mediate cough reflex rather than the axon reflex that leads to production of neuropeptides and neurogenic inflammation.[233] Alternatively, as suggested in Chapter 5, low levels of neural mediators detected may be because neuropeptide signalling in the airways is often transient and highly localised,[569] which may make it difficult to detect in sputum. It is also possible that increased levels of sputum neural mediators are only detectable in more severe and intractable disease[285,287] or during exacerbations[515] and may not be detectable in well-controlled asthmatics. Finally, the panel assessed in Chapter 5 may have missed some potentially important functional mediators. In particular, previous studies have suggested that neuropeptide S,[570] and VIP,[289] may be implicated, particularly in children with mild-moderate asthma and during rest. However, due to the limited sample volume available, these mediators were not measured in the present study.

7.2.5 Remodelling and inflammation-associated mediators were increased in EA but not NEA

Increased sputum levels of several inflammatory and remodelling mediators such as ECP and periostin were found in asthma, and IL-1β, periostin, VEGF-A, and ECP in EA, but not in NEA. This suggests that whilst multiple processes may be occurring concurrently in EA, on the basis of mediators and inflammatory cells, there is no evidence of this in NEA.

The finding of increased periostin levels in EA is consistent with previous studies, which showed that it is associated with TH_2-mediated inflammation,[437] suggesting its potential as a biomarker for EA. However, increased periostin is not specific to only EA as it is also elevated in other atopic diseases like dermatitis,[571] and allergic rhinitis,[572] and in conditions that are not associated with TH_2 inflammation or atopy, such as rhinovirus infections[573] or bronchiolitis.[574]

While there have been a few studies showing increased VEGF levels in EA,[575,576] particularly associated with asthma severity,[577] little is known about its role in asthma. Further studies are warranted to determine the specific mechanisms through which VEGF may influence the development and severity of asthma.

Although Chapter 5 suggests that periostin and VEGF are "remodelling"- associated mediators (based on previous studies),[531,578] these mediators are also associated with TH_2 inflammation. As with nociceptin, it is unclear whether increased levels of these mediators are actually produced by airway leukocytes, as reported previously,[579] or are produced by structural cells. As observed in Chapter 5, VEGF in particular is associated with macrophages, neutrophils and eosinophils, suggesting that VEGF may originate from these cells.

No evidence of increased levels of remodelling-associated mediators was found in NEA. This may potentially be due to the absence of severe or neutrophilic asthma in this population, both of which have previously been associated with increased levels of MMP-1, MMP-9,[520] TIMP-1[519] and TGF-β.[517] Despite this, further investigation of the involvement of airway remodelling in NEA may be warranted, as active remodelling may not have occurred at the time of assessment (especially when most NEA were well controlled) or may not be detectable using sputum mediator assessment. For example, alterations in ASM mass or thickening of the subepithelial basement membrane would only be detected using more invasive techniques such as bronchoscopy or histopathological examination.[463]

7.2.6 A subgroup of asthmatics respond to IB but not salbutamol

In Chapter 6, both salbutamol and IB were effective bronchodilators in a large proportion (65%) of asthmatics, although salbutamol was associated with a slightly greater response than IB. This was expected, as previous studies showed similar results,[540,541,549] and as such, provides a rationale for why salbutamol is currently advocated as the first-line reliever medication in international guidelines.[2] However, we found that a small group of asthmatics (8.4%, n=7) responded to IB but not salbutamol. Compared to the other asthmatics, this group had relatively low ACQ7 scores (0.3 vs 1.0; $p<0.05$) and showed no evidence of AHR (0%, n=0 vs 52%, n=28; $p<0.05$), but were otherwise similar in terms of inflammatory and clinical characteristics. Similar findings have previously been reported in 10-20% of asthmatics.[541,549]

This is an intriguing finding and may have important implications. In particular, it suggests that a "one size fits all" approach and reliance on clinical guidelines (i.e. the routine use of SABA alone or in combination with ICS)[2] may not adequately address the needs of a subgroup of asthmatics. Although the relative size of this group is small in the current study, given the high prevalence of asthma, many patients (both nationally and globally) may potentially benefit from using IB - either alone or in combination with salbutamol. If replicated in future studies, identifying this differential response could potentially serve as a treatable trait, enabling a more personalised approach to asthma management. Additionally, treatment response could help further define asthma phenotypes, either as traits within existing phenotypes or as distinct entities.

It is currently unclear why anticholinergic bronchodilators such as IB might be more effective in some asthmatics. However, it is possible that asthmatics with a non-inflammatory underlying mechanism (i.e. neural mechanisms), may benefit from IB. An alternative

explanation could be that IB targets smooth muscle muscarinic receptors in a similar manner to how β-agonist bronchodilators act on smooth muscle β-adrenergic receptors. Hence, the increased IB response may be due to different receptor sensitivity and the direct effect on smooth muscle, rather than different underlying mechanism of bronchoconstriction.

7.3 Strength and limitations

Many of the strengths and limitations of the specific methodological approaches used have been discussed comprehensively in earlier chapters. The more general strengths and limitations of the research described in this thesis are explored further below.

One of the major strengths is the comprehensive assessment of a broad and diverse range of pathophysiological and clinical parameters in young adults with and without asthma. In particular, neural pathways were investigated using a range of methods, and airway inflammation was assessed using induced sputum and FeNO measurement, both of which are validated for use in research and clinical practice.[307,580] This combined approach - assessing both inflammatory and non-inflammatory pathways, and the potential interactions between them - is highly novel, with many previous studies relying on fewer tests to assess underlying mechanisms, with the majority focussing on inflammation or airflow limitation. However, although comprehensive, assessment of non-inflammatory and neural pathways was limited to assessment of capsaicin response, HRV, and sputum mediator measurement. Further tests (as discussed in 7.4) could provide additional information about the underlying causes of asthma.

A significant strength is that the population size of this study was relatively large (130 asthmatics and 79 non-asthmatics) compared with many previous studies examining non-inflammatory mechanisms in asthma, such as those assessing airway sensory reactivity,[480] or autonomic activity.[364] Therefore, although a complete dataset was not available for all tests or samples for all participants, the study generally had adequate statistical power to detect meaningful associations and minimise bias. It also allowed some *ad hoc* sensitivity analyses to be conducted to assess the robustness of findings (e.g. restricting analyses to those who had AHR or used ICS medication to provide a more stringent definition of asthma).

Another strength is that the population studied was unselected and therefore represented a 'snapshot' of airway pathophysiology in the general population, rather than relying on asthmatics recruited from a secondary- or tertiary- care setting, which would have favoured selection of asthmatics who have more severe asthma and are likely to use more medication. The majority of asthmatics studied (78.4%) were stable or well-controlled at the time of assessment. One of the advantages of studying this group was that most had asthma as their sole medical condition (i.e. other chronic inflammatory disorders or co-morbidities were excluded) and therefore were not taking other medications, which could have affected results in some of the tests used. Furthermore, given the young age of participants, they were unlikely to have had much exposure to occupational allergens and irritants previously associated with airway inflammation[557] or sensory nerve reactivity,[581] potentially limiting confounding. However, as participants were stable and well-controlled at the time of assessment it is unclear how applicable and generalisable our findings are to the broader heterogeneous asthma population, particularly those with severe asthma or undergoing exacerbation.

Throughout this thesis, asthma was identified on the basis of standardised questionnaire responses (specifically based on questions about wheezing/whistling in the chest and/or asthma medication-use in the last 12 months), rather than using more objective measures such as lung function measurements, BDR, or AHR, as often advocated in international clinical guidelines.[2] This approach could be considered to be both a strength and a limitation. When considering the strength of this approach, and as described in chapters 3, 4, 5 and 6, a symptom-based approach has high sensitivity and specificity and can be more appropriate for identifying physician-diagnosed asthma than some objective measures.[496] Questionnaire-based approaches are also widely acceptable, inexpensive, and convenient to conduct in large populations, requiring no special equipment. Despite their strength, questionnaire-based

approaches also have limitations. In particular, they rely on self-reported data, which may be subject to recall bias. It is also possible that some asthmatics may have had previous, but not current asthma, or reported symptoms (i.e. wheeze) that were not actually related to asthma as has been previously described.[64] However, the use of more stringent criteria based on objective measures may exclude many asthmatics in a community-based setting, and potentially lead to bias towards certain asthma phenotypes or more severe disease.[582] Furthermore, identification of asthma based on objective measures is especially problematic in younger individuals as spirometry and BDR are often "normal" in young asthmatics,[583] even in severe asthma,[75] and particularly in patients undergoing treatment.[584] Recent GINA guidelines no longer recommend using baseline lung function parameters to identify asthma but instead advocate the use of documented expiratory airflow limitation and excessive variability in lung function- preferably at a time when asthma is not controlled and FEV_1 is reduced, and preferably before starting controller treatment.[2] While this approach is optimal, it is difficult in large population-based studies in the general community with limited clinical supervision. In particular, there is a risk of a deterioration in asthma control with treatment reduction or removal, and this was therefore not possible.

Another limitation was that, although assessing a range of pathways using different tests and approaches, the individual studies described in Chapters 3, 4, and 5 assessed these pathways or tests individually, rather than in combination. This was because, in many cases, there was not a complete dataset for all tests or samples for all participants; for example, many individuals had HRV data but no capsaicin challenge data, or vice versa. As a result, it was often not possible to examine associations between different tests (such as between HRV analysis and capsaicin challenge response), or the interaction between neural pathways and airway remodelling or inflammation. The exceptions are the examination of associations between neural pathways and inflammation in Chapters 3 and 4, and the association between

mediators associated with inflammatory, neural and remodelling pathways in Chapter 5. As discussed in 7.4.1, a comprehensive multidimensional analysis combining data representing aspects of the clinical, immunological, and non-inflammatory pathways would potentially provide a greater understanding of the heterogeneity and underlying pathophysiology of asthma, and the interplay between different processes in individual patients.

The study population was recruited from either a previous birth cohort study (NZA2CS study)[484] or through separate community-based recruitment. It is possible that recruiting through two different sources may have contributed to differences in population characteristics. In particular, asthmatics recruited from the NZA2CS birth cohort study were actively followed-up and provided with asthma educational material (as well as a written asthma action plan) throughout their involvement in the cohort. This may have modified their knowledge, treatment adherence, and/or self-management of asthma symptoms and attacks. As a result, these participants may potentially have had better outcomes such as more adequately controlled asthma, fewer exacerbations, or lower levels of airway inflammation compared with those recruited from the general community. However, it is unlikely that recruitment source may have affected study findings, as the current study was conducted several years after participants were actively engaged in the NZA2CS study, and although not described in each chapter, analyses were repeated and stratified by recruitment source. This generally showed similar results to those observed when the group were assessed as a whole and did not find significant differences between characteristics of asthmatics recruited from the two sources, suggesting that the results were robust and not biased by recruitment.

The majority of the studies described in this thesis were cross-sectional. In particular, all tests which assessed neural pathways or soluble mediators were only conducted at one single point in time. As a result, in most cases, it was not possible to assess temporal variability and

reproducibility of these findings. While some tests such as capsaicin response and HRV analysis, have been shown to have a high degree of reproducibility,[383,507] assessment of mediators in biological fluids has been suggested to show considerable variability over time.[585] Hence a single measurement of mediators may not be adequate, and it is possible that at the time of sampling, mediator levels may not be representative of a situation involving an acute exacerbation, or a well-controlled period. The same limitation also applies to inflammatory cells in the airways. As discussed in section 2.4.1.3, several studies have reported considerable longitudinal variation in sputum eosinophil and neutrophil differential cell counts,[467,556] despite other studies finding that inflammatory phenotypes are relatively stable.[180,422] As with assessing mediators, a single assessment may therefore not be or adequate for the assessment of asthma inflammatory phenotypes or, indeed, the assessment of any type of pathophysiological features in asthma. However, it is possible that phenotypes may actually not be static, and that any temporal variance observed may reflect the phenotype at that particular time, similar to temporal changes in asthma symptoms and/or airway obstruction. If true, it is important to assess associations with other outcomes around the same time as when sputum induction testing is conducted as was done in the current study, thus reducing the risk of phenotype misclassification. Whether other outcomes (e.g. sensitivity to a capsaicin challenge) show the same temporal variance remains unclear and requires further longitudinal assessments.

Another issue that may affect the interpretation of the results is that 76% of asthmatic participants were using asthma medication (β-agonists and/or ICS). Both medications can alter capsaicin response,[392] HRV parameters, airway inflammation and sputum mediator levels,[586] all potentially through different pathways. Ideally, participants who used medication that have the potential to alter test outcomes should have withhold treatment prior to these tests. However, as participants were recruited from the general community, this was

not an option due to safety concerns. Although this is a limitation, results suggest that this may not have materially affected the results described in this thesis. In particular, there were no significant differences in treatment between EA and NEA suggesting that differences observed between these two groups are unlikely due to medication-use, although the NEA group was slightly less likely (not statistically significant) to use asthma medication (either β-agonist or ICS). To further clarify the role of asthma medication, a sensitivity analysis was conducted including only asthmatics who used asthma treatment (as described in chapters 3, 4 and 5). These analyses found similar results to the main study findings, indicating that our findings were robust and unlikely to be affected by asthma medication.

7.4 Recommendations for future research

Although this research has provided important insights into the role of alternative (e.g. neural) pathways in asthma, particularly in NEA and in young well controlled asthmatics, the limitations (as described above), and the relative scarcity of literature in this area means that further research is needed. Based on the work described in this thesis, there are several avenues that future research could take.

7.4.1 Multidimensional approaches

As discussed in section 2.1.6, most previous studies assessing underlying asthma pathology have been relatively one-dimensional, often focusing on one specific mechanism i.e. inflammation. While these studies have provided considerable insight, they have often failed to capture the full extent of asthma heterogeneity. To address this issue, future studies should ideally adopt a more comprehensive and multidimensional approach; integrating multiple factors and pathologies, and involving assessment of a range of clinical, pathological, pathophysiological, genetic, and molecular characteristics.

As an example of this approach, a multi-centre study (involving the Research Centre for Hauora and Health, Massey University, Wellington, and three other centres representing low and middle-income countries) is currently underway. This study aims to assess various aspects of asthma including clinical characteristics, inflammatory markers and underlying mechanisms such as non-inflammatory (e.g. neural) and inflammatory pathways in 640 young people across four centres, with considerable variation in demographics, income, exposures, asthma prevalence, and asthma characteristics between centres. Other examples of large-scale, multicentre studies that adopt a multidimensional approach include the "Severe

Asthma Research Program" (SARP)[587] and the Unbiased BIOmarkers for the Prediction of Respiratory Disease Outcomes" (U-BIOPRED) project.[588] These studies involve the integration of various dimensions of data including clinical features, inflammatory biomarkers, genomic and genetic data, imaging techniques such as CT scan and, treatment response to gain a comprehensive understanding of severe asthma and identify specific traits associated with different asthma phenotypes. However, although multidimensional, these studies are still primarily focussed on the role of inflammation in asthma pathology.

Larger sized multidimensional studies such as these (when compared with many earlier studies) but with increased focus on alternative pathologies (i.e. non-inflammatory mechanism) - possibly using techniques such as unsupervised cluster analysis[141] or principal component analysis[589]- are likely to enable a more comprehensive examination of the underlying mechanisms in different asthma phenotypes whilst at the same time providing greater power. Data from such multidimensional studies are also likely to provide clearer answers to some of the questions raised by the research in this thesis and could be used to identify and define novel phenotypes or identify new potential treatable traits.[561]

7.4.2 Assessment of sensory nerve reactivity

As described in Chapter 3, capsaicin challenge testing showed an enhanced sensory nerve response in NEA, suggesting that $TRPV_1$ may be important in this phenotype. However, as only capsaicin was used in these tests, it is not clear whether enhanced sensory nerve reactivity is limited to the involvement of $TRPV_1$ receptor pathways[590] or if other sensory pathways may also play a role. Several studies in guinea pigs have shown considerable heterogeneity in sensory nerve response to different agents such as citric acid, nicotine, or capsaicin, which activate different afferent fibres such as delta-fibres or RAR receptors.[591,592]

However, very few have assessed the heterogeneity in sensory nerve response in humans. Future studies could assess multiple aspects of sensory nerve reactivity using different tussive agents that act through different TRP channels or pathways. While citric acid and capsaicin are known to act through the $TRPV_1$ expressing C-fibres, histamine and mannitol act through the Aδ-fibres, and adenosine triphosphate acts through both.[593] This approach (i.e. sensory reactivity testing using multiple agents) would clarify whether asthmatics, particularly NEA, show heterogeneity in response to different tussive agents. One could speculate that patterns of response to different stimuli may differentiate between different asthma phenotypes, or identify specific response-based phenotypes, with specific novel treatable traits (as described above). Such studies may also further the understanding of the specific afferent nerves and neural pathways mediating cough and cough reflex sensitisation.

7.4.2.1 Identifying specific exposures and molecular mechanisms underlying sensory nerve reactivity

In addition to assessing different agents that activate specific afferent nerves or receptors, future studies could assess environmental stimuli that may sensitise the cough reflex to other tussive stimuli. To do this, birth cohort or longitudinal infant studies may be the most appropriate approach. After baseline assessment (soon after birth), this population would be followed for ongoing assessment of symptoms, infections, living conditions, and environmental exposures (air pollution, tobacco smoke, etc). This could identify specific exposures associated with different aspects of sensory nerve reactivity and related asthma symptoms. A similar cohort approach could be conducted in an occupational setting to identify specific occupational exposures (e.g. dust, metal fumes, etc) leading to sensory reactivity.

Finally, to determine if enhanced sensory nerve reactivity is due to central or peripheral activation, future studies could assess neural activation during cough challenge using neuroimaging techniques (e.g. fMRI) or neurophysiological assessments (e.g. nerve conduction studies). Using these or similar approaches, it may be possible to determine the specific neural pathways and brain regions involved in processing cough signals and activated in response to tussive challenge agents, which would potentially unravel the mechanisms of enhanced sensory nerve reactivity, (e.g. the interplay between central and peripheral activation) and provide novel therapeutic targets to explore for cough control (discussed in section 7.4.2.2).

7.4.2.2 Developing therapeutic targets

Future studies aimed at identifying novel pathways or treatable traits, as discussed above, will be useful in guiding the development of tailored therapies for newly identified phenotypes, such as those responding to particular tussive challenges. Although speculative, therapies targeting the mechanisms responsible for neural sensitisation could potentially reduce the heightened state of responsiveness of afferent nerves seen in sensory nerve hyperreactivity. This would be more attractive than inhibiting nerve activation, which could possibly suppress a key airway defence mechanism.[454] Alternatively, specifically targeting the peripheral receptors of sensory nerves may be a better approach for potentially treating airway sensory reactivity. There are already some promising therapeutics in development for the treatment of cough such as $TRPV_1$ antagonists, selective cannabinoid agonists, and P2X3 antagonists.[594] These could be assessed in the treatment of asthma, in particular in the NEA group that appears most associated with increased sensory nerve reactivity.

7.4.3 Assessment of autonomic activity

Although we found no evidence of autonomic imbalance in young asthmatics with relatively well-controlled asthma, it is possible that given the complexity of the ANS, HRV measurement alone is not a sensitive enough tool to accurately interrogate the function of specific ANS branches. Future studies using a more comprehensive battery of tools may allow better evaluation of the autonomic activity. An example of such an approach would be conducting the Ewing test battery. This is a collection of five standardised tests including heart rate response to deep breathing, Valsalva maneuvre, heart rate response to standing, blood pressure response to standing, and sudomotor function testing. This battery evaluates various aspects of autonomic function, including cardiovascular reflexes, sweating, and sympathetic function.[351] In addition to this battery of tests, neuroimaging techniques such as an electroencephalogram (EEG) or fMRI could potentially be used to study brain activation patterns in response to asthma-related challenges or symptom perception.[476] Alternatively, as HRV is only a proxy indicator of ANS airway regulation (as discussed above and in 7.2.3), measurement of airway resistance, suggested to specifically represent bronchial vagal activity of the bronchi,[595] may be another useful approach.

As many previous studies have been relatively small and involved selected groups,[257,364] future studies assessing autonomic activity in asthma would ideally involve a large, heterogenous population of asthmatics. Finally, as β-agonist use may be associated with changes in HRV measurements (Chapter 3), ideally studies would be conducted during periods when β-agonists have not been used.

7.4.4 Nociceptin assessment

As discussed in Chapter 5, increased sputum nociceptin levels were found in asthma, indicating a potential involvement of nociceptin in asthma pathology, particularly with eosinophilic inflammation. Further investigations are needed to understand the precise role of nociceptin in asthma pathology and determine the cellular source of nociceptin in the airways. Although, it is currently difficult to assess whether nociceptin is produced by airway nerves without conducting biopsies, future studies using techniques such as flow cytometry, immunohistochemistry, in situ hybridisation could potentially be used to determine whether airway inflammatory cells express the N/OFQ receptor or secrete nociceptin, and to assess cellular nociceptin secretion patterns. A recent example of such an approach assessed real-time nociceptin release from single blood-derived granulocytes using transfected Chinese Hamster Ovary cells.[596]

Further understanding of the exact role of the nociceptin receptor and nociceptin secretion in asthma may also potentially serve as a platform to develop novel pharmacologic agents. Future clinical trials assessing the effect of exogenous nociceptin in reduction of inflammation or bronchoconstriction (as has previously been suggested in mice[525] and guinea pigs)[528] could be undertaken. If such studies clearly showed that nociceptin has clinical bronchodilatory or immunomodulatory effects, then putative asthma therapies could involve nociceptin receptor agonists (to upregulate nociceptin secretion) or administration of exogenous nociceptin (to reduce bronchoconstriction and airway inflammation).

7.4.5 Assessment of airway remodelling

As discussed in 2.3.2, current methods for assessment of airway remodelling are generally very difficult and invasive (e.g. biopsy), or may not only represent remodelling (e.g. mediator assessment). As an alternative, non-invasive imaging technologies such as such as HRCT have been proposed for assessment of remodelling.[341] However, these methods have not been fully validated for this purpose and are difficult to conduct in large populations or community-based settings. Despite these challenges, in the absence of feasible alternatives, further development, validation, and use of such imaging methods described in asthma populations with different underlying pathologies and phenotypes may at least improve our understanding of airway remodelling and its role in the development of asthma.

7.4.6 Assessing differential treatment response

As discussed in Chapter 6, the identification of a sub-group of asthmatics responding to IB rather than salbutamol warrants further studies, possibly in larger populations, to identify asthmatics who may benefit from IB treatment. This is important, as it may potentially be a large number of asthmatics globally (even if the relative proportion is small). Larger studies would also provide more power to identify traits that may be associated with this differential response. In particular, it is not clear which (if any) pathophysiological characteristics may be associated. If larger studies consistently showed that a subgroup of asthmatics showed a better response to IB, future studies could employ differential treatment to define a new characteristic for phenotyping, or as a treatable trait.

7.4.7 Longitudinal assessments

As discussed in section 7.3, cross-sectional studies have a number of limitations, and as asthma is by nature a highly variable disorder,[2] the complexities underlying this variability are unlikely to be determined using a single assessment. Inflammatory phenotypes or underlying mechanisms (either alone or in combination) may change over time either due to the variable nature of asthma, or due to e.g. ICS use, specific exposures, viral infections, or exacerbations.[470] If possible/feasible, future studies would ideally use a longitudinal study design. For example, a detailed assessment of clinical, inflammatory, and non-inflammatory characteristics could be assessed at multiple time points (with at least one assessment prior to treatment) to better understand the nature of this variability and relationships between clinical and pathophysiological characteristics. However, such an approach comes with additional logistics and significant financial costs.

7.4.8 Standardisation and collaboration

A considerable challenge in the assessment of many aspects of asthma, but in particular unconventional pathways such as neural mechanisms, is the paucity of standardised approaches and guidelines. In addition, few large or international collaborative studies have been conducted specifically focusing on these pathways. Using sensory nerve reactivity as an example, a number of different methods have been used, limiting comparisons between studies.[380] Cough challenge testing is one of the most well characterised methods used, but several factors have hindered its use in clinical practice or large-scale research; these include lack of standardisation, the need for specialised equipment and technical expertise. However, if the procedure and agents used, were validated and standardised across several centres, it would allow valid assessment of patterns of cough response and prevalence of sensory nerve

reactivity across different geographical locations and different asthma phenotypes, thus contributing to more robust findings that may have wider applicability. As noted above, RCHH (with the author's involvement) is currently involved in a multicentre study conducting a standardised capsaicin challenge protocol to evaluate sensory nerve reactivity in asthma across different countries (both high and low/medium income countries) and asthma populations. Similar multicentre collaborations would be useful for generating more robust scientific evidence regarding the importance of non-inflammatory pathways, such as sensory nerve reactivity, in asthma.

7.5 Conclusions

This thesis describes a body of work examining how pathways or markers associated with neural function, airway remodelling, and inflammation contribute to the pathophysiology of different inflammatory phenotypes. The research herein contributes to the relatively small body of literature assessing non-inflammatory mechanisms in asthma. It also highlights the importance of recognising the heterogeneity underlying asthma pathology, and the need for personalised approaches to improve asthma management and outcomes, particularly for those with NEA. The study findings provide support for the following conclusions, which directly relate to the aims of this thesis (Chapter 1):

1) Differences in some aspects of neural regulation exist between EA, NEA, and non-asthmatics. In particular, airway sensory nerve reactivity may play an important role in the pathophysiology of NEA, and nociceptin may be involved in the pathophysiology of asthma, particularly EA. However, autonomic nervous activity was not associated with asthma, EA, or NEA, when compared with non-asthmatics (Aim I).

2) Sensory nerve reactivity or autonomic activity are not associated with specific inflammatory or clinical characteristics in either EA or NEA (despite the observed association with NEA). However, levels of some sputum neural mediators are significantly associated with airway inflammation markers (Aim II).
3) Some remodelling mediators (such as periostin and VEGF-A) may be involved in EA, but do not appear to be important in NEA (Aim III).
4) Airway remodelling and inflammation appear to occur concurrently, as observed in EA (Aim IV).
5) There was no evidence of a differential response to IB in NEA (Aim V).

Some of the approaches highlighted and used in this thesis, such as classifying and characterising asthmatics on the basis of physiological responses to specific agents, or employing multiple approaches to assess alternative pathophysiology, may prove more valuable in identifying novel treatable traits or pathophysiological mechanisms than focusing on inflammation alone, as we have done for the past half century. It is hoped that by adopting such a comprehensive approach in future research we will be able to improve understanding about the primary causes of asthma and pave the way for improved personalised treatment options and associated reduced asthma burden.

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Appendix 1 Study population

The study population and data collection methodology are described briefly in each of the papers (chapters 3,4,5, and 6). This appendix provides additional background information related to the study population; in particular, recruitment sources, participant selection, data collection methods, and inclusion of participant data in specific chapters.

1.1. Recruitment

Recruitment for the non-inflammatory mechanisms in asthma study (HRC 14/474), upon which the research in this thesis was based, was conducted between 2015 and 2018. It was designed as a cross-sectional assessment of non-inflammatory mechanisms in 120 asthmatic and 60 non-asthmatic adolescents. These were recruited primarily from The New Zealand Asthma and Allergy Cohort Study (NZA2CS). This was then supplemented with separate community-based recruitment from the Wellington region.

1.1.1. The New Zealand Asthma and Allergy Cohort Study (NZA2CS)

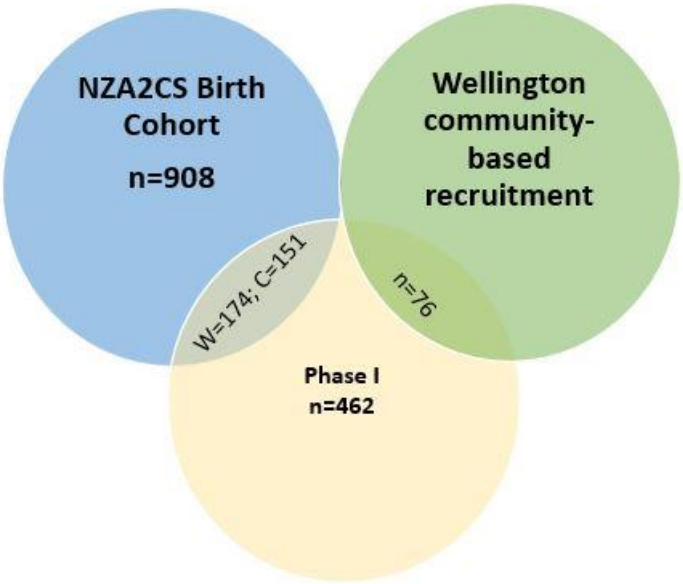
The NZA2CS was initiated as a prospective birth cohort study involving 1000 infants and taking place from 1997 to 2001 in Wellington and Christchurch, New Zealand. Pregnant mothers were recruited through participating midwives. All mothers gave written informed consent (prior to delivery) for their children to participate in this study, and to subsequently monitor their health and development. This involved regular assessments in the form of interviews, questionnaires, and a variety of specific tests. The initial consent covered assessments for the first five years, with subsequent consent for the six and eight-year assessments being obtained prior to assessments.

Demographic information (including age, gender, ethnicity, and contact details) was available for 908 NZA2CS participants. Of these 353 were based in Wellington, 367 in Christchurch,

109 in the lower North Island (outside Wellington), and 79 in the South Island (outside Christchurch). Invitations to participate in the non-inflammatory mechanisms in asthma study were sent by post to the 908 participants, along with a questionnaire. The invitation was mailed up to 3 times and non-respondents were contacted by phone (when a phone number was available). Of the 908 postal invitations, completed questionnaires were received from 386 (42%) participants, of which 174 (45%) were from Wellington, 151 (39%) from Christchurch and 61 (15%) from outside Wellington and Christchurch (Appendix Figure 1.1). A further 99 (11%) participants declined to take part. The remaining 46% failed to return the questionnaire despite multiple reminders and mailouts.

1.1.2. Community recruitment

Early in the recruitment process, it became apparent that recruitment solely through the NZA2CS cohort would not provide enough participants for the non-inflammatory mechanisms in asthma study. Therefore, to supplement numbers, additional participants were recruited through community-based recruitment in the Wellington region. This involved the use of study posters and information sheets in general practices and paediatric respiratory clinics, and face-to-face recruitment at sports and community events/venues or in commercial settings (e.g. supermarkets). Through this approach, a further 76 participants were recruited (Appendix Figure 1.1). Due to the nature of community-based recruitment, exact figures of individuals approached or targeted for recruitment were not available.



Appendix Figure 7.1. Study recruitment sources. W: Wellington, C: Christchurch

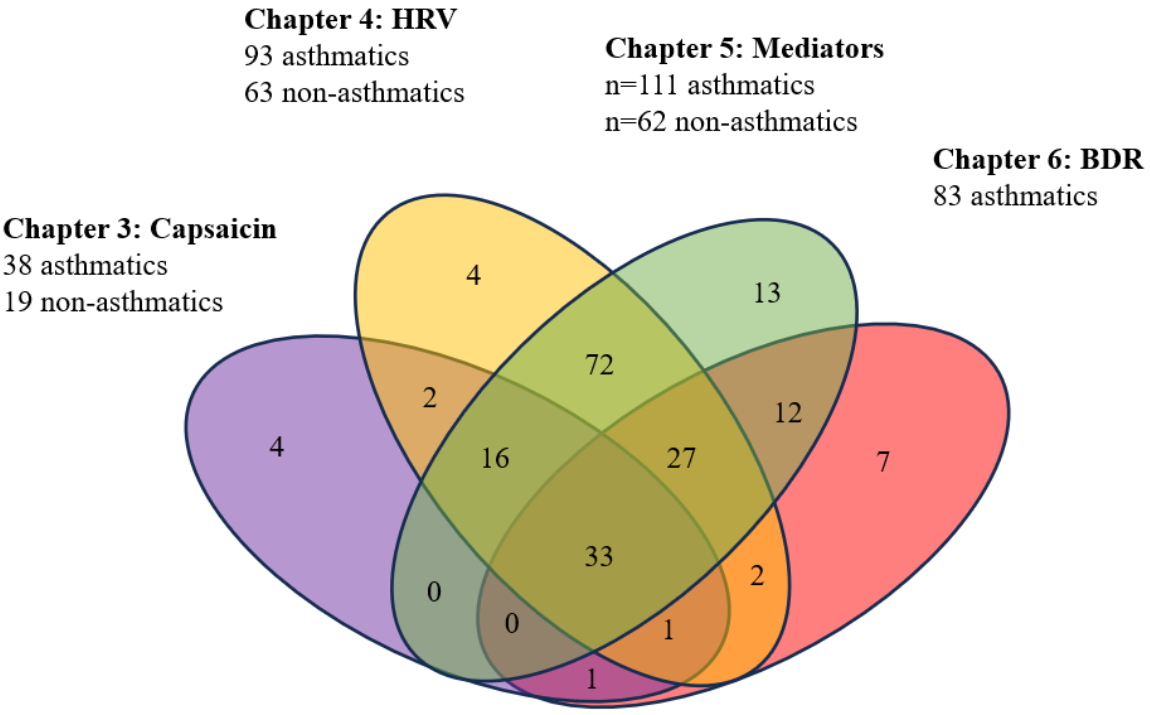
1.2. Participant selection for clinical assessments

From the combination of both recruitment sources, a total of 462 participants were available. Participants were screened on the basis of their questionnaire responses to determine their asthma status. They were then contacted and invited at random until a subset of 130 asthmatics and 79 non-asthmatic, non-smoking adolescents agreed to attend at least one clinical assessment, at either the Wellington School of Medicine or Christchurch Hospital. As such, clinical assessments were ultimately conducted with more participants than described in the original non-inflammatory mechanisms in asthma study plan.

The initial clinical assessment involved a battery of tests, including lung function testing (spirometry), combined hypertonic saline airway hyperreactivity (AHR) testing and sputum induction, skin-prick testing, exhaled NO measurement, and heart rate variability measurement. Asthmatic participants also underwent a bronchodilator response assessment and completed an asthma control questionnaire (ACQ7) during this assessment. These tests

were repeated in all asthmatics who agreed to undergo a second assessment (n=98). In a subset of participants, a blood sample (n=111 asthmatics, n=70 non-asthmatics) was collected. Capsaicin challenge testing (to assess airway sensory nerve reactivity) was also conducted in one further clinical assessment for 40 asthmatics and 20 non-asthmatics.

Participant inclusion in the analyses for each of the papers (Chapters 3,4,5, and 6) was based on data availability for the relevant tests. Appendix Figure 1.2 provides a schematic of the availability and overlap of data for participants included in each chapter.



Appendix Figure 1.2 Data availability for participants included in each chapter

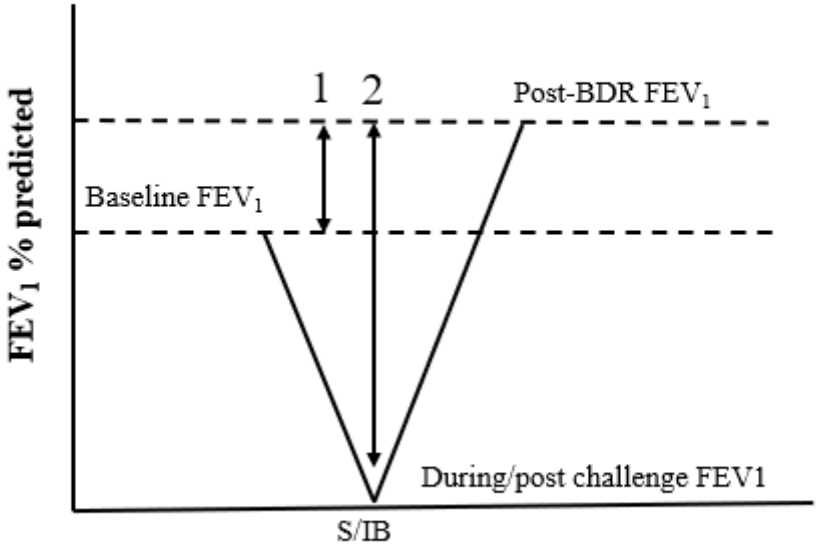
Appendix 2 Correlations between mediators

Appendix Table 2.1: Correlation between mediators normalised to the number of leukocytes/ml sputum in participants with asthma

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
	Correlation coefficient (r)																			
1. IL-1β	1																			
2. IL-6	0.58*	1																		
3. IL-8	0.80*	0.48*	1																	
4. IL-13	0.77*	0.51*	0.68*	1																
5. ECP	0.24	0.25*	0.29*	0.15	1															
6. Histamine	0.51*	0.36*	0.63*	0.55*	0.48*	1.00														
7. NE	0.48*	0.25*	0.53*	0.50*	0.17	0.50*	1													
8.Acetylcholine	0.66*	0.44*	0.70*	0.78*	0.25	0.66*	0.57*	1												
9. NKA	0.19	0.22*	0.19	0.13	0.08	0.37*	0.17	0.31*	1											
10. CGRP	0.58*	0.36*	0.57*	0.71*	0.003	0.44*	0.50*	0.62*	-0.03	1										
11. Nociceptin	0.24*	0.41*	0.32*	0.31*	0.47*	0.40*	0.14	0.47*	0.51*	0.01	1									
12. NGF-β	0.55*	0.57*	0.38*	0.58*	0.16	0.30*	0.33*	0.51*	0.12	0.53*	0.34*	1								
13. Substance P	0.50*	0.26*	0.56	0.81*	0.35*	0.57*	0.54*	0.87*	0.04	0.58*	0.50*	0.37*	1							
14. MMP-1	0.54*	0.56*	0.49*	0.40*	0.28*	0.24	0.36*	0.45*	0.23*	0.28*	0.29*	0.32*	0.52*	1						
15. MMP-9	0.72*	0.32*	0.69*	0.60*	0.12*	0.38*	0.57*	0.53*	0.12	0.63*	0.07	0.31*	0.53*	0.33*	1					
16. TIMP-1	0.56*	0.40*	0.55*	0.44*	0.22	0.25	0.44*	0.43*	-0.009	0.44*	0.18	0.46*	0.59*	0.57*	0.59*	1				
17. VEGF-A	0.52*	0.40*	0.55*	0.43*	0.14	0.33*	0.28*	0.70*	0.13	0.40*	0.25	0.30*	0.59*	0.69*	0.61*	0.63*	1			
18. Elastin	0.44*	0.46*	0.51*	0.37*	0.43*	0.55*	0.50*	0.57*	0.34*	0.39*	0.36*	0.38*	0.58*	0.50*	0.39*	0.32*	0.41*	1		
19. Periostin	0.06	0.09	0.14	0.06	0.51*	0.40*	0.08	0.12	0.25	0.06	0.31*	0.11	0.17	0.35*	0.07	-0.01	0.21	0.36*	1	
20. TGF-β	0.55*	0.47	0.58*	0.60*	0.30	0.53*	0.53*	0.67*	0.21	0.32*	0.57*	0.46*	0.33*	0.05	0.43*	0.41*	0.42*	0.30*	0.06	1

Data are presented as Spearman's correlation coefficient. * P<0.05

Appendix 3 Variables used in bronchodilator response (BDR) assessment



Appendix Figure 3.1 Bronchodilator response assessment variables. (1) comparing baseline FEV_1 (prior to the combined hypertonic saline challenge and sputum induction test) with post-bronchodilator FEV_1; and (2) comparing post-challenge FEV_1 (the last FEV_1 obtained prior to treatment with salbutamol or IB) with post-bronchodilator FEV_1.