

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**NON-INVASIVE ASSESSMENT OF AIRWAY
INFLAMMATION IN ASTHMA**

**A thesis by publications presented in partial fulfilment of the requirements
for the degree of**

Doctor of Philosophy

In

Public Health

Massey University, Wellington

New Zealand

Collin Richard Brooks

2013

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.

Acknowledgements

There are a very large number of people who are responsible for getting me to this point, and I am indebted to each and every one of them. In particular, I'd like to thank:

- My main supervisor, Professor Jeroen Douwes - for his enthusiasm, drive and guidance throughout my PhD, particularly when it came to producing the manuscripts necessary for this thesis, and for giving me enough rope to chase different avenues in the lab when things weren't working out with some of the studies I was involved in. It was Jeroen who first encouraged me to undertake a PhD at CPHR.
- My co-supervisor, Associate Professor Ian Hermans - for being open-minded enough to let a Massey technician into the Malaghan fold in the quest to find iNKT cells in the airways, and for his always considered opinion, support and guidance in all areas of immunology (when I actually got the chance to see him; a lot of my time at the Malaghan Institute was spent sat at the flow cytometer outside normal working hours)
- Doctor Christine van Dalen – for introducing me to the wonderful world of sputum! Also, for being a constant source of support and guidance, walking me through the assessment of airway inflammation (particularly anything to do with neutrophils), for supervising the clinical assessments, and for cajoling me through when all seemed lost...
- Tiz Harding – for being a force of nature when it comes to recruitment, and running the clinical assessments like clockwork. You are always a joy to work with.
- My current office co-dweller Jonathan Coakley - for listening, and letting me bounce ideas around. And for introducing me to Shuggie Otis. Just please stop with the Neil Young. Please.
- Prachee Gokhale - for taking up the slack with the other lab-based studies when I was overwhelmed, and for being constantly cheerful.
- Hils (Hilary Nuttall) - for getting things to work when I can't, and generally making the place tick.
- Doctors Amanda Eng and Fiona McKenzie - for christening me 'Marv'.
- Shirley-Belle Brogan - for her hard work with the FENO measurements and assessments.
- Professor Neil Pearce (now at the London School of Hygiene & Tropical Medicine), and everyone else at the CPHR (I apologise for not mentioning you all by name) - for always being friendly and supportive, and for accepting a 'lab rat' (and an English one at that) into their epidemiological realm with open arms.

- Professor Graham Le Gros and everyone at the Malaghan Institute of Medical Research - for trusting me to play with their very expensive toys nicely. It has been an absolute pleasure to be involved with research up on the hill, even in a fringe capacity. Particular thanks go to Ian's group for keeping me up to date with immunology, Kathryn Farrand and Kylie Price for showing me the ways of the flow cytometer, and Doctor Liz Forbes-Blom for listening to me when I start banging on about non-eosinophilic asthma or macrophages.
- Anyone I have ever 'borrowed' reagents from at the Malaghan Institute. There are more than one or two of you...
- Doctor Jodie Simpson – for letting me use Australian data when I insisted that neutrophilia and aging were associated regardless of asthma status, but couldn't get the numbers in Wellington to put an adequately sized dataset together.
- Doctors Rob Weinkove and Peter Ferguson – occasional wearers of velvet and constant late-night scientific thinkers. With or without a beer, I salute you...
- The Karori Magpies, Wellington Phoenix and Bolton Wanderers FC (in that order of importance) - for providing much (un)necessary distraction during the course of my PhD studies.
- All the study participants, without whom this work could not have been done.
- The Health and Research Council, Asthma Foundation of New Zealand and Massey University for funding.
- I am completely and utterly indebted to my family and friends, home and away - for their unwavering support and patience during the process, despite the tyranny of distance, for overlooking my sometimes grim demeanour, and for quickly learning not to ask me how the PhD was going.
- Finally, and most importantly, Suzy. Who has put up with a lot, but has always been encouraging and supportive, as well as pragmatic (in that most Yorkshire of ways). I love you. And the cats. But mostly you.

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	angiopoeitin
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 lymphopietin
VEGF	vascular endothelial growth factor
YKL	chitinase-3-like protein 1