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NON-INVASIVE ASSESSMENT OF AIRWAY INFLAMMATION IN ASTHMA

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Abstract

Inflammation is a central feature in current definitions of asthma. Despite this, airway inflammation remains infrequently assessed in either population-based studies or clinical practice. In this thesis, conventional and novel non-invasive methods (based on exhaled nitric oxide (FENO) measurement and sputum induction) were used to assess airway inflammation and examine the presence, characteristics and stability of inflammatory asthma phenotypes in a general population sample, which included very young and very old individuals.

It was shown that FENO measurement could be easily and cost-effectively conducted, and that flow cytometric analysis of sputum leukocyte populations is a feasible alternative to conventional manual cell counts. In particular, flow cytometric analysis was shown to be well suited to the detection of rare cell populations, and provided data suggesting that airway invariant natural killer T cells may not be a key player in asthma pathophysiology and that basophils may be a useful indicator of allergic airway inflammation in asthma.

When examining inflammatory asthma phenotypes, it was shown that less than 50% of asthmatics (both children and adults) had evidence of eosinophilic inflammation, although in one small study, altered treatment resulted in phenotype changes in more than 50% of asthmatics studied. Neutrophilic airway inflammation was rare, and was statistically significantly associated with age. Approximately half of all the asthmatics studied had no detectable evidence of airway inflammation at the time of assessment.

In conclusion, the methods developed and validated for the non-invasive assessment of airway inflammation allow more detailed investigations of asthma aetiology in population-based studies. However, a single assessment of airway inflammation may not be adequate for valid identification of inflammatory asthma phenotypes. The results of the studies described in this thesis suggest that 50% of asthmatics may have eosinophilic airway inflammation, with the remainder having no airway inflammation. Further investigations of non-inflammatory mechanisms are therefore warranted, as a better understanding of the mechanisms and the associated environmental exposures involved may guide the development of more effective therapies and control measures for this common phenotype.

“If you know the enemy and know yourself, you need not fear the result of a hundred battles. If you know yourself but not the enemy, for every victory gained you will also suffer a defeat. If you know neither the enemy nor yourself, you will succumb in every battle”

— Sun Tzu, the Art of War

“If we knew what it was we were doing, it would not be called research, would it?”

— Albert Einstein

Author's declaration

This thesis was produced according to Massey University's 'Thesis-by-Paper' Requirements. That is, it is largely based on research that is published, in-press, submitted for publication, or is in final preparation for submission. Each individual chapter is set out in the style of the journal to which it has been submitted. Consequently, some of the submitted chapters are relatively succinct, there is some repetition (particularly in the Methods sections) and there are small stylistic differences between chapters. To supplement the relative brevity of some of the chapters, the appropriate sections of the literature review have been extended.

The submitted manuscripts include other authors, including my PhD supervisors and in some cases, collaborators in different institutes in New Zealand and Australia. However, for each chapter, my input was greatest. I was the lead investigator for all the studies described (with the exception of chapter 7), involved in oversight of study design, recruitment, work co-ordination and data collection, data analysis and preparation of the manuscripts. In some cases, I was also involved in preparation of the grant applications and ethics applications prior to the study. I was, however, assisted and supported by my co-authors for all the studies herein.

In regard to chapter 7, the clinical assessments and data compilation had been conducted prior to my involvement. I was, however, heavily involved in the data analysis and responsible for interpretation of the findings of the study, and I wrote the manuscript, with some input provided by the co-authors.

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Table of contents

Abstract.....	i
Author's declaration	iii
Acknowledgements	iv
Table of contents	vi
List of figures.....	x
List of tables.....	xii
List of abbreviations	xiv
1. INTRODUCTION.....	1
2. LITERATURE REVIEW	11
2.1 Introduction.....	11
2.2 Asthma	12
2.2.1 A historical perspective.....	12
2.2.2 Modern definitions.....	15
2.2.3 Diagnosis and assessment	17
2.2.4 Assessment of asthma in research studies	23
2.2.5 Epidemiology: Time trends and global patterns	27
2.2.6 Treatment	31
2.3 The immunopathological basis of asthma	33
2.3.1 Introduction.....	33
2.3.2 Atopy and allergy.....	34
2.3.3 TH ₂ inflammation	36
2.3.4 Innate immunity	43
2.3.5 Impaired innate immune response in allergic asthma.....	47
2.3.6 Mechanisms underlying the protective effect of microbial exposure	48
2.3.7 Innate immunity in non-allergic asthma	50
2.4 Heterogeneity in asthma.....	51
2.4.1 Introduction.....	51
2.4.2 Approaches to classifying asthma.....	52
2.4.3 Phenotyping asthma on the basis of airway inflammation	56
2.4.4 Characteristics of eosinophilic and non-eosinophilic asthma.....	59
2.4.5 Stability of inflammatory phenotypes.....	67
2.4 Assessment of airway inflammation and pathology in asthma.....	70
2.4.1 Introduction.....	70
2.5.2 Bronchoscopic assessment: Bronchial wash, Biopsy and BAL.....	73
2.5.3 Induced sputum.....	75

2.5.4 Exhaled air and exhaled breath condensate	80
2.5.5 Fraction of exhaled nitric oxide (FENO)	81
2.5.6 Nasal lavage	84
2.5.7 Systemic markers of inflammation	85
2.5.8 Assessment of airway remodelling	87
2.6 Summary.....	88
3. MEASUREMENT OF EXHALED NITRIC OXIDE IN A GENERAL POPULATION SAMPLE: A COMPARISON OF THE MEDISOFT HYPAIR FENO AND AEROCRINE NIOX ANALYSERS.....	91
3.1 Introduction.....	92
3.2 Methods.....	94
3.3 Results	96
3.4 Discussion.....	101
4. IDENTIFYING LEUKOCYTE POPULATIONS IN FRESH AND CRYOPRESERVED SPUTUM USING FLOW CYTOMETRY	107
4.1 Introduction.....	108
4.2 Materials and Methods.....	110
4.3 Results	114
4.4 Discussion.....	122
5. INVARIANT NATURAL KILLER T CELLS AND ASTHMA: IMMUNOLOGICAL REALITY OR METHODOLOGICAL ARTEFACT? ..	129
5.1 Introduction.....	130
5.2 Methods.....	132
5.3 Results and discussion	135
6. SPUTUM BASOPHILIA AS AN ALTERNATIVE TH₂-INFLAMMATORY BIOMARKER IN ASTHMA	139
6.1 Introduction.....	140
6.2 Methods.....	142
6.3 Results	145
6.4 Discussion.....	148

7. RELATIONSHIP BETWEEN AIRWAY NEUTROPHILIA AND AGEING IN ASTHMATICS AND NON-ASTHMATICS	153
7.1 Introduction.....	154
7.2 Methods.....	156
7.3 Results	159
7.4 Discussion.....	166
8. NON-EOSINOPHILIC ASTHMA IN CHILDREN	171
8.1 Introduction.....	172
8.2 Material and Methods	174
8.3 Results	179
8.4 Discussion.....	190
9. ASTHMA PHENOTYPES: PREVALENCE, STABILITY AND AIRWAY NEUTROPHIL FUNCTION FOLLOWING TREATMENT CHANGES	197
9.1 Introduction.....	198
9.2 Materials and methods	200
9.3 Results	207
9.4 Discussion.....	214
10. DISCUSSION AND CONCLUSIONS	221
10.1 Introduction.....	221
10.2 Summary of main findings.....	221
10.3 Methodological considerations	222
10.4 Study implications.....	228
10.5 Strengths and limitations	234
10.6 Recommendations for future research.....	237
10.7 Conclusions.....	246
REFERENCES.....	249
Appendix 1: Publications contributed to during PhD study.....	295

Appendix 2: Statement of contributions for published papers included in thesis	
.....	296

List of figures

FIGURE 2.1. Conventional model of the relationship between the different components of asthma.....	16
FIGURE 2.2. Features of TH2-mediated inflammatory processes in allergic asthma.	37
FIGURE 2.3. Sputum cytopspins showing four inflammatory subtypes of asthma.....	58
FIGURE 2.4. Environmental exposures and pathways likely to be associated with the inflammatory phenotypes of asthma.....	63
FIGURE 2.5. Overlap syndrome	66
FIGURE 2.6. Sputum induction.	76
FIGURE 3.1. Correlation between FENO measurements detected with the HypAir FENO and NIOX analysers (based upon the mean of three measurements).....	97
FIGURE 3.2. Bland-Altman plot showing the relationship between FENO levels detected with the two instruments.....	98
FIGURE 3.3. Example of longitudinal variation in FENO measurements from 2 study participants	100
FIGURE 4.1. Flow cytometric gating of induced sputum.....	116
FIGURE 4.2. Correlations and Bland-Altman plots comparing flow cytometric and light microscopy differential cell counts for induced sputum	118
FIGURE 4.3. Correlations and Bland-Altman plots comparing flow cytometric differential cell counts for fresh and cryopreserved induced sputum	121
Supplementary Figure 4.1. Correlations and Bland-Altman plots comparing flow cytometric differential cell counts when gating all versus viable only induced sputum	127
Supplementary Figure 4.2. Correlations and Bland-Altman plots comparing flow cytometric differential cell counts of viable cells only versus light microscopy differential cell counts for induced sputum	128
FIGURE 5.1. Correlation between blood-derived T cells identified as iNKT cells using 6B11 antibody or loaded CD1d tetramers.....	134
FIGURE 5.2. The effect of differential gating on the detection of iNKT cells.	138
FIGURE 6.1. Assessment of basophils in induced sputum using flow cytometry... ..	147
FIGURE 7.1. Correlations and linear regressions of sputum neutrophil percentages against age.....	162

FIGURE 7.2. Distribution of sputum neutrophil percentages for asthmatics/ non-asthmatics in different age groups.	165
FIGURE 8.1. Sputum eosinophil percentages in (A) asthmatic and non-asthmatic children and (B) non-asthmatic, non-eosinophilic asthma and eosinophilic asthma.	180
FIGURE 8.2. Sputum neutrophil percentages in (A) asthmatic and non-asthmatic children and (B) non-asthmatic, non-eosinophilic asthma and eosinophilic asthma.	181
FIGURE 8.3. Percentage of sputum eosinophils in asthma when stratified for ICS use	182
FIGURE 8.4. Sputum eosinophil and neutrophil percentages in asthma groups stratified according to severity.	184
FIGURE 9.1. Flow chart showing the study plan.	201
FIGURE 9.2. Clinical and sputum sample inflammatory parameters before/after change in asthma management in asthma when stratified into EA/NEA subgroups.	212
FIGURE 9.3. Sputum sample neutrophil functional parameters (as assessed using flow cytometry) with optimal/ suboptimal treatment.	213

List of tables

TABLE 2.1. Different approaches to phenotyping asthma	54
TABLE 2.2. The advantages and disadvantages of the different methods used to assess airway inflammation.	72
TABLE 3.1. Characteristics of the study population.	96
TABLE 3.2. FENO measurements for different population subgroups obtained with the NIOX and HypAir FENO.	99
TABLE 3.3. Specifications and running costs of the NIOX and HypAir FENO	102
TABLE 4.1. Participant characteristics.	114
TABLE 4.2. Sample characteristics and percentage of leukocyte populations determined using DCC and FCM	117
TABLE 4.3. Mean coefficient of variance (CV) values for inter-observer variation during differential cell count.....	119
TABLE 4.4. Viability of leukocyte populations in induced sputum (IS) as determined by flow cytometry (FCM)	119
TABLE 5.1. Clinical characteristics of participants who successfully completed sputum induction.....	135
TABLE 6.1. Clinical and sputum sample characteristics of participants at baseline visit.....	145
TABLE 7.1. Demographics and sputum analysis data stratified on the basis of age 160	
TABLE 7.2. Multiple linear regression models with sputum neutrophil percentage as dependent variable	161
TABLE 7.3. Number of individuals with >95th percentile of age-specific reference value of percentage sputum neutrophils	163
TABLE 7.4. Number of individuals with >95th percentile of age-specific reference value of absolute number of sputum neutrophils	164
TABLE 8.1. Characteristics of study population	179
TABLE 8.2. Markers of inflammation and endotoxin levels in sputum.....	185
TABLE 8.3. Differences in clinical characteristics between eosinophilic and non-eosinophilic asthmatics.	186
TABLE 8.4. Associations between cell, cytokine, and LPS levels in sputum and clinical characteristics.....	187
TABLE 8.5. Correlations between cell, cytokine, and LPS levels in sputum.	188

Supplementary table 8.1. Clinical characteristics and sputum markers of inflammation in the 4 inflammatory phenotypes previously described	189
TABLE 9.1. Clinical characteristics and sputum sample inflammatory characteristics of all participants.....	208
TABLE 9.2. Alterations in clinical/inflammatory characteristics and inflammatory phenotypes during changes in asthma treatment.	211

List of abbreviations

α -GalCer	alpha-galactosylceramide
AAAAI	American Academy of Allergy, Asthma & Immunology
ACQ	asthma control questionnaire
ACT	asthma control test
AHR	airway hyperreactivity/hyperresponsiveness
ANG	angiopoietin
aOR	adjusted odds ratio
APC	allophycocyanin
APC	antigen-presenting cell
ASM	airway smooth muscle
ATS	American Thoracic Society
BAL	broncho-alveolar lavage
BCG	Bacille Calmette-Guerin
BDNF	brain-derived neurotrophic factor
BDR	bronchodilator reversibility / response
BHR	bronchial hyperreactivity/hyperresponsiveness
BTS	British Thoracic Society
CCL	CC chemokine ligand
CD	cluster of differentiation
CI	confidence interval
CLCA1	calcium-activated chloride channel regulator 1
COPD	chronic obstructive pulmonary disease
CT	computerised topography
CXC	CXC-subfamily chemokine
CysLTs	cysteinyl leukotrienes
CysLTR	cysteinyl leukotriene receptor
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell

DCC	differential cell count
Der P1	<i>Dermatophagoides pteronyssinus</i> group 1
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
DTE	dithioerythritol
EAACI	European Academy of Allergy and Clinical Immunology
EA	eosinophilic asthma
EBC	exhaled breath condensate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ECP	eosinophil cationic protein
ECRHS	European Community Respiratory Health Study
EDN	eosinophil-derived neurotoxin
EGF	epithelial growth factor
ELISA	enzyme linked immunosorbent assay
EMTU	epithelial-mesenchymal trophic unit
EOA	early-onset asthma
FcεR1	high-affinity immunoglobulin E receptor 1
FCS	foetal calf serum
FEV1	forced expiratory volume in one second
FENO	fraction of exhaled nitric oxide/fractional exhaled nitric oxide
FITC	fluorescein isothiocyanate
fMLP	formyl-methionyl-leucyl-phenylalanine
FoxP3	forkhead box protein 3
FSC	forward scatter
FVC	forced vital capacity
GF	growth factor
GINA	Global Initiative for Asthma
GM-CSF	granulocyte-macrophage colony stimulating factor

GWAS	genome-wide association study
HDM	house dust mite
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary axis
HRCT	high resolution computerised topography
ICS	inhaled corticosteroids
IFN- γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphocyte
JAM	junction adhesion molecule
iNKT	invariant natural killer T cell
IS	induced sputum
ISAAC	International Study of Asthma and Allergies in Childhood
LABA	long acting β_2 -agonist
LBP	lipopolysaccharide-binding protein
LOA	late-onset asthma
LPS	lipopolysaccharide
LTB4	leukotriene B4
LTC4	leukotriene C4
LTRA	leukotriene receptor antagonist
mAb	monoclonal antibody
MBP	major basic protein
MCP	monocyte chemotactic protein
MGA	mixed granulocytic asthma
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
mRNA	memory ribonucleic acid
MMP	matrix metalloprotease/metalloproteinase

moDC	monocyte-derived dendritic cell
MPO	myeloperoxidase
NA	neutrophilic asthma
NE	neutrophil elastase
NEA	non-eosinophilic asthma
NF- κ -B	nuclear factor kappa B
NGF	nerve growth factor
NK	natural killer
NOS	nitric oxide synthase
OR	odds ratio
ORMDL3/GSDMB	orosomucoid-like 3/gasdermin B
OVA	ovalbumin
PAF	population-attributable fraction
PAF	platelet-activating factor
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD15	provocative dose leading to a 15% reduction in FEV ₁
PE	phycoerythrin
PEF	peak expiratory flow
PerCP	peridinin-chlorophyll protein complex
PGA	paucigranulocytic asthma
PMA	phorbol 12-myristate 13-acetate
PPV	positive predictive value
PRR	pathogen recognition receptor
qPCR	quantitative polymerase chain reaction
RANTES	regulated on activation, normal T cell-expressed and secreted protein
RNA	ribonucleic acid

ROC	receiver-operator characteristics
ROR	Receptor tyrosine kinase-like orphan receptor
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RSV	respiratory syncytial virus
SABA	short acting β_2 -agonist
SCF	stem cell factor
SCG	sodium chromoglycate
SD	standard deviation
SELDI-TOF	surface-enhanced laser desorption/ionisation-time-of-flight
SPF	specific pathogen free
SPT	skin prick test
SSC	side scatter
TCR	T cell receptor
TGF- β	transforming growth factor beta
TH ₁	T helper 1
TH ₂	T helper 2
TIMP-1	Tissue inhibitor of metalloproteinase 1
TLR	Toll-like receptor
TNF- α	tumour necrosis factor alpha
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
VEGF	vascular endothelial growth factor
YKL	chitinase-3-like protein 1

1. Introduction

There has been a considerable amount of research published about asthma over the last fifty years. A simple keyword search in Pubmed (1) (using ‘asthma’) as of 14th May 2013 recovers more than 131,000 articles. From as few as 223 articles published on the topic in 1960, there have been more than 4,000 new academic articles about asthma published every year since the turn of the millennium. This increase in research parallels, and is overwhelmingly due to, the global increase in the prevalence in asthma and allergy observed during the same period, most clearly in high-income countries, but with an increasing impact in low-income countries (2).

Throughout this intensive period of research, asthma symptoms have regularly been documented in association with airway inflammation. In particular, bronchial biopsies have often shown evidence of increased infiltration of inflammatory cells (leukocytes) associated with allergy, such as eosinophils, mast cells and type-2 helper T lymphocytes (TH₂ cells) in the airways of asthmatics (3). This has led to inflammation becoming one of the defining characteristics of asthma, as prescribed by international bodies such as the Global Initiative for Asthma (GINA) (4). It has also led to the majority of animal-model based systems being developed solely to assess the role of allergic airway inflammation in the pathophysiology of asthma (5). However, whilst assessment of current symptoms, symptom history, atopy status, lung function, bronchodilator response (BDR) and airway hyperreactivity (AHR) may be routinely conducted when assessing asthma, this has proved more difficult with inflammation.

There are a number of reasons for this, but most importantly, until relatively recently, sampling of the airways (by bronchial biopsy-based techniques) has been a highly

technical and invasive procedure limited to well-equipped secondary/tertiary care facilities and research institutes. This has resulted in the categorisation of asthma on the basis of clinical characteristics such as severity and control, or as atopic/non-atopic asthma on the basis of the presence or absence of positive skin prick or specific immunoglobulin (Ig)-E tests; approaches which do not clarify the underlying pathophysiology of airway disease. It has also led to certain assumptions about the nature of ‘asthma’, which are based on the results of relatively small studies conducted on highly-selected populations using bronchial biopsy. These assumptions largely revolve around the concept that asthma is almost universally an ‘allergic’ inflammatory disease, associated with allergen exposure, sensitisation, and IgE-mediated inflammatory responses involving the leukocytes mentioned above. It is only recently that a greater acceptance of the heterogeneity associated with asthma has become commonplace, and that this paradigm of asthma as a single “disease” with a solely allergic pathology has been generally discounted (6). There have even been calls to discard the term “asthma” altogether, as it has been claimed to be an impediment to progressive research, and an obstacle to the development of appropriate biological and clinical definitions and interventions (7). The respiratory community’s greater acceptance of the heterogeneity of asthma is due in no small part to data produced using less invasive methods to assess airway inflammation in the general community, and in large populations of asthmatics.

Measurement of the fraction of exhaled nitric oxide (FENO) and investigation of induced sputum (IS), the approaches developed and used in this thesis, have become two of the most widely used non-invasive methods to assess airway inflammation over the last two decades. Increased levels of FENO, first described in asthma by

Alving et al more than twenty years ago (8), are often found in association with allergy and eosinophilic airway inflammation (9). Induced sputum allows sampling of inflammatory cells and soluble mediators in the proximal airways (10). Studies using IS in particular have identified several different inflammatory subgroups, or ‘phenotypes’, of asthma. These are often described in terms of granulocytic inflammation; eosinophilic (EA) or non-eosinophilic (NEA) (11), neutrophilic (NA), paucigranulocytic (PGA) or mixed granulocytic asthma (MGA) (12), depending upon the preponderance of granulocyte populations in the airways.

Measurement of FENO and assessment of IS have been well characterised and standardised by international clinical bodies such as the American Thoracic Society (ATS) and European Respiratory Society (ERS) (13,14). However, the uses of these (and other) methods to assess airway inflammation are under constant development, with the major aims being to improve understanding of underlying pathology, and to guide treatment and management of airway disease, and asthma in particular.

Measurement of FENO has been developed for use in asthma diagnosis, targeted management and prediction of future asthma outcomes, and in assessment of other airway conditions, such as chronic obstructive pulmonary disease (COPD) (15). Other recent developments include gene expression profiling of bronchial epithelial brushings, which has led to the description of TH₂-high and low phenotypes (16). Similar gene expression analyses of different asthma inflammatory phenotypes have also been described when assessing leukocytes in IS (17). Another method of assessing leukocytes in IS is flow cytometry, which allows monoclonal antibody-based identification and labelling of cells from the airways at an individual level (18). The use of such approaches to identify and characterise the underlying pathology in

asthma may be especially important for treatment and management, as individuals with non-eosinophilic asthma appear less responsive to inhaled corticosteroids (ICS) (11); one of the current mainstays of asthma therapy. Additionally, approximately 10% of asthmatics are estimated to have severe asthma that does not respond adequately to any kind of treatment (19), and severe, difficult-to-treat asthma may be seen in all of the inflammatory phenotypes described above. The cause of this lack of response to treatment is not yet entirely clear.

Thus, even when considering the impact these non-invasive methods have made so far in investigation of airway inflammation, there is still room for improvement. Despite the many years of study and considerable investment that has been made researching asthma, there are still many questions left unanswered. As suggested in a recent editorial in the Lancet;

“Progress in understanding asthma and its underlying mechanisms is slow; treatment can be difficult and response unpredictable; and the prevention or cure is still a pipedream. Asthma, one of the most important chronic diseases, remains a genuine mystery” (20)

The particular questions this thesis will approach are:

What are the pathological processes underlying different asthma phenotypes, and what are the most important molecular pathways and cells involved?

What is the prevalence of asthma phenotypes in the general population, and what are their characteristics?

Is there a difference between these asthma phenotypes in childhood and adulthood?

What is the ‘best’ or ‘most appropriate’ way to investigate the pathological process underlying asthma in the general population setting?

In New Zealand alone, it is estimated that one in six adults and one in four children experience asthma symptoms, a population prevalence that is second only to the UK. Asthma is also the leading cause of hospital admission for children in New Zealand (21). However, to date, only a relatively small number of studies have investigated the prevalence and characteristics of asthma in the general New Zealand community. The New Zealand component of the International Study of Asthma and Allergies in Childhood (ISAAC) comprehensively assessed the prevalence of asthma, rhinoconjunctivitis and eczema symptoms in children in different centres in New Zealand (22,23), but the study protocol did not allow for assessment of airway inflammation and, therefore, did not determine the prevalence of different inflammatory phenotypes. Research groups in Wellington and Dunedin have used FENO: as an indicator of airway inflammation; providing reference values for FENO in the community (24); assessing FENO response to ICS in asthma (25); examining the relationship between atopy and FENO in children (26); and using FENO as a proxy of eosinophilic inflammation during cluster analysis of clinical phenotypes of airways disease (27). However, these studies used FENO measurement alone when assessing airway inflammation and did not confirm the nature of underlying pathophysiology using alternative techniques.

To the author’s knowledge, only two research centres in New Zealand have used IS to examine airway inflammation in asthma, providing important data about the stability of inflammatory phenotypes of asthma and their responses to ICS (28-30). However,

further work must be conducted to clarify the importance, prevalence and characteristics of inflammatory phenotypes of asthma in New Zealand. Such work is clearly of importance locally, but also adds to the relatively small body of research examining inflammatory asthma phenotypes in the general population on an international level.

This thesis develops FENO measurement and assessment of IS as methods for the non-invasive assessment of airway inflammation in asthma, and uses these methods to investigate the different types of inflammation and inflammatory cell populations in adult and childhood asthma in New Zealand. In addition, this thesis contributes to a better understanding of flow cytometry as a standardised method to investigate airway inflammation in asthma, particularly to assess the presence and importance of relatively rare cell populations such as invariant natural killer T cells and basophils and the importance and functionality of airway neutrophils in asthma.

The aims and objectives of this research are:

- Provide a background literature review on asthma focussing on research relevant to this thesis (chapter 2)
- Develop and utilise methods to assess inflammation in the airways in the general population (chapters 3 & 4)
- Assess the importance of specific cell populations (with particular focus on neutrophils, invariant natural killer T cells and basophils) in asthma using these methods (chapters 5, 6 & 7)

- Determine the prevalence and characteristics of inflammatory phenotypes of asthma in the New Zealand general population, in both adults and children (chapters 8 & 9)
- In a group of asthmatics, determine the stability of asthma inflammatory phenotypes during changes in asthma treatment (chapter 9)

The structure of this thesis is therefore as follows.

Chapter 1

This chapter provides a brief introduction to the thesis.

Chapter 2

This chapter provides a review of the asthma literature, and background information relevant to the questions asked above. This chapter contains excerpts from several published reviews in which I was either first author or co-author; *Brooks, Pearce & Douwes, Curr Opin All Clin Immunol, 2013 (31)*; *Douwes, Brooks & Pearce, ERJ, 2011 (32)*; *Douwes, Brooks, van Dalen & Pearce, Curr All Asthma Rep, 2011 (33)*; *Douwes, Brooks & Pearce, JOECH, 2010 (34)*; *Douwes, Brooks & Pearce, Expert Rev Clin Immunol, 2010 (35)*; *Simpson, Brooks & Douwes, Paed Respir Rev, 2008 (36)*.

Chapter 3 *Measurement of exhaled nitric oxide in a general population sample; a comparison of the Medisoft HypAir FENO and Aerocrine NIOX analyser (This chapter has been published - Brooks et al, J Asthma, 2011, 48 (4)324-8)*

This chapter describes a study using a recently marketed and cost-effective nitric oxide analyser (Medisoft HypAir FENO) to measure FENO levels in both adult asthmatics (n=20) and non-asthmatics (n=86) in the general population. The relationship of FENO levels with atopy and health status is assessed, and results are compared with an alternative FeNO analyser (Aerocrine NIOX).

Chapter 4 *Identifying leukocyte populations in fresh and cryopreserved sputum using flow cytometry (This chapter has been published – Brooks et al, Cytometry B Clin Cytom, 2013, 84 (2)104-13)*

This chapter describes a study setting up and validating a flow cytometric approach for identification and assessment of the major leukocyte populations derived from the airways of 18 asthmatics and 23 non-asthmatics. Flow cytometric data are compared with data obtained using the conventional approach (light microscopy). The effects of cryostorage on the subsequent detection of leukocyte populations using flow cytometry is also described.

Chapter 5 *Invariant natural killer T cells and asthma: immunological reality or methodological artefact? (This chapter has been published – Brooks et al, J Allergy Clin Immunol, 2010, 126 (4)882-5)*

Experiments in mice have suggested that invariant natural killer T (iNKT) cells may be important in the pathology of asthma. Clinical studies have, however, provided conflicting results. This chapter describes a study that uses flow cytometry to investigate the importance of invariant natural killer T cells in asthmatic inflammation compared with non-asthmatics, and clarifies some technical issues involved in the detection of this very rare lymphocyte population.

Chapter 6 *Sputum basophila as an alternative inflammatory biomarker in asthma*
(Brooks et al - manuscript in preparation for submission)

In this chapter, which again uses a novel flow cytometric approach to investigate IS, levels of basophils in the airways are measured and compared with levels of sputum eosinophils and other important clinical and immunological parameters in asthma.

Chapter 7 *Relationship between airway neutrophilia and ageing in asthmatics and non-asthmatics* *(This chapter has been published – Brooks et al, Respiriology, 2013, epub ahead of print)*

This chapter: (i) examines the association between airway neutrophilic inflammation and ageing in both asthmatics and non-asthmatics in New Zealand and Australia (194 asthmatics and 243 non-asthmatics aged between 8 years and 80 years); (ii) determines which other factors are independently associated with increased airway neutrophils; and (iii) suggests age-adjusted reference values that may be useful when identifying inflammatory phenotypes of asthma.

Chapter 8 *Non-eosinophilic asthma in children* *(Brooks et al - manuscript in preparation for submission)*

This chapter assesses the prevalence and characteristics of inflammatory phenotypes of asthma (n=77) amongst New Zealand adolescents (aged between 12 and 17 years) in the general population. Inflammatory and physiological parameters are compared with non-asthmatics (n=68), and the association between inflammatory phenotypes, leukocyte populations, levels of inflammatory mediators in sputum supernatant, lung function, AHR, and atopy are investigated.

Chapter 9 *Inflammatory phenotype stability and airway neutrophil function during treatment changes in asthma (Brooks et al - manuscript in preparation for submission)*

In this chapter, inflammatory phenotype prevalence and characteristics are assessed in 50 adult asthmatics and 39 non-asthmatics. This study examines changes in asthma and asthmatic inflammation in a sub-group of asthmatics and, in particular, investigates changes in neutrophil function (using flow cytometry) with altered control of asthma, in addition to examining the effects of treatment optimisation on phenotype stability and airway inflammation in a subgroup (n=24) of asthmatics.

Chapter 10 Provides an overall synthesis of the study results described in this thesis. The main results and conclusions are reported, the strengths and limitations of the various studies are discussed, and further recommendations for clinical research approaches are made.

2. Literature Review

2.1 Introduction

Considering the breadth and volume of asthma literature available, it is not feasible to provide a complete or comprehensive overview of all aspects of asthma. Instead, this Review will provide some background and context specifically for the subsequent chapters of this thesis. In particular, this Review will give an overview of:

1. The principal features of asthma. These include a history of asthma; how asthma is currently diagnosed, defined and treated; details of the most recognisable physiological components of the asthma syndrome; the epidemiology of asthma; and the risk factors and protective factors that may be associated with asthma. In particular, the ‘hygiene hypothesis’ is introduced.
2. The immunopathology of asthma. As asthma is often considered to be primarily an allergic disease, the concepts of atopy and allergy are introduced and the mechanisms underlying allergic airway inflammation are described. Additionally, the concept of microbial interaction with the innate immune system causing or protecting against different types of asthma, and the potential mechanisms underlying the hygiene hypothesis, are described.
3. The immunopathological heterogeneity of asthma. Different approaches to clinical categorisation of asthma are briefly described, before a more comprehensive discussion about different immunopathological asthma

phenotypes and their characteristics. In particular, asthma phenotypes associated with the presence or absence of different airway granulocyte populations (eosinophils and neutrophils) are considered, as is the relative longitudinal stability of these phenotypes.

4. The various methods currently used to assess asthma pathophysiology. The more invasive bronchoscopic approaches are discussed before descriptions of less invasive methods (such as induced sputum and measurement of exhaled nitric oxide).

2.2 Asthma

2.2.1 A historical perspective

Asthma is not a modern phenomenon. Asthma-like symptoms have been described in medical writings from several ancient civilisations (37,38), and the word "asthma" itself is derived from the Greek word “aazein”, which translates as, "to exhale with an open mouth, to pant" (39). The renowned Greek clinician Aretaeus of Cappadocia provided one of the earliest recorded descriptions of asthma in the 1st century BC (39):

“heaviness of the chest; sluggishness to one’s accustomed work and to every other exertion; difficulty of breathing when running on a steep road; they [the patients] are hoarse and troubled with cough”

Since this very early description, numerous authors and commentators have continued to provide insight and develop paradigms about the nature and causes of asthma. For

example, in his “Treatise on Asthma” (discussed in (40)), Moses Maimonides (1135-1205), counseled for the need for improved air quality.

“The concern for clean air is the foremost rule in preserving the health of one’s body and soul...”

Similarly, the Italian physician Gerolamo Cardano (1501-1576) observed a relationship between exposure to the environment and the symptoms of asthma, and admonished his patient, the Archbishop of St Andrews, to remove his feather bedding, have no fires in the fireplace, and live a “simple” life, with “miraculous effect” (41).

In the 17th century, Sir John Floyer (1649-1734) documented accounts of symptoms, treatment and prevention of asthma, and described exacerbating exposures such as pollution, cold air, and stress (42). At around the same time, Italian physician Bernardino Ramazzini (1633-1714) described a relationship between organic dust and asthma (43), providing an early description of the association between microbial exposures and respiratory symptoms. Henry Hyde Salter (1823-1871) and Sir William Osler (1849-1919) were amongst the first to describe what could be considered key features of asthma (compared to other causes of dyspnea) in the mid to late 19th century (44,45). These include hyperresponsiveness to stimuli such as cold air and chemical irritants, broncho-constriction, and structural changes to the airways, now described as remodelling.

Stress has long been associated with asthma (34) and, until the early twentieth century, asthma was widely viewed as a psychosomatic disorder. Henry Hyde Salter wrote that *“asthma is essentially, and with perhaps the exception of a single class of cases, exclusively a nervous disease; the nervous system is the seat of the essential*

pathological condition” (46). This belief was widespread until relatively recently; for example, in 1933 Stolkind stated that asthma was “...*of nervous origin, generally hereditary, and due for the most part to the influence of the psychic factor*” (47).

From the late nineteenth and early twentieth century, there was increasing acceptance that rather than being solely a neurotic disorder, asthma may be associated with allergen exposure and involved inflammatory processes. An association between environmental exposures and respiratory symptoms had been observed for centuries (e.g. Blackley’s work on pollen exposure and hayfever (48)), but it was around this time that it was shown that exposure to extracted proteins led to symptoms of hay fever (49), and inhalation of allergen was associated with increased asthma exacerbations (50). In 1916, Isaac Chandler Walker observed that some of his asthma patients showed a positive response to dermal challenges with proteins derived from animals, foods and bacteria (51-53). With the discovery of “reagin” in 1922 (later characterised as immunoglobulin E (IgE) in 1966 (54)), and the introduction of routine allergen-specific skin prick testing, it was shown that this response to dermal challenges was associated with exposure to a particular allergen and occurred in combination with eosinophil recruitment to affected tissues i.e. the hallmarks of an allergic response (as further discussed in section 2.3 of this Review).

However, despite this recognition that allergen exposure was important in asthma, even in the early part of the twentieth century, it was also recognised that this allergen-driven asthmatic response was not universal. In particular, in a series of studies conducted in the early twentieth century, Rackemann categorised asthma into two general groups; extrinsic asthma (associated with allergen sensitivity and

exposure) and intrinsic asthma (more likely to be associated with a respiratory infection or stress) (55-57). Furthermore, although early post-mortem studies of fatal asthma showed some key pathological features of asthma, such as influx of inflammatory cells into the airway lumen, excessive mucus, epithelial disruption, smooth muscle hypertrophy, goblet cell hyperplasia and thickening of the subepithelial basement membrane (reviewed in (58)), considerable heterogeneity in the nature of airway inflammation was observed (59). Thus, even from the earliest investigations into the pathophysiology of asthma, there was evidence for more than a single type of underlying immunopathology.

2.2.2 Modern definitions

The definition of asthma has changed even in the last 40 years. In 1975, the definition of asthma given by the World Health Organisation was;

“Asthma...is a disease characterized by recurrent attacks of breathlessness and wheezing, which vary in severity and frequency from person to person. In an individual, they may occur from hour to hour and day to day, and is due to inflammation of the air passages in the lungs and affects the sensitivity of the nerve endings in the airways so they become easily irritated. In an attack, the linings of the passages swell, causing the airways to narrow and reducing the flow of air in and out of the lungs” (60).

The most recent definition given by the Global Initiative for Asthma (GINA) is that asthma is; *“a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway*

hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airway obstruction within the lung that is often reversible either spontaneously or with treatment” (4).

Alternatively, asthma has been described as... *“a complex syndrome with many clinical phenotypes in both adults and children. Its major characteristics include a variable degree of airflow obstruction, bronchial hyperresponsiveness and airway inflammation” (61)*

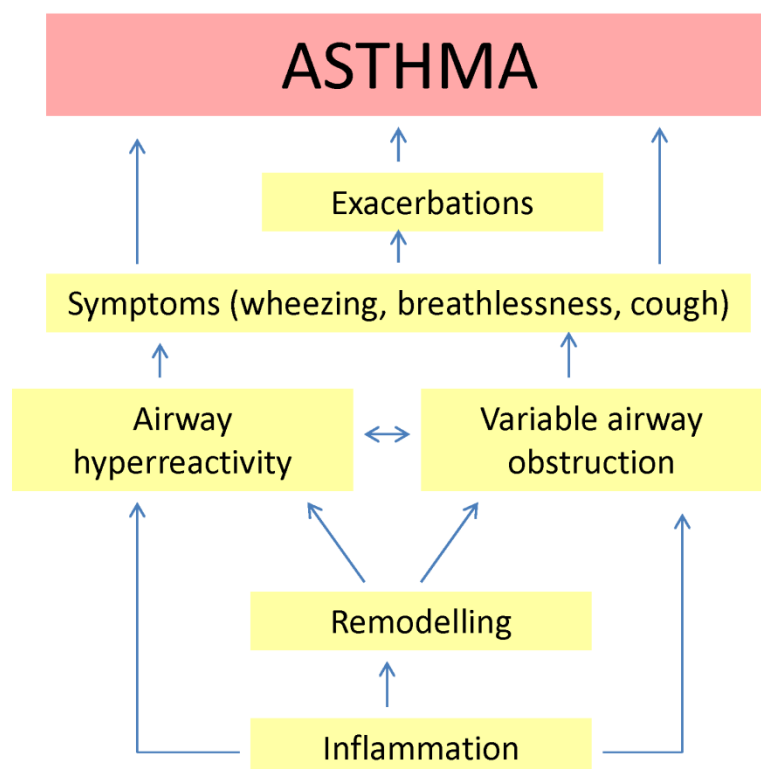


FIGURE 2.1. Conventional model of the relationship between the different components of asthma, in which inflammation is primarily responsible for the physiological changes leading to asthma symptoms (Modified from (62))

Thus, the current definitions of asthma generally describe four main features; symptoms, airway obstruction, hyperresponsiveness and inflammation (63). However these definitions do not describe a single primary characteristic by which asthma can be diagnosed or clearly distinguished from other respiratory diseases (64). This difficulty in identifying asthma is not a recent issue. For example, more than half a century ago, Frankland suggested that; *“The terms asthma, bronchitis, asthmatic bronchitis and emphysema, may in many countries refer to variations of the same complaint. This may explain to some extent why the English disease ‘bronchitis’ is so common here (UK), while in America it is (allergic) asthma that is so common.”* (65)

Therefore, although there are common themes underlying the various definitions and descriptions of asthma available, they are not necessarily entirely consistent with one another, or exclusive to asthma, as described below. Furthermore, the more recent definitions emphasise the involvement of inflammation in asthma (Figure 2.1). However, until recently inflammation has rarely been assessed in a clinical setting, and there appears to be a large number of asthmatics in studies over the last two decades in whom inflammation was not clearly evident, or did not conform to expected patterns (see section 2.4). The importance of inflammation in asthma in particular will be the focus of section 2.3.

2.2.3 Diagnosis and assessment

There is currently no single test available for making a definitive diagnosis of asthma. However, practical guidelines aiding the clinical diagnosis and management of asthma, in both children and adults, are available from international respiratory

associations, such as the American Thoracic Society (ATS) (62) or British Thoracic Society (BTS) (66). A clinical diagnosis is usually made using a combination of clinical history, physical symptoms, lung function testing to assess airway obstruction (conducted using spirometry or peak flow meters), and airway/bronchial hypereactivity (AHR or BHR; conventionally measured using histamine or methacholine bronchial challenge) over time. Pre and post-bronchodilator treatment during lung function assessment may also be used. This test allows identification of the reversible airway obstruction often seen in asthma (bronchodilator reversibility: BDR) that distinguishes it from the irreversible airway obstruction observed with another obstructive airway condition, chronic obstructive pulmonary disease (COPD).

Airway obstruction

Variable airway obstruction has long been identified as an important component of asthma. Indeed, up until relatively recently, it was at the core of the definition of asthma. In 1959, The CIBA foundation defined asthma as “...*a disease characterised by wide variation over short periods of time in resistance to flow in the airways of the lungs*” (67). The measurement of airway obstruction is readily conducted using spirometry. Spirometry is a simple, reproducible procedure used for assessment of a range of respiratory conditions, including asthma (68) and COPD (69), and can be routinely used to assess lung function in children as young as six years old (70). It involves the measurement of volume and airflow over time during exhalation and following a full inhalation (71). For the assessment of airflow limitation, forced expiratory volume in 1 second (FEV₁) and forced vital capacity (FVC) are the most commonly used variables derived from spirometric assessment and the values obtained are often compared with a set of predicted values derived from a normal

population, such as those published by the National Health and Nutrition Examination Survey (NHANES) III (72-74) or the New Zealand Respiratory Survey (75). To allow standardisation of spirometric procedures, international guidelines have been produced (76). In the general population, the FEV₁/FVC ratio is generally over 0.8, and values below this may be indicative of airflow obstruction (68).

In asthma, a single measurement of FEV₁ alone has been used to predict increased risk of future asthma attacks (77). Furthermore, several studies have shown an association between inflammatory cells and spirometric parameters such as FEV₁ or FEV₁/FVC (78,79), although data are often conflicting (80-82). The relationship between spirometric data and inflammation appears to be less clear in children with asthma, as some studies have shown that even in the presence of severe asthma symptoms, children are less likely to have significantly reduced lung function than asthmatic adults (83,84). Importantly, as asthma is characterised by *variable* airflow obstruction, a reduction in lung function as assessed by spirometry at any one time may not be observed in asthma, or may not correlate with symptoms (85).

Recent guidelines have emphasised the importance of measuring the bronchodilator-induced reversibility of airway obstruction (BDR) when assessing lung function in asthma (4) (see above). BDR has been described as an improvement in FEV₁ of at least 12% or 200ml within 15-20 minutes of administration of a short-acting β_2 -agonist (28,68), although some researchers advocate the use of >15% improvement in FEV₁ after treatment (86). However, similar to symptoms, AHR and baseline lung obstruction, BDR is not always stable on the intra-patient level, and may show temporal variability. Some patients may not display BDR when their asthma is well

controlled, and paradoxically others may not display BDR until their asthma has been brought under control (87). Moreover, although asthma is generally associated with reversible airflow obstruction (indeed it has been considered a 'hallmark' of asthma (88)), it has been estimated that between 35-50% of asthmatics have some degree of irreversible obstruction (89). Additionally, BDR may be present in non-asthmatics; for example, in one European multicentre primary care study of 1947 non-asthmatic/non-COPD adults presenting with acute cough (of less than 28 day persistence), 12% showed BDR (90).

As a convenient alternative to spirometry when assessing airflow obstruction, peak flow meters may be used. Peak flow meters are cheap; can be taken away by an individual and used at home on a regular basis with little training; and provide a measure of peak expiratory flow (PEF). PEF diurnal variation of >20% or an increase in PEF of $\geq 15\%$ after bronchodilator administration is suggested as supportive of an asthma diagnosis in some guidelines. However, these guidelines also suggest that PEF is less sensitive than spirometric assessment (4).

Airway Hyperreactivity (AHR)

Airway hyperreactivity (AHR or bronchial hyperresponsiveness (BHR)) describes an increased bronchoconstrictive response to a range of stimuli and is often used as an indicator of variable airway limitation when assessing asthma. The association of AHR with asthma was first reported in the first half of the twentieth century. In particular, Alexander and Paddock reported that asthma attacks could be precipitated by subcutaneous injections of pilocarpine (91) and Weiss showed that asthmatics had increased symptoms and reduction in FVC induced by intravenous histamine

compared with non-asthmatics (92). Airway hyperreactivity as an objectively measured response to particular doses of stimuli was first described by Curry in 1946 during a series of experiments with dosed histamine inhalation (93). Airway hyperreactivity is now commonly assessed in research settings using direct bronchial provocation tests with methacholine or histamine (94). These agents act directly on the smooth muscle (95). More recently, hypertonic saline (96) and mannitol (97,98) have been used in bronchial challenge tests as alternative indirect agents, acting through intermediate inflammatory pathways to cause bronchoconstriction. In these tests, subjects inhale increasing doses of the agent in question, with lung function measurement after each increasing dose. Asthma is often characterised by a more rapid decrease in lung function with increasing doses of the respective agent (hyperreactivity) and at much lower doses than induce an effect in non-asthmatics (hypersensitivity). Airway hyperreactivity is generally reported in terms of dose or volume of the challenging agent causing a drop in FEV₁ of between 15 and 20%.

Whilst regularly described as a key characteristic of asthma, AHR is not always observed in asthma (95) and can be observed in non-asthmatic individuals, particularly children and infants, or during viral infection in otherwise healthy individuals (99,100). For example, in a Japanese study of 154 children aged between 5 and 6 years, it was not possible to distinguish asthmatics from non-asthmatics on the basis of AHR to methacholine (101). Individuals with other obstructive respiratory conditions have also been reported to show AHR (102,103) and there is evidence that there is diurnal variation in AHR (104). Furthermore, there may be poor correlation between AHR and severity of asthma (80), lung function (105) and eosinophilic airway inflammation (106), although there are equivocal reports (81,82). The choice

of agent used in AHR testing may also lead to varied results, as has been observed in one study in children in which histamine was compared with mannitol, in which only 72% of those who responded to histamine also responded to mannitol (107). Finally, to add a further degree of complexity, it is possible that methacholine and allergen-induced AHR arise from independent mechanisms. In particular, evidence from a murine model suggests that allergen induced AHR was associated with normal smooth muscle contraction but exaggerated airway closure (with a 28% reduction in visible lung area), whilst methacholine challenge in the hyperresponsive A/J strain of mouse resulted in excessive smooth muscle contraction, with only 10% reduction in lung area, observed largely in the central airways (108). Therefore, the AHR results observed during formal AHR testing may not necessarily be indicative of what occurs during natural environmental exposures.

Airway remodelling

Alongside inflammation, airway remodelling is believed to be the major pathological cause of chronic airway obstruction and AHR in asthma. Airway remodelling was first comprehensively described by Huber and Koessler in autopsy studies of fatal asthma in 1922 (59). Initial epithelial damage in asthma leads to a series of events leading to regeneration, remodelling and repair. This involves a process of epithelial shedding, basement membrane thickening, hyperplasia and hypertrophy of smooth muscle, alterations in the extracellular matrix (including increased deposition of matrix proteins collagen I, III, V, fibronectin and proteoglycans), and alterations in blood vessels (109). Remodelling sites in the airways of asthmatics are thus largely focussed on the epithelium, smooth muscle, extracellular matrix and associated fibroblasts (110) (described together as the epithelial mesenchymal trophic unit

(EMTU (111)) and associated vascular (112) and neural (113) systems. An increase in airway smooth muscle (ASM) mass in particular is likely to be one of the main causes of airway narrowing in asthma (114). In support of this, bronchial thermoplasty (heat killing and thus reduction of ASM) has proven an effective therapy in some severe asthmatics (115).

Over time, and with inadequate asthma control, chronic inflammation is commonly believed to be the primary cause of airway remodelling (116), and is associated with increase rate of lung function decline and irreversible airway obstruction (117).

Despite this, airway remodelling can also be observed in childhood asthma from a very early age (118,119), suggesting that remodelling may actually occur in parallel with, rather than simply as a consequence of, inflammation and that inflammation may not actually be necessary for remodelling (120). Indeed, it has been speculated that remodelling is simply a result of ineffective repair of damaged epithelium (121). Furthermore, there is recent evidence that airway remodelling can occur in the absence of airway inflammation (122) and may even occur under solely the mechanical stress of bronchoconstriction (123).

2.2.4 Assessment of asthma in research studies

Clinical assessment

Despite the tests available for measurement of airflow obstruction and AHR described in section 2.2.3, there are several disorders that can be confused with or mimic aspects of “classical” asthma at assessment, including COPD (which is discussed in more detail in section 2.4.4), dysfunctional breathing (124), eosinophilic bronchitis (125),

vocal cord dysfunction (126), bronchiectasis and even congestive heart failure (reviewed in (127)). As pointed out in the adage accredited to Chevalier Jackson (1865-1968), “*all that wheezes is not asthma*” (128).

Furthermore, asthma is an extremely variable multicomponent condition, in which all four major features mentioned above may or may not be present at any given time; i.e. one individual may present with severe symptoms and very little inflammation or airway hyperresponsiveness, while another may have few symptoms but significant inflammation and AHR (106). There may not even be the presence of signs or symptoms (characteristically periodic difficulty breathing and cough, often in conjunction with audible respiratory whistling or wheezing) at the time of assessment. Moreover, although inflammation is believed to be responsible for the structural and functional airway alterations observed and, as such, is at the core of the current definition, there are a number of reports showing a relatively weak association between airway inflammation and symptoms or objective measures of lung function (80-82,85,129-132). In some cases of asthma, there may actually be no clear evidence of inflammation at the time of assessment (12,133).

A considerable difficulty when assessing asthma in clinical research studies is that a physician's original diagnosis of asthma cannot always be subsequently verified at a later date. This may be due to the variability often observed in asthma, successful management (leading to an improvement in AHR, lung function and symptoms), or the use of alternative criteria at subsequent assessment when confirming that an individual has asthma. For example, in a retrospective study of 86 patients diagnosed with asthma in general practice in Gothenburg, Sweden, an allergologist determined

that 59% had bronchial asthma, 7% had a combination of asthma/COPD, and 34% did not have asthma (134). In a primary care setting in particular, it is common for asthma medications to be prescribed without assessment of objective lung function parameters and tests such as spirometry or methacholine challenge for AHR may not be available. It has been suggested that up to 30% of asthma diagnoses may be inaccurate because of this lack of objective testing (135,136). For example, in a recent study of 262 diagnosed asthmatics from 12 general practices (GPs) in the UK, 82 asthmatic patients (31%) had no evidence of airflow obstruction or AHR. Of the remainder, 87 (33%) had AHR, whilst only 31 patients (12%) had BDR upon assessment (137). Similarly, other studies have reported the absence of AHR in subsequent tests of asthmatic subjects (138). To avoid reliance on one test (such as AHR or BDR) over another when assessing asthma, it has been suggested that algorithms using multiple measures might be useful. In a study of 566 adults being assessed for respiratory symptoms, details of symptoms, lung function, BDR and AHR were collected and used to determine asthma diagnosis using a scoring system (139). However, this approach has yet to be commonly used, and is not currently part of international guidelines.

Importantly, using very specific outcomes (such as spirometric or bronchial challenge measurements) to select asthmatic participants in clinical research studies may potentially lead to results that are not necessarily representative of asthmatics as a whole. In one study, it was estimated that only 4% of current asthmatics in the general population meet the inclusion criteria for eligibility in asthma medication randomised-control trials (140). In both clinical trials and research studies, BDR and AHR are often used to define asthma, but not all groups use the same criteria or cut-off values

for either of these parameters, meaning that asthma populations in different studies may not be comparable (e.g (28,86)). Furthermore, ongoing changes in guidelines regarding description and treatment of asthma (141) add yet more difficulty in the standardisation of asthma definitions used in clinical research studies.

Epidemiological assessment

When assessing and defining asthma in large epidemiological studies (where objective measurements such as spirometry and AHR cannot always be conducted), a common approach is to use symptom and history questionnaires, due to their low cost, convenience and the large sample sizes possible. Examples of two well characterised and validated questionnaires used for the assessment of asthma prevalence in multiple populations include the questionnaires developed for the International Study of Asthma and Allergies in Children (ISAAC) (2,142) and the European Community Respiratory Health Survey (ECRHS) (143,144). Although there is no definitive symptom of asthma, wheeze alone or wheeze in combination with other symptoms may be a sufficiently valid indicator for asthma when compared to a clinician's diagnosis and may possibly be a more appropriate marker of asthma than objective measures such as AHR (145-147) (as discussed in section 2.2.3). In general, data produced using questionnaires related to symptoms correlated strongly with clinical diagnosis (148) and provided moderately to highly repeatable results in both adults (149) and children (150).

However, as with clinical assessment of asthma, identifying asthma in epidemiological studies is not always straightforward. This is primarily due to the absence of a “gold standard” criterion for the identification of asthma, but also

because of the variability in presence and severity of symptoms, and symptoms and history that are not unique to asthma but also present in other conditions as described previously. This may result in misclassification; in particular, there may be potential problems with recall, recognition and interpretation involved in the use of symptom and history questionnaires, as has been reported when based on parental reports of childrens' symptoms. For example, one study comparing parental reports of childrens' wheezing with clinicians' reports found less than 50% agreement (151,152). Similarly, a recent Korean report suggested that although the ISAAC written questionnaire was a useful tool in assessing symptoms in children 10-12 years old, parent-reported questionnaires underestimated prevalence (153). Moreover, the method used for assessment may also affect results. Although there is evidence that there is good agreement between the written and video-based ISAAC questionnaires for comparing asthma symptoms (154), one study showed only moderate agreement (155).

2.2.5 Epidemiology: Time trends and global patterns

Although asthma has been observed for millenia, there has been a dramatic increase in the worldwide prevalence of asthma over the last half century (156,157). It is estimated that up to 300 million people worldwide have asthma (4) and one in six adults and one in four children are currently affected by asthma in New Zealand (21). Furthermore, it is estimated that 5-10% of asthmatics have severe uncontrolled disease, despite using high doses of inhaled and oral corticosteroids; this small proportion is thought to be responsible for 50% of total health costs related to asthma (19,158).

It is possible that at least part of this increase in asthma prevalence can be explained by increased asthma recognition and overdiagnosis (136). However, studies using the same questionnaire-based methodology in the same community over time (such as the ISAAC (2,142) and the ECRHS (143) amongst others (159,160)) have also reported substantial recent increases in asthma prevalence worldwide, suggesting that increased asthma prevalence is not simply an artefact of increased recognition.

Although this increase in asthma has been observed globally, there appears to be considerable regional variability. The ISAAC study (assessing 6-7 year olds and 13-14 year olds across 120 centres in 50 countries), in particular, reported asthma symptom prevalence ranging from 1.6% to 35.3% (2,157,161). In this study, increased asthma symptom prevalence was generally observed in more developed countries, with some exceptions, such as Latin America (162). Across Europe, lower prevalence was observed in Eastern and Southern Europe (163) and, in general, Africa and Asia also showed low asthma prevalence, although higher prevalence was reported in more developed nations in these areas. Asthma prevalence may have peaked or even begun to decline in affluent countries (2,164), whilst continuing to rise in less affluent countries (2,165). The reasons for this global increase and regional decrease observed recently are unclear. In particular, it is not clear if the change in asthma prevalence has been due to changes in one particular phenotype or is consistent across all asthma phenotypes.

Risk factors and protective exposures

The increase in asthma prevalence observed clearly cannot be due to genetics alone due to the rapidity of the increase (166), although genetic susceptibility appears

important, as shown by heritability and genome wide association studies (167). Several possible explanations for the global increase and regional decrease in asthma prevalence have been proposed (33,168), many of which are ascribed to various characteristics associated with living in developed countries; the so-called “Westernisation package” (166). Within the size and scope of this thesis, it is not feasible to comprehensively review all these risk and protective factors, but the reader is directed to recent reviews by Brooks *et al* (31) and Douwes *et al* (33) for further discussion. Briefly, important risk factors for asthma include: atopy and allergy ((169-172) and discussed in section 2.3); a family history of asthma or allergy (173,174); certain childhood respiratory infections (175-178); psychological and emotional stress (reviewed in (32,34)); gender (179,180); paracetamol and antibiotic use (181-186); indoor and outdoor pollution (187-189); occupational exposures (190,191); some bacterial exposures (192-195); obesity (196); and poor diet (197-199).

Protective factors include: certain infections (200-202); exposure to some non-infectious bacteria and their components (discussed in section 2.3; (203-207)); farming exposure (reviewed in (35)) (207-211); animal exposure (210-216); and vitamin D (217-219).

Over the last twenty years, possibly the most studied protective factor mentioned above is that provided by microbial exposure. The protective effect of microbial exposure and infections against the subsequent development of asthma and allergy has been dubbed ‘the hygiene hypothesis’.

The hygiene hypothesis

The increase in asthma and allergy observed over the last fifty years contrasts strongly with a reduced prevalence of infectious diseases, resulting from improved hygiene, public health measures, treatment, and vaccination programmes. Evidence suggesting that the more sanitary modern environment may increase susceptibility to allergy and asthma was originally observed in studies showing that overcrowding, unhygienic conditions and larger family size were associated with a lower prevalence of atopy, eczema, hayfever, and asthma (220,221). This is commonly referred to as the ‘hygiene hypothesis’, (222) which proposes that; ‘...*the apparent rise ... (in asthma and allergic disease prevalence) ... could be explained if allergic diseases were prevented by infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally*’.

Increased infections and exposures to microorganisms and/or their components, particularly early in life, have therefore been proposed as an explanation for these findings. Since 1989, there have been numerous studies investigating the effect that bacterial or viral infections, or exposure to microbial components such as endotoxin, may have in modulating immune response to allergens and therefore provide protection against allergies and asthma. In particular, there have been a considerable number of studies that have suggested that the increased microbial exposure associated with farm living is associated with a reduced tendency to develop asthma and allergy. These studies are discussed in detail elsewhere (31,33,35), and the potential mechanisms involved in this protective effect are discussed in section 2.3.

Importantly, when considering the heterogeneity of asthma, some of these risk factors are associated with increased risk in some asthma phenotypes but, paradoxically, may also be protective for other types of asthma. For example, whilst microbial exposures appear to have protective effects against the development of allergy and thus allergic asthma (as described above), it has long been acknowledged that exposures to high levels of microbial agents are also capable of producing asthma-like symptoms and exacerbating respiratory diseases, as observed with some occupational exposures (223). In particular, exposure to organic dust containing bacterial endotoxin (amongst other microbial components), induces an acute fever and coughing response in the short-term and may result in bronchitis and fixed airway obstruction in the long term (224). Thus, it appears that exposure to microbial components at a particular time in development and at an optimal dose, may be protective against allergy and allergic asthma, whilst high levels such as those observed in occupational exposures lead to the development of *non-allergic/non-atopic* asthma and COPD (further discussed in section 2.4).

2.2.6 Treatment

In section 2.4 of this Review, the idea that different phenotypes of asthma may have variable responses to different asthma medications is discussed. As with risk and protective factors, it is not feasible to comprehensively discuss all aspects of asthma medication available in the context of this Review (the reader is referred to (225) for an overview). However, a brief review is provided to introduce the therapies commonly used.

Conventional therapy for asthma targets either smooth muscle tone or inflammation.

The most common form of treatment used currently is a combination of inhaled

corticosteroid, addressing the inflammatory component of the disease, and

bronchodilator therapy, relaxing the smooth muscle and opening the airways.

Bronchodilators, such as the long acting β_2 -adrenoceptor agonists (LABA) e.g.

formoterol and salmeterol, and short acting β_2 -adrenoceptor agonists (SABA) e.g.

salbutamol and terbutaline, are prescribed as “relievers” and act by binding to the β_2 -

adrenoceptor on smooth muscle, inducing smooth muscle relaxation, and reducing

bronchoconstriction (226). Alternatively, recent data suggest that anticholinergics,

such as tiotropium, may be useful in patients who do not respond adequately to β_2 -

adrenoceptor agonists (227).

Inhaled corticosteroids (ICS; commonly prescribed as “preventers”) are primarily

used to target inflammation in asthma; specifically, they are thought to reduce TH₂-

inflammation in asthma by reducing the nuclear factor (NF)- κ B dependent production

of cytokines and chemokines (228). ICS use produces improvements in lung function

and symptom scores and reduction in frequency of exacerbations (229,230) in

approximately two-thirds of patients (231). However, as many as 30-45% of

asthmatics fail to have a significant improvement in lung function even with high ICS

doses (232,233) and, in some asthmatics, there may be non-uniform or no response to

ICS (233). Furthermore, several adverse health effects have been reported in

association with long-term ICS or oral corticosteroid use (234-237).

Other agents used for treating inflammation in asthma include the mast cell stabiliser

sodium cromoglycate (238), leukotriene modifiers (239) and, more recently,

monoclonal antibodies and biologics targeted at mediators of allergic inflammation

(240); these include antibodies against IgE (omalizumab) (241) and the cytokines IL-4 and IL-5 (mepolizumab and reslizumab) (242). All these agents target different cells and mediators associated with allergic inflammation and, in most cases, have been found to have limited efficacy in some asthmatics. As discussed in section 2.4, there is evidence that some phenotypes of asthma are not driven by allergic mechanisms and may not respond to such agents. In particular, a small number of recent studies have suggested that macrolide antibiotics such as clarithromycin may potentially be useful in the treatment of phenotypes of asthma that are not mediated by allergic mechanisms (243); discussed in section 2.4.

2.3 The immunopathological basis of asthma

2.3.1 Introduction

The current definitions of asthma place considerable importance on the role of inflammation in asthma pathology. In recent decades, the generally accepted paradigm has been that airway inflammation in asthma is associated with atopy, allergy and TH₂-mediated processes. However, there has been ongoing debate about this (33,244,245), and it is now increasingly accepted that asthma is a heterogeneous syndrome that may be associated with different types of inflammation (6,174,246,247).

In this section, the concepts of allergy and atopy are briefly discussed, alongside a review of the underlying mechanisms and cells involved in allergic, TH₂-mediated inflammation. In particular, the cell populations discussed in the chapters of this thesis

are introduced. Finally, the innate immune system and its possible role in both protection (i.e. the hygiene hypothesis) and pathology of asthma are discussed.

2.3.2 Atopy and allergy

The term ‘atopy’ is used to describe sensitisation followed by the generation of specific IgE antibodies against a range of common environmental allergens (248). It is also used to indicate the predisposition to produce increased levels of specific IgE after exposure to common environmental allergens and to develop type I immediate hypersensitivity (i.e. allergic) reactions against these allergens. Atopy is commonly assessed by either allergen-specific skin prick tests (SPTs), or measurement of serum allergen-specific IgE. However, these two methods do not necessarily correlate with each other and the presence of increased IgE does not mean that clinical symptoms may be evident (249,250). Only in the situation where acute or chronic symptoms result from this type I hypersensitivity response is the term ‘allergy’ generally used. In asthma, these symptoms include cough, wheezing, chest tightness and shortness of breath (251).

Asthma is strongly associated with atopy. Epidemiological studies have clearly shown associations amongst atopy, asthma and other allergic conditions such as allergic rhinitis (169,170); however, it is important to recognise that not all atopics develop asthma (252,253). In support of this, it has been estimated that 40% or more of the population in developed countries is atopic, but only about 7% manifest this as allergic asthma (254).

Allergen sensitisation

It is still not entirely clear why sensitisation to otherwise innocuous antigens occurs in some subjects and not others. Many factors are likely to play a role in the development of clinically relevant sensitisation, including allergen type, dose, timing, route and duration of exposure. Furthermore, subject genotype, phenotypic status of the physiological environment and cells exposed to allergens, and co-exposure to other possibly modulatory agents, such as pollution, microbial components and viruses are all likely to be important (reviewed in (255)). Most clinically important allergens are proteins; a number of these, including the house dust mite (HDM) allergens *Der P1* and *Der F1*, are proteases with the capacity to affect epithelial barrier function (256), allowing them greater access to subepithelial privileged sites and thus access to the cells that may guide induction of an allergic response.

The initiation process in allergic sensitisation is believed to be mediated by dendritic cells (DCs), a population of specialist antigen-presenting cells (APCs) localised within the airway epithelium and associated mucosa (257). These cells have the capacity to uptake and process allergens (258), travel to local draining lymph nodes, and present allergen-derived peptides via the major histocompatibility complex (MHC) II molecule to specific T cell receptors (TCRs) on CD4 T cells; this results in the selective expansion and activation of allergen-specific TH₂ cells. This process requires a number of signals (259,260), but a source of IL-4 is essential for TH₂ polarisation of naïve T cells to occur, as IL-4 induces activation of the TH₂-specific transcription factors STAT6 and GATA3 (compared with T-bet in TH₁ cells). There is, however, some controversy about the source of this IL-4 (261-264).

TH₂-lymphocytes secrete a group of cytokines the genes for which are clustered on chromosome 5q31-33; these include interleukins IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (255). Production of IL-4 and IL-13, in particular, by TH₂ cells induces IgE isotype switching in B lymphocytes. Originally known as 'reagin' (265), the IgE class of antibodies was first identified in the late 1960s (54,266), and has been shown to be the major instigating molecule of allergic tissue inflammation.

2.3.3 TH₂ inflammation

In allergic asthma, symptoms are associated with initial IgE-mediated allergen sensitisation, followed by a well-characterised inflammatory response to subsequent allergen exposure (Figure 2.2). Based on data originally derived from murine models and subsequently confirmed in clinical studies, this involves an early-phase response (within minutes of exposure), largely driven by IgE/allergen cross-linking of the high affinity IgE receptor (FcεR1) on basophils and mast cells. Upon cross-linking, mast cells degranulate and release the potent mediators histamine, cysteinyl leukotrienes, prostaglandins, and a range of cytokines and enzymes, leading to airway smooth muscle constriction, mucus hypersecretion and vascular leakage (267).

The early phase is followed (generally within a few hours) by a later phase response, with subsequent TH₂-cytokine-driven eosinophilic airway inflammation (driven through IL-5, which is critical for eosinophil growth and survival (268), B cell IgE production, and mucus production (driven through IL-4 and IL-13) (269,270); Figure 2.2 above). After allergen exposure in susceptible humans and mice, neutrophil influx into the airways occurs relatively quickly and is followed by eosinophil, macrophage

and T lymphocyte infiltration, with the last predominantly TH₂ lymphocytes (271,272). Although other effector cells are clearly important (and are briefly described below), eosinophils are the most characteristic effector cells associated with the pathology of allergic asthma in the airways.

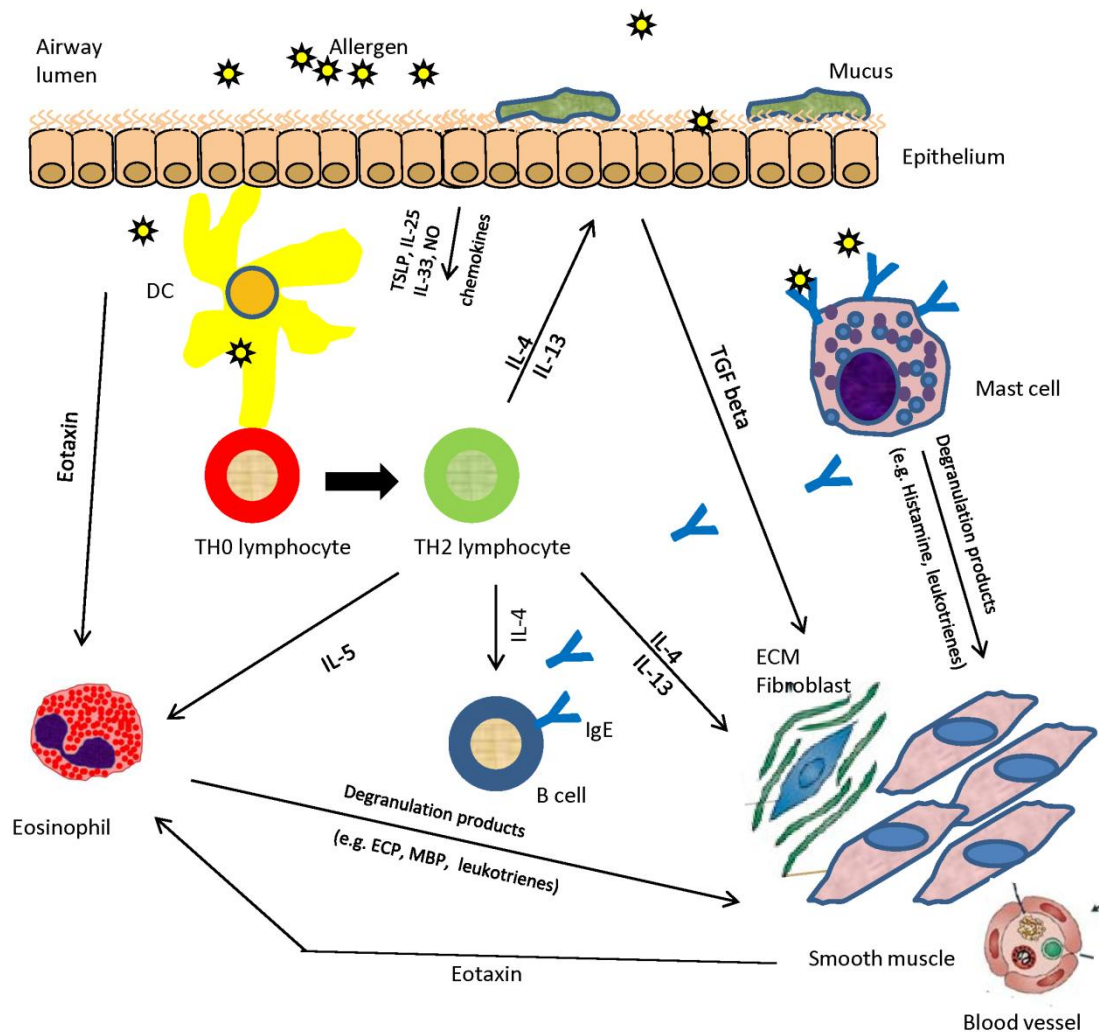


FIGURE 2.2. Features of TH₂-mediated inflammatory processes in allergic asthma. TH₂-polarisation of T cells leads to increased production of the TH₂ cytokines IL-4, IL-5 and IL-13, which in turn leads to IgE isotype switching in B cells, eosinophil accumulation and survival, mucous overproduction, and epithelial and smooth muscle changes. Crosslinking of allergen and mast cell bound allergen specific IgE leads to mast cell degranulation, histamine release, and smooth muscle contraction.

Eosinophils

Eosinophils are myeloid-derived granulocyte leukocytes associated with parasitic infections as well as allergy (reviewed in (273)). First identified by Ehrlich in 1879 on the basis of distinct staining with the acidophilic dye eosin (274), they are relatively rare in peripheral blood, but can be found in large numbers in the airways of many uncontrolled asthmatics (see section 2.4; (275)). Although often considered simple ‘effector’ cells, there is increasing evidence that eosinophils are involved in regulation of local immunity and remodelling/repair (276) and are capable of modulating other leukocyte functions (273,277).

Eosinophils are recruited into the airways and/or sites of injury on a chemotactic gradient (often in response to chemokines such as eotaxin, RANTES (regulated on activation normal T cell expressed and secreted), also known as chemokine ligand (CCL) 5) and members of the macrophage inflammatory protein (MIP) and monocyte chemottractant protein (MCP) families (278), through a well-defined leukocyte adhesion pathway (279). Once in the airways, activated eosinophils release a plethora of potent inflammatory granules and mediators, including reactive oxygen species (ROS), major basic protein (MBP), peroxidase, eosinophil-derived neurotoxin (EDN), platelet activating factor (PAF), cysteinyl leukotrienes (CysLts) and eosinophilic cationic protein (ECP), which together induce microvascular leakage, airway smooth muscle contraction, increased mucous secretion, symptoms and AHR (280).

Eosinophils also produce a vast array of cytokines, chemokines and growth factors (273). These include TH₂ cytokines (e.g. IL-4, IL-5, IL-9 and IL-13) as well as pro-inflammatory cytokines (tumour necrosis factor (TNF)- α , IL-1 β , IL-6, and IL-8) and regulatory cytokines (TGF- β and IL-10) (281). As well as promoting and maintaining

inflammation through these mediators, eosinophils from asthmatics can also induce smooth muscle cell proliferation through a direct cell-contact mediated release of cysteinyl leukotrienes (282).

Dendritic Cells

Dendritic cells have been shown to play an important role in asthma pathogenesis, by initiating and perpetuating T cell responses in asthma (257,283,284). There are two major subsets of DCs in humans: conventional myeloid mDCs and plasmacytoid pDCs (257). Both subpopulations of DCs may be particularly important in asthma. Myeloid DCs appear to be critical for allergic sensitisation (285) whereas pDCs are involved in tolerance to allergens (286); these different DC subsets can either increase (285) or decrease (287) eosinophilic inflammation in asthma models respectively. Human studies have shown that after allergen challenge, increased numbers of both mDCs and pDCs can be found in the peripheral blood and sputum of asthmatic subjects (288-290).

Mast Cells

As mentioned above, mast cells are critical for the early-phase response to allergen in asthma and their continuous activation and increased numbers may be of considerable importance in the late-phase asthmatic response and establishment of AHR (291,292). In chronic asthma, there are increased mast cells numbers associated with airway smooth muscle in the large and small airways (293), and mast cell numbers are increased in BAL in symptomatic asthma (294) and in sputum after allergen challenge in atopic asthma (295).

Basophils

Basophils (as with eosinophils, originally identified by Ehrlich in 1879 (274)) can be characterised by their expression of high affinity FCεR1 receptors and histamine production upon stimulation and, as such, share similarities with mast cells; however, unlike mast cells, they can be found in the circulation (296). Whilst often considered terminally differentiated effector cells, it has been shown that they are capable of cytokine production, in particular IL-4 and IL-13 (297-299). Basophils are recruited through similar mechanisms as eosinophils and, as such, are likely to increase in parallel with eosinophils during allergic inflammation; indeed, they can be detected in the airways in asthma (300,301) and increase after allergen challenge in induced sputum (295) and during exacerbations in bronchoalveolar lavage fluid (BALF) (302).

Macrophages and monocytes

Macrophages and monocytes (mononuclear phagocytes) are primarily involved in phagocytic clearance of foreign and endogenous matter and can be found in abundance in the airways, making up more than half of leukocytes sampled in induced sputum (12,303,304). Macrophages can be loosely characterized as either classically (M1) or alternatively (M2) activated (305). Both animal (306) and human *in vitro* studies (307) suggest that M2 macrophages may be important in the genesis and maintenance of allergic inflammation; however, to date, there are only limited data supporting this hypothesis (16,308). There are, nonetheless, other lines of enquiry suggesting there are some differences in macrophage populations in asthma compared with non-asthmatics. For example, one study showed that bronchial macrophages from mild asthmatics show a decreased CD64 expression and decreased phagocytic

capability compared to healthy controls, correlating with the proportion of eosinophils in the lung (309).

Lymphocytes

B cells are important in allergic asthma principally due to their production of allergen-specific IgE antibodies (3), but various clinical and experimental studies have shown that it is TH₂ CD4⁺ T cells that are essential for the immunopathogenesis of allergic asthma (reviewed in (310)). Although these classical TH₂ cells are considered to be the primary source of cytokines involved in allergic airway inflammation, recent evidence has suggested that innate lymphoid cells may also be important in the regulation of respiratory immunity (reviewed in (311)). Murine studies suggest that they are associated with allergic inflammation, AHR and smooth muscle contraction (312,313), but the importance of these cells in human asthma remains unclear. In contrast to CD4 cells, allergen-specific cytotoxic CD8⁺ T cells may be associated with reduced inflammation and AHR (314,315), possibly through targeted perforin-dependent killing of allergen-presenting DCs (316).

The role of regulatory T cells (Tregs) in asthmatic inflammation in humans is unclear. T regs (characterised by CD25 and forkhead box protein 3 (FoxP3) expression) have a primarily immunoregulatory role and limit immune responses to both allergens and pathogens via cell-contact dependent pathways and production of immunosuppressive cytokines and growth factors such as IL-10 and TGF- β (reviewed in (317)). They therefore may be important in controlling inflammation in asthma, as has been found in animal models (318,319).

Natural killer (NK) cells promote allergen-induced allergic inflammation in animal models, with depletion of NK cells resulting in reduced pulmonary eosinophil and T cell infiltration (320,321). In human studies, conflicting results have been found, with asthmatics having a higher peripheral NK cell number and NK cytotoxicity (322,323) but a lower proportion of NK cells in the sputum (324). Type I invariant natural killer T cells (iNKTs) are a small population of innate-like T cells that have been shown to play an important role in allergic inflammation and asthma in some animal models (325-327), although there is conflicting evidence (321,328). One group has suggested that iNKT cells act specifically through pathways affecting AHR rather than inflammation *per se* (329). Evidence for the involvement of iNKT cells in human asthma remains more controversial (330) and is discussed in more detail in chapter 5.

Limitations of animal models of TH₂ inflammation

When considering the findings of many animal model-based studies examining TH₂-mediated allergic disease pathology (through which the involvement of many of the above processes and cell populations was first observed), it must be remembered that asthma is a disease specific to humans. Few animals, such as horses (331) and cats (332) are naturally affected by an asthma-like syndrome; however, the animals most commonly used in models of asthma are not. Because of this, there has been ongoing debate about the benefits of using animal models in asthma research (5,333-339). Nonetheless, mouse models have been particularly useful for elucidating and examining particular aspects of TH₂-mechanisms underlying *allergic* disease. However, over-interpretation of the results from mouse experiments and a lack of acknowledgement of the complexity of the disease and environmental exposures in

humans means that translation of findings from the mouse to improvements in human therapy has been fraught with difficulty (340).

A plethora of acute allergen exposure mouse models have been used (338). Many of these have involved peritoneal sensitization to large doses of antigen (e.g. ovalbumin) in association with an adjuvant such as alum (aluminium hydroxide), followed by antigen challenge. This is unlikely to represent the variability and complexity of sensitization and/or challenge in a human setting (335). Furthermore, the most commonly used mouse strain for these experiments is the BALB/c, due to its propensity to develop a TH₂-response (341). This adds to a 'TH₂-bias' evident in murine-model derived data. Despite this bias, murine models have provided considerable insight into the pathways and processes involved in acute allergic airway inflammation and AHR, as described above. However, chronic models are still needed to assess other asthma characteristics such as remodelling and persistent airway obstruction (discussed in (342)). Nonetheless, even in chronic multiple allergen exposure models, some characteristics of human asthma (such as substantial airway smooth muscle increases and mast cell recruitment) may not be reproduced (335,341).

2.3.4 Innate immunity

Many of the data produced using murine models suggest that asthma is primarily initiated and driven by components of the adaptive immune system, such as TH₂ cells and DCs (3). However, recent evidence from several sources suggests that innate immunity may also be important and that the immunopathology associated with asthma is a result of complex interaction between components (cells and mediators) of both adaptive and innate immunity and the environment (195,343-345). For example,

there is evidence that a TH₂-polarised environment in the airways may lead to impaired innate responses to bacteria and viruses (346,347). There is also an increasing body of evidence suggesting that interaction between components of the innate immune system (such as the Toll-like receptors (TLRs)) with microorganisms may be protective against allergies and asthma; this may be the mechanism underlying the hygiene hypothesis. Finally, there is evidence that some types of asthma can occur in the absence of a clear indication of allergic or atopic inflammation (as described in section 2.4). Innate immune pathways, involving the identification of microbial components and pollutants by receptors such as the Toll-like receptors (TLRs), and the associated neutrophil influx into the airways, may be particularly important in such cases (36,245).

The innate immune system

The innate immune system is a multi-component defence system providing a rapid response to invasive stimuli and signals via a limited number of germline-encoded host receptors. Innate immunity is particularly important in the lung, a compartment with a large mucosal surface area constantly exposed to environmental particles and bacteria. Innate immune responses are triggered by the recognition of conserved molecules of both endogenous and exogenous origin, such as pathogen-associated and danger-associated molecular patterns (PAMPs and DAMPs) (348). These molecules are recognised by a limited number of receptors called pattern recognition receptors (PRRs) (349). Signalling PRRs recognise PAMPs and subsequently activate signal transduction pathways that induce a variety of immune response genes potentially relevant to asthma pathogenesis, such as those responsible for inflammatory cytokines, co-stimulatory molecules and reactive nitrogen and oxygen radicals. As

such, several PRRs have been suggested to play a role in the pathogenesis of asthma (36,343). The best known of the PRRs are the TLRs.

Toll-like receptors

The TLRs are a highly conserved family of homologous signalling membrane PRRs expressed on many inflammatory cell types implicated in asthma pathogenesis (36), as well as structural cells in the airways such as epithelial (350) and airway smooth muscle cells (351). TLRs are capable of binding specifically to various endogenous ligands (DAMPs) and bacterial, viral, fungal and protozoal-specific ligands (PAMPs) (352). The ligation of the TLR receptor results in signalling through adaptor proteins particular to each TLR, including MyD88, Trif and MAL/Tirap, resulting in NF- κ B activation and subsequent upregulation of inflammatory gene expression (349). TLRs 2, 3, 4 and 7 have been of particular interest in the area of asthma immunology (36). The specificities of the TLRs for different bacterial, fungal and viral components have been previously reported (e.g. (36)). For the purpose of this review, only TLR4, as possibly the most well studied TLR, will be discussed further. TLR4 is essential for responses to bacterial endotoxin or lipopolysaccharide (LPS) (353) and has also been implicated in the innate immune response to viruses such as respiratory syncytial virus (RSV) (354).

In response to LPS stimulation of TLR4 (which also involves CD14 and LPS-binding protein (LBP) (355), NF κ B activation results in the increased production and release of innate immune mediators (such as TNF- α , IL-6, IL-1 β , IL-8, ROS and PAF) by structural cells and inflammatory cells such as neutrophils, an important effector cell

in the innate immune response described in more detail below. These mediators stimulate host cells to initiate an acute phase response and remove invading bacteria or viruses. Neutrophils can also directly respond to endotoxin exposure by increased phagocytosis and release of chemokines including IL-8, which leads to further neutrophil recruitment (356).

Neutrophils

Neutrophils are short-lived polymorphonuclear leukocytes that play an essential 'first line of defence' role in innate immunity, particularly against bacterial or fungal infections (357). They can be found in large numbers in the airways, although considerably more are detected in the proximal than distal airways (358). Neutrophils are important phagocytic cells but also produce and release a number of mediators associated with inflammation that are implicated in the pathology of asthma. Many of these mediators, such as reactive oxygen species (ROS), myeloperoxidase (MPO), neutrophil elastase (NE) and cathepsin G, are critical for microbial killing, but can also result in considerable tissue damage if neutrophil activation is insufficiently regulated through programmed apoptosis and efferocytosis (359). Other neutrophil-derived enzymes such as matrix metalloproteases (MMPs; including MMP-8 and -9 and MMP-25), which are involved with tissue repair and extracellular matrix (ECM) maintenance, have been associated with airway remodelling and development of AHR (360). Some neutrophil products (for example, LTB₄, MIP-1α, IL-1β, and IL-8) are also neutrophil chemoattractants, meaning that neutrophil activation results in the influx of yet more neutrophils and thus prolonged and more severe inflammation (361). There is increasing evidence that neutrophils may be a particularly important

cell population in the pathology of some types of non-allergic asthma (discussed in section 2.3.7).

2.3.5 Impaired innate immune response in allergic asthma

An appropriate innate response to microbial components can be dysregulated in the presence of TH₂-cytokines, such as IL-4, which may be particularly important in allergic asthma. In *in vitro* studies using a monocytic cell line, Fiset *et al* observed that exposure to IL-4 induced a transient STAT-6-dependent suppression of TLR4 gene and protein expression and reduction in LPS-induced NFκB activation (362), suggesting that a TH₂ environment may impair responses to LPS. Other studies also show that TH₂ cytokines reduce LPS-induced cytokine production and CD14 expression (363,364). In agreement with this, there are data showing that asthmatics may have an impaired antibacterial TH₁ response to the effects of environmental endotoxin exposure. For example, a recent study on 13 allergic asthmatic individuals and 18 non-allergic non-asthmatics found that airway inflammatory response to LPS inhalation was blunted in asthmatic participants. Although similar increases in IL-6, TNF-α and neutrophil percentages were observed in both groups, significantly reduced total leukocyte and neutrophil recruitment and IL-1β and IL-18 levels were observed in asthmatics (347). A similar phenomenon has also been observed in nasal explants from atopics compared with non-atopics (365). In further support of this apparent innate immune impairment in allergic asthma, there is evidence suggesting that allergic asthmatics may also have impaired innate-mediated antiviral responses (particularly bronchial epithelial type-1 interferon production), leading to their increased susceptibility to respiratory infection (346,366) and increased exacerbations (367-369).

2.3.6 Mechanisms underlying the protective effect of microbial exposure

Microbial exposure-induced activation of PRRs is hypothesised to lead to downstream suppression of TH₂ cell expansion and therefore TH₂-mediated diseases, including allergic asthma, hay fever and eczema (370). Indeed, this can be observed in the laboratory, where LPS exposure induces a reduction in IL-5 expression from T cells *ex vivo* (371). Although the exact mechanisms responsible for this innate-mediated suppression of TH₂-inflammation are currently unclear, the effect of LPS exposure may be dose-specific. For example, in one *in vitro* study, treatment of DCs with low dose LPS and antigen resulted in a TH₂-type response, whilst use of a high dose of LPS plus antigen stimulated a subsequent TH₁ response (372,373). This was also originally proposed to underlie the hygiene hypothesis. In particular, the hygiene hypothesis was believed to function simply through altering the TH₁/TH₂ balance i.e. growing up in a more hygienic environment with less microbial exposure would enhance atopic (TH₂) immune responses, whereas microbial pressure would drive the response of the immune system – which is known to be skewed in an atopic TH₂ direction during foetal and perinatal life – into a TH₁ direction and away from its tendency to develop atopic immune responses (370). This model now appears to be too simplistic, as demonstrated by parasitic infections which are associated with powerful TH₂ responses, but protective against atopy (202). Additionally, the increase in TH₂ allergic diseases observed over the last half century is paralleled by a similar increase in some TH₁-mediated and autoimmune diseases (374).

These apparently paradoxical findings have led to an alternative explanation for the increased prevalence in asthma and allergies involving altered immunoregulatory

mechanisms. In particular, microbial modulation (either directly or indirectly) of regulatory T cells (T regs) may result in reduced expression of both TH₁ and TH₂ immunity as shown in both humans and murine models (319). For example, in newborns exposed to farming *in utero* (known to be associated with a lower prevalence of allergies and asthma as mentioned in section 2.2.5), an increase in number and function of cord blood FoxP3+ Treg cells has been described, with a corresponding decrease in TH₂ cytokine production (375). Similar findings have been observed in mice, in which perinatal administration of the probiotic *L. paracasei* NCC2461 prevented allergic inflammation in sensitized/challenged offspring, required TLR2/4 signaling, and was associated with increased Fox P3 mRNA expression in the lung (376). Finally, as another potential mechanism, it has been reported that bacterial exposure in the airways may protect against allergic inflammation without induction of either Treg or TH₁ cells, but with alterations in DC activation status (377).

Immune modulation mediated by early life bacterial exposure, as well as protecting against allergies (as suggested by the hygiene hypothesis above), may also modulate components of the immune system to more appropriately respond to infectious agents, such as those that may possibly cause airway inflammation through innate, non-allergic mechanisms, as discussed below (31). For example, in a murine model of *Streptococcus pneumoniae* infection, early-life exposure to microbial components prior to infection was associated with airway colonization by non-pathogenic bacteria (such as lactobacilli), increased airway expression of TLR2, 4 and 9, and decreased neutrophil recruitment (378). Most importantly in this model, early-life exposure to microbial components was associated with prolonged survival from pneumonia (with

increased expression of IFN- γ , IL-4 and MCP-1) following *S. pneumoniae* infection. Thus, lactobacilli colonisation may be responsible for increased pathogen resistance and protection against pathogen-mediated non-allergic asthma, in addition to a more appropriate response to allergens.

2.3.7 Innate immunity in non-allergic asthma

As mentioned in section 2.2, respiratory exposure to bacteria and bacterial components such as endotoxin is associated with asthma-like symptoms, bronchitis, and a reduction in lung function in several occupational exposures (223,379,380). This response (which can also be produced by stimuli as diverse as particulates, pollution and viruses) appears to be largely driven by components of the innate immune system (36). As described above, environmental exposure to agents such as endotoxin leads to stimulation of TLR receptors on structural and inflammatory cells in the airways; this, in turn, leads to the increased production of a range of inflammatory mediators including TH₁ cytokines and IL-8. This IL-8 production leads to the influx of large numbers of neutrophils that, upon activation, are capable of producing a plethora of mediators associated with AHR and remodeling. Such neutrophilic airway inflammation has been regularly observed in occupational studies assessing individuals exposed to large amounts of organic dust (380,381). However, it has also been observed in some general population studies of non-allergic asthma (382-384). The cause of this neutrophilic asthma phenotype is currently unclear, and is discussed in more detail in section 2.4. However, it is possible that some environmental exposures may lead to chronic neutrophilic inflammation. Furthermore, there is some evidence that airway colonisation with bacteria such as *Mycoplasma*, *Haemophilus influenzae* and Chlamydial species may be observed in

some non-allergic asthmatics (385), and there is increased proteobacterial colonisation in the airways in asthma compared with healthy individuals (386), suggesting that chronic bacterial exposure (and thus chronic TLR ligation) in the airways may be associated with innate-mediated neutrophilic asthma phenotypes.

This apparent paradox, that microbial exposure may protect against certain types of asthma and also be associated with inflammation in other asthma phenotypes, has been previously reviewed by Douwes *et al* (35) and Simpson *et al* (36) and is likely to be associated with a number of factors, including the nature, dose, timing and the status of the exposed individual. In particular, it is possible that a bimodal effect of microbial exposure may be responsible; in some individuals, exposure to high doses of microbial components may protect against allergic asthma but lead to an increased risk of non-allergic asthma, or even COPD, both of which have been associated with neutrophilic airway inflammation. Thus, there is considerable evidence that asthma may be caused by either allergic (TH₂- mediated) or non-allergic (innate-mediated) mechanisms in the airways, the identification of which is the topic of the subsequent section of this Review.

2.4 Heterogeneity in asthma

2.4.1 Introduction

An important issue in the study of asthma has been how to most appropriately categorise or ‘phenotype’ the heterogeneity evident within the disease. Although various approaches to phenotyping asthma have been used to date, they have generally provided little insight into the underlying pathological basis of the disease in

population-based studies. This lack of immunopathological phenotyping, in particular, is likely to have been one of the reasons for the inconsistent results observed with therapies targeted at TH₂-mediated inflammation (340), in studies examining risk and protective factors for asthma development and in intervention studies (6). Moreover, the limited characterisation of asthma phenotypes in population-based studies may also be one of the reasons why genetic studies have shown inconsistencies or only weak associations with asthma susceptibility genes. As Gibson states in a 2009 editorial (387), *“I have long puzzled over how scientists can define a person’s genotype to the level of a single nucleotide yet accept a phenotype characterisation in the same individual that is as (im)precise as the answer to the question “Do you wheeze?”*

As noted in Section 2.3, asthma is often considered as primarily an allergic disease. Although there is no doubt that a large proportion of asthma sufferers have allergic asthma, there are also many individuals who are non-atopic or have no allergy symptoms (244,254). It is also likely that there is a geographical variation in the distribution of atopic and non-atopic phenotypes of asthma, with the non-atopic asthma possibly being more prevalent in low-income countries (for example (388)). In this section, some of the approaches used in clinical categorisation of asthma are briefly described, followed by a more in-depth discussion about the nature and characteristics of different inflammatory asthma phenotypes.

2.4.2 Approaches to classifying asthma

There have been several approaches used to categorise asthma in both clinical and research settings (Table 2.1). These include stratification on the basis of severity (4),

level of control (4), age of onset (389-391), chronic airflow obstruction, exacerbations, response to treatment (392,393) or type of asthma trigger (reviewed in (246,247)).

A recently developed approach to identifying discrete groups of asthmatics is to combine characteristics, using multivariate statistical modelling, which has been described by one commentator as '*a step in the right direction*' in the identification of asthma phenotypes (394). Rather than relying on one single variable to identify groups, between nine (27) and nineteen (395) different variables have been used to 'cluster' groups. This clustering approach has been used in both adult (395-397) and childhood asthma (398,399). Statistical methods used have included latent class analysis (395), principal class analysis (400), k-means cluster analysis (396) and the Gower distance measure (27).

However, the need to characterise asthma phenotypes on a pathological rather than clinical basis has long been recognised (401) and is primarily driven by the need for improved treatment options (392,402). Clearly, some asthmatics respond to conventional treatment whilst others do not (232), with non-responders more likely to have a pathology associated with the absence of allergy (384,403) (described in section 2.4.4). Furthermore, the identification of pathological phenotypes (reviewed in more detail below) may also provide further insight into causal exposures, and thus allow the identification and development of appropriate control measures and interventions.

TABLE 2.1. Different approaches to phenotyping asthma
(* based on a combination of parameters)

Clinical phenotypes	Trigger-related phenotypes	Demographic phenotypes	Pathological phenotypes
Severity (intermittent, mild persistent, moderate persistent, severe persistent)	Specific allergen (environmental or occupational)	Age of onset	Inflammatory phenotypes (eosinophilic asthma, non-eosinophilic asthma, neutrophilic asthma, paucigranulocytic asthma, mixed granulocytic asthma)
Control (well controlled, partly controlled, uncontrolled)	Exercise	Obesity-related	
Exacerbation frequency	Aspirin or NSAIDs	Sex-related	
Treatment response (responsive/unresponsive)	Cold		TH ₂ high or TH ₂ low
Reversibility (reversible, partially reversible, irreversible)	Emotional stress		Evidence of remodelling
AHR	Menarche		
Atopic, non-atopic			
Intrinsic, extrinsic*			
Unsupervised clusters*			

Phenotyping asthma on the basis of allergy and atopy

As described in Section 2.2.1, in the early to mid-twentieth century, Rackemann recognised that some asthma patients did not show signs of allergy to “extrinsic” environmental factors such as allergens. This form of asthma was more likely to be found in women in adulthood, was often preceded by respiratory infection, was associated with severe symptoms, and importantly, showed a poor response to treatment. He described this type of asthma as ‘intrinsic asthma’ (56,57,404-406), due to the absence of any clear identifiable external component and speculated that

respiratory infection may be a pivotal initiator in its development. Categorisation on this basis of extrinsic and intrinsic asthma continued into the late twentieth century, until a series of relatively small clinical studies found that airway levels of TH₂-cytokine IL-4 and IL-5 mRNA were similarly high in both intrinsic and extrinsic asthmatics (407,408). These results suggested that asthma was likely to be a TH₂-mediated allergic disease, and led to a reduction in popularity for classifying asthma into intrinsic or extrinsic. Despite these findings, another study conducted at the same time showed distinct immunological differences between the two types of asthma, including increased expression of IL-5 upon *ex vivo* stimulation with house dust mite (HDM) only in extrinsic asthma, and increased expression of IFN- γ and IL-2 (but not IL-4) in bronchoalveolar fluid (BALF) from intrinsic asthmatics (409) (discussed in (410)).

As an alternative to this extrinsic and intrinsic classification, asthma has regularly been defined as atopic and non-atopic asthma in population-based studies, based upon the availability of convenient objective measures such as skin-prick-test (SPT) positivity, or specific or total IgE measurements (411,412). For example, one seminal study of 656 asthmatics found that 102 (16%) individuals were skin test negative to 22 allergens. These SPT-negative individuals were more likely to be aged over 30yrs at time of onset and less likely to report childhood eczema and rhinitis (413). As such, non-atopic asthmatics showed some similarities to the intrinsic asthmatics identified by Rackemann. However, as with intrinsic asthma, studies in the 1990s also found similar levels of IL-4 and IL-5 mRNA in non-atopic/atopic asthmatics (407,408), suggesting that both atopic and non-atopic asthma were mediated by TH₂ inflammation.

A problem with categorising asthma on the basis of atopy is the assumption that if asthma symptoms exist in the presence of a positive allergen test, inflammation in the airways is therefore driven by allergic mechanisms. Conversely, it also assumes that , in the absence of positive allergy tests, asthma must be caused by non-allergic pathology. There is evidence that both of these assumptions may not be entirely correct (408,414). Given these potential problems with classification on the basis of atopy, and the ongoing development of methods for conveniently and safely assessing pathology in the organ of interest (i.e. the lungs) over the last two decades, there has been an increased emphasis placed upon phenotyping asthma on the basis of airway immunopathology.

2.4.3 Phenotyping asthma on the basis of airway inflammation

In 1889, Gollasch observed eosinophils in expectorated samples and secretions derived from some individuals with asthma and hay fever (415). Since then, asthma has been most commonly associated with eosinophilic inflammation, with historic post-mortem and more recent bronchial-biopsy studies regularly showing an increase in airway eosinophils (and associated Charcot-Leyden crystals) in asthmatics compared with non-asthmatics (416). This type of asthma is referred to as eosinophilic asthma (EA) and studies based upon assessment of eosinophil differential counts in induced sputum variously describe EA as asthma with the presence of greater than between 1 and 3% sputum eosinophils (11,86,383,384,417). These thresholds are often based on arbitrary values determined from non-asthmatic reference populations (303,304,358). EA is generally believed to be representative of atopic, TH₂-mediated asthma. In support, many studies have shown that atopic

asthmatics have increased serum IgE levels and increased airway eosinophils (for example (418)).

However, it is clear that eosinophilic airway inflammation is not omnipresent in asthma. As early as 1958, Brown *et al* reported that an absence of eosinophils in a spontaneous sputum smear was associated with a poorer response to corticosteroids (419). Numerous studies, using bronchial biopsy, BAL or induced sputum to assess airway inflammation, have identified asthma phenotypes with little evidence of eosinophilic inflammation in either the airway lumen or airway wall (11,245,420,421), even during exacerbations (422). This type of asthma has since generally been designated non-eosinophilic asthma (NEA). Although there appears to be variability in the prevalence of NEA in different populations, one review of 16 separate studies suggested that NEA may represent approximately 50% of all adult asthma cases (245) and other studies have suggested between 25-50% (11,384,422), with the lower figure observed in ICS-untreated groups (86). Similar findings have been observed in children (423).

On the basis of evidence of neutrophilic involvement in some types of asthma (as discussed in section 2.3.7), a limited number of studies have further stratified asthma on the basis of airway eosinophils *and* airway neutrophils. In the prototypical study describing this approach, Simpson *et al* assessed airway inflammatory patterns in 93 non-smoking adult asthmatics and found sputum airway eosinophilia ($\geq 1.01\%$ eosinophils) in 41% of cases; 20% with elevated neutrophil levels ($\geq 61\%$ neutrophils) but normal eosinophil numbers; 8% with an elevation of both granulocyte populations; and the remainder (31%) with no signs of excessive granulocytic

inflammation. The authors suggested that, on this basis, asthma could be categorised into four inflammatory subtypes: the previously described eosinophilic asthma (EA) phenotype divided into EA and mixed granulocytic asthma (MGA), and the NEA phenotype divided into neutrophilic asthma (NA) and paucigranulocytic asthma (PGA) (12) (Figure 2.3). However, as there are few population-based data currently available regarding these phenotypes, the main focus of subsequent sections is on the differences between EA and NEA.

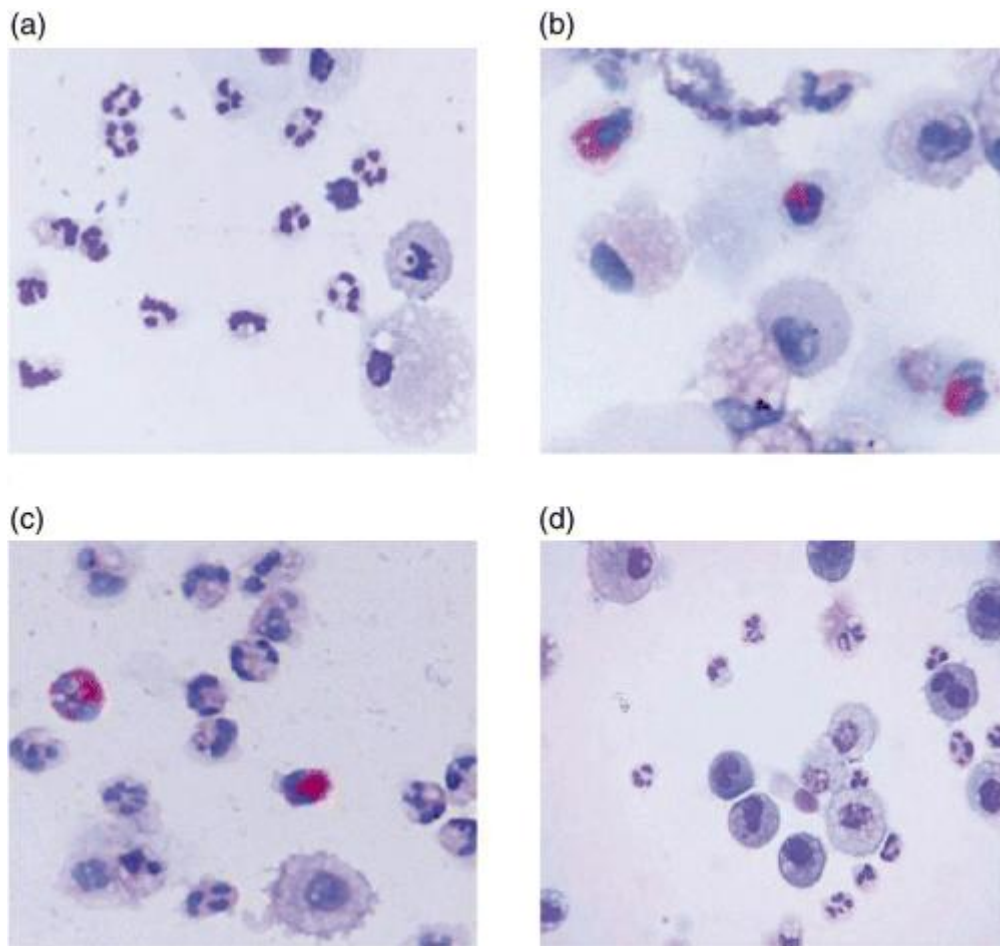


FIGURE 2.3. Sputum cytopspins showing four inflammatory subtypes of asthma: (a) neutrophilic asthma; (b) eosinophilic asthma; (c) mixed granulocytic asthma; (d) paucigranulocytic asthma. Used with permission from (12).

2.4.4 Characteristics of eosinophilic and non-eosinophilic

asthma

There have been contradictory findings reported regarding the clinical characteristics of EA and NEA in adults. Some studies have shown eosinophilic airway inflammation in adult asthma in association with increased exacerbation frequency (384), increased severity (424), decreased asthma control (425) and increased AHR (426,427) when compared with NEA. Despite this, NEA has been observed across the spectrum of clinical severity (ranging from mild (11) to severe refractory asthma (420)) and several studies have shown very few clinical differences between EA and NEA (12,403).

Studies in children have also produced equivocal results, with some showing increased severity in EA, and others showing no differences. For example, in a study of 146 asthmatic and 37 healthy children aged between 6 and 17yrs, Gibson *et al* found that increased sputum eosinophilia was associated with increased frequency of asthma attacks and increased use of ICS (83). Similarly, Lovett *et al* also found that in 84 asthmatic children, EA was associated with more symptoms (wheeze, night-time waking and reduced quality of life), more SABA use, and reduced FEV₁, despite similar use of ICS (428). However, as with adults, other studies in children did not necessarily find a significant link between eosinophilia and asthma severity (84,426).

Despite this sometimes contradictory evidence regarding their relationship with severity, EA is more likely to be associated with atopy (11) and NEA is more likely to be associated with smoking history (429), long disease duration (430) and obesity (431,432). This suggests that there are some similarities of EA/NEA with the

previously described intrinsic/extrinsic and atopic/non-atopic phenotypes, respectively.

Finally, EA has also been reported to be particularly responsive to treatment with ICS. This has been observed in both adults (11,433) and children (434). However, eosinophilic airway inflammation can still be detected in severe or well controlled asthma, even with the use of inhaled and oral corticosteroids (130,435); therefore, successful treatment does not necessarily result in the absolute clearance of eosinophils from the airways. Importantly, NEA is commonly associated with poor response to standard corticosteroid-based therapies (11,403,436,437); for example, a study of 67 symptomatic asthmatics observed that those with NEA (17/67; 25%) were less likely to show improvement with ICS, in terms of symptoms, lung function and AHR, than those with EA (438).

Immunological and pathological characteristics of eosinophilic asthma

Unsurprisingly, airway eosinophilia is associated with several distinct immunological and pathological characteristics generally observed in allergic inflammatory processes. Increased sputum eosinophil numbers in asthma are correlated with increased sputum protein (439) and mRNA (440) expression of the TH₂ cytokines IL-4, IL-5 and IL-13. Exhaled nitric oxide (FENO) is also increased with eosinophilic inflammation, as it has regularly been shown to correlate (to a greater or lesser degree) with sputum eosinophil count (13,133). Similarly, sputum levels of galectin-10 (441), eosinophil cationic protein (ECP) (423) and eosinophil-derived neurotoxin (EDN) (442) can all be observed with increased eosinophils. The presence of an increased number of CD3, CD4 and CD8 T cells, mast cells (420), TGF- β expressing

cells (443) and 15-lipoxygenase (444) has also been reported in some studies of severe refractory asthmatics with EA.

In terms of remodelling, a thickened sub-basement membrane has been observed in EA compared with NEA (403,420,445). However, this pathological difference is not found in all studies; in one report, Baraldo *et al* conducted bronchial biopsies on 21 NEA, 34 EA, and 25 non-asthmatic children under the age of ten years and found that airway remodelling (thickening of basement membrane, angiogenesis, loss of epithelium) was similar in EA and NEA (446), suggesting that the pathological characteristics of NEA may differ in children and adults.

Immunological and pathological characteristics of non-eosinophilic asthma

Very little is currently known about the underlying mechanisms of NEA, but it has been suggested to have both a different immunopathology and aetiology compared with EA (447,448). In particular, an *ex vivo* assessment of cytokine production from sputum cells derived from 20 eosinophilic and 28 non-eosinophilic asthmatics found that less IL-4 and more TNF- α was produced in NEA (439), suggesting, firstly, that NEA is more likely to be a 'TH₂-low' condition and, secondly, that NEA may involve an innate-like pathology.

Molecular methods support the presence of a TH₂-low phenotype of airway inflammation in asthma. In a series of recent studies, a TH₂-molecular signature (i.e. gene expression) in epithelial cells taken from bronchial brushings was used to define

major subphenotypes of asthma (16,449,450). The TH₂-associated genes CLCA1, periostin, and serpin B2 (451,452) were expressed at similar levels to healthy controls in approximately half of the asthmatics studied, compared with high levels in TH₂-high allergic asthmatics. Individuals with the TH₂-low phenotype had lower airway eosinophils and serum IgE than TH₂-high asthma, suggesting that this TH₂-low inflammatory phenotype may be representative of the NEA phenotype (453).

In addition to the TH₂-low phenotype, there has previously been speculation that NEA may be the result of innate-mediated, neutrophil-driven inflammation, similar to that observed with exposure to organic dusts, pollution, and microbial exposure ((245,380); see also section 2.3.7). In support of this, some studies have reported evidence of increased airway neutrophilia, sputum IL-8 and myeloperoxidase levels in NEA (383), which led to the aforementioned subdivision of NEA into paucigranulocytic asthma and neutrophilic asthma (12). However, there are also several studies showing that neutrophils are not necessarily important in NEA (403,420,454,455) and, in some populations, there is little or no evidence of a distinct neutrophilic asthma phenotype in NEA (28,29).

The pathophysiological mechanisms involved in paucigranulocytic asthma are not clear (Figure 2.4). It is possible that the underlying inflammation may be below detectable levels or below cutoff values using current methods or it may be that, rather than inflammation, a disorder of the smooth muscle (456) or epithelium (121) or even alterations in neurogenic control, may be the underlying cause (34).

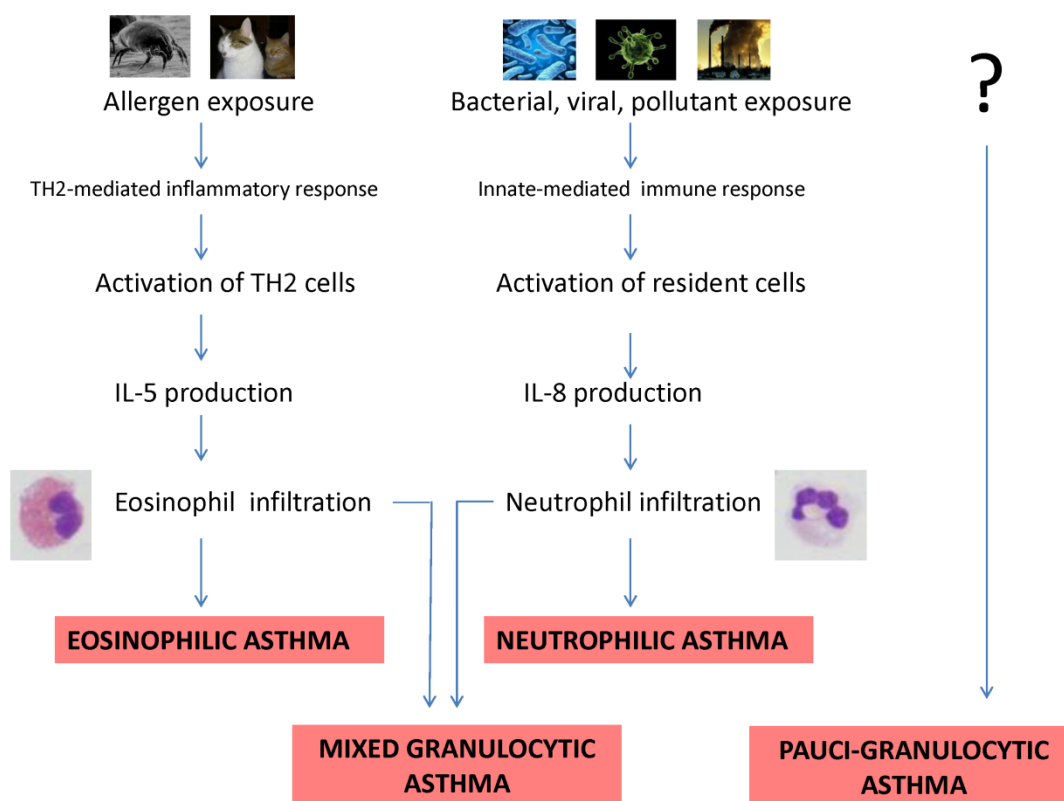


FIGURE 2.4. Environmental exposures and pathways likely to be associated with the inflammatory phenotypes of asthma identified in (12)

Neutrophilic asthma

Although very little is known about the mechanisms associated with PGA, there have been a number of studies conducted over the last decade examining the NA phenotype. In such studies (using induced sputum), NA has variously been defined as >61% neutrophils (based on the 95th percentile of a healthy population (12)) or >77% neutrophils based on 2 standard deviations (SD) of a healthy population mean (303), although it remains unclear as to what are ‘normal’ levels of neutrophils in different populations (457). In some studies, NA has been reported to have a prevalence of

between 10-30% of stable asthmatics (12,384,458). These patients were more likely to be female, non-atopic and to have adult onset asthma (384).

As described in section 2.3.7, NA involves an innate-mediated IL-8-driven neutrophil influx and activation (Figure 2.4), as well as impaired clearance of debris and dead cells (459,460). In one study of 49 asthmatics, it was found that NA (which represented 14% of asthmatics) was associated with increased mRNA levels of the innate receptors TLR2, TLR4 and CD14, as well as TNF- α (458). In addition to this increased innate gene expression in airway neutrophils, NA may also be associated with altered gene expression in blood neutrophils (461). There are also reports of increased sputum levels of IL-8 and IL- β in adult NA (12,383,436,458,462) and 6-8 times higher levels of endotoxin found in NA than other asthma phenotypes (458). Neutrophilic asthma in children has also been found in association with raised endotoxin, as well as increased IL-8 and MMP-9 (463).

Evidence of increased innate immune system and bacterial involvement in NA has been reported in a number of studies. For example, colonisation by bacteria such as *Chlamydia pneumoniae* has been shown to be associated with elevated neutrophils and IL-8 in BALF from asthmatic children (464), as has chronic infection with bacteria such as *Mycoplasma pneumoniae* (465) or *Chlamydia pneumonia* (466) in adults. Furthermore, the association between neutrophilic inflammation and bacterial exposure can also be seen in murine models. For example, in one particular murine study assessing the effects of ovalbumin administration in conjunction with bacterial exposure, chronic *Haemophilus influenzae* infection was associated with steroid-resistant IL-17-dependent neutrophilic airway infiltration (467,468).

In addition to the inflammatory differences observed, NA appears less responsive to ICS than EA (384,424). This has led to suggestions that alternative interventions and therapies should be used for this asthma phenotype, although, to date, few studies have specifically targeted treatment towards NA. However, blockade of TNF- α (469) and use of macrolide antibiotics (243,466) have both been suggested as possible NA-specific therapies.

Overlap syndrome: the middle-ground between asthma and COPD

Although some differences have been reported (470), NA shares a number of inflammatory and physiological features with COPD; this, in some cases, makes it difficult to accurately distinguish the two conditions. In particular, neutrophilic, TH₁-associated CD8⁺ airway inflammation is a hallmark of COPD (471-477). As with NA, this increase in neutrophils in COPD is also associated with increased gene expression of TLR2 and 4 mRNA in airway samples and peripheral neutrophils (476,478). Furthermore, some of the characteristics of both NA and COPD, such as increased recruitment of neutrophils into tissues such as the lung, can be observed during the natural ageing process (457,479,480), meaning that discrimination of NA and COPD is more difficult in older subjects.

Despite the similar inflammatory profiles observed, it should be possible to identify COPD by the presence of irreversible airway limitation, as described in current international guidelines (4,481). However, there is evidence of irreversible, or only partially reversible, airway obstruction in some asthmatics (482); conversely, many COPD patients may show a measurable degree of airway obstruction reversibility

(483-485). Asthma and COPD have therefore been described as overlapping syndromes, sharing some clinical features and environmental triggers (486) (Figure 2.5). This concept, that asthma and COPD may be considered as just different components of airway disease (reviewed in (487)), was proposed in 1961 as the *Dutch hypothesis*, and has been the subject of some more recent debate (488,489).

There are a number of commonalities between the inflammatory characteristics of airway cell patterns found in patients with overlap syndrome and with both asthma and COPD (490). Some studies have suggested that, as asthma becomes more severe and chronic, more TH₁ and CD8⁺ cells with the capacity to produce cytokines such as TNF- α and IFN- γ are recruited to the airways (440,491,492), leading to a immunopathology similar to that observed in COPD. It is therefore possible that in older patients, NA may not necessarily be a distinct asthma phenotype, but could possibly be the result of long-term asthma chronicity, and may represent features of COPD, asthma and normal ageing.

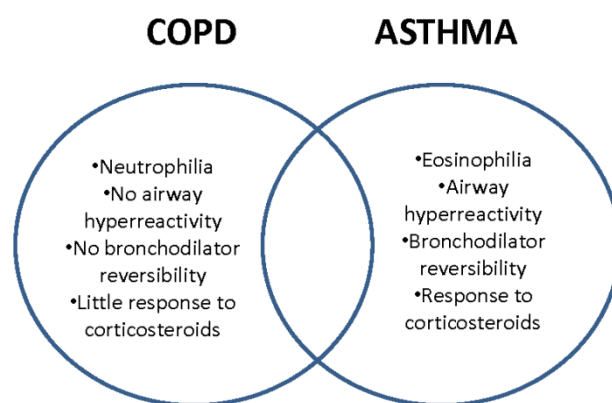


FIGURE 2.5. Overlap syndrome. Some patients have a number of features generally associated with either chronic obstructive pulmonary disease or asthma.

2.4.5 Stability of inflammatory phenotypes

The longitudinal stability of inflammatory asthma phenotypes has only recently begun to be fully investigated. Early studies suggested that EA and NEA were relatively stable, with one study showing that NEA was reproducible in both the short (4 weeks, assessed in 40 asthmatics) and long-term (1-5 years, assessed in 18 asthmatics) (12). Similarly, Berry *et al* found no evidence of EA on repeat sampling of NEA (403). Other studies are consistent with these findings (493-495). However, at least one study conducted a decade ago provided evidence of inflammatory phenotype instability in up to 70% of asthmatics (436), and several recent studies (discussed below) further suggest that, in some situations and different populations, inflammatory asthma phenotypes are prone to changing over time.

There are a number of possible reasons for longitudinal changes in inflammatory phenotype in asthma. Firstly, alterations in treatment status may play a role. For example, in a recent study of 94 patients, ICS withdrawal led to an increase in EA prevalence, from 39% to 67% (28). This study also found that 40-50% of NEA cases showed significant improvements in asthma control and AHR with ICS use, although this is a feature commonly ascribed only to EA. In subsequent work, the same research group found that only 1 of 8 (12.5%) patients identified as NEA, after a 4 week run-in period in the absence of ICS, remained NEA throughout a 6-week period of placebo treatment. Furthermore, of 26 NEA patients (again identified after a 4 week run-in period in the absence of ICS), all provided at least one eosinophilic sample after 12 weeks of placebo (29). Studies assessing sputum at multiple points over a 12-month period have also showed that inflammatory profile commonly changed over time in moderate-to-severe patients receiving ICS (496). Thus,

corticosteroids may mask EA in some cases and, therefore, the prevalence of NEA may be less than previously thought.

Secondly, in addition to changes in ICS treatment, there may be spontaneous changes in inflammatory phenotype occurring, regardless of treatment; for example, in 128 asthma patients assessed more than once, D'silva *et al* found that only 23% maintained the same inflammatory phenotype, with EA reproduced only in 18% of patients (417). Similarly, during salmeterol monotherapy (i.e. no ICS treatment) over a 6-month period in 30 steroid-naïve asthmatics, Bacci *et al* found that, despite generally low numbers of eosinophils, transient eosinophilia occurred in 40% of cases (497), once again suggesting the temporal instability of the NEA phenotype. In another recent study assessing the stability of childhood asthma inflammatory phenotypes, 37/59 children (63%) changed phenotype during longitudinal analysis. Of these, 24 changed between NEA and NA. This change was not related to ICS dose, control, or FENO change (498).

Finally, asthma exacerbation is also associated with changes in inflammation and inflammatory phenotype. Although asthma exacerbations can be associated with eosinophilia (as occurs with environmental allergen exposure (499)), many exacerbations are associated with respiratory virus infection; for example, Pizzichini *et al* showed, in a study of 6 asthmatics and 6 non-asthmatics with confirmed respiratory viral infection, that exacerbations were particularly associated with neutrophilia and that this neutrophilia was more profound in asthmatics (500). Similarly, Wark *et al* showed that acute episodes of asthma caused by viral infections were associated with increased sputum neutrophils and levels of sputum NE (501).

Asthma exacerbations may be associated with different inflammatory profiles in different age groups; Wang *et al* compared inflammatory phenotypes in asthmatic adults (29 stable, 22 acute) and children (aged 7 to 17yrs; 49 stable, 28 acute), in which acute asthmatics were defined as presenting to hospital with an asthma exacerbation (502): during stable *adult* asthma, 27.6% (8) were NA, 17.2% (5) EA, 3.5% (1) MGA and 51.7% PGA. During acute asthma, however, this changed to 81.9% (18) NA and 18.2% (4) MGA, with no EA or PGA observed. In contrast, in *children* with stable asthma, 49% were PGA, 28.6% EA, 2% MGA and 20.4% NA. In acute asthmatic children, EA increased to 50%, NA dropped to 7.1%, MGA increased to 35.7% and only 7.1% PGA was observed. This study suggests that asthma exacerbation may be associated with neutrophilic inflammation in adults and eosinophilic inflammation in children.

In summary, results from studies examining inflammatory phenotype stability have been mixed but it is possible that, in some situations, accurate identification of asthma inflammatory phenotypes may be confounded by factors such as ICS use, smoking, and respiratory infections (503). However, despite the mixed results observed in the studies to-date, the absence of detectable eosinophilia in symptomatic, corticosteroid-naïve patients in several studies (including one large study of ICS-naïve 995 asthmatics, in which 47% had no evidence of eosinophilia during repeated testing (495)) suggest that in at least some patients, NEA is a ‘real’ phenomenon, and not simply the result of ICS treatment.

2.4 Assessment of airway inflammation and pathology in asthma

2.4.1 Introduction

Early studies of inflammation in asthma required post-mortem investigations of the airways (58,59). The subsequent advent of bronchoscopy as a viable technique to investigate pathology in the airways of living asthmatics has significantly added to knowledge of the inflammatory and structural changes associated with asthma (416). However, its invasive nature generally limits its use to small, selected clinical populations. Furthermore, bronchoscopic techniques are ill-suited to the longitudinal studies necessary to assess ongoing inflammation, the natural history of asthma, or ageing-related changes.

Over the past two decades, several non-invasive techniques have been developed to assess airway inflammation in asthma. The introduction of hypertonic-saline induced sputum (IS) (78,504) in particular has provided a more convenient and less-invasive methodology to assess airway inflammation. Other studies have suggested that measurement of blood granulocyte levels may be indicative of airway inflammation (505). Alternatively, rather than measure cells in the airways or blood, levels of compounds associated with airway inflammation may be detectable in serum, urine, and exhaled breath (506,507). Exhaled nitric oxide (9) and exhaled breath condensate (EBC) measurements (508), in particular, have provided further simple alternative methods of non-invasively assessing airway inflammation. However, as described below, a number of these methods are yet to be fully validated and characterised, and

the validity and usefulness of some of these techniques, such as FENO and EBC as sampling methods for airway inflammation are still unclear.

In this section, the various methods for assessment of airway inflammation are discussed. The advantages and disadvantages of the different methods are summarised in Table 2.2. As they are the main methods used throughout this thesis, induced sputum and FENO measurement are described in greater detail than the other methods.

TABLE 2.2. The advantages and disadvantages of the different methods used to assess airway inflammation.

Method	Advantages	Disadvantages
Bronchoscopy	<ul style="list-style-type: none"> Both tissue samples and cells/mediators obtained from mucosal tissue and airway lumen Provides information relating to structural changes Samples can be used for downstream or <i>in vitro</i> analysis 	<ul style="list-style-type: none"> Only available in secondary/tertiary care Expensive Invasive Cannot be used in large population-based studies Risk of breathing difficulties, bleeding, hypoxia or pneumothorax Requires specialised equipment and personnel Difficult to conduct repeated measurements BAL - obtained from one lung segment, can be contaminated with blood. Low numbers of granulocytes. Diluted by saline Biopsy - Only from larger airways. Cell count reproducibility is low Hours to days for results
Induced sputum	<ul style="list-style-type: none"> Relatively non-invasive and safe Cells/mediators obtained from proximal airways Samples can be used for downstream or <i>in vitro</i> analysis Can be conducted repeatedly Can be used in moderately large population-based studies 	<ul style="list-style-type: none"> Risk of bronchoconstriction Varied success rate (approx 80%) Variable sample quality and yield Time-consuming and labour intensive The procedure may affect results Requires some expertise and specialised equipment Cannot be conducted in a primary care setting Hours for results
Measurement of exhaled nitric oxide	<ul style="list-style-type: none"> Non-invasive and safe Can be conducted in very young children Immediate results Can be conducted repeatedly Can be used in large population-based studies 	<ul style="list-style-type: none"> Equipment is often expensive and limited to research/secondary care Only one mediator is assessed Measurement affected by a large number of variables, particularly steroid use and atopy
Exhaled air/ Exhaled breath condensate	<ul style="list-style-type: none"> Non-invasive and safe Can be conducted in very young children 	<ul style="list-style-type: none"> Equipment is expensive and limited to research/secondary care Time to results varied depending upon mediator measured Methods still under development Very low levels of some mediators
Nasal lavage	<ul style="list-style-type: none"> Non-invasive and safe Simple, inexpensive procedure Cells/mediators obtained from upper airways Can be conducted repeatedly Can be used in large population-based studies 	<ul style="list-style-type: none"> Variable sample quality and yield Processing can be time-consuming Hours for results Upper airway inflammation may not be indicative of processes in the lower airways

2.5.2 Bronchoscopic assessment: Bronchial wash, Biopsy and

BAL

There are reports of bronchoscopy being conducted on asthmatics from as early as 1914 (509) but it wasn't until the mid-1980s that biopsy-derived reports of the histological features of asthma in the airways were published (510). There are now guidelines for the practice of bronchoscopic sampling in airway disease (511-513), even in cases of severe asthma (514).

Bronchoscopy can be conducted using a rigid or fibre-optic bronchoscope. Bronchial biopsies are performed using cupped biopsy forceps through the bronchoscope; these allow assessment of submucosal inflammation and structural changes and, as such, are particularly useful for investigation of airway remodelling (515). BAL involves the instillation and recovery of sterile saline into the sub-segmental (distal) bronchi during bronchoscopy and allows sampling of cellular and non-cellular components of the epithelial lining fluid. Cells and soluble mediators from the recovered solution (obtained from the distal airways and alveolar surfaces) can subsequently be analysed. Bronchial brushings are easier and less invasive than both BAL and biopsy, and involve insertion of a cytology brush through an endotracheal tube (516). This approach allows assessment of epithelial cells in particular, and has been used to harvest, and subsequently culture, primary bronchial epithelial cells *in vitro* (517).

Together, these bronchoscopy-based methods have provided considerable insight into asthma pathology. In particular, early biopsy data clearly showed the characteristic mucosal and submucosal inflammation present in asthma (416) and early BAL and bronchial biopsy sampling showed increased levels of eosinophils and eosinophil

degranulation products in the airways (518,519). From the beginning of the use of these biopsy-based methods, it was important to determine differences in asthmatic compared with non-asthmatic airways to clarify the pathology of the disease. Early, relatively small studies provided BAL reference values for healthy patients (520). A more recent review of 9 BAL studies by Balbi *et al*, which included 760 individuals, provided more up-to-date reference values (358). In general, data derived from bronchoscopic studies are reproducible in the short and long term (521,522); for example, a BAL study of 295 healthy volunteers, which included 47 individuals who had 2-5 repeat visits over several years, found that BAL results were unaffected by the age or gender of the individual or the season of sampling (523). Conversely, some studies have suggested that reproducibility may be poor (524,525).

There are some problems involved with bronchoscopy. In particular, fibreoptic bronchoscopy is an invasive and uncomfortable procedure, and there are risks of hypoxia and bleeding from the biopsy site, as well as post-procedural wheeze or pneumothorax (526). Furthermore, it is generally not suitable for repeated use, and, for some patients, such as children, general anaesthesia may be required (527).

Another issue with the assessment of BAL and bronchial biopsy is the relative scarcity of granulocytes (particularly neutrophils) in samples collected in this way, as these cells are non-tissue resident cells. Thus luminal sampling, such as that achieved using induced sputum, may provide a more appropriate means of assessing and describing granulocytic inflammation in the airways.

2.5.3 Induced sputum

Microscopic and macroscopic assessment of spontaneously produced sputum has been conducted since the mid-19th century (528). However, assessment of spontaneous sputum is generally limited by the unpredictability of sample production, quantity and quality (504). This unpredictability led to the development of controlled sputum induction techniques (529), which were further optimised in the late 1980s and early 1990s (78,504,530) and are now used to assess a multitude of respiratory disorders (531,532).

Sputum induction by inhalation of hypertonic saline (Figure 2.6) (78), and more recently mannitol (533-535), is a relatively non-invasive, repeatable, and safe procedure (536-538) that has been standardised and validated (14,539,540). In general, it involves inhalation of a nebulised 4.5% hypertonic saline solution for increasing intervals from 30 seconds to a total of approximately 15 minutes (426). Lung function is measured at each interval and subjects are given inhaled β_2 -agonist if there is a predicted FEV₁ reduction of > 15%. At the end of the procedure, participants are asked to cough up sputum, which is then collected and processed. There are, however, variations in the method across studies. These include: using either selected or whole sample (536,541-543); different concentrations of hypertonic saline (303,304); varied induction time; and use of different reducing, mucolytic agents in sample preparation (dithioerythritol (DTE) or dithiothreitol (DTT), used to break the disulphide bonds in mucin molecules and release the cellular portion of the sample (544,545)).

Sputum induction can be conducted repeatedly in the same individual (for example, assessment of inflammatory changes due to allergen challenge (546,547)) and has been shown to be generally reproducible (539). It can be used in diverse population groups, including children as young as seven years old (84,548-551), with a reported success rate of 68-100% (552,553).

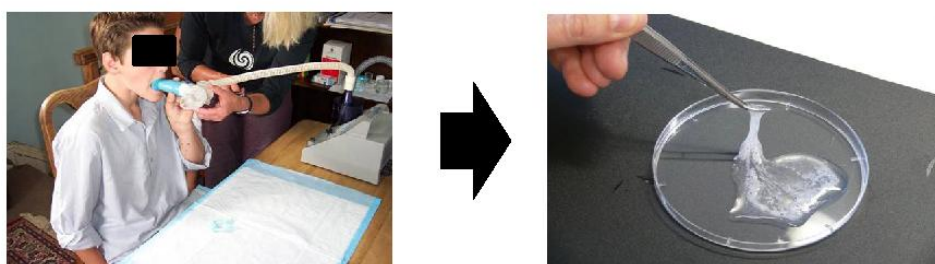


FIGURE 2.6. Sputum induction. The process involves inhalation of nebulised hypertonic saline for set intervals, and if successful, leads to production of a sputum sample which can be processed and analysed.

Induced sputum has been regularly used for the measurement of leukocyte population absolute number and differential counts, as well as levels of soluble mediators in COPD (554) and asthma (532,555). Gene expression can also be measured in IS samples e.g. assessment of cytokine or TLR mRNA levels (17,440,556). More novel developments in the assessment of induced sputum include approaches such as proteomic analysis of the liquid phase of induced sputum using surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (557) and assessment of the presence of flagellated protozoa in asthma (558).

Induced sputum samples are obtained from the proximal, central airways, as confirmed in experiments introducing radiolabelled aerosols into the lungs of volunteers (559), although extending the length of sputum induction may also allow sampling of the distal airways (560). It is believed that hypertonic saline induces sputum production by increasing the osmolarity of airway lining fluid; this, in turn, increases the permeability of the bronchial mucosa and mucus production (561), although this is not entirely clear. It is also possible that inhalation of hypertonic saline results in mast cell degranulation and airway afferent nerve stimulation (562,563), which may also stimulate production of sputum.

Despite induced sputum being sampled at a different site from bronchoscopy-based investigations, findings observed in induced sputum have been reported to closely correlate with bronchial washings by some researchers (564). However, they are less well correlated with bronchoalveolar lavage (565) and still less with bronchial biopsy (566,567). As noted above, this is likely to be at least partly due to the absence of granulocytes as resident cells in airway tissue as observed with bronchoscopic studies.

As with bronchoscopy, a number of studies have produced reference values of leukocyte populations and inflammatory mediators to allow comparison between healthy and diseased populations (303,304,358). These studies determined that, in general, the most common leukocyte population found in non-asthmatic induced sputum is the macrophage (358), although neutrophils (ranging from 17% to 80%) can be found in high numbers in some, particularly older, individuals (457). A small population of lymphocytes (less than 5%) is generally observed. As stated previously, eosinophils are found in increased numbers in many asthmatics and, infrequently,

mast cells may also be found (426). The presence of bronchial epithelial cells may also be increased in asthma (568), although this finding is inconsistent between studies.

As well as its use in examining asthma immunopathology in research studies, methods based on induced sputum have been intensively utilised in the management of asthma in recent years. In particular, successful treatment of asthma with ICS or systemic corticosteroids is associated with a decrease in sputum eosinophils, an effect that is more visible in sputum than bronchial biopsies. There are now several reports showing that titration of ICS based on sputum eosinophil counts is a more appropriate method for asthma management than using either symptom-based or FENO-based approaches (433), although there is some debate about this, and it may not be appropriate in some populations, such as children (569,570).

Whilst it has been shown to be useful for the assessment of airway inflammation and management of ICS treatment, induced sputum has some limitations. Although less invasive and requiring less expertise than bronchoscopy, sputum induction remains difficult to conduct in large population-based studies, as it is time-consuming and requires specialised equipment and trained staff. The procedure itself may induce changes in inflammatory cell populations, particularly increases in sputum neutrophils and eosinophils, within a 24 to 48 hour period (571,572), suggesting that repeated visits within a short period of time may lead to artefactual findings. A further source of variation with assessment of IS is that, like asthma symptoms, asthma inflammation may be circadian rhythm-dependent; for example, one study of 11 mild asthmatics showed that, in the morning compared with the evening, patients had

significantly reduced FEV₁ and increased BDR, which was associated with increased total sputum leukocytes, and eosinophils in particular (573).

As stated above, there is some interlaboratory variation in processing and handling of samples, so it is not always possible to compare results from different studies. There can also be considerable inter- and intra-individual variability in the quality, quantity and viability of sputum produced; in a proportion of cases, sputum cannot be successfully induced at all. According to various reports, adequate sputum samples can be obtained in approximately 60-90% of cases (553), although a >93% success rate has been reported (551). In some sputum samples, there is also significant squamous cell contamination, making reading and interpretation of slides difficult. It has therefore been suggested that if there is greater than, for example, 50% squamous epithelial cell contamination, the procedure should be repeated (574).

Excessive contamination with saliva can also result in reduced total leukocyte counts, as well as substantial reductions in measured levels of soluble mediators such as ECP and IL-8, although excessive saliva doesn't appear to affect differential cell counts (575). In addition, the presence of proteases within saliva may lead to degradation of some cytokines in sputum supernatant (576). Even the agents used in sample homogenisation may result in altered detection of sputum components. The use of DTT is associated with significantly reduced levels of TNF α and MPO (577) and changes in fluorescent intensity of the surface molecules CD16, CD2, CD45 and CD14 on some leukocyte populations during flow cytometric analysis (578).

Finally, induced sputum samples need to be processed in a relatively short time. Rapid fixation techniques, such as the use alcohol or formaldehyde (579), or freezing (580,581) have been suggested to alleviate rapid sample degradation.

2.5.4 Exhaled air and exhaled breath condensate

Exhaled air contains several gases including nitric oxide (508) (discussed below), ethane and pentane (582,583), all of which have been shown to be increased in asthma. Analytical methods for assessing distinct profiles of these gases, using electronic nose nano-sensors (enose) or by gas-chromatography and mass-spectrometry (GC-MS), could allow discrimination of different airway diseases, as has been described for well-defined COPD and asthma (584) and may even be used to assess pathological features within one disease. For example, a recent study by Fens *et al* found several compounds of interest in exhaled breath that correlated with sputum cell counts for eosinophils (8 compounds) and neutrophils (17 compounds) in COPD (585). As is found in asthma, increased markers of eosinophilia in COPD predicted ICS responsiveness and therefore EBC assessment may allow the guidance of appropriate therapy.

Alongside volatile gases detected in exhaled breath, several inflammatory compounds and mediators can be detected in EBC; this requires cooling exhaled air to the point at which water vapour can be collected. These include hydrogen peroxide, prostaglandins, 8-isoprostane and cytokines (586,587).

Although the assessment of exhaled air has been suggested to hold considerable potential as a non-invasive ‘inflammometer’ in the monitoring of airway disease

(588,589), particularly in regard to its completely non-invasive nature, there are difficulties associated with EBC. In particular, many compounds and mediators are present at extremely low levels: often below, or at the very limit of, current methods of detection. Furthermore the equipment and expertise required for analysis is very rarely available in a primary or secondary care setting, and is currently limited to specialised research institutes. Finally, to date, there have been few longitudinal or large population-based studies investigating the use of exhaled gases (with the exception of FENO) or EBC in guiding treatment and management of asthma and, therefore, these methods require further study and validation.

2.5.5 Fraction of exhaled nitric oxide (FENO)

Increased levels of exhaled FENO were first described in exhaled air by Gustaffson in 1991 (590). Since then, the measurement of FENO has been increasingly recognised as a convenient and cost-effective means of non-invasively assessing airway inflammation in asthma (591). FENO is produced from L-arginine by NO synthases, which occur as either constitutive or inducible (iNOS) isoforms in epithelial cells and various inflammatory cells in the airways (592). iNOS in particular is upregulated in response to inflammatory stimuli (593), and has been confirmed as the principal source of FENO (594).

As with other methods for assessment of airway inflammation, standardised guidelines and recommendations (13) and reference values for FENO in normal healthy populations have been published (595-598). FENO measurements in such studies are generally highly consistent and reproducible in both adults and children (599). In fact, unlike most other methods discussed, FENO can be measured in

infants, allowing an indication of early developmental airway inflammation. As infants cannot perform a constant exhalation manoeuvre, various approaches have been used to measure FENO in this group, including offline collection of breath exhaled during normal-tidal breathing using an inert bag (600) or online measurement (601) during sleep.

Increased FENO has often been associated with disease severity, airway eosinophilia and AHR in asthma (133,602-605). Several studies have observed correlations between sputum or BAL eosinophil differential counts and FENO levels, in steroid-naïve, steroid-treated and healthy non-atopic groups (602,606,607), and in childhood asthma (434,608). It has even been suggested that measurement of FENO may be used to titrate ICS dose in asthma (609) or to guide asthma diagnosis; for example, in a study of 47 patients with symptoms suggestive of asthma, Smith *et al* found that FENO measurements had a stronger asthma diagnostic capability (88% sensitivity) than peak flow or spirometric assessment (between 0-47% sensitivity) (610).

It may also be possible to use FENO measurement to identify EA/NEA or steroid-resistant phenotypes, as increased FENO levels may be particularly associated with atopic or eosinophilic asthma. For example, Dweik *et al* found that a high FENO phenotype (>35ppb) was associated with greatest sputum eosinophilia (611), but that patients with non-atopic or NEA appeared to have normal FENO levels, regardless of disease severity (535). Similarly, in a recent meta-analysis, Donohue *et al* showed that FENO measurement was valuable in identifying patients more likely to respond to ICS (612).

However, findings of increased FENO in association with asthma are not always consistent. For example, FENO levels may not strongly correlate with sputum eosinophils in patients with severe prednisone-dependent asthma (613), and in other studies, FENO was not correlated with sputum eosinophils but was correlated with bronchial biopsy eosinophils (614). It is likely that, as epithelial cells (rather than inflammatory cells) are the major airway FENO source, increased FENO indicates similar, but not the same, underlying pathological processes. Importantly, in terms of the guidance of asthma management, ICS therapy has been shown to decrease FENO even if sputum eosinophils are not correspondingly reduced (615).

FENO has been proposed to be a useful indicator of airway inflammation, but it is not unique to asthma. Increased FENO levels are also observed in eosinophilic bronchitis (616), allergic rhinitis (617), respiratory infections (618), and bronchiectasis (619). Furthermore there are a large number of variables that influence FENO levels in both asthmatics and the general population (620), including gender (621,622), weight (623), age (598), respiratory infection (618), smoking, recent caffeine intake (624), exercise, drugs (625,626), and recent mannitol inhalation (533), meaning that considerable variability can be observed on both an inter- and intra-individual basis.

Possibly the most important variable associated with increased FENO is atopy, with or without allergy symptoms (597,622,627-630). In particular, individuals with allergy and atopy are more likely to have elevated FENO levels regardless of the presence or severity of asthma, leading to suggestions that it is not actually a particularly useful biomarker in asthma and that, like skin-prick testing, elevated FENO may not necessarily be indicative of immunopathology in asthma (628).

Finally, there are some difficulties inherent in the technical measurement of FENO; for example, substantial amounts of nitric oxide are produced in the paranasal sinuses (631), meaning that the soft palate must be firmly closed when measuring lower airway NO (632).

2.5.6 Nasal lavage

Nasal lavage is a simple, non-invasive technique that can be conducted relatively quickly. It involves administration of a pre-warmed solution (usually 0.9% saline) into the nasal cavity. After a predetermined time the recovered fluid is collected, and can be used to investigate nasal inflammation, particularly involving soluble inflammatory mediators or granulocyte populations (633). Although this approach has been used specifically to investigate seasonal or perennial allergic rhinitis (634,635), occupational exposures (636,637) or infectious rhinitis (638), it has also been suggested as a possible indicator of inflammation in the lower airways; for example, Amorim *et al* assessed sputum and nasal samples from 130 adult asthmatics, and found a significant correlation between nasal and lower airway eosinophilia ($r=0.67$; $p<0.001$), with nasal eosinophilia being predictive of airway eosinophilia (receiver operator characteristics (ROC) 85%, $p<0.001$) (639).

These findings are consistent with the unified airways disease theory; that inflammation in one part of the airways is reflected in other areas (640). However, data produced using nasal lavage may be confounded by sampling individuals with allergic rhinitis but no lower airway involvement or allergic airway inflammation with no upper airway involvement. It is, therefore, not entirely clear how useful nasal lavage will be in the assessment of *lower* airway inflammation.

2.5.7 Systemic markers of inflammation

It has been proposed that assessment of cells or protein in peripheral blood or even urine may be a useful alternative method to investigate inflammation in the airways. In particular, a range of systemic markers generally associated with eosinophilic airway inflammation have been assessed. Firstly, increased peripheral blood eosinophilia is commonly observed in allergic asthma; for example, in a study of 80 adult asthmatics, Yap *et al* found that when serum eosinophil counts doubled, the odds of a corresponding increase in sputum eosinophils also doubled (641). Similarly, a paediatric study has shown that increased blood eosinophils are associated with reduced lung function and increased symptoms (642) and, from data obtained using sputum induction, a reduction in blood eosinophils may also be indicative of successful ICS treatment (643). One study has extended this approach to attempt to phenotype 381 well characterised asthmatics on the basis of conventional blood eosinophil and neutrophil counts above the 75th percentile of normal values (505). The authors observed high blood eosinophils ($\geq 250/\text{mm}^3$) in approximately 50% of asthmatics; this finding was associated with increased serum IgE and reduced FEV₁. Furthermore, high blood neutrophils ($\geq 5000/\text{mm}^3$) in the low blood eosinophil group were more likely to be associated with COPD-like symptoms in non-smokers, suggesting that this blood-phenotyping approach may identify similar characteristics to the EA/NEA airway phenotypes. However, other studies have found that blood eosinophil counts may not necessarily be indicative of airway inflammation. For example, in 88 children with severe asthma, 86% had normal blood eosinophils but, of these, 84% had airway eosinophilia as measured by BAL (644), and increased blood eosinophils can often be observed in atopy in the absence of asthma (645).

Measurement of serum protein levels associated with eosinophils has also been assessed as an indicator of airway inflammation. For example, serum ECP is an indicator of exacerbation in persistent asthma (646). Recent studies assessing other surrogate biomarkers of eosinophilic airway inflammation have also reported that measurement of serum periostin may be a particularly promising alternative to measuring sputum eosinophil differential counts; for example, Jia *et al* examined several systemic biomarkers of inflammation (FENO, blood eosinophils, serum periostin, YKL-40 and IgE) in 67 symptomatic asthma patients and found that, of the aforementioned indicators, periostin was the best single indicator of sputum eosinophilia (647). Serum periostin has subsequently been used to as a marker of TH₂-mediated inflammation to identify asthma patients more likely to respond to omalizumab in clinical trials (648). Alternatively, rather than assess TH₂ mediated inflammation, other serum proteins, such as C-reactive protein (CRP), have been reported to be increased in association with COPD, and therefore could be measured to help differentiate asthma from COPD (649,650). However, raised serum CRP has also been reported in neutrophilic asthma (651), and occupational asthma studies have shown increased CRP levels in individuals exposed to organic dust (652,653); these findings, taken together, suggest that raised CRP may be associated with innate-mediated neutrophilic inflammation, rather than COPD in particular.

Urinary metabolites of lipid peroxidation (F2-isoprostanes) and protein bromination (bromotyrosines) have been reported to be increased in asthma, suggesting that in future this may provide another means of assessing airway inflammation (654); for example, one study using a urine metabolic profiling approach (assessing urine from 41 atopic asthmatic children and 12 age-matched controls), found that asthmatics had

significantly lower urine levels of urocanic acid, Ile-Pro fragment and methyl-imidazoleacetic acid than non-asthmatics (655).

With the further development of these and other proteomic approaches, such as protein microarray analysis (656), it is possible to simultaneously assess levels of multiple mediators in biological samples, including serum, urine, BAL fluid, and induced sputum (657). This may provide further insight into protein signatures associated with different pathological processes and phenotypes in asthma. However, all these systemic biomarker approaches have yet to be comprehensively validated and blood eosinophil counts and levels of serum eosinophil-related proteins, in particular, may not be representative of airway pathology or be specific for asthma (658).

2.5.8 Assessment of airway remodelling

It is possible that the lack of response to conventional or TH₂-based therapies in some asthmatics may be due to asthma symptoms being associated with tissue remodelling rather than active inflammation. This has led to an increased interest in the assessment of airway remodelling in asthma (340). However, there are several difficulties involved in the assessment of airway remodelling and there are currently few methods to conveniently and non-invasively assess it. Bronchial biopsies allow investigations of airway structure but are highly invasive and difficult to conduct on large numbers of patients (659). This has led to the development of assays measuring soluble mediators associated with airway remodelling and ECM degradation in BAL, serum, sputum supernatant, or even urine. Such mediators include transforming growth factor (TGF)- β , epidermal growth factor (EGF), vascular endothelial growth factor (VEGF),

TNF- α and MMP-9 (reviewed in (660)). For example, increased MMP-9 has been reported in BAL fluid and induced sputum from severe asthmatics (661). In particular, mediators associated with vascular remodelling may be important in the pathogenesis of severe asthma. In one study of 38 patients with severe refractory asthma, Tseliou *et al* found significantly elevated sputum supernatant levels of the mediators angiopoietin (ANG) 1 and 2, which correlated with levels of MMP-2, VEGF, and IL-13, involved respectively in tissue remodelling, angiogenesis and TH₂-inflammation (662). A previous study had also observed an increase in ANG-1 sputum levels in asthmatics with suboptimal treatment (663).

Alternatively, as decline in lung function is related to airway remodelling (664), low post-bronchodilator FEV₁/FVC can be used as a proxy of airway remodelling (665). More recently, high resolution computed tomography (HRCT) or endobronchial ultrasound have been used, both of which show good agreement with data derived from spirometry (666-668). However, as with serum and proteomic assays, these approaches have yet to be fully validated, and may not be specific to airway pathology.

2.6 Summary

Despite considerable investment and investigations over several decades, asthma still cannot be simply defined or described. Although often believed to be driven by allergic mechanisms, atopic sensitization accounts for less than one-half of cases. Asthma is a complex, variable and multicomponent syndrome, involving interactions between the environment and numerous components of the immunological,

physiological, anatomic, genetic, and neurological systems of the affected individual. These interactions may be affected by timing, dose, and nature of the exposure, as well as health status at the time of the exposure. There are also a myriad of protective and risk factors involved in both the development and exacerbation of asthma; these may protect against asthma in one individual, whilst causing asthma in another. Furthermore, there is still some way to go in accurately identifying causal exposure and high-risk populations in asthma. To do this, one has to firstly identify the nature of the particular ‘asthma’ in question.

Conventional methods used in the clinical and epidemiological identification of asthma have generally provided little insight into the underlying pathology of asthma within a given individual and thus provided little information about important mechanisms or causal exposures. They also provide little insight into the potential efficacy of asthma therapy. It is important to target therapies at patients who are likely to benefit, whilst at the same time developing more appropriate therapies for those whose needs are currently unmet. The need for such a personalised approach to asthma therapy was identified decades ago (669), and is one of the key rationales behind inflammatory phenotyping (670).

There are now relatively novel methods available for the simple and non-invasive assessment of airway inflammation in asthma; these are expected to facilitate an improved understanding of asthma pathophysiology. This thesis will explore and develop the use of two of these approaches, whilst assessing the nature of airway inflammation in a New Zealand community setting.

3. Measurement of exhaled nitric oxide in a general population sample: a comparison of the Medisoft HypAir FENO and Aerocrine NIOX analysers

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Background and objective: Measuring the fraction of nitric oxide in exhaled breath (FENO) is increasingly utilised to assess airway inflammation in asthma. The primary aim of this study was to compare exhaled nitric oxide measurements obtained using two devices from different manufacturers i.e. the recently marketed portable and electrochemical-based Medisoft HypAir FENO, and the well established chemiluminescence-based Aerocrine NIOX analyser, in an unselected population.

Methods: FENO measurements were conducted in 106 subjects (86 healthy; 20 asthmatic; 56.6% atopic). Atopy and health status were assessed by skin prick tests and questionnaire respectively.

Results: The two instruments showed strong correlation over a wide range of FENO measurements (8 to 261.3ppb with the Hypair, 5.6 to 156.8ppb with the NIOX; $r = 0.98$; $p < 0.0001$). This correlation was observed in the population as a whole, as well as in healthy non-atopics, healthy atopics and atopic asthmatics when considered separately. The measurements on the HypAir FENO were consistently 1.6 times (95% CI: 1.11-2.05) higher than those obtained with the NIOX.

Conclusions: FENO measurements obtained with the HypAir FENO correlated well with the NIOX, but were approximately 1.6 times higher. Therefore a conversion factor is required if results are to be compared with the NIOX instrument.

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3.1 Introduction

Airway inflammation is considered a hallmark of asthma but it is difficult to measure, particularly on the scale required for large population studies. Bronchial biopsy, bronchoalveolar lavage and induced sputum can be used, but these methodologies are invasive, time-consuming, and require highly specialised staff. The measurement of the fraction of nitric oxide in exhaled air (FENO) has been increasingly recognised as a convenient and cost-effective means of non-invasively assessing airway inflammation in asthma. Increased FENO has been shown to be associated with disease severity, airway eosinophilia and bronchial hyperresponsiveness in asthmatics (8,602,607) and corticosteroid therapy has been shown to decrease FENO (615). Although measuring FENO shows promise in the diagnosis and management of asthma, other factors not directly related to asthma such as smoking, atopy, height and gender affect FENO levels (596,620,621,671).

As measurement of FENO becomes more widely accepted, there is a greater requirement for convenient, portable and inexpensive devices suitable for use in primary-care, community-based or occupational settings. Several instruments are now available utilising either chemiluminescent or electrochemical NO measurement technologies. Many of these are bulky, expensive and have high-ongoing running costs, and also require technical expertise in set-up and calibration, limiting their use to specialist centres.

Previous studies have compared NO analysers, (672-678) but as new instruments are introduced to the market it is important to determine how comparable they are. To our knowledge only one other recent study has compared the recently marketed HypAir

FENO (Medisoft; Sorinnes, Belgium) electrochemical analyser with more established instruments (679), such as the NIOX (Aerocrine; Solna, Sweden), which has been approved for use by the US Food and Drug Administration (680). The NIOX instrument is based upon chemiluminescent detection of FENO, which is considered by many researchers in the field as the “gold standard”. In the current study we compare FENO measurements in 106 subjects using both devices. Such data is required if any valid comparisons between clinical or epidemiological studies using different machines are to be made.

3.2 Methods

A population of 107 healthy and asthmatic individuals were recruited from colleagues of the three research institutes involved. The study was approved by the regional ethics committee and written informed consent was obtained for each participant. FENO was measured for each individual using the two analysers in an alternating manner, with the Hypair FENO first, as dictated by its longer analysis time. Both instruments were calibrated and used according to the manufacturer's instructions, and work in conjunction with a personal computer. The software supplied by either manufacturer provided both audio and visual feedback allowing the participant to maintain a constant exhaled breath flow rate. Three acceptable measurements were taken with each instrument at a flow rate of 50 ml/s within a 15 minute period according to ATS/ERS guidelines (13). The mean of the three values was used, and data was log-transformed to normalise distribution prior to analysis. Further FENO measurements were collected using a second Aerocrine NIOX instrument in a small group of individuals (n=15) to investigate intra-manufacturer variation. Also, in two individuals, one non-atopic and one atopic, FENO was measured with both analysers on 14 different days over a 30-day period, to provide insight into longitudinal variation between instruments.

Atopy was defined as a positive skin prick test to at least one of a panel of eight commercially available allergens, which included house dust mite (*Dermatophagoides pteronyssinus*), tree pollen mix, grass pollen mix, cat and dog dander, *Alternaria tenius*, Penicillium mix (Hollister-Stier Laboratories, Spokane, WA, USA) and Cladosporium (Stallergenes SA, Antony, France). Histamine and saline were used as positive and negative controls. A mean wheal size of 3 mm or

greater for any one or more allergens was considered positive. Clinical and demographic data (sex, age, height, weight) were collected at assessment, and asthma, allergy symptoms, recent upper respiratory tract infection and general health status were determined using a questionnaire. Asthma was defined as a positive answer to the questions “have you had wheezing or whistling in the chest at any time in the last 12 months?”, “have you ever had asthma?”, and “was the diagnosis confirmed by a doctor?” Bland-Altman plots and Pearson’s correlation (using log-transformed data) were utilised to determine both agreement and comparability of FE_{NO} measurements collected with the two instruments. Differences between different population groups were assessed using the Student’s t-test. Coefficient of variation (CV) values were calculated to assess intra-individual measurement variation for each instrument. All statistical analyses were conducted using Prism 5 (Graphpad Software, San Diego, CA).

3.3 Results

Population characteristics are reported in Table 3.1. Just over half of the participants were atopic, with the majority (90%) being sensitised to house dust mite. Twenty individuals (18.9%) had received a physician's diagnosis of asthma and reported current asthma symptoms. The majority of these asthmatics (90%) were also atopic. The asthma group was treated with various therapeutic regimens: untreated (20%), short-acting β -agonist only (35%), inhaled corticosteroids (ICS) only (5%), or a combination of short-acting β -agonist and ICS (40%).

TABLE 3.1. Characteristics of the study population. Unless otherwise indicated data are presented as mean (SD).

Number (n)	106
Female, (n,%)	69 (65.1)
Age (year)	35 (11)
Height (cm)	170.5 (9.3)
Weight (kg)	72.6 (15.8)
BMI	24.8 (4.6)
Atopy (n, %)	60 (56.6)
Asthma (n, %)	20 (18.9)
Allergic rhinitis (n, %)	48 (45.3)
Reported upper respiratory tract infection within 4 weeks of testing (n, %)	17 (16)
Current smokers (n, %)	6 (5.7)

One hundred and six participants were able to provide three acceptable manoeuvres on each of the two machines. One individual was unable to adequately perform the test on the HypAir FENO analyser and was excluded from further analysis. FENO levels ranged from 8 to 261.3 ppb (geometric mean 34 ppb) with the HypAir FENO analyser, or 5.6 to 156.8ppb (geometric mean 21.8 ppb) with the NIOX.

Variation over the 3 measurements taken per individual was similar for both the HypAir FENO and NIOX, with mean overall CV values of 5.9% (SD 4.4%) and 6.1% (SD 4%) respectively. As expected, for both instruments it was observed that mean CV values increased with lower FENO measurements (to 7.6% and 8.5% for FENO measurements under 20ppb detected with the HypAir FENO and NIOX respectively).

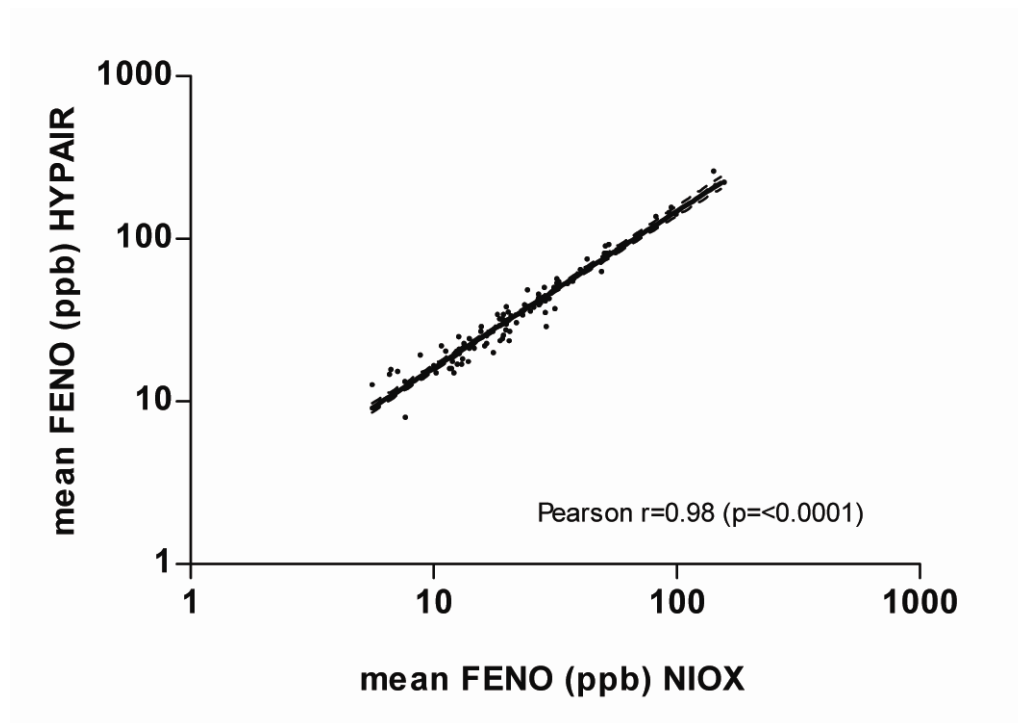


FIGURE 3.1. Correlation between FENO measurements detected with the HypAir FENO and NIOX analysers (based upon the mean of three measurements)

There was a high degree of correlation between the 2 analysers ($r=0.98$; $p<0.0001$; Figure 3.1). Bland-Altman analysis also showed good overall agreement between both devices, although the HypAir FENO provided consistently higher results than the NIOX (ratio 1.58; 95% CI 1.11-2.05; Figure 3.2). Similar to the CV values, the magnitude of difference between the two instruments was observed to increase when very low FENO measurements were obtained. This effect was particularly apparent when FENO levels detected using the NIOX system were less than 9ppb (seven

individuals; 6.6% of sample). In five of these seven cases, the average readings obtained with the HypAir FENO were more than double those obtained with the NIOX (2.15-2.35 times greater; Figure 3.2).

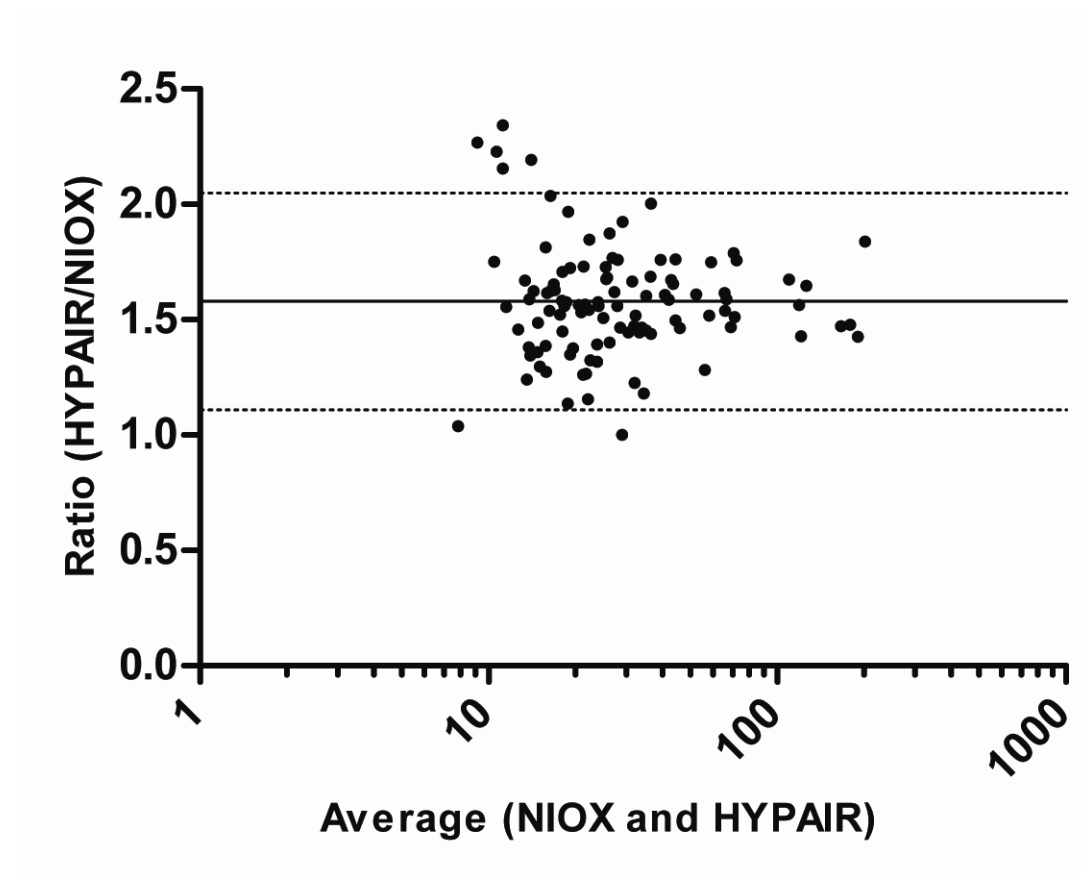


FIGURE 3.2. Bland-Altman plot showing the relationship between FENO levels detected with the two instruments expressed as ratio against mean for all measurements. Ratio 1.58 (95% CI 1.11-2.05)

Geometric mean FENO levels for the different participant groups (healthy atopic, healthy non-atopic, asthma atopic and asthma non-atopic) are shown in Table 3.2 by type of analyser. The ratio expressing the difference between both analysers was independent of atopy and asthma status. FENO levels were significantly higher in atopics compared to non-atopics ($p=0.0003$ and $p=0.0011$ with the NIOX and HypAir FENO respectively when considering only non-asthmatics; and $p<0.0001$ - irrespective of instrument used for asthmatics and non-asthmatics combined). There

was, however, one small difference between both devices when comparing atopic asthmatics with atopic non-asthmatics, with the NIOX showing statistically significantly higher FENO levels in atopic asthmatics ($p=0.0391$), whereas with the HypAir FENO this difference did not quite reach statistical significance ($p=0.0629$).

TABLE 3.2. FENO measurements for different population subgroups obtained with the NIOX and HypAir FENO. Unless otherwise indicated data are expressed as geometric mean (95% CI).

Group	Number	NIOX NO (ppb) Geometric mean (95% CI)	HypAir FENO NO (ppb) Geometric mean (95% CI)	Mean Ratio (relative to NIOX)(SD)
Healthy atopic	42	24.6 (20-30.3)	37.5 (30.1-46.7)	1.5 (0.2)
Healthy non-atopic	44	15.7 (13.8-17.8)	24.99 (22.1-28.2)	1.6 (0.3)
Asthma atopic	18	37.4 (23.9-58.5)	57.7 (37.7-88.4)	1.6 (0.2)
Asthma non-atopic	2	18.1 (N/A)	32.7 (N/A)	1.8 (0.4)
Total/mean	106	21.8 (19.1-25)	34 (29.7-38.9)	1.6 (0.2)

The results obtained on the two separate NIOX analysers ($n=15$) yielded almost identical results (within 2ppb in all cases; data not shown) suggesting that the difference between the two instruments was a real finding, and not due to a fault specific to the NIOX analyser. Longitudinal assessment of FENO measurement using both instruments in 2 individuals (1 healthy atopic, 1 healthy non-atopic) showed some variation over time, but time trends were independent of type of instrument used (Figure 3.3).

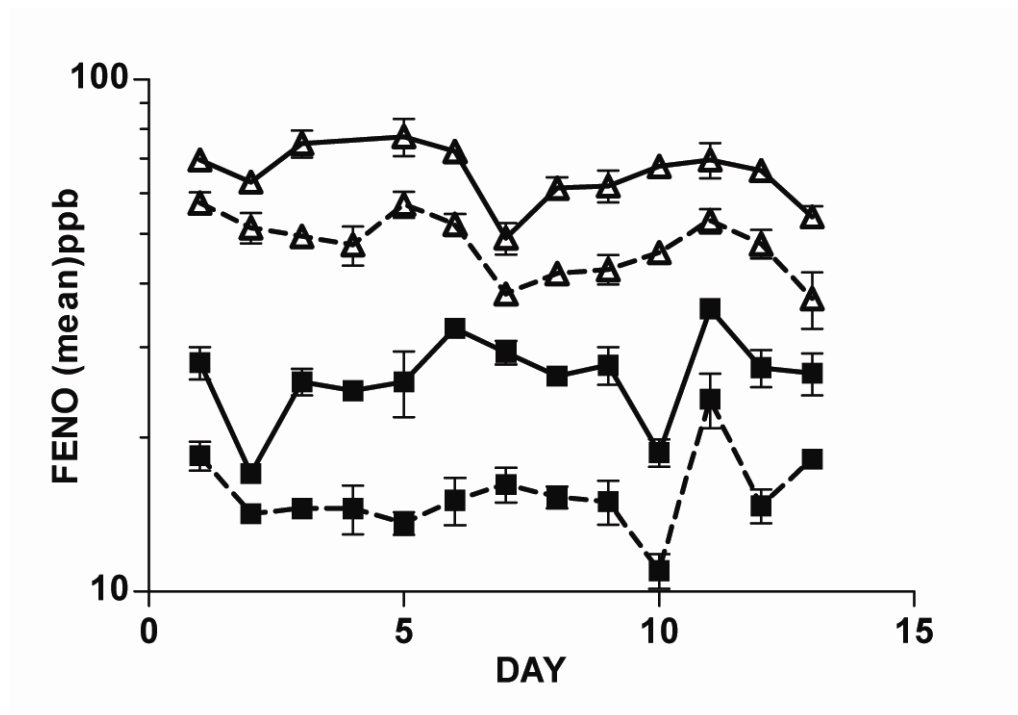


FIGURE 3.3. Example of longitudinal variation in FENO measurements from 2 study participants (open triangles = atopic male, closed squares = non-atopic male) using either the HypAir FENO (solid line) or NIOX (broken line), including SD of 3 measurements.

3.4 Discussion

The results of our study show a very strong correlation between FENO measurements obtained with the Medisoft HypAir FENO analyser and the Aerocrine NIOX analyser over a broad range of detectable NO levels regardless of asthma or atopy status.

FENO measurements obtained with the Medisoft HypAir FENO analyser were, however, significantly higher than those obtained with the Aerocrine NIOX. This difference was approximately 60% and was largely consistent irrespective of FENO levels, although when very low FENO measurements were detected the difference increased to greater than 100%.

Since the completion of this study, the only other study comparing these two particular instruments (alongside three others) has been published. In a mixed population of 85 subjects (consisting of asthma and COPD patients, and healthy volunteers), Korn *et al* (679) found that the measurements obtained with the Medisoft HypAir FENO were approximately 70% higher than those detected with the NIOX. This is strongly comparable to our study, which showed FENO levels measured with the Medisoft HypAir FENO to be 60% higher. The close corroboration between these two studies strongly suggests that the differences observed are due to the technologies used, rather than instrument faults, or variations in calibration and instrument use by the researcher. However, although we ensured that all instruments were used and calibrated in accordance with the manufacturers' guidelines, we were unable to eliminate this possibility due to the lack of availability of a second HypAir FENO analyser.

In contrast to the results obtained in our study, Korn *et al* found that the differences between the two instruments were less consistent as determined by Bland-Altman analysis, and suggest that a correction factor could not be used. They also showed only a moderate correlation (Spearman Rho 0.854) compared to the strong correlation found in our study (Spearman Rho 0.97/Pearson Rho 0.98). The reasons for these differences are unclear. It is possible that the randomisation between the five analysers tested and multiple measurements conducted may have resulted in greater variation and inconsistency in detected FENO levels between instruments. This is feasible, as multiple exhalation manoeuvres result in lower observed FENO measurements (681).

TABLE 3.3. Specifications and running costs of the NIOX and HypAir FENO (all prices obtained from the New Zealand supplier and converted to US dollars (\$) at the time of manuscript preparation; prices are likely to vary across regions). Service and calibration schedules as suggested by manufacturer.

	NIOX	HypAir FENO
Portability	Requires PC connection	Requires PC connection
Dimensions (cm)	50x30x40	14x21x33
Weight (kg)	40	10
Unit cost (\$)	58,344	22,168
Servicing cost (schedule)	8540 (18 months)	1618 (12 months)
Calibration schedule	Every 2 weeks	Every 6 months
Cost per filter	9.5	4

The introduction of FENO analysis in large-scale community and primary care settings has been limited by the high cost and inconvenience associated with the majority of these instruments. The HypAirFENO is more portable and cheaper to either purchase or run than the NIOX analyser (Table 3.3). However, the time taken by the HypAir FENO to analyse and report a single FENO measurement is

considerably longer than the NIOX (approximately 2 minutes). Such a delay may be inconvenient in an epidemiological or occupational setting, particularly since some individuals may have difficulty in obtaining an adequate measurement in the first instance. As the CV values of the 3 repeated measurements of the HypAir FENO were very low (mean CV 5.9%), it may be possible to reduce the number of repeated measurements to obtain an accurate result. In the case of the only other portable electrochemical analyser on the market, the Aerocrine NIOX MINO, it has been suggested that use of a single measurement is adequate (672).

The Medisoft HypAir FENO generated substantially higher FENO values compared to the NIOX instrument. A number of previous studies comparing the NIOX and the NIOX MINO (see above) have also found higher FENO results with an electrochemical, portable instrument, albeit to a lesser magnitude (672,677). Large differences (of greater than 50%) between FENO results obtained with other analysers have also been described (674). In the current study this phenomenon was only apparent when FENO measurements were very low (less than 10ppb as measured by the NIOX). As both manufacturers suggest that their instruments measure down to 0 ppb, it is currently unclear if this is due to the different NO detection technology used (chemiluminescent versus electrochemical) or the fact that the instruments are less accurate in the very low measurement range as suggested by the higher CV values for both instruments at levels below 10ppb.

Currently, there is no evidence suggesting that low FENO measurements are clinically significant, and given the overall strong correlation with the NIOX instrument we

don't consider the more than average increase in FE_{NO} at the low end of the measurement range to be a problem.

A related issue with the Medisoft instrument is the included package software which interprets the degree of airway inflammation from the FENO results obtained. Any FENO reading above 25ppb is designated "significant inflammation", which may be misleading as the values obtained are consistently higher than those obtained with the NIOX. A possibly more appropriate (but arbitrary) cut-off point would be determined as 37.7ppb using the conversion determined in the current study, but further studies using this new instrument would be required to define a more valid cut-off point.

Finally, it is unclear if FENO values detected with the HypAir FE_{NO} may change over time, or due to instrument wear. For the duration of this study the good correlation between the FENO values obtained using the two instruments shown suggests that this did not occur.

To conclude, we show that the Medisoft HypAir FENO provides comparable results with those obtained with the Aerocrine NIOX over a wide range of exhaled nitric oxide measurements, albeit at (consistently) higher levels. Therefore whilst care must be taken when comparing FENO results using different machines in different studies, the use of a conversion factor may allow comparisons to be made. The relative simplicity, compact size and relatively low running costs of the Medisoft HypAir FENO may make it a suitable candidate for FENO measurement in primary care and epidemiological studies.

Acknowledgements

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4. Identifying leukocyte populations in fresh and cryopreserved sputum using flow cytometry

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Background: Airway inflammation is commonly assessed by sputum induction followed by a differential cell count (DCC) using light microscopy. This method is prone to inter-counter variability and poor reproducibility. We aimed to develop a more objective method using flow cytometry (FCM).

Methods: 56 sputum inductions were conducted in 41 adults (23 asthmatics). Sputum was processed, a cytospin prepared for DCC, and the remainder immunolabelled for FCM using CD45, CD14 and CD16-specific antibodies to distinguish major leukocyte populations. Aliquots of 15 samples were frozen at -80°C to assess the effects of cryostorage. DCC and FCM were compared, and viability of individual cell populations was determined by FCM.

Results: FCM and DCC, and fresh and frozen samples, were significantly correlated, $R=0.54$ to 0.87 ; all $p<0.0001$, and $R=0.57$ to 1 ; $p<0.005$, respectively. There was a significant neutrophil loss after cryostorage (from median 30.5% to 17.4% of total leukocytes; $p<0.0001$). Cell viability was higher for lymphocytes compared to granulocytes or macrophages ($p<0.001$). With the exception of the expected higher levels of eosinophils ($p<0.005$) no significant difference in cell differentials or viability was observed between asthmatics and non-asthmatics using either DCC or FCM.

Conclusions: FCM is a suitable means of assessing leukocyte populations in induced sputum. Sample storage at -80°C prior to FCM is feasible, but may be detrimental to neutrophils, although good correlations were still observed between fresh and frozen samples. Large differences in viability were found between individual cell populations suggesting that viability dye use may be necessary.

4.1 Introduction

Induced sputum (IS) is a commonly applied technique for assessing airway inflammation in respiratory disease (504,547), and primarily involves conducting differential cell counts (DCCs) of leukocytes using light microscopy. However, there are significant problems associated with this including inter-observer variability, poor reproducibility, and the low cell numbers routinely counted. Assessment is particularly problematic when dealing with poor quality samples containing high levels of squamous-epithelial cells or debris.

These issues can potentially be overcome using flow cytometry (FCM). FCM allows thousands of events to be assessed per sample, and provides the ability to gate out squamous-epithelial cells and debris, thus providing a means to remove cytopsin reader bias and minimise small cell population bias. However, there is little detailed information available regarding FCM gating for leukocyte populations in IS, although strategies for bronchoalveolar lavage (BAL) have been published (682,683). To-date most reports using FCM to analyse IS have focussed on one particular cell subset, such as macrophages (309,684), neutrophils (685) or lymphocytes (686-689). This may be due in part to the specific difficulties associated with the FCM analysis of IS, including contamination with non-viable cells, or the use of DTT (dithiothreitol) for mucus dispersal, which affects FCM detection of certain cell markers (578).

To our knowledge only one recent study (690) has attempted to correlate cytopsin DCC and FCM data for all major cell populations in IS. In addition, although freezing has been reported as a suitable storage strategy prior to assessment of sputum lymphocyte subsets (686) and DCC (580,581), the effects of freezing on FCM

assessment of other leukocyte populations in IS has not been reported. Furthermore, little is known about the viability of *individual* cell populations in IS, as light microscopic assessment only allows *overall* cell viability to be determined.

The aims of this study therefore were to: (i) develop a robust strategy to detect leukocyte populations in IS using FCM and compare this to DCC data, (ii) assess the effects of cryostorage on FCM analysis, and (iii) determine the viability of *individual* cell populations. Finally, to test the utility of this approach, we wanted to (iv) compare results obtained using DCC and FCM in samples obtained from asthmatics and non-asthmatics.

4.2 Materials and Methods

Subjects

Adult participants (n=54; 29 asthmatics and 25 non-asthmatics) were recruited from staff and the general population. All completed a respiratory health questionnaire, lung function test and at least one hypertonic saline sputum induction. Asthma was defined as a physician's diagnosis of asthma, and/or asthma medication use within 12 months of assessment. Exclusion criteria included a respiratory infection within the last four weeks, forced expiratory volume in 1 second (FEV₁) <75% predicted or an irreversible drop in FEV₁ >15% during sputum induction. The study protocol was approved by the Upper South A Regional Ethics Committee, New Zealand (URA/08/08/056). Fully informed consent was obtained from all participants.

Sputum induction

Sputum induction was conducted as described previously (426) with some modifications. Briefly, participants were pre-treated with 400 µg salbutamol. Spirometry was performed according to American Thoracic Society guidelines (691), before and 15 minutes after salbutamol. Sodium chloride (Baxter Healthcare, Auckland, New Zealand; 4.5% w/v) vapour was produced using an ultrasonic nebuliser (DeVilbiss Ultraneb 2000), and administered orally for increasing intervals from 30 seconds to 4 minutes, to a total of 16 minutes. FEV₁ was obtained by spirometry at each interval. At the end of the session participants were encouraged to produce a sputum sample into a sterile plastic container. The procedure was

discontinued if the participant declined, or if a decline $>15\%$ FEV₁ could not be reversed by salbutamol administration during the procedure.

Sputum processing

Sputum samples were processed by plug selection (542). In several cases (n=7) this was not possible due to a dispersed mucoid appearance, and the whole sample was processed (530). Total cell count and viability (trypan-blue exclusion) were determined using light microscopy. The sample was centrifuged (350 x g, 8 minutes), and the cell pellet resuspended in RPMI 1640 medium (Invitrogen, Auckland, New Zealand) containing 10% foetal calf serum (FCS, Invitrogen) and 1% penicillin-streptomycin (Invitrogen) at 1×10^6 cells/ml.

Identification of cell populations by differential cell count

Cell suspension (75 μ l) was centrifuged in a Labofuge 400 cytospin column (Heraeus, Hanau, Germany) at 44 x g for 5 minutes. The slide produced was air dried, fixed and stained with the Diff-Quik® fixative and stain set (Dade Behring, Deerfield, IL). 400 non-squamous cells were counted using a DME light microscope (Leica Microsystems, Wetzlar, Germany). 29 slides (51.8%) were counted separately by a second researcher and compared with the original counts to determine inter-observer variation.

Identification of cell populations by flow cytometry

Cell suspension (100 μ l) was used for each FCM stain, conducted in 96 well plates. Cells were washed in FCM buffer (phosphate-buffered saline (PBS) containing 1% FCS, 0.01% sodium azide and 2 mM ethylenediaminetetraacetic acid (EDTA; both Sigma-Aldrich, Auckland, New Zealand; also for all subsequent wash steps), incubated with polyclonal IgG (Intragam, Commonwealth Serum Laboratories, Sydney, Australia) at 2 mg/ml (10 minutes, 4°C) to block non-specific binding as described previously (692), washed again, then labelled in a total volume of 25 μ l (25 minutes, 4°C). Antibodies used were CD45-APC (clone HI30), CD14-FITC (clone M5E2) and CD16-PE (clone 3G8) (all BD Biosciences, San Jose, CA). All antibodies were optimally titrated and matching isotype controls were used as appropriate. Cells were washed again and resuspended in FCM buffer containing 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) at a final concentration of 0.5 μ g/ml. FCM data acquisition was performed on a LSR II flow cytometer (BD Biosciences), equipped with 5 lasers. FITC was detected using a 515/20 bandpass filter with the 488 nm laser, PE using a 575/26 filter with the 532 nm laser, APC using a 670/14 filter with the 640 nm laser and DAPI using a 450/50 filter with the 355 nm laser. Data were collected using FACSDIVATM software (BD Biosciences). Instrument voltage settings were kept the same throughout the study. Instrument compensation was set-up using fluorescently-labelled antibodies and Compbeads (BD Biosciences) according to manufacturer's instructions. Flowjo software (Treestar Incorporated, Ashland, OR) was used for FCM analysis.

Freezing and thawing of induced sputum samples

For 15 randomly selected sputum samples, a further aliquot of between 5×10^5 and 2×10^6 cells was cryopreserved. Samples were centrifuged at $350 \times g$ for 5 minutes, resuspended in 1 ml freeze medium (FCS containing 10% dimethyl sulfoxide (DMSO: Sigma-Aldrich), placed in a cryostorage container (cooling rate of $1^\circ\text{C}/\text{minute}$) and transferred to a -80°C freezer. Samples were stored between 1 and 3 months. Prior to FCM analysis, frozen samples were thawed rapidly by adding 10 ml pre-warmed RPMI 1640, counted, centrifuged ($350 \times g$, 8minutes), then resuspended and processed for FCM analysis. A further antibody panel was used in thawed samples to assess the effect of cryostorage on the major CD4/8 T cell subsets (CD45, CD4/8 BD Biosciences multitest).

Statistical analysis

Spearman's rank correlation and Bland-Altman plots were used to compare FCM/DCC data, and fresh/frozen samples. Additional comparisons were made using the paired t-test or Wilcoxon matched-pairs test. Differences between groups were assessed using the unpaired t-test or Mann-Whitney u-test. Data normality was determined using the D'Agostino & Pearson omnibus test. Multi-group comparison was conducted using Kruskal-Wallis with Dunn's post-test. All statistical analyses were conducted using Prism 5 software (Graphpad Software, La Jolla, CA).

4.3 Results

A total of 56 sputum samples adequate for analysis by both FCM and DCC were produced by 41 individuals, representing 75.9% of participants (23 asthmatics and 18 non-asthmatics). Repeat visits were conducted once with 5 participants (4 asthmatic, 1 non-asthmatic), twice with one participant (1 asthmatic), and 4 times with two participants (2 non-asthmatics) as part of a study assessing stability of airway inflammation (data not shown). Thirteen participants (6 asthmatics and 7 non-asthmatics) were unable to provide an adequate sample for FCM and/or DCC, and were excluded. Demographic data is provided in Table 4.1.

TABLE 4.1. Participant characteristics. Expressed as median (IQR) or number (percentage)

	All participants	Asthmatics	Non-asthmatics
Number	41	23	18
Gender (F/M)	22/19	12/11	10/8
Age	30 (24-42.5)	28 (25-44)	31.5 (23-42.25)
Atopy	28 (68.3%)	19 (82.6%)	9 (50%)
FEV1 (% predicted)	96 (86-102.5)	93 (81-99)	100 (92-108.8)
FEV1/FVC	0.83 (0.79-0.86)	0.8 (0.72-0.82)	0.86 (0.84-0.87)
Δ FEV1 (pre/post bronchodilator)	4 (1.25-7)	6 (4-11.5)	3 (0.75-4.25)
Current ICS use	15 (36.6%)	15 (65.2%)	0 (0%)
Current smoker	2 (4.9%)	1 (4.3%)	1 (5.6%)

Identification of leukocyte populations by FCM

FCM was conducted by gating on forward scatter (FSC)-H and FSC-A to exclude doublets, followed by gating on CD45 expression to discriminate leukocytes from contaminating squamous-epithelial cells. Subcellular debris was then excluded on the basis of FSC and side scatter (SSC). Specific populations were identified on the basis

of marker expression and/or autofluorescence (693), as well as size and granularity.

Using this approach, lymphocytes were identified as $CD45^{\text{high}}$, $CD14^{-}$ (SSC^{low}); macrophages as $CD45^{\text{high}}$, autofluorescence^{high}, $CD14^{+}$, $CD16^{+}$ (SSC^{high}); monocytes as $CD45^{\text{high}}$, autofluorescence^{low}, $CD14^{+}$, $CD16^{+}$ or $-$ (SSC/FSC^{mid}); neutrophils as $CD45^{\text{mid}}$, $CD14^{\text{dim}}$, $CD16^{+}$; and eosinophils as $CD45^{\text{mid}}$, $CD14^{\text{dim}}$, $CD16^{\text{dim}}$.

Representative gating is shown in Figure 4.1. An alternative gating strategy was attempted which also excluded non-viable DAPI+ events prior to leukocyte gating (as is commonly conducted with FCM), but this resulted in overestimation of lymphocytes and underestimation of macrophages when compared to either DCC or the FCM strategy above (supplementary Figure 4.1& 4.2 online: shown at end of chapter 4).

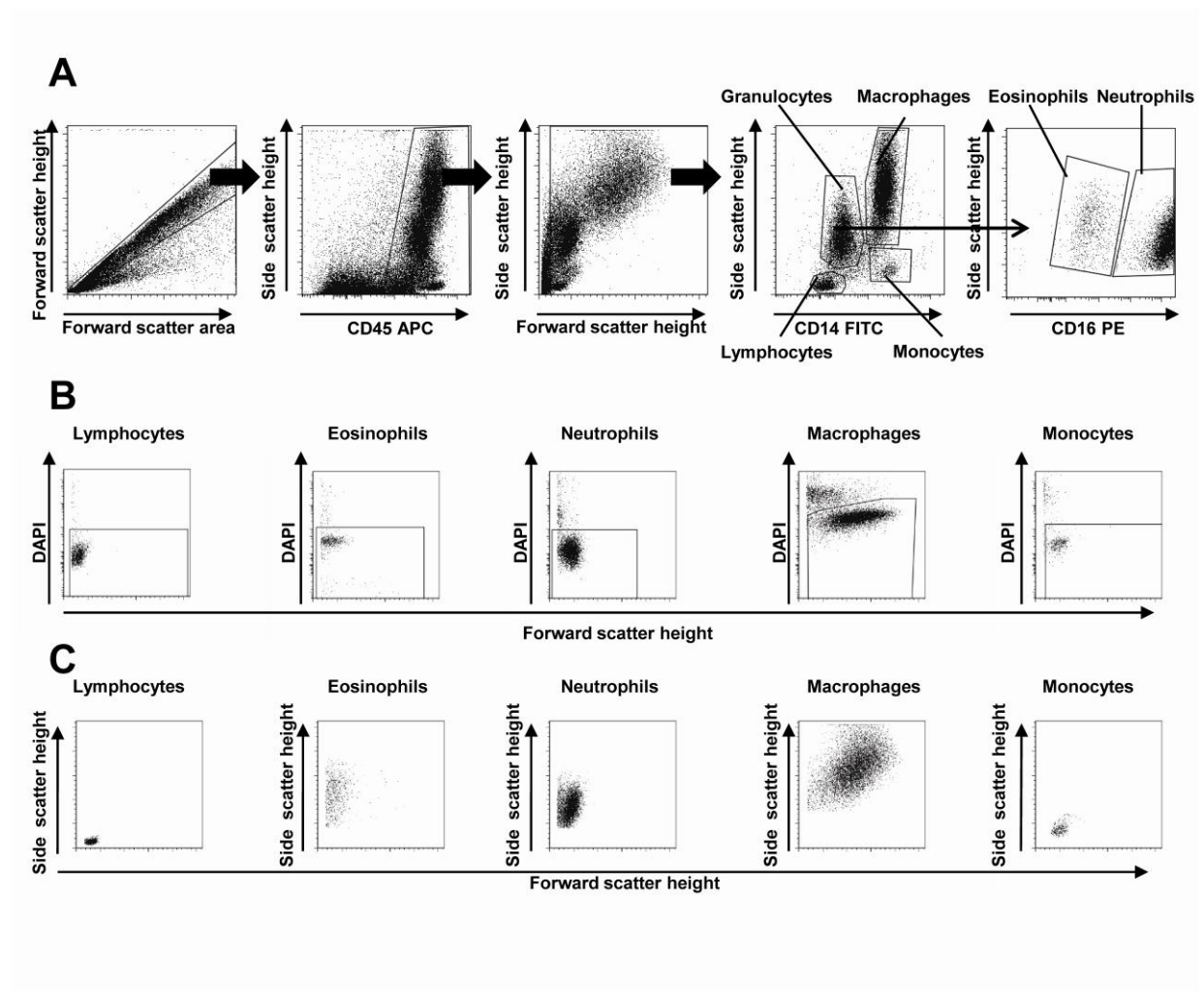


FIGURE 4.1. Flow cytometric gating of induced sputum. (previous page). A: Representative sequential gating of leukocyte populations in induced sputum from an asthmatic participant. (Left to right) Firstly, doublets were excluded, then CD45- events, then subcellular debris. Leukocytes were identified on the basis of CD14 expression and side scatter, and granulocytes on the basis of CD16 expression. B: Viability of individual leukocyte populations was determined on the basis of DAPI staining. C: Leukocyte population characteristics were confirmed by gating on the basis of FSC and SSC.

Comparison of FCM and DCC data in fresh sputum samples

We observed significant correlations between FC and DCC for all cell populations ($p < 0.0001$), with correlation coefficients of 0.87 for neutrophils, 0.83 for macrophages/monocytes (combined in FCM to allow for comparison to DCC), 0.84 for eosinophils and 0.54 for lymphocytes (Figure 4.2). In contrast to previous findings (694), the strength of these correlations was not improved by the exclusion of samples with relatively high squamous-epithelial cell contamination, or processing whole rather than selected sputum (data not shown). Neutrophils and macrophages represented a slightly greater percentage of leukocytes by DCC (medians of 30.2 and 60.8 respectively, compared to 26.0 and 57.9 by FCM; Table 4.2). This increase was small but significant in both cases (paired t-test; $p = 0.005$ and 0.001 respectively). Conversely, lymphocytes were detected as a greater percentage of leukocytes using FCM rather than DCC (5.7% vs 1.7%; $p < 0.0001$). Eosinophil percentage was similar using either method (0.5% and 0.6%).

TABLE 4.2. Sample characteristics and percentage of leukocyte populations determined using DCC and FCM (expressed as median (IQR)), p value determined by T test or Mann-Whitney test (†) between asthmatics and non-asthmatics

Parameter	DCC and light microscopy				FCM			
	All participants	Asthmatics	Non-asthmatics	P value	All participants	Asthmatics	Non-asthmatics	p value
Total non-squamous cells/ml ($\times 10^6$)	1.70 (1.22-2.52)	1.60 (1.22-3.24)	1.76 (1.15-2.12)	0.43 †	-	-	-	-
Sputum volume selected (ml)	1.75 (1-2.25)	2.00 (1.00-2.25)	1.68 (1.00-2.31)	0.92 †	-	-	-	-
Total non-squamous cells ($\times 10^6$)	2.98 (1.48-5.25)	2.84 (1.49-6.45)	3.15 (1.46-5.06)	0.83 †	-	-	-	-
Viability non-squamous cells (%)	68.88 (57.23-81.66)	70.98 (62.75-81.71)	66.60 (53.38-81.51)	0.36	-	-	-	-
% Squamous cells (of all)	10.34 (4.56-19.41)	11.94 (4.62-22.32)	8.23 (4.48-17.62)	0.33 †	-	-	-	-
% Neutrophils (of non-squamous)	30.15 (18.29-43.5)	27.23 (18.29-42.64)	30.19 (19.15-43.58)	0.49	26.02 (14.13-39.05)	25.21 (15.53-38.84)	28.97 (10.25-39.83)	0.73
% Lymphocytes (of non-squamous)	1.73 (1.15-2.59)	1.76 (1.00-2.21)	1.69 (1.21-3.02)	0.66	5.71 (3.07-8.34)	4.55 (3.02-7.35)	7.05 (3.06-9.57)	0.15
% Macrophages (of non-squamous)	60.81 (46.60-76.48)	62.65 (49.41-75.88)	60.40 (46.30-79.35)	0.72	57.90 (42.96-71.84)	62.12 (42.85-71.77)	54.69 (43.22-72.64)	0.72
% Eosinophils (of non-squamous)	0.48 (0-1.65)	1.30 (0.24-3.97)	0.24 (0.00-0.50)	0.0004 †	0.57 (0.28-0.58)	1.63 (0.41-3.76)	0.32 (0.21-0.62)	0.007†
% Col epithelial cells (of non-squamous)	1.57 (0.57-7.11)	1.68 (0.63-3.91)	1.46 (0.46-12.52)	0.85 †	-	-	-	-
Monocytes (SSC mid CD14+CD16+)	-	-	-	-	1.66 (1.13-2.62)	1.62 (1.20-3.69)	1.70 (0.96-2.67)	0.46†
Monocytes (SSC mid CD14+CD16-)	-	-	-	-	1.23 (0.84-1.94)	1.15 (0.76-2.20)	1.32 (0.90-1.88)	0.69†

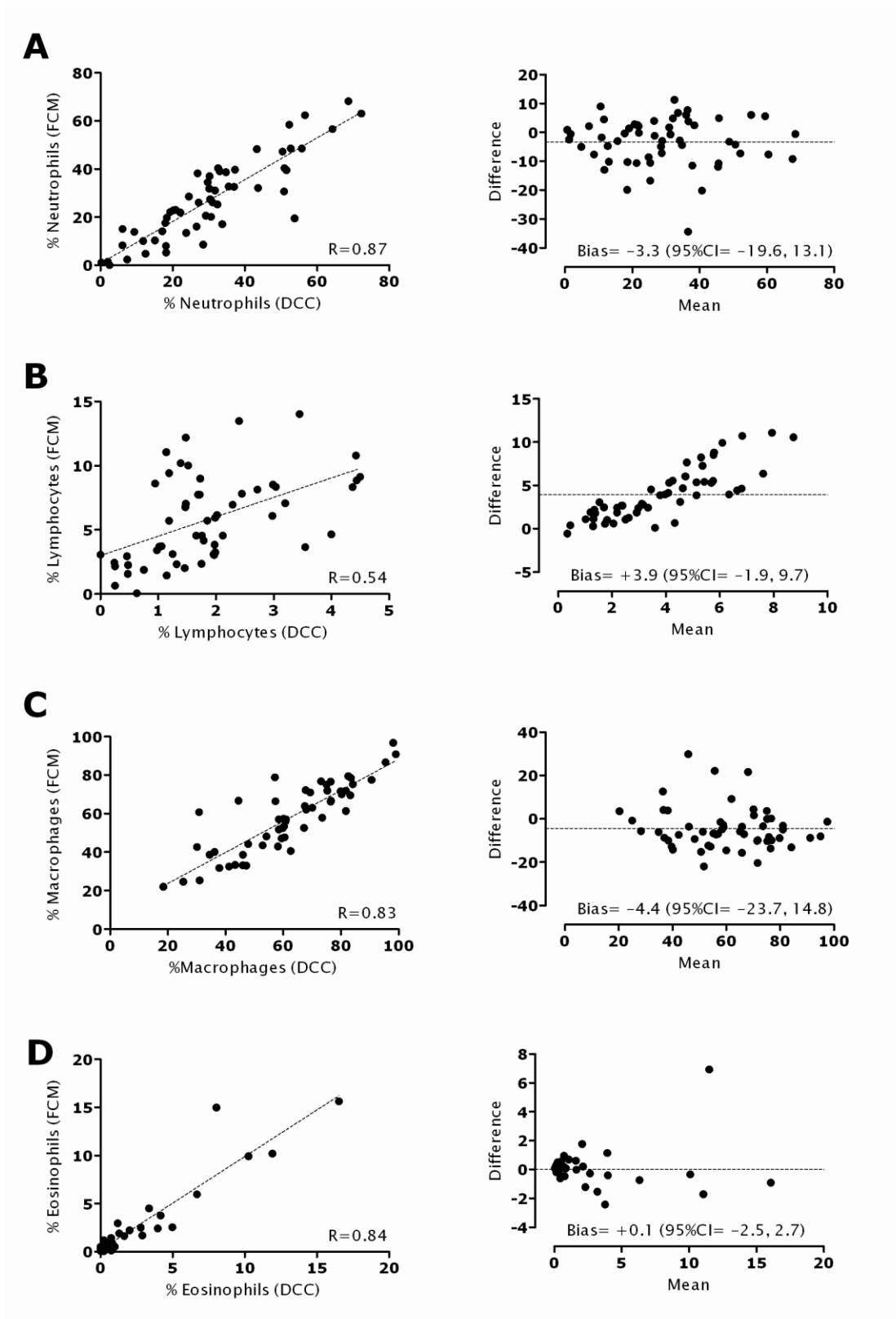


FIGURE 4.2. Correlations (left) and Bland-Altman plots (right) comparing flow cytometric and light microscopy differential cell counts for induced sputum (A) neutrophils (B) lymphocytes (C) macrophages and (D) eosinophils. (CI=confidence interval)

Despite the significant correlations observed between FCM and DCC, there was some inter-observer variation when conducting DCC on less frequent leukocyte populations, with a coefficient of variance (CV) of 60.9% for eosinophils and 32.9% for lymphocytes, compared to a CV of 7.1% for the more abundant macrophages/monocytes (65.2 % of leukocytes) (Table 4.3).

TABLE 4.3. Mean coefficient of variance (CV) values for inter-observer variation during differential cell count (DCC) (n=29). (SD: standard deviation)

Cell population	% leukocytes	DCC	
		SD	CV (%)
Neutrophils	27.31	3.70	16.55
Lymphocytes	2.21	0.65	32.89
Macrophages/monocytes	65.17	3.60	7.05
Eosinophils	2.58	0.71	60.94

Viability of leukocyte populations by FCM

Individual leukocyte populations were further gated based on their uptake of DAPI into viable/non-viable populations (Figure 4.1B). Significant differences in viability were observed, with lymphocytes significantly more viable than all other cell populations identified (median 98.59% viability; $p < 0.001$), while macrophages had the lowest overall viability (71.93%; $p < 0.001$; Table 4.4).

TABLE 4.4. Viability of leukocyte populations in induced sputum (IS) as determined by flow cytometry (FCM) (expressed as median (IQR), p value determined by Mann-Whitney test)

Cell population	% viability (DAPI low to negative cells)			
	All participants	Asthmatics	Non-asthmatics	p value
Neutrophils	87.54 (79.94-91.65)	88.11 (82.09-90.37)	84.49 (63.77-93.67)	0.64
Lymphocytes	98.59 (96.47-99.24)	98.45 (96.23-99.00)	98.82 (96.96-99.43)	0.11
Macrophages/monocytes	71.93 (59.56-80.55)	73.21 (60.13-83.99)	65.87 (56.45-79.56)	0.20
Eosinophils	88.09 (72.78-93.82)	88.70 (82.75-94.80)	87.51 (68.73-90.60)	0.30

Assessment of the effects of cryostorage on FCM analysis of sputum samples

Significant correlations were observed between FCM data obtained with fresh and frozen samples (all $p < 0.001$) for all leukocyte populations. The correlation coefficients ranged from 1 for ratio of CD4+/CD8+ T cell subsets to 0.57 for macrophages/monocytes (Figure 4.3). Although we observed only minor reductions in most cell populations after freeze-thawing, a significant loss was seen with neutrophil percentage, from a median of 30.54% of total leukocytes (interquartile range (IQR) 18.78, 34.01) to 17.44% (11.19, 20.41); $p < 0.0001$), although a strong correlation was observed between fresh and frozen samples (Figure 4.3). The majority of cryopreserved samples did not contain a significant number of eosinophils, so no comparative data for eosinophils is available.

Analysis of sputum samples from asthmatics and non-asthmatics

There were no differences between asthmatics and non-asthmatics for any light microscopic assessment, such as total cell yield or viability. A significantly increased eosinophil proportion was observed in asthmatics using FCM (1.63% vs 0.32%; $p = 0.007$) which corresponded to the increased eosinophil differential also observed using DCC (1.30% vs 0.24%; $p = 0.0004$; Table 4.2).

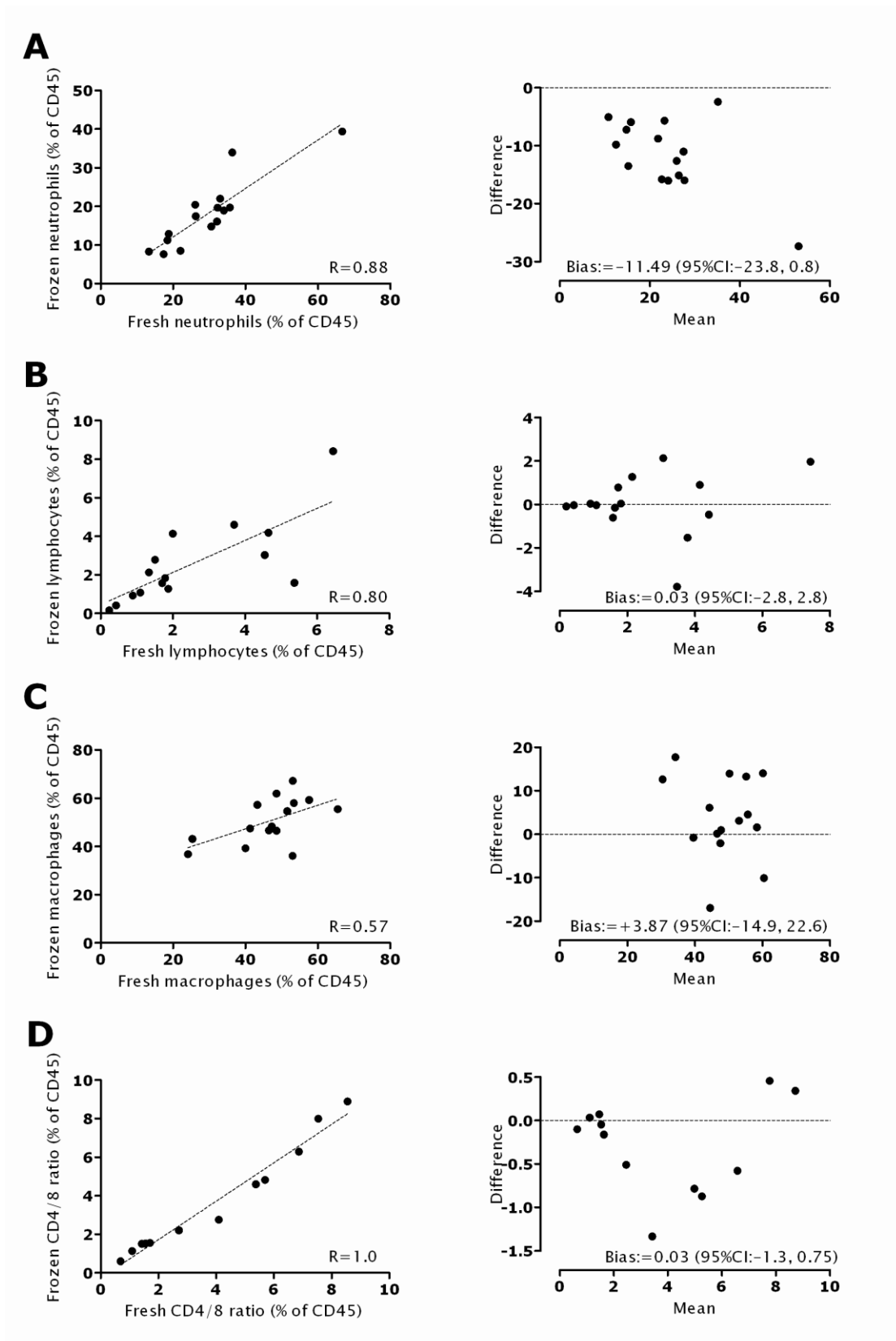


FIGURE 4.3. Correlations (left) and Bland-Altman plots (right) comparing flow cytometric differential cell counts for fresh and cryopreserved induced sputum (A) neutrophils (B) lymphocytes (C) macrophages and (D) CD4/CD8 lymphocyte ratio. (CI=confidence interval)

4.4 Discussion

In this study we described a robust FCM strategy (using a panel of three markers: CD14, CD16 and CD45, alongside the viability dye DAPI) to identify the major leukocyte populations in IS. The differential leukocyte count determined by FCM correlated well with DCC for all the cell populations studied i.e. lymphocytes, neutrophils, monocytes/macrophages and eosinophils. A strong correlation was also observed when comparing FCM analysis of fresh and cryopreserved sputum samples, although neutrophil numbers were significantly reduced after thawing. We were able to determine the viability of the individual leukocyte cell populations and found the lowest viability levels (approximately 70%) for macrophages and the highest (approximately 99%) for lymphocytes. Finally, analyses of induced sputum from asthmatics and non-asthmatics by FCM and DCC were comparable, with a higher eosinophil percentage seen in asthmatics by both methods.

Several approaches to FCM analysis of airway leukocyte populations in IS and BAL have been attempted. Some studies suggested that sputum leukocytes could be differentiated by size and granularity alone (695). We found it extremely difficult to differentiate leukocyte populations on this basis, and therefore used the pan-leukocyte marker CD45+ prior to subsequent analysis. A similar approach was also used in two recent publications. Lay *et al* (694) describe work conducted over the last decade, including use of an antibody panel similar to that used in our laboratory. However, while comprehensively describing gating strategies for several cell populations, only neutrophil correlation data ($R=0.82$) was provided. Vidal *et al* (690) describe a study of 38 patients, using CD45, CD66b, CD125 and CD14. When comparing FCM and DCC, this group found correlation coefficients of 0.73 for neutrophils, 0.75 for eosinophils and 0.53 for macrophages, but no reported values for lymphocytes. These correlations are similar but not as strong as the correlation coefficients

we observed ($R=0.87$ for neutrophils, 0.84 for eosinophils, 0.83 for macrophages and 0.54 for lymphocytes). Vidal *et al* express caution about the use of CD14 and CD16 to identify macrophages and neutrophils due the co-expression of these markers on both. Interestingly, we did not find an obvious overlap. CD14 was expressed at low levels on granulocytes in comparison with macrophages. When CD14 was assessed in combination with SSC, discrete populations representing granulocytes, lymphocytes and macrophages could be observed. CD16 expression could then be used to confirm the identity of neutrophils and eosinophils, as has previously been described in blood analysis (696).

Two studies have compared leukocyte populations in BAL using FCM and DCC. Both (682,683) describe slightly different methods, but show strong correlations between FCM and DCC for lymphocytes, macrophages and neutrophils. However, BAL does not have the same issues with poor viability as IS, and leukocyte proportions in BAL are different to IS (697); results therefore cannot be directly compared. Nonetheless, both studies observed a tendency for DCC to underestimate lymphocytes and overestimate macrophages, similar to that described above.

Our study offers several advances on the studies discussed above. Firstly, the strong correlations between FCM and DCC suggest that our approach compares well (in some cases better) for identification of major leukocyte populations, compared to previous studies (690,694). Secondly, we measured populations before and after cryostorage. Although it has been suggested that freezing has no effect on subsequent DCC counts (581), deterioration of neutrophils and macrophages after freezing has been reported (682). We observed a significant reduction in neutrophil percentage after freezing (from 30.5% to 17.4% of leukocytes), suggesting that FCM analysis conducted after freezing may bias results. Despite

this, neutrophil loss during freezing was consistent, with a strong correlation between fresh and frozen samples observed ($R=0.88$). Therefore comparison between cryopreserved samples in a study may be feasible, although results are not directly comparable with those obtained from fresh samples.

Finally, we routinely used a dye (DAPI) to identify viable cells. A similar approach has been conducted in BAL analysis using 7-aminoactinomycin (682). When conducting functional experiments (such as oxidative burst assays) a viability dye is likely to be essential for avoiding staining artefacts, particularly when assessing the relatively low viability macrophage population. Since conducting this study, we have adapted our protocols to use the LIVE/DEAD® fixable blue reagent (Invitrogen), which adds further convenience by allowing downstream fixation.

Some previous studies have only analysed 10,000 events per run during FCM analysis (690). However, the large amount of debris/non-cellular material in IS may negatively affect results with such a limited number of events. For example, using the gating strategy described in our study, we observed a median of 94.4% non-doublets per sample, of which 30.6% were CD45+, and 61.7% of which were larger than debris. If only 10,000 events were run on such a sample, only 1,781 events would be leukocytes. While this number is greater than that used routinely in DCC, it may produce some imprecision in detected levels of eosinophils or lymphocytes, which regularly represent 0-5% of total leukocytes. We therefore routinely conducted analysis on >100,000 events per sample during this study.

When comparing data obtained from asthmatics and non-asthmatics we observed no significant differences by either DCC or FCM, with the exception of increased eosinophils in

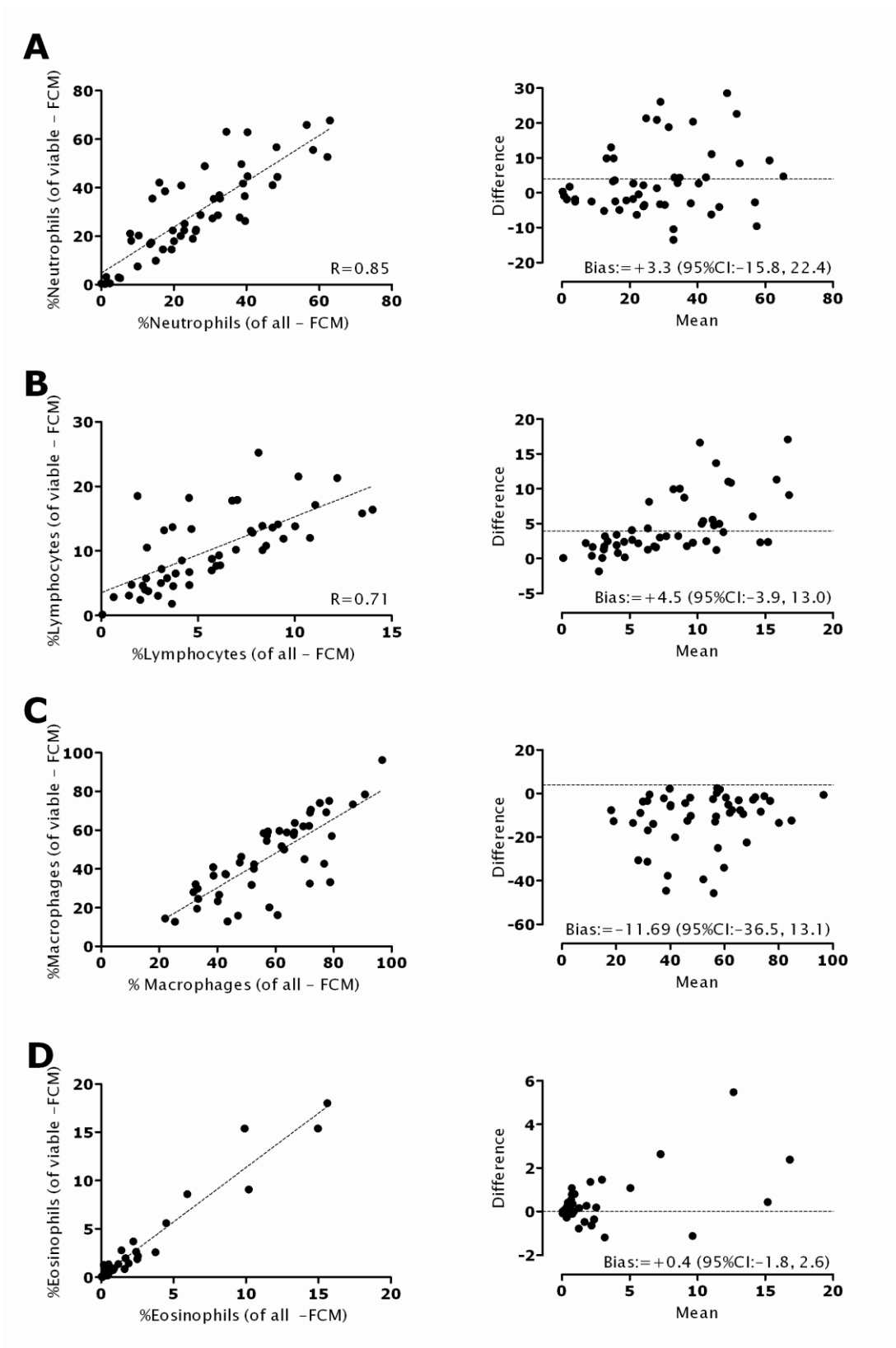
asthmatics using either technique (median 1.3% eosinophils using DCC and 1.64% eosinophils using FCM). Although some studies have reported considerably higher IS eosinophil percentages in asthmatics (e.g. Fahy *et al* reported a mean of 8.1% in 18 asthmatics (530)), our findings are consistent with those of Munoz *et al* (698) and Grootendorst *et al* (567), who reported a median IS eosinophil percentage of approximately 1% in studies assessing 84 adult asthmatics and 18 mild-to-moderate adult asthmatics respectively. The regular use of inhaled corticosteroids in 65% of asthmatics in our study may also have resulted in a lower eosinophil percentage than would be observed in studies assessing steroid-naïve patients.

Interestingly, using FCM we observed that 5 asthmatic individuals (21.7%) had a CD16⁺ monocyte population making up more than 4% of their total IS leukocyte population, compared to only 1 individual (5.6%) in the non-asthmatic group. Although not statistically significant, this may be indicative of active inflammation, and could (like eosinophils) be another indicator of inflammatory airway disease. Indeed, one study (699) has shown an increase in a population of small macrophages in another respiratory disorder, chronic obstructive pulmonary disease (COPD).

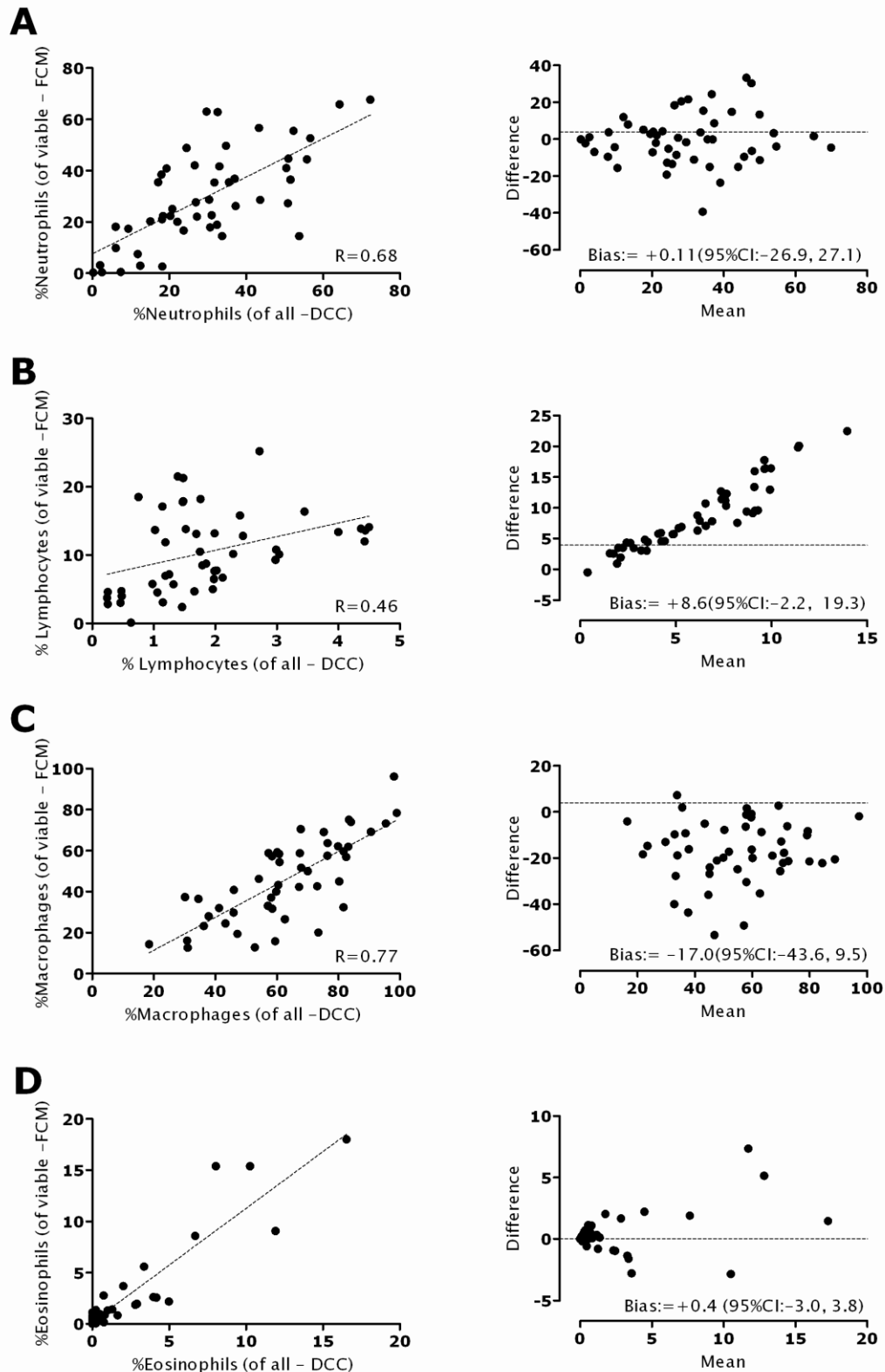
There are several advantages of FCM compared with DCC analysis of sputum. Using DCC, counts of cell populations with low frequency (such as lymphocytes and eosinophils) are prone to considerable variation and imprecision, as shown by the lower correlation coefficients and increased CV values observed when assessing these populations. DCC is also prone to underestimating lymphocyte percentages. Both issues been previously described (682), and the variation in particular is unsurprising, as only 400 leukocytes are counted as standard during DCC, whilst we routinely assessed more than 100,000 cells using FCM.

Furthermore, the use of well-defined, specific antibodies in combination with distinct FSC/SSC characteristics allows accurate and objective identification of each leukocyte population using FCM. Slide preparation and staining for DCC can be variable, often making accurate identification of cell populations difficult and subjective. However, it must be acknowledged that there are also some limitations with FCM. It takes longer to prepare IS samples for analysis, needs access to a flow cytometer, the use of more expensive reagents, and requires more technical expertise.

In summary, we have described a robust method of FCM analysis of IS that correlates strongly with the conventional approach using light microscopy, and thus provides a convenient alternative method of enumerating sputum leukocytes. We show that this approach can be used with cryopreserved samples, and therefore may be useful for epidemiological studies in which immediate analysis of sputum samples is difficult. Although the current study was limited to four fluorophores, FCM analysis of IS could be extended to identify several more important cell populations, such as DCs and lymphocyte subsets (e.g. (288,692), within a single antibody panel, as has been recently conducted with a 12-colour panel in peripheral blood (700). Such an approach could be of considerable benefit for the detailed immunophenotyping of IS in respiratory diseases such as asthma.



Supplementary Figure 4.1. Correlations (left) and Bland-Altman plots (right) comparing flow cytometric differential cell counts when gating all versus viable only induced sputum (A) neutrophils (B) lymphocytes (C) macrophages and (D) eosinophils. (CI=confidence interval)



Supplementary Figure 4.2. Correlations (left) and Bland-Altman plots (right) comparing flow cytometric differential cell counts of viable cells only versus light microscopy differential cell counts for induced sputum (A) neutrophils (B) lymphocytes (C) macrophages and (D) eosinophils. (CI=confidence interval)

5. Invariant natural killer T cells and asthma: immunological reality or methodological artefact?

Collin R Brooks, Robert Weinkove, Ian F Hermans, Christine J van Dalen, Jeroen Douwes

Background and objective: Several animal model-based studies have suggested an important role for invariant natural killer T cells (iNKTs) in airway inflammation in asthma, but evidence from clinical studies has been equivocal. In this study we assessed the presence of iNKT cells in induced sputum from asthmatics and non-asthmatics using a stringent cytofluorometric gating strategy, and compared our method with a previously published two-step strategy.

Methods: Twenty-five adult asthmatics and 19 non-asthmatics underwent clinical assessment, including hypertonic saline sputum induction, spirometry, and skin prick testing. Sputum cells were processed and stained for differential cell count, or for flow cytometric assessment of iNKT cells.

Results: No significant difference in iNKT cell frequency was found using the stringent gating strategy: (0.07% (0-0.17%) versus 0.06% (0-0.20%); median (IQR) $p=0.75$, for asthmatics and non-asthmatics respectively. However, a reanalysis of our data using the two-step gating strategy showed that up to 16.9% of the $CD3+SSC^{low}$ population now identified as iNKT cells. However, a similar increase in iNKT numbers was observed in asthmatics and non-asthmatics, and no significant difference was observed between groups (3.38% (1.07-5.71%) versus 2.29% (1.09-3.35%); $p=0.18$. Asthma severity was not associated with increased sputum iNKT cells.

Conclusions: This study suggests that the reported role of iNKT cells in human asthma aetiology may be related to analytical problems associated with flow cytometric gating strategies, rather than representing a true immunological mechanism.

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(extended from published short report)

5.1 Introduction

Invariant natural killer T (iNKT) cells can be identified by their expression of a semi-invariant variable region of the T cell receptor (TCR) (V α 24-J α 18/V β 11 in humans) (701), and display specificity for glycolipid antigens in the context of the non-classical and highly conserved MHC class I molecule CD1d (702). iNKT cells were first characterised by their recognition of the prototypical glycolipid ligand α -galactosylceramide (α -GalCer), which leads to the production of substantial quantities of cytokines such as IL-4, TNF- α and IFN- γ (701). A number of studies in different animal species have suggested that iNKT cells may play an important role in allergic inflammation and asthma (325,327,703-705), and airway hyperreactivity (AHR) in particular (329). Despite this, other studies have shown that iNKT cells may be dispensable for allergic airway inflammation in murine models (321,328).

Evidence for the involvement of iNKT cells in human asthma remains controversial. An initial study by Akbari *et al* suggested that iNKT cells made up the majority (up to 63%) of BAL-derived T cells in asthmatics as determined by flow cytometry (706). However, subsequent studies have shown that iNKT cells make up a tiny fraction of T cells in the lung of either asthmatics or non-asthmatics (330). There is also some evidence that the methods used to detect iNKT cells in the aforementioned study by Akbari *et al* were suboptimal, and may have been detecting macrophages or non-viable cells as methodological artefacts (707,708).

Matangkasombut and co-workers in a recent issue of the Journal of Allergy and Clinical Immunology provided further evidence of higher iNKT cell numbers (up to 64% of all CD3⁺ T cells) in bronchoalveolar lavage fluid (BALF) of severe asthmatics compared to well-

controlled asthmatics, who in turn had higher numbers than non-asthmatics (709). If true, this may have major implications for our understanding of asthma pathology.

Some of the discrepancies between studies may be due to the use of different cytofluorimetric gating strategies when assessing iNKT cells in BALF. The use of a simple two-step CD3+SSC^{low} strategy (as utilised by Matangkasombut *et al* (709)) allows exclusion of large cells such as macrophages, but is not capable of excluding non-specific binding to non-viable cells and debris. To investigate the effectiveness of this two-step strategy, we compared the detection of iNKT cells in induced sputum (IS) of 25 physician-confirmed asthmatics and 19 non-asthmatics using flow cytometry (FC) against a more stringent approach which involved gating out doublets and non-viable cells.

5.2 Methods

Fifty-four subjects provided informed consent and underwent spirometry, skin-prick testing and sputum induction using hypertonic saline as described previously (426). Asthma was defined as a physician's diagnosis of asthma, and/or asthma medication use within 12 months of assessment. Exclusion criteria included a respiratory infection within the last four weeks, forced expiratory volume in 1 second (FEV_1) <75% predicted or an irreversible drop in FEV_1 >15% during sputum induction. Four asthmatics and five non-asthmatics failed to provide an adequate sputum sample and were excluded, and we did not collect a sample from one severe asthmatic whose FEV_1 value was too low to safely undergo hypertonic saline challenge. The study protocol was approved by the Upper South A Regional Ethics Committee, New Zealand (URA/08/08/056). Fully informed consent was obtained from all participants.

Spirometry

Spirometric measurements were conducted for all participants according to American Thoracic Society (ATS) criteria (691) using an Easyone spirometer (NDD Medizintechnik AG, Zurich, Switzerland). Participants were pre-treated with 400 µg salbutamol, and spirometry was performed before and 15 minutes after salbutamol. NHANES III equations (72) were used for lung function parameters.

Sputum induction

Sodium chloride (Baxter Healthcare, Auckland, New Zealand; 4.5% w/v) vapour was produced using an ultrasonic nebuliser (DeVilbiss Ultraneb 2000), and administered orally over intervals for a total of 16 minutes. FEV_1 was obtained by spirometry at each interval. At the end of the session participants were encouraged to produce a sputum sample into a sterile

plastic container. The procedure was discontinued if the participant declined, or if a decline $>15\%$ FEV₁ could not be reversed by salbutamol administration during the procedure.

Sputum processing

Sputum samples were processed by plug selection (542). Induced sputum was incubated with Sputasol, filtered through a 60 μ M filter, and total cell count and viability (using trypan-blue exclusion) were determined using light microscopy. The sample was centrifuged (350 x g, 8 minutes), and the cell pellet resuspended in RPMI 1640 medium (Invitrogen, Auckland, New Zealand) containing 10% foetal calf serum (FCS, Invitrogen) and 1% penicillin-streptomycin (Invitrogen) at 1×10^6 cells/ml.

Identification of cell populations by differential cell count

Cell suspension (75 μ l) was centrifuged in a Labofuge 400 cytopspin column (Heraeus, Hanau, Germany) at 44 x g for 5 minutes. The slide produced was air dried, fixed and stained with the Diff-Quik® fixative and stain set (Dade Behring, Deerfield, IL). 400 non-squamous cells were counted using a DME light microscope (Leica Microsystems, Wetzlar, Germany).

Identification of cell populations by flow cytometry

Cell suspension (100 μ l) was used for each FCM stain, conducted in 96 well plates. Cells were washed in FCM buffer (phosphate-buffered saline (PBS) containing 1% FCS, 0.01% sodium azide and 2 mM ethylenediaminetetraacetic acid (EDTA; both Sigma-Aldrich, Auckland, New Zealand; also for all subsequent wash steps), incubated with polyclonal IgG (Intragam, Commonwealth Serum Laboratories, Sydney, Australia) at 2 mg/ml (10 minutes,

4°C) to block non-specific binding, washed again, then labelled in a total volume of 25 µl for 25 minutes on ice. iNKT cells were stained with either PE-conjugated CD1d-tetramers loaded with α -galactosylceramide (a gift from Vincenzo Cerundolo, University of Oxford, UK), or PE-conjugated clone 6B11 antibody, which recognizes the CDR3 region of the invariant V α 24JaQ TCR chain. Other reagents used were anti-CD3-FITC, anti-CD19-PerCP and anti-CD45-APC (BD Biosciences). DAPI was added to allow exclusion of dead cells. FC analyses were conducted using a BD LSR II and FlowJo software, with a median of 115,892 (IQR 83,973-198,337) events analysed per sample. Preliminary experiments comparing 6B11-positive and tetramer-positive staining of peripheral blood-derived CD3⁺ lymphocytes showed strong correlation between these iNKT-specific reagents ($r^2=0.97$: Figure 5.1). They also showed that blood-derived iNKT cells could be successfully detected with either reagent when spiked into a sputum sample at less than 10 cells per 200,000 sputum cells (data not shown).

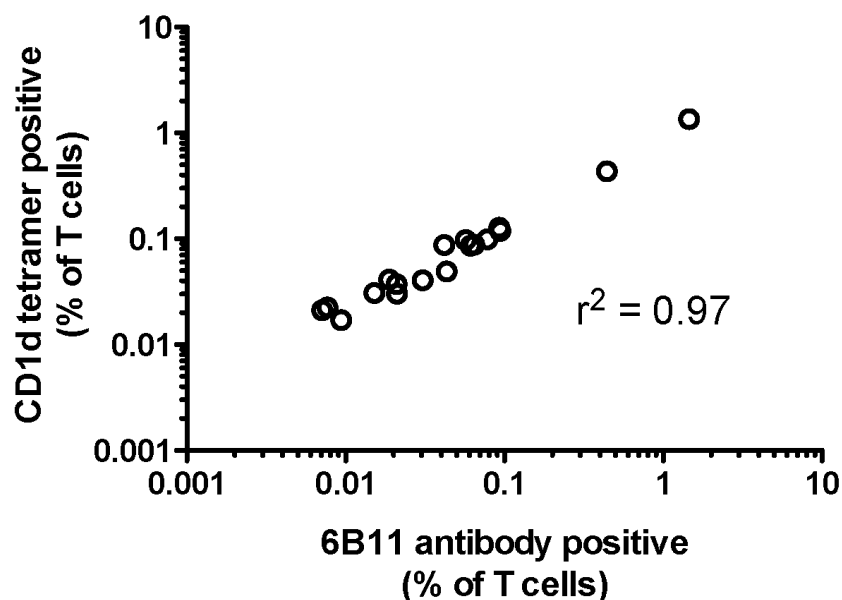


FIGURE 5.1. Correlation between blood-derived T cells identified as iNKT cells using 6B11 antibody or loaded CD1d tetramers

5.3 Results and discussion

The clinical characteristics of individuals successfully completing sputum induction (81.5%) are presented in Table 5.1.

TABLE 5.1. Clinical characteristics of participants who successfully completed sputum induction (expressed as number, percentage or median/IQR). Asthma classification based on GINA guidelines. P values calculated using the Mann-Whitney U test or Fisher's exact test.

	Asthma	Non-asthma	P value
Number (female)	25 (14)	19 (11)	
Asthma severity (% , No.)			
Intermittent	32% (8)		
Mild persistent	24% (6)		
Moderate persistent	44% (11)		
Age	28 (25.5-41)	33 (24-43)	0.8495
Atopy (% , No.)	80% (20)	52.6% (10)	0.1007
FEV ₁ (% predicted)	93 (82-98)	102 (93-111)	0.0062
FEV ₁ /FVC	0.8 (0.71-0.81)	0.85 (0.83-0.87)	0.0040
% sputum eosinophils	0.99 (0.24-3.67)	0 (0-0.46)	0.0008

Using the more stringent cytofluorimetric gating strategy we found little evidence of an increased presence of iNKT cells in asthmatics. In particular, in no sample did more than 0.57% of all T cells express the invariant V α 24-J α 18 TCR using either tetramer or 6B11, and no significant difference in iNKT cell frequency between asthmatics and non-asthmatics was found (0.07% (0-0.17%) versus 0.06% (0-0.20%); median (IQR) p=0.75; Figure 5.2A).

However, a reanalysis of our data using the gating strategy published by Matangkasombut *et al* (709) showed very different results, with up to 16.9% of CD3+SSC^{low} population now labelled tetramer or 6B11 positive in asthmatics. A similar increase in iNKT numbers was detected in non-asthmatics, such that (as with the previous gating strategy) no significant difference was observed between both groups (3.38% (1.07-5.71%) versus 2.29% (1.09-

3.35%); $p=0.18$; Figure 5.2B). Subsequent analysis after subdividing asthmatics on the basis of severity (see Table 5.1) or control also showed no statistical differences when using either gating strategy. Further examination of CD3⁺ tetramer/6B11⁺ events detected using the two-step strategy in both IS and peripheral blood confirmed that most were DAPI positive and therefore non-viable, and were smaller in size (FSC) than expected for viable lymphocytes (Figure 5.2C). Exclusion of these non-viable events led to detection of similar numbers of iNKT cells (less than 0.5% of CD3⁺SSC^{low} events) compared to the more stringent method. Using a similar approach for iNKT cell analysis we found equally discordant results for eighteen peripheral blood samples of non-asthmatic volunteers (Figure 5.2C).

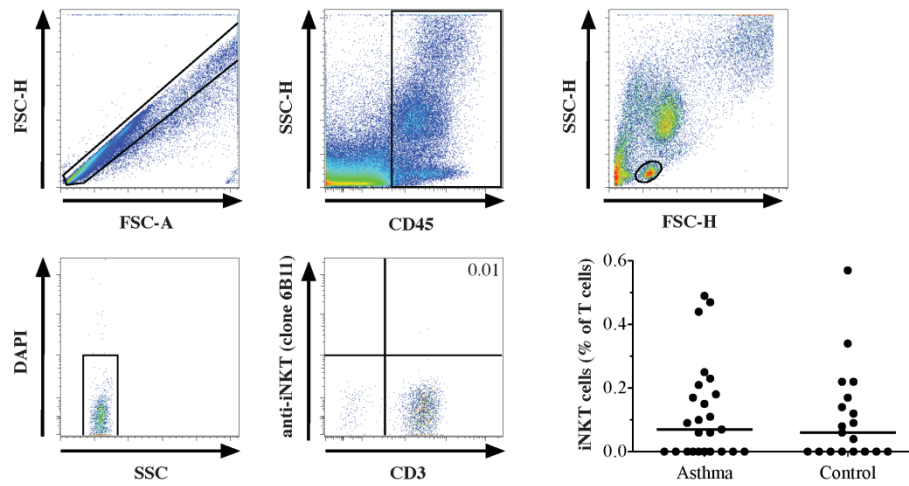
These results suggest that iNKT reagents bind in a non-specific fashion to non-viable cells. Suboptimal gating strategies may therefore lead to overestimation of iNKT cell numbers due to the misidentification of non-viable cells as iNKT cells. Our data also highlights the requirement for adequate precautions to be taken when using flow cytometry to detect any rare cell populations, not only iNKT cells, as non-specific binding to dead cells and debris may have significant effects on observed cell frequencies.

Our findings have several limitations and results should therefore be interpreted with caution. Firstly, the asthmatic population consists of mild-to-moderate asthmatics only, and results may be different for severe asthmatics, although five asthmatics in the present study (20%) had poorly controlled asthma. Secondly, cells were obtained from induced sputum and therefore have generally lower viability (68% in our study; no significant difference between population groups) than BALF. However, our experiments with peripheral blood (Figure 5.2C) show that reagent binding to dead cells and debris is still apparent in high-viability tissues. Thirdly induced sputum samples represent cell populations present in the central

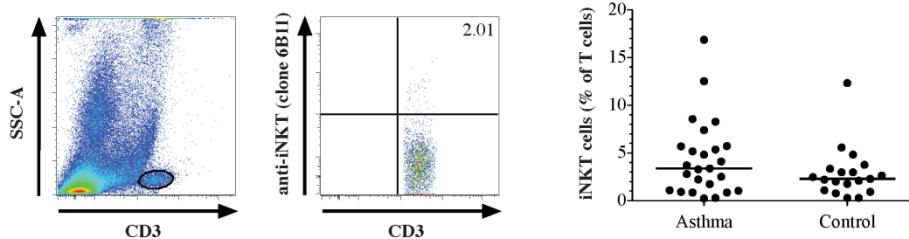
rather than distal airways. Finally, we conducted some of our experiments using the 6B11 antibody rather than the CD1d tetramer. However, preliminary experiments showed that this antibody gave comparable results to the tetramer (as previously reported (710)), and in many cases improved the quality of staining by reducing non-specific staining. The use of unloaded tetramers as controls as applied by others (706,709) could be perceived as a strength. However, loaded and unloaded tetramers may vary in their non-specific binding propensity, particularly if they do not come from the same lot or have been handled differently (711). Therefore, unless it can be demonstrated that this is not the case, there is a real possibility that the methodological issues described above are not adequately addressed by the use of unloaded tetramers.

In summary, our study emphasises the importance of stringent gating strategies in analysing iNKT cells and suggests that simple gating strategies may lead to false positive results possibly explaining the mixed evidence regarding the role of iNKT cells in asthma. Our study also further supports previous evidence (707,708) suggesting that iNKT cells are not increased in the central airways in mild-to-moderate asthma. Whilst we found no difference in iNKT cells between asthmatics and non-asthmatics we do not rule out the possibility, as suggested by animal models, that iNKT cells may play a role in at least some asthma phenotypes. However, until the methodological difficulties surrounding the detection of iNKT cells in lung-derived tissue are adequately addressed, the true importance of iNKT cells in the pathology of human asthma will remain unknown.

A: Stringent gating, induced sputum



B: Two-step gating, induced sputum



C: Gating comparison, peripheral blood

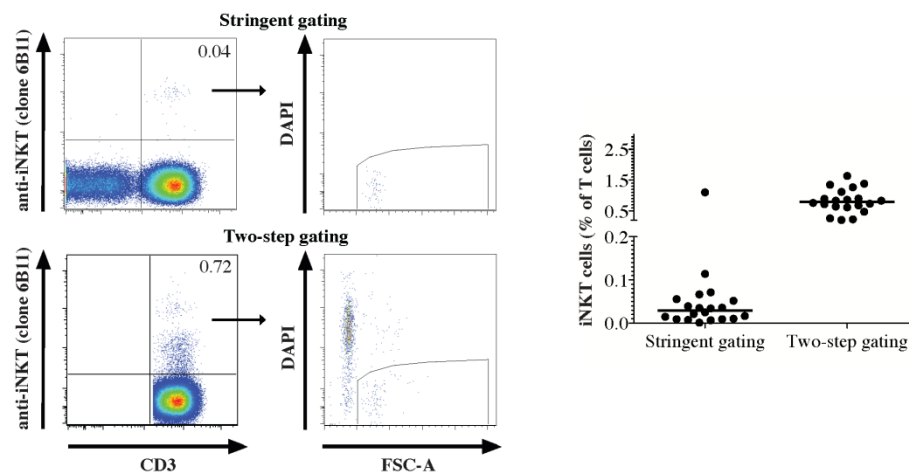


FIGURE 5.2. The effect of differential gating on the detection of iNKT cells. Dot plots showing a stringent gating strategy (A) and two-step strategy (B) in a representative sample, and the overall percentage of iNKT cells detected. Similar discrepancies were observed with peripheral blood samples (C). Median values are represented by a horizontal bar.

6. Sputum basophilia as an alternative TH₂-inflammatory biomarker in asthma

Collin R Brooks, Christine J van Dalen, Ian F Hermans, Jeroen Douwes

Background and objective: Airway eosinophilia is often used to identify asthma associated with TH₂-mediated pathology, but sputum eosinophil percentage is prone to variability and may be reduced by inhaled corticosteroid (ICS) treatment. In this study, we assessed sputum basophils using flow cytometry as an alternative marker of TH₂-mediated airway inflammation.

Methods: 16 asthmatic and 7 non-asthmatic adults underwent spirometry, skin-prick testing, exhaled nitric oxide (FENO) measurement and sputum induction using hypertonic saline. Twelve asthmatics provided a sample when their asthma was controlled (optimal treatment) and when their asthma control medication had been removed or reduced (suboptimal treatment).

Results: Sputum basophils were higher in asthmatics than non-asthmatics (suboptimally-treated asthma median 0.16% (IQR 0.1-0.47%); optimally treated asthma 0.18% (0.12-0.24); non-asthmatic subjects (0.02% (0.01-0.03); both $p < 0.05$). However, there was no significant difference between optimal and suboptimal treated asthma ($p = 0.15$). Basophil numbers correlated with sputum eosinophil numbers, as well as clinical parameters such as exhaled nitric oxide and lung function (all $p < 0.05$).

Conclusions: Basophil numbers in sputum could be assessed as an alternative biomarker of TH₂-inflammation in the airways in asthma, although further population-based studies are required to validate this.

In preparation for submission

6.1 Introduction

Eosinophilic airway inflammation is common in asthma (3). Assessment of eosinophil numbers in induced sputum (IS) can be used to guide therapy, as the eosinophilic asthma (EA) phenotype appears more responsive to inhaled corticosteroid (ICS) treatment (11,403). However, sputum eosinophils may be increased in non-asthmatic conditions, such as allergic rhinitis (712,713) or eosinophilic bronchitis (125) and show considerable longitudinal variability (496). In addition, sputum eosinophil numbers are often reduced by ICS treatment (28,29). This means that, depending upon the conditions, IS eosinophils in asthma may be undetectable or below arbitrary cutoffs at any given assessment, leading to asthma being erroneously identified as non-eosinophilic asthma (NEA). This asthma phenotype is less responsive to ICS and may have a different underlying pathology to that observed in TH₂-mediated eosinophilic disease (245).

Basophils are effector cells in the TH₂-mediated immune response. Although relatively rare in the periphery (<1% of blood leukocytes), they are capable of migration to sites of allergic inflammation (714). Basophils can be characterised by their expression of the high affinity IgE receptor (FcεR1) and upon cross-linking of this receptor, release a number of inflammatory mediators and TH₂ cytokines involved in allergic inflammation, including histamine, IL-4, IL-13 and leukotriene C4 (715). Previous studies of airway IS and biopsy samples have found that basophil numbers are increased in the airways of asthmatics and increase during asthma exacerbations (302) or after allergen inhalation challenge (295). Therefore they could potentially be used as an alternative marker of TH₂-mediated inflammation in asthma. Previous studies examining basophils in IS have been largely based on the morphological and metachromatic staining characteristics of these cells under light microscopy. This approach is subjective and, due to the extremely low number of basophils

observed (504), may be prone to error and underestimation of basophil numbers when compared with alternative approaches, such as flow cytometry.

We have recently developed and validated a flow cytometric approach to the assessment of IS in asthma (716), which allows us to detect rare inflammatory cell populations (692). In the current study, we used this method to assess sputum basophil numbers in asthmatic and non-asthmatic subjects. Furthermore, we examined the association between IS basophil and eosinophil differential counts and between basophils and clinical characteristics of asthma, including exhaled nitric oxide (FENO), % predicted forced expiratory volume in one second (FEV₁), and bronchodilator reversibility (BDR). Finally in a small group of asthmatics we determined if basophil numbers change as a result of alterations to ICS treatment.

6.2 Methods

Eighty-nine subjects were recruited as part of another study at the Centre for Public Health Research, Wellington (approved by the Lower South Regional Ethics Committee, New Zealand). All provided informed consent and underwent spirometry, skin-prick testing, exhaled nitric oxide (FENO) measurement and sputum induction using hypertonic saline (as described in Chapter 9). Of these subjects, 16 asthmatics (who were involved in a nested treatment-change study) and 7 non-asthmatics (selected at random) provided at least one IS sample with enough cells remaining for use in the current study. Twelve asthmatics successfully produced a sample when their asthma was optimally controlled and also when their asthma control medication had been removed or reduced (Chapter 9). Two asthmatics produced an adequate sample only during well controlled asthma, and the remaining two asthmatics produced a sample only during suboptimally controlled asthma.

Spirometry

Spirometric measurements were conducted for all participants according to American Thoracic Society (ATS) criteria (691) using an Easyone spirometer (NDD Medizintechnik AG, Zurich, Switzerland). Participants were pre-treated with 2400 µg salbutamol, and spirometry was performed before and 15 minutes after salbutamol. NHANES III equations (72) were used for lung function parameters.

Exhaled nitric oxide (FENO) measurement

FENO measurements were conducted according to ATS/ERS guidelines (13) using a Hypair FENO analyser (Medisoft, Sorinnes, Belgium) as previously described (717).

Sputum induction

Sodium chloride (Baxter Healthcare, Auckland, New Zealand; 4.5% w/v) vapour was produced using an ultrasonic nebuliser (DeVilbiss Ultraneb 2000), and administered orally over intervals for a total of 16 minutes as described previously (426). FEV₁ was obtained by spirometry at each interval. At the end of the session, participants were encouraged to produce a sputum sample into a sterile plastic container. The procedure was discontinued if the participant declined, or if a decline >15% FEV₁ could not be reversed by 200µg salbutamol administration during the procedure.

Sputum processing

Sputum samples were processed by plug selection (542). Induced sputum was incubated with dithiothreitol, filtered through a 60µM filter, and total cell count and viability (using trypan-blue exclusion) were determined using light microscopy. The sample was centrifuged (350 x g, 8 minutes) and the cell pellet resuspended in RPMI 1640 medium (Invitrogen, Auckland, New Zealand) containing 10% foetal calf serum (FCS, Invitrogen) and 1% penicillin-streptomycin (Invitrogen) at 1×10^6 cells/ml.

Identification of cell populations by differential cell count

Cell suspension (75 µl) was centrifuged in a Labofuge 400 cytospin column (Heraeus, Hanau, Germany) at 44 x g for 5 minutes. The slide produced was air dried, fixed, and stained with the Diff-Quik® fixative and stain set (Dade Behring, Deerfield, IL). 400 non-squamous cells were counted using a DME light microscope (Leica Microsystems, Wetzlar, Germany).

Identification of cell populations by flow cytometry

Cell suspension (100 μ l) was used for each FCM stain, and staining was conducted in 96 well plates. Cells were washed in FCM buffer (phosphate-buffered saline (PBS) containing 1% FCS, 0.01% sodium azide and 2 mM ethylenediaminetetraacetic acid (EDTA; both Sigma-Aldrich, Auckland, New Zealand; also for all subsequent wash steps), incubated with polyclonal IgG (Intragam, Commonwealth Serum Laboratories, Sydney, Australia) at 2 mg/ml (10 minutes, 4°C) to block non-specific binding, washed again, then labelled as previously described (716). Basophils were stained for 30 minutes on ice with antibody panels containing anti-CD45-APC-Cy7, anti-CD14-APC, anti-CD16-Alexa 700, anti-CD123-PE, anti-FcER1-FITC, and anti-HLA-DR-PerCP (all BD Biosciences, San Jose, CA; all titrated to optimal concentrations). Samples were washed, incubated with live/dead fixable blue dye kit (Molecular Probes; to identify live cells) on ice for 30 minutes, and then washed again. Labelled aliquots were resuspended in 150 μ l FACS buffer, and 150 μ l neutral buffered formalin (Sigma-Aldrich) was added to fix samples. Flow cytometric analyses were conducted using a LSR II flow cytometer (BD Biosciences), data were collected using FACSDIVA™ (BD Biosciences) and analysed using FlowJo software (Treestar Inc, Ashland, ORE).

Statistical analysis

Associations between sputum basophils and eosinophils, FENO, FEV₁-% predicted, BDR and FEV₁/FVC were assessed using Spearman's rank correlation. Differences between groups were assessed using the Mann-Whitney u-test, or Wilcoxon matched-pairs test for paired data. Multi-group comparison was conducted using the Kruskal-Wallis test with Dunn's post-test. All statistical analyses were conducted using Prism 5 software (Graphpad Software, La Jolla, CA).

6.3 Results

The baseline clinical characteristics of participants are presented in Table 6.1.

TABLE 6.1. Clinical and sputum sample characteristics of participants at baseline visit (expressed as number, percentage or median/IQR). P values calculated using the Mann-Whitney U test or Fisher's exact test.

	Asthma	Non-asthma	P value
Number (female)	16 (8)	7 (5)	
Age	39.5 (32.3-55.5)	40.0 (34.0-57.0)	0.710
Atopy % (No)	94% (15)	29% (2)	0.003
FEV ₁ (% predicted)	92.2 (86.7-98.7)	94.2 (86.4-96.6)	0.730
FEV ₁ /FVC	0.67 (0.63-0.72)	0.77 (0.74-0.83)	0.004
Bronchodilator reversibility (% change)	13.0 (5.3-16.2)	3.2 (1.7-4.8)	0.021
Sputum eosinophils %	2.2 (1.3-7.2)	0.0 (0.0-0.9)	0.0002
Sputum neutrophils %	35.7 (13.7-53.2)	37.5 (27.5-45.4)	0.816
Sputum cell viability %	78.1 (67.1-87.1)	87.6 (70.0-91.1)	0.19
Exhaled nitric oxide (ppb)	98.0 (45.8-165.0)	34.7 (32.0-46.6)	0.030

Basophils in IS were identified as a CD45^{dim}, non-debris/ SSC^{low}, live/dead blue^{low}, HLA-DR^{low}, CD16^{low}, CD14^{low}, CD123+, FceR1+ cell population, consistent with previous studies conducted with peripheral blood (718,719). Preliminary testing in peripheral blood showed a small leukocyte population conforming to these characteristics (Figure 6.1A). Representative gating of this cell population in IS from one asthmatic (6.1B) and one non-asthmatic subject (6.1C) is also shown. Basophil numbers (expressed as a percentage of viable CD45+ leukocytes in IS) were significantly higher in asthma, (suboptimally treated asthma median 0.16% (IQR 0.1-0.47%); optimally treated asthma 0.18% (0.12-0.24)) than in non-asthmatic subjects (0.02% (0.01-0.03); both p <0.05; Figure 6.1D). When optimal and suboptimally controlled asthma were compared, there was no statistically significant

difference in basophil numbers, however paired analysis suggested that there was a non-significant trend towards an increase in basophil percentage in suboptimally treated asthma ($p = 0.15$; Figure 6.1E).

To examine the relationship between basophils and other inflammatory parameters (sputum eosinophils and FENO) as well as clinical parameters (lung function), we conducted a Spearman's correlation analysis. When only data from asthmatics were assessed, we did not find a significant correlation between basophils and any of the other parameters measured, although there was a statistically non-significant association observed with eosinophils ($p=0.086$) and BDR ($p=0.17$). However when data for all participants (asthmatics and non-asthmatics) were analysed together, the percentage of sputum basophils significantly correlated with sputum eosinophils (Figure 6.1F), as well as exhaled nitric oxide (Figure 6.1G), bronchodilator reversibility (change in $FEV_1\%$ predicted after ventolin administration) (Figure 6.1H), $FEV_1\%$ predicted (Figure 6.1I), and FEV_1/FVC (data not shown) (all $p < 0.01$).

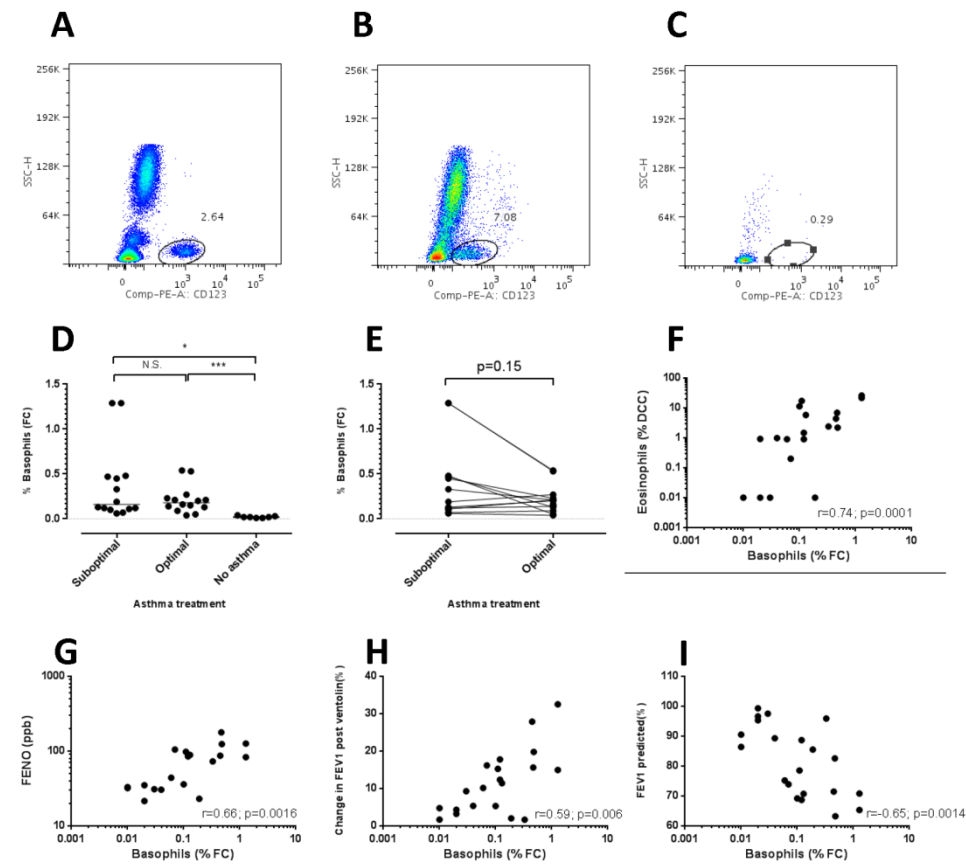


FIGURE 6.1. Assessment of basophils in induced sputum using flow cytometry. Dot plots showing gating representative plots (after sequential gating as described in Methods) in (A) peripheral blood, (B) 1 asthmatic and (C) 1 non-asthmatic. (D) Difference between basophils percentage in optimally-controlled asthma, suboptimally-controlled asthma and non-asthma (expressed as % viable leukocytes; line-median). (E) Paired data showing basophil percentages in patients when optimally and sub-optimally controlled. (F) Association between sputum percentage eosinophils (as measured by differential slide count) and sputum basophils for all subjects. (G) Association between exhaled nitric oxide (FENO) and sputum basophils for all participants. (H) Association between change in FEV1% predicted after ventolin administration (BDR) and sputum basophils for all patients. (I) Association between FEV1% predicted and sputum basophils for all patients.

6.4 Discussion

These results (using the relatively novel approach of assessing basophils in IS by flow cytometry) support previous reports showing increased airway basophils in asthma measured using more conventional means (295,302). In addition, we showed that basophil numbers in IS are statistically significantly associated with IS eosinophils and FENO, in addition to a number of lung function parameters, such as FEV₁-% predicted, FEV₁/FVC and BDR.

Although one previous study had observed that basophils may be associated with physiological alterations in the airways, such as AHR (295), to the authors' knowledge this is the first report showing an association with airway eosinophils, FENO, BDR and lung function.

To identify basophils, we used markers and gating strategies that have been previously documented. Monoclonal antibodies against CD123 (IL-3 receptor alpha) were used to identify plasmacytoid DCs and basophils. This marker is unaffected by atopy status (719), in contrast with surface IgE and FcEr1 expression. Excluding cells which express high levels of CD14, CD16 and HLA-DR, and subsequent inclusion of cells expressing FcEr1 (high affinity IgE receptor) allowed identification of basophils. To confirm this identification, the population identified as basophils were found to have lower SSC characteristics than eosinophils or mast cells, and identification of a corresponding cell population was clearly observed in blood, in agreement with previous reports (718,719,720,721).

The percentages of basophils we observed in IS were similar to previous reports using light microscopic assessment of basophils in IS (295,504). To to our knowledge only one previous study has measured basophils in IS using flow cytometry (722). This study used the markers CD63 and IgE to identify IS basophils, and observed that a significantly higher number of

basophils (between 0-15% of IS cells) could be observed in athletes (with or without asthma) compared with non-athletes. The high basophil percentages reported by Sastre *et al* contrast with our findings and those described previously, which suggested that basophils are present at less than 1% of IS leukocyte numbers, even during allergen challenge (295). There are a number of possible reasons for this discrepancy. Surface-bound IgE can be detected on various APCs including DCs and monocytes, eosinophils and mast cells, and its expression is affected by atopy status (723). Furthermore, up to 80% of cells positively labelled in peripheral blood using anti-IgE are non-basophils, suggesting it is unsuitable as a specific basophil marker (724). CD63 is a tetraspanin located on the membrane of the late endosome which relocates to the cell surface during granule secretion in a number of different leukocyte populations. CD63 expression can be seen clearly in activated basophils, and correlates with histamine release (725). As such it is measured as part of the basophil activation test (BAT) (726), but again it is not a specific marker of basophils. In addition, monoclonal antibodies may non-specifically bind to dead cells, leading to erroneous results if a viability dye is not used (692). As Sastre *et al* also report large numbers of bronchial epithelial cells (which in IS are generally identifiable as non-viable), it is possible that labelling of non-viable epithelial cells may be partly responsible for the high basophil numbers reported.

Our findings have some limitations. Firstly, this was preliminary work nested within a larger study (Chapter 9), and only a relatively small number of asthmatics and non-asthmatics recruited had adequate IS cells remaining for flow cytometric analysis. The relatively large number of cells required to investigate cell function in the larger study meant that only samples with relatively large cell yields could be used. This potentially adds selection bias; for example, larger sputum cell yields may possibly be associated with greater airway inflammation. However, our data shows that there is not a significant difference between

sputum total cell count between asthmatics and non-asthmatics, suggesting this is unlikely to be an issue.

Secondly, we were unable to unequivocally confirm the identity of the cell population assessed to be basophils on a morphological basis. Light microscopic assessment of the corresponding May Grunwald Giemsa stained slides did not provide clear evidence of basophil presence, possibly due to their very low frequency (less than median 0.18% of leukocytes in asthma), and the lack of use of a specific metachromatic dye, such as toluidine blue. Despite this, the basophil specific flow cytometric staining strategy we used has been well characterised in peripheral blood (719,720), and our experiments with peripheral blood showed a corresponding population that did not have the flow cytometric characteristics of similar leukocytes, such as eosinophils or mast cells. The use of an antibody specific for CD117 (also known as mast/stem cell growth factor receptor, or c-kit) may have allowed further distinction between mast cells (positive) and basophils (negative) (715), but we did not believe it necessary as the cells detected had lower SSC than mast cells. FACS sorting of an IS sample followed by appropriate staining and light microscopic assessment could have confirmed the presence of basophils, but was not available at the time of the study.

Finally, it remains unclear to what extent basophil numbers are reduced in optimally controlled asthma compared with suboptimally controlled asthma. Our experiments showed that median basophil numbers in asthmatics remained significantly higher than non-asthmatics with or without the use of ICS, suggesting that misclassification would not occur to the same degree as observed with eosinophils (28,29). However, in the absence of cut-off values from a reference population, this remains unconfirmed.

In conclusion, this preliminary study using flow cytometry has shown that basophils are increased in the sputum of asthmatics compared to non-asthmatics, that basophil number is associated with corresponding changes in other inflammatory and physiological parameters, and trends towards an increase in uncontrolled compared with controlled asthma.

Measurement of basophils in IS could therefore be conducted to assess TH₂ inflammation in asthma, and may be useful either as an alternative or additional biomarker of TH₂-mediated asthmatic inflammation. However it remains unclear as to whether basophils are more useful than eosinophil counts, and further studies are required to assess the stability of basophil numbers over time and in response to ICS treatment. Investigations of rare cell populations (such as basophils) during changes in asthma control may provide further insight into underlying inflammatory mechanisms in asthma.

7. Relationship between airway neutrophilia and ageing in asthmatics and non-asthmatics

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Background: Increased sputum neutrophilia has been observed in asthma, but also during normal ageing in asthmatics and non-asthmatics. It remains unclear what constitutes “normal” neutrophil levels in different age groups.

Methods: We assessed the relationship between age and airway neutrophils using data derived from studies of 194 asthmatics and 243 non-asthmatics (age range: 6yrs-80yrs). Regression analyses were used to assess the relationship between age and sputum neutrophils adjusted for confounders including asthma status, atopy, sex, smoking and current use of inhaled corticosteroids. Age-corrected reference values for different age groups were determined using the 95th percentile of non-asthmatic participants.

Results: Age was positively associated with sputum neutrophils in both asthmatic and non-asthmatic adults (0.46% neutrophil increase/year (95% CI 0.18, 0.73) and 0.44%/yr (0.25, 0.64) respectively), but no association was found in the <20yr age category. Individuals with high sputum neutrophil counts (>95th percentile of non-asthmatic subjects' counts for any given age group) were statistically significantly more likely to be asthmatic (OR=2.5; 95% CI 1.3, 5.0), with the greatest effect observed in the older age group. Other factors that independently contributed to increased sputum neutrophil levels included atopy in non-asthmatic adults, male sex and current use of ICS in asthmatic adults. Age-specific reference values for neutrophil percentage were: under 20 years-76%, 20-40 years-62%, 40-60 years-63%, over 60 years-67%.

Conclusions: Airway neutrophilia is associated with age in adults, with a neutrophilic asthma phenotype present in older adults. The use of appropriate age-specific reference values is recommended for future studies aimed at elucidating the role of neutrophils in asthma.

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7.1 Introduction

Whilst asthma is classically characterised by eosinophilic airway inflammation, increased neutrophil numbers have been shown in fatal status asthmaticus (727), occupational asthma (380) and during severe asthma exacerbations (728). Moreover, airway neutrophilia is evident in asthmatics in the absence of eosinophilia, and is associated with both decreased lung function and poor response to ICS (384,420). Nonetheless, the importance of neutrophils in the immunopathogenesis of asthma remains largely unclear.

A critical requirement for identifying increased airway neutrophilia is to determine what “normal” neutrophil numbers are. Some studies designed to provide reference values for differential leukocyte counts in induced sputum included few older subjects (303,304) limiting their applicability. Recent studies in both asthmatics (729,730) and non-asthmatics (457,731) have shown that differential sputum neutrophils increase with advancing age. This is particularly relevant when comparisons are being made between different age groups. For example, some COPD studies showed increased neutrophilic airway inflammation compared with controls (471,732). However, the reference population was significantly younger than the study population, and part of the difference in neutrophil levels may therefore have been explained by age differences alone. Similarly, we have proposed four inflammatory asthma phenotypes, with two phenotypes classified based on a fixed cut-point of >61% sputum neutrophils (designated neutrophilic asthma and mixed granulocytic asthma). However, those with high neutrophil levels were significantly older than those with neutrophil levels <61% (eosinophilic and paucigranulocytic asthma) (12). Thus, at least part of the difference in neutrophils observed between both phenotypes may be due to age. Nonetheless, there is evidence that neutrophil increases are associated with persistent airway obstruction in asthmatics even after adjusting for age (733), and also with an increase in sputum levels of

neutrophil products implicated in airway remodelling, such as neutrophil elastase, IL-8, and MMP-9 (462,730,734). This suggests that neutrophils may play an active role in asthma pathogenesis.

To date the association between age and airway neutrophils has only been reported in adults with no studies including children and adolescents. In this study we examined the relationship between age and airway neutrophils in a large population of asthmatic and non-asthmatic adults and children, and assessed which other characteristics predict airway neutrophilia. Furthermore, we determined age-specific reference values for sputum neutrophils to avoid misclassification of the neutrophilic asthma phenotype, particularly in older populations.

7.2 Methods

Study population

This study involved a retrospective assessment of cross-sectional data obtained from 264 non-asthmatics and 231 asthmatics recruited for studies at either Massey University, Wellington, New Zealand (692,735), or the John Hunter Hospital, Newcastle, Australia (12,83,243,478,551), between 1995 and 2010. Recruitment was conducted either by advertisement, from the Respiratory Ambulatory Care Service at the John Hunter Hospital, or for many adolescents studied (n=145) from five schools that had participated in the ISAAC Phase III survey (736). Studies were approved by the appropriate ethics committee, and all participants (or children's parents) provided written informed consent. Participants underwent a similar core protocol involving detailed physical and questionnaire-based assessment of smoking, medication and respiratory health status, spirometry, skin prick testing and sputum induction. Sputum was analysed for inflammatory cell counts. Subjects with asthma were asked to withhold their rescue medications for at least 6 hours (short-acting β -agonist) or 24 hours (long-acting β -agonist) prior to evaluation. Participants who had a respiratory infection within four weeks were asked to return at a later date. Assessments were conducted by a respiratory physician. Current smokers and individuals with evidence of other respiratory morbidities were excluded from further study.

Asthma definition used

Asthma was defined as physician-diagnosed asthma, with asthma symptoms and/or asthma medication use within 12 months (736). Non-asthmatics reported no asthma symptoms, asthma medication use, no diagnosis or evidence of respiratory disease and had normal

spirometric results. In the Australian studies, physician's diagnosis of asthma was confirmed by demonstrated airway-hyperreactivity to hypertonic saline.

Skin prick testing

Skin prick testing was conducted using a panel of aeroallergens. For Australian studies these included *Aspergillus fumigatus*, *Alternaria tenuis*, HDM (*Dermatophagoides pteronyssinus*), Cockroach mix and grass mix (Bayer Australia Ltd, Pymble, NSW, Australia). For New Zealand studies allergens used were HDM, tree mix, grass mix, cat and dog dander, *Alternaria tenuis* and Penicillium mix (Hollister-Stier Laboratories, Spokane, WA, USA). Positive atopy status was determined as the presence of at least one weal >3mm. Histamine and saline were used as positive and negative controls respectively. Atopy was not assessed in 37 of the non-asthmatics in the Australian studies.

Spirometry

Spirometric measurements were conducted according to American Thoracic Society (ATS) criteria (691), using a spirometer from either KoKo PD Instrumentation (Louisville, CO, USA) (Australia) or Easyone spirometer (NDD Medizintechnik AG, Zurich, Switzerland) (New Zealand). NHANES III equations were used for lung function parameters.

Sputum induction, processing and analysis

Sputum induction (combined with bronchial provocation assessment to determine PD 15), sputum processing, and differential and total cell enumeration were conducted as described previously (426), except in one New Zealand study (692), in which participants (n=54) were

pre-medicated with a β -2 agonist (salbutamol) prior to sputum induction as part of reversible airway obstruction assessment.

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 described as median and interquartile ranges (IQR) unless stated. The population was stratified into either adults (>20 years), children/adolescents (<20 years); or quartiles on the basis of 20-year age categories. Univariate analyses were conducted using Mann-Whitney u-testing, linear regression or Spearman's correlation. Fisher's exact test was used for categorical data, and Kruskal-Wallis with Dunn's post test analysis was used for multiple group comparison. Multiple linear regression analyses were performed to assess associations between sputum neutrophils and age whilst controlling for potentially confounding variables. Variables were selected for multivariate analysis if they showed an association ($p < 0.10$) with sputum neutrophil percentage, or if these variables were previously shown to be related to neutrophils, and depending upon the model included gender, previous smoking status, asthma, atopy and use of inhaled corticosteroids (ICS). Logistic regression analyses were used for dichotomous outcomes with adjustment for potential confounders (age, gender and previous smoking).

7.3 Results

From a total of 495 individuals (264 non-asthmatics, 231 asthmatics), 437 (243 non-asthmatics, 194 asthmatics) were included in the final analyses. Participant exclusion was due to an inadequate sputum sample (no sample or excessive contamination with squamous epithelial cells) (n=21; 9 asthma, 12 no asthma), current smoking (n=36; 27 asthma, 9 no asthma) or unrecorded asthma status (n=1). Demographics and clinical characteristics are described in Table 7.1.

Sputum neutrophil percentage and absolute neutrophils/ml of sputum increased with increasing age (Table 7.1), which equated to 0.27%/year (95% CI: 0.17, 0.38; $p < 0.0001$) (Figure 7.1A) for all participants. For asthmatics and non-asthmatics separately these estimates were 0.37%/year (95% CI: 0.20, 0.53; $p < 0.0001$) and 0.19%/year (95% CI: 0.05, 0.32; $p = 0.09$) (Figure 7.1B). Exclusion of the <20yr group, which showed considerable variation and a non-significant reduction in neutrophil percentage per year (Figure 7.1C), suggested that there was no difference in estimated change of neutrophil percentage per year when all adults were analysed (0.47%/year (95% CI: 0.31, 0.63)) or divided into asthmatics (0.46%/year (95% CI: 0.18, 0.73)) and non-asthmatics (0.44%/year (95% CI: 0.25, 0.64)) (all $p < 0.0001$) (Figure 7.1D). Similarly no difference was observed when adults were stratified into males (0.46%/year (95% CI: 0.17, 0.75) and females (0.45%/year (95% CI: 0.25, 0.64), or if previous smokers were excluded from analyses (data not shown). Significant associations were also observed when neutrophils were considered as absolute number per ml/sputum rather than percentages, but both univariate analyses and analyses controlling for potential confounders showed weaker associations than when using percentages (data not shown)

TABLE 7.1. Demographics and sputum analysis data stratified on the basis of age, expressed as median (IQR) or number (percentage) unless stated. (TCC/ml=total non-squamous cells per ml sputum, $\times 10^6$. Viability=percentage viability of non-squamous cells. TN= total neutrophils $\times 10^4$ per ml of sputum. Mann-Whitney test ***=p<0.0001, **=p<0.01, *=p<0.05 compared to corresponding non-asthmatic group)

	Group									
	Less than 20yrs		20-40yrs		40-60yrs		Greater than 60yrs		ALL	
	No asthma	Asthma	No asthma	Asthma	No asthma	Asthma	No asthma	Asthma	No asthma	Asthma
Number	111	72	59	34	41	40	32	48	243	194
Age	15.0 (12.8-16.1)	15.8 (15.0-16.6)	27.6 (23.2-34.2)	27.7 (24.8-35.3)	50.6(43.3-55.2)	52.5 (47.7-56.4)	65.6 (62.3-70.2)	66.12 (66.2-70.1)	22.5 (15.1-43.54)	34.57 (16.3-59.9)***
Sex (female/male)	42/69	13/59**	46/13	22/12	29/12	26/14	15/17	28/20	132/111	89/105
FEV ₁ % predicted	101.7 (93.2-107.4)	94.7 (86.4-103.2)***	102.6 (96-111.3)	94 (83.5-104.4)***	103.7 (93.9-114)	75 (53.1-85.6)***	100.4 (92.6-111.7)	71.4 (54.5-87.1)***	102.3 (94.1-110.4)	86.2 (73-97.2)***
FEV ₁ /FVC (%)	81.9 (75-87.5)	81.4 (75.5-84.8)	85.9 (83-88.3)	79.9 (71.2-82)***	82 (79.1-85)	68 (57.3-76)***	78 (74-80)	65 (56.1-73)***	82 (76.6-87)	75.2 (66.2-81.9)***
Smoking status(prev%)	0	0	9 (15.3)	5 (14.7)	12 (29.3)	5 (12.5)	18 (56.3)	14 (29.2)	39 (16.1%)	24 (12.4%)
ICS use (%)	-	28 (41.8)	-	24 (70.6)	-	34 (85)	-	43 (89.6)	-	129 (66.5)
Atopy/ no atopy (%)	43/37 (53.8)	60/12 (83.3)***	23/31 (42.6)	31/3 (91.2)***	21/19 (52.5)	25/15 (62.5)	8/24 (25)	38/10 (79.2)***	95/111 (46.12)	154/40 (79.4)***
TCC/ml	2.79 (1.4-5.1)	4.0 (1.6-8.2)	1.7 (1.3-2.4)	1.8 (1-4.1)	1.9 (0.9-2.7)	2.9 (1.2-7.4)*	2.0 (0.9-3.8)	2.5 (1.3-6.8)	2.1 (1.3-4.1)	2.8 (1.3-6.8)**
Viability (%)	78 (68-88)	79.5(70.7-89.1)	71.4 (56.4-78.9)	63.6 (55.1-78.7)	66.7 (50-85)	72.1 (58-82.7)	79.3 (66.9-91)	81.7 (66.5-88.9)	75 (61.1-86.7)	75 (60.1-87.1)
% Neutrophils (%N)	21.2 (11.5-44.1)	16.1 (5.8-33.9)	18.3 (10.1-30.4)	25.3 (15.4-32.8)	29.7 (20.4-51.4)	29.1 (16.1-52.1)	38.6 (23.1-58.2)	35.4 (15.2-67.1)	26.3 (12.6-42.9)	24.2 (12.2-45.4)
95 th percentile (%N)	75.57	89.46	61.61	70.34	63.25	79.63	67.25	94.75	69.44	86.5
Neutrophils $\times 10^4$ ml (TN)	57.2 (13.3-154.5)	42.1 (10.2-182.2)	36.3 (14.7-58.4)	35.3 (18.8-94)	40.9 (18.7-111.8)	86.7 (29.1-224.1)	56.41 (28-217.1)	93 (29.1-357.1)	45.9 (17.2-117.6)	56 (21-183.9)*
95 th percentile (TN)	735	1751	162	589	319	1587	442	931	461	1047
% Eosinophils	0 (0-0.6)	1.31 (0-8)***	0 (0-0.3)	1.71 (0.4-3.8)***	0 (0-0.3)	1.3 (0-4.5)***	0.3 (0-0.5)	1 (0.1-2.4)***	0 (0-0.5)	1.3 (0.3-4)***
Eosinophils $\times 10^4$ ml	0 (0-2.2)	2.6 (0-47.8)***	0 (0-0.6)	2.5 (0.5-10.1)***	0 (0-0.4)	4.8 (0.1-20.8)***	0.4 (0-1.1)	1.6 (0.1-9.8)**	0 (0-1)	2.6 (0-16.3)***

TABLE 7.2. Multiple linear regression models with sputum neutrophil percentage as dependent variable (unstandardised co-efficients). All individuals – model adjusted $R^2=0.09$; $P<0.0001$. All adults - model adjusted $R^2=0.15$; $p<0.0001$. Individuals<20yrs – model adjusted $R^2=0.01$; $p<0.28$. All non-asthmatic adults – model adjusted $R^2=0.16$; $p<0.0001$. All asthmatic adults – model adjusted $R^2=0.13$; $P<0.002$. (*Number of participants with observations available for variables included)

Variable	All Individuals (n=395*)		Individuals<20yrs (n=147*)		All adults (n=248*)		Non-asthmatic adults (n=126*)		Asthmatic adults (n=106*)	
	Neutrophil change (%) per variable unit (95% CI)	p	Neutrophil change (%) per variable unit (95% CI)	p	Neutrophil change (%) per variable unit (95% CI)	p	Neutrophil change (%) per variable unit (95% CI)	p	Neutrophil change (%) per variable change (95% CI)	p
Age	0.33 (0.2,0.47)	<0.001	3.14 (-0.66, 6.94)	0.11	0.43 (0.26,0.61)	<0.001	0.48 (0.26,0.69)	<0.001	0.40 (0.021,0.76)	0.04
Sex	-3.86 (-8.56,0.84)	0.11	-3.01 (12.36,6.34)	0.53	-3.88 (-9.61,1.83)	0.18	3.67 (-3.43,10.77)	0.31	-13.96 (-24.57, -3.36)	0.01
Smoking status (never/previous)	-0.22 (-7.06,6.61)	0.95	-	-	0.59 (-5.77,6.95)	0.85	3.21 (-4.41,10.83)	0.41	-1.25 (-14.04,11.54)	0.85
Atopy (yes/no)	1.18 (-3.87,6.23)	0.65	-6.68 (-15.85,2.49)	0.15	5.63 (-0.23,11.48)	0.06	6.56 (0.07,13.05)	0.05	3.94 (-8.17,16.05)	0.52
Asthma (yes/no)	-2.65 (-9.46,4.16)	0.44	-0.57 (-10.49,9.35)	0.91	-9.37 (-19.38,0.63)	0.07	-	-	-	-
ICS use (yes/no)	4.68 (-2.84,12.20)	0.22	-4.60 (16.74,7.55)	0.46	12.50 (2.09,22.90)	0.02	-	-	20.38 (3.93,36.84)	0.02
Constant	22.89 (14.00,31.77)	<0.001	-14.37 (-74.28,45.54)	0.64	14.53 (0.28,28.78)	0.05	-1.40 (-18.56,15.76)	0.87	12.83 (-15.17,40.83)	0.37

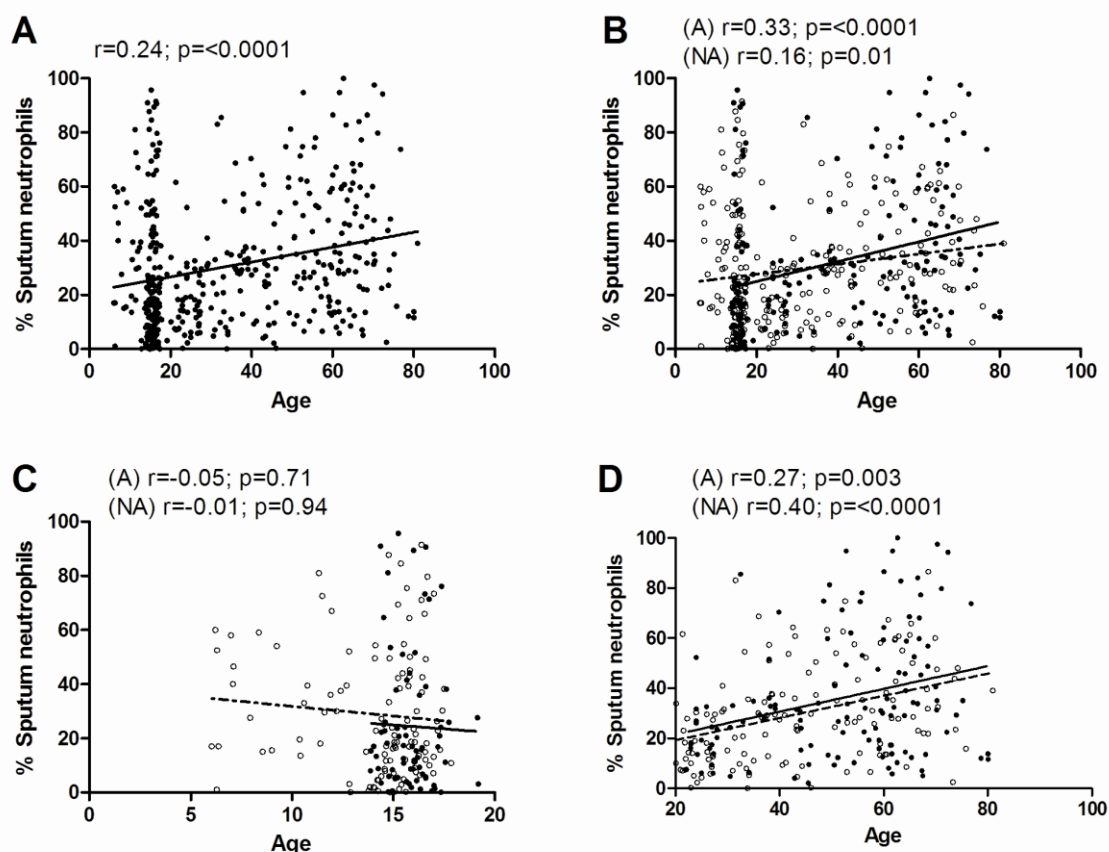


FIGURE 7.1. Correlations and linear regressions of sputum neutrophil percentages against age for: all study participants (A), all study participants divided on the basis of asthma/ no-asthma (B), participants under 20 yrs divided on the basis of asthma/no-asthma (C), and all adult participants divided on the basis of asthma/no-asthma (D). (For B,C and D, filled circle/solid line= asthma, empty circle/dashed line= no-asthma) (A=asthmatic, NA=non-asthmatic)

In support of previous findings (733), we observed in univariate analysis that neutrophil percentage was inversely associated with a reduction in both % predicted FEV₁ and FEV₁/FVC. This was particularly significant when only adults were included in analysis (Spearman's $r = -0.21$; $p = 0.0007$ and $r = -0.26$; $p = <0.0001$ respectively).

Controlling for asthma status, atopy, sex, smoking and current ICS use in multivariate analysis did not significantly alter the observed association between neutrophil percentage and age in adults (Table 7.2). In addition to age, factors independently associated with

increased neutrophils in multivariate analysis included atopy in non-asthmatic adults ($p=0.05$), and male sex and current use of ICS in asthmatic adults ($p=0.01$ and 0.02 respectively; Table 7.2). In a multivariate model including all adults, asthma was associated with a lower percentage of sputum neutrophils, although this did not reach significance ($p=0.07$; Table 7.2).

There was no significant difference in neutrophil percentages within age groups between asthmatics and non-asthmatics (Table 7.1, Figure 7.2), although the asthma group had increased total cell counts as well as slightly significantly higher absolute numbers of neutrophils (Mann-Whitney u-test; $p=0.046$; Table 7.1).

Interestingly, despite the lack of difference in neutrophil percentages between asthmatics and non-asthmatics, those with high sputum neutrophil differential counts (greater than 95th percentile of non-asthmatic values for any given age group) were significantly more likely to be asthmatic, with the strongest association for the oldest age group (Table 7.3). When using cut-off values derived from 95th percentile of absolute neutrophils (rather than neutrophil percentage), we found a similar association (Table 7.4).

TABLE 7.3. Number of individuals with >95th percentile of age-specific reference value of percentage sputum neutrophils/number of individuals with <95th percentile per age group (95th percentile=75.57% for those <20yrs, 61.61% for 20-40yrs, 63.2% for 40-60 yrs and 67.25% for those >60yrs) (adj: adjusted for age, sex and smoking, * = $p<0.05$, **= $p<0.005$)

Age-group	Asthma	No asthma	OR (95%CI)	Adj OR (95%CI)
<20yrs	6/66 (8.3%)	6/106 (5.4%)	1.59 (0.49-5.14)	1.40 (0.40-4.93)
20-40yrs	2/32 (5.9%)	3/56 (5.1%)	1.17 (0.19-7.35)	1.35 (0.18-9.94)
40-60yrs	6/34 (15%)	3/38 (7.7%)	2.24 (0.52-9.64)	2.03 (0.43-9.52)
>60yrs	12/36 (25%)	2/30 (6.25%)	5.00 (1.04-24.12)*	4.22 (0.84-21.01)
All	26/168 (13.4%)	14/229 (5.8%)	2.53 (1.28-4.99)**	2.12 (1.04-4.29)*

TABLE 7.4. Number of individuals with >95th percentile of age-specific reference value of absolute number of sputum neutrophils/number of individuals with <95th percentile per group (95th percentile=735x10⁴/ml for those <20yrs, 162x10⁴/ml for 20-40yrs, 319x10⁴/ml for 40-60 yrs and 442x10⁴/ml for those >60yrs) (adj: adjusted for age, sex and smoking, * = p<0.05, **=p<0.005)

Age-group	Asthma	No asthma	OR (95%CI)	Adj OR (95%CI)
<20yrs	4/45 (8.2%)	5/92 (5.2%)	1.26 (0.47-3.37)	0.94 (0.37-5.94)
20-40yrs	5/27 (15.6%)	2/52 (3.7%)	3.63 (0.32-41.54)	7.21 (0.37-141.73)
40-60yrs	9/29 (23.7%)	1/33 (2.9%)	7.06 (0.81-61.56)	4.82 (0.51-45.99)
>60yrs	11/37 (28.9%)	1/29 (3.3%)	8.16 (0.99-67.26)	6.83 (0.79-58.91)
All	29/138 (17.4%)	9/206 (4.2%)	2.74 (1.37-5.49)**	2.27 (1.10-4.67)*

Although 61% has been previously been determined as the 95th percentile of airway neutrophil percentage in non-asthmatic populations (and thus indicative of neutrophilic inflammation), an overall 95th percentile value for sputum neutrophils in non-asthmatics of 69.43% was observed. Using a similar 95th percentile cut-off approach, we observed age-specific reference values of 75.57% for those <20yrs, 61.61% for 20-40yrs, 63.2% for 40-60 yrs and 67.25% for those >60yrs (Table 7.1, Figure 7.2).

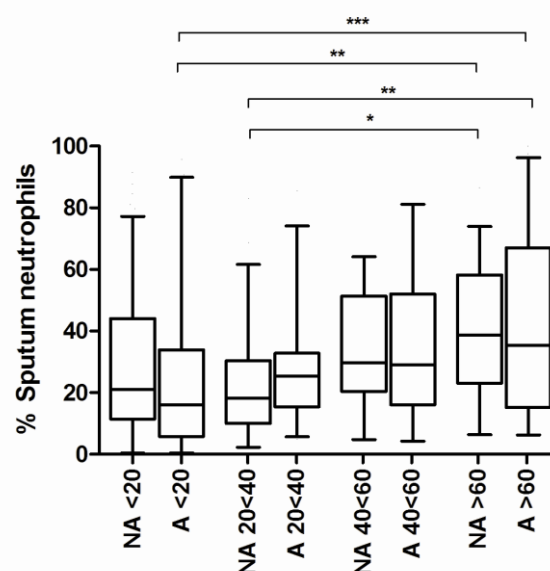


FIGURE 7.2. (previous page). Distribution of sputum neutrophil percentages for asthmatics/non-asthmatics in different age groups. (Line= median, box=IQR, whiskers=5 to 95th percentile values; * $p=0.01$ to 0.05 , ** $p=0.001$ - 0.01 and *** $p<0.0001$) (A=asthmatic, NA=non-asthmatic)

7.4 Discussion

In this cross-sectional study we found a positive association between age and sputum neutrophils in both asthmatic and non-asthmatic adults. Overall, there was little difference in sputum neutrophil levels between asthmatics and non-asthmatics within age groups, although individuals with high sputum neutrophil differential counts (greater than 95th percentile) were significantly more likely to be asthmatic, with this effect being most pronounced for older age groups.

To the authors' knowledge, this is the first report comprehensively comparing this association in both asthmatics and non-asthmatics in a large population, ranging from childhood to old age. Our findings are consistent with those previously described separately for asthmatics and non-asthmatic adults. For example, Thomas *et al* reported a significant correlation ($r=0.58$; $p<0.001$) between sputum neutrophils and age in healthy volunteers ($n=66$) (457), whilst in a study investigating older (50-70yr; $n=6$) and younger (20-40yr; $n=12$) asthmatics (734), a trend was shown for an increased percentage of sputum neutrophils in the older group, and a corresponding increase in IL-8, MMP-9 and neutrophil elastase (730). The correlation observed for adults in our study ($r=0.35$; $p<0.0001$) was not as strong as that reported by Thomas *et al*, but was similar to those observed previously in asthmatics ($r=0.27$ to $r=0.38$) (28,729,737). To the authors' knowledge, no studies reporting on age and neutrophil levels in children are available.

Sex and ICS use had a significant effect on sputum neutrophil differential independent of age in asthmatics, whilst atopy had a significant effect on neutrophil percentage in non-asthmatics. In a recent longitudinal study ICS use was shown to increase sputum neutrophils in asthmatics (28), possibly through mechanisms leading to impairment of apoptosis (738).

Woodruff *et al* (729) and Shaw *et al* (733) have previously established that decreased lung function was independently associated with sputum neutrophil percentage, alongside age, male sex, ethnicity and use of ICS, although to our knowledge, a direct relationship between male sex and sputum neutrophils has not previously been described. A similar gender effect was observed with serum MMP-9 levels (produced by neutrophils), which were significantly increased in elderly men (not women) with decreased FEV₁ (739). However, the aforementioned study did not specifically assess this in asthma, or determine if it was associated with neutrophilic airway inflammation. The association between atopy and airway neutrophilia in non-asthmatic adults was unexpected. However, one previous cross-sectional study comparing food allergy patients (without respiratory symptoms), asthmatics and controls found a significantly increased proportion of airway neutrophils in the food allergy patients (740), suggesting that allergic/atopic mechanisms may be associated with subclinical neutrophilic airway inflammation. It is unclear if this is related to food allergy specifically, or atopy in general.

A 61% cut-off value has been commonly used to define “normal” neutrophil numbers, and is based on the 95th percentile value of sputum neutrophil percentages in mixed-age healthy populations (12). Other studies reporting sputum neutrophil reference values examined relatively young groups (mean age 34-38yrs) (303,304). As shown in the current study airway neutrophil levels are dependent on age and the previous 61% cut-off may not be entirely appropriate for some groups, particularly the very young or very old. The 95th percentile of neutrophil differential from non-asthmatic subjects in discrete 20 year age groups as determined in the current study (under 20yrs-75.57%, 20 to 40yrs- 61.61%, 40-60yrs- 63.25% and over 60yrs- 67.25%) may guide the development of more valid age-specific reference values.

There were some limitations with this study. Firstly, it was cross-sectional, with only one assessment per participant. The variation we observed in each age group might be due, at least in part, to assessing individuals when they have particularly high or low neutrophil numbers, as intra-individual variation in sputum neutrophil percentages has previously been reported in longitudinal studies (29,496,498). Nonetheless, this would contribute to random error (independent of age) and is therefore unlikely to explain the specific trends with age which we and others have shown.

Due to analysing data from several smaller studies, there were relatively small numbers in some age groups and limited age variance in the youngest group in particular. Furthermore, individual studies that provided data for this study were conducted over several years in two different centres, and it is possible that there may be some variation due to slightly different procedures. However, training and sputum processing methodology was identical in both centres. The definition of asthma used (physician diagnosis) was the same in both centres, and although BHR measurement was used to confirm diagnosis only in Australia, the questionnaire-based approach used in New Zealand has previously been validated against BHR (146). We were unable to control for the different centres due to different demographic groups studied (predominantly younger and male in New Zealand, and older in Australia). Although we adjusted for gender in multivariate models, and observed no significant differences in univariate analysis when we stratified the population into males and females, we cannot exclude the possibility that the significantly higher number of males in the youngest age group compared to other groups may have affected our results. Furthermore we were unable to assess asthma severity, bronchodilator response or number of exacerbations for all asthmatic participants, due to study variations and the current use of ICS in the

majority (66.5%) of asthmatics studied. Finally, it is possible that some of the older asthmatic subjects may have asthma/COPD overlap, as although individuals with a diagnosis or evidence of COPD were excluded at assessment, the component of irreversible airway obstruction in some elderly asthmatics is well characterised (486). Despite this, when we conducted analyses excluding participants with FEV1/FVC ratio of less than 0.7 (based on Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) guidelines), we observed a similar significant association between neutrophilia and ageing (data not shown).

There are various immunological changes observed with ageing in the airways (479), and these occur in parallel with structural and physiological changes, such as reduced elastic recoil of the lungs and increased alveolar size (741). It is currently unclear as to whether increased neutrophilia is actually a cause or effect of this process, previously described as “inflamm-ageing” (742). There is evidence that increased neutrophilia may occur in response to bacterial colonisation in asthma (458) and COPD (743), and macrolide antibiotics have been shown to reduce both IL-8 levels and neutrophil numbers in severe refractory asthma (243). Although it remains unclear as to exactly how important airway neutrophilia is in some types of asthma, the consistent relationship between airway obstruction, age and airway neutrophilia observed in this study and previously (729,733,737,744), especially in older asthmatic populations warrants further investigation. Furthermore, we cannot explain the considerable variation, and lack of association observed between neutrophil percentage and age in the youngest age group. Indeed, inclusion of this group weakened the association between airway and neutrophilia and ageing observed when adults were analysed alone. We believe this requires further attention.

In conclusion, this study showed similar increases in sputum neutrophil percentages with ageing in both asthmatics and non-asthmatic adults, whilst the under 20 year old age group showed substantial variation and no clear relationship with age. Our study suggests that appropriate age-adjusted reference values may be useful when describing respiratory diseases characterised by airway neutrophilia.

Acknowledgements

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8. Non-eosinophilic asthma in children

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Background: Neutrophilic inflammation has been implicated in non-eosinophilic asthma (NEA) in adults, but relatively little is known about NEA in children.

Aim: To assess the prevalence and both clinical and inflammatory characteristics of NEA and, in particular, to determine the importance of neutrophilic airway inflammation in childhood asthma.

Methods: Airway inflammation (assessed by analysis of induced sputum), sputum endotoxin, airway hyperreactivity, atopy (skin prick testing) and lung function were measured in 68 non-asthmatics and 77 asthmatic children (aged 12 – 17 years). Respiratory symptoms were assessed using a validated questionnaire.

Results: The proportion of NEA (defined as sputum eosinophil levels $<2.5\%$) was 54%. In this group, atopy, sputum neutrophil, eosinophil, ECP, endotoxin, neutrophil elastase (NE) and IL-8 levels were not statistically significantly different from non-asthmatics. In contrast eosinophilic asthma (EA) was associated with statistically significantly elevated sputum ECP and IL-8 levels, and was more strongly associated with atopy. The majority of NEA had no evidence of any detectable inflammation and only 14% of NEA had sputum neutrophilia $\geq 61\%$, compared with 11% of EA and 15% of non-asthmatics. Small and non-statistically significant differences in FEV₁ were found between EA and NEA; no significant differences in symptom prevalence and severity were found (83% of EA and 79% of NEA were classified as moderate to severe asthmatics respectively), although there was a trend toward increased severity in EA (49% classified as severe compared with 33% in NEA).

Conclusions: NEA is very common among asthmatic children. Despite differences in inflammatory profile, EA and NEA appear have to similar clinical characteristics. Neutrophils do not appear to play a major role in NEA in children and the underlying inflammatory mechanisms of NEA remain unclear.

In preparation for submission

8.1 Introduction

Until recently, asthma has generally been regarded as an allergic disease involving TH₂-mediated eosinophilic airways inflammation (3). However, there is increasing evidence that non-allergic inflammatory mechanisms may also be important (36). We have previously proposed that, at most, only 50% of asthma cases are attributable to eosinophilic airway inflammation and that the remainder of non-eosinophilic asthma (NEA) may be due to neutrophilic inflammation (245). Other studies in adult asthmatics have also suggested a possible role for neutrophils in NEA (12,383,384). However, increased neutrophilic inflammation in NEA has not been a universal finding (403,420) and neutrophils may be important in only a fraction of NEA. We have subsequently described the presence of neutrophil-dominant asthma phenotypes representing 28% of adult asthmatics in one study: neutrophilic asthma (NA: $\geq 61\%$ neutrophils and $< 2.5\%$ eosinophils) and mixed granulocytic asthma (MGA: $\geq 61\%$ neutrophils and $\geq 2.5\%$ eosinophils) (12). These phenotypes were associated with increased levels of neutrophil-associated products including interleukin (IL)-8, neutrophil elastase (NE) and matrix metalloprotease (MMP)-9 (383,462); increased gene expression of innate immune receptors (toll-like receptor (TLR) 2, TLR4 and CD14) and elevated airway levels of bacterial endotoxin (458).

Some studies in adult asthmatics have suggested that eosinophilic asthma (EA) is associated with increased clinical severity (130,729), exacerbation frequency (436) and increased airway hyperreactivity (AHR) (426) compared with NEA. Conversely, other studies have shown that the EA and NEA phenotypes have similar clinical features (12,384,420). Despite the lack of consensus about the clinical characteristics of EA and NEA in adults, some distinct pathological differences have been shown: NEA appears to be less atopic; to have normal

sub-epithelial layer thickness (420); and, perhaps most importantly, to have a poor short-term response to treatment with inhaled corticosteroids (ICS) (384). Furthermore, there is evidence that asthma phenotypes characterised by neutrophilic involvement may be successfully treated using macrolide antibiotics (243). Thus EA and NEA may represent distinct pathological phenotypes with potentially different causative environmental exposures and triggers. Appropriate treatment, intervention and prevention strategies may be dependent upon identification of these inflammatory phenotypes.

In the limited number of studies assessing NEA in children there are similar conflicting data to that observed in adults. EA is associated with statistically significantly reduced lung function (83) and uncontrolled asthma (568) in some studies, but there are also reports of no substantial clinical differences between EA and NEA (446,745). Similar equivocal findings have been reported about the importance of neutrophils in NEA in childhood (498,502,745,746). The aims of the current study were therefore to: 1) assess the relative importance of the NEA phenotype in asthmatic children; and 2) assess whether the EA and NEA phenotypes in childhood asthma differ with regard to atopic sensitisation, lung function, airway hyperreactivity, medication use, asthma severity, and airway levels of neutrophils and other non-cellular inflammatory markers, as well as bacterial endotoxin.

8.2 Material and Methods

Study design and population

Subjects were recruited from five Wellington schools that had previously participated in the Wellington ISAAC Phase III survey of asthma prevalence, which involved students aged 13-14 years (736). We invited all participants who had given a positive response to the following two questions: “*Have you had wheezing or whistling in the chest in the past 12 months?*”, and “*Have you ever had asthma?*” (n=244); we also invited a random sample of those who gave a negative response to both questions (n=595). Those who agreed to participate (136 with and 134 without symptoms) completed a second questionnaire based on the ISAAC Phase II (736) survey, which included additional questions on medication use for the 12 month and two week periods prior to assessment. Ethical approval for the study was obtained from the Wellington Ethics Committee (00/03/010). Written consent was obtained from the school principals and from each child and his/her parents.

Asthma definition

Asthmatics had wheezing or whistling in the chest in the past two weeks or had used medication for wheezing or asthma in the past 12 months. Non-asthmatics had no wheezing/whistling in the chest in the last 12 months, no past history of asthma, no nocturnal cough in the last 12 months apart from that associated with cold or chest infection, and no asthma medication use in the past 12 months. Sixteen subjects were excluded because they did not meet the definitions, due either to a history of asthma but no current symptoms or an ambiguous questionnaire response. A further eight subjects were excluded because they were unable to be contacted again after indicating a willingness to participate in the study.

Skin prick testing

Atopy was defined as a positive skin prick test to at least one of a panel of eight commercially available allergens, which included house dust mite (*Dermatophagoides pteronyssinus*), tree pollen mix, grass pollen mix, cat and dog dander, *Alternaria tenius*, Penicillium mix (Hollister-Stier Laboratories, Spokane, WA, USA) and Cladosporium (Stallergenes SA, Antony, France). Histamine and saline were used as positive and negative controls. Fifteen minutes after testing, a mean wheal size of 3 mm or greater for any one or more allergens was considered positive, once any reaction to the negative control was subtracted.

Spirometry

Lung function tests were conducted using portable EasyOne™ Spirometers (ndd Medizintechnik AG, Zurich, Switzerland). Subjects performed three acceptable reproducible (within 5%) manoeuvres following standard procedures (691). Highest values for forced vital capacity (FVC), forced expiratory volume in one second (FEV₁) and peak expiratory flow (PEF) were used in the analyses. Predicted values for lung function parameters were determined using the NHANES III equation (72). Eleven subjects were unable to perform adequate spirometry, and 11 were excluded because of medication use such as β_2 -agonist within 6 hours prior to assessment.

Classification of asthma severity

Asthma cases were defined as mild, moderate or severe based on the frequency of wheezing attacks (mild, 0-3; moderate, 4-12; severe, >12), sleep disturbance due to wheeze (mild, < 1 night/week; moderate, ≥ 1 night/week), speech limitation by wheeze in the past 12 months (severe), daily symptoms in last two weeks (moderate), daily use of β_2 agonist (moderate), and FEV₁ (mild, $\geq 80\%$ predicted; moderate, $\geq 60\%$ and $< 80\%$; severe, $< 60\%$). Asthma was also defined as intermittent or persistent, depending upon the absence or presence of weekly symptoms, respectively. All definitions were based on previously published guidelines (747). Subjects were assigned an asthma severity category according to the highest severity class for any one of these variables.

Hypertonic saline challenge with sputum induction

Bronchial provocation testing, and sputum induction with 4.5% saline using a DeVilbiss 2000 ultrasonic nebulizer (DeVilbiss Health Care Inc, Somerset, PA, USA) and a two-way valve (Hans Rudolph Inc, Kansas City, USA), were performed on all subjects with baseline FEV₁ greater than 75 % predicted, as previously described (426). The procedure was stopped after 11.5 min or earlier if an adequate sputum sample was obtained. If the FEV₁ fell below 15% of baseline, the procedure was stopped. The subject was then given 200 μ g salbutamol, and the procedure was continued after 5 minutes. Airway hyperreactivity (AHR) was calculated from the dose-response curve and defined as a $\geq 15\%$ drop in FEV₁ from baseline. Seventy eight (35%) subjects failed to provide an adequate sputum sample, therefore sputum induction was successful in 145 cases (65%). Complete data were available for 77 (53%) asthmatics and 68 (47%) non-asthmatics.

Sputum processing and cell counts

Sputum plugs were dispersed using dithiothreitol (Sputasol, Oxoid Ltd, Basingstoke, Hampshire, England) (542). The suspension was filtered through a 60µm filter (Millipore, County Cork, Ireland) and total cell count and viability performed. Following centrifugation, supernatant was aspirated and stored at -20°C, and a cytospin was prepared from the cell pellet, air dried and then stained with May-Grunwald-Giemsa stain. A differential cell count of 400 non-squamous cells was made.

Asthma phenotype classification

Subjects with a sputum eosinophil count $\geq 2.5\%$ were classified as having EA; those with an eosinophil count $< 2.5\%$ were classified as having NEA (553). Further phenotyping on the basis of neutrophil count $\geq 61\%$ was conducted as described previously (12).

Sputum fluid phase mediator assessment

Determination of IL-8 was by ELISA (R&D Systems, Minneapolis MN USA). Active NE was measured using n-methoxysuccinyl-l-alanyl-l-alanyl-prolyl-l-valyl-p-nitroanilide (Sigma, St Louis, MO USA) as described previously (748). Eosinophil cationic protein (ECP), IL-8, NE and ECP have previously been validated for assessment in induced sputum (458,462,575).

Sputum endotoxin measurement

Endotoxin in sputum supernatant was measured using the quantitative kinetic chromogenic *Limulus* Amebocyte Lysate (LAL) assay (Kinetic QCL 50-650U; BioWhittaker) according to

the manufacturer's instructions. Inhibition or enhancement of the LAL assay was not detectable at sample dilutions of 50 times or higher, as previously described (458).

Statistical analyses

Data were analysed using SAS (SAS Institute, Cary, NC) and STATA 11 (Statacorp, College Station, TX) statistical software. Lung function measures were expressed as mean values with 95% confidence intervals (95% CI). Sputum cell counts, inflammatory markers and endotoxin were expressed as median values with 25th and 75th-percentiles. Chi-square and t-tests were performed to test differences in prevalence and mean levels, respectively. The Wilcoxon ranked-sum test was used to determine the significance of differences between groups for non-parametric data. Prevalence odds ratios were calculated using logistic regression analyses for those analyses involving dichotomous health outcomes, linear regression analyses were used for continuous outcomes. The population attributable fraction (PAF) was calculated to assess the proportion of asthma attributable to eosinophilia using the formula: $PAF = p (OR-1)/OR$ as previously (244), where p = prevalence of eosinophilia ($\geq 2.5\%$ eosinophils) and OR is the odds ratio that summarises the association between eosinophilia and asthma.

8.3 Results

Asthmatic children had a slightly but significantly lower FEV₁ and FEV₁/FVC, and significantly higher prevalence of atopy and airway hyperreactivity than non-asthmatics (Table 8.1). Approximately 40% of the asthmatics were classified as severe, 40% as moderate and 20% as mild asthmatic.

TABLE 8.1. Characteristics of study population

	Non-asthmatics N=68	Asthmatics N=77
Age (SD)	15.0 (1.04)	15.1 (1.01)
Males	72.1%	76.6%
Ethnicity		
European	75.0%	68.8%
Non-European	25.0%	31.2%
Lung function		
FVC %-predicted (Mean, SD)	101.1% (12.0)	102.3% (12.8)
FEV ₁ %-predicted (Mean, SD)	100.9% (11.4)	96.4% (11.9)*
FEV ₁ /FVC %-predicted (Mean, SD)	100.2% (7.6)	94.4% (7.9)**
Atopy ^a	36.8%	80.5%**
Airway hyperreactivity ^b	7.6%	49.4%**
Asthma severity		
Mild	-	19.4%
Moderate	-	40.3%
Severe	-	40.3%
Asthma persistence		
Intermittent	-	53.2%
Persistent	-	46.8%

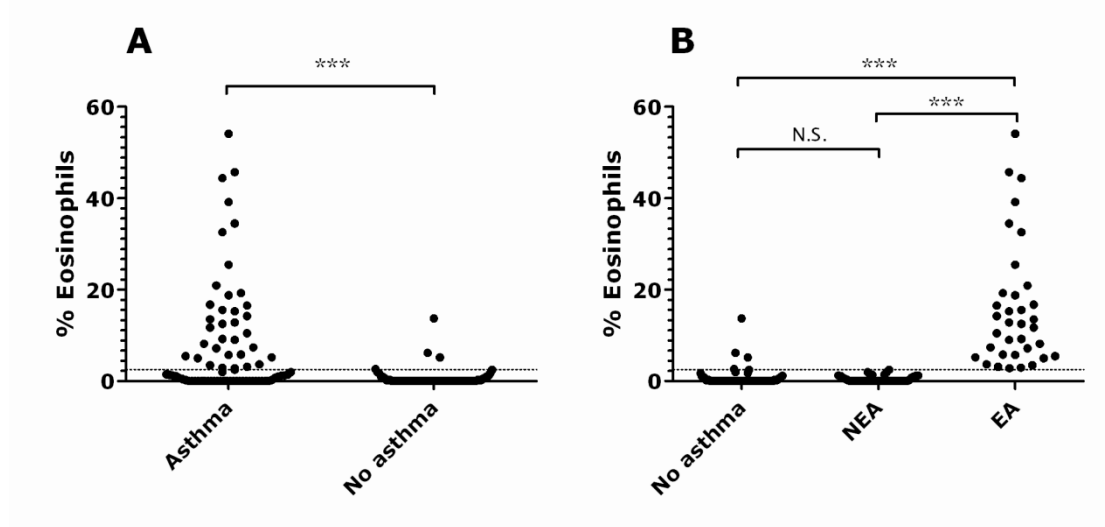


FIGURE 8.1. Sputum eosinophil percentages in (A) asthmatic and non-asthmatic children and (B) non-asthmatic, non-eosinophilic asthma and eosinophilic asthma (***) $p < 0.0001$)

Fifty four percent ($n=42$) of asthmatics had sputum eosinophil levels $<2.5\%$ and were designated NEA, with the remaining 46% (sputum eosinophils $\geq 2.5\%$) designated EA. Taking into account that 6% ($4/68$) of non-asthmatics had elevated eosinophil levels (Figure 8.1A), the proportion of asthma cases attributable to eosinophilia was 43%. When comparing markers of inflammation between groups, as expected, eosinophil and eosinophil cationic protein (ECP) levels were statistically significantly higher in EA than NEA or non-asthmatics (Table 8.2) and, additionally, those with EA were more likely to be atopic (Table 8.3). Eosinophil levels were not higher in NEA compared to non-asthmatics (Figure 8.1B). Sputum neutrophil levels were not significantly different between asthmatics and non-asthmatics (Figure 8.2A); indeed, in non-asthmatics the proportion with $\geq 61\%$ sputum neutrophils ($10/68$: 14.7%) was actually higher than in asthmatics. Neutrophil levels were also not different between EA and NEA (Figure 8.2B).

Using the inflammatory phenotyping approach of Simpson *et al* (12), 31 (40%) children had raised eosinophils in the absence of raised neutrophils. Ten asthmatic (13%) children had sputum neutrophil levels $\geq 61\%$, of which 6 (8%) were classified as NA and 4 (5%) MGA. The remaining asthmatics ($n=36$; 47%) had no clear evidence of granulocytic inflammation and could be classified as PGA (paucigranulocytic asthma). Supplementary Table 8.1 shows the inflammatory and clinical characteristics of asthmatics stratified into these inflammatory phenotypes. Due to the small numbers of NA and MGA, further analyses were conducted using NEA and EA classifications only.

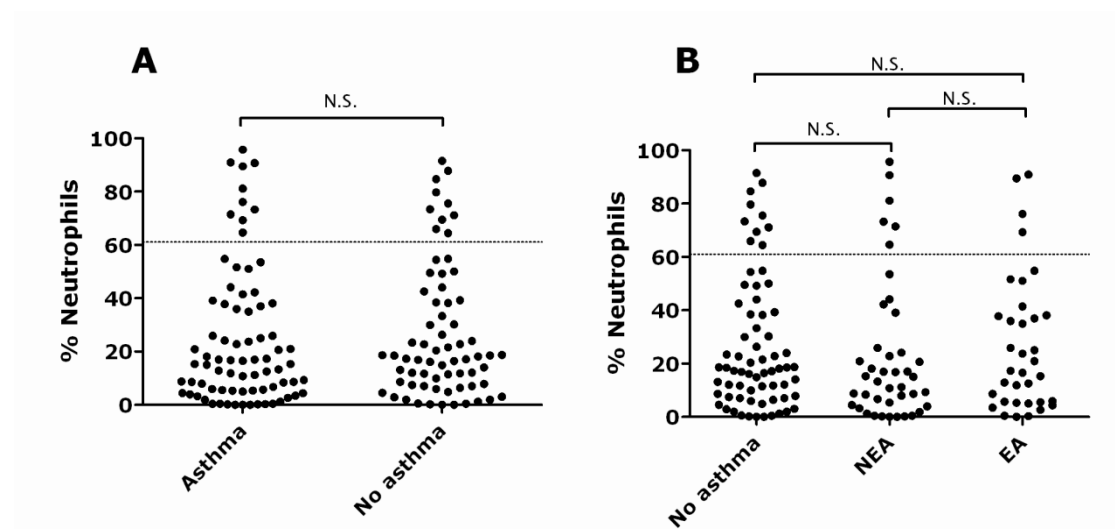


FIGURE 8.2. Sputum neutrophil percentages in (A) asthmatic and non-asthmatic children and (B) non-asthmatic, non-eosinophilic asthma and eosinophilic asthma

Mean lung function in NEA was marginally higher than in EA, but this was not statistically different (Table 8.3). Similarly, the prevalence of AHR, asthma medication use and most symptoms were lower in NEA, but these differences were small and not statistically significant. Although EA were slightly more likely to use ICS, there were no significant differences in sputum eosinophil percentage when asthmatics were stratified by recent ICS

use (Figure 8.3). The prevalence of moderate-to-severe asthma was similar between groups (83% in EA; 79% in NEA), although 49% of EA were classified as severe asthmatics compared with 33% in NEA (Table 8.3). Similarly, the EA group was more likely to have persistent asthma than NEA (52% compared with 43%: Table 8.3), although this was not statistically significant. Severity was not associated with significantly increased eosinophils or neutrophils in all asthmatics (including EA and NEA) (Figure 8.4 A-B) or EA or NEA separately (Figure 8.4 C-F). Despite this, sputum eosinophils were inversely

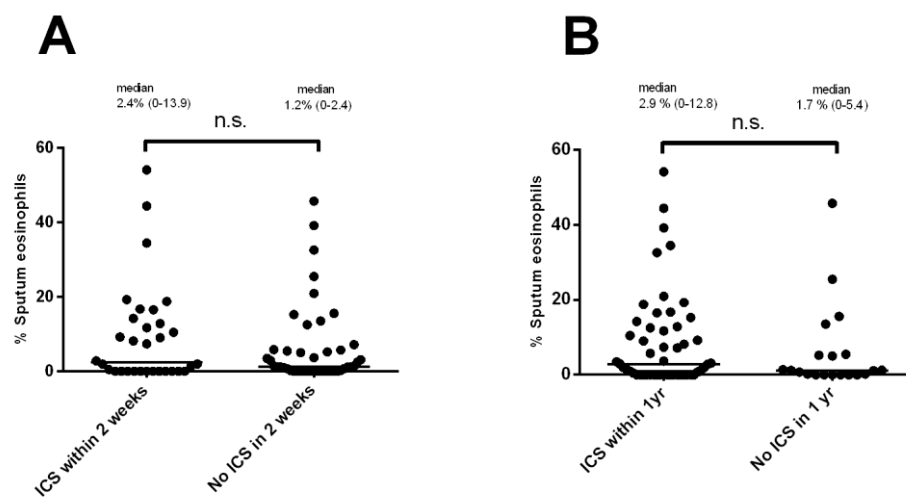


FIGURE 8.3. Percentage of sputum eosinophils in asthma when stratified for ICS use (A) ICS use within the last two weeks (B) ICS use within 1 year

associated with lung function, reaching statistical significance for total eosinophils and FEV₁ %-predicted (Table 8.4). Borderline statistically significant associations ($p < 0.10$) were also found for % eosinophils and FEV₁ %-predicted as well as total eosinophils and FEV₁/FVC-% predicted (Table 8.4). Conversely, neutrophils were positively associated with FEV₁-% predicted and FEV₁/FVC-% predicted.

Mediators associated with granulocytic inflammation (ECP, NE and IL-8) were inversely associated with FEV₁/FVC-% predicted, but not with any of the other lung function variables (Table 8.4). Although non-detectable in the majority of samples, NE was correlated with neutrophil levels but not with eosinophils (Table 8.5). No differences in NE levels were found between asthmatics and non-asthmatics or between EA and NEA. An alternative neutrophil-associated mediator, IL-8, was elevated in EA, but not in NEA but, despite this, IL-8 was correlated with both eosinophil and neutrophil levels. Levels of ECP were strongly correlated with eosinophils, expressed as either percentage or total number of cells (Table 8.5), and both sputum eosinophils and ECP were associated with atopy and airway hyperreactivity (Table 8.5). Finally, no differences were found in sputum endotoxin (LPS) levels for any of the studied sub-groups (Table 8.2) and, whilst endotoxin was weakly correlated with NE ($r=0.17$; $p<0.05$; Table 8.5), it was not associated with lung function, atopy or airway hyperreactivity (Table 8.4). All regression coefficients and ORs were very similar when the analyses were restricted to only the asthmatics but results were generally no longer statistically significant. Adjusting analyses for asthma status did not significantly change the results.

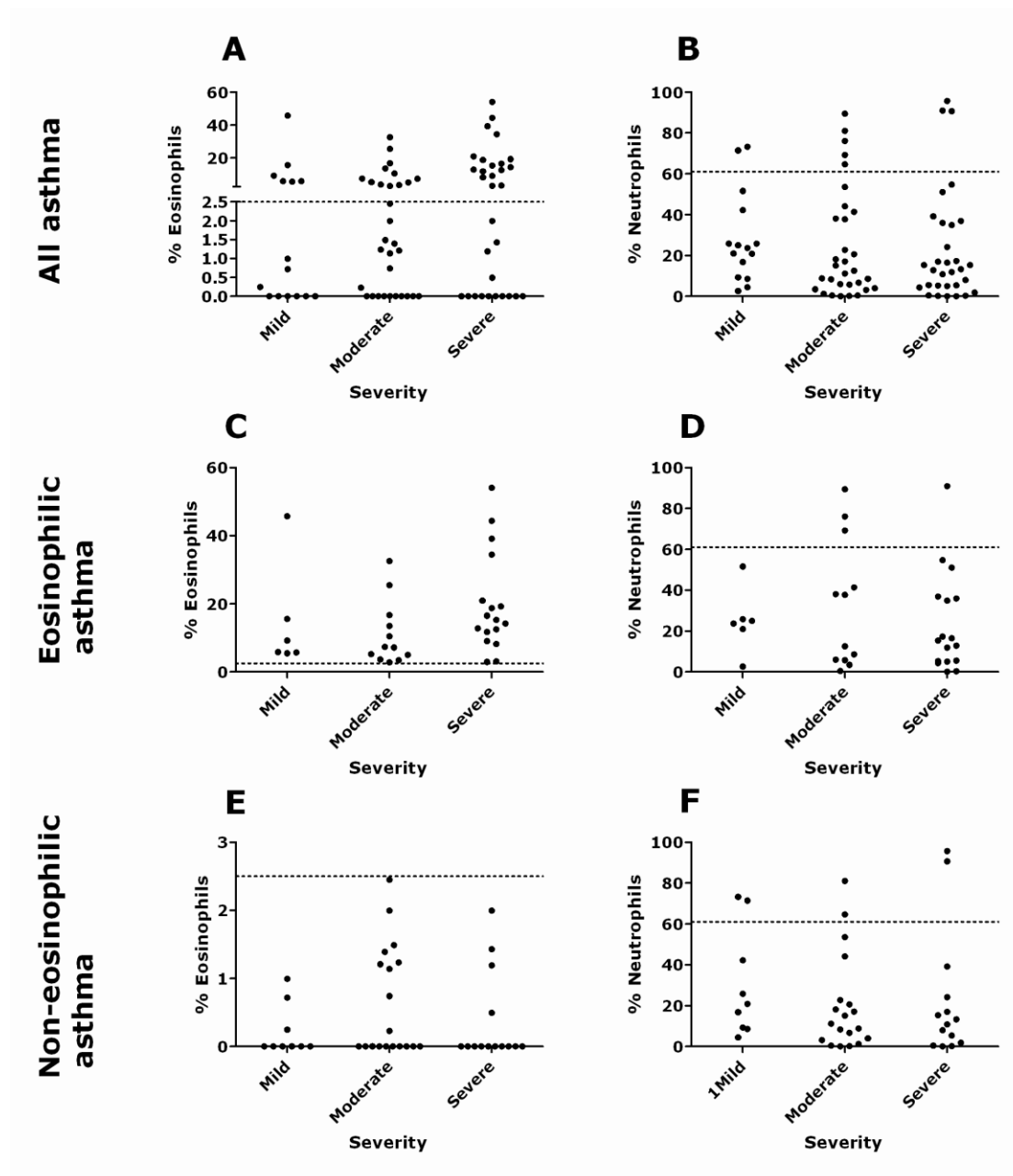


FIGURE 8.4. Sputum eosinophil and neutrophil percentages in asthma groups stratified according to severity. (A, B) all asthma, (C, D) eosinophilic asthma, (E, F) non-eosinophilic asthma (***) $p < 0.0001$

TABLE 8.2. Markers of inflammation and endotoxin levels in sputum. Expressed as median (inter-quartile range IQR). * P<0.05; ** P<0.01 asthmatics versus the reference population , † P<0.05; †† P<0.01 non-eosinophilic versus eosinophilic asthmatics

	Non- asthmatics N=68	All asthmatics N=77	Eosinophilic asthmatics N=35	Non-eosinophilic asthmatics N=42
% Eosinophils	0.0 (0.0-0.2)	1.5 (0.0-10.5)**	12.5 (5.7-19.3)**	0.0 (0.0-1.0) ††
Total eosinophils (x 10 ⁴ /ml)	0.0 (0.0-3.8)	28.1 (0.0- 228.7)**	229.9 (89.3- 385.7)**	0.0 (0.0-6.8) ††
% Neutrophils	18.3 (7.7-40.9)	15.3 (5.4-37.8)	17.3 (5.6-38.1)	14.2 (4.4-25.9)
Total neutrophils (x 10 ⁴ /ml)	262.4 (49.0- 716.0)	216 (40.6- 941.8)	242.6 (74.0- 1233.7)	170.1 (28.8-856.4)
ECP (ng/ml)	9.0 (2.1-34.3)	44.6 (9.6- 193.8)**	168.1 (70.3- 231.9)**	12.1 (3.4-49.8) ††
NE (µg/ml)	43.8 (43.8- 337.2)	43.8 (43.8- 193.8)	43.8 (43.8-43.8)	43.8 (43.8-316.6)
IL-8 (ng/ml)	117.8 (36.1- 342.0)	190.5 (52.1- 715.9)	295 (94.8-1008.8)*	163 (46.9-466.3)
Endotoxin (EU/ml)	1.4 (0.7-2.8)	1.2 (0.6-3.0)	1.4 (0.6-3.0)	1.1 (0.5-2.3)

TABLE 8.3. Differences in clinical characteristics between eosinophilic and non-eosinophilic asthmatics. Analyses were adjusted for age, sex and ethnicity. * $P < 0.05$; ^a positive SPT against one or more common allergens. ^b $\geq 15\%$ drop in FEV₁ from baseline following a 4.5% saline challenge. ^c ORs were undefined because either all or none of the subjects in the EA group were positive for the specific symptom or condition

	Eosinophilic asthmatics (EA), N=35	Non-eosinophilic asthmatics (NEA), N=42	NEA versus EA N=77
	Mean (SD)	Mean (SD)	Δ %-predicted (95% CI)
Lung function			
FVC %-predicted	102.0 (14.1)	102.5 (11.8)	0.5 (-5.4 – 6.4)
FEV ₁ %-predicted	93.8 (10.6)	98.6 (12.5)	4.8 (-0.5 – 10.1)
FEV ₁ /FVC %-predicted	92.5 (7.3)	96.1 (8.1)	3.6 (0.01 – 7.1)*
	%	%	OR (95% CI)
Atopy ^a	100.0	64.3	undefined ^c
Airway hyperreactivity ^b	57.1	42.9	0.5 (0.2 – 1.2)
Asthma medication			
No asthma medication in last 12 months	0.0	23.8	undefined ^c
ICS in the last 12 months	74.3	54.8	0.4 (0.1 – 1.2)
ICS in the last 2 weeks	48.6	31.0	0.6 (0.2 – 1.5)
β 2-agonist in the last 12 months	100.0	69.1	undefined ^c
β 2-agonist \geq once a day in last 2 weeks	65.7	42.9	0.4 (0.2 – 1.1)
Wheezing attacks in the last 12 months	60.0	66.7	1.7 (0.6 – 4.6)
0-3 times	31.4	16.7	0.4 (0.1 – 1.4)
4-12 times	8.6	16.7	1.3 (0.3 – 6.0)
>12 times			
Sleep disturbance due to wheeze in last 12 months (nights/week)			
Never	54.3	66.7	2.1 (0.8 – 5.6)
< than once a week	40.0	31.0	0.6 (0.2 – 1.7)
\geq once a week	5.7	2.4	0.4 (0.0 – 5.2)
Speech limited by wheeze in last 12 months	28.6	19.1	0.5 (0.2 – 1.6)
Asthma severity			
Mild	17.1	21.4	1.7 (0.5 – 6.1)
Moderate	34.3	45.2	1.7 (0.6 – 4.7)
Severe	48.6	33.3	0.4 (0.2 – 1.2)
Asthma persistence			
Intermittent	48.6	57.1	1.5 (0.6 – 3.9)
Persistent	51.4	42.9	0.7 (0.3 – 1.8)

TABLE 8.4. Associations between cell, cytokine, and LPS levels in sputum and clinical characteristics. Analyses were adjusted for age, sex and ethnicity.

	% Eosinophils	Total Eosinophils	% Neutrophils	Total Neutrophils	ECP (ng/ml)	Neutrophil elastase (µg/ml)	IL-8 (ng/ml)	Endotoxin (LPS; EU/ml)
<i>Regression coefficients (95% CI)^c</i>								
FVC %-predicted	-0.27 (-1.42 - 0.89)	-0.05 (-0.16 - 0.06)	0.53 (0.13 - 0.92)**	0.00 (-0.00 - 0.01)	0.28 (-1.11 - 1.66)	0.03 (-0.00 - 0.07)	0.09 (-0.00 - 0.18)	-0.14 (-0.34 - 0.07)
FEV ₁ %-predicted	-0.56 (-1.64 - 0.51)	-0.10 (-0.21 - 0.01)	0.45 (0.08 - 0.83)*	0.00 (-0.00 - 0.01)	-0.55 (-1.85 - 0.75)	-0.00 (-0.04 - 0.03)	0.04 (-0.05 - 0.12)	-0.12 (-0.31 - 0.08)
FEV ₁ /FVC %-predicted	-0.25 (-0.98 - 0.48)	-0.05 (-0.12 - 0.03)	-0.03 (-0.29 - 0.23)	0.00 (-0.01 - 0.01)	-0.76 (-1.64 - 0.12)	-0.03 (-0.06 - -0.01)*	-0.04 (-0.10 - 0.02)	0.02 (-0.11 - 0.16)
<i>OR (95% CI)^d</i>								
Atopy ^a	31.74 (2.55 - 394.35)**	1.18 (1.01 - 1.36)*	0.95 (0.88-1.03)	1.00 (1.00 - 1.00)	1.60 (0.97 - 2.63)*	1.00 (1.00 - 1.01)	1.00 (1.00 - 1.02)	1.03 (0.95 - 1.11)
Airway hyperreactivity ^b	1.25 (0.99 - 1.58)	0.99 (0.97 - 1.02)	0.98 (0.90 - 1.06)	1.00 (1.00 - 1.00)	1.12 (0.88- 1.43)	1.00 (1.00 - 1.00)	1.00 (0.96 - 1.01)	0.99 (0.95 - 1.03)

* P<0.05; ** P<0.01. ^a positive SPT against one or more common allergens

^b ≥15% drop in FEV₁ from baseline following a 4.5% saline challenge

^c Decrease/increase in % predicted lung function per 5% cells, 10 units of total cells, 100 units of ECP and IL-8, 10 units of NE, or one unit of LPS

^d Decrease/increase in risk per 5% cells, 10 units of total cells, 100 units of ECP and IL-8, 10 units of NE, or one unit of LPS

TABLE 8.5. Correlations between cell, cytokine, and LPS levels in sputum. * p<0.05; ** p<0.01.

	% Eosinophils	Total Eosinophils	% Neutrophils	Total Neutrophils	ECP	Neutrophil elastase	IL-8	LPS
% Eosinophils	-	0.98**	0.12	0.17	0.59**	-0.09	0.23**	0.05
Total Eosinophils	-	-	0.22*	0.27**	0.66**	0.07	0.29**	0.00
% Neutrophils	-	-	-	0.84**	0.20*	0.28**	0.28**	0.12
Total Neutrophils	-	-	-	-	0.40**	0.28**	0.40**	0.01
ECP	-	-	-	-	-	0.26**	0.48**	-0.02
Neutrophil elastase	-	-	-	-	-	-	-0.13	0.17*
IL-8	-	-	-	-	-	-	-	-0.11
LPS	-	-	-	-	-	-	-	-

SUPPLEMENTARY TABLE 8.1. Clinical characteristics and sputum markers of inflammation in the 4 inflammatory phenotypes previously described (12).
P<0.05; ** P<0.01 EA versus PGA (n too small for NA and MGA)

	Eosinophilic Asthmatics (EA) (31)	Neutrophilic asthmatics (NA) (6)	Mixed granulocytic asthmatics (MGA) (4)	Paucigranulocytic asthmatics (PGA) (36)
Age (Mean, SD)	15.4 (1.0)	15.2 (1.0)	15.5 (1.3)	14.9 (1.0)
Male (%)	81	100	100	67
FEV ₁ %-predicted (Mean, SD)	100.6 (14.1)	101.6 (9.5)	112.5 (10.3)	98.1 (13.0)
FEV ₁ /FVC %-predicted (Mean, SD)	92.8 (7.5)	95.2 (7.0)	90.3 (5.0)	96.2 (8.4)
Atopy (%)	100	50	100	67
Airway hyperreactivity (%)	65	50	0	42
% Eosinophils (median, IQR)	13.5 (7.2, 20.9)**	0.6 (0, 1.3)	4.4 (3.1, 6.9)	0.0 (0, 0.7)
Total eosinophils (median, IQR)	176.1 (81.3, 357.1)**	16.4 (0, 32.9)	317.3 (235.7, 885.5)	0 (0, 6.8)
% Neutrophils (median, IQR)	15.3 (5.3, 36.0)	77.2 (70.0, 92.0)	82.8 (71.00, 90.6)	10.0 (3.4, 20.0)
Total neutrophils (median, IQR)	186.3 (49.4, 573.5)	5564.0 (2497, 8632)	5554.0 (3467, 21099)	156.6 (24.6, 579.6)
ECP (ng/ml) (median, IQR)	148.1 (50.7, 223.5)**	32.1 (18.4, 139.5)	906.1 (228.4, 919.4)	10.7 (2.5, 48.5)
Neutrophil elastase (µg/ml) (median, IQR)	43.8 (43.8, 43.8)	501.2 (43.8, 1045)	2099.0 (1124, 2577)	43.8 (43.8, 270.6)
IL-8 (ng/ml) (median, IQR)	273.0 (85.4, 925.2)	464.0 (171.0, 1060)	9924.0 (772.8, 18139)	125.5 (30.5, 427.0)
Endotoxin (EU/ml) (median, IQR)	1.5 (0.6, 3.0)	1.5 (1.2, 4.2)	0.9 (0.4, 3.2)	1.0 (0.5, 2.3)

8.4 Discussion

In this study we found that NEA was more common than EA in children, and was not associated with increased neutrophil levels. As expected, atopy was more prevalent in EA (100%) than NEA (64%) or non-asthmatics (37%). Whilst those with NEA had slightly higher lung function, fewer symptoms and lower AHR, none of these differences were statistically significant. The proportion of moderate-to-severe asthma in both groups was similar, although severe asthma was slightly (but not significantly) increased in EA. Finally, no statistically significant differences were found between NEA and non-asthmatics with regard to any of the inflammatory markers studied, in particular those associated with neutrophilic inflammation. Thus, we saw little evidence of airway inflammation in NEA, despite similar symptoms and clinical parameters to those observed in EA.

Fifty four percent of asthmatic children had asthma symptoms in the absence of raised sputum eosinophils. This is comparable with previous studies in both adults (12,245) and children (428), which have reported approximately 50% NEA prevalence. However, lower NEA prevalence has also been found. In particular, in a group of 247 *steroid-naïve* asthmatic children, Lee *et al* (423) reported an EA prevalence of 64% and NEA of 36%. As the asthmatics in the current study population were not steroid-naïve (in contrast with (423)), it is possible that the use of ICS in the NEA group may have suppressed eosinophil numbers (29). However, only 32% of the NEA group had used ICS in the last two weeks, and only 54% had used ICS in the last year, suggesting that a large proportion of asthmatics in the current study had no evidence of significant eosinophilic inflammation in the absence of ICS treatment. Furthermore, we did not observe that ICS use was associated with statistically significantly different sputum eosinophil numbers.

Taking into account that 6% of non-asthmatics had sputum eosinophils $\geq 2.5\%$, the proportion of asthma cases attributable to eosinophilia (PAR) was only 43%, despite 80% of the asthmatic population being atopic. This is in agreement with our previous findings, which showed that, at most, 50% of all asthma cases are attributable to eosinophilic airway inflammation (245). Another recent study with similar atopy prevalence (86%) in childhood asthma yielded an even lower PAR (25%; (423)). It therefore appears that the majority of childhood asthma may not involve eosinophilic inflammatory mechanisms, and that categorisation of asthma as atopic or non-atopic may not be truly indicative of the underlying pathology.

We found little evidence that neutrophilic inflammation is involved in NEA in children. Indeed, raised sputum neutrophils were observed at a similar frequency in EA, NEA and non-asthmatics. There was therefore a low prevalence of the previously described neutrophil-dominant phenotypes NA (8%) and MGA (5%). Previous studies of adult asthmatics found higher prevalence of NA and MGA, which together make up approximately 30% of adult asthma (12,502). To our knowledge, only two studies to date have shown evidence of airway neutrophilic inflammation in childhood asthma but, in both studies, sputum neutrophils were relatively low. In particular, Li *et al* reported that raised neutrophils were associated with cough frequency in asthma, but overall neutrophil numbers were low with a median level of 8.3% (IQR 6.1-18.3%) (746). Drews *et al* reported significantly raised neutrophils in non-atopic asthmatics than in atopic asthmatics and non-atopic non-asthmatics, but found only 18% sputum neutrophils in this group compared with 9% in atopic asthmatics and 13% in controls (745). These values for airway neutrophil percentages are considerably lower than those reported in healthy adult reference populations (303,304). Other studies have also suggested that neutrophilic asthma in children is either non-existent or has very low

prevalence (498,502). Taken together, these studies suggest that neutrophilic involvement is less important in childhood than in adult asthma. As has been shown previously, NA and MGA are both associated with older age (12) and airway neutrophilia increases with age in both asthmatics and non-asthmatics (749). As a consequence, neutrophilic asthma, and the pathology associated with persistent neutrophilic inflammation, may therefore be more prevalent in older asthmatic populations than children. Furthermore, environmental exposures associated with airway neutrophilia, such as smoking and occupational exposure to particulates or pollution (380,430), are more likely to be important in adult populations than in children.

In the absence of overt inflammation, it is currently unclear as to what causes asthma symptoms in NEA. There is a possibility that eosinophilic inflammation is occurring at airway sites that are not sampled by sputum induction (604) or that the use of a 2.5% sputum cutoff for eosinophilia (as derived from healthy populations (553)) could misclassify some asthmatics as NEA when they actually have TH₂-mediated eosinophilic inflammation. This may be due to temporal fluctuation in eosinophil counts (496), changes in disease status (502) or alterations in transepithelial exit of eosinophils into the airway lumen (750). However, in 26/42 (62%) of the NEA group, we did not observe even a single eosinophil when analysing sputum samples, suggesting that it was unlikely that eosinophilic airway inflammation was involved. Another possibility is that misclassification of children as asthmatics may have occurred, as status was determined on the basis of self-reported symptoms and history using a questionnaire. However, the tool used has previously been shown to compare well with objective markers of asthma (146). In addition, the prevalence of moderate-to-severe symptoms was similar between EA and NEA. Therefore, in some children with NEA, asthma symptoms may be driven by alternative (non-inflammatory) mechanisms, which may have

neurogenic (32) or structural (751,752) origins. Assessment of such mechanisms is currently unachievable using conventional approaches such as assessment of induced sputum.

We observed no significant clinical differences between EA and NEA, in agreement with some previous reports (446,745). As with adults (130,729), there are, however, reports showing that eosinophilic inflammation is associated with a more severe phenotype in childhood asthma, with more frequent asthma episodes, significantly reduced lung function (83,428), increased use of oral corticosteroids (434), and more poorly controlled asthma (568). Lee *et al* (423) observed that moderate-to-severe asthma was more common in EA than NEA but also found that higher sputum eosinophils or neutrophils, in EA or NEA respectively, was associated with increased asthma severity. In contrast, we found that increased eosinophils in EA or increased neutrophils in NEA were not associated with increased asthma severity and moderate-to-severe symptoms had similar prevalence in both phenotypes. We did, however, observe a non-significant trend towards a higher prevalence of severe symptoms in the EA group.

When assessing associations between inflammation and lung function, we found a significant inverse association for total eosinophils and FEV₁ %-predicted. We also observed a positive (but non-statistically significant) association between neutrophils and FEV₁-% predicted and FEV₁/FVC-% predicted. The mechanism for these associations is unclear. One possible explanation for the lack of strong association between lung function and airway inflammation in asthma is that lung function parameters are often relatively higher in asthmatic children than in adults and it is therefore difficult to assess asthma severity on the basis of lung function in children (735,753).

Increased sputum ECP was detected in EA (and correlated with eosinophil numbers) and, as such, is indicative of eosinophilic inflammation, as shown in both sputum and peripheral blood (423). The finding of significantly increased IL-8 in EA sputum was more unexpected, as increased levels of this chemokine are generally associated with neutrophil rather than eosinophil influx (361) but may simply be due to increased IL-8 production by epithelial cells in response to general inflammation. Finally, we observed no differences in bacterial endotoxin levels in sputum supernatant amongst any of the groups studied, in contrast with our previous findings of (non-significantly) increased endotoxin levels in adult NA compared with other asthma groups and non-asthmatics (458). However, endotoxin levels measured in children were >100 fold lower than those observed in adults. The reasons for this are unclear but it is possible that the higher levels observed in adults may represent increased bacterial colonisation of the airways due to dysregulation of the innate immune system during natural ageing (754).

A limitation of this study was that only one visit per participant was conducted. In adults, some repeated sputum induction studies indicated that the NEA phenotype was reproducible (12,493,494). In contrast, other studies in children and adults suggest that inflammatory phenotypes are prone to considerable temporal variability (496,498,502), with a number of reports showing an important role for ICS treatment; for example, Hancox *et al* found that phenotype changes occurred frequently (in some cases in 100% of the adult asthmatics studied) in response to changes in therapy (29). Other studies suggest that ICS treatment may also affect classification of inflammatory phenotypes in children (434,755). Despite these findings, NEA has been demonstrated in patients with and without corticosteroid treatment and eosinophilic airway inflammation may persist in patients with well-controlled asthma receiving corticosteroid treatment (418,568), suggesting that EA and NEA can occur

independent of ICS treatment. Whether any temporal changes in inflammatory phenotype may have affected our results is unclear.

In summary, at a single assessment, we found that NEA represented approximately 50% of asthma cases in a population sample of children. Critically, NEA was not associated with neutrophilic inflammation or any of the other markers of inflammation assessed. This suggests that targeting therapeutic interventions towards neutrophilic inflammation in NEA (at least in children) may be of little benefit. A better understanding of the disease mechanisms underlying NEA, and the associated environmental exposures, is necessary to appropriately guide the development of more effective therapies and control measures for NEA, which makes up a considerable proportion of childhood asthma.

9. Asthma phenotypes: prevalence, stability and airway neutrophil function following treatment changes

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Background & aims: Asthma is increasingly categorised into inflammatory phenotypes on the basis of sputum granulocyte differential counts. The aim of the study was to assess the prevalence and characteristics of these phenotypes and examine the effects of treatment changes on phenotype stability, inflammation and neutrophil function in adult asthmatics.

Methods: Fifty asthmatics and 39 non-asthmatics were assessed using questionnaires, skin prick testing, spirometry, exhaled nitric oxide (FENO) measurement and sputum induction. Twenty-one asthmatics underwent further assessment following cessation/ sub-optimisation (-inhaled corticosteroids (-ICS)/long-acting β -agonists (-LABA)) or starting/optimisation of treatment (+ICS/LABA). Sputum samples were assessed by cell counts, flow cytometric analyses and ELISA.

Results: We observed a prevalence of 40% eosinophilic asthma (EA) and 60% non-eosinophilic asthma (NEA), of which 8% was neutrophilic asthma (NA). EA was associated with higher FENO, reduced lung function and greater bronchodilator reversibility (BDR) (all $p < 0.05$) than other groups. Altered treatment was associated with inflammatory phenotype change in 11/21 (52%). Optimal treatment was associated with reduced EA prevalence (38% versus 62%), decreased FENO (median 51 versus 89 ppb; $p < 0.01$), decrease in ACQ7 (0.57 versus 1.71; $p < 0.0001$), increased FEV₁- % predicted (79.3% versus 75.6%; $p < 0.05$) and reduced BDR (8.3% versus 11.9%; $p < 0.01$), when compared with assessment during sub-optimal treatment. These differences, apart from ACQ7, were seen only in EA. Optimal treatment was also associated with a statistically significantly lower percentage of eosinophils in EA ($p < 0.05$) and of neutrophils in NEA ($p = 0.047$), as well as with enhanced neutrophil phagocytosis ($p < 0.05$) in EA only. Neutrophil oxidative burst was statistically significantly higher in optimally treated asthma ($p < 0.05$).

Conclusions: NEA is a common asthma phenotype associated with less severe disease and only a small proportion of cases demonstrate evidence of neutrophilic inflammation. Altered treatment was shown to result in changes in phenotype in a substantial proportion of asthmatics, suggesting that phenotype classification based on a single measurement may not be valid for any specific individual. This study also showed that optimised treatment may lead not only to a reduction in eosinophils, but also to changes in neutrophil numbers and function.

In preparation for submission

9.1 Introduction

Airway inflammation is a defining feature of asthma (4), with many studies suggesting an important role for eosinophilic, TH₂-mediated immunopathology (3). However, there is evidence that asthma may occur in the absence of airway eosinophils in approximately 50% of cases (245). Non-eosinophilic asthma (NEA) can be observed across the spectrum of severity and is associated with distinct pathological features (420). Furthermore, NEA appears less responsive to treatment with inhaled corticosteroids (ICS) (11,384,403). Identification of inflammatory asthma phenotypes may therefore help guide asthma treatment.

We have previously hypothesised that NEA may be the result of innate-mediated neutrophilic airway inflammation (245), associated with microbial exposure. In support, some studies have reported increased sputum neutrophil numbers, levels of associated mediators (such as interleukin (IL)-8, matrix metalloproteinase (MMP)-9 and neutrophil elastase (NE)), and bacterial endotoxin in NEA (383,458,756). One particular study of 96 adult asthmatics has therefore suggested that asthma phenotypes may be defined on the basis of levels of eosinophils *and* neutrophils ($\geq 1\%$ eosinophils and $\geq 61\%$ neutrophils), and found raised neutrophil numbers in 28% of cases (12). Conversely, other studies in both adults and children have shown little evidence of neutrophilic inflammation in NEA (403,420,454,455), suggesting that neutrophilic asthma (NA) may be important only in a small proportion of NEA, or may only be present in some populations. The importance of neutrophilic inflammation is in asthma in the general population, and in NEA in particular, therefore remains unclear.

Initial studies suggested that inflammatory phenotypes were relatively stable (12,494), although recent reports suggest that changes over time may be common (29,496,498). ICS treatment in particular may have a critical impact on inflammatory phenotype as it may result in the reduction of sputum eosinophils (757) and, possibly, an increase in sputum neutrophils (28). Moreover, neutrophil involvement may be different during poorly controlled asthma, as observed in adults undergoing exacerbation (502). The effects of control and ICS treatment on asthma phenotypes have not generally been taken into account in previous studies in which airway inflammation was assessed only once and mostly during periods of stable asthma. In addition, most studies have been conducted in hospital or outpatient settings, in which asthma is more likely to be treated with high ICS doses (494); this may have led to an underestimation of the prevalence of EA. Also, assessing airway inflammation has often been limited to conducting differential cell counts and measuring soluble mediators. More recent methods, such as flow cytometry (694,716), may allow greater definition of underlying inflammatory mechanisms and the investigation of the functional status of airway leukocytes, such as neutrophils, in asthma.

In this study, we assessed the frequency and characteristics of inflammatory asthma phenotypes in a general population sample. In a subset of asthmatics we also examined phenotype stability and changes to clinical and inflammatory parameters during changes in asthma treatment. In particular, neutrophil numbers and function were measured to assess the effect of changes to patients' asthma treatment on this cell population.

9.2 Materials and methods

Study population

Non-smoking adult participants (n=97; 53 asthmatics and 44 non-asthmatics, age range 17-65) were recruited through advertisements in newspapers and primary care clinics.

Asthmatics had a physician's diagnosis of asthma and wheezing within 12 months; non-asthmatics had no previous or current diagnosis of asthma, no history of wheeze or nocturnal cough (other than that due to respiratory infection) within 12 months and forced expiratory volume in 1 second (FEV_1) > 75% predicted. Participants who had a respiratory infection within four weeks were asked to return at a later date. All participants underwent a respiratory health questionnaire, skin prick testing (SPT), spirometry before and after bronchodilator administration (bronchodilator reversibility; BDR), exhaled nitric oxide (FENO) measurement and sputum induction. Eighty-nine subjects successfully produced sputum at this assessment (91.8%), and were included in data analyses examining phenotype prevalence and characteristics referred to in the remainder of this paper as the “prevalence study”.

In a subset of the asthmatics, we also examined the effects of optimisation and sub-optimisation of treatment on phenotype stability and neutrophil function referred to in the remainder of this paper as the “change-in-treatment study”. Figure 9.1 shows a flow chart of the study design. The prevalence study and the nested change-of-treatment study were approved by the Upper South A and Lower South Regional Ethics Committee, New Zealand, respectively. All subjects gave written informed consent.

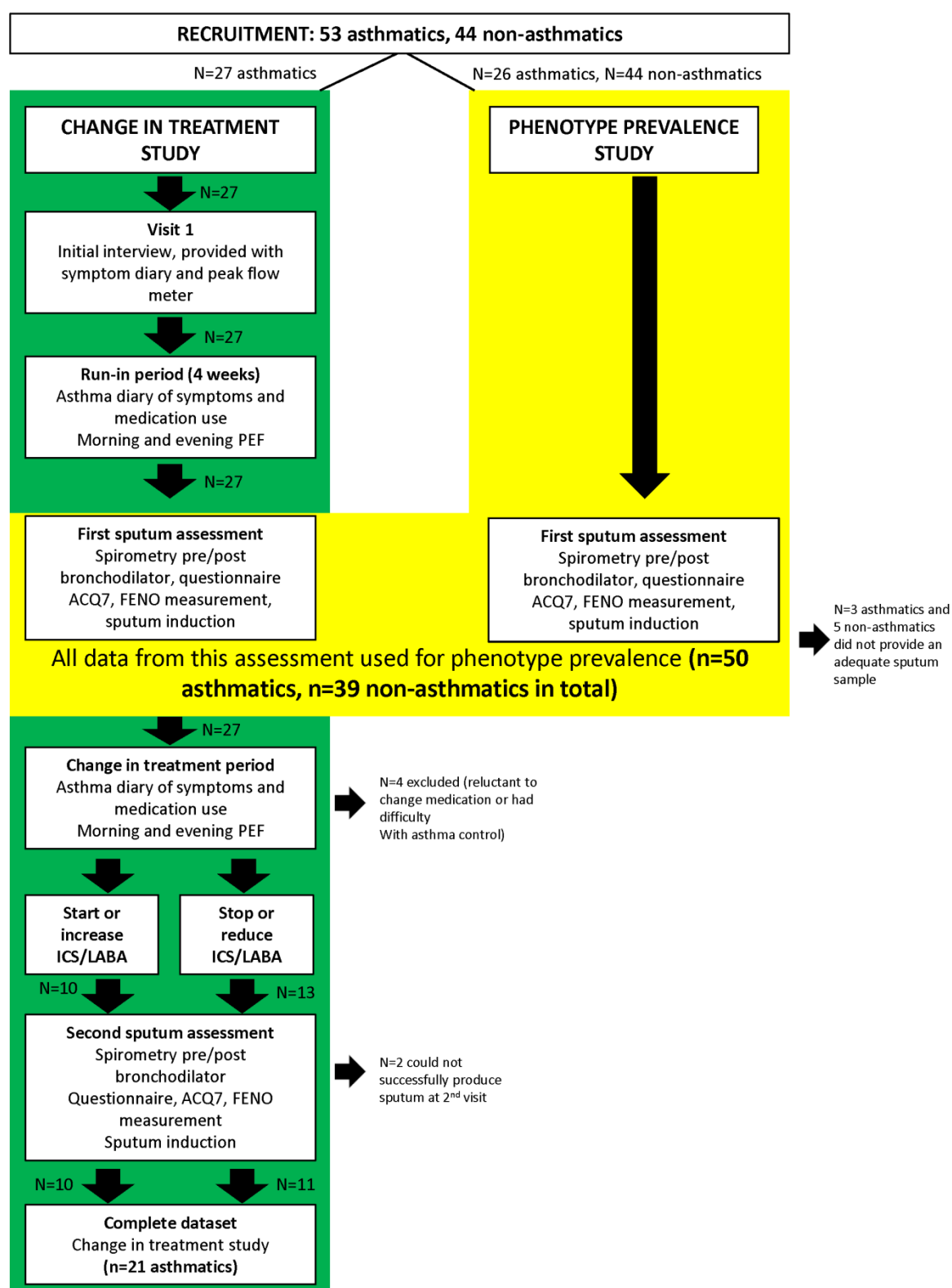


FIGURE 9.1. Flow chart showing the study plan: recruitment of participants, and the assessments involved in the phenotype prevalence (yellow) and change-in-treatment components (green) of the study.

Change-in-treatment study

Twenty-seven randomly selected asthmatic subjects were included in the change-in-treatment study. Participants were interviewed, given a peak flow meter and diary card, and asked to record their morning and evening peak expiratory flow (PEF), symptoms and medication use over a 4 week 'run-in' period. They then returned for the general respiratory assessment (see Figure 1) as described above. An assessment of asthma control was made based on the results of asthma control questionnaire (ACQ) 7 score (758,759), PEF and symptom diary. Four subjects left the study after this assessment as they were reluctant to change asthma treatment, or had difficulty maintaining asthma control. A change in asthma treatment was made for the remaining 23 subjects according to the National Asthma Education and Prevention Program (NAEPP) Expert Panel Report 3 (EPR 3) guidelines (62). Subjects with adequately controlled asthma had their treatment sub-optimised to a reduced dose of ICS (n=10) or to no ICS (n = 3). Of these subjects, 8 also used long-acting β_2 -agonists (LABA); of these, 3 stopped LABA treatment only, 2 reduced ICS/LABA, and 3 stopped both ICS and LABA. Subjects with inadequately controlled asthma had their treatment optimised; they commenced ICS (n = 4) or received an increased ICS/LABA dose (n = 6).

Subjects undergoing treatment optimisation returned for a further assessment after 4-6 weeks. Subjects undergoing treatment sub-optimisation were given a 3-day prescription of prednisone 20mg and instructed to contact the investigators if they had a fall in morning PEF over 7 days > 10% of baseline, a drop in morning or evening PEF of > 20% on 2 consecutive days, an increase in daily use of reliever > 3 puffs above baseline, an increase in night-time wakening > 3 nights or more/week above baseline, or distressing or intolerable asthma symptoms. If none of these occurred, they returned within 4-6 weeks for further assessment. Throughout this period, reliever medication was used as required, and participants monitored

their PEF twice daily and recorded their asthma symptoms and reliever use. After this period of change-in-treatment, subjects attended for a final visit with a repeat of all previous tests (Figure 9.1). Asthma treatment was then readjusted for each patient according to asthma control before discharge from the study. Two subjects undergoing treatment sub-optimisation were excluded from the change-in-treatment study analyses as they were unable to provide an adequate sputum sample at this visit.

Skin prick testing

SPT was conducted using a panel of aeroallergens; house dust mite, tree mix, grass mix, cat and dog dander, *Alternaria tenuis* and Penicillium mix (Hollister-Stier Laboratories, Spokane, WA, USA). Positive atopy status was determined as the presence of at least one weal >3mm. Histamine and saline were used as positive and negative controls respectively.

Spirometry

Spirometric measurements were conducted for all participants according to American Thoracic Society (ATS) criteria (691), using an Easyone spirometer (NDD Medizintechnik AG, Zurich, Switzerland). NHANES III equations were used for lung function parameters.

Exhaled nitric oxide (FENO) measurement

FENO measurements were conducted according to ATS/ERS guidelines (13) using a Hypair FENO analyser (Medisoft, Sorinnes, Belgium) as previously described (717).

Sputum induction and processing

Sputum induction was conducted as described previously (426) with some modifications. Briefly, participants were pre-treated with 400µg salbutamol; spirometry was performed before and 15 minutes after salbutamol. Aerosolised sodium chloride (4.5% w/v) was produced using an ultrasonic nebuliser (DeVilbiss Ultraneb 2000) and administered orally for increasing intervals from 30 seconds to 4 minutes, to a total of 16 minutes. At the end of the session, participants were encouraged to produce a sputum sample in a sterile plastic container. Sputum samples were processed by plug selection (542). Total cell count and viability (trypan-blue exclusion) were determined using light microscopy. The sample was centrifuged (350xg, 8 minutes), supernatant aspirated, and the cell pellet resuspended in RPMI 1640 (Invitrogen, Auckland, New Zealand), 10% foetal calf serum (FCS, Invitrogen), and 1% penicillin-streptomycin at 1×10^6 cells/ml.

Identification of cell populations by differential cell count

Cell suspension (75µl) was centrifuged in a cytopsin column at 44xg for 5 minutes. The slide produced was air dried, fixed with methanol and stained with the Diff-Quik stain set (Dade Behring, Deerfield, IL). Four hundred non-squamous cells were counted by light microscopy. Asthmatics with $\geq 2\%$ sputum eosinophils were classified EA (of which those with $\geq 61\%$ neutrophils were classified as mixed granulocytic asthma (MGA)) and $< 2\%$ eosinophils as NEA (of which $< 2\%$ eosinophils and $\geq 61\%$ neutrophils were classified NA, and $< 2\%$ eosinophils and $< 61\%$ neutrophils as paucigranulocytic asthma (PGA)), based on previous reports (12,383).

Measurement of inflammatory mediators in sputum supernatant

Sputum was analysed for levels of IL-8 and MMP-9 using sandwich ELISA and for bacterial endotoxin using the kinetic Limulus amoebocyte assay, as described previously (458).

Neutrophil elastase (NE) was also measured as described previously (748) but was undetectable in >90% of samples. Results are therefore reported as undetected/detected only. Endotoxin and NE were measured only in the change-in-treatment study due to study reagent limitations.

Flow cytometric assessment of sputum

For the change-in-treatment study only, remaining cells were used for flow cytometric determination of neutrophil function. For measurement of neutrophil respiratory burst (paired samples available for 7 asthmatics), three 100µl (2×10^5) cell aliquots per subject were added to wells of chilled low-adherence 96-well plates with 0.5µM dihydrorhodamine (DHR)123 (Molecular Probes, Eugene, OR). Plates were incubated (37°C, 5 mins) to allow DHR123 uptake and immediately quenched on ice. One aliquot was kept on ice (negative control) and one aliquot was incubated (37°C, 30 mins) with 1ng phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and then quenched on ice (activated sample). Data are expressed as percentage of DHR positive neutrophils after PMA stimulation minus negative control. For assessment of phagocytosis (paired samples available for 16 asthmatics), two 100µl (2×10^5) cell aliquots per subject were added to wells of chilled low-adherence 96-well plates alongside 10:1 concentration of opsonized Texas Red-labelled zymosan A microbeads (Molecular Probes). One aliquot was kept on ice (background control) and one aliquot was incubated (37°C, 60 mins) and then quenched on ice (positive sample). Data are presented as percentage of bead-positive neutrophils after incubation at 37°C minus percentage bead-

positive neutrophils in the background control sample. Following incubation on ice for 30 mins, samples were washed, incubated with live/dead fixable blue (Molecular Probes) on ice for 30 mins and washed again with FACS buffer. All cell aliquots were subsequently washed and antibody labelled to identify neutrophils by FC as described previously (716). Labelled aliquots were resuspended in 150µl FACS buffer; 150µl neutral buffered formalin (Sigma) was added for sample fixation. Samples were stored on ice and analysed on a BD LSRII flow cytometer within 3 hours. Appropriately-labelled compensation beads (BD) and unlabelled cells were used for setting up compensation on the flow cytometer.

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 described as median and interquartile ranges (IQR) unless otherwise stated. Univariate analyses were conducted using either student's t-test or Pearson's correlation for parametric data, or Mann-Whitney u-testing or Spearman's correlation for non-parametric data. In the change-in-treatment study, data were analysed using the paired t-test or Wilcoxon's matched pairs test as appropriate. Fisher's exact test was used for categorical data, and Kruskal-Wallis with Dunn's post test analysis was used for multiple group comparison.

9.3 Results

As expected, asthmatics had higher sputum eosinophil differential counts ($p<0.0001$), FENO levels ($p=0.01$), atopy prevalence ($p<0.0001$), BDR ($p=0.0007$), and lower FEV₁-% predicted and FEV₁/FVC (both $p<0.005$) compared with non-asthmatics (Table 9.1). When categorised on the basis of sputum granulocyte differential counts, 40% of all asthmatics were classified as EA and 60% NEA, of which 8% were NA and 52% were PGA. None was classified as MGA. EA was associated with statistically significantly reduced lung function, greater reversible airway obstruction and poorer ACQ7 score (all $p<0.05$) than PGA (Table 9.1). Eosinophilia correlated with FENO levels ($r=0.74$; $p<0.0001$) and the EA group had higher FENO levels than the other asthmatic groups ($p<0.05$). With the exception of increased eosinophils in the EA group and increased neutrophils in the NA group, we observed few differences in inflammatory parameters among phenotypes (Table 9.1). However, the PGA group had higher numbers of eosinophils than the non-asthmatics ($p<0.05$). We also observed a statistically significant correlation between neutrophil percentage and levels of soluble sputum IL-8 and MMP-9 when all asthmatics were assessed ($p=0.02$ - 0.002 ; data not shown).

The demographics of the participants for the change in treatment study were not different from those observed in the prevalence study, with the exception of statistically significantly lower lung function (Table 9.2). Following treatment change, 10 subjects demonstrated a change in asthma control based on a combination of ACQ7, change in asthma symptoms and reliever medication (loss of control, 2: improvement in control, 8). The remaining 11 subjects did not demonstrate a change in asthma control (data not shown). Treatment optimisation led to a reduction in EA; of the 10 subjects, EA prevalence reduced from 9 (90%) at first assessment to 6 (60%) at 2nd assessment (Table 9.2). Similarly, sub-optimisation led to a doubling of EA

TABLE 9.1. Clinical characteristics and sputum sample inflammatory characteristics of all participants. Asthmatics categorised based on Simpson et al 2006 at baseline visit. BDR=Change in FEV1% pred post bronchodilator. Median (IQR) or number (%) † Significantly greater than PGA, ¥ Significantly greater than NA, § Significantly greater than EA, by Kruskal-Wallis with Dunns post-test *Significantly greater than no asthma (p<0.05) by Mann-Whitney U test.

	Eosinophilic Asthma (EA)	Neutrophilic asthma (NA)	Paucigranulocytic asthma (PGA)	All asthma	No asthma
Number (% of asthma)	20 (40%)	4 (8%)	26 (52%)	50	39
Sex (female)	8 (40%)	4 (100%)	17 (68%)	30 (60%)	27 (69.2%)
Age	33.5 (27-43)	60.5 (40.5-64)	35 (26-47.5)	35.5 (27-51.8)	37 (27-47)
Atopy	20 (100%)	3 (75%)	21 (80.8%)	44 (88%)*	16 (41%)
ACQ7	1.14 (0.86-1.86)†	0.86 (0.25-1.89)	0.57 (0.29-1.00)	0.86 (0.43-1.57)	-
FEV1 % predicted	87.25 (79.08-93.90)§	92.70 (84.45-100.9)	94.30 (85.25-100.4)	91.6 (83.5-98.5)*	96.6 (90.5-105.5)
FEV1/ FVC	0.69 (0.64-0.82)§	0.72 (0.68-0.79)	0.79 (0.69-0.84)	0.71 (0.65, 0.75)*	0.83 (0.77-0.86)
BDR	13.97 (5.99-19.33)†§	3.32 (-0.92-13.88)	4 (0-6.00)	5.9 (2.06-13.75)*	3.0 (1.0-5.0)
FENO (ppb)	139.5 (95-195.6)†¥§	28.35 (18.43-86.5)	26.65 (18.63-48.5)	49.5 (22.4-134.5)*	33 (27.3-38.5)
ICS use (%)	16 (80%)	4 (100%)	18 (72%)	38 (76%)	-
TCC/ml	1.88 (1.2-2.84)	6.07 (0.8-6.12)	1.29 (0.73-2.39)	1.40 (0.92-2.84)	1.42 (0.90-2.05)
Viability %(non-squamous cells)	68.57 (57-81.3)	80.74 (69.44-88.24)	65.15 (58.39-74.33)	69.38 (59.5-76)	74.45 (57.5-83)
Sputum eosinophils %	5.8 (3.52-11.78)†¥*	0.12 (0-1.04)	0.47 (0-1.33)*	1.358 (0.24-4.61)*	0 (0-0.25)
Total sputum eosinophils x 104 ml	12.31 (4.88-29.20)†*	1.48 (0-7.89)	0.65 (0-1.48)*	1.53 (0.37-8.81)*	0 (0-0.32)
Sputum neutrophils %	26.90 (17.43-40.19)	63.14 (61.46-67.47) †§	24.24 (13.02-33.14)	27 (17.21-42.82)	32.1 (19.27-45.43)
Total sputum neutrophils x10 4 ml	48.75 (20.79-96.24)	390.5 (49.03-419.4)	31.27 (11.18-55.04)	33.94 (15.7-81.6)	47.51 (10.90-73.10)
Sputum macrophages %	62.25 (48.93-67.97)	32.37 (30.46-35.59)	68.88 (56.07-77.08) ¥	63.95 (49.76-74.58)	60.49 (45.55-78.44)
Total sputum macrophages x 10 4 ml	109.7 (67.18-158.7)	185.2 (28.98-188.5)	90.14 (46.87-146.2)	96.80 (53.16-160.6)	80.94 (41.71-125.9)
Sputum Lymphocytes%	2.61 (1.69-4.37) †	2.36 (1.16-3.99)	1.47 (0.83-2.32)	1.94 (0.98-3.14)	2.12 (1.25-3.45)
Total sputum lymphocytes x 10 4 ml	4.70 (2.24-8.96) †	5.93 (1.39-18.09)	1.83 (0.72-2.92)	2.37 (1.15-6.24)	3.15 (1.28-6.22)
Sputum IL-8 (ng/ml)	2.36 (1.52-3.39)	2.33 (1.47-3.39)	1.77 (1.01-3.08)	2.19 (1.26-3.18)	1.62 (1.66-2.33)
Sputum MMP-9	1063 (374-2126)	1595 (1084-2943)	528.9 (185.8-1040)	693.7 (374-1363)	736.7(334.5-1340)

prevalence (from 18% to 36%). Ultimately, 11/21 (52.4%) of asthmatics changed phenotype during the course of the study, and 14/21 (66.7%) could be categorised as EA on at least one assessment, although this reduced to 8 (38.1%; Table 9.2) during optimised treatment.

Table 9.2 shows the changes in inflammatory and clinical parameters observed when stratified on the basis of assessment during optimised or sub-optimised treatment. There was significantly lower BDR ($p=0.002$), a significant increase in FEV₁-% predicted ($p=0.026$), and decrease in FENO ($p=0.002$) and ACQ7 ($p<0.0001$; Table 9.2) with treatment optimisation. Although there was a reduction in the number of subjects with EA and NA with treatment optimisation, there was no significant change in either sputum neutrophil or eosinophil numbers (despite a non-significant trend to higher eosinophil percentage with suboptimal treatment), or in IL-8 and MMP-9 (Table 9.2). However, sputum bacterial endotoxin levels significantly decreased in the optimisation group, and more subjects had detectable sputum NE with sub-optimised treatment (Table 9.2). Due to the low proportion of NA/MGA phenotypes, further analyses were conducted using EA/NEA designations only.

When the treatment groups were stratified on the basis of $\geq 2\%$ eosinophils at either visit as EA/NEA (with NEA containing NA and PGA as previously described(12)), statistically significant lung function and clinical changes were present only in the EA subjects (Figure 9.2A-D). The exception to this was an observed decrease in ACQ7 in NEA. However, there was a significant decrease in eosinophils in the optimally treated EA groups (Figure 9.2E, $p < 0.05$) and a significant decrease in neutrophils in the optimally treated NEA (Figure 9.2F, $p = 0.047$) compared with suboptimal treatment.

Assessment of changes in sputum neutrophil functional parameters showed an increase in oxidative burst after PMA stimulation ($p < 0.05$) and an increase in the numbers of neutrophils phagocytosing beads ($p < 0.05$) (Figure 9.3A-B) with optimised treatment. When phagocytosis was stratified on the basis of optimisation in the EA/NEA groups, a borderline trend towards an increase ($p = 0.052$) in phagocytosis with optimal treatment was observed only in the EA group (Figure 9.3C). We observed a loss of neutrophils during *in vitro* stimulation and therefore compared this loss between suboptimal and optimal treatment, to assess relative ‘fragility’ of neutrophils at different levels of asthma control. We found no statistically significant difference (data not shown).

TABLE 9.2. Alterations in clinical/inflammatory characteristics and inflammatory phenotypes during changes in asthma treatment. ICS dose – average daily dose ICS over recording period; in mcg budesonide equivalent. Data expressed as number (percentage) or median (IQR). Δ difference between median values of paired groups. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ when compared with matched group using paired t-test/Wilcoxon matched-pairs test depending upon normality of data.

	Start or increase ICS/LABA (n=10)			Reduce or decrease ICS/LABA (n=11)			All (n=21)		
	Visit 1	Visit 2	Δ	Visit 1	Visit 2	Δ	Visit with sub-optimised treatment	Visit with optimised treatment	Δ
Female (%)	5 (50%)	-	-	7 (64%)	-	-	12 (57%)	-	-
Age	36.5 (32.8, 45.8)	-	-	40 (28.0, 56.0)	-	-	37.0 (51.0, 54.0)	-	-
Atopy	9 (90%)	-	-	11? (100%)	-	-	20 (95%)	-	-
ICS dose (mcg, budesonide equiv.)	0 (0, 140)	1000 (800, 1000)	1000**	560 (250, 800)	0 (0, 475)	-560**	0 (0, 250)	500 (800, 1000)	500***
ACQ7	1.71 (1.07, 2.18)	0.71 (0.57, 0.97)	-1.00**	0.57 (0.57, 1)	1.29 (1, 1.71)	0.72**	1.71 (1.07, 2.07)	0.57 (0.57, 1.00)	-1.14***
FEV1 % predicted	72.7 (62.5, 82.8)	79.5 (69.8, 82.6)	6.8	78.7 (74.8, 90.2)	80.8 (70.2, 89.8)	2.1	75.6 (69.6, 89.4)	79.3 (74.1, 85.8)	3.7*
FEV1/FVC	0.66 (0.64, 0.70)	0.71 (0.64, 0.78)	0.05	0.73 (0.65, 0.82)	0.70 (0.61, 0.83)	-0.03	0.67 (0.64, 0.76)	0.71 (0.65, 0.78)	0.04
BDR (post-bronchodilator reversibility)% change	15.4 (9.3, 21.8)	11.0 (7.7, 13.9)	-4.4**	5.0 (1, 8.6)	8.9 (1.9, 13.7)	3.9	11.9 (5.9, 17.4)	8.3 (3.6, 11.8)	-3.6**
FENO (ppb)	114.5 (86-182.5)	73.5 (24.7-113.8)	-41**	44.0 (21.0, 81.0)	73.0 (34.0, 89.0)	29	89.0 (47.0, 139.5)	51.0 (23.8, 94.0)	-38**
EA (%)	9 (90%)	6 (60%)	-3 (-30%)	2 (18.2%)	4 (36.4%)	2 (18.2%)	13 (61.9%)	8 (38.1%)	-5 (-23.8%)
NEA (%) of which:	1 (10%)	4 (40%)	3 (30%)	9 (81.8%)	7 (63.6%)	-2 (-18.2%)	8 (38.1%)	13 (61.9%)	5 (23.8%)
NA (%)	1 (10%)	1 (10%)	0 (0%)	0 (0%)	3 (27.3%)	3 (27.3%)	4 (19.0%)	1 (4.8%)	-3 (-14.3%)
PGA (%)	0 (0%)	3 (30%)	3 (30%)	9 (81.8%)	4 (36.4%)	-5 (-45.5%)	4 (19.0%)	12 (57.1%)	8 (38.1%)
Sputum eosinophils %	9.1 (3.9, 17.9)	3.7 (0.7, 10.2)	-5.4	1.4 (0.7, 1.6)	0.9 (0, 5.8)	-0.5	4.4 (0.7, 15.3)	1.5 (0.8, 4.8)	-2.9
Sputum neutrophils %	29.3 (9.2, 46.5)	29.2 (17.3, 43.4)	-0.1	35.7 (11.9, 44.4)	42.1 (19.9, 63.7)	6.4	35.8 (17.5, 53.9)	30.4 (16.4, 42.2)	-5.4
TCC/ml	2.35 (1.54, 3.72)	2.38 (1.74, 3.69)	0.03	1.19 (0.56, 1.58)	0.90 (0.72, 2.08)	-0.29	1.74 (0.82, 2.44)	1.58 (1.11, 3.07)	-0.16
Viability % (non-squamous cells)	68.3 (56, 84.1)	77.3 (64.5, 84.4)	9	69.4 (64, 77.6)	89.3 (69.5, 87.1)	19.9	74.8 (60.3, 83.8)	75.9 (64.0, 79.9)	1.1
Sputum IL-8 (ng/ml)	2.19 (1.00, 3.01)	1.87 (1.16, 4.81)	-0.32	2.27 (1.01, 3.03)	2.00 (1.06, 3.26)	-0.27	2.19 (1.11, 3.11)	2.05 (1.12, 3.57)	-0.19
Sputum MMP-9	539 (261, 2685)	891 (396, 1326)	352	1094 (219, 1438)	1261 (336, 1964)	167	666 (292, 2042)	1094 (326, 1408)	428
NE detected (%)	1 (10%)	0 (0%)	-1 (-10%)	0 (0%)	3 (27.3%)	3 (27.3%)	4 (19%)	0 (0%)	-4 (-19%)
Endotoxin (EU/ml)	1816 (1141, 2399)	1298 (515, 1857)	-518*	2255 (774, 3583)	1865 (1445, 2949)	-390	1865 (1226, 2577)	1730 (559, 2650)	-135

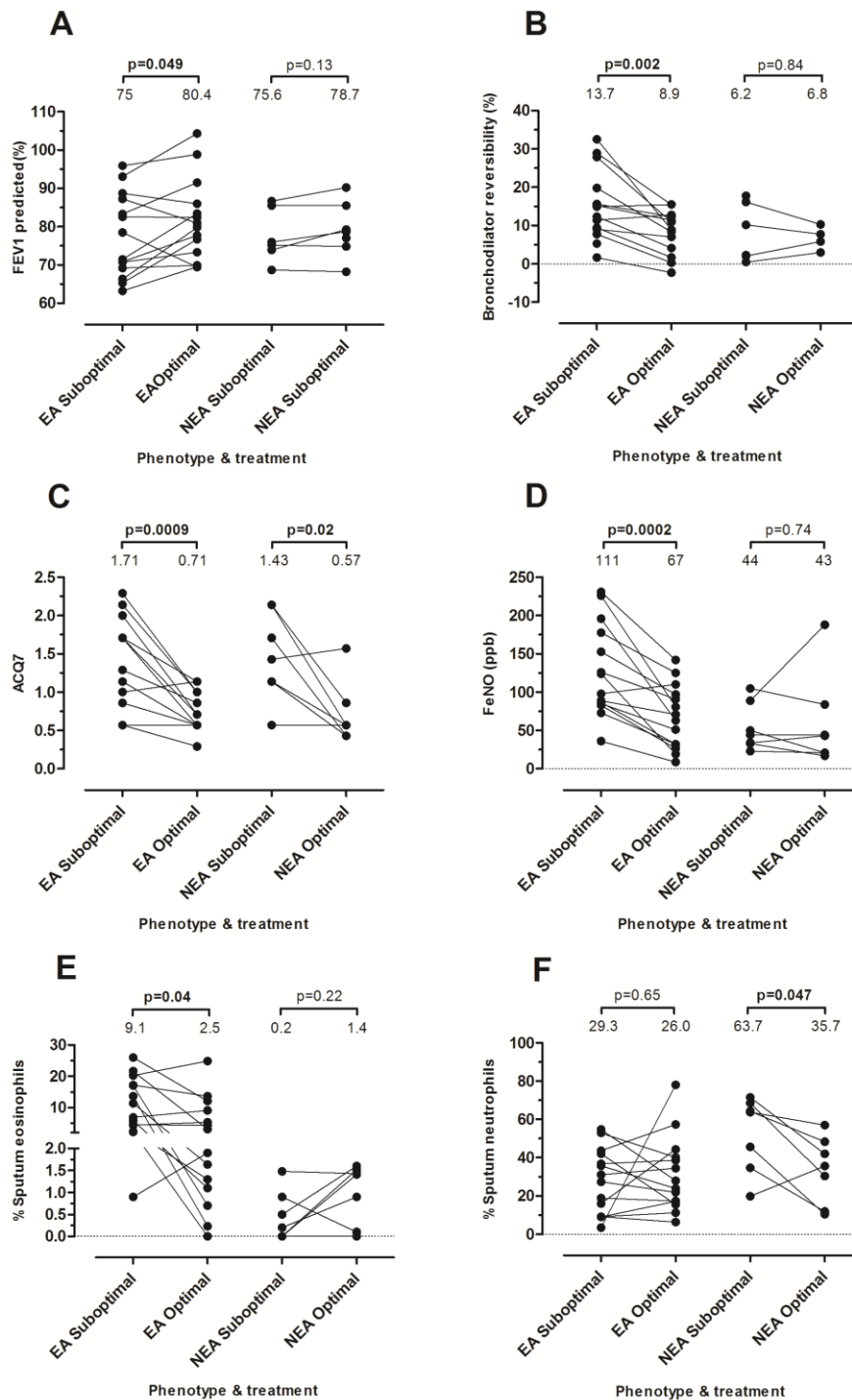


FIGURE 9.2. Clinical and sputum sample inflammatory parameters before/after change in asthma management in asthma when stratified into EA/NEA subgroups. A – FEV1 % predicted, B- change in FEV1 % predicted post-bronchodilator (BDR), C – ACQ7, D- exhaled NO levels, E – change in % sputum eosinophils, F-change in % sputum neutrophils. Median data values are expressed at the top of each group. Bold p values indicate significance ($p < 0.05$) using paired t-test or Wilcoxon’s matched pairs test.

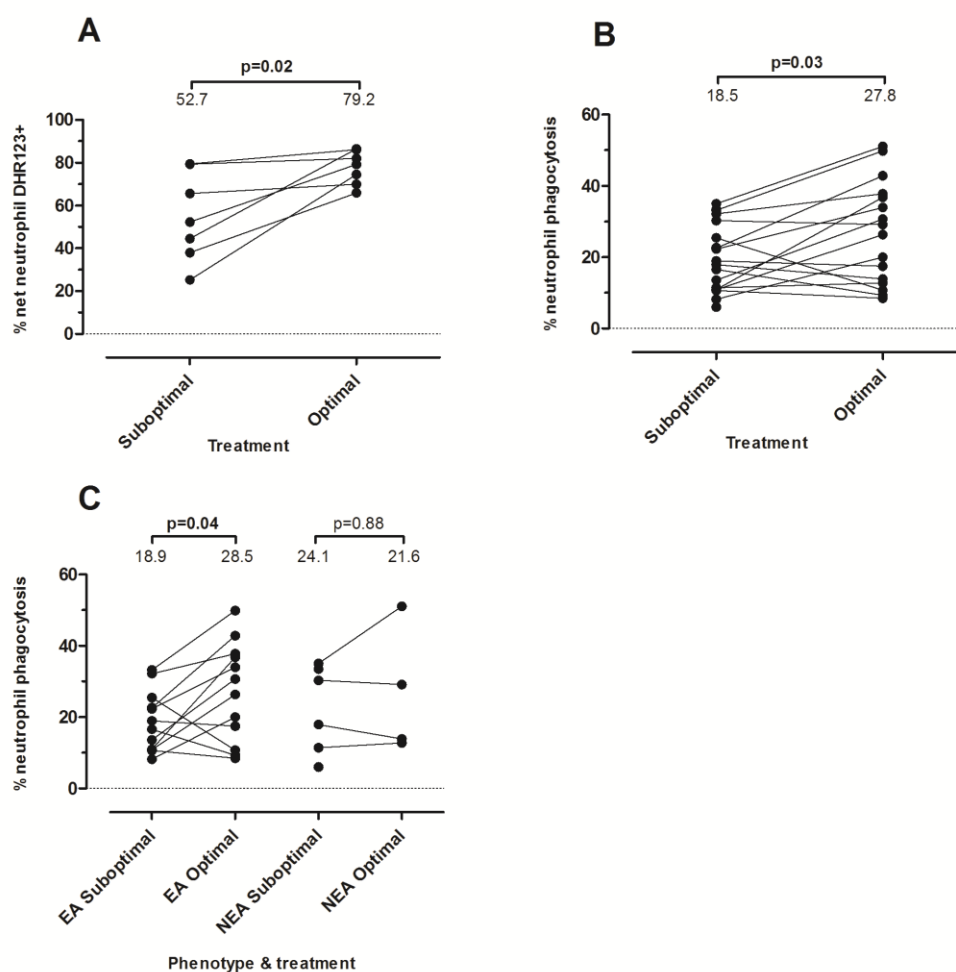


FIGURE 9.3. Sputum sample neutrophil functional parameters (as assessed using flow cytometry) with optimal/suboptimal treatment. A – change in % sputum neutrophils undergoing oxidative burst after stimulation with PMA, B –change in % sputum neutrophils that are capable of phagocytosing fluorescent zymosan beads in culture at 37°C, C- change in % sputum neutrophils that are capable of phagocytosing fluorescent zymosan beads in culture at 37°C when stratified into EA/NEA. Median data values are expressed at the top of each group. Bold p values indicate significance ($p < 0.05$) using paired t-test or Wilcoxon’s matched pairs test.

9.4 Discussion

In this study, we observed a low prevalence of neutrophil-dominant phenotypes (8% NA), with EA (40%) and PGA (52%) observed in the majority of asthmatics. EA was associated with statistically significantly increased airway obstruction, ACQ7 score and BDR.

Optimised treatment was associated with clinical improvement, although not necessarily with reclassification as controlled asthma. More than half of the asthmatics changed phenotype with altered treatment; in particular, suboptimal treatment was associated with an increased prevalence of EA and NA, whereas optimal treatment was associated with an increase in PGA. Sputum eosinophil numbers were decreased in optimally treated EA and sputum neutrophil numbers were decreased in optimally treated NEA. Finally, sputum neutrophil function was significantly increased with optimised treatment.

Previous studies assessing adult asthmatics reported a prevalence of the neutrophilic phenotypes NA and MGA of approximately 30% (12,502). In contrast, we found NA in only 8% of asthmatics. Raised sputum neutrophils were observed at a similar frequency in asthmatics (EA or NEA) and non-asthmatics and levels of neutrophil-associated mediators were also similar between groups. This is in agreement with studies previously conducted in New Zealand (28,29). We speculate that the higher prevalence of neutrophilic phenotypes described in other centres (12) may be in part due to specific environmental exposures, such as those associated with particular occupations or heavy industry (380,653,760-762). Furthermore, NA has previously been reported in association with older age groups (12,749). In agreement, we observed NA only in older individuals in the cross-sectional study, with 3/4 asthmatics classified as NA being >60yrs of age. The remaining individual had NA at only one assessment (69% sputum neutrophils, which was reduced to 30% at the 2nd assessment).

In contrast with earlier findings (12), we did not find that lung function was reduced in NA compared with PGA. Indeed, only EA had significantly lower FEV₁, FEV₁/FEV and higher ACQ7 scores and BDR, which is consistent with several previous reports (130,729,763). We observed that PGA (which made up the majority of NEA) was associated with statistically significantly higher eosinophil percentages than found in non-asthmatics, even though levels were below the 2% cut-off used to define EA. It is therefore possible that the presence of relatively low numbers of sputum eosinophils may not necessarily be indicative of different asthma pathology but may represent a less overt form of EA, or overlapping phenotypes (247).

In the change-in-treatment study we were unable to establish that there had been a clear change in asthma control based on a combination of ACQ7, asthma symptoms and reliever use between the suboptimal and optimal treatment stages in many participants (58%; data not shown). Despite this, the statistically significant changes that we observed in clinical parameters (improvement in BDR, increased FEV₁-% predicted and exhaled FENO), as well as an overall decrease in ACQ7 and in eosinophils in EA with optimised treatment indicate that there was a change in at least some aspects of asthma control.

Whilst it has been shown by others that NEA was relatively stable at both one-month and 5-year assessments (12), more than half (52%) of asthmatics in this study undergoing treatment change altered phenotype. Similar findings have been previously reported; for example, Hancox *et al* found that NEA/EA changes occurred in 50 to 100% of adult asthma cases studied (n=54) in response to changes in ICS therapy (29), a finding also described in children (498). ICS use may therefore lead to the erroneous identification of EA as NEA, as ICS has been shown to be associated with a reduction in sputum eosinophil numbers and

prevalence of EA in this study and elsewhere (28,29). In particular, we found that EA reduced from 62% to 38% prevalence with optimised treatment. Thus, our findings support previous suggestions that inflammatory phenotype classification based upon a single sputum assessment is unlikely to be valid for all asthmatics (764).

Although some previous reports have shown ICS to be less effective in NEA compared with EA (11,384,403), we observed improvements in ACQ7 score in the NEA group with optimised treatment. At least three other studies have shown that symptoms improve in NEA with ICS treatment, although EA remains associated with a better response (28,29,429). As noted above, it is possible that some NEA subjects have a degree of ICS-responsive but undetectable eosinophilic inflammation. Alternatively, ICS may act through suppression of non-TH₂ mediated pathways in NEA, such as suppression of epithelial cytokine production (765).

ICS use has previously been found to lead to an increase in sputum neutrophils and neutrophil survival (28), possibly via impairment of apoptosis (738). We observed the opposite. A non-significant decrease in sputum neutrophils was observed in optimal compared with suboptimal treatment when analysing data from all asthmatics, but when the NEA group was considered alone, treatment optimisation was associated with a statistically significant reduction in sputum neutrophils. Although there was no change in IL-8 and MMP-9 levels, we also observed a significant reduction in sputum endotoxin levels and fewer samples with detectable levels of sputum NE with optimised treatment. Both these markers have previously been associated with neutrophilic inflammation in asthma (458). Whilst this reduction in sputum neutrophils may have been due to reduced neutrophil viability, we did not find a difference between viability of the total cell population between asthmatic and non-

asthmatic subjects or differences in neutrophil survival between the optimal and suboptimal treatment states. Furthermore, although increased neutrophil survival may possibly account for the improvement in neutrophil function observed, it would not explain the decrease in neutrophil number in NEA with optimal treatment. However, it is possible that ICS use may have led to the reduced production of IL-8 by bronchial epithelial cells, as has been shown *in vitro* (765), and was therefore associated with reduced neutrophil influx into the airways.

In addition to the decrease in neutrophils observed in optimally-treated NEA, we observed that 3/9 (33%) individuals with PGA changed to NA during treatment sub-optimisation. This increase in neutrophil-dominant inflammation contrasts with findings of previous studies; these studies suggested that asthma exacerbations after tapered ICS withdrawal are associated with eosinophilic inflammation (425,766). However, one previous report suggested that sudden (rather than tapered) ICS withdrawal, as conducted in our study, may result in neutrophilic exacerbations (767). It is therefore possible that the nature of airway inflammation post-ICS withdrawal may be dependent upon the kinetics of withdrawal, which may, in turn, affect the kinetics of inflammatory cell influx.

As noted above, we observed that neutrophil function was enhanced in optimally treated asthma, with the greatest effect observed in EA. Although earlier studies have investigated function in sputum phagocytes (309,460,695), we believe that this is the first study assessing changes in neutrophil function associated with treatment changes in asthma. Although it is unclear if the improvement in neutrophil function observed is a direct effect of treatment, particularly as subjects underwent different changes in medication on the basis of their current control and treatment, we speculate that improved neutrophil function may be one of the mechanisms by which asthma treatment leads to a reduction in eosinophilic inflammation,

possibly through improved efferocytosis (460). There is however a degree of incongruity in our data when considered together. They suggest that both enhancement in neutrophil cell function and reduction in neutrophil number may be associated with optimised ICS treatment. This may also possibly be the basis of improvement in symptoms with ICS treatment in chronic obstructive pulmonary disease (COPD), where the neutrophil is the predominant inflammatory cell in the airways (472). However, larger investigations are required to more fully understand the role of the neutrophil in asthma, and also the effect of medication on neutrophil function.

There were some limitations with this study. Firstly, although asthmatics reported a doctor's diagnosis of asthma and recent symptoms, it is possible that some may have an incorrect diagnosis. Despite this, previous studies phenotyping asthma defined asthma primarily on the basis of current symptoms (12) and this approach has been shown to be better in some cases than objective measures such as airway hyperreactivity (146). Secondly, as the majority of asthmatics were undergoing ICS therapy, and there were safety concerns about total ICS withdrawal in some cases, it is possible that successful ICS treatment may be "cloaking" physiological responses, as well as eosinophilic inflammation. Thirdly, the relatively large number of cells required to investigate cell function meant that only samples with larger cell yields could be assessed. For this reason, respiratory burst was assessed in relatively few samples (n=7 for respiratory burst, 16 for phagocytosis assessment). This may have resulted in selection bias; for example if large sputum volumes are obtained only from subjects with greater airway inflammation. However, our data suggest that there is no significant difference between sputum total cell count between asthmatics and non-asthmatics and that this is unlikely to be an issue.

In conclusion, in this general population sample of asthmatics, NEA is a common asthma phenotype associated with less severe disease, with only a small proportion of all cases demonstrating evidence of neutrophilic inflammation. Altered treatment was shown to result in changes in phenotype in a substantial proportion of asthmatics suggesting that phenotype classification based on a single measurement is unlikely to be valid. This study also showed that optimised treatment may lead, not only lead to a reduction in eosinophils, but also to changes in neutrophil numbers and function.

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10. Discussion and conclusions

10.1 Introduction

This thesis was based on a series of studies developing and utilising FENO measurement and induced sputum for the non-invasive assessment of airway inflammation. These methods were used specifically to investigate the different types of inflammation and inflammatory cell populations associated with adult and childhood asthma in New Zealand. In particular, this thesis has contributed to the development and validation of flow cytometry as a standardised method to investigate induced sputum in asthma. Furthermore, as there has been very little similar research conducted in New Zealand, this thesis has provided important data about the prevalence, characteristics and stability of different inflammatory phenotypes of asthma in the general population. The studies described also add to the relatively small body of research assessing inflammatory asthma phenotypes in the general population internationally. The main findings of the individual studies are summarised below, followed by some considerations of the methodology used and the implications of the research. This is followed by a discussion of their strengths and limitations and suggestions for future studies.

10.2 Summary of main findings

The main findings of the studies reported in this thesis showed that:

- FENO measurement can be easily and cost-effectively conducted in the general population (Chapter 3);
- Flow cytometric analysis of leukocyte populations in sputum is feasible, and comparable to the conventional method involving manual differential cell counts (Chapter 4);

- Flow cytometric analysis is a suitable method to assess rare cell populations and cell function in cells derived from the airways (Chapters 5, 6 and 9);
- Proximal airway iNKT cells are unlikely to be a key player in the pathology of human asthma (Chapter 5);
- Basophils may be a suitable alternative indicator of allergic airway inflammation in asthma (Chapter 6);
- A ‘one size fits all’ approach to phenotyping based on cut-off values of airway granulocytes derived from healthy populations may not be appropriate when investigating certain populations, such as older asthmatics (Chapter 7)
- In cross-sectional assessment, approximately 50% of asthmatics (both children and adults) have evidence of eosinophilic inflammation (Chapters 8 and 9)
- More than 50% of adult asthmatics may change phenotype with altered treatment (Chapter 9)
- Sputum neutrophil function appears to be improved by optimal treatment/ICS use (Chapter 9)
- Neutrophilic airway inflammation does not appear to play a major role in asthma in the general population in New Zealand and around half of all asthmatics may not have detectable evidence of airway inflammation (Chapters 8 and 9)

10.3 Methodological considerations

Both induced sputum and FENO measurement have a number of strengths to support their use in the assessment of airway inflammation in population-based studies. However, as discussed in Chapter 2.5, they also have some weaknesses. Both strengths and weaknesses of these methods will be discussed below.

FENO measurement

In population-based studies, measurement of FENO using a portable analyser (as described in Chapter 3) is a more feasible proposition than induced sputum assessment. The specific benefits of FENO measurement over induced sputum for population-based studies include: a high success rate; instant results; and low costs. In the study described in Chapter 3, we observed a very high success rate, obtaining FENO measurements for >99% of the participants, which is considerably higher than that generally reported with assessment of sputum (553,768), including our own studies in which sputum induction testing was used (Chapters 4 to 9). FENO measurement could also be optimised to make it more convenient for population-based studies. Based on the low CV values obtained in our study (less than 6%), the use of a single measurement (rather than the three advocated in international guidelines (13)) would probably suffice, meaning that a FENO assessment could be conducted in seconds rather than minutes. Finally, the purchase and running costs of using the Hypair FENO were reasonable and less than half that of the costs involved with using the alternative Aerocrine NIOX FENO analyser.

However, whilst measurements conducted using the Hypair FENO instrument correlated well with those obtained with the ‘gold standard’ NIOX instrument, they were approximately 60% higher. Large differences between FENO results obtained with other analysers have also previously been reported (674), suggesting that FENO results from different instruments, and hence different studies, cannot necessarily be directly compared.

In addition, it remains unclear as to whether high FENO levels are necessarily indicative of airway immunopathology. As previously discussed, we and others observed that atopy is a more important determinant of high FENO levels than asthma (Chapter 3). Increased FENO may therefore be a result of non-pathological TH₂-mediated processes, independent of the perceptible symptoms or pathological changes associated with asthma. Furthermore, although high FENO levels have been shown to be statistically significantly associated with sputum eosinophils in several studies (602,607), including the study described in Chapter 9, there are other studies showing no significant correlation (613,614), suggesting that high FENO in asthma does not necessarily indicate high sputum eosinophils. Finally, FENO levels are affected by a number of factors (described in Chapter 2.5.5), and are often reduced by ICS use in asthma (769), suggesting that these factors would have to be adequately adjusted for in cross-sectional epidemiological studies.

Induced sputum

Sputum induction is difficult to conduct in large field studies where laboratory facilities are not readily available (e.g. in occupational settings), or when there are time or labour constraints. This is largely because the overall process of obtaining a sample, plug selection and slide preparation is labour intensive and requires considerable expertise. Moreover, sample processing has to be conducted within a few hours of sputum induction because of rapid sample degradation due to the presence of salivary enzymes. These factors have resulted in the limited use of sputum induction in large field-based epidemiological studies to date (421,770) and have meant that epidemiological assessment of induced sputum-derived data has generally involved retrospective analysis of data that has often been collected in smaller studies conducted over a number of years (Chapter 7 (495,771)).

In an attempt to address some of the difficulties associated with the use of sputum in epidemiological studies, experiments were conducted to determine the differences in leukocyte populations in induced sputum before and after cryostorage (Chapter 4). If comparable, this would allow sample collection and storage in the field. Samples could then be run together in batches at a later date rather than individually on the day of collection, minimising set-up time, costs, and reducing inter-run variation. We observed that cryostorage at -80°C prior to analysis was feasible, but may be detrimental to some sputum leukocyte populations, particularly neutrophils. Despite this, good correlations were still observed between fresh and frozen samples, and therefore cryopreservation of sputum samples may be a convenient alternative to immediate processing and labelling of samples for analysis. However, although cryostorage did not affect the relative frequencies of leukocytes (as shown by the significant correlations observed), it resulted in a reduction in total cell number, due to cell damage and loss of viability during the freeze/thaw procedure. Cryostorage of induced sputum in epidemiological studies may therefore only be practical for sputum samples with relatively high cell yields, which may add possible selection bias.

Cytofluorimetric analysis of induced sputum

The addition of flow cytometry to the analysis of induced sputum has some major advantages, such as the detection of rare cell populations reported in Chapters 5 and 6, and assessment of cell function reported in Chapter 9. In particular, the use of specific antibodies in combination with distinct size and granularity characteristics allowed accurate and objective identification of each leukocyte population within a sample. Another advantage of flow cytometric analysis is that the viability of the individual leukocyte cell populations may be determined. This is particularly important, as reagents used in flow cytometry (including

antibodies and tetramers) may bind in a non-specific fashion to non-viable cells, which are common in tissue as variable as induced sputum. The combination of non-specific binding and suboptimal gating strategies may have substantially effects on observed cell frequencies, and could be the cause of the implausibly high numbers of iNKT cells detected in some previous studies in asthma (as suggested by the results described in Chapter 5). To avoid these problems, our data strongly suggest that adequate precautions (i.e. use stringent gating strategies) are required when using flow cytometry to detect any rare cell populations in difficult or variable tissues.

Whilst it has its advantages, cytofluorimetric assessment of induced sputum also adds to a considerable amount of time (in some cases, up to an additional 4 hours per sample), labour, equipment and skill demands to a process that is already difficult to conduct in large populations. Downstream analysis of data produced by flow cytometry further added to time requirements. This is not conducive to the analysis of large numbers of patients (particularly when analysing only 1 to 2 samples at a time). Furthermore, although sputum induction has been reported to have >80% success rate ((551,553), and as observed in our studies), success rates can be extremely variable; for example, a recent study examining non-invasive biomarkers during exacerbations of respiratory disease reported that out of 107 sputum inductions, only 74 (69%) produced a sample and, of these, 62 (58%) provided suitable material for a basic sputum leukocyte cell count (768). It is likely that low cell yields in some sputum samples may make it extremely difficult to conduct both flow cytometry and a manual differential cell count, reducing the number of samples from which a complete dataset can be obtained. Taken together, these issues suggest that sputum induction and cytofluorimetric analysis of freshly obtained sputum samples in particular, may not be feasible in large epidemiological studies unless there is access to considerable staffing and

technical resources. In fact, this approach may only be suitable for more detailed investigations of nested sub-populations within a larger study.

Validity of data obtained using induced sputum

Finally, an issue that was not addressed in this thesis is the validity of data derived from sputum induction for identifying inflammatory phenotypes. In particular, although sputum eosinophilia is considered a hallmark of TH₂-mediated allergic asthma, it is not specific to asthma alone. For example, eosinophils can be found in the lower airways in subjects with allergic rhinitis with no evidence of asthma or airflow limitation (712,713,772). Furthermore, increased airway eosinophilia is observed in cases of eosinophilic bronchitis (125), in which cough, but no other asthma-like symptoms, may be observed. It has therefore been suggested that the presence of eosinophils may not necessarily imply a detrimental immunopathological process (750).

Sputum eosinophil percentage may also not be indicative of eosinophilic inflammation occurring at other airway sites, such as the mucosa or distal airways. For example, a high sputum eosinophil percentage in severe asthma is not necessarily associated with high bronchial biopsy counts and vice-versa (604,614). In another example, 50% of asthmatics previously identified as NEA by sputum induction had evidence of eosinophilic inflammation in the distal airways (773). Therefore, like FENO, increased sputum eosinophils may not necessarily confirm that asthma is driven by allergic mechanisms and the absence of eosinophils does not necessarily preclude TH₂-mediated pathology.

10.4 Study implications

The studies in this thesis used a combination of well-characterised methods (with some additional novel approaches) to assess airway inflammation in asthma in the general population in New Zealand. There have been relatively few such studies in New Zealand, or even internationally, examining the presence and characteristics of inflammatory phenotypes of asthma in the general population. Even fewer data are available regarding the presence of these phenotypes in very young or very old populations, or from studies that have used flow cytometry to examine cells in induced sputum. Such studies are particularly important when considering the increasing awareness of the heterogeneity of the mechanisms underlying asthma. For example, a recently published PRACTALL consensus report (jointly released by the AAAAI and EAACI) states that *'...one of the major obstacles to understanding the causes of asthma and improving treatment is the failure to understand the underlying disease mechanisms in individuals with different types of disease'* (6). This report emphasises the need for improved identification of inflammatory phenotypes of asthma, which will likely result in tailored/personalised approaches to prevention, intervention, management and treatment, rather than the current 'one size fits all' approach based on control and severity. In the sections below, the main findings and implications related to airway inflammation in asthma, as described in this thesis, will be discussed.

iNKT cells in asthma

As reported in Chapter 5, no evidence for the involvement of invariant NKT cells in asthma was found, and very few of these cells could be detected in either the EA or NEA. After initial reports suggested a role for iNKT cells in asthma, it was proposed that the iNKT axis could possibly be targeted for development of therapeutic approaches (774). Our study

indicates that this is unlikely. In particular, our data suggest that it is important that the methods used to examine the role of rare cell populations in asthma are robust and well validated.

Eosinophilic and non-eosinophilic asthma

Eosinophilic airway inflammation was associated with greater severity in adults than NEA, and was evident in approximately 40-50% of asthmatics (of all ages) at the time of assessment. This figure may, however, be inaccurate due to the effects of ICS treatment (as reported previously (28,29,496,775)) and temporal variability (discussed below).

Furthermore, the absence of sputum eosinophils does not necessarily confirm the absence of TH₂ immunopathological processes, as discussed above. Despite this, the studies described in Chapters 8 and 9 suggest that approximately 50% of asthmatics studied had asthma symptoms in the absence of sputum eosinophils at the time of assessment. This implies that a large proportion of asthmatics in the New Zealand general population may not have airway inflammation conforming to the conventional allergic paradigm of asthma and therefore may not be optimally served with treatment targeting TH₂-mediated inflammation.

Furthermore, no clear evidence of any kind of inflammation was observed in the majority of NEA. Indeed, one of the most important findings of this thesis was the apparent lack of importance of neutrophilic airway inflammation in asthma in NEA in the general population. A similar lack of evidence for an NA phenotype has been described in studies conducted in Dunedin, New Zealand (28,29). These findings are in contrast with previous reports that suggested an important role for innate immunity and neutrophilic inflammation in NEA

(245,383). It is therefore possible that NA may be detectable only in some geographical areas or in association with specific environmental exposures (380); further studies would be required to confirm this. In particular, objective measurements may be required to determine the environmental determinants associated with NA.

Little is currently known about the cause of persistent neutrophilia in the airways in NA. It has been suggested that it may be due to exposure to ozone, pollutants, bacterial components or viruses (36,245). As neutrophils are extremely short-lived cells, it is likely that the cause will be persistent or ongoing, such as occupational exposures, chronic bacterial infections, localised airway structural defects or systemic immune alterations. Bacterial infections may occur throughout life, and occupational exposures may occur throughout adulthood. However, airway defects and changes can be observed in older subjects in particular. These defects may be associated with increased bacterial colonisation and airway neutrophilia. Airway neutrophilia has been shown to be associated with age in a number of studies to-date, but it is still unclear as to whether this increased neutrophilia is actually a cause or effect of the changes observed with ageing. Either way, the evidence described in Chapter 7 suggests that it is important to adequately control for the effect of age when identifying asthma variants associated with increased airway neutrophilia.

On the basis of the studies described in this thesis, defining asthma as either EA or NEA may be the most appropriate current approach to inflammatory phenotyping using sputum, as very little evidence of either the NA or MGA phenotypes was found. The NEA phenotype was predominantly paucigranulocytic i.e. there was no evidence of increased granulocytic infiltration into the airway lumen.

Non-inflammatory mechanisms?

As noted above, a consistent observation throughout these studies was that inflammation (at least in a form that can be detectable with the methods used) was not present at the time of assessment in a large proportion of asthmatic subjects. There are several possible reasons for this; it may be that: (i) sampling was at an irrelevant site; (ii) sampling was at an inappropriate time; (iii) inappropriate or inadequate indicators of inflammation were measured; or (iv) asthma symptoms are not necessarily caused by inflammation.

With regard to (i), a major problem with all current means of assessing airway inflammation is that, when considered alone, they do not provide a definitive answer regarding airway inflammation. Pathological changes may be occurring in the mucosa or submucosa with no detectable effect on mediators in the lumen and increased proximal luminal mediators may be found in the absence of mucosal or distal inflammation; for example, a high sputum eosinophil percentage in severe asthma is not necessarily associated with high bronchial biopsy eosinophil counts and vice-versa (614). In some instances, even systemic markers of inflammation may be more useful than assessing airway inflammation using induced sputum. A recent report investigating 111 asthmatics (22 totally controlled, 47 well controlled, 42 controlled) found that asthma control was more associated with reduction in blood and airway wall eosinophils than sputum eosinophils (776). Adding further complexity, it has been speculated that an increase in the number of eosinophils in the lumen may not be indicative of ongoing inflammation, but could simply be due to resolution of inflammation (750), and that evidence of eosinophil lysis (presence of extracellular eosinophil granules) rather than simply the presence of eosinophils, may be a better indicator of airway pathology (777). Although both methods used in this thesis are well-characterised, FENO and induced

sputum are capable of sampling only specific compartments of the airways. Induced sputum may not necessarily provide a measure of inflammation in the mucosa or distal airways, whilst exhaled FENO appears to be only *generally* indicative of eosinophilic inflammation and is affected by atopy in the absence of asthma.

Regarding (ii), it is increasingly apparent from a number of studies that airway inflammation in asthma is temporally affected by a number of factors and that when assessing sputum “... *a single measurement alone will not suffice*” (764). Cross-sectional assessment may simply not be indicative of persistence of an inflammatory phenotype, despite previous suggestions to the contrary (12,494). In particular, the use of ICS in a large number of the asthmatics studied may lead to the misclassification of inflammatory phenotypes, as ICS has clearly been shown to reduce airway eosinophils. In support, alteration in treatment in the nested study described in Chapter 9 led to a reduction in eosinophils in EA and a change in inflammatory phenotype in over 50% of participants. ICS use has also been shown to increase sputum neutrophils in asthmatics (28), although a recent meta-analysis shows that ICS use may result in a *reduction* in airway neutrophils in COPD (778); furthermore, in Chapter 9, we observed a decrease in neutrophils in some asthmatics with NEA with optimised treatment. The interaction between neutrophils and ICS may therefore not be as simple as previously thought.

When considering (iii), it is difficult to discern what the most ‘useful’ biomarker of airway inflammation in asthma is. Measurement of sputum eosinophils is widely reported and accepted but may be subject to considerable temporal variability. An alternative approach may be to identify biomarkers that represent TH₂-mediated inflammation, but are not as variable as airway eosinophils. Sputum basophils may be such an alternative marker of TH₂-

mediated inflammation, although the study described in Chapter 6 did not have the sample size to adequately confirm whether their numbers were more stable than eosinophils. Other less variable markers of TH₂-inflammation may be derived from structural and long-lived resident cells that are less prone to rapid changes than granulocytes, such as epithelial cells (e.g. periostin, produced during IL-4/13 stimulation of epithelial cells (451)) or macrophages (e.g. evidence of previous eosinophil phagocytosis (779)).

With regard to point (iv), in the absence of clear evidence of inflammation in approximately 50% of asthmatic subjects, the cause of asthma symptoms remains unknown. Although evidence from humans and animal models suggests that both adaptive and innate immunity play an important role in asthma pathogenesis, it is not clear what immunological mechanisms (if any) are responsible for NEA. As suggested in Chapters 8 and 9, smooth muscle, epithelial or neurogenic involvement (possibly resulting from, or in association with, remodelling) may lead to symptoms in the absence of current inflammation. Airway remodelling is clearly important in asthma pathology (as described in Chapter 2.2.3), but there are currently only limited methods to conveniently and non-invasively assess it (Chapter 2.5.8). In particular, when considering the importance of autonomic airway innervation in maintaining bronchial tone, very little research has been conducted to assess the role of airway nerves in different phenotypes of asthma (780). As it is possible that the lack of response to conventional or TH₂-based therapies in some asthmatics may be because their symptoms are primarily due to remodelling, or altered muscle or neural processes, rather than active inflammation (as suggested by Holgate (340)), there is clearly a need for the measurement of the different aspects of airway remodelling in asthma, particularly in NEA. Moreover, the mechanisms responsible for airway remodelling in the absence of inflammation are not clear and further studies are required to more fully characterise such

mechanisms. Possible candidates include the mechanical process of bronchoconstriction itself (123); a recent rodent-based study has provided evidence that early-life viral bronchiolitis may actually lead to remodelling on a macroscopic level. This involved structural lesions in the small airways resulting in abnormal airway branching and reduction in luminal area, persisting into maturity (781). It has yet to be confirmed if a similar process occurs in humans.

10.5 Strengths and limitations

The studies described in this thesis have a number of strengths. Firstly, whilst adding to the relatively limited body of data regarding inflammatory phenotypes in asthma, the studies described in Chapters 4, 5, 6, and 9 also demonstrated how flow cytometry may be successfully used during investigations of airway inflammation. Secondly, the assessment of phenotype stability conducted in Chapter 9 emphasised the importance of the effects of treatment on the phenotype observed, and suggests that a single examination is not enough to identify inflammatory phenotypes. Thirdly, the populations studied within this thesis were unselected and therefore represented a ‘snapshot’ of airway inflammation in asthma in the general population, rather than in a secondary- or tertiary-care setting, in which asthmatics may be more likely to be both more severe and using higher doses of ICS. Finally, the study populations covered a wide age range. Participants were generally aged between 13 and 65 years but in some of the studies were as young as 6yrs and as old as 80yrs, allowing assessment of the importance of age in determining inflammatory phenotypes.

In the studies described in this thesis, asthma was identified on the basis of standardised questionnaire, rather than spirometry or AHR. This inclusiveness could be considered both a strength and a weakness. In some cases, it is possible that misclassification may have occurred. In particular, the relatively subjective nature of a questionnaire-based approach means that some ‘asthmatic’ subjects may have had: previous (but not current) asthma; incorrectly perceived asthma symptoms; or another condition associated with wheezing. However, the use of stringent criteria based on objective measures would exclude the majority of asthmatics in the community (140). Furthermore, the absence of objective measurements during assessment does not preclude asthma (66), as many subjects with mild or intermittent asthma (commonly observed in the general population) often have normal lung function and BDR measurements at the time of assessment (782). Also, as described in Chapter 2.2.4, a questionnaire-based approach has to been shown to be more appropriate for identifying physician-diagnosed asthma than some objective measures, such as AHR (146). Ultimately, the use of the questionnaire to define asthma may have resulted in some of the asthmatics included in our studies not corresponding to the ‘classical’ idea of asthma. However, in light of the recent PRACTALL consensus report which states that *‘it will be necessary to classify patients according to the underlying disease mechanism rather than relatively crude clinical characteristics such as BDR or BHR’* (6), the absence of an objective measure in the definition used may have been a strength and is unlikely to have resulted in invalid results.

Most of the studies described within this thesis were limited to one single assessment per participant (with the exception of the nested study described in Chapter 9) and the majority of asthmatics studied were stable or well controlled at the time of assessment, in accordance with the ethical guidelines used. As described above, a single measurement may not be

enough to accurately identify inflammatory phenotypes of asthma. In particular, as reported in Chapter 9, ICS use was associated with a reduction in both EA and NA. It is therefore possible that inflammation may have been suppressed in asthmatics currently using ICS, who made up a substantial proportion of the asthmatics studied (approximately 40% in Chapter 8 and 76% in Chapter 9). It may therefore be more appropriate to assess airway inflammation when asthma is sub-optimally controlled or treated (as in Chapter 9) or during exacerbation. However, this approach may lead to different problems when identifying phenotypes. In particular, environmental exposures responsible for exacerbations may be associated with different inflammatory profiles (i.e. viral or bacterial infection may lead to airway neutrophilia or allergen exposure may lead to eosinophilic inflammation), regardless of the underlying pathology of asthma. It is therefore possible that different inflammatory phenotypes may be detected at different times with varied environmental exposures in the same individual, suggesting that studies investigating inflammatory phenotypes during exacerbation may also have to objectively identify and measure the cause of the exacerbation.

Finally, due to limitations of resources and recruitment, the number of subjects involved in each study was relatively small, although similar to comparable published studies. It is possible that, in some studies (such as those described in Chapters 8 and 9), greater numbers may have resulted in statistically significant findings when we observed a non-significant trend. Examples of such non-significant trends include those observed with increased severity of EA in adolescents in Chapter 8 and the increase in FEV₁ predicted (%) observed in NEA with optimal treatment in Chapter 9. Ideally, future studies examining the nature of inflammation in asthma should be larger, and thus provide adequate power for statistical analyses.

10.6 Recommendations for future research

On the basis of the findings of the studies described in this thesis, and other recent publications and developments in the field of asthma research, there are a number of recommendations that should be considered when conducting future studies of inflammatory asthma phenotypes.

Multiple assessments

As alluded to throughout this thesis, a single assessment may not be definitive for the assessment of inflammation and inflammatory phenotypes in asthma. There are now a number of studies available showing considerable intra-individual variation in differential counts of sputum eosinophils and neutrophils. Ideally, assessment of asthma should be conducted at multiple time points (preferably including prior to ICS treatment) to comprehensively characterise variability of inflammation, airway obstruction and symptoms within a given individual. Whilst assessing longitudinal changes in airway inflammation, it may also be useful to investigate the role of particular environmental or occupational exposures with specific asthma phenotypes. Such investigations (particularly in centres that have reported NA) may clarify the aetiology of NA and explain why NA has been infrequently observed in New Zealand.

Asthma in the elderly

Until recently, few studies have specifically focussed on asthma in the elderly. Indeed, this group is generally excluded from clinical trials as they often have co-morbidities or use multiple medications, and do not necessarily conform to the ‘normal’ definitions of asthma

used in clinical trials (140). Despite this, it is estimated that 5-15% of those over 65 years old have asthma (783). This probably underestimates the number, as asthma in the elderly is under-diagnosed, and they are more likely to be diagnosed with COPD (784). Difficulty with diagnosis is a particular problem when assessing airway disease in older populations, as there are often inflammatory, pathological or clinical characteristics of both asthma and COPD. This has led to the description of ‘overlap syndrome’ ((486); discussed in Chapter 2.4.4). Regardless of the diagnostic label, this older asthma/COPD/overlap population are likely to grow, as the population as a whole, and asthmatics in particular, get older.

When assessing airway inflammation in older patients, our data suggest that increased airway neutrophilia is a normal feature of ageing in both asthmatics and non-asthmatics. This should be taken into account in any future studies assessing the importance of neutrophilic inflammation. In particular, with an ageing population, it will become more important to accurately differentiate a disease state from what is ‘normal’ at any given age, meaning that appropriate reference values and guidelines are required. This is made more difficult by the considerable variation and overlap of respiratory conditions such as asthma and COPD and the associated physiological and clinical parameters in older adults as noted above. However, as Moores states in a 2008 discussion about accurate identification of different respiratory diseases, (127) “...*Does it matter? Maybe what we really need to understand is reversing what’s there...what’s the inflammatory process, and targeting the inflammatory process rather than worrying about whether it’s asthma with COPD or COPD with asthma.*”

As the inflammatory process in airway disease in the elderly may be neutrophilic, this population may be particularly well suited to therapies targeting neutrophils (243). Further

work is also required to fully elucidate the cause of increased neutrophilia with ageing and, also, to determine the actual importance of TH₂-mediated processes in elderly asthmatics.

Assessment of airway remodelling

Biopsy-based sampling allows localised assessment of airway tissue remodelling, but bronchial biopsies are highly invasive and difficult to conduct. There are assays available for measuring mediators indirectly associated with airway remodelling (reviewed in (660)), but the majority have yet to be fully validated, and are involved in numerous other biological pathways (and therefore not specific to airway remodelling). Recent data suggest that high resolution computed tomography (HRCT) (666,667), ultra-short echo-time (TE) magnetic resonance imaging (MRI) (785) or endobronchial ultrasound (668) may be useful alternative approaches for measuring remodelling, but these methods have yet to be fully validated for this purpose and, again, are difficult to conduct in population-based studies. Thus, there are currently only limited methods to conveniently and non-invasively assess airway remodelling in asthma. Further development and use of such methods should be focussed on the 50% of asthmatics without clear evidence of inflammation, as it is possible that airway remodelling (affecting one or all of the epithelial, mesenchymal, muscle or neural compartments), rather than inflammation, may be responsible for asthma symptoms in these cases.

Sputum induction

Previous studies have attempted to use a simple method of sputum induction using hypertonic saline in epidemiological studies (426) but the procedure remains time-consuming, and difficult to conduct on a routine basis. Despite this, sputum induction remains possibly the

most well characterised method for non-invasive airway investigation of the inflammatory mechanisms and phenotypes associated with respiratory symptoms. Recent studies have suggested that mannitol dry powder (MDP) may be a more simple and convenient method to induce sputum than using a nebuliser and hypertonic saline; it also allows concurrent measurement of AHR (533-535) . Moreover, in some populations (such as children), assessment using MDP has been shown to be simpler, quicker and better tolerated than an equivalent test with hypertonic saline or methacholine (533). Although it has yet to be fully validated, mannitol has the potential to be particularly useful in field studies, as application simply involves inhalation of the contents of a MDP capsule through a delivery device (Osmohaler®).

The other major issue with induced sputum when conducting epidemiological studies (i.e. that samples need to be processed in a relatively short time) may be circumvented using rapid fixation techniques (579), or freezing (Chapter 4, (580,581); to the author's knowledge, none of these solutions has been used on a large scale to-date.

Polychromatic flow cytometric analysis of induced sputum

At the time of study, there were only a limited number of fluorophores and reagents available for assessment of multiple flow cytometric markers within a single sample. This meant that several independent antibody panels with a limited number of fluorophores were required per sample, with each panel requiring a separate aliquot of cells, different handling and further validation. As technology and reagents have developed, it is now possible to investigate large numbers of markers simultaneously. Flow cytometric analysis could currently be extended to

identify several more cell populations within a single antibody panel, as has been conducted with a 12-colour panel in peripheral blood (700). However, as noted above, flow cytometry may not be a suitable method for use in large population studies as it is labour and resource intensive.

Standardisation and collaboration

There is an increasing awareness that many current methods used for classifying asthma have been inadequate for identifying patients who are likely to benefit from particular treatment and management regimens. Furthermore, many important and heavily cited studies examining the pathophysiology of asthma have assessed only relatively small numbers of asthmatics; preselected populations on the basis of asthma severity, atopy, BDR or BHR; and have speculated that observations from such studies could be ascribed to the majority of asthmatics. However, this generalisation, and a lack of identification of the underlying pathophysiology of asthma, can (and has) led to equivocal or ambiguous results in clinical trials of TH₂-targeted therapies. As noted by Lotvall (6):

‘The outcomes of many clinical trials in asthma have been biased by adopting inclusion and exclusion criteria that require subjects to conform to the generic description of the disease...trials performed in this way do not necessarily establish whether a studies medication works equally well among all patients with the asthma syndrome’

Inclusion on the basis of non-pathological criteria in clinical trials increases the ‘noise to signal’ ratio and decreases the chance of obtaining significant results when using agents that target specific pathways. It is also possible that one of the (many) reasons why genetic studies have shown inconsistencies, or only weak associations, with asthma susceptibility genes has

been the limited phenotyping of the populations studied. Therefore, one of the key points of the PRACTALL report suggests that *‘population-based studies aimed at identifying genetic and environmental links to asthma must ... focus on more homogeneous groups rather than groups of patients with asthma with clearly different disease features, because they then are likely to include patients with different pathophysiologies’* (6).

These limitations have led to the recent development of international programs, consensus reports and calls for multicentre collaborations. For example, iCAALL is a collaborative program between the AAAAI, ACAAI, EAACI and the World Allergy Organization, primarily designed to co-ordinate delivery of information in the area of asthma and allergy using international consensus statements (ICONS) providing evidence-based information about e.g. eosinophilic disease or food allergy (786,787). On a more practical level, PRACTALL is a consensus report from AAAAI and EAACI specific to the identification of asthma endotypes and how this process can be used in clinical studies and drug design to target patients most likely to benefit (6). The PRACTALL report suggests that the most appropriate approach to identify asthma endotypes may be the use of clustering multiple variables that describe different components of the disease, such as immunology, physiology, triggers, treatment response or symptoms. There has been a recent increase in the number of studies using such statistical modelling techniques, including cluster analysis or factor analysis, to allow non-hierarchical assessment of asthma subgroups based upon multiple parameters (e.g. (27,395,396)). However, the PRACTALL report also acknowledges that, to be useful, such an approach would have to be confirmed by detailed pathophysiological assessment and replication.

Sputum induction is one of the few methods available to non-invasively examine asthma pathophysiology but, as described, is unlikely to be easily and conveniently used in a standardised way in very large populations within single institutions. However, if the procedure was fully standardised between several centres (in a similar way to that conducted in Chapter 7), it could be used to compile datasets representative of hundreds to thousands of participants and allow comparison of the prevalence and characteristics of inflammatory phenotypes of asthma across different geographical locations.

Use of biomarkers in pre-selection of asthmatics, and development of new biomarkers

The airways are not an easy source of tissue for assessment of inflammation in large numbers of subjects. Based on the aforementioned PRACTALL report (6), simple non-invasive biomarkers may be particularly useful for the pre-selection of relatively homogeneous groups within large populations. One possible strategy to identify subgroups would be to firstly include all patients with wheeze, on the basis of a standardised, validated questionnaire. Subjects could then be examined on the basis of relatively conventional methods, such as clinical, demographic and occupational assessment and history, lung function testing, SPT or total IgE measurement, complete blood count, AHR and BDR, and so on. All subjects could then be assessed using FENO measurement as a preliminary measure of TH₂-eosinophilic pathology. In particular, preliminary assessment of steroid-naïve inflammatory phenotypes of asthma could be conducted on a simplistic basis, such as FENO high (TH₂-mediated EA) and FENO low (TH₂-low NEA) (535) (with due consideration to the limitations associated with FENO previously described).

Small clusters representing the extremes of the measured variables (e.g. high FENO/ high reversibility or late-onset/low FENO/irreversible component) could then be selected for more thorough assessment using e.g. a classification tree. In particular, labour-intensive procedures such as sputum induction or BAL could be conducted on subgroups of these relatively 'homogeneous' groups, to determine if there are indeed consistent pathophysiological characteristics. These individual subgroups could then be assessed for common characteristics, such as response to treatment, disease history, genetics, and environmental triggers. This approach may potentially allow development of specific treatment and interventions for different asthma subgroups.

In addition to FENO, other systemic, serum, urine, and metabolomic biomarkers also show considerable promise for identifying asthma phenotypes in epidemiological studies. However, they are still some way from being fully validated for this purpose. The use of blood counts to describe granulocyte inflammatory patterns corresponding to previously described asthma inflammatory phenotypes (505) is particularly appealing, as samples can be collected from all patients in a simple and well-established manner (phlebotomy). Although measurement of blood granulocytes may be indicative of different types of airway inflammation, more work is needed to assess agreement between granulocyte counts in the different compartments: blood versus airways. Similarly, the recent use of periostin as a systemic indicator of allergic airway inflammation (788) appears promising but it is as yet unclear if this marker will simply be another indicator of atopy and allergy, rather than representing airway pathology in particular.

As an alternative to systemic biomarkers, exhaled breath and EBC had been heralded as amongst the best available methods to directly and non-invasively sample the lower airways, but may not have lived up to their initial promise. Of the variables determined when analysing EBC, pH has been one of the clearest indicators of asthma (789) and asthma exacerbation (587), but this can be influenced by non-respiratory disease and sample storage. Furthermore, with the exception of pH, total protein and H_2O_2 , many markers of interest (in particular cytokines (588)) cannot be detected in more than 50% of EBC samples (768,790) .

Nasal lavage (NAL) has been suggested to be another potentially useful method for the assessment of airway inflammation in asthma (639) and is considerably easier to conduct in population-based studies than sputum induction. However, nasal lavage obtains samples from the upper, not lower, airways. Further studies are required to confirm the validity of NAL data in individuals with allergic rhinitis but no lower airway involvement, or allergic lower airway inflammation with no upper airway involvement.

Despite the current limitations, the technology available for assessing exhaled air, EBC, NAL and systemic protein/metabolites is under constant development. With methods such as proteomic profiling (657) for example, it may soon be possible to identify distinct inflammatory phenotypes on the basis of proteomic signatures. Alternatively, microarrays could be used to examine gene expression signatures in asthma phenotyping (791). Furthermore, novel biomarkers are also continually being identified and developed. For example, increased levels of the proteins neopterin and IP-10 in sputum supernatant have recently been shown to indicate asthma exacerbation (768). However, limited access to the relevant biological tissues (i.e., samples from the airways) is always likely to hinder

assessment in large populations and therefore additions and improvements to the current methods for non-invasive assessment of airway inflammation in asthma are required. Once again, it still remains unclear which are the ‘best’ biomarkers in different situations, and there is no ‘gold standard’ non-invasive biomarker(s) available to help fully resolve the underlying mechanisms, or even to identify asthma.

10.7 Conclusions

It is increasingly clear that asthma is a heterogeneous condition and that different pathophysiological mechanisms may be important in different groups of asthmatics. As inflammation is generally believed to be the key mechanism responsible for asthma symptoms, simple, cost-effective, non-invasive and accurate methods for identifying airway immunopathology in asthma phenotypes are required.

In this thesis, FENO measurement and induced sputum were the methods used. Both methods have their strengths and weaknesses and, as such, data produced using these methods should be interpreted with caution. In particular, the use of a data from a single assessment to define inflammatory phenotype may be inappropriate in some circumstances. Despite their limitations, both methods provide a convenient means to investigate the association between asthma pathology and clinical characteristics in the general population. The addition of flow cytometry, in particular, increases the resolution of investigations of induced sputum, and allows detection of rare cell populations associated with pathology; it is likely to be useful for smaller, in-depth studies of asthmatic subgroups.

Eosinophilic asthma represented between 40 and 50% of asthmatics studied. However, neutrophilic asthma was relatively rare outside older age groups and approximately half of all subjects with current asthma symptoms had no evidence of any kind of airway inflammation. In the absence of inflammation, the causes and mechanisms underlying NEA remain unknown. Although it is possible that the methods used were not adequate for assessing inflammatory mechanisms in NEA, it is also possible that *non-inflammatory* mechanisms may be important. Further investigations of remodelling, in particular, epithelial, neural, muscular and vascular involvement in NEA, are warranted. A better understanding of the disease mechanisms underlying NEA and the associated causal environmental exposures, is necessary to appropriately guide the development of more effective therapies and control measures for NEA, which makes up a considerable proportion of asthma. Also, in the absence of detectable inflammation in a large proportion of asthmatics in the general population and the paucity of knowledge about the specific mechanisms underlying this phenotype, it may perhaps be more appropriate to return to a definition of asthma as a functional disorder, rather than specifically an *inflammatory* disorder of the airways.

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Appendix 1: Publications contributed to during PhD study

1. Weinkove R, **Brooks CR**, Carter JM, Hermans IF, Ronchese F. Functional invariant natural killer T-cell and CD1d axis in chronic lymphocytic leukemia: implications for immunotherapy. *Haematologica* 2013;98(3):376-84.
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5. **Brooks CR**, van Dalen CJ, Hermans IF, Douwes J. Identifying leukocyte populations in fresh and cryopreserved sputum using flow cytometry. *Cytometry B Clin Cytom* 2013;84(2):104-13.
6. **Brooks CR**, Siebers R, Crane J, Noss I, Wouters IM, Sander I, Raulf-Heimsoth M, Thorne PS, Metwalie N, Douwes J. Measurement of β -(1,3)-glucan in household dust samples using *Limulus* amoebocyte assay and enzyme immunoassays: an inter-laboratory comparison. *Environ. Sci: Processes Impacts* 2013;15:405-11.
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***Appendix 2: Statement of contributions for published papers
included in thesis***



MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal 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: Collin Brooks

Name/Title of Principal Supervisor: Prof Jeroen Douwes

Name of Published Research Output and full reference:

Measurement of exhaled nitric oxide in a general population sample: a comparison of the Medisoft HypAir FENO and Aerocrine NIOX analysers. *Journal of Asthma* 2011; 48 (4):324-328.

In which Chapter is the Published Work: 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 90%
and / or
- Describe the contribution that the candidate has made to the Published Work:

Candidate's Signature

Date

Principal Supervisor's signature

Date



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Name of Candidate: Collin Brooks

Name/Title of Principal Supervisor: Prof Jeroen Douwes

Name of Published Research Output and full reference:

Identifying leukocyte populations in fresh and cryopreserved sputum using flow cytometry. Cytometry Part B (Clinical Cytometry) 2013;84B: 104-113

In which Chapter is the Published Work: 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 90%
and / or
- Describe the contribution that the candidate has made to the Published Work:

Candidate's Signature

Date

Principal Supervisor's signature

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Name of Candidate: Collin Brooks

Name/Title of Principal Supervisor: Prof Jeroen Douwes

Name of Published Research Output and full reference:

Invariant natural killer T cells and asthma: immunological reality or methodological artefact? *Journal of Allergy and Clinical Immunology* 2010; 126 (4):882-885

In which Chapter is the Published Work: 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 90%
- and / or
- Describe the contribution that the candidate has made to the Published Work:

Candidate's Signature

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TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal 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: Collin Brooks

Name/Title of Principal Supervisor: Prof Jeroen Douwes

Name of Published Research Output and full reference:

Relationship between airway neutrophilia and ageing in asthmatics and non-asthmatics.
Respirology 2013; March: epub ahead of print

In which Chapter is the Published Work: 7

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 90%
- and / or
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are found in patients who were treated by eradicating the underlying cause of their eosinophilia.¹⁻³ However, our measurements of circulating IL-33 levels did not support this mechanism.

In the serial tests of 6 patients with IAEP, their symptom scores were remarkably improved within 5 to 10 days of admission (Fig 2). The levels of ECP and CRP measured within 5 to 10 days and 15 to 30 days after admission were significantly lower than those on the second and third days after admission. However, IL-33 levels during the first 3 days after admission were not significantly different from those observed within 5 to 10 days or 15 to 30 days after admission. Notably, IL-33 levels varied between individuals and did not correlate with symptom scores. Two of the 6 patients with IAEP (patients 1 and 2; Fig 2) presented high levels (>1000 pg/mL) of IL-33 regardless of the reduction of their symptom scores, and 1 of the 6 patients with IAEP (patient 3) presented continuously low levels. These results suggest that circulating IL-33 is not a useful severity marker for IAEP. A plausible explanation for this is that an individual heritable trait could affect the concentration of circulating IL-33 more so than the disease state. For example, *in vivo* IgE concentrations are more strongly related to individual genetic variation than to specific IgE response states.⁸

Our study for the first time identified elevated circulating IL-33 levels in patients with eosinophilia. However, on comparing IL-33 levels with previous eosinophilia-related markers and CRP levels, we found that IL-33 levels were less reliable for detecting PE or for the resolution of an eosinophil-mediated immune response and indicators of disease activity of IAEP. Further studies are required to identify the individual levels of each IL-33 form (full-length pro-form and cleaved forms) or inactive IL-33 by sST2 binding. Plasma ECP levels presented higher PE detection rates than other markers, and their progression correlated with clinical improvement of patients with IAEP. The ECP trends in this study were similar to findings of previous studies of eosinophilic disease.⁵⁻⁷ The data presented here strongly support the use of ECP, rather than IL-33, as a marker of PE and IAEP activity.

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Invariant natural killer T cells and asthma: Immunologic reality or methodologic artifact?

To the Editor:

A role for invariant natural killer T (iNKT) cells in the cause of asthma has been shown in mice,¹ but the evidence in human subjects is equivocal.²⁻⁵ Matangkasombut et al,⁶ in a recent issue of the *Journal*, provided further evidence of higher iNKT cell numbers (up to 64% of all CD3⁺ T cells) in bronchoalveolar lavage fluid of subjects with severe asthma compared with those seen in subjects with well-controlled asthma, who in turn had higher numbers than seen in nonasthmatic subjects. If true, this might have major implications for our understanding of asthma's pathology.

Some of the discrepancies between studies might be due to the use of different cytofluorimetric gating strategies when assessing iNKT cells in bronchoalveolar lavage fluid. It has already been suggested^{4,5} that the iNKT cells identified in the initial study² are likely to be alveolar macrophages on the basis of forward and side scatter characteristics. The use of a simple, 2-step CD3⁺SSC^{low} strategy (as subsequently used by Matangkasombut et al⁶) allows exclusion of large cells, such as macrophages, but is not capable of excluding nonspecific binding to nonviable cells and debris. To investigate the effectiveness of this 2-step strategy, we compared the detection of iNKT cells in induced sputum (IS) of 25 subjects with physician-confirmed asthma and 19 nonasthmatic subjects by using flow cytometry against a more stringent approach, which involved gating out doublets and nonviable cells.

TABLE I. Clinical characteristics of participants who successfully completed sputum induction

	Asthmatic subjects	Nonasthmatic subjects	P value
No. (female)	25 (14)	19 (11)	
Asthma severity (no.)			
Intermittent	32% (8)		
Mild persistent	24% (6)		
Moderate persistent	44% (11)		
Age (y)	28 (25.5-41)	33 (24-43)	.8495
Atopy (no.)	80% (20)	52.6% (10)	.1007
FEV ₁ (% predicted)	93 (82-98)	102 (93-111)	.0062
FEV ₁ /FVC ratio	0.8 (0.71-0.81)	0.85 (0.83-0.87)	.0040
Sputum eosinophils (%)	0.99 (0.24-3.67)	0 (0-0.46)	.0008

Values are expressed as numbers, percentages, or medians (IQRs). Asthma classification is based on Global Initiative for Asthma guidelines. *P* values were calculated by using the Mann-Whitney *U* test or Fisher exact test. *FVC*, Forced vital capacity.

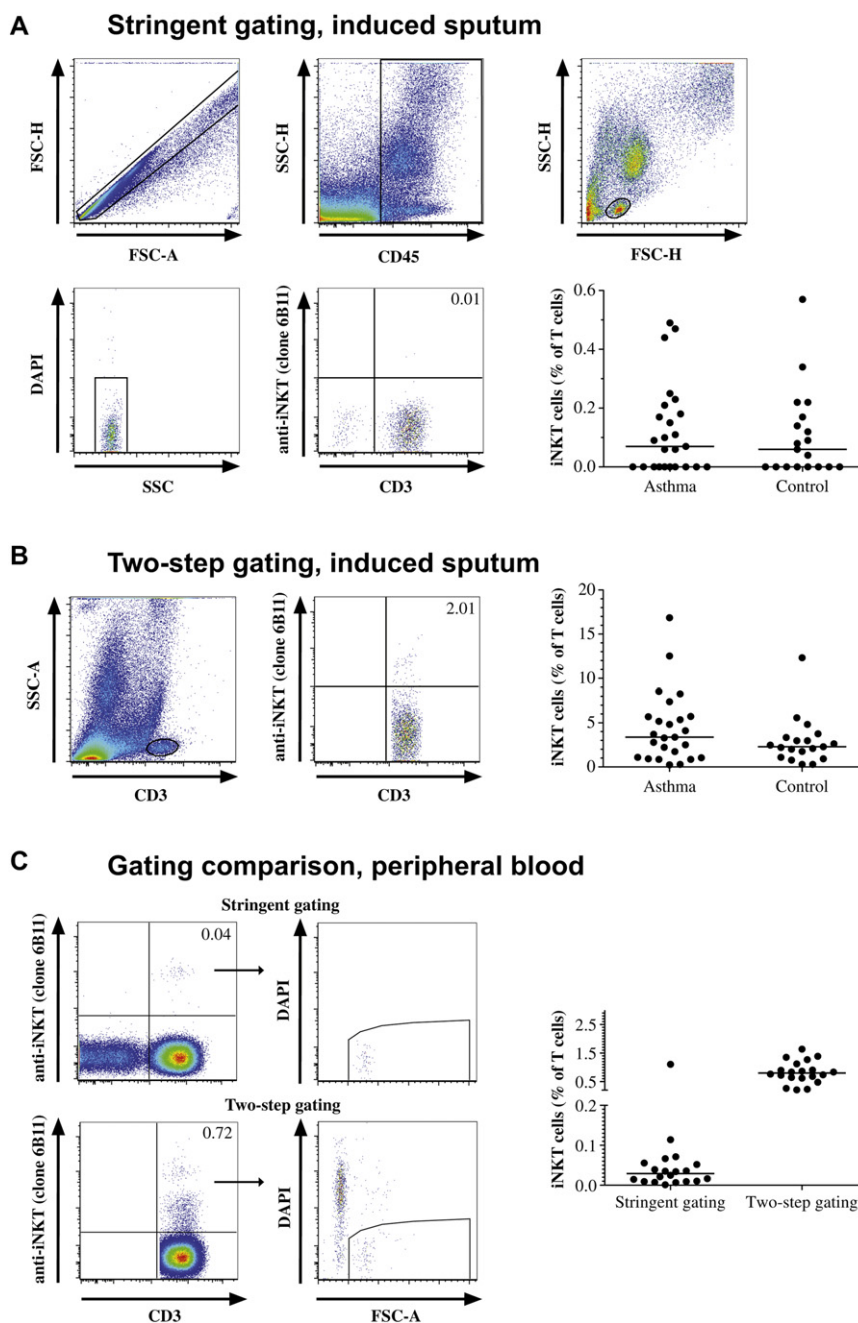


FIG 1. The effect of differential gating on the detection of iNKT cells. **A** and **B**, Dot plots showing a stringent gating strategy (Fig 1, **A**) and 2-step strategy (Fig 1, **B**) in a representative sample and the overall percentage of iNKT cells detected. Similar discrepancies were observed with peripheral blood samples (**C**). Median values are represented by a horizontal bar. DAPI, 4'-6-diamidino-2-phenylindole; FSC, Forward scatter; SSC, side scatter.

Fifty-four subjects provided informed consent and underwent spirometry, skin prick testing, and sputum induction with hypertonic saline.⁷ Four asthmatic subjects and 5 nonasthmatic subjects did not provide an adequate IS sample and were excluded, and we did not collect a sample from a subject with severe asthma whose FEV₁ value was too low to safely undergo hypertonic saline challenge. The clinical characteristics of subjects successfully completing sputum induction (81.5%) are presented in Table I. The study was approved by the Upper South A Regional Ethics

Committee, New Zealand. IS was incubated with dithiothreitol (Sputasol; Oxoid Ltd, Hants, England), filtered through a 60- μ m filter, counted, and assessed for viability by using trypan blue. Cells obtained were washed and resuspended in RPMI. Cells for flow cytometry were blocked with human polyclonal IgG and stained for 30 minutes on ice. iNKT cells were stained with either phycoerythrin-conjugated CD1d tetramers loaded with α -galactosylceramide (a gift from Vincenzo Cerundolo, University of Oxford, Oxford, United Kingdom) or phycoerythrin-conjugated

clone 6B11 antibody, which recognizes the CDR3 region of the invariant V α 24-J α Q T-cell receptor chain. Other reagents used were anti-CD3–fluorescein isothiocyanate, anti-CD19–peridinin-chlorophyll-protein, and anti-CD45–allophycocyanin (BD Biosciences, Franklin Lakes, NJ). 4'-6-Diamidino-2-phenylindole was added to allow exclusion of dead cells. Flow cytometric analyses were conducted with a BD LSR II and FlowJo software (TreeStar, Inc, Ashland, Ore) with a median of 115,892 events (interquartile range [IQR], 83,973–198,337 events) analyzed per sample. Preliminary experiments comparing 6B11⁺ and tetramer-positive staining of peripheral blood–derived CD3⁺ lymphocytes showed strong correlation between these iNKT-specific reagents ($r^2 = 0.97$). They also showed that blood-derived iNKT cells could be successfully detected with either reagent when spiked into a sputum sample at less than 10 cells per 200,000 sputum cells (data not shown).

Using the more stringent cytofluorimetric gating strategy, we found little evidence of an increased presence of iNKT cells in asthmatic subjects. In particular, in no sample did more than 0.57% of all T cells express the invariant V α 24-J α 18 T-cell receptor by using either tetramer or 6B11, and no significant difference in iNKT cell frequency between asthmatic and non-asthmatic subjects was found (median of 0.07% [IQR, 0% to 0.17%] vs 0.06% [IQR, 0% to 0.20%]; $P = .75$, Mann-Whitney U test; Fig 1, A). However, a reanalysis of our data using the gating strategy published by Matangkasombut et al⁶ showed very different results, with up to 16.9% of the CD3⁺SSC^{low} population now labeled tetramer positive or 6B11⁺ in asthmatic subjects. A similar increase in iNKT numbers was detected in nonasthmatic subjects, such that (as with the previous gating strategy) no significant difference was observed between groups (median of 3.38% [IQR, 1.07% to 5.71%] vs 2.29% [IQR, 1.09% to 3.35%]; $P = .18$; Fig 1, B). Subsequent analysis after subdividing asthmatic subjects on the basis of severity (Table I) or control also showed no statistical differences when using either gating strategy. Further examination of CD3⁺ tetramer/6B11⁺ events detected by using the 2-step strategy in both IS and peripheral blood confirmed that most were 4'-6-diamidino-2-phenylindole positive and therefore nonviable and were smaller in size (forward scatter) than expected for viable lymphocytes (Fig 1, C). Exclusion of these nonviable events led to detection of similar numbers of iNKT cells (<0.5% of CD3⁺SSC^{low} events) compared with the more stringent method. Using a similar approach for iNKT cell analysis, we found equally discordant results for 18 peripheral blood samples of nonasthmatic volunteers (Fig 1, C).

These results suggest that iNKT reagents bind in a nonspecific fashion to nonviable cells. Suboptimal gating strategies might therefore lead to overestimation of iNKT cell numbers because of the misidentification of nonviable cells as iNKT cells. Our data also highlight the requirement for adequate precautions to be taken when using flow cytometry to detect any rare cell populations and not only iNKT cells because nonspecific binding to dead cells and debris might have significant effects on observed cell frequencies.

Our findings have several limitations, and results should therefore be interpreted with caution. First, the asthmatic population consists of subjects with mild-to-moderate asthma only, and results might be different for subjects with severe asthma, although 5 asthmatic subjects in the present study (20%) had poorly controlled asthma.

Second, cells were obtained from IS and therefore have generally lower viability (68% in our study, with no significant difference between population groups) than bronchoalveolar lavage fluid. However, our experiments with peripheral blood (Fig 1, C) show that reagent binding to dead cells and debris is still apparent in high-viability tissues.

Third, IS samples represent cell populations present in the central rather than distal airways.

Finally, we conducted some of our experiments with the 6B11 antibody rather than the CD1d tetramer. However, preliminary experiments showed that this antibody provided comparable results to the tetramer⁸ and in many cases improved the quality of staining by reducing nonspecific staining. The use of unloaded tetramers as controls, as applied by others,^{2,6} could be perceived as a strength. However, loaded and unloaded tetramers might vary in their nonspecific binding propensity, particularly if they do not come from the same lot or have been handled differently.⁹ Therefore unless it can be demonstrated that this is not the case, there is a real possibility that the methodologic issues described above are not adequately addressed by the use of unloaded tetramers.

In summary, our study emphasizes the importance of stringent gating strategies in analyzing iNKT cells and suggests that simple gating strategies might lead to false-positive results, possibly explaining the mixed evidence regarding the role of iNKT cells in asthma. Our study also further supports previous evidence^{4,5} suggesting that iNKT cells are not increased in the central airways in subjects with mild-to-moderate asthma. Although we found no difference in iNKT cell numbers between asthmatic and nonasthmatic subjects, we do not rule out the possibility, as suggested by animal models, that iNKT cells might play a role in at least some asthma phenotypes. However, until the methodologic difficulties surrounding the detection of iNKT cells in lung-derived tissue are adequately addressed, the true importance of iNKT cells in the pathology of human asthma will remain unknown.

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Corrections

With regard to the May 2009 article by Ohgiya et al (*J Allergy Clin Immunol* 2009;123:1157-62), the accession number is incorrect as given. The correct accession number is AB453156.

With regard to the “Anaphylaxis” chapter in the 2010 Primer on Allergic and Immunologic Diseases (*J Allergy Clin Immunol* 2010;125:S161-S181), several points should be corrected or clarified: On page S173, in the 2nd line of the 1st paragraph, the phrase “...too low for children weighing more than 30 kg...” should read “...too low for **some** children weighing more than 30 kg...” In the 6th line of the same paragraph, the sentence “...as ascertained by using computed tomographic scans...” should read “...as ascertained by using **ultrasound or** computed tomographic scans...” Finally, on page S174, lines 19-20 of the 1st paragraph, in the phrase “...enhances blood flow in the coronary arteries because its beta-2 adrenergic action leads to increased myocardial,” “beta-2” should be “beta-1.”