THE IDENTIFICATION OF BIOMARKERS TO PREDICT RESPONSE TO TREATMENTS IN ASTHMA

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Thesis submitted to the University of Nottingham for the degree of

Doctor of Philosophy

May 2019

Table of Contents

Abstract ..... *xii*

Aims and Hypothesis ..... *xiv*

Acknowledgements ..... *xv*

Declaration ..... *xvi*

Abstracts and Publications ..... *xviii*

List of Tables ..... *xix*

List of Figures ..... *xxii*

Abbreviations ..... *xxvi*

CHAPTER 1 GENERAL INTRODUCTION *1*

1.1 BACKGROUND INFORMATION **2**

1.2 DIAGNOSING ASTHMA **3**

1.2.1 Respiratory symptoms 3

1.2.2 Lung function tests 4

1.2.2.1 Spirometry 4

1.2.2.2 Reversibility test 6

1.2.2.3 Oscillometry 7

1.2.2.4 Bronchial provocation tests 12

1.2.3 Fractional exhaled nitric oxide 12

1.2.4 Allergy tests 13

1.2.5 Asthma severity 13

1.2.5.1 Mild asthma 14

1.2.5.2 Moderate asthma 14

1.2.5.3 Severe asthma 14

1.3 ASTHMA MANAGEMENT 15

1.3.1 Pharmacological management of asthma 18

1.3.1.1 Corticosteroids 19

1.3.1.2 Beta agonists 21

1.3.1.3 Combination inhalers 21

1.3.1.4 Leukotriene receptor antagonists 22

1.3.1.5 Anticholinergics 23

1.3.1.6 Theophylline 23

1.3.1.7 Antibiotics 24

1.3.1.8 Interferon-beta 25

1.3.1.9 Biologic treatments 25

1.3.2 Self management 26

1.4 IMMUNE RESPONSE IN ASTHMA 27

1.5 CYTOKINES INVOLVED IN ASTHMA PATHOGENESIS 29

1.5.1 Cytokines secreted by T cells 30

1.5.2 Pro-inflammatory cytokines 31

1.5.3 Growth factors 32

1.5.4 Chemokines 33

1.5.5 Cytokines secreted from non-immune cells 33

1.6 ASTHMA CLASSIFICATIONS AND PHENOTYPES 35

1.6.1 Classifications 36

1.6.1.1 Early-onset asthma 36

1.6.1.2 Late-onset asthma 36

1.6.1.3 Atopic asthma 36

1.6.1.4 Non-atopic asthma 37

1.6.1.5 Other possible classifications 37
1.6.2 Phenotypes 38
1.6.2.1 Eosinophilic asthma 38
1.6.2.2 Neutrophilic asthma 39
1.6.2.3 Mixed eosinophilic neutrophilic asthma 40
1.6.2.4 Paucigranulocytic asthma 40
1.7 SMALL AIRWAY INVOLVEMENT 41
1.7.1 Small airway physiology 41
1.7.2 Techniques to assess small airway involvement 42
1.7.3 Treatment options 42
1.8 AIRWAY REMODELLING 43
1.9 AIRWAY HYPERRESPONSIVENESS 44
1.10 EXACERBATIONS 46
1.10.1 Exacerbation prevention 47
1.11 OTHER FACTORS INFLUENCING ASTHMA 48
1.12 GLUCOCORTICOIDS 50
1.12.1 Glucocorticoid receptor 52
1.12.1.1 Structure and isotypes 52
1.12.1.2 Receptor activation and signaling 53
1.12.2 Mechanisms of glucocorticoid action 54
1.12.3 Steroid treatment and asthma 55
1.12.3.1 Pharmacokinetic and pharmacodynamic factors of ICSs 56
1.12.4 Adverse side effects 58
1.12.5 Corticosteroid resistance in asthma 59
1.13 BIOMARKERS 60

1.13.1 Fractional exhaled nitric oxide 61
1.13.2 Serum periostin 64
1.13.3 Eosinophils 65
1.13.3.1 Blood eosinophils 66
1.13.3.2 Sputum eosinophils 67
1.14 FUTURE THERAPIES AND DRUG TARGETS 67
1.14.1 Monoclonal antibodies 68
1.14.2 AntI-IgE monoclonal 69
1.14.3 Anti-IL-5 monoclonals 69
1.14.4 Anti-IL-4 receptor alpha monoclonal 72
1.14.5 Future therapies 73
CHAPTER 2 MATERIALS AND METHODS 74
2.1 LABORATORY MATERIALS AND METHODS 75
2.1.1 Buffers and reagents 75
2.1.2 Enzyme-linked immunosorbent assay 75
2.1.3 Antibody microarray 78
2.1.3.1 Plate preparation 79
2.1.3.2 Printing 80
2.1.3.3 Slide processing 80
2.1.3.4 Slide scanning 82
2.1.4 Venepuncture collection 83
2.1.5 Blood processing 83
2.1.5.1 Serum processing 83
2.1.5.2 Sodium heparin processing 83
2.1.6 Sputum processing 85
2.2 CLINICAL MATERIALS AND METHODS 86

2.2.1 Spirometry 86
2.2.2 Fractional exhaled nitric oxide 87
2.2.3 Forced oscillation technique (FOT) 87
2.2.4 Sputum induction 88
2.2.5 Venepuncture 89
2.2.6 Questionnaires 90
2.2.6.1 Asthma Control Questionnaire 90
2.2.6.2 Leicester Cough Questionnaire 91
2.2.6.3 Medical Research Council Dyspnea Scale 91
2.2.6.4 Asthma Control Test 92
2.2.7 Information storage 92
2.2.8 Statistical analysis 92

CHAPTER 3 DEVELOPMENT AND OPTIMIZATION OF CYTOKINE QUANTIFICATION ASSAYS 93

3.1 BACKGROUND INFORMATION 94
3.1.1 Enzyme-linked immunosorbent assay 94
3.1.2 Microarray 95

3.2 METHODOLOGY: MICROARRAY 100

3.3 RESULTS: MICROARRAY 104
3.3.1 Optimizations of the antibody microarray on PLL slides 107
3.3.2 Buffer and slide surface optimizations 111
3.3.2.1 Aminosilane 111
3.3.2.3 Epoxysilane 116
3.3.2.4 Poly-l-lysine 120

3.4 CONCLUSIONS: MICROARRAY 124

3.5 METHODOLOGY: ELISA 125

3.5.1 Optimization of the 384 well ELISA 125

3.5.2 Optimization of the protocol 127

3.5.3 Optimization of bovine serum albumin 128

3.5.4 Validation of 384 well standard curves with 96 well curves 129

3.6 CONCLUSIONS: ELISA 135

3.7 CYTOKINE ANALYSIS 136

3.7.1 Cytokines and biomarkers 138

3.8 DISCUSSION 140

3.8.1 Microarray 140

3.8.2 ELISA 142

3.8.3 Summary 144

CHAPTER 4 DOES A LOW EXHALED NITRIC OXIDE LEVEL EXCLUDE A CLINICAL BENEFIT FROM INHALED CORTICOSTEROIDS IN SUSPECTED ASTHMA; A RANDOMISED, PLACEBO CONTROLLED TRIAL 145

4.1 BACKGROUND INFORMATION 146

4.2 AIMS 147

4.3 STUDY DESIGN 148

4.4 ETHICAL APPROVAL 149

4.5 STUDY PARTICIPANTS 149

4.5.1 Inclusion criteria 149

4.5.2 Exclusion criteria 149

4.6 STUDY SETTING AND PARTICIPANT RECRUITMENT 150

4.7 STUDY INTERVENTION 150

4.8 OUTCOME MEASURES 151

4.8.1 Primary outcome 151

4.8.2 Secondary outcome 151

4.9 POWER CALCULATION 151

4.10 ANALYSIS 151

4.11 RANDOMIZATION SEQUENCE GENERATION, ALLOCATION AND BLINDING 155

4.12 PROTOCOL 155

4.13 RESULTS 158

4.13.1 Baseline demographics 160

4.13.2 Primary outcome 162

4.13.2.1 ACQ7 162

4.13.3 Secondary outcomes 164

4.13.3.1 LCQ 164

4.13.3.2 MRC 164

4.13.3.3 FEV_1 165

4.13.4 Fractional exhaled nitric oxide levels 170

4.13.4.1 Baseline FeNO levels and response to treatment 170

4.13.4.2 Changes in FeNO levels 171

4.13.5 Baseline factors that predict response to treatment 174

4.13.6 Baseline serum cytokine levels 179

4.14 DISCUSSION 184

4.14.1 Using FeNO to predict response to corticosteroids 184

4.14.2 Study limitations 186

4.14.3 Conclusions 189

CHAPTER 5 AN EVALUATION OF INCREASED DOSES OF CORTICOSTEROID IN POORLY CONTROLLED ASTHMA 190

5.1 BACKGROUND INFORMATION 191

5.2 AIMS 192

5.3 STUDY DESIGN 193

5.4 ETHICAL APPROVAL 193

5.5 STUDY SETTING AND PARTICIPANT RECRUITMENT 193

5.5.1 Inclusion criteria 193

5.5.2 Exclusion criteria 194

5.6 STUDY INTERVENTION 194

5.7 OUTCOME MEASURES 194

5.7.1 Primary outcome 194

5.7.2 Secondary outcome 194

5.8 POWER CALCULATION 195

5.9 ANALYSIS 195

5.10 PROTOCOL 195

5.11 RESULTS 198

5.11.1 Baseline demographics 198

5.11.2 Primary outcome 202

5.11.2.1 Inhaled vs. oral corticosteroids 215

5.11.2.2 Defining response to an increased dose of corticosteroid 219

5.11.2.3 Identifying responders to an increased dose of corticosteroid 219

5.11.2.4 Baseline characteristics of responders to an increased dose of corticosteroid 219
..... 222
5.11.3 Long term impact 223
5.11.4 Secondary outcomes 223
5.11.4.1 Cytokine levels in blood serum and plasma 223
5.11.4.2 Cytokine levels in sputum supernatant 224
5.12 DISCUSSION 231
5.12.1 Mechanisms of corticosteroid resistance 234
5.12.2 Study limitations 236
5.12.3 Summary 237
CHAPTER 6 AN EVALUATION OF RESPONSE TO TREATMENT WITH MEPOLIZUMAB IN PATIENTS WITH SEVERE ASTHMA 238
6.1 BACKGROUND INFORMATION 239
6.2 AIMS OF THE AUDIT 242
6.3 STUDY DESIGN 242
6.4 ETHICAL APPROVAL 243
6.5 STUDY SETTING AND PARTICIPANT RECRUITMENT 243
6.5.1 Inclusion criteria 243
6.5.2 Exclusion criteria 244
6.6 STUDY INTERVENTION 244
6.7 OUTCOME MEASURES 244
6.7.1 Primary outcome 244
6.7.2 Secondary outcomes 244

6.8 SAMPLE SIZE 245

6.9 PROTOCOL 245

6.10 ANALYSIS 247

6.11 RESULTS 249

6.11.1 Baseline demographics 251

6.11.2 Primary outcome 261

6.11.2.1 Response to 12 months of treatment 261

6.11.2.2 Responders vs non-responders 270

6.11.3 Secondary outcomes 272

6.11.3.1 Baseline features of responders and non-responders 272

6.11.3.2 Response to treatment 274

6.11.3.3 Features of early response 284

6.11.4 Serum cytokine levels 292

6.11.4.1 Mepolizumab assay validation 297

6.12 DISCUSSION 298

6.12.1 Limitations 301

6.12.2 Conclusion 303

CHAPTER 7 DISCUSSION 304

References 310

APPENDICES 334

A: Consent Form 335

B: Participant Information Sheet 336

C: REC Favourable Opinion 340

Abstract

Background: Asthma is a complex heterogeneous disease of the airways characterized by non-specific symptoms such as cough, wheeze and shortness of breath. Despite this known heterogeneity asthma patients are generally treated the same way. As such, patients are at risk of taking medications they either do not require or will not benefit from. The identification of biomarkers capable of aiding in disease phenotyping is essential to ensure patients receive treatments that will be of the most benefit to them.

Aim: To personalize asthma treatments by identifying clinical and physiological characteristics that can reliably identify the patients who will and will not benefit from traditional corticosteroid and novel biologic treatments.

Method: Three clinical studies were conducted to describe and characterize individual responses. Two studies evaluated responses to corticosteroids, while the third study investigated responses to treatment with mepolizumab, an anti-IL-5 monoclonal antibody. Clinical features as well as systemic cytokine levels were documented for each study.

Results: The first study determined that a low level of fractional exhaled nitric oxide (≤27 ppb) excluded a clinical benefit from initiating treatment with an inhaled corticosteroid in steroid-naïve patients with suspected asthma in the short-term. The second study identified that patients with poorly controlled asthma have varying responses to increased doses of corticosteroid. This study failed to identify specific markers that were predictive of response with the exception that those on a low level of maintenance inhaled corticosteroid were most likely to benefit from an increased dose. The third study found that 61.5 % of

patients responded to treatment with mepolizumab. The responders were significantly older at diagnosis, experienced fewer exacerbations in the previous year, were on a slightly higher daily maintenance dose of systemic corticosteroids and reported better controlled asthma than the non-responders.

Conclusions: The work performed in this thesis demonstrated a high level of heterogeneity in response to asthma treatments which supports the need to personalize treatments in asthma by identifying biomarkers predictive of response so that the right treatments can be given to the right patients maximizing benefit and minimizing unnecessary treatment effects.

Aims and Hypothesis

Asthma is a complex heterogeneous condition that affects approximately 334 million people worldwide. Due to the heterogeneous nature of asthma patients exhibit varying levels of response to the available treatments. As such the overall aim of the work performed here was to identify biomarkers that could reliably identify patients who would or would not benefit from treatment with either corticosteroids or mepolizumab.
The aims and hypothesis of each clinical study are as follows:

Chapter 4: The aim of the study "Does a low exhaled nitric oxide level exclude a clinical benefit from inhaled corticosteroids" was to determine if a low level of nitric oxide (≤27 ppb) would exclude a clinical benefit from treatment with an inhaled corticosteroid in the short-term. The hypothesis was that treatment with budesonide and placebo would be equivalent in terms of the Asthma Control Questionnaire.

Chapter 5: The aim of the study "An evaluation of increased doses of corticosteroid in poorly controlled asthma" was to describe the pattern of response to an increased dose of corticosteroid and determine if there were any baseline features that could have predicted response. The hypothesis was that patients would exhibit varying levels of response to treatment and retrospectively we may be able to identify markers of response.

Chapter 6: The aim of the audit entitled "An evaluation of response to treatment with mepolizumab in patients with severe asthma" was to describe the baseline features of the responders and non-responders. The hypothesis was that patients would exhibit varying levels of response and it may be possible to identify baseline features or early markers that could predict response retrospectively.

Acknowledgements

The work performed in the thesis would not have been possible without the many patients who gave their time to advance our understanding of asthma.

I would like to thank my supervisor, Professor Tim Harrison, for allowing me the opportunity to learn from him and for his patience answering my many questions along the way.

I would like to thank my supervisor, Dr Lucy Fairclough, for presenting this opportunity to me and for her guidance throughout my work. Additionally, I would like to thank Dr Paddy Tighe for his support, time and hands-on support in the lab.

I would like to thank the staff in the Nottingham Respiratory Research Unit for their guidance as I learned the intricacies of conducting clinical research. I would especially like to thank Liz Dark for teaching me venepuncture and for supporting me during clinical visits. Thank you to Holly Scratchered for her enthusiasm and teaching me lung function. I would also like to thank Katherine Smith for her help ensuring all my paperwork was in order. Thank you to the LowNo team, Karen Shaw and Clair Parrish, for their work in primary care practices and Carly Clayton and Helen Bailey for their help storing samples. Additionally, I would like to thank Shani Hutchinson for her constant support, guidance and friendship throughout my time here.

Finally, I would like to thank my family and friends at home for supporting my decision to move to the UK to continue my academic career. I would like to thank my Uncle Grant who made it possible for me to move here, my mother for her ongoing support and Dave for chauffeuring my samples and I around, putting up with my long hours and my very occasional complaints.

Declaration

The initial protocol for the antibody microarray had been developed in-house by previous students. I was responsible for all optimizations to the microarray and enzyme-linked immunosorbent assay (ELISA) that are outlined in this thesis to develop an assay that could be used to quantify cytokine concentrations in human serum. I performed all assays on clinical samples (serum, plasma and sputum) myself.

When I joined the Nottingham Respiratory Research Unit (NRRU) the protocol for the clinical study "Does a low exhaled nitric oxide level exclude a clinical benefit from inhaled corticosteroids" had already been written. I assisted in modifying the protocol to include venepuncture at the first visit for cytokine analysis. I was involved in patient recruitment by mailing study posters and information letter to GP practices and patients from the NRRU's database and patient registries in primary care practices. I also performed telephone screens of prospective patients. Once the study started, I participated in research visits that were conducted within the NRRU and occasionally in practices within Nottingham. I was responsible for processing the majority of the blood samples to separate and store the serum. I analyzed all samples for cytokine concentrations and performed all statistical analysis with some guidance from medical statisticians, Iain Stewart and Tricia Mckeever.

The idea for the study "An evaluation of increased doses of corticosteroid in poorly controlled asthma" arose from discussions with my supervisor, Professor Tim Harrison. I wrote the protocol, participant information sheet and consent form. I was responsible for obtaining all required ethical approvals and sponsorship agreements. I managed the project, created the case report form, saw all patients in clinic, processed, stored and analyzed the blood samples. I performed all statistical analysis.

I was involved in the design of the audit that was conducted in conjunction with an NHS mepolizumab clinic in the NRRU. I was responsible for obtaining consent from the majority of the patients who agreed to an additional blood sample. I participated in the weekly clinics performing venepuncture and/or lung function tests as required. The injection itself was given by an NHS nurse. Blood samples were processed and stored by the Biobank. I was responsible for analyzing the samples for cytokine concentrations. My colleague, Paula Almeida, and I co-managed the database. I performed all statistical analysis.

Abstracts and Publications

Abstracts

Joint Respiratory Research Day Leicester UK, 2018: Poster Presentation

An Evaluation of Cytokine Profiles in Severe Eosinophilic Asthmatics Treated with Mepolizumab
Lissa Sutherland, Paula Almeida, Tina Wilkinson, Charlotte Dobson, Lucy Fairclough and Tim Harrison.

ATS Dallas, Texas, USA 2019: Poster Presentation

A Low Exhaled Nitric Oxide Level Excludes a Short-term Benefit from Inhaled Corticosteroids in Suspected Asthma
Lissa Sutherland, Karen Shaw, Clair Parrish, Tricia McKeever, Iain Stewart, Dominick Shaw and Tim Harrison

Publication

Sutherland G. Lissa and Harrison W. Tim. Can a temporary increase in corticosteroid dosage reduce asthma exacerbations?. Expert Review of Respiratory Medicine 2018, 12(12): 993-995.

List of Tables

Table 1.1 Low, medium and high daily doses of inhaled corticosteroids 20

Table 1.2 Overview of the cytokines evaluated in this thesis 34

Table 1.3 Pharmacokinetic properties of commonly used inhaled corticosteroids 58

Table 2.1 Cytokine and antibody concentrations 77

Table 2.2 Composition of printing buffers 79

Table 3.1 Interassay Percent coefficient of variation for aminosilane slides 115

Table 3.2 Interassay percent coefficient of variation for epoxysilane slides 119

Table 3.3 Interassay percent coefficient of variation for poly-l-lysine slides 123

Table 3.4 Serum cytokine concentrations 137

Table 4.1 Schedule of events for study visits 157

Table 4.2 Baseline demographics 161

Table 4.3 Length of time from asthma diagnosis to visit 1 162

Table 4.4 Secondary outcome treatment responders 169

Table 4.5 Fractional exhaled nitric oxide (FeNO) levels 173

Table 4.6 Baseline factors to predict response to treatment: considered independently 176

Table 4.7 Baseline factors to predict response to treatment: regression model 177

Table 4.8 Baseline factors to predict response to treatment: final model 178
Table 4.9 Budesonide group- cytokine profile raw data 180
Table 4.10 Placebo group- cytokine profile raw data 181
Table 5.1 Overview of the information obtained as part of the participants routine care and study specific information 197
Table 5.2 Baseline demographics 200
Table 5.3 Baseline clinical features 201
Table 5.4 Treatment response 214
Table 5.5 Sputum cell count 215
Table 5.6 Baseline characteristics of the responders 221
Table 5.7 Baseline clinical characteristics 222
Table 5.8 Serum cytokine concentrations 225
Table 5.9 Plasma cytokine concentrations 226
Table 5.10 Sputum supernatant cytokine concentrations 227
Table 6.1 Audit schedule of events 246
Table 6.2 Baseline patient demographics 252
Table 6.3 Baseline patient characteristics 252
Table 6.4 Baseline clinical characteristics 253
Table 6.5 Summary of responders to treatment with mepolizumab .. 271
Table 6.6 Mean baseline demographics of responders and non-responders 273
Table 6.7 Mean baseline clinical characteristics of responders and non-responders 274

Table 6.8 Individual responses at baseline and 12 months of treatment 276
Table 6.9 Individual responses to the asthma control questionnaire (ACQ) at each visit 286
Table 6.10 Individual responses to the asthma control test (ACT) at each visit 289
Table 6.11 Individual responses to forced expiratory volume over one second (FEV_1) at each visit 290
Table 6.12 Individual responses to airway reactance (AX) at each visit 291
Table 6.13 Summary of serum cytokine concentrations at baseline.. 294

List of Figures

Figure 1.1 Spirometry 6

Figure 1.2 Forced oscillation technique (FOT) 11

Figure 1.3 Asthma diagnostic algorithm 13

Figure 1.4 A stepwise approach to the pharmacological management of asthma 15

Figure 1.5 Cytokines involved in the pathogenesis of asthma.....30

Figure 1.6 Hypothalamic-pituitary-adrenal (HPA) axis 51

Figure 1.7 Genomic structure of the glucocorticoid receptor.....53

Figure 1.8 Progression of monoclonal antibodies 69

Figure 2.1 Enzyme-linked immunosorbent assay (ELISA) 78

Figure 2.2 Microarray slide processing 82

Figure 3.1 Epoxysilane surface chemistry.....97

Figure 3.2 Aminosilane surface chemistry 97

Figure 3.3 Poly-l-lysine surface chemistry 98

Figure 3.4 Slide processing 103

Figure 3.5 Cytokine microarray on poly-l-lysine (PLL) slides following the previously optimized protocol- combined blocks..... 105

Figure 3.6 Cytokine microarray on poly-l-lysine (PLL) slides following the previously optimized protocol- individual blocks..... 106

Figure 3.7 Cytokine standard curves printed at 50 µg/mL..... 108

Figure 3.8 Cytokine standard curves printed at 100 µg/mL..... 109

Figure 3.9 Cytokine standard curves printed at 200 µg/mL..... 110

Figure 3.10 Cytokine standard curves on aminosilane slides 114

Figure 3.11 Cytokine standard curves on epoxysilane slides 118
Figure 3.12 Cytokine standard curves on poly-l-lysine slides..... 122
Figure 3.13 Optimization of coating buffer and volume for 384 well ELISA 126
Figure 3.14 384 well ELISA protocol optimization 127
Figure 3.15 Bovine serum albumin optimization 128
Figure 3.16 Comparison of standard curves in 96 and 384 well plates 132
Figure 3.17 Intraassay coefficient of variation for 384 well assays ... 133
Figure 3.18 Interassay coefficient of variation for 384 well assays ... 134
Figure 3.19 Fractional exhaled nitric oxide (FeNO) and serum IL-13 139
Figure 3.20 Blood eosinophils and IL-5 139
Figure 4.1 Equivalence test 154
Figure 4.2 Consort diagram..... 159
Figure 4.3 Two, one-sided equivalence test (TOST) ACQ7 163
Figure 4.4 Asthma Control Questionnaire (ACQ7) Mean ACQ7 scores of the budesonide and placebo groups are sown at each visit. Decreasing scores represent an improvement in control. Error bars represent the standard deviation. 163
Figure 4.5 Two, one-sided equivalence test (TOST) LCQ 166
Figure 4.6 Leicester cough questionnaire (LCQ) 166
Figure 4.7 Medical research council dyspnea scale (MRC) 167
Figure 4.8 Two, one-sided equivalence test (TOST) FEV_1 168
Figure 4.9 Forced expiratory volume over one second (FEV_1) 168

Figure 4.10 Baseline fractional exhaled nitric oxide (FeNO) divided into subgroups 171
Figure 4.11 Fractional exhaled nitric oxide (FeNO) levels..... 172
Figure 4.12 Cytokine profile heat map 183
Figure 5.1 Asthma control questionnaire (ACQ) 7 206
Figure 5.2 Blood eosinophils..... 207
Figure 5.3 Fractional exhaled nitric oxide (FeNO) 208
Figure 5.4 Forced expiratory volume over one second (FEV_1)..... 209
Figure 5.5 Total airway resistance at 5 Hz (R5)..... 210
Figure 5.6 Large airway resistance at 20 Hz (R20) 211
Figure 5.7 Small airway resistance at 5-20 Hz (R5-20) 212
Figure 5.8 Airway reactance (AX)..... 213
Figure 5.9 Asthma control questionnaire 7 (ACQ7) 216
Figure 5.10 Blood Eosinophils..... 217
Figure 5.11 Fractional exhaled nitric oxide (FeNO) 217
Figure 5.12 Small airway resistance (R5-20 Hz)..... 218
Figure 5.13 Airway reactance (AX) 218
Figure 5.14 Serum cytokine profiles 228
Figure 5.15 Plasma cytokine profiles 229
Figure 5.16 Sputum cytokine profiles 230
Figure 6.1 Consort diagram..... 250
Figure 6.2 Body mass index (BMI) at baseline 254
Figure 6.3 Distribution of clinical characteristics at baseline..... 260
Figure 6.4 Blood eosinophils..... 263

Figure 6.5 Fractional exhaled nitric oxide (FeNO) 263
Figure 6.6 Asthma control questionnaire (ACQ) 264
Figure 6.7 Asthma control questionnaire (ACQ) levels of control..... 265
Figure 6.8 Asthma control test (ACT) 266
Figure 6.9 Forced expiratory volume over one second (FEV_1)..... 267
Figure 6.10 Forced Oscillation Technique (FOT) 269
Figure 6.11 Blood eosinophils..... 279
Figure 6.12 Fractional exhaled nitric oxide (FeNO) 279
Figure 6.13 Asthma control questionnaire (ACQ) 5 280
Figure 6.14 Asthma control questionnaire (ACQ) 6 280
Figure 6.15 Asthma control questionnaire (ACQ) 7 281
Figure 6.16 Asthma control test (ACT)..... 281
Figure 6.17 Forced expiratory volume over one second (FEV_1) 282
Figure 6.18 Total airway resistance (R5)..... 282
Figure 6.19 Large airway resistance (R20) 283
Figure 6.20 Small airway resistance (R5-20) 283
Figure 6.21 Airway reactance (AX)..... 284
Figure 6.22 Concentrations at Baseline..... 293
Figure 6.23 Serum Cytokine concentrations over 12 months..... 296
Figure 6.24 Mepolizumab enzyme-linked immunosorbent assay 297

Abbreviations

Ach	Acetylcholine
ACTH	Adrenocorticotropic hormone
AE	Adverse events
AHR	Airway hyperresponsiveness
AR	Airway remodelling
ASM	Airway smooth muscle
ACQ	Asthma Control Questionnaire
ACT	Asthma Control Test
AU	Arbitrary fluorescence unit
BMI	Body mass index
BSA	Bovine serum albumin
BTS	British Thoracic Society
BPT	Bronchial provocation test
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CRH	Corticotropin-releasing hormone
cGMP	Cyclic guanosine monophosphate
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DBD	DNA binding domain
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
EIA	Exercise induced asthma
ECM	Extracellular matrix
FBC	Full blood count
FEF	Forced expiratory flow
FEV_1	Forced expiratory volume over one second
FeNO	Fractional exhaled nitric oxide
FOT	Forced oscillation technique
FVC	Full vital capacity
GCP	Good clinical practice
GI	Gastrointestinal
GPR	Genepix Result File
GINA	Global Initiative for Asthma Guidelines
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GC	Glucocorticoids

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HSP	Heat shock protein
HRP	Horseradish peroxidase
HAMA	Human anti-mouse antibody
HFA	Hydrofluoroalkane
HSD	Hydroxysteroid dehydrogenase
HPA	Hypothalamic-pituitary-adrenal
Ig	Immunoglobulin
Z	Impedance
iNOS	inducible nitric oxide synthase
ICS	Inhaled corticosteroid
ILC	Innate lymphoid cell
IFN	Interferon
IL	Interleukin
LCQ	Leicester Cough Questionnaire
LTRA	Leukotriene receptor antagonist
LBD	Ligand binding domain
LABA	Long-acting beta agonist
MART	Maintenance and Reliever Therapy
MRC	Medical Research Council Dyspnea Score
MAPK	Mitogen-activated protein kinase
mAbs	Monoclonal antibodies
NICE	National Institute for Health and Care Excellence
NTD	N-terminal transactivation domain
NHS	National Health Service
NOS	Nitric oxide synthase
NF-kB	Nuclear factor- kappa-light-chain-enhancer of B cells
OCS	Oral corticosteroid
OD	Optical density
PIS	Participant information sheet
ppb	Parts per billion
PEF	Peak expiratory flow
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PMT	Photomultiplier tube
MDI	Pressurized metered dose inhaler
X	Reactance
RD	Reagent diluent

Treg	Regulatory T cell
RRA	Relative receptor affinity
RV	Residual volume
R	Resistance
Fres	Resonant frequency
SABA	Short-acting beta agonist
sGC	Soluble guanylate cyclase
Th	T helper
TMB	Tetramethylbenzidine
TSLP	Thymic stromal lymphopoietin
TGF	Transforming growth factor
TNF	Tumour necrosis factor
UK	United Kingdom
URTI	Upper respiratory tract infection
VEGF	Vascular endothelial growth factor

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND INFORMATION

Asthma is one of the most prevalent diseases, affecting approximately 334 million people worldwide (1). It has been estimated that in the United Kingdom (UK) alone approximately 5.4 million people have been diagnosed with asthma. However, whether all of these patients have been diagnosed correctly is a topical issue. Asthma costs the National Health Service (NHS) an estimated 1.1 billion pounds every year and approximately one person dies from asthma every 90 seconds worldwide (2-4).

Asthma is a heterogeneous inflammatory disease affecting both the large and small airways. It is characterized by chronic inflammation, airway hyperresponsiveness (AHR) and reversible airflow obstruction which results in symptoms of chest tightness, cough and wheeze (5). Periods of increased inflammation and bronchoconstriction are likely to accompany the patient's chronically inflamed state, these periods are known as exacerbations or attacks (6).

Asthma varies between patients both in its clinical and biological presentation as well as in its response to treatment (7). Numerous disease phenotypes based on clinical presentation, inflammatory cell profiles and exacerbation triggers have been identified (8). It is still unknown how many different phenotypes there are and the classifications for the existing phenotypes are inconsistent in the literature. Understanding asthma phenotypes and the mechanisms

driving them (endotypes) is critical in order to generate new and personalized treatments in asthma.

1.2 DIAGNOSING ASTHMA

Currently, there is no definitive test to diagnose or exclude a diagnosis of asthma which can make it difficult to distinguish from other respiratory conditions such as chronic obstructive pulmonary disease (COPD) or chronic cough. As a result, asthma is often over-diagnosed resulting in patients receiving unnecessary medications. Conducting a physical examination and collecting a thorough patient history including respiratory incidents in childhood and atopic status in addition to conducting lung function and blood tests are necessary to ensure the diagnosis of asthma is correct (9).

1.2.1 RESPIRATORY SYMPTOMS

According to the 2018 Global Initiative for Asthma guidelines (GINA), when a diagnosis of asthma is being considered the patient should exhibit at least two of the following respiratory symptoms: cough, wheeze, shortness of breath or chest tightness (9). Symptoms are likely to vary in intensity and over time often depending on the season. They may peak at different times throughout the day typically worsening at night, in the early morning or on exertion. Viral infections, changes in temperature, exercise, exposure to allergens, smoke or strong smells may trigger an increase in symptoms (10).

1.2.2 LUNG FUNCTION TESTS

Lung function tests are important for diagnostic purposes and to track changes over time (11). In asthma, lung function is variable. Individuals can fluctuate from relatively normal lung function to severe obstruction. As such, lung function tests may need to be performed on more than one occasion. The more poorly controlled the patient is the more variable their lung function is likely to be (9).

1.2.2.1 Spirometry

Airway diseases affecting measures of lung function produce either a restrictive or obstructive pattern. Restrictive diseases are characterized by reduced lung volume and an inability to fully inhale. Obstructive diseases, such as asthma, are characterized by a reduction in airflow and sometimes by an inability to fully exhale due to gas trapping (10). In asthma, air flow is reduced due to smooth muscle contractions narrowing the bronchioles (12).

Spirometry is the most commonly performed lung function test. Detailed information regarding air volume and flow including forced expiratory volume over one second (FEV_1), full vital capacity (FVC), and peak expiratory flow (PEF) are obtained. Spirometry can help distinguish between restrictive and obstructive diseases. In healthy individuals, the ratio of FEV_1/FVC is between 75 % and 80 %. A reduced FEV_1/FVC ratio of less than 70 % is representative of airflow limitation from an obstructive disease such as asthma. Conversely, a ratio greater than 80 % indicates the presence of a restrictive airway disease (13) (**Figure**

1.1). It is important that spirometry is performed correctly as poor technique can also result in a reduced ratio and thus can contribute to an incorrect diagnosis (14). During the test, a flow-volume curve is generated. The morphology of the curve can give further insights into the patients' condition (12). For an accurate reading, tests should be performed until there are three measurements with a maximum variability of 5 % between the measurements (11). Currently, spirometry is considered to be the gold standard test to evaluate lung function, disease severity and monitor response to treatment.

However, spirometry requires significant effort from the patient and competent coaching from the technician in order to obtain an accurate reading. As such, spirometry is not ideal for patients who are unwell or unable to perform the required forced expiratory technique (i.e. children) (15). In these patients, alternative techniques to assess lung function may be required.

In 2017, Meneghini and colleagues conducted a study to determine the accuracy of spirometry for detecting asthma in the general population. They determined that spirometry had high specificity, 90 %, but low sensitivity, 23 %, and suggested that spirometry alone is not sufficient to detect asthma in the general population and that symptoms in conjunction with a bronchial challenge test may be more accurate (16).

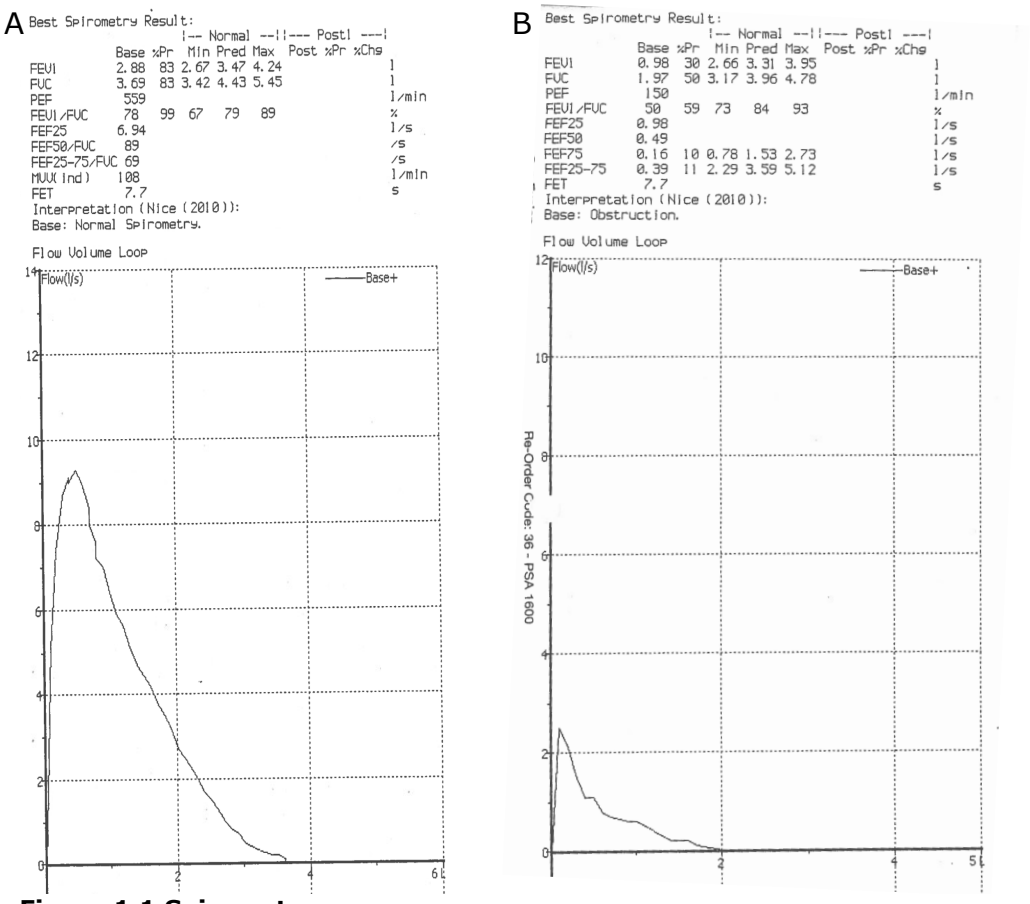


Figure 1.1 Spirometry

A. Normal spirometry as indicated by the forced expiratory volume over one second (FEV_1) to full vital capacity (FVC) ratio is between 75-80 %. **B**. Obstructed spirometry indicating a possible diagnosis of asthma where the FEV_1/FVC ratio is less than 70. The flow volume curve is also dramatically reduced compared to normal spirometry.

1.2.2.2 Reversibility test

Reversibility represents a rapid improvement in FEV_1 and or PEF. In order to determine if the patient has reversible airflow obstruction, baseline spirometry is conducted, then a bronchodilator (usually salbutamol) is administered and spirometry is repeated 20 minutes later when the medication has reached its maximum concentration. An improvement of ≥12 % and ≥200 mL in FEV_1 post bronchodilator represents significant reversibility and is suggestive of asthma (12). More confidence can be

put in this diagnosis if reversibility is >15 % or >400 mL (14). However, reversibility in asthmatic patients can be difficult to see unless the patient is steroid naïve (1). It is important that patients withhold bronchodilator medication before spirometry is performed otherwise a negative result cannot confirm fixed airflow obstruction and as such a diagnosis of asthma could not be ruled out (11). This test cannot definitively distinguish between asthma and COPD however if the patient does demonstrate marked reversibility asthma is the more likely diagnosis as COPD is characterized by fixed airflow obstruction (13).

1.2.2.3 Oscillometry

Forced oscillation technique (FOT) passes sound waves into the lungs during tidal breathing which measures respiratory impedance (17). Impedance (z) is made up of resistance (R) and reactance (X).

Resistance is defined as the change in pressure over flow. Therefore, resistance to airflow in the lungs increases as the airways narrow and reflects bronchial obstruction (17). Lung resistance reflects the airways and surrounding tissues. In asthma, inflammation and bronchoconstriction reduce the diameter of the airways which increases resistance (18).

During oscillometry, impulses of varying frequencies are superimposed on tidal breathing. Impulses with a frequency greater than 20 Hz travel short distances and remain in the large central airways, whereas frequencies less than 15 Hz travel further and reach into the small distal airways and lung parenchyma (19). When interpreting FOT results, R at

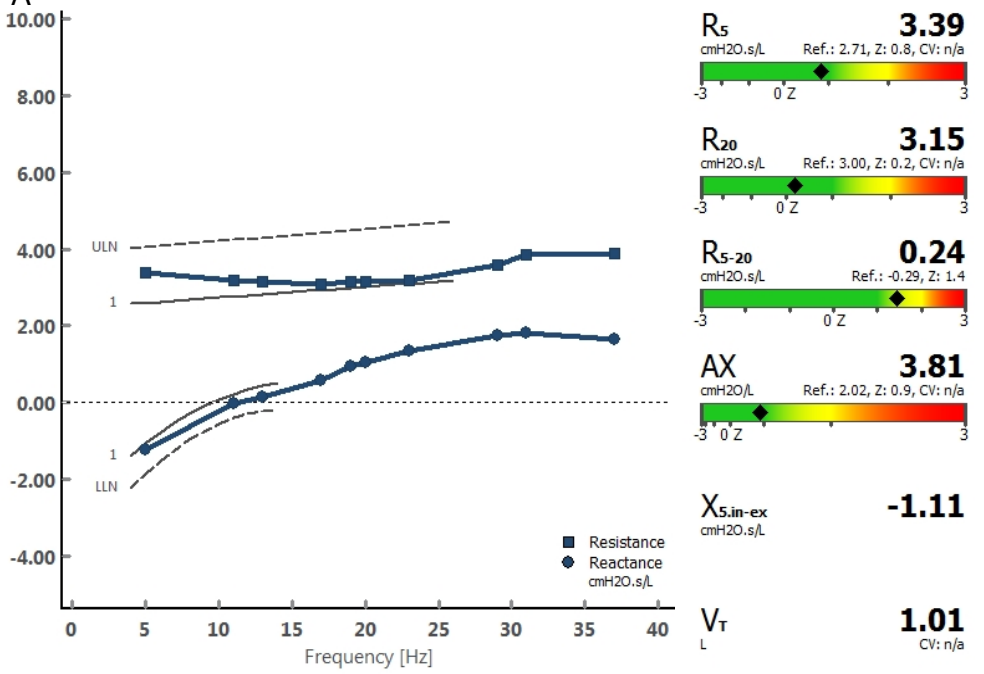
5 Hz (R5) reflects the overall airway resistance as the impulses are of a low enough frequency to travel throughout the lung. R20 reflects resistance of the large central airways as the frequency is too large to pass into the small airways. Therefore, resistance of the small airways is ascertained by subtracting resistance of the large airways from resistance of the whole lung (R5-20) (20). In healthy individuals, resistance is not dependent on frequency so oscillometry results in a straight line (see **Figure 1.2**). If obstruction exists in the central airways resistance will increase across all frequencies and the line will be elevated. If obstruction exists in the small airways this is reflected in the low-frequency impulses only resulting in an elevated resistance reading at 5 Hz. A study performed on pediatric patients by Kim and colleagues found that resistance at 5 Hz reflected basal lung function and was more capable of differentiating between children with asthma and healthy children than spirometry as resistance was increased in those with asthma even if their FEV_1 was normal (21).

Reactance (X) is made up of inertance, which reflects changes in pressure of moving air, and capacitance which reflects elastic properties, lung compliance and the ability to store energy which occurs primarily in the lung periphery. Inertance and capacitance oppose each other and are dependent on frequency. Capacitative pressure losses are represented as negative values whereas inertive pressure losses are positive (22). At lower frequencies, the capacitative pressure losses are greater than inertive pressure losses so the resulting oscillometry values

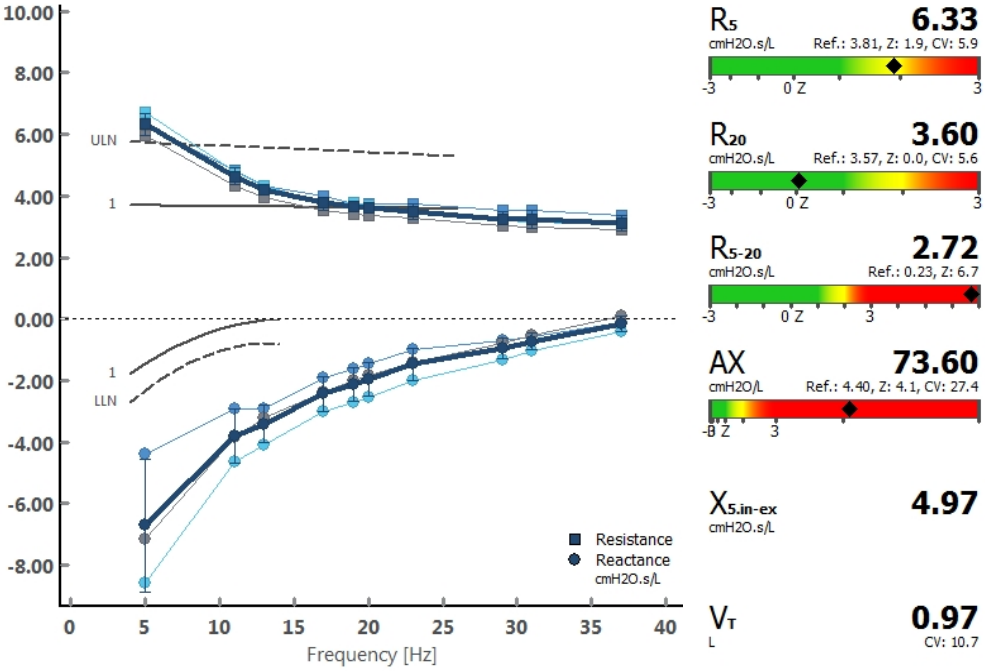
are negative whereas at higher frequencies, the inertive pressure losses are greater and the resulting values are positive. The frequency at which capacitative and inertive pressure losses are equal is referred to as the resonant frequency (Fres) (23). In healthy individuals, Fres occurs between 6-11 Hz. The area of reactance (AX) represents the low-frequency reactance between 5 Hz and Fres and so reflects the small airways (20). With small airway obstruction (SAO) this area becomes greater and Fres occurs at a higher frequency. AX is linked with R5-20 as they both reflect the small airways. A pediatric study revealed that reactance was as sensitive and specific as traditional measures of assessing AHR and therefore may be a suitable alternative for bronchial provocation tests (21).

Oscillometry is conducted during tidal breathing thereby requiring minimal effort and coordination making it much easier to perform than spirometry. Additionally, oscillometry produces a more in-depth and segmented analysis of the lungs allowing the location of the obstruction to be visualized more accurately. However, oscillometry is performed less frequently and does not have the predicted values or standard values for judging significant improvements that spirometry has which can make the results more difficult to interpret.

A


10.00
8.00
6.00
4.00
2.00
0.00
-2.00
-4.00
ULN
1
1
LLN
Resistance
Reactance
cmH2O.s/L
0
5
10
15
20
25
30
35
40
Frequency [Hz]
R5
cmH2O.s/L
3.39
Ref.: 2.71, Z: 0.8, CV: n/a
-3
0 Z
3
R20
cmH2O.s/L
3.15
Ref.: 3.00, Z: 0.2, CV: n/a
-3
0 Z
3
R5-20
cmH2O.s/L
0.24
Ref.: -0.29, Z: 1.4
-3
0 Z
3
AX
cmH2O/L
3.81
Ref.: 2.02, Z: 0.9, CV: n/a
-3
0 Z
3
X5.in-ex
cmH2O.s/L
-1.11
VT
L
1.01
CV: n/a

B


10.00
8.00
6.00
4.00
2.00
0.00
-2.00
-4.00
-6.00
-8.00
ULN
1
1
LLN
Resistance
Reactance
cmH2O.s/L
0
5
10
15
20
25
30
35
40
Frequency [Hz]
R5
cmH2O.s/L
6.33
Ref.: 3.81, Z: 1.9, CV: 5.9
-3
0 Z
3
R20
cmH2O.s/L
3.60
Ref.: 3.57, Z: 0.0, CV: 5.6
-3
0 Z
3
R5-20
cmH2O.s/L
2.72
Ref.: 0.23, Z: 6.7
-3
0 Z
3
AX
cmH2O/L
73.60
Ref.: 4.40, Z: 4.1, CV: 27.4
-3 Z
3
X5.in-ex
cmH2O.s/L
4.97
VT
L
0.97
CV: 10.7

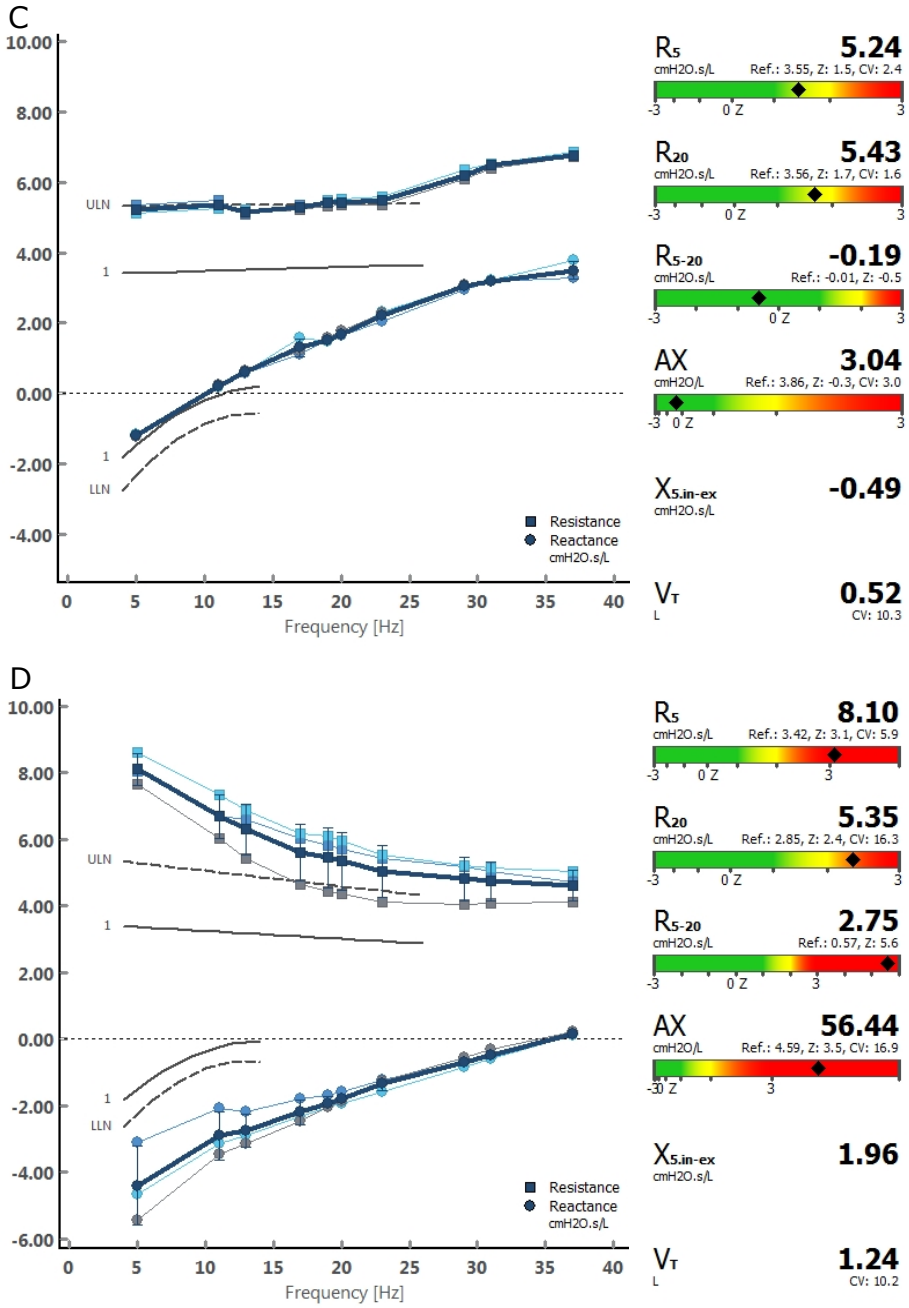


Figure 1.2 Forced oscillation technique (FOT)

Resistance is represented on the top half of each graph with squares and reactance is represented with circles on the bottom half of each graph. **A**. Normal lung function as indicated by a straight resistance line and a resonance frequency (Fres) occurring between 6-11 Hz. **B**. Small airway obstruction as indicated by increased resistance at low frequencies, which is reflected in higher resistance between R5 and R20 (R5-20) and the area of reactance (AX) and an Fres that is well past 11 Hz. **C**. Large airway resistance as indicated by increased resistance across all frequencies, especially at higher frequencies, which is reflected in a higher R20. **D**. Small and large airway resistance as indicated by the resistance output elevated across all frequencies, which is reflected in increased R5, R20, R5-20, AX and Fres that is well past 11 Hz.

1.2.2.4 Bronchial provocation tests

Documenting variable airflow limitation is key to correctly diagnosing asthma. However, due to its variable nature, this may not be present at the time of testing. In this case, bronchial provocation or challenge tests can be performed. Challenge tests measure airway responsiveness. For this test, the patient inhales a pharmacological agent such as histamine, mannitol or methacholine that triggers bronchoconstriction. Patients with asthma are likely to have hyperresponsive airways and are therefore more likely to experience bronchoconstriction at a lower dose (24, 25). However, once corticosteroid treatment has been initiated, airway responsiveness may return to normal making it is less easy to exclude a diagnosis of asthma based on a negative result.

1.2.3 FRACTIONAL EXHALED NITRIC OXIDE

Fractional exhaled nitric oxide (FeNO) is a non-invasive measure of T2 inflammation which is usually accompanied by high levels of eosinophils and is common in atopic asthma (26). High FeNO values (>50 ppb) are associated with high levels of IL-13 and in steroid naïve patients are a predictive biomarker of positive response to corticosteroid treatment, whereas values of less than 25 ppb indicate that the patient is less likely to respond to corticosteroids (27). An elevated FeNO in conjunction with asthma symptoms and an FEV_1/FVC of <70 % can help confirm a diagnosis of asthma and likely represents a phenotype that will respond well to corticosteroids (28). As FeNO values increase it has improved specificity but decreased sensitivity for diagnosing asthma. As such,

FeNO alone is not sufficient to either rule in or rule out asthma (29) however, FeNO is included in the NICE algorithm to diagnose asthma (**Figure 1.3**).

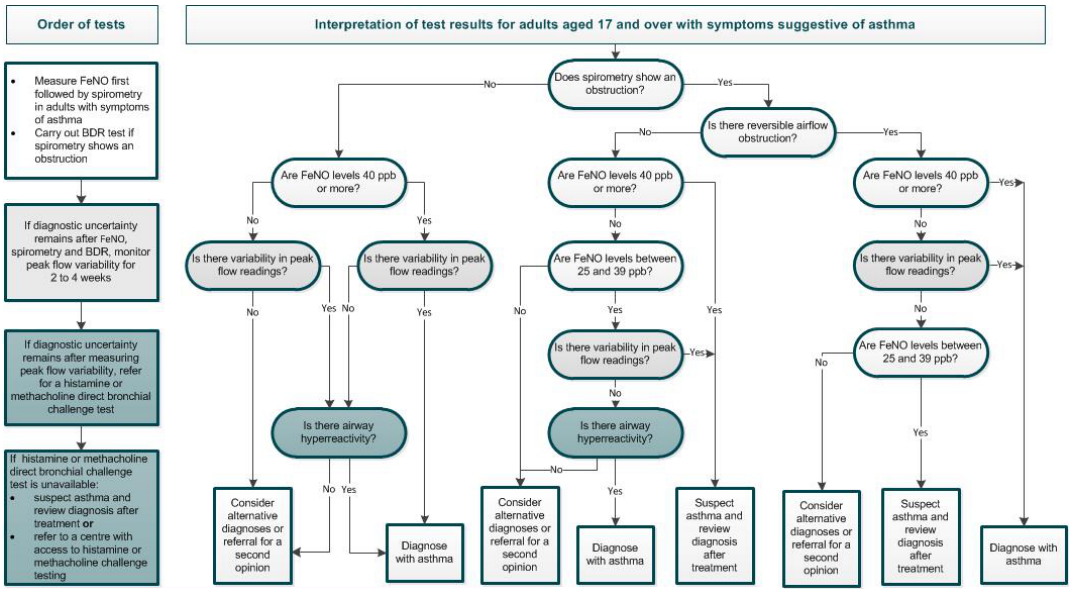


Figure 1.3 Asthma diagnostic algorithm

National Institute for Health and Care Excellence (NICE) asthma diagnostic algorithm (30).

1.2.4 ALLERGY TESTS

Allergy tests are not specific for asthma, however, a positive result in conjunction with symptoms suggestive of asthma does increase the likelihood that the patient could have an allergic asthma phenotype. Allergy tests can be performed by either a skin prick test or by measuring levels of specific immunoglobulin (Ig) E in serum (14).

1.2.5 ASTHMA SEVERITY

There are no objective markers of asthma severity. Severity is determined post-treatment and is based on the level of treatment required for the patient to achieve control over their symptoms (31).

"Control" is the extent to which asthma can be observed or has been minimized by treatment and is discussed further in **Section 1.3** (9). Severity is assessed after the patient has been on their controller therapy for a few months and their dose has been adjusted to reflect the minimum dosage required to achieve control (32, 33).

1.2.5.1 Mild asthma

Mild asthma is defined as asthma that is well controlled with an as-needed reliever (a short-acting β_2-agonist (SABA)) alone, or in conjunction with a low dose controller. Controllers are usually inhaled corticosteroids (ICSs) however, there are many alternative treatment options which are discussed in **Section 1.3**. Mild asthma is represented by steps 1 and 2 of **Figure 1.4** (9).

1.2.5.2 Moderate asthma

Moderate asthma is well controlled with a low dose ICS/ long-acting β_2-agonist (LABA) as a controller with a reliever taken as needed. Moderate asthma is represented by step 3 in **Figure 1.4** (9).

1.2.5.3 Severe asthma

Severe asthma requires high dose ICSs usually combined with a LABA to prevent loss of control or remains uncontrolled despite high dose treatment (9, 32, 34). In order for a diagnosis of severe asthma to be given common issues such as poor medication adherence and inhaler technique need to be assessed and comorbidities should be evaluated. Severe patients may be recommended for add-on therapies such as

biologics depending on their phenotype and exacerbation history (14). Severe asthma is represented in steps 4 and 5 of **Figure 1.4**.

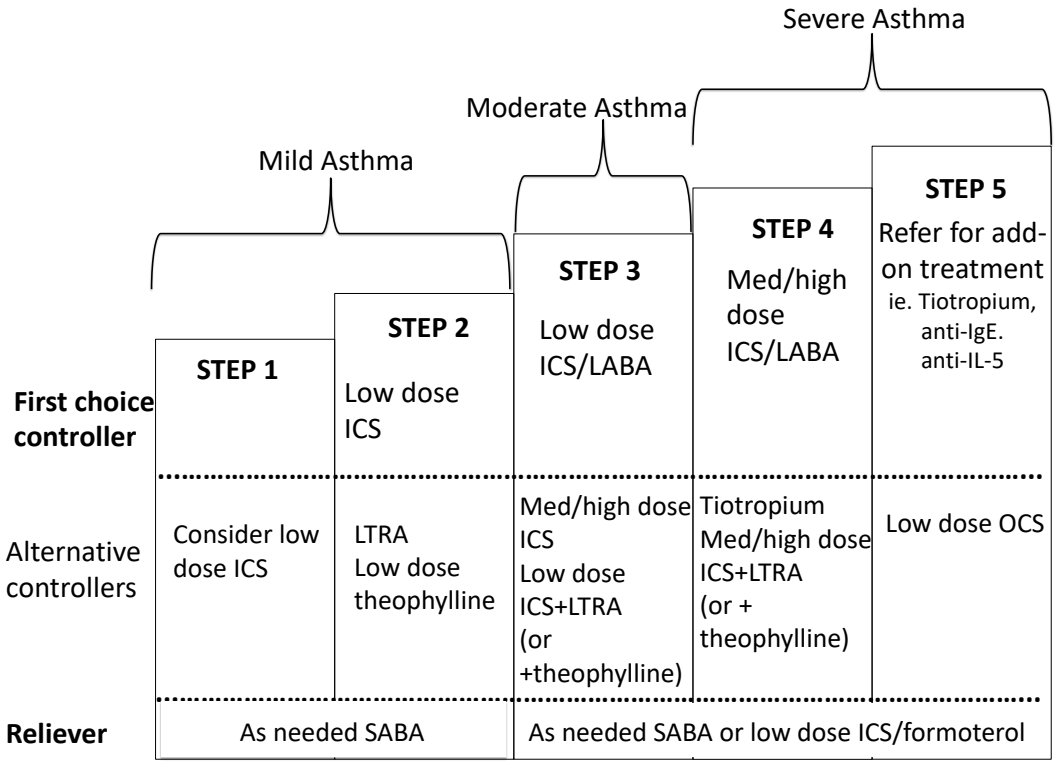


Figure 1.4 A stepwise approach to the pharmacological management of asthma

Mild asthma is controlled with an as-needed reliever alone or with the addition of a low-dose controller as represented by steps 1 and 2. Moderate asthma is controlled with an as-needed reliever plus a low-dose controller as represented by step 3. Severe asthma is either controlled with high-dose controller therapy and a reliever as required or remains symptomatic despite high-dose treatment. Severe asthma is represented by steps 4 and 5. Figure adapted from the Global Initiative for Asthma Global strategy for asthma management and prevention, updated 2018 (9).

1.3 ASTHMA MANAGEMENT

The primary goals of asthma management are to achieve control, minimize symptoms and reduce the risk of future exacerbations whilst minimizing adverse reactions from treatment. The 2018 GINA guidelines

define asthma control as the extent to which the manifestations of asthma can be observed in the patient, or have been reduced by treatment (9). Control is comprised of symptoms and the risk of future adverse events.

Asthma symptom control is assessed based on the frequency of symptoms (day and night), the need for rescue medication and activity limitations over the previous four weeks. Questionnaires such as the Asthma Control Questionnaire (ACQ) can be useful to obtain a baseline measure from which response to treatment can be gauged (14). However, it is important to note that these methods may not reflect changes in airway inflammation, obstruction or AHR. As such, a study by Galvan and colleagues suggested that adding spirometric FEV_1 reversibility to the currently used measures of control may be beneficial in assessing control and determining the best course of treatment for the patient (35).

Exacerbations should not be taken into consideration when determining the level of control as they can occur in patients with minimal symptoms (36). Responsiveness to treatment dictates the amount of treatment required for the patient to achieve control and is indicated by how easily control is achieved once treatment has been started. There are three primary classifications of responsiveness. One, responsive asthma, which results in significant improvements in measures of asthma control with minimal treatment. Two, difficult-to-treat asthma as indicated by only a partial reduction of symptoms and three, treatment-resistant

severe asthma which is further broken down into two groups; those whose achieve control but require the highest amount of treatment to get there and those who remain uncontrolled or only partially controlled despite the highest levels of treatment (33, 36).

Determining if the patient is at risk of experiencing an adverse event such as; an exacerbation, fixed airflow limitation or treatment side effects is the second part of assessing control. Symptoms alone are not a sufficient indicator of future risk. This is likely because there is a known dissociation between symptoms and inflammation. Risk factors for experiencing an exacerbation include having experienced an exacerbation in the previous 12 months, poor symptom control along with increased reliever use, an inadequate dose of ICS, a drop in FEV_1, smoking or sputum eosinophilia (37, 38). The 2018 GINA guidelines suggest that experiencing one or more of these factors increases the risk of an exacerbation even when symptoms are well controlled. Risk factors for developing fixed airflow limitation include inadequate ICS use, smoking or exposure to other toxic chemicals, reduced FEV_1, sputum eosinophilia or chronic overproduction of mucus. Risk factors for developing adverse treatment side effects include maintenance or frequent use of systemic corticosteroids or long-term treatment with high-dose ICSs (9).

Treatment non-adherence and poor inhaler technique are two major issues in asthma management that can severely affect the ability of the clinician to determine the severity of the disease and the patients'

response to treatment. There are several reasons for non-adherence such as the fear of developing adverse side-effects, the adverse side effects themselves, or simply forgetfulness. Studies have shown that educating patients on the importance of daily treatment as well as electronic reminders can help to improve treatment adherence by approximately 20 % (39).

Incorrect inhaler technique leads to more of the drug being deposited into the mouth and throat and subsequently being swallowed meaning less is deposited in the lungs. This can cause asthma to appear more severe than it actually is. As such, inhaler technique training for each device should be provided when the inhaler is first prescribed and checked at subsequent follow-up visits.

1.3.1 PHARMACOLOGICAL MANAGEMENT OF ASTHMA

Asthma therapies fall into three categories; controllers, relievers and add-on. Controllers are used daily as maintenance therapy to treat the underlying disease-causing inflammation. Reliever medications are fast-acting bronchodilators used as required to treat acute symptoms. This class of medication does not address the underlying inflammation and therefore cannot be used independently to treat asthma (40). Treatment guidelines around the world recommend a SABA for fast relief and a controller (usually an ICS) for long-term symptom control (41). Add-on treatments may be required in patients with severe asthma who remain poorly controlled despite treatment with a high-dose controller (1).

There are many different types of asthma medications, the three main classes are: corticosteroids, β_2-agonists, and leukotriene modifiers (40).

1.3.1.1 Corticosteroids

Corticosteroids are the cornerstone of asthma management. Primarily, ICSs are used which are glucocorticoids (GC), that have a strong anti-inflammatory effect by triggering activation of genes that encode anti-inflammatory proteins (40). Low dose ICSs, up to 400 mcg/day of budesonide or equivalent, are recommended to control mild asthma, if asthma symptoms remain uncontrolled a medium dose, >400-800 mcg/day of budesonide or equivalent, of ICS in combination with a LABA, is recommended. If asthma symptoms still remain uncontrolled, asthma is referred to as severe and is treated with high dose ICSs, greater than 800 mcg/day of budesonide or equivalent, in conjunction with a LABA and possibly other add-on treatments including biologic agents (**Table 1.1**) (9, 42).

ICSs are the most effective class of controller medication for asthma and are recommended from step two of the GINA guidelines (43). Treatment with ICS has consistently been shown to reduce inflammation, improve symptoms, quality of life and lung function as well as reduce the likelihood of experiencing an exacerbation (44, 45).

Elevated FeNO (>50 ppb) and sputum eosinophils (≥3 %) have shown to be reliable biomarkers predictive of a positive response to ICS therapy (28, 46). While ICS treatment is less likely to be effective in patients with a low FeNO (<25 ppb) (47).

Systemic corticosteroids can be used to maximize lung function, evaluate steroid responsiveness or for rescue during life-threatening exacerbations (41). Only the minimum dose of corticosteroid that is required for the patient to achieve control should be prescribed as use is associated with serious side effects such as; suppression of the hypothalamic-pituitary-adrenal (HPA)-axis, glaucoma, fluid retention and weight gain (48). Side effects and steroids are discussed in more detail in **Section 1.12**.

Table 1.1 Low, medium and high daily doses of inhaled corticosteroids

Estimated clinical comparability of commonly prescribed inhaled corticosteroids. Adapted from 2018 GINA guidelines (9).

Medication	Daily Dose (mcg)		
	Low	**Medium**	**High**
Beclometasone dipropionate (CFC)	200-500	>500-1000	>1000
Beclometasone dipropionate (HFA)	100-200	>200-400	>400
Budesonide (DPI)	200-400	>400-800	>800
Ciclesonide (HFA)	80-160	>160-320	>320
Fluticasone furoate (DPI)	100	n/a	200
Fluticasone propionate (DPI)	100-250	>250-500	>500
Fluticasone propionate (HFA)	100-250	>250-500	>500
Mometasone furoate	110-220	>220-440	>440
Triamcinolone acetonide	400-1000	>1000-2000	>2000

1.3.1.2 Beta-agonists

Beta-agonists are very effective bronchodilators that work by stimulating airway smooth muscle (ASM) relaxation resulting in increased airflow. Bronchodilators have either a fast or slow onset and can be either short-acting (SABAs) or long-acting (LABAs) (40). Beta-agonists alone are not sufficient to treat asthma as they do not address the underlying disease-causing inflammation and therefore should only be used in conjunction with a corticosteroid (49). LABAs can be used alongside an ICS as controller therapy to prevent the emergence of asthma symptoms (41). The addition of a LABA to an ICS has been shown to result in improvements in symptoms and lung function as well as reduce the likelihood of experiencing an exacerbation (50).

1.3.1.3 Combination inhalers

Combination inhalers deliver an ICS and LABA simultaneously. They are recommended for patients on step three or higher of the GINA guidelines and have been shown to significantly decrease the occurrence of severe exacerbations, reduce symptoms and improve lung function (9, 32, 51-55). Airway inflammation and bronchoconstriction are key characteristics of exacerbations which highlights the importance of initiating bronchodilation in conjunction with reducing inflammation at the first sign of deteriorating control. Combination inhalers simplify treatment regimens by having only one inhaler that is used regularly and ensures that neither medication can be used without the other. This simple approach may also help to increase treatment compliance (56).

Combination inhalers with formoterol can be used for both maintenance and reliever therapy (MART). Formoterol is a potent fast-acting LABA that begins to have bronchodilator effects within one to three minutes of inhalation, which is comparable to the SABA salbutamol. These characteristics allow combination inhalers with formoterol to be used to provide rapid relief in addition to daily maintenance care. MART inhalers have been shown to significantly increase the time to the first severe exacerbation and reduce the occurrence of exacerbations at lower or equal daily doses of corticosteroid than fixed dose regimes (51, 53, 56).

1.3.1.4 Leukotriene receptor antagonists

Leukotrienes are synthesized from arachidonic acid which is released from mast cells and causes bronchoconstriction as well as excessive mucus production. Leukotriene receptor antagonists (LTRAs) prevent leukotrienes from binding to their receptors on ASM. During asthma exacerbations, leukotriene production is up-regulated (57). Therefore, LTRAs such as montelukast can be useful in treating acute and chronic asthma to reduce inflammation and initiate bronchodilation (40). LTRAs have been shown to reduce the risk of exacerbations by approximately 50 % compared with placebo in steroid-naive patients (58). However, LTRA's do not appear to be as effective as ICSs as those treated with an LTRA were approximately 60 % more likely to experience an exacerbation than those treated with an ICS (59).

1.3.1.5 Anticholinergics

The parasympathetic system is the primary branch of the nervous system to innervate the airways. The most common neurotransmitter is acetylcholine (Ach), which upon binding to muscarinic receptors, is responsible for initiating bronchoconstriction (60). Due to unknown mechanisms, Ach release is upregulated in patients with asthma. This causes AHR and reflex bronchoconstriction. Tiotropium bromide is a long-acting anticholinergic agent, commonly used in COPD, that binds selectively to muscarinic receptors and initiates bronchodilation (60, 61). Patients who remain symptomatic despite treatment with an ICS and LABA require treatment with an alternative mechanism of action. In 2015, the GINA guideline approved tiotropium as an add-on treatment for patients at steps four and five (32). Clinical studies have revealed that tiotropium consistently improves lung function, reduces symptoms and the risk of exacerbations in patients who remain symptomatic on ICSs and LABA therapy regardless of baseline features such as age, body mass index (BMI), smoking status or lung function (62-65).

1.3.1.6 Theophylline

Theophylline is a methylxanthine derivative that has bronchodilator and immunomodulatory actions (66). Currently, it is recommended as a controller medication from steps two to four of the GINA guidelines. However, theophylline can result in negative side-effects such as headaches, nausea, vomiting and abdominal discomfort and therefore should be used with caution (67-69).

1.3.1.7 Antibiotics

Some patients with asthma have little evidence of eosinophilic inflammation and instead have high levels of neutrophils and interleukin (IL)-8 in their airways (70). As these patients do not display eosinophilia, ICSs are relatively ineffective. Macrolides are a class of antibiotics that also have immunomodulatory and anti-inflammatory effects and have been shown to reduce neutrophils and IL-8 levels in the airways (71). Brusselle and colleagues showed that using azithromycin as an add-on therapy significantly reduced the number of exacerbations and or lower respiratory tract infections as well as exacerbations alone compared with placebo in a subgroup of patients with non-eosinophilic asthma as indicated by blood eosinophil levels ≤200 cells/μL at baseline (72). Recently, Gibson and colleagues found that adding azithromycin to patients on an ICS and LABA significantly reduced the rate of severe exacerbations regardless of inflammatory phenotype (73). This study was longer, included more subjects, gave twice the dose of azithromycin and used a larger cut off value of blood eosinophils of 300 cells/μL rather than 200 cells/μL which could account for the conflicting results. It is important to note that this study included the need for antibiotics in their definition of an asthma exacerbation. Other studies have failed to show a clinical benefit with add-on macrolide therapy (74). Further studies are needed to identify reliable biomarkers to determine which patient phenotypes are likely to benefit from macrolides.

1.3.1.8 Interferon-beta

Previous research has shown that asthmatic patients who are treated with corticosteroids produce insufficient amounts of the antiviral interferon (IFN)-β, which leaves them vulnerable to viral infections which is a common exacerbation trigger (75). Djukanovic and colleagues administered inhaled IFN-β to asthma patients with a history of cold-induced exacerbations within 24 hours of developing cold symptoms. They observed an increase in morning PEF values, reduced viral load in sputum and a reduced need for oral corticosteroids and/or antibiotics (76). However, a subsequent study evaluating the same patient group was unable to reproduce these findings (77) indicating the need for further investigation.

1.3.1.9 Biologic treatments

Asthma consists of numerous phenotypes each with their own endotype (mechanism driving the disease) and as such patients display heterogeneous responses to traditional therapies. Identifying which therapies will be most beneficial on an individual basis continues to be a topical issue in clinical practice. The development of medications targeted to each phenotype is required to implement personalized medicine in asthma. There are currently four biologics approved for add-on use in asthma with several more undergoing clinical trials. Biologic therapies are discussed in detail in **Section 1.14**.

1.3.2 SELF MANAGEMENT

Patient education is an important aspect of asthma care. Education providing disease information, self-monitoring guidance, regular medical reviews and a written action plan has shown to be effective in reducing exacerbations and emergency hospital visits (78). Written action plans should be personalized to each patient and include exacerbation triggers, when and how to modify medication as well as how to recognize when to seek further medical attention. Medication adjustments based on personalized action plans have been shown to be as effective as physician adjusted medications in terms of hospital admissions and unplanned visits to the doctor (79). Plans should be discussed and agreed upon with the patient to clarify the need for each treatment, which may also help to increase compliance.

A major focus of the self-management plan should be on how to identify worsening control so that exacerbation prevention can be initiated as early as possible. Prevention plans should include increasing the dose of ICS by four to five times when control beings to deteriorate (80). The plan also needs to detail when and how treatment can safely be returned to the maintenance routine. Self-management education results in clinically significant improvements in health outcomes as seen by a reduction in the need to seek emergency medical attention as well as fewer days off work or school due to asthma (79).

1.4 IMMUNE RESPONSE IN ASTHMA

Airway inflammation is one of the hallmarks of asthma. It contributes to airway remodeling (AR) and precedes the appearance of asthma symptoms. Inflammation is linked with airway dysfunction via cytokine release and AR (81). Recently it has been suggested that asthma could result from defects in the epithelial barrier which is responsible for anatomic and functional integrity as well as immunological competence (82). In the asthmatic lung the apical tight junctions, which are crucial for maintaining the physical and functional structure of the airway mucosa, are defective which weakens the barrier against external stimuli such as allergens, viruses and pollutants (83). These stimulants activate pathogen recognition receptors triggering epithelial cells to secrete regulatory cytokines: thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 which stimulate T helper (Th) type 2 and innate lymphoid type 2 (ILC2) cells (84). Following Th2 activation T2 inflammatory cytokines such as IL-4, IL-13 and IL-5 which increase FeNO and periostin production and recruit eosinophils to the airways where they release mediators that further amplify the Th2 response and contribute to AR (85).

There is a large association between asthma and allergy. In atopic individuals, allergens specifically aeroallergens such as house dust mite, invade the mucosal epithelium where they bind to innate receptors on dendritic cells (DCs). The DCs then migrate to secondary lymph nodes where they present the allergens to T cells and in the presence of IL-4

naive T cells are encouraged to differentiate into Th2 cells (81). The Th2 cells then migrate to the site of inflammation where they secrete pro-inflammatory cytokines, specifically, IL-4, IL-5 and IL-13. Allergen-specific IgE on the surface of mast cells and basophils will also bind the allergen triggering receptor cross-linking causing the cells to release their preformed granules which further potentiates the Th2 response. Th2 asthma can also occur without allergy and IgE activation. In these cases, inflammation is thought to be initiated by innate pathways that cause ILC2s to be activated and release IL-5 and IL-13 promoting eosinophilia and AHR (86).

While asthma is commonly associated with Th2 inflammation and eosinophilic infiltrations this is not always the case. Inflammatory profiles can vary between patients and even over time as they are susceptible to the effects of smoking, corticosteroids, pollutants and respiratory infections (87). Neutrophilic asthma is linked with respiratory infections which activate nuclear factor-kappa-light-chain-enhancer of B cells (Nf-κB) establishing a pro-inflammatory state that increases IL-8 production which recruits neutrophils to the airways (86). Sputum analysis is a non-invasive method to reveal the inflammatory profile in the airways which can be either eosinophilic, neutrophilic, mixed eosinophilic and neutrophilic or paucigranulocytic (88). Distinguishing between inflammation caused by different cell types is important for determining which treatments will be most beneficial for the patient.

1.5 CYTOKINES INVOLVED IN ASTHMA PATHOGENESIS

Cytokines are critically involved in recruiting, activating and maintaining the inflammatory cells that contribute to chronic airway inflammation in asthma. Inflammatory cells and cytokines can help to distinguish asthma from other chronic inflammatory conditions. Asthmatic bronchioles often become infiltrated with Th2 cells, eosinophils and degranulating mast cells. Inflammation causes structural changes such as basement membrane thickening due to increased collagen deposition and angiogenesis which contributes to AR (34). Some of the key cytokines involved in asthma pathogenesis are outlined in **Figure 1.5**. A summary of the cytokines evaluated in this thesis is presented in **Table 1.2**.

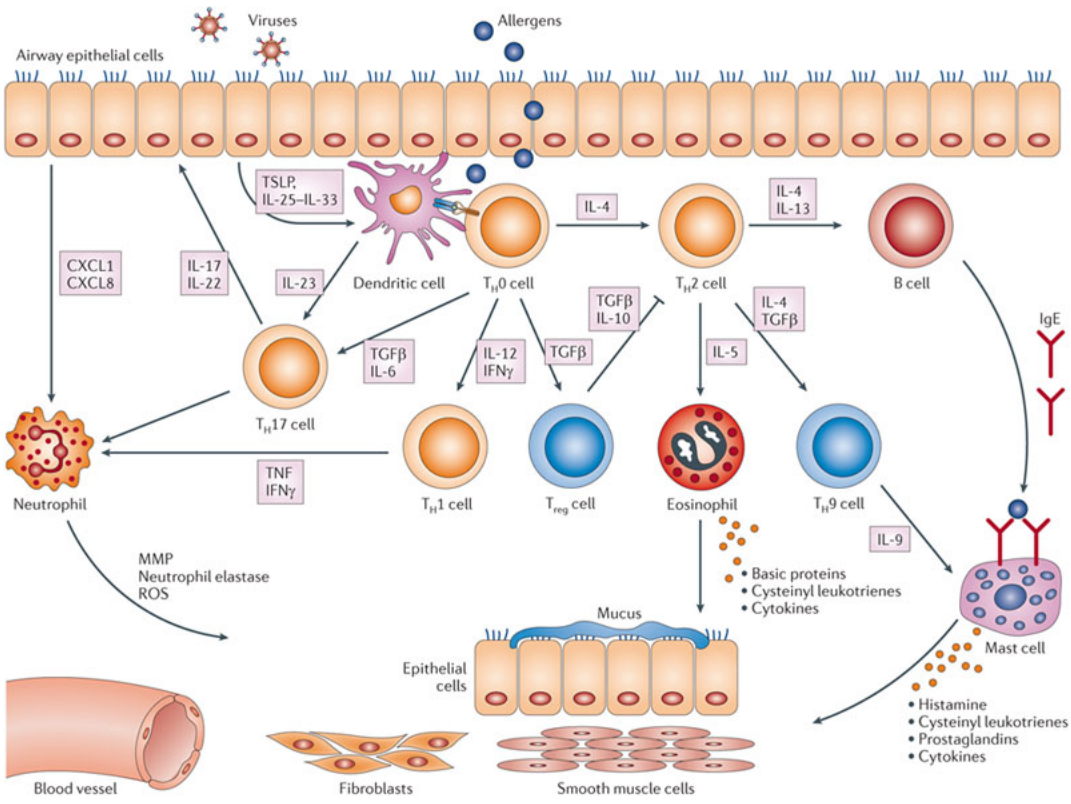


Figure 1.5 Cytokines involved in the pathogenesis of asthma

The majority of cytokines involved in asthma are Th2 inflammatory cytokines. Epithelial cells produce pro-inflammatory cytokines that influence the type of antigens dendritic cells present to T cells which promotes the development of a Th2 environment and potentiates the inflammatory response. Figure from Pelaia et al., 2012 (89).

1.5.1 CYTOKINES SECRETED BY T CELLS

The majority of cytokines involved in asthma are Th2 cytokines. Asthmatic airways show increased numbers of Th2 $CD4^+$ T cells which secrete IL-4, IL-5, IL-9 and IL-13 (90). IL-4 encourages T cell differentiation into Th2 cells. In allergic asthma, IL-4 and IL-13 promote B cell class switching to produce IgE in response to allergen exposure and increase the production of NO and periostin in the lungs (91). IL-5 is crucial for the activation, differentiation, migration and survival of

eosinophils (92). Blocking IL-5 with a humanized antibody results in a marked reduction in eosinophils and fewer exacerbations (93). IL-9 contributes to the overproduction of mucus and eosinophilic inflammation in response to IL-13 (94).

Th1 cytokines are less common in asthmatic inflammation. The Th1 transcription factor, *T-bet,* is under-expressed in asthmatic inflammation, whereas it is overexpressed in COPD (95, 96). As a result, Th1 cytokines such as IFN-α, IFN-γ and IL-12 are under-expressed in asthma (34). IFN-α treatment in patients with severe corticosteroid-resistant asthma has shown to improve lung function, cause a shift in the T cell phenotype towards Th1 and increase production of the regulatory cytokine IL-10 (97).

Th17 cells are proinflammatory cells that produce IL-17 which helps to recruit macrophages and neutrophils into tissues. These cells are increased in asthma (98). Neutrophilic inflammation is linked with high levels of IL-17A which induces the release the neutrophil chemoattractants CXCL1 and CXCL8 (IL-8) from airway epithelial cells (99).

1.5.2 PRO-INFLAMMATORY CYTOKINES

Pro-inflammatory cytokines amplify and maintain inflammation by activating NF-κB which increases the expression of inflammatory genes. IL-1β is a key inflammatory cytokine that is overexpressed in asthma. Antagonizing the IL-1R results in reduced AHR (34). Airway mast cells and epithelial cells can secrete tumour necrosis factor (TNF)-α which

induces AHR likely by increasing the production of Th2 cytokines (100). In asthmatic patients blocking TNF-α has been shown to reduce AHR, decrease exacerbations and increase lung function (101, 102). IL-6 is released by activated mast cells and is thought to promote Th17 and Th2 expansion (103). Anti-inflammatory cytokines such as IL-10 and IL-12 are reduced in asthma.

1.5.3 GROWTH FACTORS

Growth factor cytokines contribute to AR by activating structural cells as well as promoting cell survival and proliferation (34). Eosinophil, neutrophil and macrophage survival is linked with granulocyte-macrophage colony-stimulating factor (GM-CSF) which is produced in response to inflammation by macrophages and epithelial cells. GM-CSF from epithelial cells acts on DCs to promote Th2 cell development (104). Transforming growth factor (TGF)-β is a pleiotropic cytokine that induces proliferation of ASM cells, extracellular matrix (ECM) repair, deposition and AR (34). Asthmatic airway eosinophils have increased levels of TGF-β1 which is correlated with increased asthma severity and sub-epithelial fibrosis (100). Vascular endothelial growth factor (VEGF) is upregulated in asthma causing increased vascularity in the airways as well as inducing vascular permeability. The increased permeability allows proteins to escape into the surrounding tissues causing edema (105).

1.5.4 CHEMOKINES

Chemokines are responsible for recruiting cells to the site of inflammation. CCL2, also known as MCP-1, attracts monocytes and is found in increased concentrations in asthmatic lungs. CCL11, CCL22 and CCL17 are part of a pro-inflammatory loop where they are produced in response to Th2 cytokines and encourage the release of Th2 cytokines. They are found in increased concentrations in BAL from asthmatic patients following allergen exposure (34, 100).

1.5.5 CYTOKINES SECRETED FROM NON-IMMUNE CELLS

Non-immune cells also secrete and respond to cytokines allowing them to contribute to ongoing inflammation. Allergic responses in the airways may be initiated by cytokines released by epithelial cells after they have come into contact with an allergen. Epithelial cells secrete CCL20 which recruits DCs into the lungs and TSLP, GM-CSF and IL-6 which contribute to DC maturation, ILC2 activation and skewing the $CD4^+$ T cell population in favour of a Th2 phenotype (106). TSLP also encourages DCs to take up and present allergens to T cells. IL-25 and IL-33 are produced by epithelial cells and are involved in promoting Th2 eosinophilic inflammation (107).

Table 1.2 Overview of the cytokines evaluated in this thesis

Cytokine	Class	Function	Prevalence in asthma	Effect in asthma
IL-4	Th2	Differentiation of Th2 cells, allergen sensitization, B cell isotype switching to IgE,	Increased in T2 high	Potentiates disease pathology
IL-5	Th2	Eosinophil activation, differentiation, maturation and survival	Increased in T2 high	Potentiates disease pathology
IL-13	Th2	Structural changes in the airways, induces IgE, AHR, FeNO, periostin and mucus hypersecretion	Increased in T2 high	Potentiates disease pathology
TSLP	Th2	Triggers release of Th2 cytokines, promotes eosinophil survival, induces IL-13 and IL-8, suppresses function of Tregs	Increased in T2 high	Potentiates disease pathology
VEGF	Th2	Induces angiogenesis and vascular leakage in airways	Increased in asthma	Potentiates disease pathology
IL-12	Th1	Differentiates and activates Th1 cells, suppresses Th2 expansion, induces IFN- γ and TNF-a release	Reduced levels	Lessens disease pathology
IFN-γ	Th1	Recruits Th1 cells to the lungs, inhibits allergic eosinophilia and AHR, induces IL-10 production	Reduced levels except in severe disease acute exacerbations	Lessens disease pathology
TNF-α	Th1	Proinflammatory, induces AHR and neutrophilic airway inflammation	Increased in asthma	Potentiates disease pathology
IL-1β	Th17	Differentiation of Th17 cells, proinflammatory	Increased in asthma	Potentiates disease pathology
IL-8	Th17	Secreted by epithelial cells to recruit neutrophils	Increased in severe asthma	Potentiates disease pathology
IL-17	Th17	Differentiation of Th17 cells, contributes to neutrophilia	Increased in asthma	Potentiates disease pathology
IL-10	Treg	Suppresses production of pro-inflammatory cytokines, inhibits production of IL-4, IL-5 and IFN-y	Reduced in asthma	Lessens disease pathology
TGF-β1	Treg	Contributes to airway remodelling by inducing proliferation of fibroblasts and ASM cells, deposition of ECM, and epithelial repair	Increased in asthma	Pleiotropic, potentiates severe disease pathology

1.6 ASTHMA CLASSIFICATIONS AND PHENOTYPES

Asthma is a complex heterogeneous disease that has many different pathologies and clinical presentations. “Asthma” is now recognized as an umbrella term that incorporates multiple phenotypes rather than being a distinct disease entity.

Phenotypes are observable characteristics that manifest due to genetic and environmental interactions (8). Asthma phenotyping began in the late 1990s with the goal of describing subgroups of patients who shared biological and clinical characteristics to better understand their disease pathology and determine which treatments they would be most likely to benefit from (108). Phenotyping patients is essential to personalize their care, understand their disease pathology and ensure they are prescribed the correct medications thereby eliminating unnecessary side effects. Phenotyping has become increasingly important with the realization that a significant proportion of patients are not responsive to the treatment options that are currently available (109). Important variables to consider include age, sex, BMI, age of asthma onset, symptomatic triggers and the number of exacerbations experienced per year (110). Phenotypes described in the literature have varied depending on the variables that were considered. For example, a study by Moore and colleagues considered 34 variables and identified five phenotypes whereas a study by Haldar and colleagues identified three phenotypes based on approximately half the number of variables (111, 112). Disease

phenotyping is inconsistent in the literature, some of the common phenotypes and classifications are described below.

1.6.1 CLASSIFICATIONS

Over the years many different subtypes of asthma have been described, some have been studied more extensively than others.

1.6.1.1 Early-onset asthma

In the majority of cases, asthma first appears in children under the age of six (113). Childhood asthma is generally a T2 high disease with eosinophilia and elevated levels of IgE and FeNO caused by allergy that is often associated with a family history of atopy (8). Due to the high levels of eosinophils, this phenotype is generally responsive to treatment with corticosteroids.

1.6.1.2 Late-onset asthma

Asthma that initially presents in adulthood occurs more commonly in females and is often linked with obesity. It is often more severe than early-onset asthma and is associated with a quicker decline in lung function (113). Occasionally late-onset asthma has been associated with allergies however, it is more frequently recognized as a T2 low disease characterized by neutrophilic infiltrations and as such corticosteroids are not always as effective in this group of patients (8, 108, 113, 114).

1.6.1.3 Atopic asthma

Asthma has traditionally been thought of as a T2 mediated disease with a strong connection to allergies mediated by type I hypersensitivity.

Atopic or allergic asthma is the most prevalent phenotype and the easiest to diagnose (88). Allergic asthma most commonly, but not exclusively, manifests early in life. Early-onset is highly linked with a family history of atopy and other allergic diseases (8). This phenotype typically presents with high FeNO and IgE levels and generally responds well to treatment with corticosteroids. For those with severe disease at step 5 of the GINA guidelines there is an option to treat with an add-on biologic against IgE; omalizumab.

1.6.1.4 Non-atopic asthma

Non-atopic or T2 low asthma is thought to be relatively common but is poorly characterized compared to T2 high asthma. Non-atopic asthma tends to show less airway obstruction and AHR than atopic asthma. This phenotype is largely made up of patients with adult-onset asthma who did not have childhood allergies and generally responds poorly to corticosteroids (8).

1.6.1.5 Other possible classifications

Brittle asthma is divided into two types and is characterized by sudden severe attacks with no known trigger (115). Type 1 is characterized by a wide range of PEF values (>40 % diurnal variation) over an extended period of time despite consistent high dose treatment (115-117). Patients with type 2 brittle asthma appear to have normal lung function that is punctuated with sudden acute attacks that occur in less than three hours (115). The prevalence of brittle asthma is not known (116).

Exercise-induced asthma (EIA) is characterized by narrowing of the airways in response to vigorous exercise (118). Symptoms typically last for 30-90 minutes if left untreated. The mechanisms behind EIA are not fully understood. Possible mechanisms include hyperventilation and exposure to dry air which could activate inflammatory pathways (115). The key diagnostic criterion is a fall in FEV_1 of at least 10 % upon exertion (110).

Obesity has been associated with developing asthma, though this relationship is complex. The diseases may be linked by lower lung volumes in obesity and the effect this has on AHR. Obese patients have poorer asthma control and a decreased response to corticosteroids when compared to asthma patients of normal weight (119).

Aspirin-sensitive asthma is generally late-onset, severe and non-atopic. Ingesting aspirin or other non-steroidal anti-inflammatories triggers bronchospasm. Approximately 10-20 % of adult asthma is aspirin-sensitive (115).

1.6.2 PHENOTYPES

1.6.2.1 Eosinophilic asthma

Eosinophilic asthma is the most prevalent and clearly defined phenotype. It is characterized by high levels of eosinophils in blood and sputum and as such generally responds well to corticosteroids. High levels of eosinophils result from T2 driven processes such as CD4 T cells and ILC2s producing high levels of IL-4, IL-5 and IL-13 (120). IL-4 and IL-13 increase NO and periostin production in the lungs while IL-5 recruits and

activates eosinophils (87). Eosinophils are granulocytic immune cells that store highly toxic substances within their granules which are useful in fighting parasitic infections and are implicated in allergic and autoimmune conditions. IL-5 and GM-CSF are important for eosinophilic growth, differentiation and migration from the bone marrow (92) which is generally associated with atopic asthma and thickening of the basement membrane (121, 122). Adjusting corticosteroid treatment to maintain sputum eosinophils below 3 % has proven to be an effective method of reducing the occurrence of exacerbations in this phenotype (120). However, not all patients with severe asthma display airway eosinophilia and it is not required for nor is it specific to a diagnosis of asthma (108). Eosinophils are also found in the airways in patients with COPD and bronchitis (92). Corticosteroids have a pro-apoptotic effect on eosinophils and thus are an effective treatment regardless of the diagnosis (123).

1.6.2.2 Neutrophilic asthma

Airway neutrophilia has been associated with reduced lung function, increased AHR and airway wall thickening (124). This phenotype is commonly seen in patients on a high-dose of corticosteroids as they can contribute to the development of neutrophilia by encouraging neutrophil activation and preventing their apoptosis. However, it is still unclear if these patients would still have neutrophilia in the absence of corticosteroid treatment (8, 125). Neutrophils are the most common form of granulocyte. They are a good indicator of inflammation as they

are one of the first cell types to be recruited (126). Asthmatic airway neutrophilia is associated with airway infections, obesity and exposure to hazardous substances such as cigarette smoke. Patients with neutrophilic inflammation tend to have a decreased response to ICSs (121, 124, 127-129). A study by Cowan et al found that treatment with ICSs increased airway neutrophilia and a switch towards the neutrophilic phenotype was noted in some patients (87). Shaw et al found that neutrophilic asthma is also less responsive to bronchodilators as these patients displayed a smaller improvement in FEV_1 than patients with eosinophilic inflammation (130).

1.6.2.3 Mixed eosinophilic neutrophilic asthma

Some patients exhibit both eosinophilic and neutrophilic infiltrations. This phenotype is poorly studied. It has been suggested to be associated with fungi allergies and bronchiectasis (8).

1.6.2.4 Paucigranulocytic asthma

Paucigranulocytic inflammation is characterized by a lack of inflammatory cells in the airways and is poorly characterized in the literature. It has been suggested that approximately 30-50 % of patients could fall into this category representing a significant proportion of patients who are not likely to respond to ICS due to their lack of eosinophils and therefore are not being effectively managed on steroid-based treatments (70).

1.7 SMALL AIRWAY INVOLVEMENT

1.7.1 SMALL AIRWAY PHYSIOLOGY

Historically asthma has been considered a disease of the large airways. However, it is now known that inflammation of the small airways (defined as having an internal diameter of <2 mm) is critical in asthma and is more prevalent in certain patients such as those with severe asthma, a longer duration of asthma, smokers and the elderly (131). Some researchers suggest that small airway involvement could represent its own phenotype (132). Characteristics of small airway obstruction include air trapping and premature closure of the airways. Air trapping and hyperinflation are indicated by an increase in residual volume (RV) and RV/ total lung capacity (TLC) (133). Small airway dysfunction is strongly associated with severe difficult-to-treat asthma and may be related to the increased occurrence of exacerbations in this population (134). In severe asthma, the basement membrane and smooth muscle layers of the small airway walls thicken and the lumen can be further blocked by mucous and an influx of inflammatory cells which leads to increased airway resistance, air trapping and difficulty breathing (135). Recently, studies have found that small airway dysfunction is not only found in severe asthma but also exists in patients with poorly controlled mild asthma (132).

1.7.2 TECHNIQUES TO ASSESS SMALL AIRWAY INVOLVEMENT

Spirometry alone is not capable of accurately assessing the small airways. FEV_1 is predominately a measure of the proximal airways; the small airways contribute the later portion of expiration. As such, small airway function is better assessed by the forced expiratory flow (FEF) between 25-75 % ($FEF_{25-75\%}$) of the FVC however, this value has a wide range of normal values, can only be used when the FVC is within normal ranges and is not reproducible which makes it an unreliable marker (133). The failure of spirometry to accurately identify small airway involvement was demonstrated in a study conducted by Perez and colleagues. They found that small airway obstruction was observable using plethysmography in over 50 % of patients with stable moderate-to-severe asthma and normal spirometry (FEV_1 and FEV_1/FVC) (133). This study demonstrated that small airways disease is more prevalent than originally believed (136, 137).

Oscillometry as discussed above (**Section 1.2.2.3**) is superior to spirometry when it comes to assessing small airway involvement as it provides a measure of overall lung impedance as well as breaking it down into the large and small airways (138). As this test is effortless it is optimal for patients of all ages and abilities.

1.7.3 TREATMENT OPTIONS

The corticosteroid ciclesonide delivered via a hydrofluoroalkane (HFA) pressurized metered dose inhaler (pMDI) containing small particles

(approximately 1.1 microns) deposits approximately 50 % of the delivered dose to the small airways (139). Compared to large particle ICSs, a fine particle spray has been shown to improve symptoms, control lung function and allow for a reduction in reliever use (7, 140).

A study designed to examine the effects of ciclesonide and montelukast as add-on therapies on small airway function in patients with stable asthma determined that ciclesonide significantly reduced alveolar nitric oxide levels and montelukast significantly reduced small airway reactance. The authors suggested that while separately both therapies improved small airway abnormalities, together they may be even more effective (141).

In patients with mild asthma, ICS/LABA combination therapies have resulted in sustained improvements in small airway function over four weeks based on significant reductions in R5-20 and AX parameters (142).

1.8 AIRWAY REMODELLING

Chronic airway inflammation that leads to airway remodeling (AR) is a pathophysiological hallmark of asthma (143). AR is represented by structural changes such as airway wall thickening, subepithelial fibrosis, angiogenesis, basement membrane thickening, airway cell hyperplasia and hypertrophy (100, 144). These changes correlate with increased asthma severity and poorer outcomes (144). Chronic inflammation is implicated in AR although the exact mechanisms are not completely understood (145). Epithelial cells are the physical barrier against inhaled

pathogens. Biopsies from patients with chronic asthma have shown that epithelial cells slough off suggesting that the epithelium is in constant repair. Abnormalities in the injury-repair process could result in fibroblasts increasing the deposition of ECM proteins below the basement membrane causing subepithelial fibrosis (146). Hoshino et al found that increasing concentrations of serum periostin, an ECM protein, correlated with increasing airway wall thickness and that ICS therapy was effective in decreasing both periostin levels and airway wall thickness (147).

1.9 AIRWAY HYPERRESPONSIVENESS

Airway hyperresponsiveness (AHR) along with symptoms, variable airflow obstruction and inflammation comprise the four domains of asthma (148). Airway responsiveness is the ability of the airways to constrict in response to a trigger (149). AHR is the predisposition of the airways to constrict excessively in response to stimuli that would cause little-to-no reaction in healthy individuals (150). AHR is a clinical feature of asthma that is present in virtually every patient and often reflects the severity of the disease. However, there is a high degree of variability both within and between individuals (151). AHR can be divided into two components: variable and persistent. The variable component pertains to the inflammation present in the airways which is heavily influenced by the environment and thus is constantly changing. The persistent component reflects structural changes to the airway such as subendothelial thickening, basement membrane thickening, matrix deposition and vascular alterations (148, 150-152). The degree of AHR

and the mechanisms causing it are evaluated clinically using either direct or indirect bronchial provocation tests (BPTs). Direct BPTs, such as methacholine, cause constriction by acting directly on receptors on ASM cells whereas indirect BPTs, such as mannitol, trigger the release of mediators from inflammatory cells already in the airways that cause bronchoconstriction (148, 152). Since sensitivity to indirect stimuli depends on the inflammation that is already present in the airways there is a strong relationship between AHR response and anti-inflammatory treatment in terms of reduction of eosinophils and FeNO (152). Direct BPTs have a high sensitivity (96.5 %) for the presence of asthma however, they have a low specificity (78.4 %) as individuals with other conditions such as atopy and allergic rhinitis may also react (152, 153). Indirect BPTs have shown to be more specific but less sensitive. Therefore, a positive response to indirect stimuli would be more useful than a positive response to direct stimuli to confirm the diagnosis of asthma however a negative response to direct stimuli can rule out a diagnosis of asthma (148).

Symptom severity does not correlate with AHR or airway inflammation. Well controlled patients still display sensitivity to challenge tests, because of this AHR may provide unique information not linked with symptoms or lung function (148). Several studies have investigated if the severity of AHR can be used to adjust treatment, but the results have been inconsistent. However, sensitivity to indirect stimuli at baseline is predictive of response to ICS treatment which suggests that indirect

challenges could be used to direct treatment and monitor asthma progression (148).

1.10 EXACERBATIONS

Asthma exacerbations are periods of increased symptoms and decreased lung function. These events are distressing for patients and account for a significant proportion of the financial burden associated with asthma care (154). As such, the primary goals of asthma management are to increase lung function, minimize current symptoms and reduce the risk of future exacerbations (155). The American Thoracic Society and European Respiratory Society proposed that exacerbations be defined as "events characterized by a change from the patient's previous status" for both clinical and investigational purposes. They suggest that severe exacerbations require immediate treatment to prevent hospitalization or death whereas moderate exacerbations are defined as events that extend beyond the patient's normal symptom variation indicating the need for a change in treatment (156). In 2014, this definition was updated to include the need for oral corticosteroids for at least three consecutive days (157). However, this definition is not fully agreed upon and many studies still use different criteria to evaluate and report on exacerbations. Such differences include; an increase in symptoms (which can be quantified by numerous different methods), a decrease in lung function (PEF or FEV_1) over varying periods of time or the need for additional treatment (systemic or an increased dose of ICS or antibiotics).

Prior to adolescence, boys are twice as likely as girls to experience an exacerbation however, during adolescence this trend reverses and women are more than twice as likely as men to experience an exacerbation (158). Common exacerbation triggers include viral and bacterial infections, as well as exposure to allergens, pollutants and chemicals. Upper respiratory tract infections (URTI), specifically rhinovirus infections, account for the majority of exacerbations in adults (159).

1.10.1 EXACERBATION PREVENTION

Numerous pharmacological treatments along with personalized action plans are used to control symptoms and prevent exacerbations. Strategies can be separated into daily maintenance treatments to prevent the occurrence of exacerbations and intervention methods that can be implemented or increased at the first sign of deteriorating control to prevent a full-blown exacerbation.

The risk of exacerbating and the severity of the exacerbation are linked with access to and/or compliance to ICSs which act by reducing the production of pro-inflammatory mediators and enhancing innate immunity (159).

Two decades ago Pauwels and colleagues demonstrated that LABAs successfully reduced the rates of mild and severe exacerbations, especially in patients already being treated with high dose ICSs (160). Combination inhalers with the LABA formoterol that can be taken daily for symptom control and can be increased when symptoms worsen and

control deteriorates have been shown to significantly increase the time to the first severe exacerbation and reduce the occurrence of exacerbations at lower or equal daily doses of corticosteroid than fixed dose regimes (53, 161, 162). Self-management plans that outline how and when to implement this have shown to be effective at reducing severe exacerbations by approximately 20 % (80).

1.11 OTHER FACTORS INFLUENCING ASTHMA

Sex hormones are not just physiologically active in reproductive organs. They can affect the function of other organs such as the lungs. Numerous lung conditions such as asthma, COPD and cystic fibrosis show sex differences in terms of disease severity and prevalence. Estrogens are pro-inflammatory and have been suggested to predispose individuals to atopy whereas androgens are immunosuppressive and thus may provide a protective effect (163). Asthma shows clear gender differences in terms of severity and prevalence. Before puberty boys are more likely than girls to have asthma (164); following puberty this trend is reversed and women are twice as likely to have asthma (165). In males, asthma severity tends to be stable from puberty until old age when testosterone levels start to decline, and asthma severity worsens (166). In females the risk of asthma peaks during periods of hormonal changes such as puberty, menarche, menstruation and menopause (165).

It is strongly accepted that there is a familial link in asthma however, to date there is no singular "asthma gene". It is hypothesized that asthma polymorphisms are context-dependent in that they predispose for the risk of developing asthma depending on environmental factors and polymorphisms in other genes (167). One of the biggest issues in genetic studies has been the lack of consistency between studies. Many genes that are identified in one study failed to show any association with asthma in another or in different populations. Continuing to identify asthma-associated genes and the ways in which genetic polymorphisms influence asthma phenotypes will be crucial in advancing treatment options and developing genetic-based therapies.

Vitamin D is fat-soluble and is obtained from dermal synthesis of vitamin D3 with UV light or sunlight as well as from diet (168). Vitamin D has significant effects on both the innate and adaptive immune system, although the mechanisms are not completely understood. Vitamin D inhibits CD4 T cell proliferation, down-regulates Th1 cytokines and IL-17 production. It has also been shown to increase IL-10 by promoting naïve T cells to become regulatory T cells (Tregs). Production of immunomodulatory TGF-β has also been shown to increase in the presence of vitamin D (169). In asthma, low levels of vitamin D have been associated with reduced lung function and increased disease severity, airway remodeling and exacerbations (170, 171). However, the literature outlining the benefit of vitamin D supplementation in asthma has been inconsistent. The benefits of vitamin D seem to be stronger in

children than in adults and even then possibly only if supplementation was given during pregnancy (168).

1.12 GLUCOCORTICOIDS

Glucocorticoids are steroid hormones derived from cholesterol which are endogenously produced and released under the control of the HPA axis into the circulatory system following environmental or physiological stimuli (172). GCs are essential for life and have a variety of functions including metabolism, cognition, immune function and development (172).

During times of stress corticotropin-releasing hormone (CRH) is released from the hypothalamus which in turn stimulates the production and release of adrenocorticotropic hormone (ACTH) from the anterior lobe of the pituitary gland. ACTH causes the adrenal cortex to synthesize and release GCs, namely cortisone. Rising levels of GCs complete a negative feedback loop and inhibit the subsequent release of ACTH (172, 173). (**Figure 1.6**). The actions of GCs are regulated in tissues by the actions of 11β-hydroxysteroid dehydrogenase enzymes (11β-HSD) and corticosteroid-binding globulin in serum. In order for GCs such as cortisone (endogenous) and prednisone (synthetic) to be physiologically active, they must be converted into their active metabolites, cortisol and prednisolone respectively by 11β-HSD1 (174). Endogenously produced GCs can be inactivated by 11β-HSD2. However, synthetic GCs are not affected by this enzyme nor do they bind to corticosteroid-binding globulin meaning that their availability is less regulated.

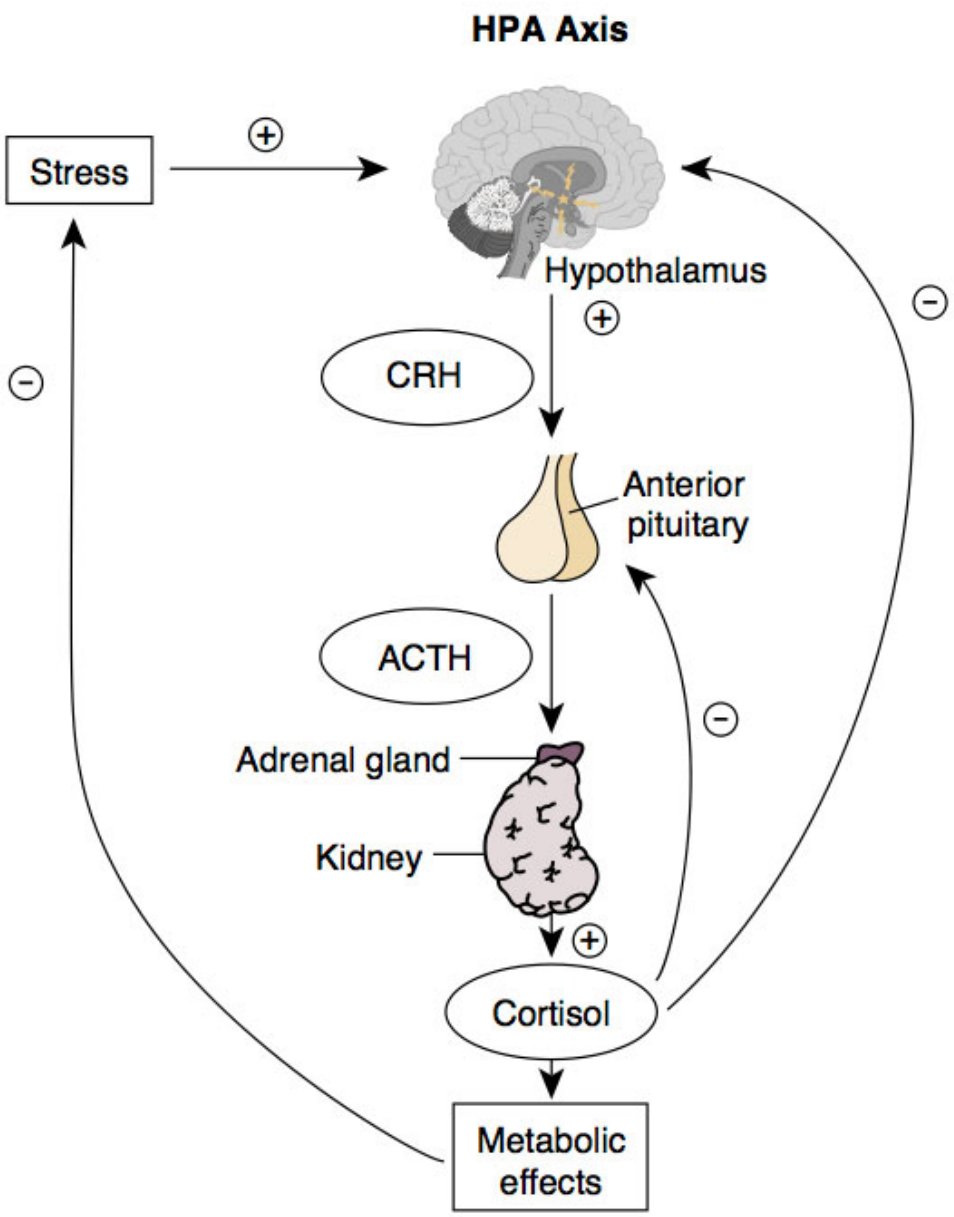


Figure 1.6 Hypothalamic-pituitary-adrenal (HPA) axis

The hypothalamus secretes corticotropin-releasing hormone (CRH) which stimulates the anterior lobe of the pituitary gland to produce adrenocorticotropic hormone (ACTH). ACTH activates the adrenal glands and stimulates the release of cortisol. Cortisol feeds back to the hypothalamus to inhibit the release of ACTH. + simulates, - inhibits (175).

1.12.1 GLUCOCORTICOID RECEPTOR

1.12.1.1 Structure and isotypes

The glucocorticoid receptor (GR) belongs to the nuclear receptor superfamily (173). It is a modular protein made of three domains: NH_2-terminal transactivation domain (NTD), central deoxyribonucleic acid (DNA)-binding domain (DBD) and COOH-terminal ligand-binding domain (LBD) (173, 176). The LBD interacts with transcriptional activating factors, the DBD is required for receptor dimerization and binds to specific DNA sequences, called glucocorticoid response elements (GREs), on target genes and the NTD binds to transcriptional factors thus mediating gene transactivation (177). The GR is the product of the *NR3C1* gene located on chromosome five which contains 9 exons. Alternative splicing of exon 9 results in two molecular isoforms: GR-α, GR-β, both of which are thought to be expressed in all human tissues. GR-α is the primary isoform which is capable of binding to GCs to alter gene expression. GR-β is lacking amino acids in the LBD which prevents it from binding GCs (172, 173) (**Figure 1.7**)**.** GR-α is located in the cytoplasm of cells until it binds its ligand where it translocates into the nucleus whereas GR-β is contained in the nucleus of cells and acts as a dominant negative inhibitor of GR-α induced gene transcription (172). Recently, it has been proposed that GR-β may have its own intrinsic transcriptional activity that is independent of GR-α. Kino and colleagues found that GR-α and GR-β were primarily responsible for regulating a distinct set of genes. However, in a small subpopulation of genes both

receptors were active and regulated each other's actions (178). The complexity of GR signaling is increased by various types of GREs which are thought to contribute to the selectivity of transcription (174).

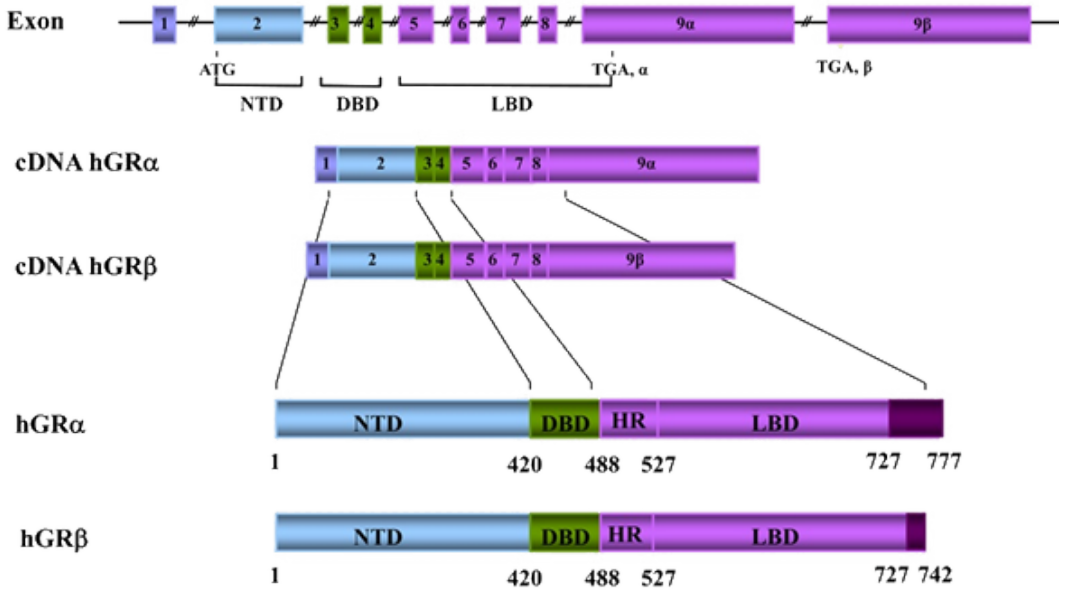


Figure 1.7 Genomic structure of the glucocorticoid receptor

Alternative splicing of exon nine results in two receptor isomers, glucocorticoid receptor (GR)-α and GR-β. The two isoforms are identical until amino acid 727 then GR-β is lacking 50 amino acids in the ligand-binding domain (LBD) which prevents it from binding to glucocorticoids (179).

1.12.1.2 Receptor activation and signaling

In the absence of ligand, GR is located in the cytoplasm bound to chaperone proteins such as heat shock proteins (hsp90). GCs are able to diffuse through the lipid bilayer of the cell and enter the cytoplasm where they bind to their receptor. Ligand binding triggers the receptor to undergo conformational changes which causes the chaperone proteins to dissociate allowing the receptor-ligand complex to move into the nucleus. In the nucleus, the receptors dimerize and bind to GREs on

corticosteroid-responsive genes to regulate their transcription (180-182). Binding to GREs triggers recruitment of transcriptional coactivator proteins (i.e. SRC-1 and cAMP response element binding protein) which allow for increased transcription (183). Gene expression and repression are influenced by changes in the chromatin structure caused by histone modifications. An increase in histone acetylation is associated with increased gene transcription whereas deacetylation is associated with reduced gene transcription (184).

1.12.2 MECHANISMS OF GLUCOCORTICOID ACTION

GCs act through several different genomic mechanisms which usually take minutes to hours to occur (176). Transactivation occurs when GR homodimers bind directly to DNA at GREs of target genes resulting in their transcription. Co-activators or co-repressors are recruited to the GREs which triggers an increase in the expression of anti-inflammatory mediators such as IL-10 and a decrease in pro-inflammatory mediators such as IL-8 (173). GRs can also bind to negative GREs (nGRE) that suppresses the transcription of the target gene.

GCs can also have effects through non-genomic mechanisms. These actions are rapid and occur within seconds to minutes of exposure as they are not dependent on changes in gene expression. Non-genomic actions occur in cell types that do not typically respond to steroids and are not affected by inhibiting gene transcription (173). As these mechanisms do not require nuclear translocation they occur either in the

cytoplasm or at the plasma membrane and are generally responsible for modulating vascular flow (173).

Post-translational modifications such as phosphorylation, ubiquitination, nitrosylation and acetylation also affect the functionality and sensitivity of the receptor (185). Phosphorylation of the GR impacts movement into the nucleus and transcription, ubiquitination encourages receptor degradation, nitrosylation impacts GC binding and acetylation increases GR sensitivity for GC (173).

1.12.3 STEROID TREATMENT AND ASTHMA

Inhaled corticosteroids, or GCs, are the most effective controller medication used to treat asthma. Even at low doses, they are able to reduce inflammation present in the airways by inhibiting recruitment of inflammatory cells, inducing apoptosis of inflammatory cells already in the airways and decreasing the production of chemotactic mediators and adhesion molecules (43). Treatment with ICSs results in a significant decrease not only in inflammation but also in symptom severity, AHR, morbidity and exacerbations as well as improving lung function (43). Inhalation is the preferred route of administration as the majority of the drug is deposited directly into the lungs where it can reduce local inflammation and minimize systemic side effects (186). During inhalation, a portion remains in the mouth and pharynx. The portion delivered into the lungs acts locally on the airways where it can be absorbed into systemic circulation and result in side effects. The portion that is deposited into the mouth and pharynx is swallowed and absorbed

into the gastrointestinal (GI) tract if it is not rinsed out. Once in the GI tract, the drug has avoided inactivation by first-pass metabolism in the liver and is free to enter systemic circulation where it can again cause side effects (187). In some cases, the dose required to control severe asthma is too great and oral steroids are required which further increases the likelihood of experiencing adverse systemic side effects.

1.12.3.1 Pharmacokinetic and pharmacodynamic factors of ICSs

Receptor binding affinity varies significantly between GCs which impacts on the safety, efficacy and side effects of each treatment as all effects are mediated by receptor binding. Binding strength is termed relative receptor affinity (RRA) with dexamethasone given a reference value of 100 (186). It is important to remember that higher RRAs do not necessarily correlate with increased clinical efficacy as increased receptor activation can also enhance systemic side effects (188).

Particle size is a key factor in determining how far into the airways the drug can reach. Small airways have a diameter of ≤2 mm. As such particle sizes of <5 μm are likely to reach the bronchi and bronchioles while sizes >5 μm will likely stay in the mouth and pharynx where they are more likely to contribute to hoarseness and can cause candidiasis (189).

The bioavailability of the drug has clear impacts on the safety profile. The proportion of the drug that is deposited in the mouth and absorbed orally will have minimal therapeutic benefit and can contribute to

systemic side effects once absorbed into circulation. Therefore, a drug with low oral bioavailability is preferred. A drug with high pulmonary bioavailability is likely to have greater clinical benefit, although it can also become absorbed into systemic circulation (190).

Drugs such as ciclesonide, which are delivered as inert prodrugs and are only activated once they reach the lungs, are advantageous in that in their inert form they are essentially inactive and cannot contribute to adverse systemic side effects (48).

The more lipophilic the corticosteroid the more easily it can pass through the phospholipid bilayer of cell membranes and is retained there for longer. In this way, increasing pulmonary retention time increases the anti-inflammatory effects of the drug without increasing adverse systemic side effects (191).

Corticosteroids are able to reversibly bind to proteins, typically albumin in intracellular and extracellular fluids as well as in circulation. Protein-binding minimizes the systemic side-effects of corticosteroids as they are not pharmacologically active when bound to protein (186).

The pharmacokinetic properties of several commonly used inhaled corticosteroids are summarized in **Table 1.3**.

Table 1.3 Pharmacokinetic properties of commonly used inhaled corticosteroids

Overview of the relative receptor affinity (RRA), bioavailability, protein binding and half-life of naturally produced glucocorticoids and several commonly used inhaled corticosteroids in asthma (190).

ICS	RRA	Oral bioavailability (%)	Protein-binding (%)	Half-life (h)
Cortisol	10	-	-	-
Dexamethasone	100	-	-	-
Ciclesonide	12	<1	99	0.4
Desisobutyryl-Ciclesonide	1200	<1	99	3.6-5.1
Beclometasone dipropionate	53	15	87	0.1
Budesonide	935	11	88	2.8
Fluticasone propionate	1800	<1	90	14.4
Mometasone furoate	2200	<1	98	4.5

1.12.4 ADVERSE SIDE EFFECTS

There are numerous adverse side effects associated with corticosteroid use. A longer duration of treatment and higher dose are correlated with adverse symptom appearance and severity. Systemic corticosteroids are most likely to result in adverse side effects, but ICSs can trigger the same effects especially with long-term use.

ICSs can result in local adverse effects such as coughing, oropharyngeal candidiasis, bronchospasms, throat irritation and dysphonia. Local side effects are not usually severe and do not cause significant morbidity.

However, as these effects are unpleasant for patients they can affect treatment compliance which can lead to uncontrolled asthma (186).

Systemic side effects are likely to be more severe. The most serious being suppression of the HPA-axis which can progress to adrenal crisis. Other systemic side effects that can occur are thought to be related to HPA-axis suppression include thinning of the skin, bruising, glaucoma, cataracts, reduced bone mineral density and osteoporosis (48). Manson and colleagues performed an analysis of 63 studies to determine the range and frequency of adverse events (AE) due to steroid treatment. They found that fractures were the most commonly reported AE and occurred more frequently in women than men and when steroids were used at higher doses (192). Long-term glucocorticoid treatment can induce systemic vascular resistance causing hypertension.

Ciclesonide has been shown to be as effective as other ICSs in terms of improving lung function, decreasing symptoms and reducing the need for rescue treatment while reducing side effects (193, 194).

1.12.5 CORTICOSTEROID RESISTANCE IN ASTHMA

Reduced sensitivity to corticosteroids occurs in approximately 5-25 % of patients with asthma and is more common in severe cases (180, 195). Steroid-resistant asthma has been defined as a failure to improve FEV_1 by >15 % following a 14-day course of oral steroids (196). Severe steroid-resistant patients account for 50-80 % of the costs associated with asthma management and as such finding a successful alternative

treatment is a priority. Resistance is more commonly linked with non-eosinophilic phenotypes characterized by increased Th1 and Th17 responses than with classical Th2 eosinophilic asthma (180). As there are multiple molecular mechanisms that can contribute to steroid resistance not only in asthma but all inflammatory conditions it is likely that effective treatments will need to be personalized to the mechanisms driving the inflammation to restore sensitivity.

1.13 BIOMARKERS

Biomarkers are features that can be reliably and objectively measured as indicators of healthy pathology, presence of disease, disease phenotype, disease pathology or response to treatment (197). The identification of biomarkers is crucial in asthma to define phenotypes and direct specific treatments to the subgroups of patients that are likely to respond which is the foundation of personalized medicine (88, 109). Ideal biomarkers are easily obtained, non-invasive, reliable and cost-effective. Biomarkers can be classified as either predictive, pharmacodynamic, diagnostic or prognostic. Predictive biomarkers provide information as to which patients are likely to benefit from a certain treatment and as such, they are influential in clinical decision making. Pharmacodynamic biomarkers provide information as to the biological response to pharmacological interventions. Diagnostic biomarkers are useful in identifying the presence or absence of a particular disease while prognostic biomarkers provide information regarding the outcome of the patients' condition (197). There are

currently four widely accepted biomarkers for asthma: FeNO, serum periostin, and eosinophil levels in blood and sputum. The use of biomarkers is essential to correctly identify disease phenotypes and to determine which treatments are likely to be beneficial so that the right drugs are given to the right patients and progress can be made towards personalized medicine (198, 199).

1.13.1 FRACTIONAL EXHALED NITRIC OXIDE

The volatile organic compounds found in exhaled breath vary depending on the presence of different diseases and therefore can serve as biomarkers to distinguish between disease phenotypes and response to treatment (200). In the lungs, nitric oxide (NO) is an important regulator of airway tone, mucin secretion, mucociliary clearance and immune function (201). NO is produced when a guanidino group of L-arginine is oxidized, a reaction which is catalyzed by the enzyme nitric oxide synthase (NOS) (202). NO synthesis is upregulated in asthma. When NO binds to its soluble receptor, guanylate cyclase (sGC), protein activation is triggered which increases the production of cyclic guanosine monophosphate (cGMP) which causes vasodilation (203). sGC expression and activity is diminished in asthma, which causes a reduction in vasodilation. This is known to contribute to AHR. *In vitro* sGC levels have shown to be regulated by inflammatory stimuli (204). Thus, low levels of sGC in asthmatic airways could be due to the presence of increased inflammatory cytokines.

FeNO is a biomarker that was initially thought to reflect eosinophilia as it correlates with sputum eosinophils (205, 206). However biologic therapies which reduce eosinophil levels by binding IL-5 have no effect on FeNO and therapies that reduce FeNO by binding to IL-13 do not reduce blood eosinophils (197, 207, 208). As such, FeNO and eosinophils reflect two different arms of T2 inflammation. FeNO is induced by IL-4 and IL-13 which signal through STAT-6 to upregulate the expression of inducible NOS (iNOS), and subsequently the production of NO, in bronchial epithelial cells. Therefore, FeNO is a biomarker of allergic asthma and reflects the extent of inflammation in the airways. Whereas eosinophils are induced by IL-5 which signals through STAT-3 and are a marker of T2 inflammation which may or may not have an allergic component (47).

FeNO is obtained non-invasively from the lower respiratory tract and can be used as a predictive biomarker of response to corticosteroids (209). A FeNO of greater than 50 ppb has been shown to be a predictive biomarker of response to corticosteroids whereas a FeNO of less than 25 ppb suggests a lack of response (210). Continuous monitoring of FeNO levels may be useful in assessing asthma control as elevated FeNO levels have been recorded during exacerbations and are linked with a loss of control and a rapid decline in lung function (211). FeNO levels are associated with allergen sensitization (212, 213). A study by Roos and colleagues found increased FeNO, NO synthase activity and iNOS expression following allergen exposure in allergic asthmatics. This

finding supported the hypothesis that in allergic asthmatics increased bronchial iNOS expression leads to increased NOS. They found FeNO was increased during exacerbations suggesting FeNO may reflect or predict loss of control (213). This finding was the first to present a mechanistic explanation for increased FeNO in response to an allergen in allergic patients. FeNO (along with serum periostin) is also used as a biomarker to predict response to anti-IL-13 therapies which have shown to improve lung function in addition to reducing exacerbation rates (207).

The relationship between FeNO, symptom control and corticosteroids is still unclear. Shaw and colleagues used FeNO measurements instead of patient symptoms and airflow obstruction tests to adjust the dose of ICS. This method did not result in significantly fewer exacerbations or reduced corticosteroid use (214). A systematic review of 26 studies determined that in terms of diagnosing asthma the specificity (0.82) of FeNO was higher than the sensitivity (0.65). For this reason, the authors suggest that while a low reading cannot exclude the diagnosis of asthma (as these patients may have asthma that is not characterized by T2 airway inflammation), its high specificity suggests that it may be a useful diagnostic tool to rule in asthma. The authors also suggest that in patients with high FeNO levels this test may negate the need for bronchial provocation tests as FeNO reflects AHR and is advantageous in that it is easy to perform, quick and does not require specialist training (215).

FeNO is an ideal biomarker to add to the physician's diagnostic tool kit as it provides immediate information regarding local inflammation which can be used to complement spirometry, which details airflow obstruction, as well as symptoms and medical history to provide a comprehensive overview of each patients condition (216). In support of this concept, a meta-analysis by Donohue and Jain evaluated the predictive value of FeNO in identifying corticosteroid-responsive asthma and determined that FeNO used together with clinical parameters resulted in significantly lower exacerbation rates than clinical parameters alone (47).

1.13.2 SERUM PERIOSTIN

Periostin is a matricellular protein secreted by epithelial cells and fibroblasts in the lungs in response to IL-4 and IL-13 (217, 218). Periostin was identified as a biomarker for asthma in a study by Woodruff and colleagues where they identified periostin (POSTN) along with chloride-channel accessory protein 1 (CLCA1) and serine peptidase inhibitor, clade B (serpinB2) as genes that were increased in asthma, specifically by IL-13 production, and could be reduced two fold by corticosteroid treatment. This was identified as the "3-gene signature" and is indicative of Th2 inflammation that responds well to ICSs as measured by an increase in FEV_1 (217, 219). Periostin is involved in subepithelial fibrosis, production of TGF-β, increased cell adhesion and thus is a major contributing factor to AR in patients with asthma (220, 221). Guquan and colleagues found that serum periostin was elevated only in asthma with eosinophilic airway inflammation, suggesting that

periostin is a systemic biomarker of asthma characterized by eosinophilic inflammation (222). Elevated periostin along with FeNO are used as predictive biomarkers for a positive response in terms of improved lung function (FEV_1) and reduced exacerbation rates following treatment with anti-IL-13 biologics in asthma (207, 223).

1.13.3 EOSINOPHILS

Asthma characterized by T2 eosinophilic inflammation generally responds well to treatment with corticosteroids and/or anti-IL-5 therapy (121, 122, 224). It has consistently been shown that adjusting ICS dose based on eosinophilic levels reduces exacerbation occurrence and severity (120, 225, 226). Blood and sputum eosinophilia are associated with basement membrane thickening and increased asthma severity (227). Not all patients present with airway eosinophilia however, this does not necessarily mean that eosinophilic inflammation is not involved in their disease pathology. It could be that corticosteroid treatment has diminished their presence. Kulkarni and colleagues conducted a study to measure ongoing eosinophilic inflammation during treatment with corticosteroids. They found that macrophage eosinophil proteins were increased in asthmatics regardless of sputum eosinophil levels. They suggest that in these patients sputum eosinophils were reduced by the corticosteroid therapy but that eosinophilia was still a part of their disease pathology and treating these patients as though they were eosinophilic may be beneficial (228). This finding may also offer an

explanation as to why patients diagnosed with non-eosinophilic asthma see improvements with corticosteroids.

1.13.3.1 Blood eosinophils

Elevated blood eosinophil levels (≥0.3 cells $x10^9$/L) are the most commonly utilized predictive biomarker of T2 high asthma and are used to identify those most likely to respond to various treatments including corticosteroids and anti-IL-5 monoclonal antibodies (229). Early studies investigating the utility of mepolizumab (anti-IL-5) in severe asthma did not stratify patients based on eosinophilia and failed to show any treatment benefit. Following this, treatment was given to those with elevated eosinophils and in these patients, mepolizumab was found to reduce the occurrence of exacerbations and allow for a reduction in their daily maintenance dose of OCS without compromising lung function. This finding highlighted the necessity of utilizing biomarkers to ensure the correct medications are given to the correct patients and subsequently blood eosinophilia (≥0.3 cells $x10^9$/L) has become a requirement in order to be approved for treatment with mepolizumab.

Blood eosinophils have been shown to be an accurate surrogate marker of sputum eosinophil levels and thus are an easy-to-measure biomarker of eosinophils in the airway (230, 231). Several studies have suggested that blood eosinophilia is linked with reduced lung function (FEV_1), more severe exacerbations and poorer disease control (232-234).

1.13.3.2 Sputum eosinophils

Inflammation in the airways is thought to cause asthmatic symptoms and variations in lung function. Sputum analysis helps to determine the inflammatory profile so anti-inflammatory treatment can be specifically tailored to each patient (88). Sputum cell counts can reveal numerous patterns including; eosinophilic, neutrophilic, paucigranulocytic or mixed granulocytic (229). Sputum eosinophilia is predictive of a positive response to corticosteroids and monitoring sputum levels has consistently found that elevated levels (>3 %) are predictive of loss of control and/or an impending exacerbation. As such, sputum eosinophil levels can be used to guide steroid therapy. Maintaining sputum eosinophils <3 % is ideal to achieve control and reduce the risk of experiencing an exacerbation (120). While monitoring sputum eosinophil levels has value in guiding steroid treatment not all patients are able to produce a sample spontaneously and inducing sputum can be challenging and uncomfortable for the patient. Additionally, processing the sample requires specialist knowledge and equipment. As such, blood eosinophil levels are often used as a surrogate marker of sputum eosinophils (230).

1.14 FUTURE THERAPIES AND DRUG TARGETS

The heterogeneity of asthma pathogenesis and responsiveness to established treatment has highlighted the need for new therapies.

Biologic agents against inflammatory mediators implicated in the pathogenesis of asthma are becoming an attractive treatment option in addition to or instead of traditional corticosteroids and bronchodilators.

1.14.1 MONOCLONAL ANTIBODIES

Biologic treatments have been revolutionary in treating patients in whom traditional therapies have been ineffective. First-generation monoclonal antibodies (mAbs) were fully murine. This was problematic as human fragment crystallizable (Fc) receptors did not recognize the murine Fc fragment and patients were developing human anti-mouse antibodies (HAMAs) causing the mAbs to be quickly cleared from circulation limiting their therapeutic time frame (235). Second-generation mAbs are partially human and are classified as either chimeric or humanized. Chimeric mAbs contain murine variable regions and human Fc regions resulting in a final product that is approximately 75 % human. Humanized mAbs are comprised of human antibodies where only the complementary determining region (CDR) is murine resulting in an antibody that is approximately 95 % human. These antibodies have slower clearance times and are less immunogenic (236). Now, third-generation mAbs are fully human. The evolution of mAbs is represented in **Figure 1.8**.

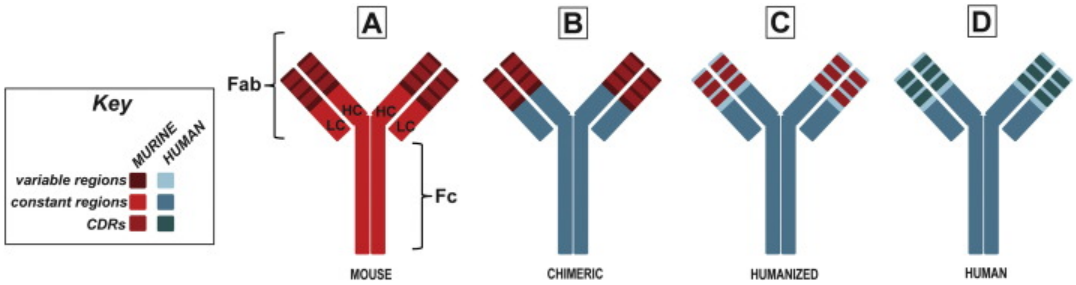


Figure 1.8 Progression of monoclonal antibodies

A. First generation monoclonal antibodies (mAbs) were made entirely from mice which triggered a reaction from the human immune system resulting in them being quickly cleared from the recipient's system. **B**. Second generation mAbs are chimeric mAbs are comprised of human fragment crystallizable (Fc) regions and murine fragment antigen-binding (Fab) regions and **C**. humanized mAbs were only the complementary determining region (CDR) is murine. **D**. Third generation mAbs are fully human (237).

1.14.2 ANTI-IGE MONOCLONAL

Omalizumab, a monoclonal antibody against IgE was the first monoclonal therapy to be approved for use in asthma. It is approved as an add-on treatment for patients with severe allergic asthma with high levels of serum IgE and as such omalizumab was the start of personalized medicine in asthma (109, 238). Omalizumab has consistently been shown to reduce symptoms and exacerbations by up to 50 %, improve asthma-related quality of life and reduce the need for ICSs in patients with elevated FeNO, periostin, blood eosinophils and IgE at baseline (239-241).

1.14.3 ANTI-IL-5 MONOCLONALS

Increased levels of IL-5 is a key marker of eosinophilic inflammation which is critical to the pathology of numerous inflammatory diseases in addition to asthma, such as hypereosinophilic syndrome, allergy and eosinophilic esophagitis (242-244). IL-5 is the primary cytokine

responsible for activating, recruiting and differentiating eosinophils which express the IL-5 receptor on their surface. The receptor is comprised of an α-chain where IL-5 binds and a β-chain that contains a signal transducing unit. Eosinophils contribute to AHR by releasing basic granule proteins and to airway narrowing by releasing leukotriene C_4 (245). Airway eosinophilia is reduced by ICS treatment. However, it has been estimated that treatment with ICSs is ineffective at reducing eosinophils in up to 50 % of patients with severe asthma (108, 246). There are currently three monoclonals that target IL-5 or its receptor that are currently used to treat severe asthma; mepolizumab, benralizumab and reslizumab.

Mepolizumab is a fully humanized IgG1/κ monoclonal antibody against IL-5. The specific binding regions of the variable domains are derived from a mouse anti-human IL-5 monoclonal antibody which has been grafted onto a human IgG1/κ antibody. It functions by binding to IL-5 with high affinity and specificity and in doing so prevents IL-5 from binding to the α-chain of the IL-5R on the surface of eosinophils (247). A study by Flood-Page and colleagues investigated the effects of mepolizumab in patients experiencing asthma symptoms despite treatment with ICSs. This study failed to show that the treatment had a significant affect on lung function or exacerbations despite decreasing blood and sputum eosinophil levels (245). However, they did not evaluate patients to see if they had elevated eosinophil levels at baseline. Following this, a number of studies were conducted to observe the effects

of mepolizumab on patients with persistent eosinophilia. Haldar and colleagues trialed mepolizumab in patients with refractory eosinophilic asthma with recurrent exacerbations. They concluded that mepolizumab was able to reduce exacerbations and improved scores on the Asthma Quality of Life Questionnaire. There was also a reduction in ICS use in the treatment group. No effect was seen on lung function, FeNO, or symptoms (93). This work was instrumental in highlighting the importance of using biomarkers to ensure the correct patients are receiving the correct medications. A large multi-center international study determined the number of exacerbations was significantly reduced as a result of treatment with mepolizumab as were blood and sputum eosinophils (208). However, some eosinophils did remain in the airways despite treatment which may be why mepolizumab has minimal effects on symptoms and lung function (34). Based on these studies elevated blood eosinophils along with frequent exacerbations are now requirements to be approved for treatment with mepolizumab (248).

Benralizumab is a humanized monoclonal antibody against the α-chain of the IL-5 receptor. The Fc portion of benralizumab is afucosylated, meaning that the oligosaccharide regions do not contain fucose, which increases the binding and functionality of the antibody (249). Over a six month period, benralizumab has been shown to reduce asthma exacerbations, increase the time to the first exacerbation and improve asthma control and quality of life questionnaire scores while allowing

patients to reduce their daily oral corticosteroid dose (75 % reduction with benralizumab vs 25 % reduction with placebo) (250).

Reslizumab is an IgG4/κ monoclonal antibody that has been humanized from a rat IgG2a antibody against IL-5 (251). Reslizumab has also been shown to decrease exacerbations and improve disease control in patients with severe eosinophilic asthma (247). To date, there have not been any direct comparisons between the three anti-IL-5 treatment options. However, a recent meta-analysis of 3,421 patients revealed that the three were comparable at equivalent doses in terms of improving control and reducing exacerbations (252, 253).

1.14.4 ANTI-IL-4 RECEPTOR ALPHA MONOCLONAL

IL-13 and IL-4 are structurally similar. They have different receptors that each possess the α-subunit of the IL-4R. Dupilumab is a humanized monoclonal antibody against the α-subunit of the IL-4R and therefore inhibits the actions of both IL-13 and IL-4. Wenzel and colleagues found that dupilumab was effective in reducing exacerbation, improving lung function, and reducing Th2 markers such as FeNO, serum IgE and eotaxin-3 compared with placebo. Patients have been shown to benefit irrespective of inflammatory biomarkers such as FeNO or blood eosinophils (254). The authors suggest that blocking the actions of both IL-4 and IL-13 may be more effective than blocking either cytokine alone (255).

1.14.5 FUTURE THERAPIES

IL-13 is crucially involved in airway remodeling (AR) by triggering epithelial cells to secrete matricellular proteins such as periostin. Lebrikizumab is a humanized IgG4 monoclonal antibody that binds to IL-13 preventing its functions. Lebrikizumab is still undergoing clinical investigations but has been shown to increase lung function as measured by FEV_1 and reduce exacerbations in patients with elevated FeNO and serum periostin levels at baseline (256).

In response to inflammation, epithelial cells produce TSLP which acts on DCs to decrease their secretion of IL-12. This encourages Th0 cells to become Th2 cells (257, 258). Gauvreau et al trialed patients with mild allergic asthma on tezepelumab (AMG 157) which is a human anti-TSLP monoclonal immunoglobulin G2λ that binds to TSLP preventing it from binding to its receptor. Their study demonstrated that tezepelumab resulted in less allergen-induced bronchoconstriction and airway inflammation both before and after allergen exposure (257). Since then, Corren and colleagues have demonstrated that tezepelumab lowers the rates of clinically significant exacerbations and improves FEV_1 irrespective of baseline blood eosinophil levels or FeNO (259).

CHAPTER 2

MATERIALS AND METHODS

2.1 LABORATORY MATERIALS AND METHODS

2.1.1 BUFFERS AND REAGENTS

All chemicals, reagents and materials were purchased from Sigma-Aldrich (UK) unless otherwise stated.

2.1.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

Cytokine Duosets® were purchased from R&D Systems® and contained a capture antibody, cytokine standard, detection antibody and streptavidin-horseradish peroxidase (HRP) substrate. All reagents were used at the concentration recommended by the manufacturer (**Table 2.1**). Once reconstituted antibodies and standards were aliquoted and stored at -20 °C until required. Enzyme-linked immunosorbent assays (ELISAs) were performed in both 96 and 384 well Nunc Maxisorp™ plates (ThermoFisher Scientific). When using 384 well plates 20 µl was added to each well and when using 96 well ELISA 100 µl was used unless otherwise stated. The exception to this was when washing the wells in between steps where wells were completely filled with wash buffer. When liquid was added to 384 well plates the plate was centrifuged face-up to ensure the liquid was at the bottom of the well and following each wash step bulk liquid was manually removed from the plate by inversion the plate was then was centrifuged face down to ensure all liquid was removed from each well.

ELISAs were performed in accordance with the manufacturer's instructions. Briefly, capture antibodies were diluted in carbonate-bicarbonate (CBC) buffer unless otherwise stated and added to the required number of wells. The plate was covered and left to incubate overnight at room temperature. The following morning the plate was washed by completely filling the wells in a wash buffer of Phosphate Buffered Saline (PBS) with 0.05 % $Tween_{20}$ (PBST) three times. Wash buffer was removed by inversion or centrifuge. Following this, the plate was blocked using 10x concentrate Reagent Diluent (RD) (R&D Systems) diluted 1 in 10 in distilled water (1x RD) and left to incubate for one hour. The plate was then washed three times. Samples were added in duplicate either neat or diluted in 1x RD until they could be read within the range of the assay. Cytokine standards were serially diluted in 1x RD and were added in duplicate alongside the samples and the plate was left to incubate for two hours. The plate was then washed three times. Detection antibodies were diluted in 1x RD and left to incubate for two hours. The plate was washed three times. HRP was diluted 1:40 in 1x RD and left to incubate in the dark for 30 minutes. The plate was washed three times. Supersensitive 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate was used as the substrate for HRP and was added and to the plate and left to incubate in the dark for 20 minutes. The reaction was stopped using 2M H_2SO_4 stop solution (**Figure 2.1**). ELISA plates were scanned using a GloMax® Discover Multimode Microplate reader (Promega) to obtain optical densities (OD) at 450 nm from each well.

Table 2.1 Cytokine and antibody concentrations

Cytokine Duosets® were purchased from R&D systems and were reconstituted and diluted to their working concentrations according to the manufacturer's instruction.

Cytokine	Capture antibody concentration (µg/mL)	Standard concentration range (pg/mL)	Detection antibody concentration (ng/mL)
IL-1β	4.0	3.91-250	200
IL-4	4.0	31.2-2000	75
IL-5	2.0	23.4-1500	125
IL-8	4.0	31.2-2000	20
IL-10	2.0	31.2-2000	75
IL-12	4.0	31.2-2000	100
IL-13	2.0	93.8-6000	90
IL-17	4.0	15.6-1000	20
IFN-γ	4.0	15.6-1000	200
TGF-β1	2.0	31.2-2000	50
TNF-α	4.0	15.6-1000	50
TSLP	0.8	31.2-2000	50
VEGF	1.0	31.2-2000	100

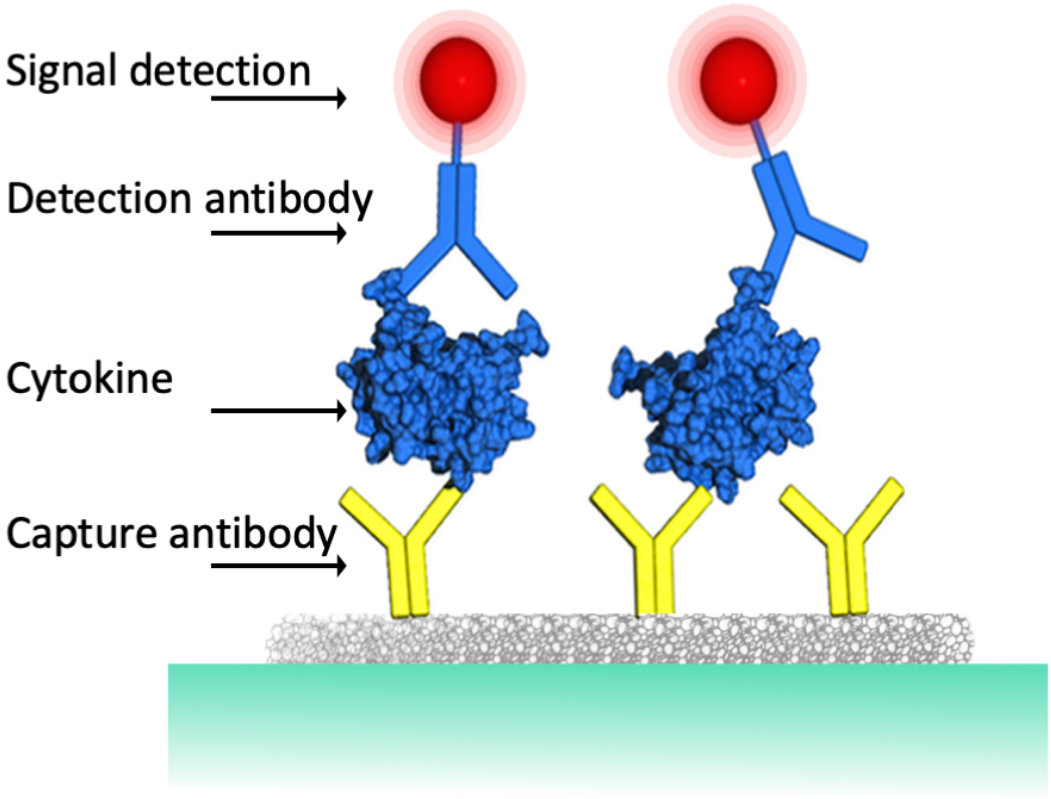


Figure 2.1 Enzyme-linked immunosorbent assay (ELISA)

Capture antibodies stick to the surface of the plate where they bind to their substrate. A detection antibody then binds to a different epitope on the substrate. Streptavidin and supersensitive 3,3',5,5'-tetramethylbenzidine bind to produce a colour change to visualize the reaction and quantify the substrate.

2.1.3 ANTIBODY MICROARRAY

All arrays were produced using Microgrid II 610 Arrayer (Digilab Inc.) using either steel (Digilab Inc.) or silicone pins (Parallel Synthesis Technologies, USA) onto 25 x 75 mm glass slides. Slides (Arrayit) were coated with either poly-l-lysine, 3-aminopropyltriethoxysilane (aminosilane), or 3-glycidoxypropyltrimethoxysilane (epoxysilane). The antibody kits used were the same as those used for ELISAs (**Section 2.1.2**). The concentrations used were the concentrations recommended

by the manufacturer with the exception of the capture antibodies which were diluted to 100 µg/mL. Cytokine capture antibodies were diluted in various printing buffers to determine the optimal buffer composition. See **Table 2.2** for buffers and their composition. The array process can be visualized in **Figure 2.2**.

Table 2.2 Composition of printing buffers

Six printing buffer compositions were tested to determine the optimal buffer for printing cytokine capture antibodies.

Printing Buffer	Composition
PBS-TBT	1x PBS, 50 mM Betaine, 50mM Trehalose, 0.1 % $Tween_{20}$
PBS-Bet	1x 50mL PBS, 50 mM Betaine
PBS- BetTw	1x PBS, 50 mM Betaine, 0.1 % $Tween_{20}$
PBS-Tre	1x PBS, 50 mM Trehalose
PBS-TreTw	1x PBS, 50 mM Trehalose, 0.1 % $Tween_{20}$
PBS- HighTre	1x PBS, 100 mM Trehalose

2.1.3.1 Plate preparation

Capture antibodies were diluted in printing buffer to 100 µg/mL. Diluted capture antibodies were aliquoted 10 µl/well into a 384 microtitre well plate (Genetix). A biotinylated anti-human IgM antibody was used as a printing control and was aliquoted 10 µl into a well. Printing buffer alone was used as a negative control and was aliquoted 10 µl into a well.

2.1.3.2 Printing

Before printing the arrayer was cleaned and all water tanks were inspected to ensure they contained sufficient water for the print. The arrayer was set to 65 % humidity and time was allowed to reach humidity before the print started. Dwell time, the length of time the pin touched the slide surface, was set to 0.4 seconds as this produced reproducible spots and a longer dwell time reduced the number of spots that could be printed due to evaporation within the arrayer. Pins were cleaned three times and dried between contact with new samples. Slides were printed and left to dry overnight on the arrayer. Arrays were printed from prepared 384 well plates using a custom grid file which informed the arrayer of where each sample was to be printed. After completion of a print, a comma-delimited text file (.csv) listing the column, row and identity of each sample was imported into the arrayer and the clone tracking wizard in the Genepix software was used to generate a map of the arrays which had been printed on the slides with the exact location of each sample and replicate. The resultant file, a Genepix array list (gal file), was required for analysis with Genepix Pro software.

2.1.3.3 Slide processing

Slides were held in place using a cassette to ensure the printed arrays could be properly processed. For all processing steps, 100 µl of liquid was added to each block and during the incubation periods slides were covered to minimize evaporation. Following the print, slides were blocked in a buffer of 3 % Bovine Serum Albumin (BSA) in distilled water for one

hour. Slides were then washed in PBST for one minute and then inverted to remove the liquid. The wash step was repeated for a total of three times. All cytokine standards were diluted in 1x RD to the highest concentration as per the manufacturer's instructions and were mixed together. The cocktail of standards was serially diluted two-fold in 1x RD seven times. 1x RD alone was added to the bottom two arrays as a negative control and to obtain the background reading. Slides were covered and incubated in the diluted standards for one hour. Slides were washed three times. Cytokine detection antibodies were mixed together and diluted to the manufacturer's instructions in 1x RD. Detection antibodies were then added to the wells and slides were covered and incubated for one hour. Slides were washed three times. Streptavidin HRP was diluted 1:40 in 1x RD and added to each well. Slides were covered and incubated in the dark for 30 minutes. Slides were washed three times. Cyanine 5 (Cy-5) dye (E-Biosciences, UK) was diluted 1:2000 in 1x RD and added to each well. Slides were covered and incubated for 15 minutes in the dark then washed three times. Finally, slides were rinsed in ultrapure water and centrifuged to dry for two minutes (**Figure 2.2**).

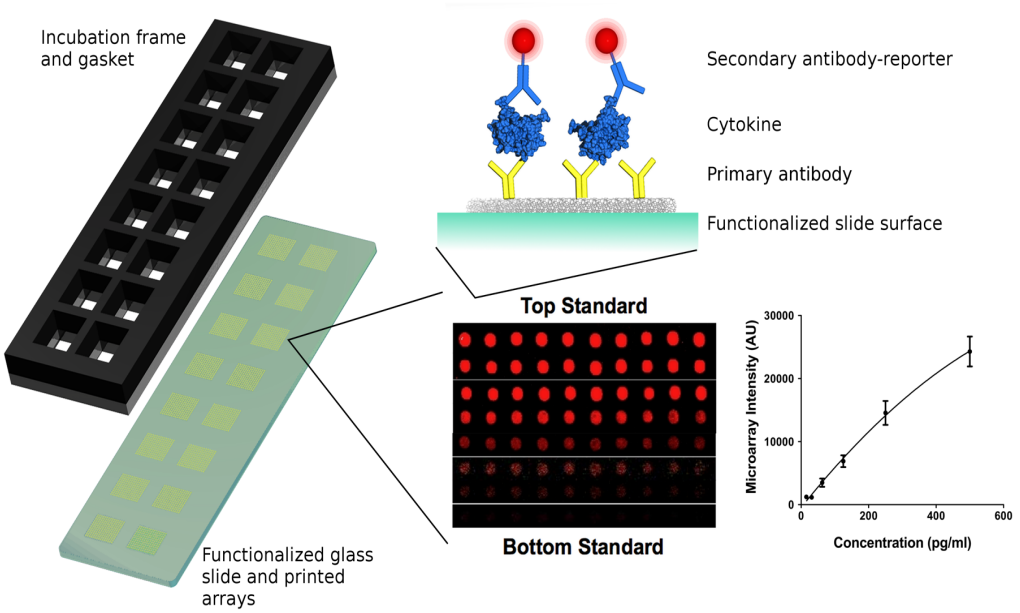


Figure 2.2 Microarray slide processing

Slides were set in cassettes to separate the slide into 16 sections to allow for different samples to be added to each slide simultaneously. Slides were blocked for one hour, washed, then probed with standards at known concentrations for one hour. Following this, slides were washed, probed with detection antibodies for one hour. Slides were washed, probed with streptavidin for 30 minutes, washed, and finally probed with cyanine dye for 15 minutes. Slides were then scanned, and standard curves were generated.

2.1.3.4 Slide scanning

After slides were processed, they were scanned using a Genepix 4200AL scanner (Axon Genepix ®) at 635 nm at 500 photomultiplier tubes (PMT) at 100 % power. Slide images were saved as 16-bit multi-image TIFF files. Numerical data encompassing the fluorescent signals detected for each spot on the array were extracted from the image using the Genepix Pro V6. This was achieved by overlaying the gal file generated following array printing onto the image and aligning it with the array. Industry

standard Genepix Result Files (GPR) files containing a detailed analysis of each spot were then generated for further analysis.

2.1.4 VENEPUNCTURE COLLECTION

Blood was collected in serum, sodium heparin, and ethylenediaminetetraacetic acid (EDTA) tubes (BD). A 5702R centrifuge (Eppendorf) was used for both serum and sodium heparin processing. Sodium heparin tubes were used to isolate polymononuclear cells (PBMCs) this process was performed using Histopaque-1077. An Olympus CX21FS1 microscope and Trypan Blue stain were used to perform all cell counts.

2.1.5 BLOOD PROCESSING

Serum and sodium heparin tubes were processed in the lab as described below and EDTA tubes were sent to the pathology department at Nottingham City Hospital (NCH) for a full blood count (FBC) analysis.

2.1.5.1 Serum processing

Serum samples were left to clot for 30 minutes to one hour after collection before processing. Two collection tubes were filled per patient. Samples were centrifuged at 1000 x g for ten minutes. The serum was pipetted off the top, samples were homogenized, separated into aliquots, and stored at -80 ^{o}C.

2.1.5.2 Sodium heparin processing

Sodium heparin samples were processed within one hour of being taken. Collection tubes were rocked for 15 minutes before processing to prevent

clotting. Fifteen mL of Histopaque-1077 was added to a 50 mL Accuspin™ tube and centrifuged at 1000 x g for 30 seconds until the histopaque moved below the filter. If necessary, this was repeated to ensure the histopaque completely filled the bottom of the tube up to the filter with no bubbles. Excess histopaque above the filter was pipetted off. Blood collection tubes were pipetted into the Accuspin™ tube and centrifuged at 1000 x g for ten minutes at 18-26 ^{o}C. The plasma layer was pipetted off and stored in 500 µl aliquots at -80 ^{o}C. The mononuclear layer was then pipetted into a separate 15 mL falcon tube and PBS was added to make up 10 mL and mixed. The sample was then centrifuged at 250 x g for ten minutes at 18-26 ^{o}C. The PBS was aliquoted off and disposed of. The pellet was resuspended in 10 mL PBS and centrifuged at 250 x g for ten minutes. The PBS was aliquoted off and disposed of. The pellet was resuspended in 1 mL. From here 20 µl was removed and mixed with 20 µl trypan blue. From this mixture, 10 µl was pipetted onto a hemocytometer and a cell count was performed by counting the white blood cells in the four large outer squares to ensure cell viability. The pellet was then resuspended in a further 9 mL of PBS for a total of 10 mL for the final wash and centrifuged at 25 x g for ten minutes. The PBS was aliquoted off and disposed of. The remaining pellet was resuspended in 500 µl of RNAlater® (ThermoFisher Scientific) and stored at -80 ^{o}C.

2.1.6 SPUTUM PROCESSING

If a spontaneous sample could not be obtained sputum was induced using saline solutions prepared with Resp-ease™ sterile 7 % hypertonic sodium chloride solution. The hypertonic solution was diluted in Water for Injections (Fannin) to concentrations of 0.9 %, 3 %, 4 % and 5 % and stored at -4 ^{o}C until required for nebulization. Once sputum samples were obtained, they were immediately stored on ice until processing, which occurred within one hour. Sputum plugs were isolated from saliva and transferred to a 15 mL falcon tube. The sputum plugs were incubated with eight volumes of the sample weight of PBS and 150 volumes of the sample weight of Protease Inhibitor Cocktail (PIC) was added to the sample. The sample was then vortexed for 15 minutes, rocked on ice for ten minutes and then centrifuged at 600 x g for ten minutes at 4 ^{o}C. Four volumes of the supernatant were aliquoted into a 15 mL falcon tube and centrifuged at 1500 x g for ten minutes at 4 ^{o}C. The supernatant was then aliquoted and stored at -80 ^{o}C. In order to obtain a further sample, four volumes of 0.2 % dithiothreitol (DTT) was added to the sample. The sample was then vortexed for 15 seconds and rocked on ice for ten minutes. The sample was then filtered into a fresh 15 mL falcon and was centrifuged at 600 x g for ten minutes at 4 ^{o}C. The supernatant was then aliquoted and stored at -80 ^{o}C. The pellet was resuspended in d-PBS to a concentration of $5x10^{5}$ cells/mL. 75 µl and 150 µl were added to poly-lysine cytoslides which were spun at 450 rpm for six minutes. Slides were air dried then fixed to poly-lysine slides using methanol and left to dry

for a further ten minutes. Slides were then stained using Rappi-Diff II stain B and C dyes (Atom Scientific) and left to dry for ten minutes following each stain. A cell count was performed to distinguish between eosinophils, neutrophils, macrophages, leukocytes and squamous cells.

2.2 CLINICAL MATERIALS AND METHODS

2.2.1 SPIROMETRY

Calibration was conducted using a 3 L syringe (Carefusion) before each session or if the machine was moved to a new environment. Calibration was performed to ensure that volume was being accurately measured at different flow rates. The spirometer was left to acclimatize to the environment for 30 minutes before calibration. A calibration syringe was used at a slow rate (2 L/min), medium rate (4 L/min) and fast rate (6 L/min) which simulated different breathing rates. Calibration was successful if results were within 100 mL as differences greater that this represented a significant difference in lung function. The calibration report was printed, signed and stored.

Spirometry was performed using a portable microlab spirometer (CareFusion). Spirometry was performed on all patients who were eligible, able, and consented to perform the test. Contraindications for spirometry included: aneurysm, glaucoma, late-stage pregnancy, perforated eardrums, hemoptysis, eye surgery, abdominal/thoracic surgery in the last three months, antibiotics or steroids in the last six

weeks for chest conditions, myocardial infarction, uncontrolled hypertension or unstable angina.

Patients were coached throughout the procedure. Patients were instructed to put on a nose clip and then inhale fully. They then created a tight seal around the mouthpiece and exhaled with as much force as possible for as long as possible. This was repeated three times or until there was a maximum of 5 % variability between attempts and the British Thoracic Society (BTS) criteria had been met.

2.2.2 FRACTIONAL EXHALED NITRIC OXIDE

Fractional exhaled nitric oxide (FeNO) was performed using a Nixo Vero® (Niox). Patients were instructed to take a deep breath in through the mouthpiece and maintain a long steady breath out for approximately six seconds through the mouthpiece. Breathing in triggered an image to appear on the screen which guided the patient through the procedure. The aim was to maintain a steady exhalation at 50 mL/s. If the exhaled breath was too strong or too soft the device would beep to help guide the patient for the duration of the test.

2.2.3 FORCED OSCILLATION TECHNIQUE (FOT)

Oscillometry was performed using a tremoFlo™ C-100 (Thorasys) with clearFlo™ F-100 Filter mouthpieces. The oscillometer was calibrated before each session or if the machine was moved to a new environment. Calibration was performed using a test load. Patients were seated upright with their chin tilted upwards at a 15^{o} angle. While the test was being

performed patients held their hands over their cheeks to ensure air was not dispersed through their mouth. A nose peg was worn during the test and the patient made a tight seal with their mouth around the mouthpiece and their tongue under the mouthpiece. The patient was instructed to breathe tidally for 15 seconds while the test was being performed. Tests were repeated three times to get an accurate reading. A Dell Notebook Latitude 3450, Intel Core 5 was used to conduct the test and visualize the results.

2.2.4 SPUTUM INDUCTION

Sputum induction was performed using the Omron NE-U17 ultrasonic nebulizer using saline at 0.9 %,3 %, 4 %, and 5 %. Sputum induction was only performed in patients who were willing, unable to produce a spontaneous sample and had an FEV_1 of greater than one liter. The saline was removed from the fridge approximately 15 minutes before use to allow it to reach room temperature.

To ensure the patient's safety spirometry was performed three times to obtain the best pre-bronchodilator FEV_1. Following this, four puffs of salbutamol were delivered via a spacer. The patient was then required to wait 20 minutes for the salbutamol to reach its maximum concentration before repeating spirometry to obtain the best post-bronchodilator FEV_1. This value was taken as the baseline FEV_1 and was used to calculate all safety cutoff points. Cutoff points were calculated for 90 % of baseline, baseline minus 200 mLs, 80 % of baseline and minus 400 mL.

Either 0.9 % or 3 % for the first nebulization depending on the patient's lung function and tolerance. The nebulizer was set to an airflow and nebulizer volume of three and time of five minutes. The bottom of the nebulizer was filled with water and 5 mL of the appropriate saline concentration was poured into the NE-U17 medication cup. The patient was advised to wear a nose clip and breathe tidally through a mouthpiece for five minutes or until they could no longer tolerate it. Upon completion, the patient was advised to blow their nose and have a drink of water to rinse their mouth and throat. The patient was then asked to cough to try and produce a sample. Following this safety spirometry was performed three times to check for a fall in FEV_1. If their best FEV_1 was ≥90 % of baseline, >200 mL of baseline and still greater than 750 mL nebulization was repeated with a higher saline concentration. If there was a fall in FEV_1 that was <90 % but >80 % of baseline, nebulization was repeated at the same concentration. This process was repeated until the patient was able to produce a sample, was unable to tolerate the procedure or a maximum of three times (either with increasing concentrations of saline solution or the same concentration). Following induction, the patient was observed until their FEV_1 returned to within 5 % of the baseline value, additional puffs of salbutamol were administered if required.

2.2.5 VENEPUNCTURE

Venepuncture was performed using a butterfly needle Safety-Lok™ (BD Vacutainer®). Venepuncture was taken from the antecubital fossa. Blood

was collected in serum vacutainers followed by sodium heparin and EDTA.

2.2.6 QUESTIONNAIRES

The Asthma Control Questionnaire (ACQ), Leicester Cough Questionnaire (LCQ), Medical Research Council dyspnea score (MRC) and Asthma Control Test (ACT) were used to determine symptom severity. Questionnaires were completed at the beginning of each visit to ensure minimal bias which could occur as a result of becoming breathless after performing lung function tests.

2.2.6.1 Asthma Control Questionnaire

The Juniper Asthma Control Questionnaire (ACQ) consisting of seven questions was provided in its original paper format. Each question was equally weighted when the overall score was calculated. Participants answered the first six questions on a seven-point scale. The first five pertained to the severity of symptoms they experienced during the past week with zero being no impairment and six being maximum impairment. The sixth question asked about their daily SABA use during the past week with zero representing no use and six representing more than 16 puffs a day. The seventh question was completed by the researcher after spirometry was performed and the FEV_1 % predicted was obtained. Overall scores were obtained by averaging either the first five questions (ACQ5 score), first six questions (ACQ6 score) or all seven questions (ACQ7 score) where appropriate. Overall scores of 0-0.75 were indicative of well-controlled asthma whereas scores over 1.5 represented

poorly controlled asthma. Scores between 0.75 and 1.5 were considered to be adequately controlled asthma. A change of ≥0.5 was required for the difference to be considered clinically significant (14, 260).

2.2.6.2 Leicester Cough Questionnaire

The Leicester Cough Questionnaire (LCQ) is a measure of chronic cough. The questionnaire consisted of 19 questions was provided in its original paper format. Each question was weighted equally when the overall scores were calculated. Participants were asked to answer all 19 questions with a seven-point Likert response scale where one was the most affected by their cough and seven was the least affected. The questions comprised three domains: physical, psychological and social (261). To obtain the final score the questions in each domain were added together and divided by the number of questions in that domain. The possible scores ranged from 3-21 where 3 represented the worst cough and 21 was no cough. An increase of ≥1.3 was required for the difference to be considered clinically significant (262).

2.2.6.3 Medical Research Council Dyspnea Scale

The Medical Research Council Dyspnea Scale (MRC) was provided in paper format to assess the effect of breathlessness on daily activities. The questionnaire consisted of five questions alongside a 1-5 stage scale to ascertain the grade of perceived clinical breathlessness. Stage one represented no breathlessness while stage five represented a disabling level of breathlessness (263). There is no accepted interval of change that represents a clinically significant improvement for this test.

2.2.6.4 Asthma Control Test

The Asthma Control Test (ACT) was used to assess how well controlled the patient's asthma was over the previous four weeks. The test was delivered in paper format and consisted of five questions. Each question was scored out of five where one represented the lowest level of control and five was perfectly controlled. Overall test scores ranged from 5 (poorly controlled) to 25 (well controlled). The minimally important difference was a change of three points (264).

2.2.7 INFORMATION STORAGE

Clinical information collected from the studies presented in chapters four and six were stored in the online database, Medrio. The study presented in chapter five involved significantly fewer participants, enabling the data to be directly entered and stored into Microsoft Excel.

2.2.8 STATISTICAL ANALYSIS

The statistical analysis performed was different for each clinical study. Briefly, microarray spot analysis was conducted using Genepix Pro to obtain the average fluorescence reading which was then entered into Graphpad Prism V7 to create eight-point standard curves. ELISA standard curves were also created in Graphpad Prism V7. Multi experiment viewer (MeV_4_8) was used to create cytokine profile heatmaps to visualize the results. All statistical analysis was performed using STATA / SE 15.1. The specific statistical method used will be covered in more detail in each chapter.

CHAPTER 3

DEVELOPMENT AND OPTIMIZATION OF CYTOKINE QUANTIFICATION ASSAYS

3.1 BACKGROUND INFORMATION

3.1.1 ENZYME-LINKED IMMUNOSORBENT ASSAY

For more than 40 years the enzyme-linked immunosorbent assay (ELISA) has been the "gold standard" method for protein quantification (265). ELISA is a plate-based assay that is easy to perform and uses an HRP-colourimetric system to detect the presence of a specific analyte in solution. It is a reliable and reproducible method for detecting and quantifying proteins. To quantify cytokines typically a sandwich ELISA is performed where a capture antibody specific for the cytokine is immobilized to a polystyrene plate by passive absorption. A high degree of specificity is ensured through the high-affinity association between an epitope of the capture antibody and its cognate binding site on the cytokine itself (266). A detection antibody, complexed with biotin, then binds to a different epitope on the cytokine. The biotin reacts with streptavidin HRP to produce a colour change which can be visualized and quantified. However, a traditional 96 well ELISA only detects one analyte at a time and requires a relatively large volume (100 µl/well) of reagents and sample. This can become costly and require a large volume of sample from each patient if numerous analytes are being analyzed as a separate ELISA would need to be performed to quantify each one.

3.1.2 MICROARRAY

Microarray technology can be used to quantify multiple analytes that are present in a sample in a multiplexed and high-throughput manner. This method is essentially a miniaturized sandwich ELISA with several benefits over the traditional plate-based ELISA: it requires significantly less material which reduces costs, requires less sample from the patients making it less invasive and it can be used to quantify numerous analytes simultaneously over a range of concentrations. Additionally, multiple repeats can be run in a single assay adding validity to the results. However, the microarray has many more variables than an ELISA, which makes it less reliable, less reproducible and requires a great amount of time to be spent optimizing and validating all components of the protocol.

In order to ensure that assays are reliable, their accuracy and precision need to be considered. Accuracy is defined as how close the obtained value is to the expected value. It is not always possible to assess this, as sometimes the expected value is unknown. In these circumstances, accuracy is replaced with sensitivity and specificity. Assay precision is the variability between replicates from the same sample. This encompasses repeatability (within assay variability) and reproducibility (between assay variability) (267). Precision is represented by the percentage coefficient of variation (CV %) which can be calculated based on a minimum of three measurements. The CV % is a measure of relative

variability and represents the ratio of the standard deviation to the mean. The formula for calculating CV % is:

$$CV = {}^{\sigma}/_{\bar{X}} * 100$$

Equation 3.1 Percentage coefficient of variation (CV %)

CV % is calculated by dividing the standard deviation (σ) which is the dispersion of the data, by the mean ($\bar{X}$) multiplied by 100.

This is more valuable than looking at the standard deviation alone as it adjusts for differences in the means (268). The Food and Drug Administration (FDA) has defined the acceptable level of variation both within and between assays. According to the 2018 FDA guidelines of Accuracy and Precision for Bioanalytical Methodology and In-Study Conduct the CV % within an assay must be <15 % and <20 % between assays (269).

Microarrays are conducted by "printing" a protein, which in this case is a capture antibody specific for a cytokine, to the surface of a glass slide. The slide surface should be level and free from impurities or scratches to allow for even printing. The coating determines how the capture antibody will bind to the slide and how stable and accessible it will be which contributes to spot morphology and intensity. In the work described here, slides coated with epoxysilane, aminosilane and poly-l-lysine were used.

Epoxysilane slides have a reactive epoxide group on the surface that binds covalently and irreversibly to a wide range of biomolecules. The epoxysilane surface chemistry is shown in **Figure 3.1**.

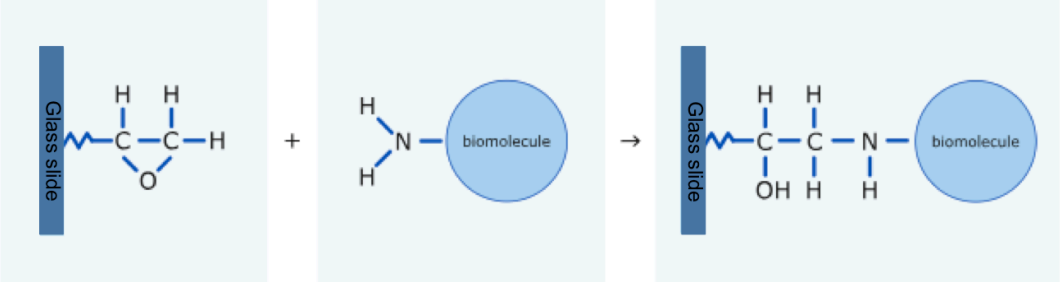


Figure 3.1 Epoxysilane surface chemistry

Epoxysilane slides provide a multipurpose reactive layer that forms covalent bonds with the capture antibodies printed onto the surface. Figure adapted from NEXTERION® (270).

Aminosilane slide surfaces provide positively charged amine groups for negatively charged phosphate groups in the protein antibody structure to form ionic bonds with. The aminosilane surface chemistry is shown in **Figure 3.2**.

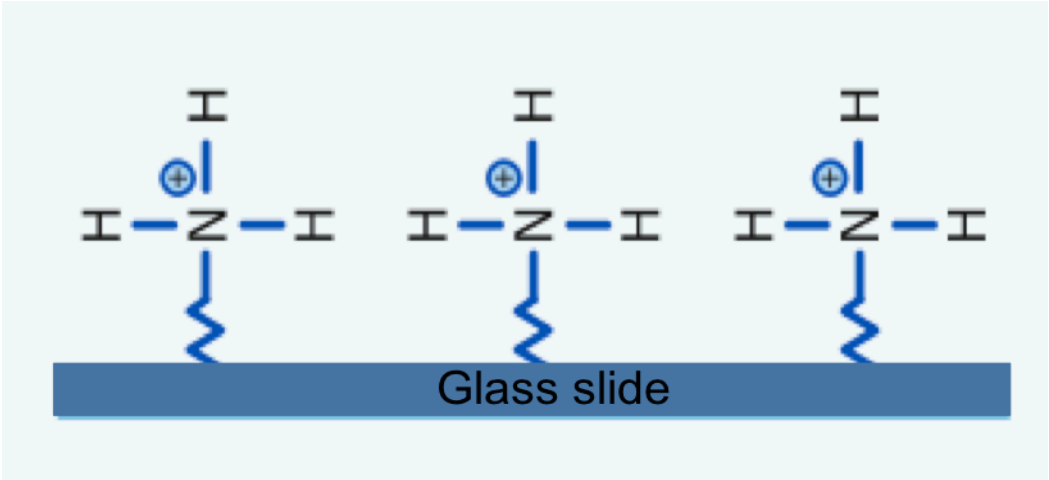


Figure 3.2 Aminosilane surface chemistry

Aminosilane slides provide positively charged amino groups for negatively charged phosphate groups on the printed capture antibodies to form ionic bonds with. Figure adapted from NEXTERION® (271).

Poly-l-lysine provides a layer of positively charged amine groups with high electrostatic binding ability. This layer binds to a wide range of negatively charged biomolecules such as proteins. The surface chemistry is shown in **Figure 3.3**.

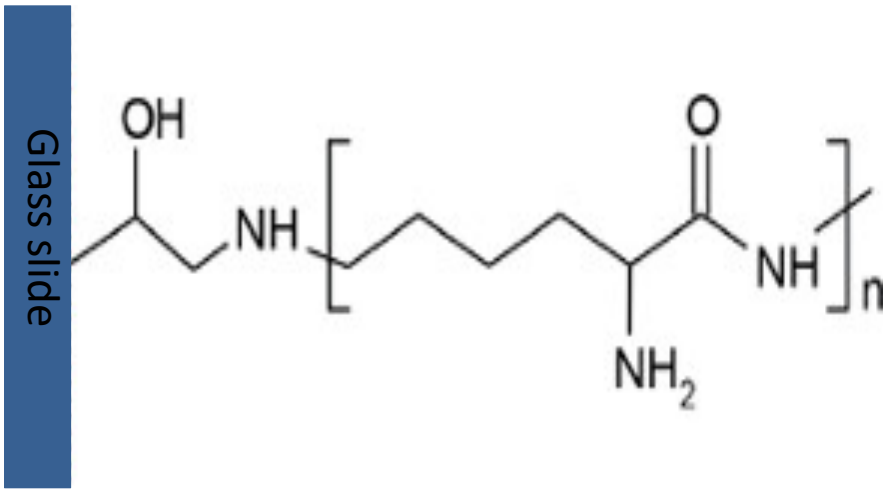


Figure 3.3 Poly-l-lysine surface chemistry

Poly-l-lysine slides provide positively charged amine groups which form electrostatic covalent bonds with the capture antibodies printed onto the slide surface. Figure adapted from PolyAn (272).

Antibodies are large proteins whose function depends on their structural conformation. The antibodies should be securely bound to the slide surface, but the binding should not interfere with the physical integrity or functionality of the antibody. The composition of the printing buffer contributes to the size and morphology of the printed spots. A printing buffer that produces inconsistent or low-intensity spots results in less reliable and reproducible findings. The composition of the printing buffer

needs to be optimized for the substance that is being printed as well as for the surface it is being printed onto. PBS is the primary component of most buffers as it maintains a neutral pH (273). Other substances such as a detergent, trehalose or betaine can also be added. $Tween_{20}$ is a detergent that can help reduce background signals and hold the spots together while drying (274). Trehalose is a protein preservative that immobilizes, stabilizes and prevents the printed antibodies from dehydrating (275). Betaine increases protein stability and the viscosity of the buffer as well as reducing evaporation (276).

In order to determine if the assay is reproducible mean signal intensities for each cytokine in each block on the same slide (intraassay) and on different slides processed on different days (interassay) need to be calculated. Additionally, the spot morphology and size also need to be considered. Spots should be circular with roughly the same diameter to ensure the same amount of protein has bound to the slide in each spot. Consequently, microarray technology requires significant time to be spent on optimizations as there are so many variables to consider.

3.2 METHODOLOGY: MICROARRAY

Cytokine detection and quantification were achieved by performing what was essentially a miniaturized sandwich ELISA where the capture antibodies were printed onto a glass slide as opposed to being added to wells of a plate as would be done with a traditional ELISA. The remainder of the protocol was conducted essentially as described by the manufacturer with a few alterations that were previously optimized in-house by S. Selvarajah to detect cytokine levels in human serum and plasma (277). The optimizations developed by S.Selvarajah are detailed below:

Assay Materials

1. R&D systems ELISA Duoset kits
2. Poly-l-lysine (PLL) slide surface

Printing Optimizations

1. Capture antibodies were diluted to 100 µg/mL in printing buffer
2. Printing buffer of 50 mM trehalose in PBS
3. Arrayer humidity set to 65 % to minimize the rate of evaporation, which could affect signal intensities, while still allowing the antibodies to bind to the slide.
4. Dwell time of 0.4 seconds for the most reproducible spots

Processing Optimizations

1. Blocking buffer of 3 % BSA in distilled water to reduce background levels resulting in the best signal to noise ratio.
2. 45-minute incubation times for cytokine standards and detection antibodies compared to the two-hour time recommended by the manufacturer
3. Tyramine amplification to increase the intensity of the signals without increasing background levels

When slides were printed, the printing buffer alone was printed as a negative control to ensure the buffer itself was not producing any signals. A printing control of highly concentrated protein (IgM) was also included at the edges of each block which served as both a positive printing control and as "landing lights" to help align the spot location (gal) file to obtain the data. Once the capture antibodies had been printed onto the slide the processing protocol was very similar to an ELISA. This protocol is described in detail in **Section 2.1.3.3**. Briefly, slides were blocked to minimize any non-specific binding during the assay. Then a cocktail of cytokine standards was serially diluted and added to all but two blocks where only reagent diluent was added as a negative control. Each standard was diluted to the maximum concentration recommended by the manufacturer (concentrations ranged from 250-6000 pg/mL, see **Table 2.1**) for this reason graphs are labeled by block numbers instead of a specific concentration when multiple cytokines are presented on the

same graph. Following this, a cocktail containing the biotinylated detection antibody was added. Next, streptavidin HRP was added which reacted with the biotin. Slides were then amplified using Tyramine. After the amplification process, streptavidin dye was added. Finally, slides were rinsed and scanned. The slide setup can be visualized in **Figure 3.4**.

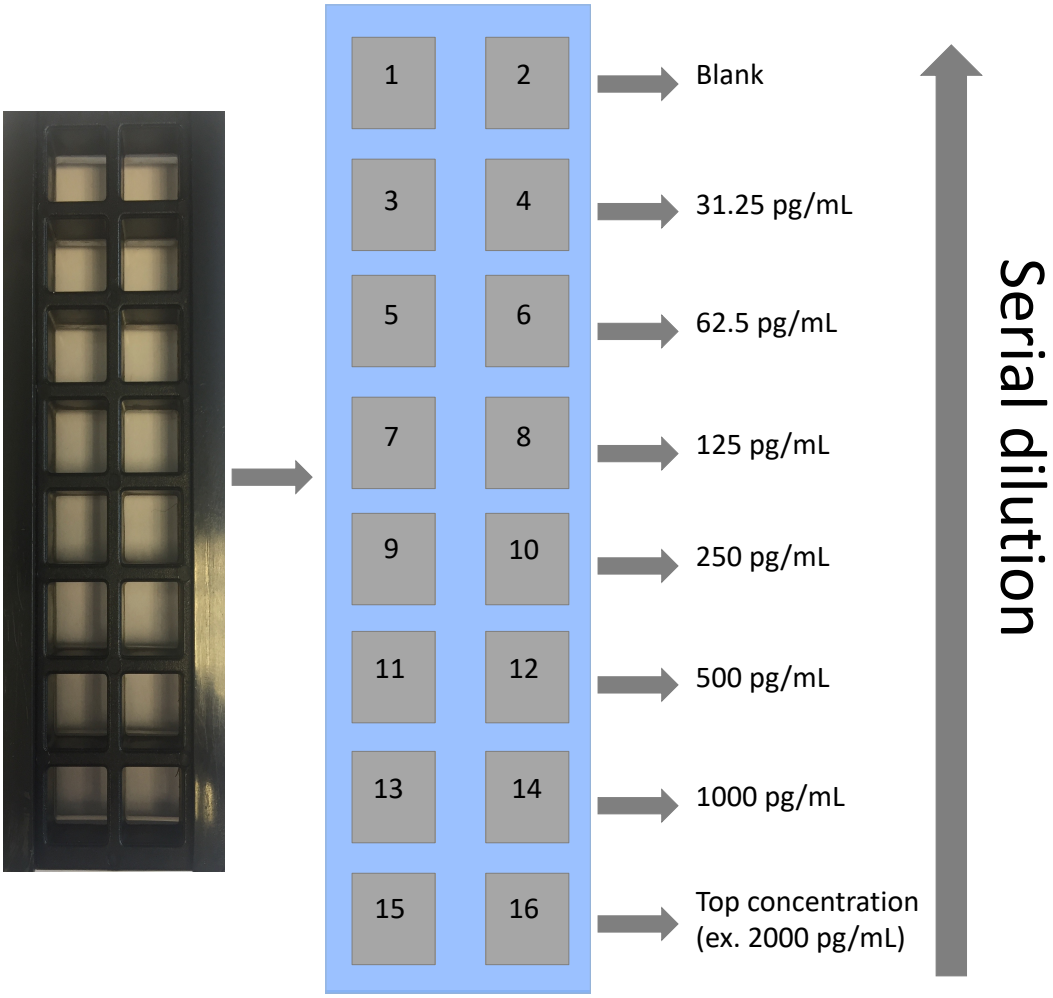


Figure 3.4 Slide processing

All blocks have identical antibody arrays. 16 pad slide holders were used to separate the slide into 16 sections so that serial dilutions of the standards could be performed on each side of the slide (i.e. the left and right sides of the slides were duplicates of each other).

The optimizations described above were performed using a set of 16 cytokines involved in the pathogenesis of COPD. These conditions were used as a starting point to optimize the assay for the 13 cytokines involved in the pathogenesis of asthma that were evaluated in this report.

3.3 RESULTS: MICROARRAY

Thirteen cytokines were printed in replicates of six in each block onto a PPL slide and were processed following S. Selvarajah optimized protocol. Standards were serially diluted and added to the left and right sides in duplicate (i.e. the top concentration was added to the top two blocks) to allow for comparison between spots printed in the same block and spots printed in the opposite block. Reagent diluent alone was added to blocks one and two as a negative control. Printing buffer alone was printed in replicates of six in each block as an additional negative control.

The assay resulted in higher background signals in some blocks than others (**Figure 3.5 A**) and significant variability in signal intensities between the cytokines. Some capture antibodies displayed a "comet tail effect" (**Figure 3.5. B** i.e IFN- γ and IL-1β) which could suggest there was too much antibody to be contained within the printing buffer and therefore smeared as the antibodies were only loosely bound to the surface. There was also a large amount of variability between the replicates of each cytokine printed at the same concentration. Mean signal intensities of the combined wells (12 replicates) are represented in **Figure 3.5 C**. Mean signal intensities for each side of the slide (6 replicates) are represented in **Figure 3.6**. While a similar pattern was observed on both sides of the slide there was considerable variability between the sides and between the replicates in each block in terms of signal intensities. The standard curves showed a "hook effect" where at the highest concentration a lower signal intensity was observed likely

due to antibody saturation from the high cytokine concentration. Therefore, the highest concentration was omitted during future experiments.

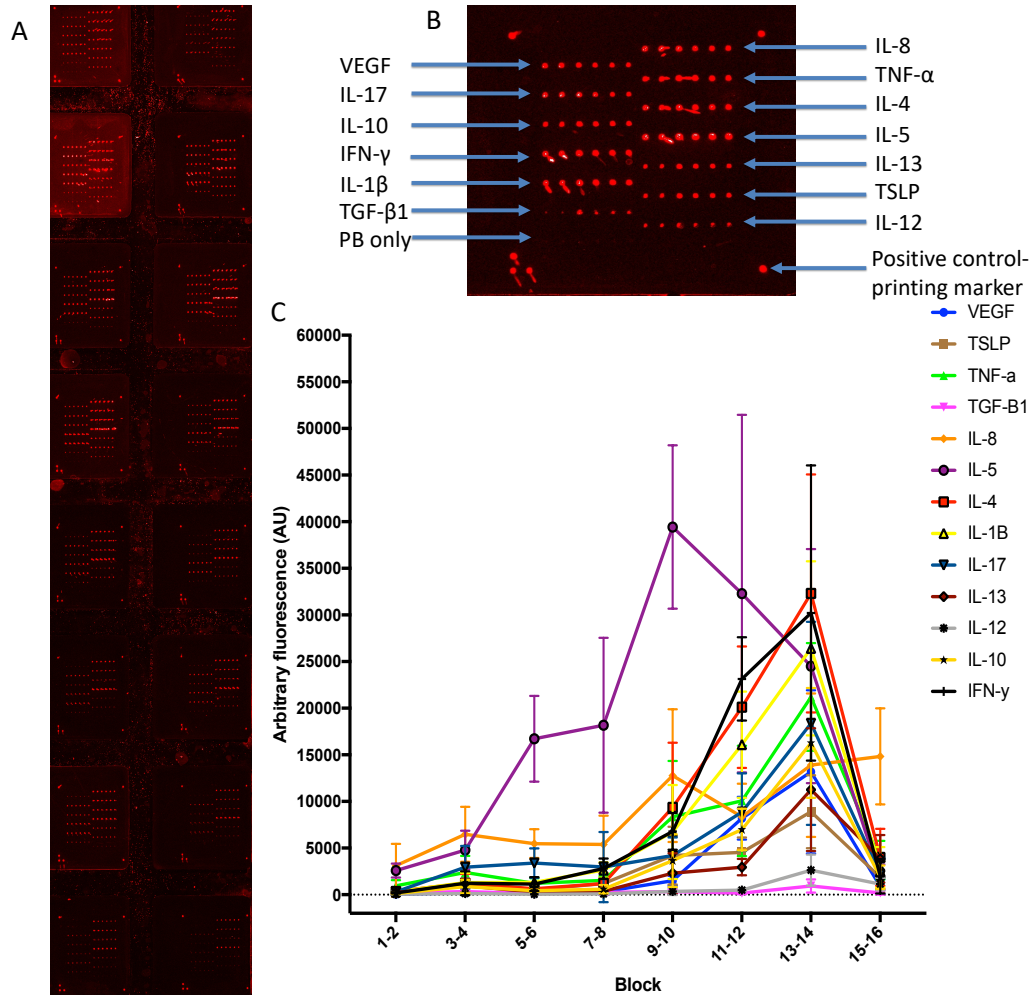


Figure 3.5 Cytokine microarray on poly-l-lysine (PLL) slides following the previously optimized protocol- combined blocks

A. All 13 cytokines were printed in replicates of six in each of the 16 blocks on PLL slides. The left and right slides are duplicates of each other. Cytokines were not added to the bottom two wells as a negative control. High background signals can be seen in the top two left blocks. Cytokine signals decrease moving down the slide. **B**. Close up of one well to see the pattern of capture antibodies printed and the comet tail effect on some spots. **C**. The resulting standard curves are represented as the mean of all spots (12 replicates). Error bars represent the standard deviation.

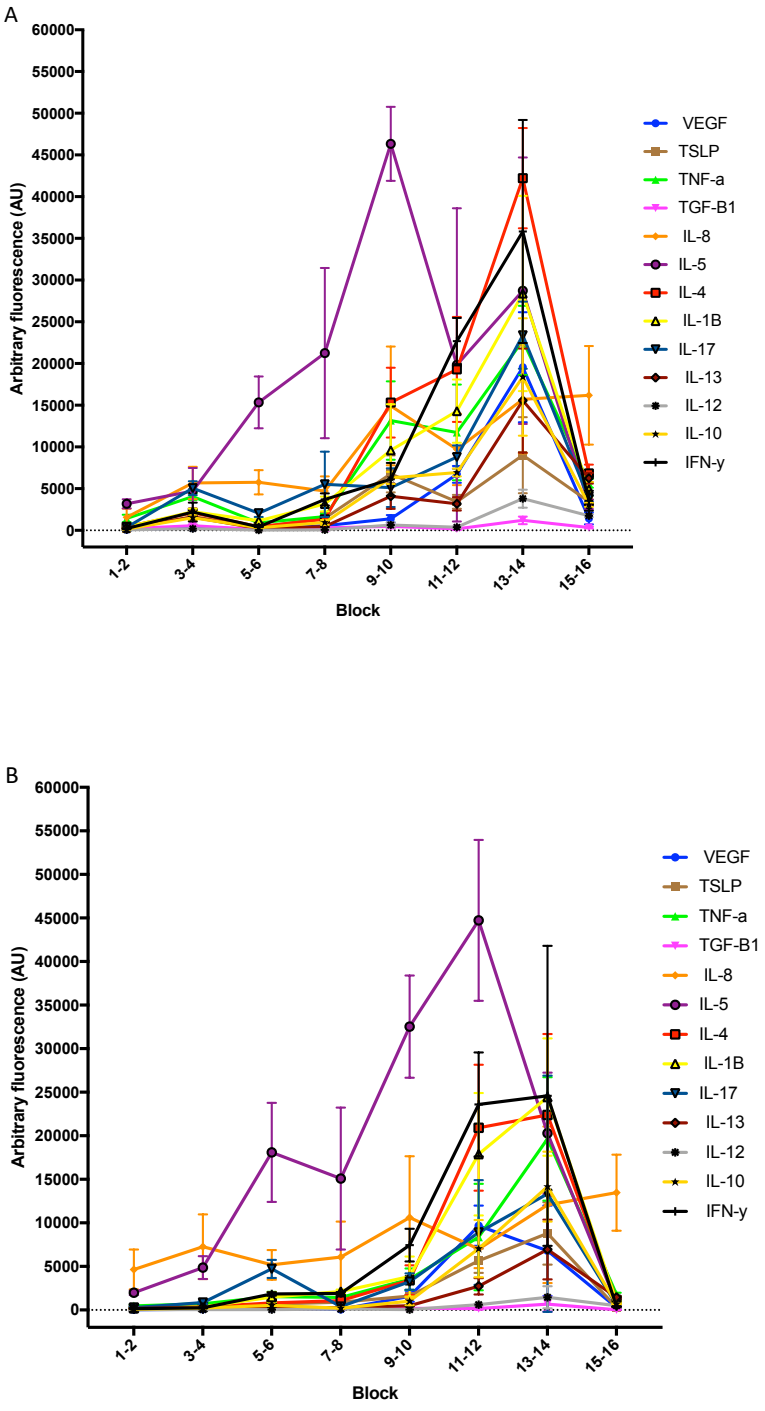


Figure 3.6 Cytokine microarray on poly-l-lysine (PLL) slides following the previously optimized protocol- individual blocks

The resulting standard curves are represented as the mean of all spots from each side of the slide (6 replicates). **A**. left side of the slide; **B**. right side of the slide. Error bars represent the standard deviation.

3.3.1 OPTIMIZATIONS OF THE ANTIBODY MICROARRAY ON PLL SLIDES

In order to address the high level of variability between the replicates, $Tween_{20}$ was added to the 50 mM trehalose printing buffer to help the spots stay together and promote uniformity while drying. Capture antibodies were diluted in both the new and the previous buffer for comparison. Cytokines were printed at 50 µg/mL (**Figure 3.7**), 100 µg/mL (**Figure 3.8**) and 200 µg/mL (**Figure 3.9**) to address the "comet tails" and both with and without amplification to address the "hook effect".

When printed at 50 µg/mL with amplification signals did not increase uniformly. Signals tended to be very flat, peak at the third highest concentration, and then drop back down. Without, amplification signals were very low with large error bars. Curves for IL-8 and IL-5 were slightly better without amplification in the trehalose-tween buffer.

As expected, when printed at 100 µg/mL more cytokines resulted in curves. However, with the exception of IL-1β, most cytokines still resulted in a flat curve when amplification was used. Without amplification, IL-5 produced a curve when printed with the trehalose buffer and IL-5 and IL-8 produced curves in the trehalose-tween buffer. When printed at 200 µg/mL the resulting curves were similar to those printed at 100 µg/mL. Curves were mostly flat when amplification was used with the exception of IL-1β. More cytokines resulted in curves without amplification though the variability was very high when printed in the trehalose only buffer.

Based on the results of these assays, optimizations were continued without amplification using capture antibodies diluted to 100 µg/mL to try and increase reproducibility.

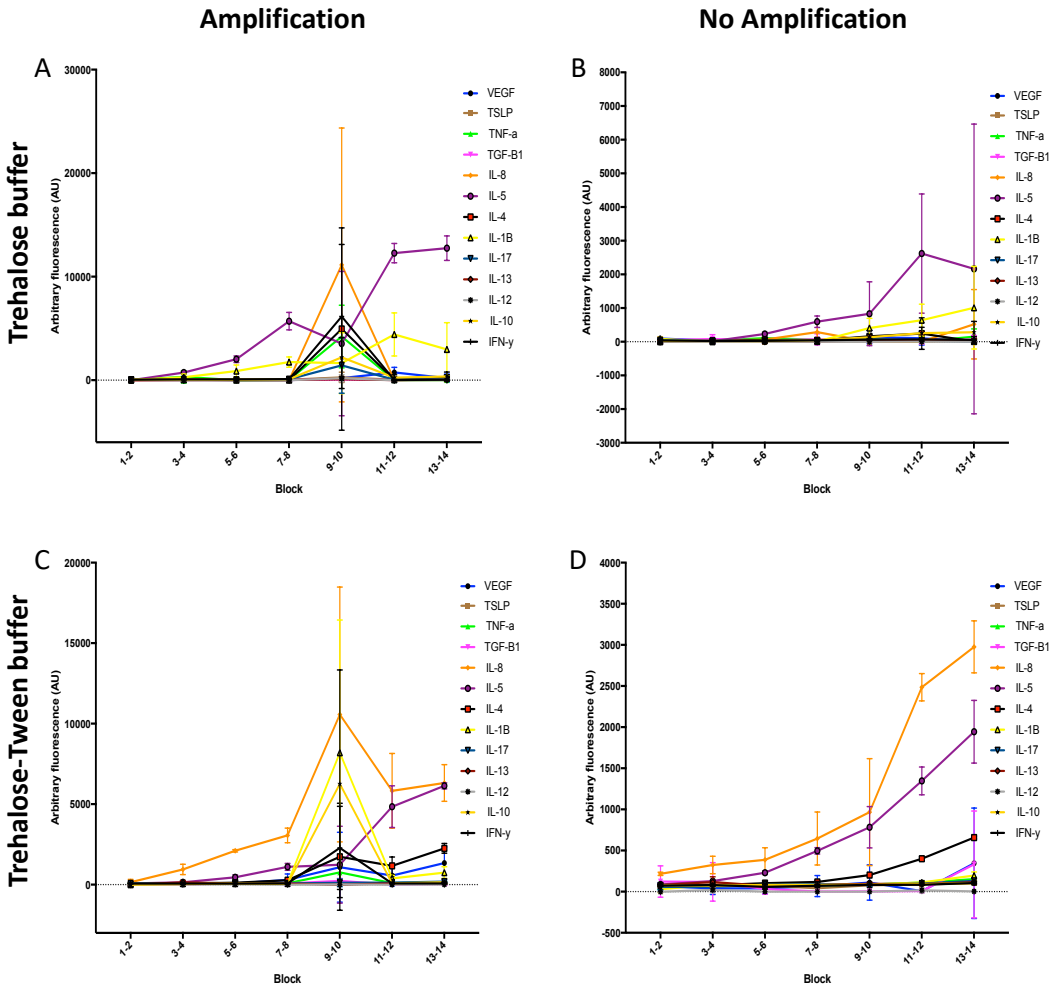


Figure 3.7 Cytokine standard curves printed at 50 µg/mL

Standard curves were generated using the mean of four replicates. The error bars represent the standard deviation. **A**. Capture antibodies were printed in trehalose buffer and were processed with amplification. **B**. Capture antibodies were printed with trehalose buffer and were processed without amplification. **C**. Capture antibodies were printed in trehalose tween buffer and were processed with amplification. **D**. Capture antibodies were printed with trehalose tween buffer and were processed without amplification. Note the Y-axis is not the same in each graph.

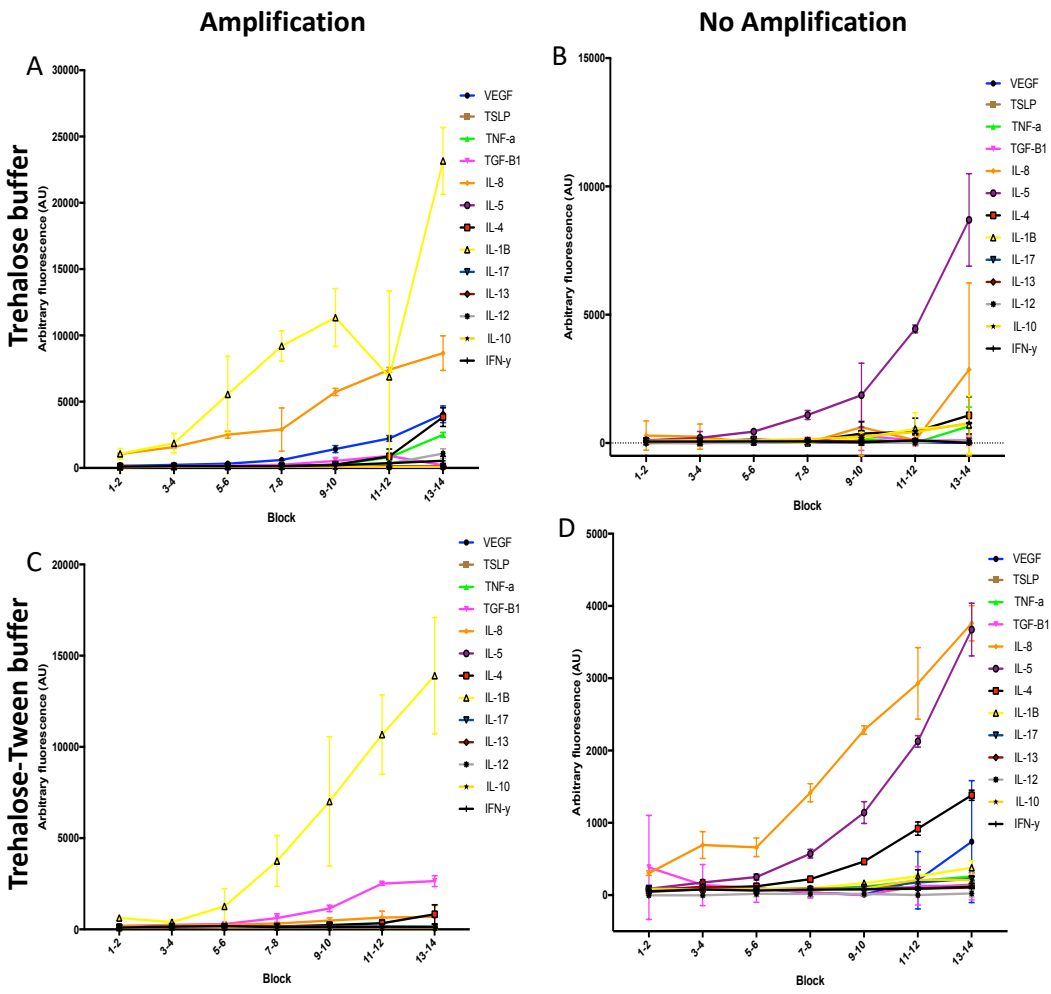


Figure 3.8 Cytokine standard curves printed at 100 µg/mL

Standard curves were generated using the mean of four replicates. The error bars represent the standard deviation. **A**. Capture antibodies were printed in trehalose buffer and were processed with amplification. **B**. Capture antibodies were printed with trehalose buffer and were processed without amplification. **C**. Capture antibodies were printed in trehalose tween buffer and were processed with amplification. **D**. Capture antibodies were printed with trehalose tween buffer and were processed without amplification. Note the Y-axis is not the same in each graph.

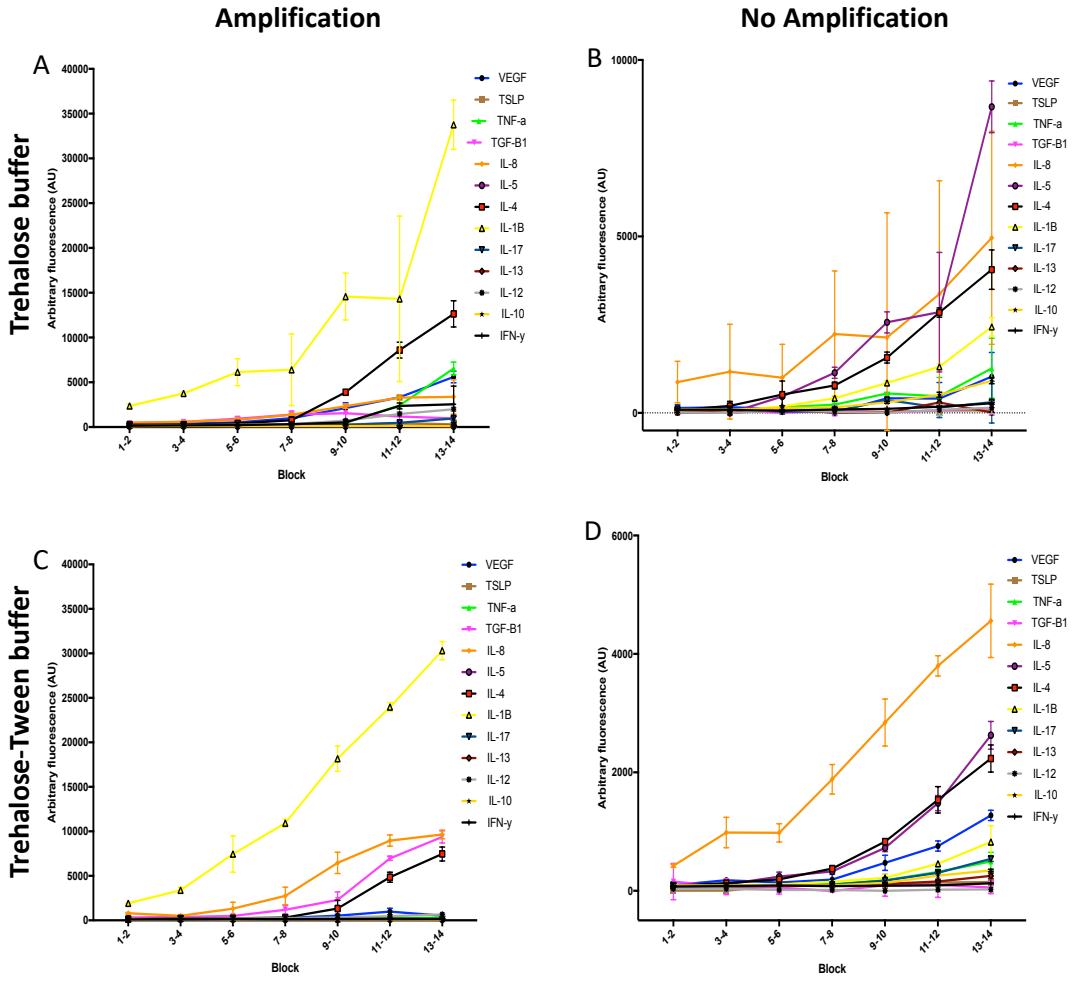


Figure 3.9 Cytokine standard curves printed at 200 µg/mL

Standard curves were generated using the mean of four replicates. The error bars represent the standard deviation. **A**. Capture antibodies were printed in trehalose buffer and were processed with amplification. **B**. Capture antibodies were printed with trehalose buffer and were processed without amplification. **C**. Capture antibodies were printed in trehalose tween buffer and were processed with amplification. **D**. Capture antibodies were printed with trehalose tween buffer and were processed without amplification. Note the Y-axis is not the same in each graph.

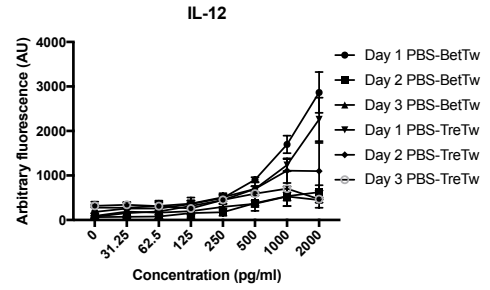
3.3.2 BUFFER AND SLIDE SURFACE OPTIMIZATIONS

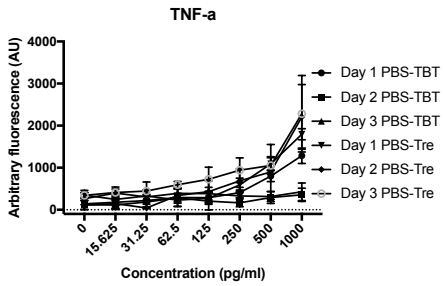
To determine the optimal printing buffer composition as well as the combination of printing buffer and slide surface, six different homemade buffers (see **Table 2.2** for buffer compositions) were tested on PLL, epoxysilane and aminosilane slides. Each cytokine was printed seven times in each buffer on one slide of the slide and then duplicated on the other side for a total of 14 spots per slide per assay. Assays were repeated on three separate days for a total of 42 spots over the three days. Multiple replicates were printed to allow for spots that had been poorly printed to be excluded from the analysis if need be. Poorly printed spots were identified with a "flag" when the GPR file was generated and were not included in the analysis. Each printing buffer was printed on its own as a negative control to ensure none of the buffer components were causing false signals. Following this, the two buffers that produced the best standard curves for each cytokine on each slide surface were selected and repeated on three separate days. Each cytokine was again printed seven times on each side of the slide for a total of 42 spots. This data is represented in **Figures 3.10-3.12** below. The procedure for processing the slides remained the same as recommended by R&D and optimized by S. Selvarajah (277).

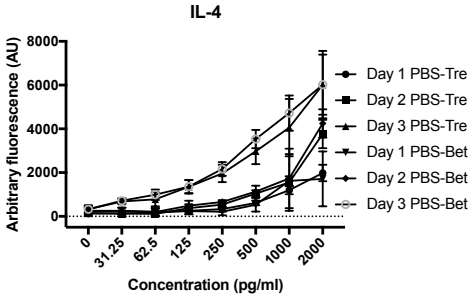
3.3.2.1 Aminosilane

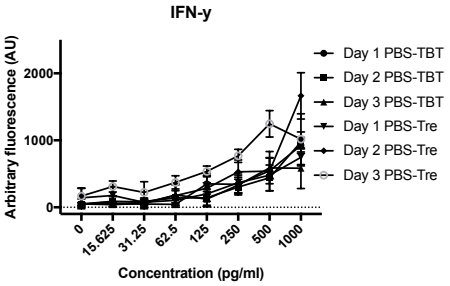
Of the six buffers tested, 50 mM trehalose (PBS-Tre) was one of the preferred buffers for 12 of the 13 cytokines (all except IL-12). None of

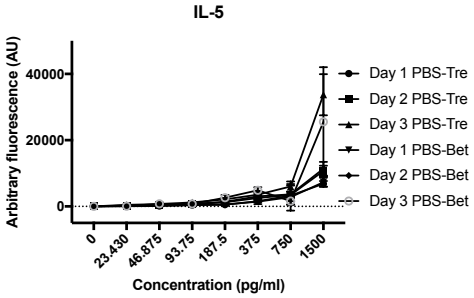
the cytokine-buffer combinations produced a curve on all three days and IL-5, IL-13, TSLP and TGF-β1 never generated a curve (**Figure 3.10**). The remaining cytokines produced curves that were generally very flat with high variability between the replicates (large error bars) and between days (large CV %s). None of the interassay CV %s were <20 % as is required by the FDA guideline. The interassay CV %s ranged from 25.00 to 382.40 % and are summarized in **Table 3.1**.

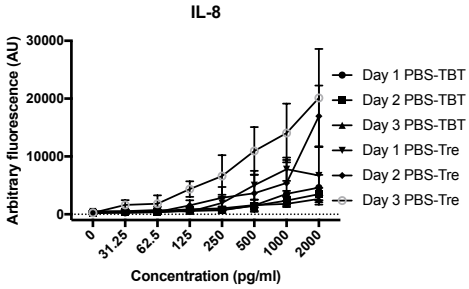

IL-12
Arbitrary fluorescence (AU)
4000
3000
2000
1000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-BetTw
Day 2 PBS-BetTw
Day 3 PBS-BetTw
Day 1 PBS-TreTw
Day 2 PBS-TreTw
Day 3 PBS-TreTw

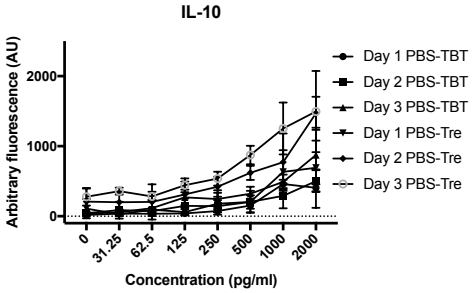

TNF-a
Arbitrary fluorescence (AU)
4000
3000
2000
1000
0
0
15.625
31.25
62.5
125
250
500
1000
Concentration (pg/ml)
Day 1 PBS-TBT
Day 2 PBS-TBT
Day 3 PBS-TBT
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre

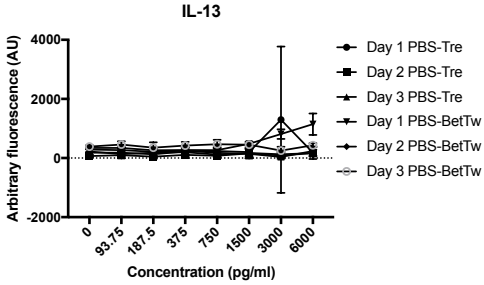

IL-4
Arbitrary fluorescence (AU)
8000
6000
4000
2000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet

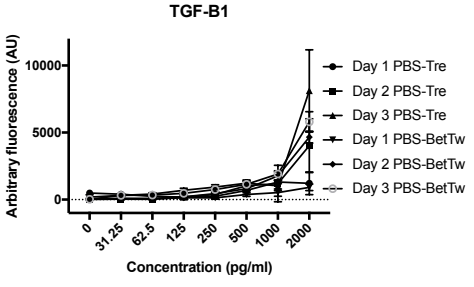

IFN-y
Arbitrary fluorescence (AU)
2000
1000
0
0
15.625
31.25
62.5
125
250
500
1000
Concentration (pg/ml)
Day 1 PBS-TBT
Day 2 PBS-TBT
Day 3 PBS-TBT
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre

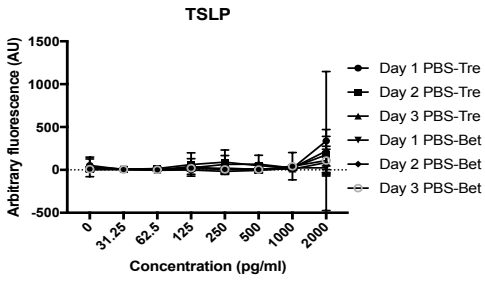

IL-5
Arbitrary fluorescence (AU)
40000
20000
0
0
23.430
46.875
93.75
187.5
375
750
1500
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet


IL-8
Arbitrary fluorescence (AU)
30000
20000
10000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-TBT
Day 2 PBS-TBT
Day 3 PBS-TBT
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre


IL-10
Arbitrary fluorescence (AU)
2000
1000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-TBT
Day 2 PBS-TBT
Day 3 PBS-TBT
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre


IL-13
Arbitrary fluorescence (AU)
4000
2000
0
-2000
0
93.75
187.5
375
750
1500
3000
6000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-BetTw
Day 2 PBS-BetTw
Day 3 PBS-BetTw


TGF-B1
Arbitrary fluorescence (AU)
10000
5000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-BetTw
Day 2 PBS-BetTw
Day 3 PBS-BetTw


TSLP
Arbitrary fluorescence (AU)
1500
1000
500
0
-500
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet

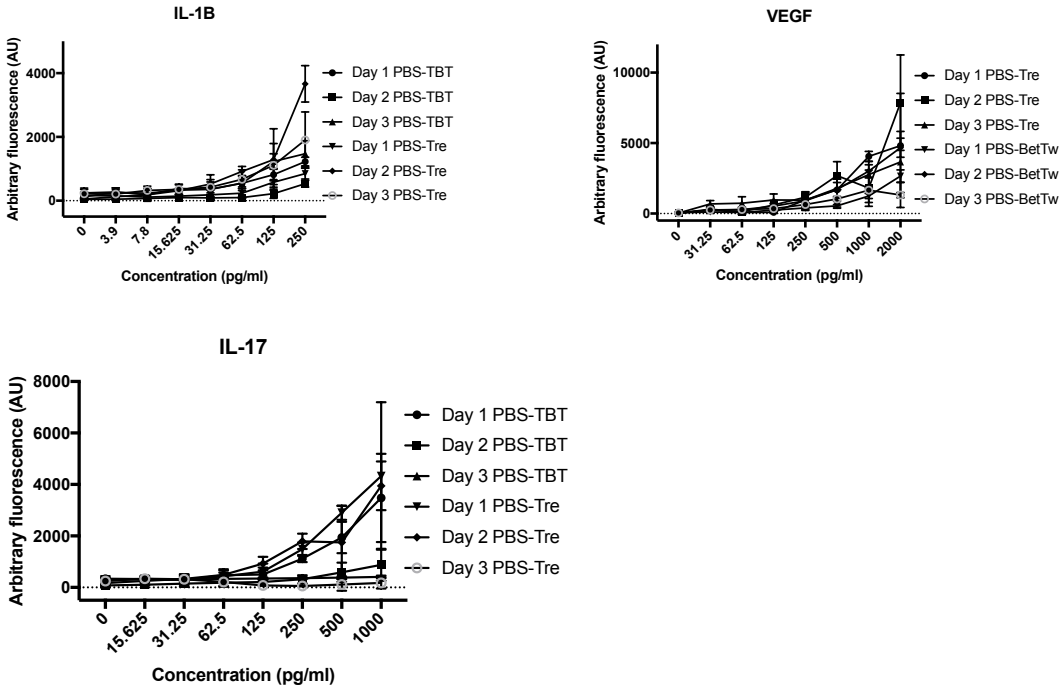


Figure 3.10 Cytokine standard curves on aminosilane slides

Each capture antibody was diluted to 100 µg/mL in the two optimal buffers for that antibody and was printed onto aminosilane slides on three separate days. Standard curves represent the mean of 14 replicates and error bars represent the standard deviation. PBS-Tre: 50mM trehalose, PBS-Bet: PBS and betaine, PBS-BetTw: PBS, betaine and tween$_{20}$, PBS-TBT: PBS, betaine, tween$_{20}$ and trehalose, PBS-TreTw: PBS, trehalose and tween$_{20}$. Note the Y-axis is not the same in each graph.

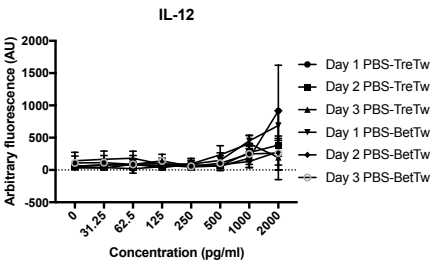
Table 3.1 Interassay Percent coefficient of variation for aminosilane slides

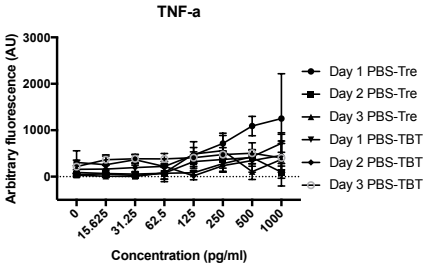
Percentage coefficient of variations (CV %) are given for each cytokine printed in two buffers over three days. CV %s were calculated by combining the 42 spots printed over three separate days. CV %: Coefficient of variation, PBS-Tre: 50mM trehalose, PBS-Bet: PBS and betaine, PBS-BetTw: PBS, betaine and $tween_{20}$, PBS-TBT: PBS, betaine, $tween_{20}$ and trehalose, PBS-TreTw: PBS, trehalose and $tween_{20}$.

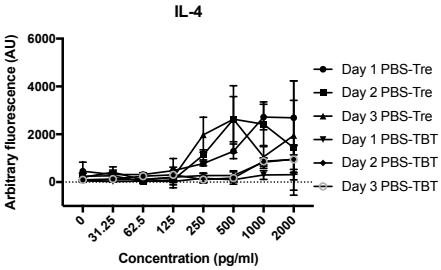
Cytokine and Buffer	CV %		
	Median	Minimum	Maximum
VEGF PBS-Tre	62.66	43.73	130.50
VEGF PBS-BetTw	55.21	45.53	61.80
TSLP PBS-Tre	234.6	117.60	335.90
TSLP PBS-Bet	200.2	121.10	324.50
TNF-α PBS-Tre	52.71	35.54	91.78
TNF-α PBS-TBT	65.23	48.79	74.11
TGF-β1 PBS-Tre	75.71	33.34	123.40
TGF-β1 PBS-BetTw	56.89	35.20	89.35
IL-8 PBS-Tre	76.96	49.48	111.20
IL-8 PBS-TBT	37.91	31.46	64.04
IL-5 PBS-Tre	56.82	33.82	120.50
IL-5 PBS-Bet	72.52	40.71	158.00
IL-4 PBS-Tre	73.43	38.77	99.50
IL-4 PBS-Bet	64.85	41.66	84.98
IL-1β PBS-Tre	70.68	46.85	88.50
IL-1β PBS-TBT	79.02	51.57	101.90
IL-17 PBS-Tre	76.72	38.92	110.20
IL-17 PBS-TBT	57.82	51.44	118.80
IL-13 PBS-Tre	85.95	72.06	382.40
IL-13 PBS-BetTw	58.98	40.07	90.88
IL-12 PBS-BetTw	72.98	48.68	97.90
IL-12 PBS-TreTw	47.79	25.00	75.52
IL-10 PBS-Tre	53.68	40.67	76.66
IL-10 PBS-TBT	60.51	40.20	124.80
IFN-γ PBS-Tre	62.12	34.86	95.96
IFN-γ PBS-TBT	87.6	35.60	101.00

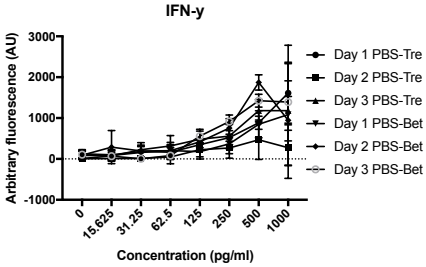
3.3.2.3 Epoxysilane

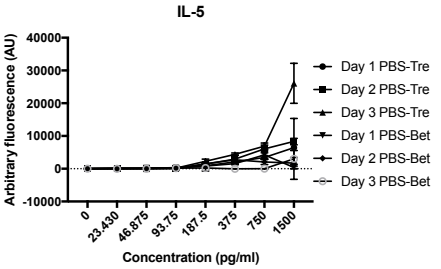
Of the six buffers tested PBS-Tre was one of the preferred buffers for 12 of the 13 cytokines (all except IL-12). The only cytokine to produce a curve was IFN-γ on day 1 printed in PBS-Tre. However, the bottom end of the curve was very flat, there was no distinction in signals between 0-62.5 pg/mL. The assays resulted in signals that were very low with some peaks in intensity and high variability between the replicates (large error bars) (**Figure 3.11**) and between days (large CV %s). None of the interassay CV %s were <20 % they ranged from 22.10 to 1127.00 % and are summarized in **Table 3.2**.

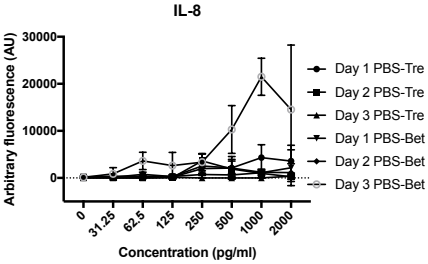

IL-12
Arbitrary fluorescence (AU)
2000
1500
1000
500
0
-500
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-TreTw
Day 2 PBS-TreTw
Day 3 PBS-TreTw
Day 1 PBS-BetTw
Day 2 PBS-BetTw
Day 3 PBS-BetTw

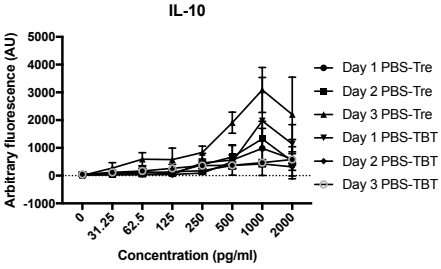

TNF-a
Arbitrary fluorescence (AU)
3000
2000
1000
0
0
15.625
31.25
62.5
125
250
500
1000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-TBT
Day 2 PBS-TBT
Day 3 PBS-TBT

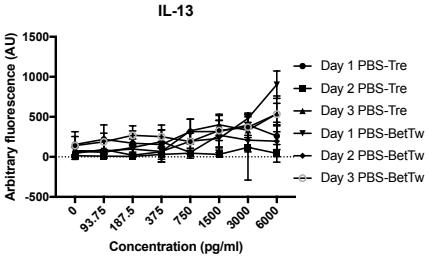

IL-4
Arbitrary fluorescence (AU)
6000
4000
2000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-TBT
Day 2 PBS-TBT
Day 3 PBS-TBT

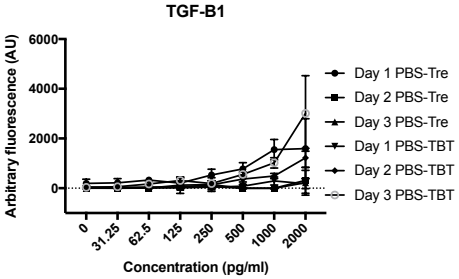

IFN-y
Arbitrary fluorescence (AU)
3000
2000
1000
0
-1000
0
15.625
31.25
62.5
125
250
500
1000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet

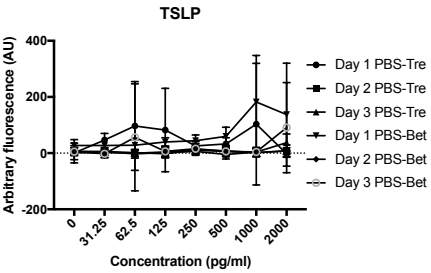

IL-5
Arbitrary fluorescence (AU)
40000
30000
20000
10000
0
-10000
0
23.430
46.875
93.75
187.5
375
750
1500
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet


IL-8
Arbitrary fluorescence (AU)
30000
20000
10000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet


IL-10
Arbitrary fluorescence (AU)
5000
4000
3000
2000
1000
0
-1000
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-TBT
Day 2 PBS-TBT
Day 3 PBS-TBT


IL-13
Arbitrary fluorescence (AU)
1500
1000
500
0
-500
0
93.75
187.5
375
750
1500
3000
6000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-BetTw
Day 2 PBS-BetTw
Day 3 PBS-BetTw


TGF-B1
Arbitrary fluorescence (AU)
6000
4000
2000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-TBT
Day 2 PBS-TBT
Day 3 PBS-TBT


TSLP
Arbitrary fluorescence (AU)
400
200
0
-200
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet

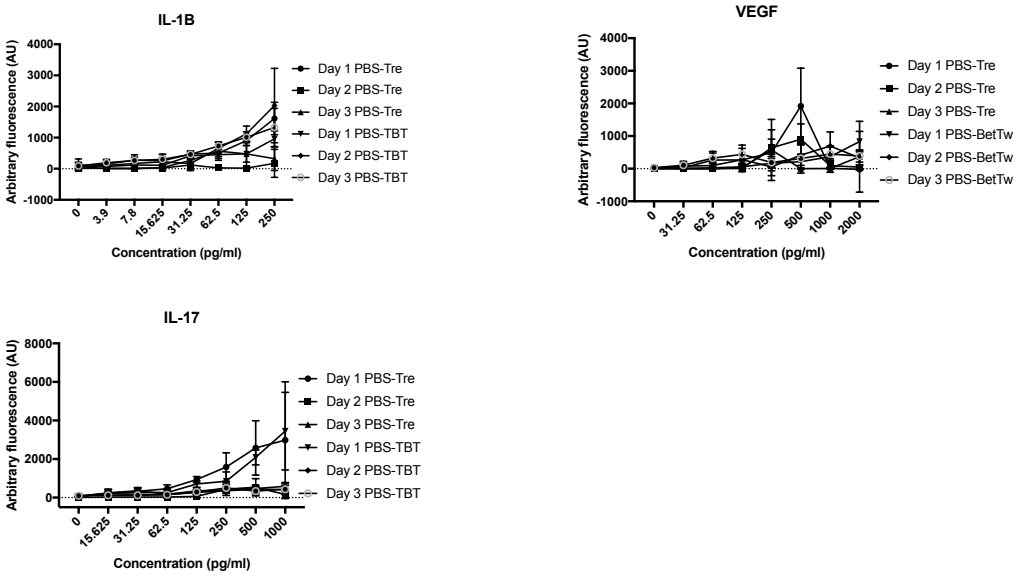


Figure 3.11 Cytokine standard curves on epoxysilane slides

Each capture antibody was diluted to 100 µg/mL in the two optimal buffers for that antibody and was printed onto epoxysilane slides on three separate days. Standard curves represent the mean of 14 replicates and error bars represent the standard deviation. PBS-Tre: 50mM trehalose, PBS-Bet: PBS and betaine, PBS-BetTw: PBS, betaine and tween$_{20}$, PBS-TBT: PBS, betaine, tween$_{20}$ and trehalose, PBS-TreTw: PBS, trehalose and tween$_{20}$. Note the Y-axis is not the same in each graph.

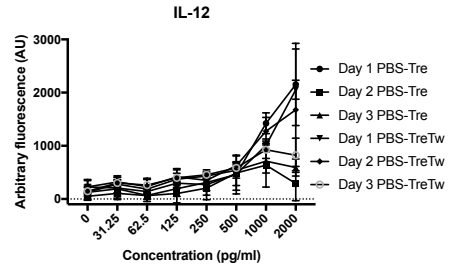
Table 3.2 Interassay percent coefficient of variation for epoxysilane slides

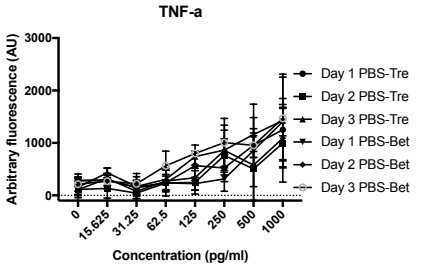
Coefficient of variations (CV %) are given for each cytokine printed in two buffers over three days. CV %s were calculated by combining the 42 spots printed over three separate days. CV %: Coefficient of variation, PBS-Tre: 50mM trehalose, PBS-Bet: PBS and betaine, PBS-BetTw: PBS, betaine and $tween_{20}$, PBS-TBT: PBS, betaine, $tween_{20}$ and trehalose, PBS-TreTw: PBS, trehalose and $tween_{20}$.

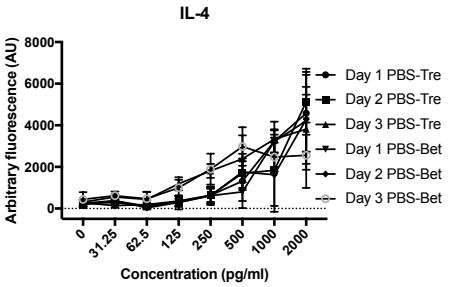
Cytokine and Buffer	CV %		
	Median	Minimum	Maximum
VEGF PBS-Tre	126.30	57.20	273.10
VEGF PBS-BetTw	89.20	64.22	138.20
TSLP PBS-Tre	219.90	131.30	1127.00
TSLP PBS-Bet	216.10	107.60	480.60
TNF-α PBS-Tre	161.50	51.52	186.90
TNF-α PBS-TBT	62.01	29.94	110.10
TGF-β1 PBS-Tre	232.80	148.5	273.60
TGF-β1 PBS-TBT	77.76	49.51	140.60
IL-8 PBS-Tre	182.50	113.10	253.10
IL-8 PBS-Bet	129.50	69.69	188.90
IL-5 PBS-Tre	103.00	26.60	261.00
IL-5 PBS-Bet	163.80	97.03	245.10
IL-4 PBS-Tre	72.27	48.31	190.90
IL-4 PBS-TBT	79.88	35.43	109.80
IL-1β PBS-Tre	158.10	98.70	224.70
IL-1β PBS-TBT	62.01	22.10	130.00
IL-17 PBS-Tre	116.70	92.36	188.40
IL-17 PBS-TBT	72.85	58.19	148.80
IL-13 PBS-Tre	139.50	86.30	208.40
IL-13 PBS-BetTw	74.01	50.24	93.16
IL-12 PBS-BetTw	87.72	62.54	100.70
IL-12 PBS-TreTw	93.00	42.96	129.50
IL-10 PBS-Tre	97.35	51.75	187.70
IL-10 PBS-TBT	80.33	37.57	119.50
IFN-γ PBS-Tre	104.40	57.72	214.10
IFN-γ PBS-Bet	100.10	28.04	179.50

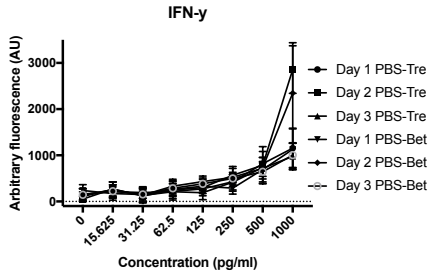
3.3.2.4 Poly-l-lysine

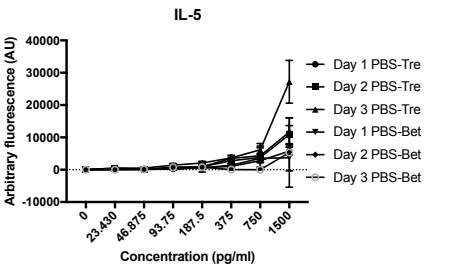
Of the six buffers tested PBS-Tre was one of the preferred buffers for 11 of the 13 cytokines (all except IL-8 and VEGF). The second most preferred buffer (9 of 13 cytokines) was PBS with betaine (PBS-Bet). None of the buffer combinations produced a curve for IL-5, TSLP or TGF-β1. The remaining cytokines resulted in better curves than when printed on aminosilane or epoxysilane. However, there was still considerable variability between the replicates (large error bars) (**Figure 3.12**). The majority of the curves were still very flat at the bottom of the curve. IL-12 printed in PBS-TreTw was the only cytokine to come close to the CV % cutoff of <20 % (20.04 %). The CV %s ranged from 20.04 to 416.90 %. The CV %s are summarized in **Table 3.3**.

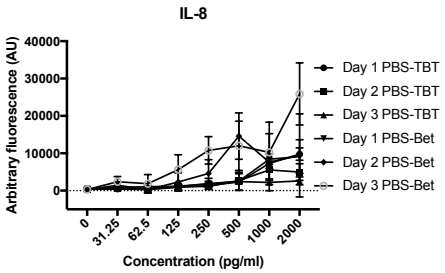

IL-12
Arbitrary fluorescence (AU)
3000
2000
1000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-TreTw
Day 2 PBS-TreTw
Day 3 PBS-TreTw

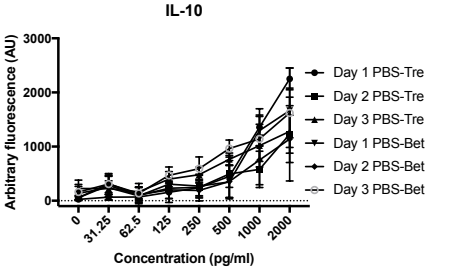

TNF-a
Arbitrary fluorescence (AU)
3000
2000
1000
0
0
15.625
31.25
62.5
125
250
500
1000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet

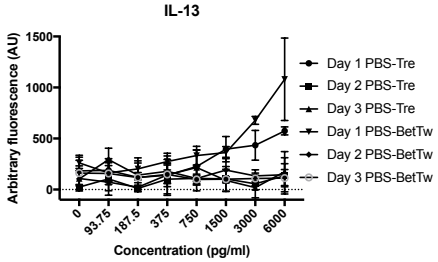

IL-4
Arbitrary fluorescence (AU)
8000
6000
4000
2000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet

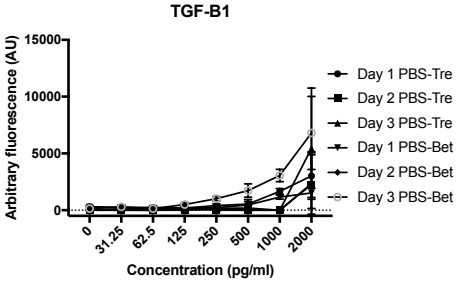

IFN-y
Arbitrary fluorescence (AU)
3000
2000
1000
0
0
15.625
31.25
62.5
125
250
500
1000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet

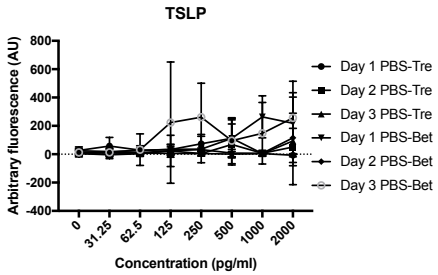

IL-5
Arbitrary fluorescence (AU)
40000
30000
20000
10000
0
-10000
0
23.430
46.875
93.75
187.5
375
750
1500
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet


IL-8
Arbitrary fluorescence (AU)
40000
30000
20000
10000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-TBT
Day 2 PBS-TBT
Day 3 PBS-TBT
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet


IL-10
Arbitrary fluorescence (AU)
3000
2000
1000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet


IL-13
Arbitrary fluorescence (AU)
1500
1000
500
0
0
93.75
187.5
375
750
1500
3000
6000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-BetTw
Day 2 PBS-BetTw
Day 3 PBS-BetTw


TGF-B1
Arbitrary fluorescence (AU)
15000
10000
5000
0
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet


TSLP
Arbitrary fluorescence (AU)
800
600
400
200
0
-200
-400
0
31.25
62.5
125
250
500
1000
2000
Concentration (pg/ml)
Day 1 PBS-Tre
Day 2 PBS-Tre
Day 3 PBS-Tre
Day 1 PBS-Bet
Day 2 PBS-Bet
Day 3 PBS-Bet

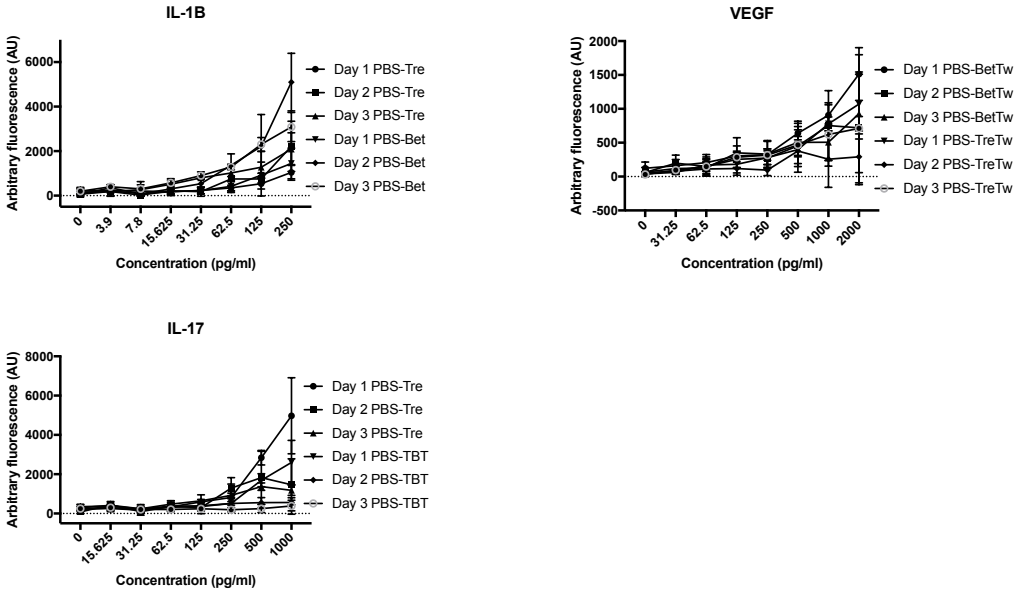


Figure 3.12 Cytokine standard curves on poly-l-lysine slides

Each capture antibody was diluted to 100 µg/mL in the two optimal buffers for that antibody and was printed onto poly-l-lysine slides on three separate days. Standard curves represent the mean of 14 replicates and error bars represent the standard deviation. PBS-Tre: 50mM trehalose, PBS-Bet: PBS and betaine, PBS-BetTw: PBS, betaine and tween$_{20}$, PBS-TBT: PBS, betaine, tween$_{20}$ and trehalose, PBS-TreTw: PBS, trehalose and tween$_{20}$. Note the Y-axis is not the same in each graph

Table 3.3 Interassay percent coefficient of variation for poly-l-lysine slides

Coefficient of variations (CV %) are given for each cytokine printed in two buffers over three days. CV %s were calculated by combining the 42 spots printed over three separate days. CV %: Coefficient of variation, PBS-Tre: 50mM trehalose, PBS-Bet: PBS and betaine, PBS-BetTw: PBS, betaine and $tween_{20}$, PBS-TBT: PBS, betaine, $tween_{20}$ and trehalose, PBS-TreTw: PBS, trehalose and $tween_{20}$.

Cytokine and Buffer	CV %		
	Median	Minimum	Maximum
VEGF PBS-BetTw	61.02	45.09	87.30
VEGF PBS-TreTw	74.81	48.63	110.90
TSLP PBS-Tre	287.20	226.90	416.90
TSLP PBS-Bet	180.80	116.50	328.50
TNF-α PBS-Tre	140.70	103.30	216.00
TNF-α PBS-Bet	62.76	49.47	114.30
TGF-β1 PBS-Tre	217.10	110.10	274.80
TGF-β1 PBS-Bet	88.68	77.12	157.30
IL-8 PBS-TBT	43.60	31.98	72.37
IL-8 PBS-Bet	74.52	62.84	193.40
IL-5 PBS-Tre	60.79	42.64	118.70
IL-5 PBS-Bet	116.90	95.59	187.30
IL-4 PBS-Tre	53.28	38.24	115.20
IL-4 PBS-Bet	66.76	47.71	127.90
IL-1β PBS-Tre	76.86	48.98	135.30
IL-1β PBS-Bet	51.60	37.02	126.80
IL-17 PBS-Tre	57.83	39.65	113.20
IL-17 PBS-TBT	47.54	34.56	114.90
IL-13 PBS-Tre	124.40	89.76	202.00
IL-13 PBS-BetTw	73.22	54.87	138.00
IL-12 PBS-Tre	87.49	52.27	132.60
IL-12 PBS-TreTw	34.94	20.04	64.79
IL-10 PBS-Tre	62.44	38.16	121.40
IL-10 PBS-Bet	62.83	45.79	101.10
IFN-γ PBS-Tre	57.49	36.97	106.40
IFN-γ PBS-Bet	62.61	34.30	91.72

3.4 CONCLUSIONS: MICROARRAY

Following these experiments, it was determined that to continue optimizations would be too costly and time-consuming as none of the cytokines produced a standard curve that was able to be reproduced over three days within the acceptable limits of variability. It was determined that cytokine levels would be measured using an ELISA as it is the accepted "gold standard" method and optimizations would be performed to miniaturize the ELISA to be performed in a 384 well plate. This would minimize costs in terms of the quantity of reagents used and additionally would require less sample from each patient than a traditional 96 well ELISA.

3.5 METHODOLOGY: ELISA

Reliable cytokine quantification can be achieved by performing a sandwich ELISA. Briefly, this is done by binding a capture antibody to the bottom of the wells of the ELISA plate. The plate is then blocked to ensure there is no non-specific binding and then the cytokine standard and/or patient sample is added. Following this, the biotinylated detection antibody is added to the wells. Next, streptavidin HRP is added which reacts with the biotin and finally, an enzymatic substrate solution is added which results in a colour change (clear to blue). In order to stop this reaction and visualize the results sulfuric acid (H_2SO_4) is added and the liquid turns from blue to yellow which can be read at 450 nm. ELISAs were conducted according to the manufacturer's instructions in 96 well plates and optimized to be miniaturized and conducted in 384 well plates to minimize the amount of reagent and patient sample used while still producing equally high signals and reliable, reproducible results.

3.5.1 OPTIMIZATION OF THE 384 WELL ELISA

According to the manufacturer's instructions, ELISAs are to be conducted in 96 well plates using PBS as a coating buffer to dilute the capture antibody. In order to ensure maximal signals and obtain the most reliable and reproducible standard curves that could also be performed in a 384 well plate an alternative coating buffer of carbonate-bicarbonate (CBC) was tested. In order to perform the optimizations cost-effectively one cytokine (TNF-α) was used as a sample for all optimizations. The capture antibody was diluted to the

concentration recommended in the manufacturer's instructions (4 µg/mL) in PBS as well as CBC. Both buffers were added to wells in volumes of 10 µl and 20 µl in triplicate to determine the optimal volume that would completely coat the bottom of the wells whilst keeping the materials used to a minimum. CBC at 10 or 20 µl resulted in more uniform curves than PBS at 10 or 20 µl and showed higher signal intensities (**Figure 3.13**). The difference in signal intensities were apparent as low as 15.625 pg/mL and continued to increase as the concentration increased. Although PBS 20 µl spiked to a similar absorbance reading as CBC 20 µl at the top concentration (0.695 OD in CBC vs 0.607 OD in PBS), the bottom of the curve was very flat and unable to differentiate between signals. Therefore, CBC at 20 µl was chosen as the coating buffer for the remainder of the experiments.

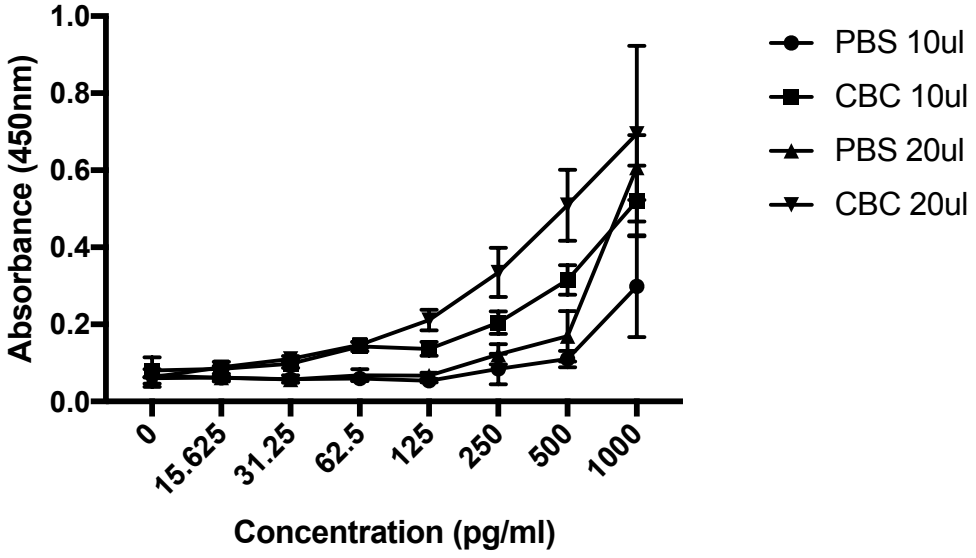


Figure 3.13 Optimization of coating buffer and volume for 384 well ELISA

TNF-α was used to determine the ideal coating buffer for the 384 well ELISA and the optimal volume to add to each well. The capture antibody for TNF-α was diluted in phosphate buffered saline and carbonate bicarbonate. The CBC buffer added 20 µl per well resulted in a steeper curve than PBS 10 µl, 20 µl or CBC with more differentiation at all concentrations. Curves represent the mean of three replicates and error bars represent the standard deviation of the mean.

3.5.2 OPTIMIZATION OF THE PROTOCOL

Following every wash step, some wash buffer remained in each well despite vigorous inversion. This was thought to be contributing to the lower signal strengths than would be expected as it would further dilute subsequent reagents added to the wells. In order to remove all residual liquid, the plate was centrifuged for ten seconds upside down following the last wash of each wash cycle. To ensure all newly aliquoted liquid went directly to the bottom of each well the plate was centrifuged face up for ten seconds after liquid was added. This was found to increase signal intensities throughout the curve (maximum 1.564 OD with centrifuge vs 0.695 OD without centrifuge) (**Figure 3.14**). Therefore, centrifuge steps were added to the protocol for all subsequent experiments.

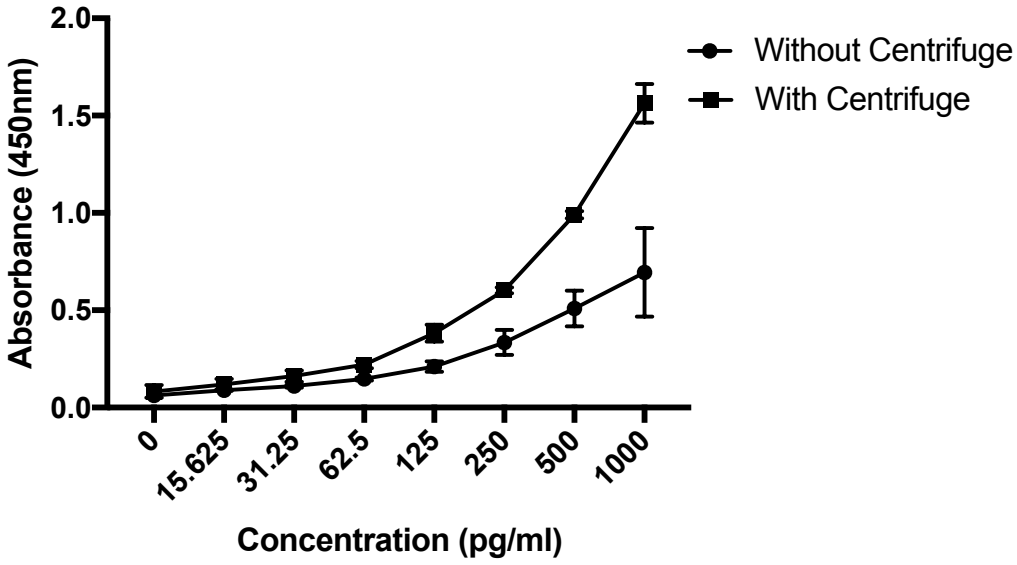


Figure 3.14 384 well ELISA protocol optimization

TNF-α was used to optimize signal strength for the 384 well ELISA to produce. The assay was conducted with and without centrifuging the plate before and after each step.

3.5.3 OPTIMIZATION OF BOVINE SERUM ALBUMIN

When cytokine kits were received from the manufacturer the cytokine standard and detection antibodies were reconstituted in BSA, and during the assays, they were diluted in BSA to the recommended concentrations as per the manufacturer's instructions. However, this was initially done using BSA purchased from ThermoFisher that was not the same as that recommended by the manufacturer. In order to determine if switching to the recommended BSA would increase signal intensities, a new kit was reconstituted and diluted using 10X concentrated BSA from R&D. The R&D BSA produced significantly higher signals than the ThermoFisher BSA throughout the curve (maximum 2.873 OD vs 1.564 OD) with high reproducibility between the replicates and therefore was used for all subsequent assays (**Figure 3.15**).

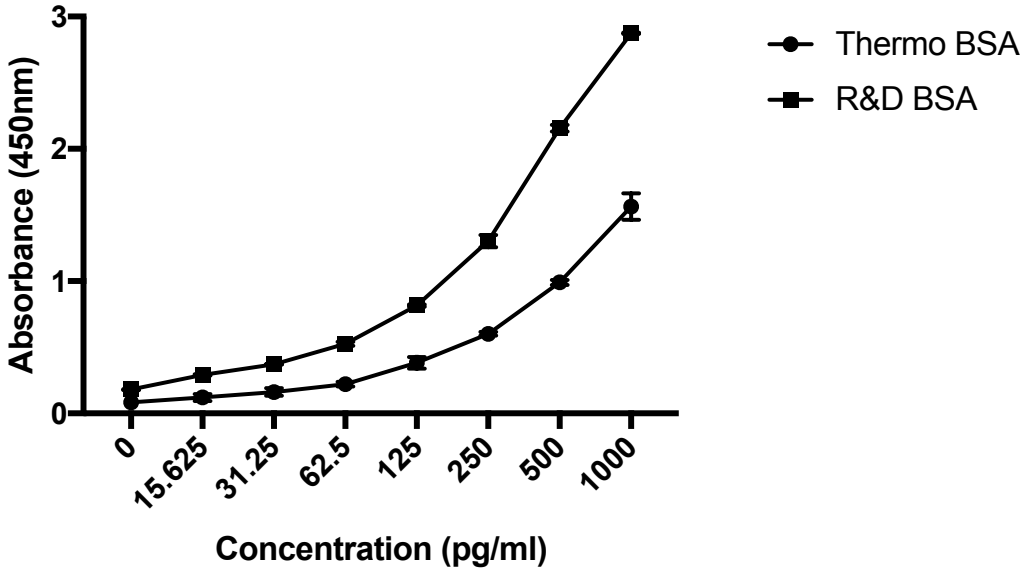


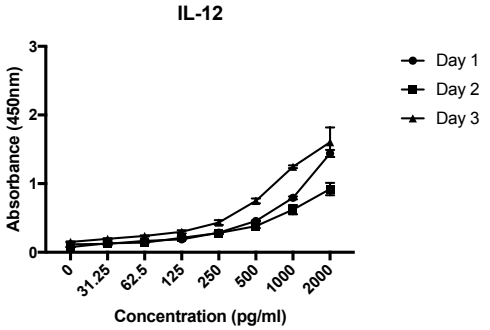
Figure 3.15 Bovine serum albumin optimization

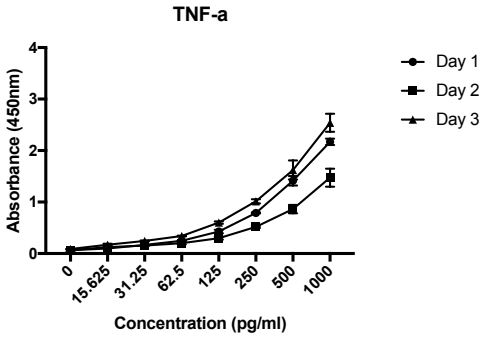
TNF-α was used as an example to determine the optimum bovine serum albumin (BSA) to use to reconstitute and dilute the components of the ELISA kits. The R&D BSA resulted in significantly higher signal intensities throughout the curve than the BSA from ThermoFischer.

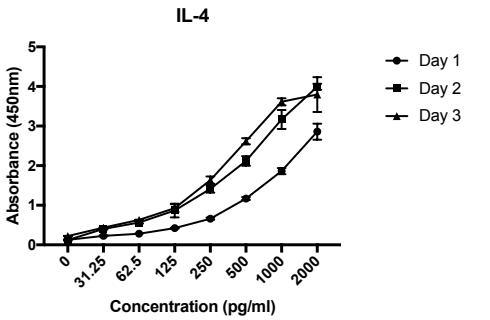
3.5.4 VALIDATION OF 384 WELL STANDARD CURVES WITH 96 WELL CURVES

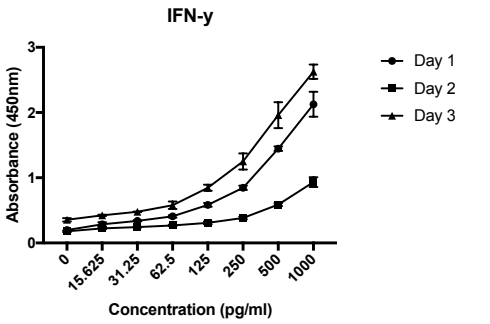
ELISAs were performed in triplicate on three separate days for all 13 cytokines in 96 and 384 well plates for reproducibility. ELISAs performed in 384 well plates were conducted according to the manufacturer's instructions incorporating the optimizations detailed above whereas the 96 well ELISAs were done strictly according to the manufacturer's instructions using kits that had previously been reconstituted in BSA from ThermoFischer, which was necessary due to the financial impact of purchasing all new kits. All 13 cytokines produced uniform standard curves that were reproducible between and within the assays. ELISAs performed in 384 well plates were more or equally as reproducible than those performed in 96 well plates and their maximum signals tended to be higher (See **Figure 3.16**). Intraassay CVs were generally within the acceptable range of variability with the exception of TGF-β1 whose mean CV from the third assay was 21.46 %. Interassay variability was also within the acceptable range with the exception of IL-12 (mean CV 23.47 %) and TNF-α (mean CV 21.63 %). Following these experiments, it was determined that the assays were ready to be used to detect cytokine levels in patient samples. (see **Figures 3.17 and 3.18**).

96 well ELISA

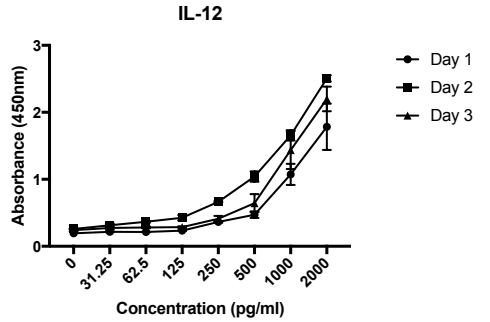


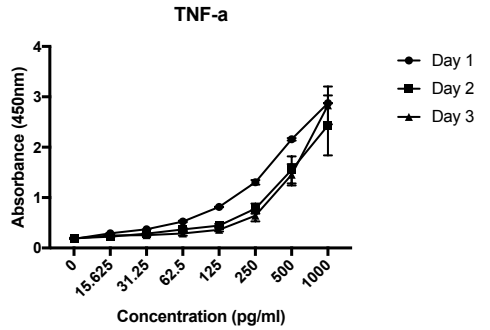


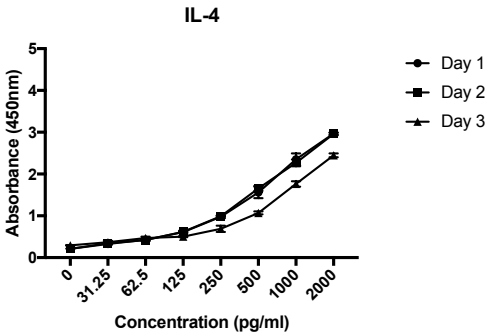


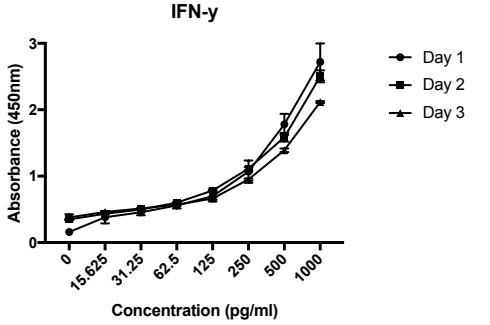


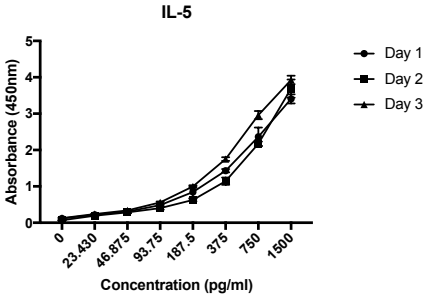
384 well ELISA

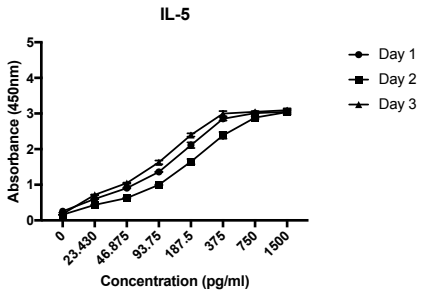


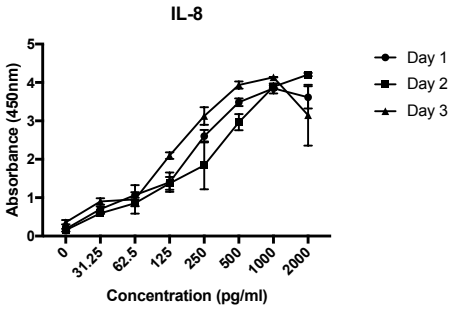


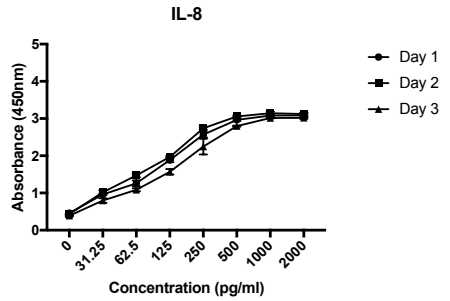


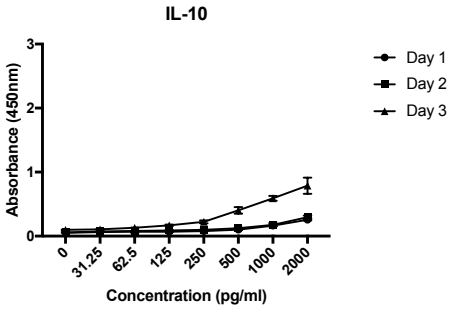


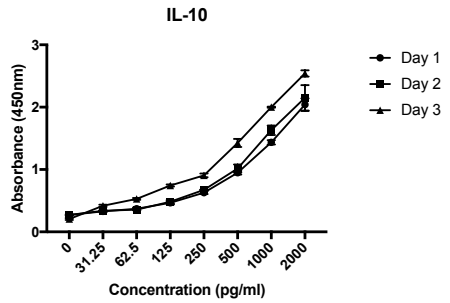

IL-5
Absorbance (450nm)
Concentration (pg/ml)
0
23.430
46.875
93.75
187.5
375
750
1500
Day 1
Day 2
Day 3

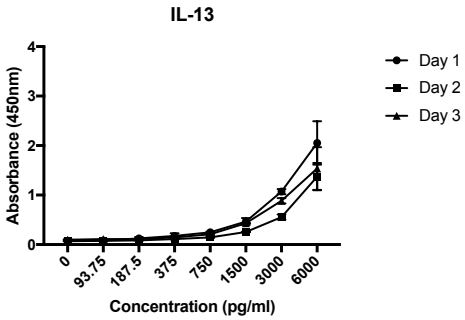

IL-5
Absorbance (450nm)
Concentration (pg/ml)
0
23.430
46.875
93.75
187.5
375
750
1500
Day 1
Day 2
Day 3

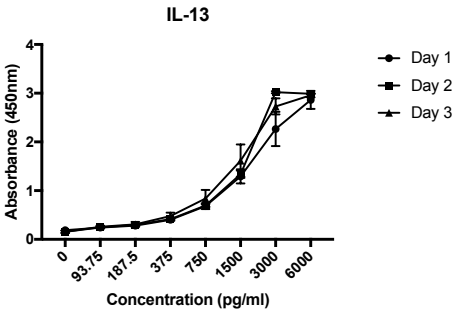

IL-8
Absorbance (450nm)
Concentration (pg/ml)
0
31.25
62.5
125
250
500
1000
2000
Day 1
Day 2
Day 3

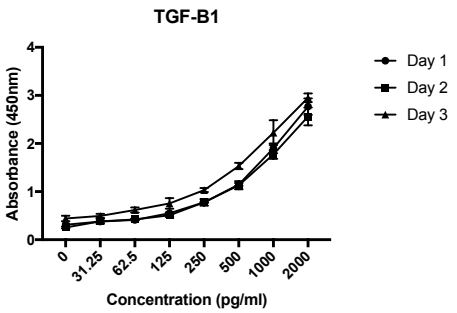

IL-8
Absorbance (450nm)
Concentration (pg/ml)
0
31.25
62.5
125
250
500
1000
2000
Day 1
Day 2
Day 3

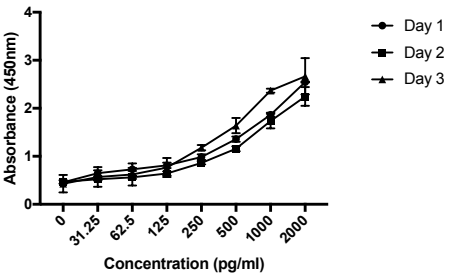

IL-10
Absorbance (450nm)
Concentration (pg/ml)
0
31.25
62.5
125
250
500
1000
2000
Day 1
Day 2
Day 3


IL-10
Absorbance (450nm)
Concentration (pg/ml)
0
31.25
62.5
125
250
500
1000
2000
Day 1
Day 2
Day 3


IL-13
Absorbance (450nm)
Concentration (pg/ml)
0
93.75
187.5
375
750
1500
3000
6000
Day 1
Day 2
Day 3


IL-13
Absorbance (450nm)
Concentration (pg/ml)
0
93.75
187.5
375
750
1500
3000
6000
Day 1
Day 2
Day 3


TGF-B1
Absorbance (450nm)
Concentration (pg/ml)
0
31.25
62.5
125
250
500
1000
2000
Day 1
Day 2
Day 3


TGF-B1
Absorbance (450nm)
Concentration (pg/ml)
0
31.25
62.5
125
250
500
1000
2000
Day 1
Day 2
Day 3

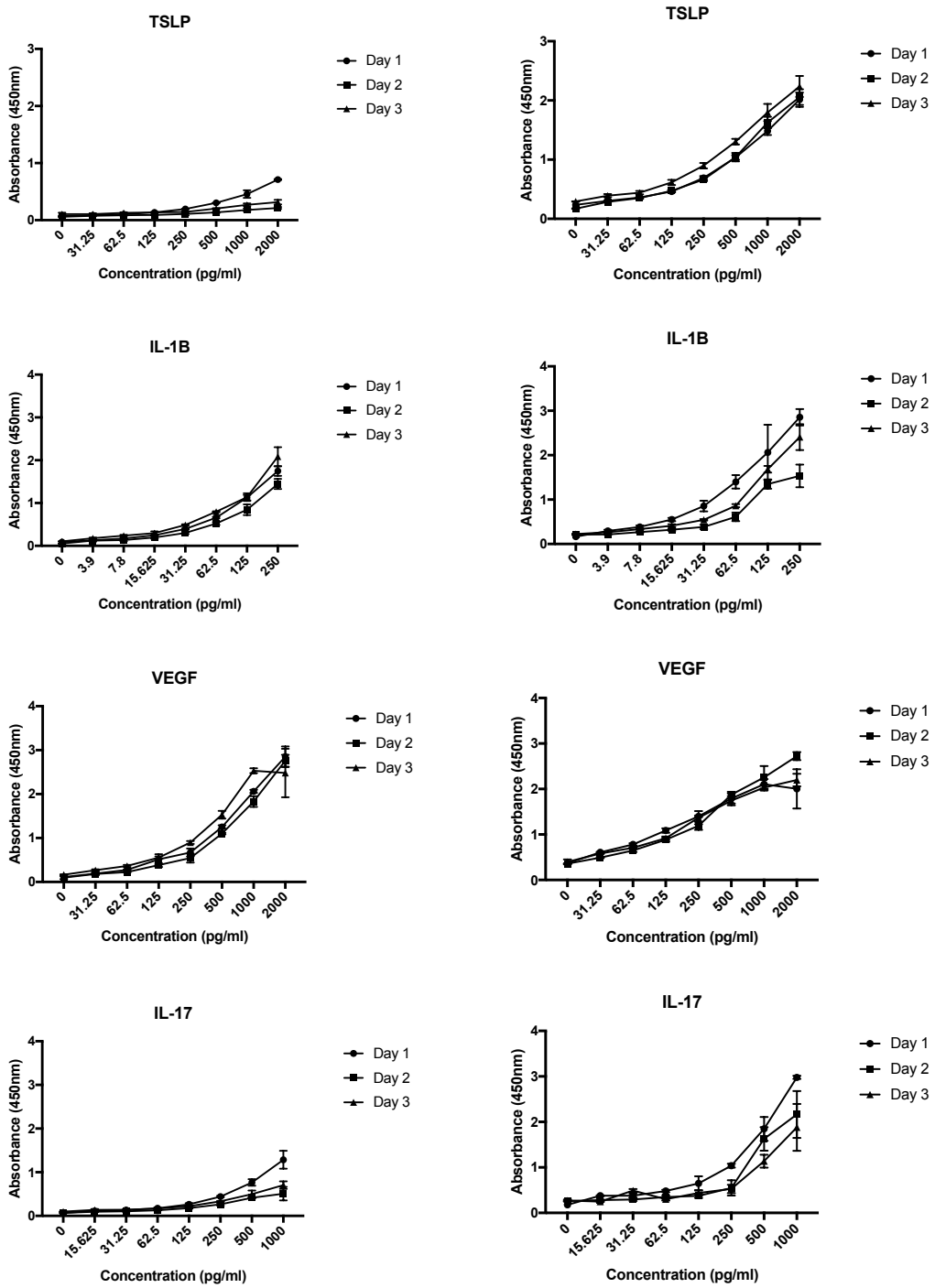


Figure 3.16 Comparison of standard curves in 96 and 384 well plates

Enzyme-linked immunosorbent assays were performed in triplicate on three separate days in order to demonstrate reproducibility of the assays. 384 well assays incorporated the optimizations detailed above whereas 96 well assays were performed strictly according to the manufacturer's instructions. Data points represent the mean and error bars represent the standard deviation of the mean.

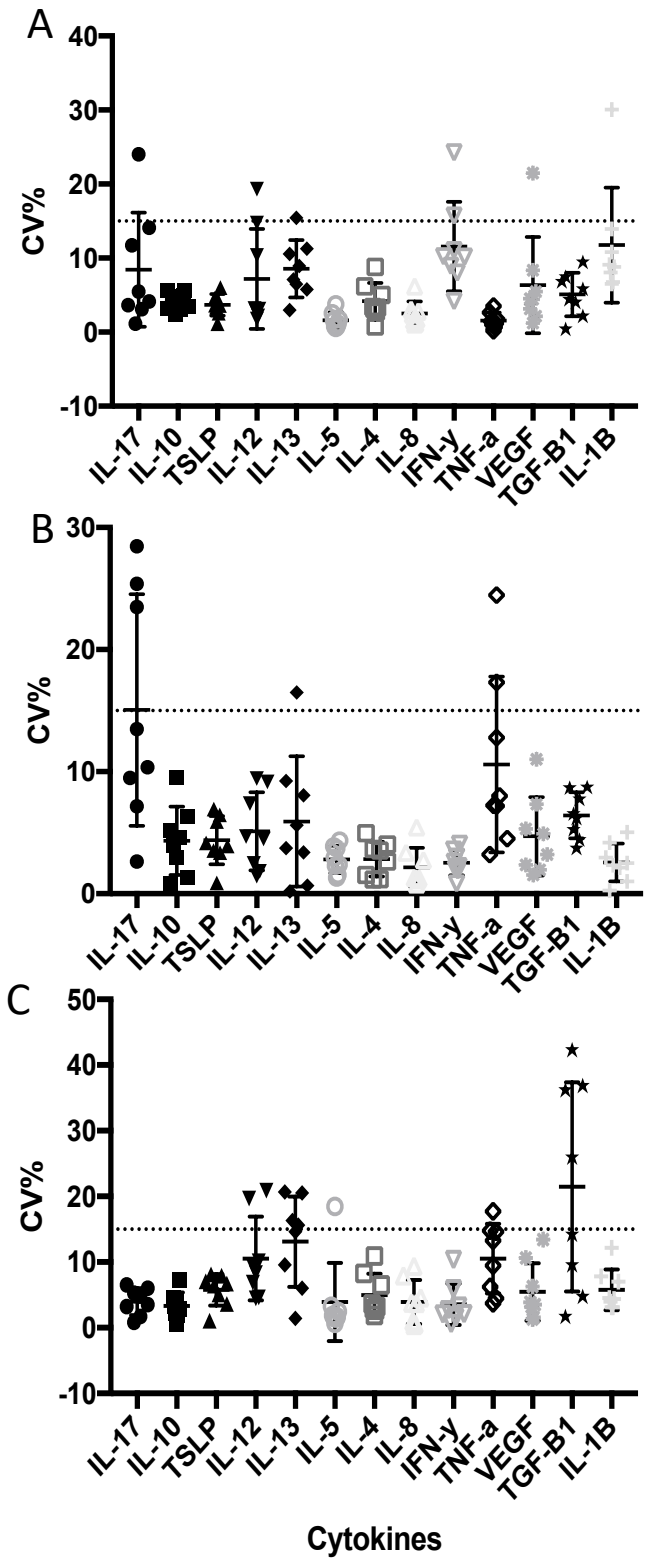


Figure 3.17 Intraassay coefficient of variation for 384 well assays

Assays were run in triplicate and were used to calculate the coefficient of variation. Each data point represents the mean of each concentration on the standard curve, the line represents the mean coefficient of variation and the error bars represent the standard deviation of the mean. On days 1 and 2 the mean for all assays was at or below the 15 % threshold for acceptable variation. On day 3 the mean for all assays except TGF-β1 (mean 21.46 %) were below the threshold. **A**. Day 1, **B**. Day 2, **C**. Day 3, CV %- coefficient of variation

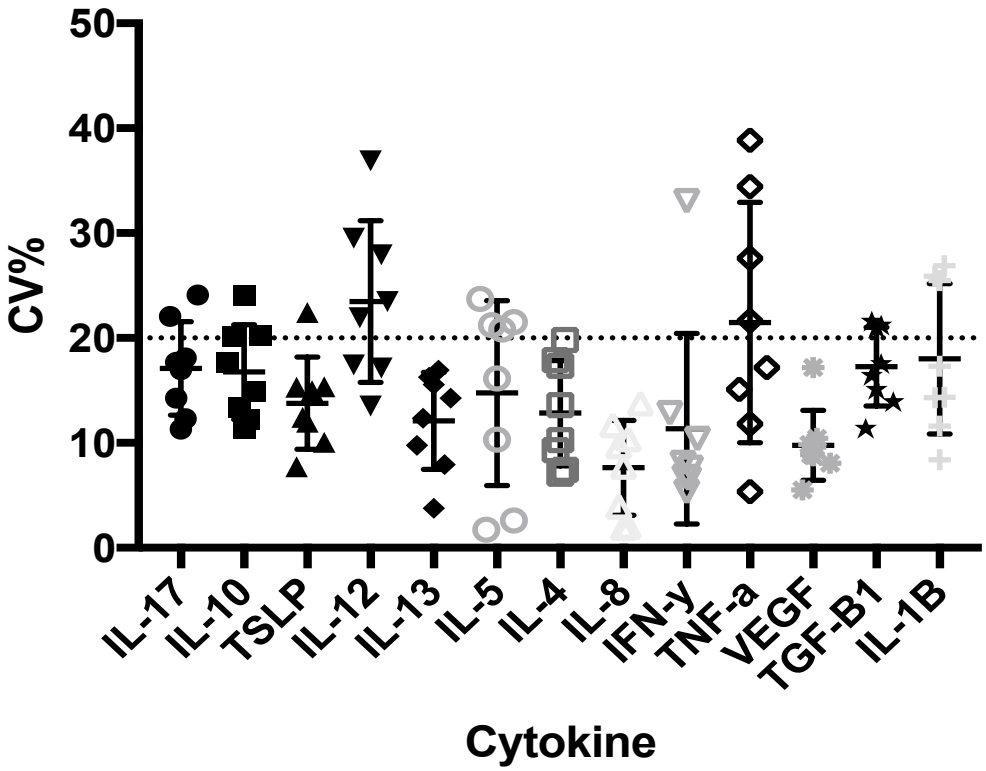


Figure 3.18 Interassay coefficient of variation for 384 well assays

Interassay variability was calculated from a total of nine data points. The mean coefficient of variation was below the acceptable threshold of ≤20 % with the exception of IL-12 (23.47 %) and TNF-α (21.63 %). CV %- Coefficient of variation

3.6 CONCLUSIONS: ELISA

The results of this work have shown that ELISAs can be miniaturized to be performed in 384 well plates with a high degree of reproducibility. Absorbance signals and reproducibility were considerably increased when; CBC buffer was used (absorbance increase from 0.6 to 0.7 OD), the plate was centrifuged (0.7 to 1.6 OD) and when BSA specific for the cytokine kits was used (1.6 to 2.9 OD).

3.7 CYTOKINE ANALYSIS

Serum samples from three clinical studies, presented individually in chapters four-six, were combined to analyze a total of 106 samples. 53 samples were from participants with mild asthma, 13 were moderate-to-severe and 40 were severe. There were no significant differences between disease severities in the concentrations of any of the 13 cytokines analyzed. TGF-β1 was the least detected cytokine regardless of disease severity. Detection levels were the lowest in the moderate-to-severe participants. (**Table 3.4**).

Table 3.4 Serum cytokine concentrations

Mean serum cytokine concentrations from three clinical studies of mild, moderate and severe asthma were analyzed via 384 well ELISA. Significance levels (p-values) were determined by ANOVA. SD: standard deviation

	Mild Asthma			Moderate-to-severe Asthma			Severe Asthma			
Cytokine	Detectable samples out of 53 (%)	Mean (pg/ mL)	SD	Detectable samples out of 13 (%)	Mean (pg/ mL)	SD	Detectable samples out of 40 (%)	Mean (pg/ mL)	SD	P-value
IL-4	13 (24.5)	3319.06	17015.98	1 (7.7)	28.02	101.04	18 (45)	1919.20	6083.01	**0.6522**
IL-5	12 (22.6)	536.15	2991.55	1 (7.7)	28.06	101.18	12 (30)	413.42	1625.11	**0.7745**
IL-13	39 (73.6)	30445.04	170710.81	3 (23.1)	4018.92	14125.50	32 (80)	11644.55	37427.97	**0.6444**
TSLP	38 (71.7)	33085.93	144107.37	8 (61.5)	1840.89	5898.79	35 (87.5)	13028.20	29432.31	**0.4608**
VEGF	19 (35.8)	1759.96	10534.68	4 (30.7)	18.59	30.96	29 (72.5)	692.84	4364.00	**0.6788**
IL-12	17 (32.1)	3486.42	16451.35	1 (7.7)	248.82	897.14	20 (50)	923.63	3275.46	**0.4433**
IFN-y	17 (32.1)	718.35	3251.17	1 (7.7)	214.09	771.92	19 (47.5)	1318.32	4703.73	**0.8344**
TNF-a	14 (26.4)	2530.34	15473.89	1 (7.7)	90.92	327.80	20 (50)	722.40	2637.97	**0.6145**
IL-1B	22 (41.5)	260.10	841.35	3 (23.1)	378.68	1360.05	24 (60)	726.52	2739.69	**0.4715**
IL-8	8 (15.1)	26.44	97.77	2 (15.4)	8.30	21.85	8 (20)	37.51	144.41	**0.7047**
IL-17	22 (41.5)	482.86	2086.48	1 (7.7)	51.89	187.10	16 (40)	307.48	996.33	**0.6435**
IL-10	16 (30.2)	1661.66	7370.26	1 (7.7)	279.46	1007.61	19 (47.5)	621.08	2291.84	**0.5176**
TGF-B1	6 (11.3)	15.654	52.33	0	N/A	N/A	3 (7.5)	18.66	104.74	**0.7404**

3.7.1 CYTOKINES AND BIOMARKERS

Fractional exhaled nitric oxide (FeNO) is a biomarker commonly used in asthma that reflects the extent of inflammation in the airways driven by IL-13. FeNO levels were compared with systemic (serum) IL-13 levels reported in the three clinical studies detailed in this report. No correlation was found between IL-13 and FeNO (Spearman's correlation; r=0.0063, **Figure 3.19**).

Blood eosinophils are a biomarker of T2 inflammation driven by IL-5 which are commonly used to identify participants who are likely to respond to corticosteroid and/or anti-IL-5 therapies. Blood eosinophil levels were compared with systemic (serum) IL-5 levels reported in the three clinical studies detailed in this report. No correlation was found between IL-5 and blood eosinophils (Spearman's correlation; r=0.0607, **Figure 3.20**).

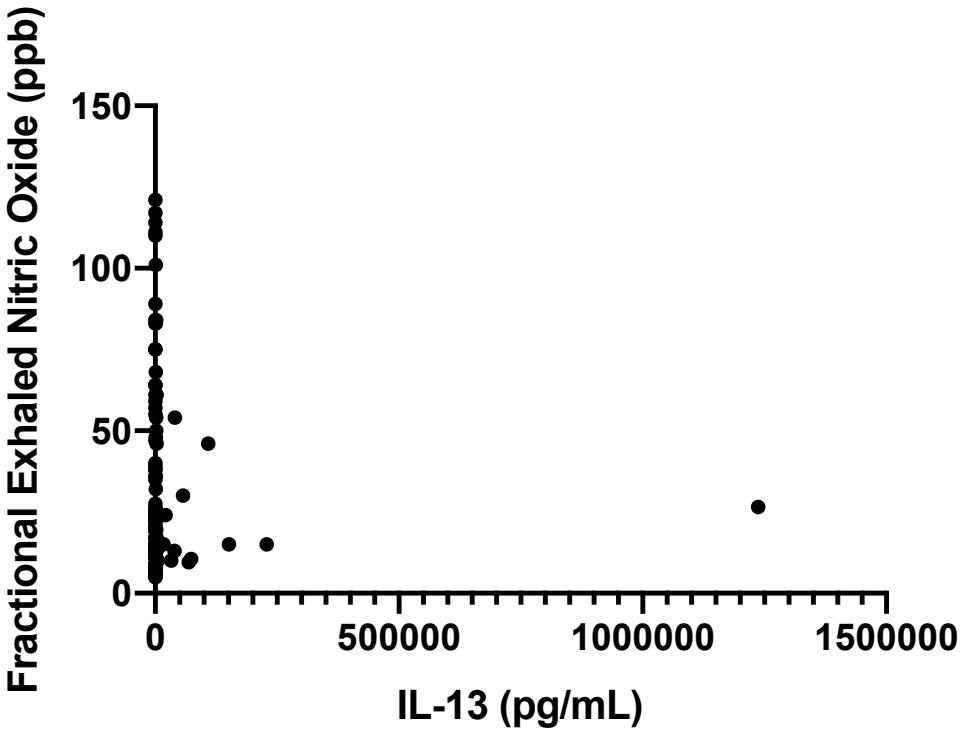


Figure 3.19 Fractional exhaled nitric oxide (FeNO) and serum IL-13

110 participants with mild to severe asthma had FeNO and serum IL-13 measured at baseline. No correlation was found (Spearman's correlation; r=0.0063)

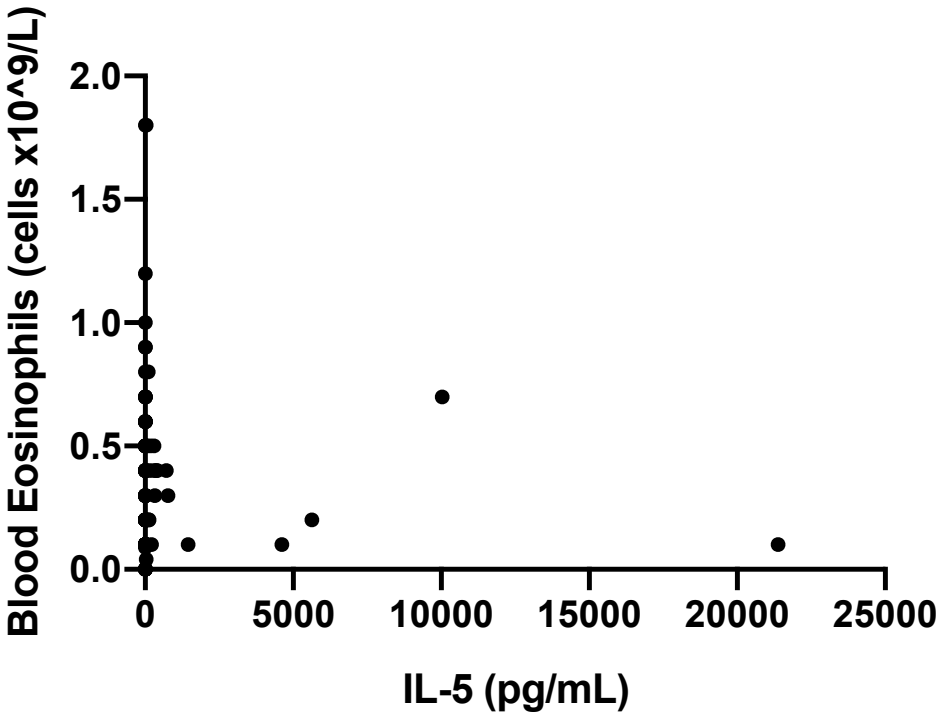


Figure 3.20 Blood eosinophils and IL-5

108 participants with mild to severe asthma had blood eosinophils and IL-5 measured at baseline. No correlation was found (Spearman's correlation; r= 0.0607).

3.8 DISCUSSION

Microarray technology can be used to quantify proteins in a multiplexed and high-throughput manner. However, due to complexities of this process, it requires considerable time to optimize and as such becomes a costly and time-consuming process. The ELISA has shown to be a reliable and reproducible method of protein quantification and when miniaturized to be performed in a 384 well plate required significantly fewer reagents which kept expenses to a minimum.

3.8.1 MICROARRAY

In the age of biomarkers, rapid and reliable quantification of protein levels has become highly desirable. This makes techniques, such as the antibody microarray, that enable analytes to be measured in a multiplexed and high-throughput manner highly desirable. However, currently there are no guidelines for the validation and optimization of this technique and there is a paucity of information in the literature. Consequently, the optimizations performed in this thesis were based on previous in-house work that was started nearly a decade ago. At that time numerous ELISA cytokine kits were used to trial cytokine quantification using microarray technology. The only kits that were able to adhere to the slide surfaces and reliably produce standard curves when used in a multiplexed manner were the cytokine duosets from R&D Systems. These same kits were used for the work performed here but with minimal success. The most likely explanation for this discrepancy is that in the nearly ten years that have passed

between the attempts the buffers that the cytokines and antibodies are preserved in has changed and is no longer compatible with the buffers used to facilitate binding to the slide surfaces. ELISA kits are optimized to bind to the sticky surface of ELISA plates, which are composed of high-density polystyrene treated with oxygen plasma. This surface is significantly different from a smooth chemically charged surface of a glass slide. Unfortunately, the buffer formulations are not disclosed by the manufacturer so there is no way to confirm that this is the case. For the purposes of this project, optimizations had to be stopped in order to have time to ensure the development of an alternative method and continuing optimizations would have placed significant financial restraints on the remainder of the project. Future work will continue to optimize this technique as being able to reliably measure numerous analytes in the same sample would save valuable time and resources and would require less sample from each patient. Future optimizations should include a buffer exchange to try alternatives that are more suited to binding glass slide surfaces. There are commercially available slides that have the analytes already affixed to the slide surface. For example, RayBiotech manufacture cytokine array kits that come with the capture antibody already bound slide and allow for processing of several hundred cytokines simultaneously (278). However, one kit containing enough materials to process eight samples costs over £4,000 which makes it largely unaffordable for most research groups. Additionally, several studies that have used a company produced microarray kit for cytokine analysis have also run their samples using

an ELISA to verify the results which adds to the overall cost and time of the project (279, 280).

In addition to the microarray, there are other multiplex techniques that can be used to quantify cytokine levels such as bead immunoassays. Bead-based assays use multiple populations of pre-stained microbeads each with its own fluorescent signature which are read by a flow cytometer. This technique allows for highly sensitive protein quantification with a minimum level of detection of approximately 1 pg/mL while only using approximately 25 µl of sample (281, 282).

3.8.2 ELISA

ELISAs are currently the gold standard method for protein quantification and the work performed here demonstrated that they are just as reliable when miniaturized to be performed in a 384 well plate. Miniaturizing the assay requires 20 % of the reagents and patient sample that would be required for the standard 96 well ELISA. Assays were found to be within the acceptable limits of variation as assessed by CV % both between and within assays. Wu and colleagues compared ELISA results of tumour antigens processed in 96 and 384 well plates. They also found that ELISAs had similar sensitivities and concentration ranges when performed in a either a 384 or 96 well plate. They also noted that some antigens showed increased sensitivity when performed in 384 well plates and suggested this may be due to an increased well-height to well-volume ratio (283).

In this work, a 20 % cutoff was used to assess the level of interassay variation which is in accordance with the Food and Drug Administration (FDA) regulations for analytical validation. Some of the cytokine standards measured by 384 well ELISA did fall slightly above this acceptable limit (TGF-β1 intrassay 21.46 % and IL-12 and TNF-α interassay 23.47 % and 21.63% respectively). The few data points that fell above the acceptable limit of variation were from concentrations at the lower end of the curve where a small difference between replicates equates to a larger difference overall. Samples that give an absorbance reading below the lowest value on the standard curve will be given a value of zero and samples that are at the very top of the curve will be diluted and analyzed again. Similarly, samples with more than 20 % discrepancy between the replicates will be reanalyzed. Therefore, sample values will be obtained from the middle section of the curve which were well under the 20 % limit for all cytokines. For this reason, the assays that did fall above the 20 % interassay limit (IL-12, TNF-α and TGF-β1) are unlikely to affect results and therefore these assays were deemed to be acceptable for the non-diagnostic, investigational work performed in this project. However, the variability does need to be recognized as a limitation.

Another limitation to measuring samples via ELISA as opposed to microarray is that readings are given in optical density absorbance which has a range of 0-3 OD whereas the microarray measures fluorescence up to 60,000 AU. Clearly, the range of the microarray offers a greater scope for the detection of protein levels. However,

samples above the top of the 3-point scale on an ELISA can be diluted until a reliable reading is obtained.

The optimizations performed here were successfully applied to analyze the concentration of 13 cytokines in serum from 106 patients with mild, moderate-to-severe and severe asthma. All 13 assays were able to detect serum cytokines levels with a high degree of specificity and sensitivity.

3.8.3 SUMMARY

In summary, the work performed here has resulted in a reliable method of cytokine quantification that is reproducible, relatively cost and time effective and easy to perform.

CHAPTER 4

DOES A LOW EXHALED NITRIC OXIDE LEVEL EXCLUDE A CLINICAL BENEFIT FROM INHALED CORTICOSTEROIDS IN SUSPECTED ASTHMA; A RANDOMISED, PLACEBO CONTROLLED TRIAL

4.1 BACKGROUND INFORMATION

Asthma is commonly over diagnosed in primary care (284, 285). This is partially due to the lack of a definitive diagnostic test and non-specific symptoms including cough, wheeze and shortness of breath. Also, asthma is variable meaning that during periods where asthma is well controlled lung function tests can often give normal results (286). Once a diagnosis of asthma has been made patients are frequently treated with an ICS. However, if the diagnosis is incorrect the ICS is unlikely to be of benefit, meaning the patient is taking a medication they do not require and therefore are at risk of developing unnecessary side effects including oral candidiasis or dysphonia. As such, a simple test that could be used to help guide clinicians in diagnosing asthma and prescribing treatments would improve patient care and help to reduce unnecessary prescription costs.

FeNO is a non-invasive biomarker that has been used to identify patients with T2 high airway inflammation who are likely to respond to an ICS (287). Nitric oxide synthase enzymes generate nitric oxide (NO) and exist in three forms: endothelial, inducible and neuronal (288). In the respiratory epithelium of patients with atopic asthma inducible nitric oxide is upregulated by Th2 cytokines IL-4 and IL-13 via STAT-6, causing increased concentrations of NO in exhaled breath (289). FeNO was traditionally thought to reflect eosinophilic inflammation however, eosinophils are recruited and activated via IL-5 which does not use STAT-6, but rather uses STAT-3, and therefore does not increase inducible nitric oxide synthase (290). Therefore, while eosinophilia does typically accompany T2 high inflammation,

FeNO does not directly reflect eosinophils but rather reflects atopy and IL-13 in the airways. This has been demonstrated by studies that have used monoclonal antibodies against IL-5 and IL-13. Lebrikizumab, a monoclonal against IL-13, has been shown to increase blood eosinophils and reduce FeNO (207) while mepolizumab, a monoclonal antibody against IL-5, reduces blood and sputum eosinophils but has no effect on FeNO (93).

As FeNO is a marker of T2 inflammation which generally responds to corticosteroids it has been used as a biomarker to identify corticosteroid responsive patients (47, 291, 292). A previous in-house study (n=74) investigating the utility of FeNO to diagnose asthma and predict response to an ICS revealed that patients with FeNO levels >33 ppb were highly likely to respond to an ICS (positive predictive value 92 %) whereas patients with levels <27 ppb were unlikely to respond to an ICS (negative predictive value 93 %) (284). This finding suggested that baseline nitric oxide levels could be used as a biomarker to identify a group of patients who may not benefit from ICS therapy. In the current study patients with a baseline level of ≤27 ppb were randomized to receive either an ICS or placebo to determine if a low FeNO at baseline could be used to identify patients with symptoms suggestive of asthma who would not benefit from initiating treatment with an ICS.

4.2 AIMS

The purpose of this in-house study was to determine if a FeNO ≤27 ppb at baseline could be used as a reliable biomarker to identify

patients with suspected asthma who would not benefit from starting treatment with an ICS in the short-term.

Our hypothesis was that treatment with budesonide and placebo would be equivalent over 12 weeks.

The aims were to answer the following questions:

Question 1. Do patients with a low FeNO respond to treatment with an ICS?

Question 2. Do the patients that do respond the treatment have any baseline features in common that could be used as predictors of response?

Question 3. Is there a cytokine pattern that can predict response to an ICS?

4.3 STUDY DESIGN

This was a randomized, double-blind, placebo-controlled, multicenter 12-week study in which patients were recruited from primary care practices across Nottinghamshire, Leicestershire and Derbyshire. The study was funded by the NHS National Institute for Health Research (NIHR), Research for Patient Benefit programme. The study inhalers were provided by AstraZeneca. The study was a Clinical Trial of an Investigational Medicinal Product (CTIMP), Clinical Trial Registration Number: NCT02771717.

4.4 ETHICAL APPROVAL

This study was sponsored by the University of Nottingham and received ethical approval from the East Midlands Leicester South Ethics Committee (REC), and Medicines and Healthcare products Regulatory Agency (MHRA). All participants completed a written consent form and the study was conducted in accordance with Good Clinical Practice (GCP) principles.

4.5 STUDY PARTICIPANTS

Adults with GP or nurse suspected asthma currently using only a SABA were invited to take part if they were either newly diagnosed or had intermittent symptoms requiring SABA treatment.

4.5.1 INCLUSION CRITERIA

The inclusion criteria for participants were:

1. Aged 18 years or over
2. Asthma suspected by a GP or practice nurse
3. Ability to give informed consent
4. FeNO $\leq$27 ppb
5. FEV_1 >70 % predicted pre-bronchodilator

4.5.2 EXCLUSION CRITERIA

The exclusion criteria for participants were:

1. Requiring treatment with oral corticosteroids on visiting the GP/ practice nurse
2. The use of oral prednisolone or antibiotics within the last four weeks
3. Currently using an ICS
4. The presence of any other clinically significant co-morbidities
5. Expectant or breastfeeding mothers

6. Hypersensitivity to budesonide and its excipients

4.6 STUDY SETTING AND PARTICIPANT RECRUITMENT

Subjects were recruited from 47 primary care practices throughout Nottinghamshire, Derbyshire and Lincolnshire as well as from the Nottingham Respiratory Research database and from poster advertisements placed in primary care practices, the University of Nottingham and Nottingham University Hospitals. The study nurses conducted spirometry clinics in primary care practices to identify potential patients. They also screened each practices' patient registry for patients with a suspected diagnosis of asthma who had only been prescribed a SABA. These patients were then sent an invitation letter and participant information sheet (PIS). Participants were recruited from May 2016 to March 2018. During this time 821 patients were seen in the spirometry clinics; 236 patients were screened for entry into the study. All potential participants were provided with a PIS. Study visits were conducted at either the patient's primary care practice or at the Respiratory Research Unit (NRRU) in Nottingham City Hospital (NCH).

4.7 STUDY INTERVENTION

Participants were randomly assigned to receive either 200 mcg Budesonide (Pulmicort) via a Turbuhaler or placebo one puff twice daily. The total daily dose of budesonide was therefore 400 mcg, which is the recommended initial treatment for mild asthma.

4.8 OUTCOME MEASURES

4.8.1 PRIMARY OUTCOME

The primary outcome was the difference in ACQ7 scores between the budesonide and placebo groups.

4.8.2 SECONDARY OUTCOME

The secondary outcomes were the difference in FEV_1, MRC and LCQ scores between the budesonide and placebo groups.

4.9 POWER CALCULATION

It was determined that 150 patients would be required to give 90 % power to demonstrate equivalence based on a difference in ACQ7 of ≥0.5 being clinically important and a standard deviation of 0.9. The initial goal was to recruit 165 patients to allow for drop-outs. This recruitment figure was increased to 180 in February 2018 due to more drop-outs than expected.

4.10 ANALYSIS

All analysis was conducted prior to unblinding. The statistically significant threshold for all tests was $p<0.05$ with 95% confidence intervals (CI).

The primary outcome was to determine if the mean difference in ACQ7 scores between the budesonide and placebo groups were equivalent. This was assessed using a two one-sided equivalence test (TOST). Equivalence tests are used when the research or alternative hypothesis (H_A) is that there is no difference between two groups and the null hypothesis (H_0) is that there is a difference between the

groups. This is in contrast to traditional hypothesis testing where the H_A is that there is a difference between the groups and the H_o is that the groups are the same. In this case, a t-test could be used.

$$H_o: \mu A - \mu B = 0$$

$$H_A: \mu A - \mu B \neq 0$$

Equation 4.1 Traditional hypothesis test

A traditional hypothesis test (t-test) assumes (H_o) that the mean (μ) of one group (A) is not statistically different from the mean of the other group (B). The research hypothesis (H_A) is that there is a difference between the means of the two groups.

In this case, the burden of proof rests on H_A to show that there is a difference between the groups. If the H_o cannot be rejected it is because there is insufficient evidence to claim that the means are different. However, this also does not provide enough evidence to claim that the means are equivalent. In order to claim equivalence, the H_o must assume that there is a difference between the groups placing the burden of proof on the H_A to show that the means are equivalent.

A TOST test is essentially two one-sided t-tests with the important difference that the maximal level of clinically acceptable difference (δ), also known as the equivalence margin, must be prespecified. A TOST has two H_0's, one for each t-test:

$$H_o\,1: \mu A - \mu B < -\delta$$

$$H_0\,2: \mu A - \mu B > \delta$$

Equation 4.2 Equivalence test

An equivalence test assumes that there is a difference between the means of two groups and the research hypothesis (H_A) is that there is no difference between the means of the two groups. There are two null hypotheses (H_0), one assumes that the difference between the two means is less than the negative clinically acceptable difference (δ) and the other assumes that the difference between the two means is greater than positive clinically acceptable difference.

A p-value is generated from each t-test and statistical equivalence can be claimed if both H_o's are rejected ($p<0.05$). As both H_o's in a TOST have a type I error rate (α) of 5 %, a confidence interval (CI: (1-2α) x 100 %) of 90 % falling within the equivalence margin ($-\delta,\delta$) demonstrates equivalency with 95 % confidence as the type I error can only be on one side (293-295) (see **Figure 4.1**). The TOST conducted to determine ACQ7 equivalence was run with a δ of 0.5, the widely validated minimal clinically significant difference in ACQ scores (260), and α of 0.05 (5%). TOSTs were also conducted for the secondary outcomes LCQ and FEV_1. The TOST for LCQ was run with a δ of 1.3, (262) and the TOST for FEV_1 was run with a δ of 0.2 L (284). The TOSTs were conducted in STATA/SE 15.0 and Minitab was used to generate the equivalence graphs.

The secondary outcomes were assessed using chi-squared (χ^2) tests (STATA/SE 15.0) to evaluate the number of responders in the budesonide and placebo groups in terms of LCQ, MRC and FEV_1. Responders were defined by an increase of ≥ 1.3 in LCQ scores, an increase of ≥ 200 mL and/or 12 % in FEV_1, and for the MRC where there is no validated minimally important clinical difference, therefore, a decrease in score was considered to be a positive

response to treatment. χ^2 tests were also used to evaluate the number of responders and non-responders in the active and placebo groups for baseline FeNO levels divided into subgroups.

Logistical regression (STATA/SE 15.0) was performed to evaluate baseline features that may predict response to treatment.

Eight-point standard curves were produced to interpolate individual cytokine levels. A cubic spline fit using 1000 points was used to find the best fit for each standard curve. This analysis was performed using GraphPad Prism 7. Heat maps were created in Multi Experiment Viewer (MeV_4_8) to visualize the results.

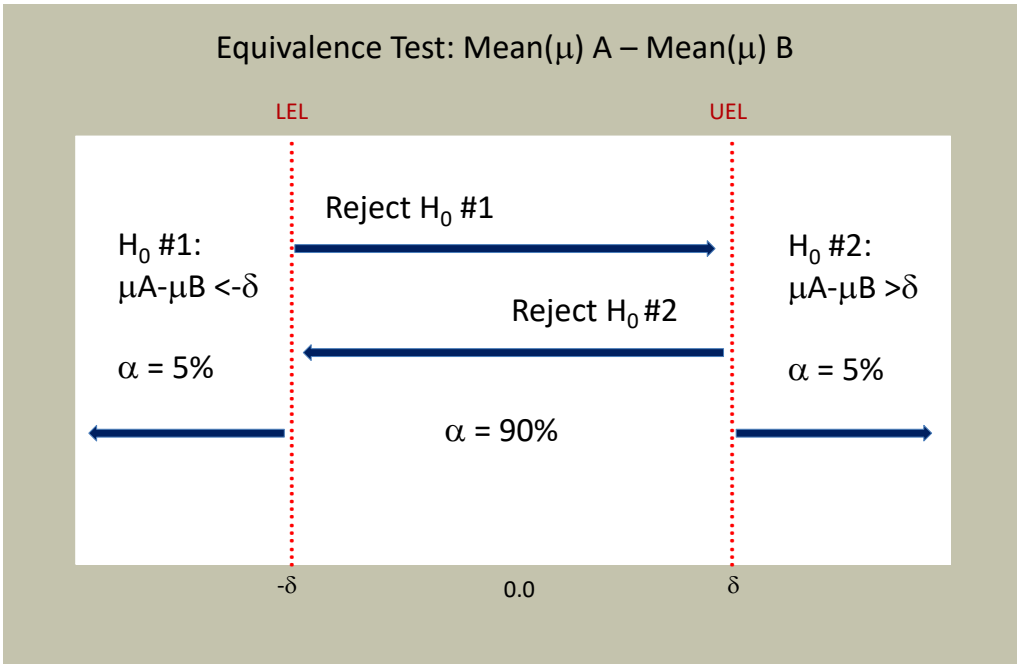


Figure 4.1 Equivalence test

The two, one-sided equivalence test (TOST) is a test composed of two one-sided t-tests, each with its own null hypotheses (H_0). The first hypothesis is that the mean (μ) of the first group (A) minus the mean of the second group (B) is less than the negative value of the maximal level of the clinically acceptable difference. The second is that the mean of group A minus the mean of group B is greater than the positive value of the maximal level of clinically acceptable difference. Both tests are conducted with a type I error rate (α) of 5 %. If both are rejected the difference between the means falls within the equivalence margin and equivalence is demonstrated with 90 % confidence intervals and demonstrates equivalency with 95 % confidence as the type I error can only be on one side.

4.11 RANDOMIZATION SEQUENCE GENERATION, ALLOCATION AND BLINDING

Patients were randomized to the budesonide or placebo group in blocks of eight with stratification according to their smoking status; current (or smoked within 12 months) or never/ex (never or quit more than one year ago). Allocation sequences were generated by the Respiratory Research Unit's database manager using a pseudo-random number generator (sealedenvelop.com). This sequence was linked to an electronic case report form (eCRF) where the participants' study data was recorded. If the participant was successfully entered into the study a coded prescription was generated for the Clinical Trials Pharmacy at NCH to dispense the correct study inhaler.

4.12 PROTOCOL

During the first visit, participants were given a PIS and were able to ask any questions they had regarding their participation in the study. All participants that were happy to take part signed a consent form and were screened to ensure they met the inclusion and exclusion criteria detailed in **Section 4.5**. As part of the baseline visit, their medical history was recorded and an average of two FeNO readings was obtained. Participants who satisfied the criteria and had an average FeNO of $\leq$27 ppb were offered a place in the study. Successful participants completed baseline spirometry with reversibility, venepuncture and ACQ7, LCQ and MRC questionnaires. Spirometry was performed using either CareFusion or Vitalograph spirometers.

During subsequent visits, each participant performed spirometry using the same spirometer that was used at baseline. At the end of the baseline visit participants were given inhaler training to ensure proper technique. Following the completion of their baseline visit participants were randomized to receive either budesonide 200 mcgs or placebo via a Turbuhaler one puff twice daily for 12 weeks.

Following randomization into the study participants returned to either their GP practice or the NRRU for follow up visits at four, eight and 12 weeks. During these visits, spirometry, FeNO and all questionnaires were repeated. Patients were asked about their medication adherence at each follow-up visit.

Once the participants completed the study, they were unblinded by the study nurse so that instructions on how to continue treatment could be sent to their GP. All other study researchers remained blinded until after the results had been analyzed.

An overview of the information collected during the study is presented in **Table 4.1**.

Table 4.1 Schedule of events for study visits

FeNO: Fractional exhaled nitric oxide, ACQ: Asthma Control Questionnaire, LCQ: Leicester Cough Questionnaire, MRC: Medical Research Council dyspnea sore, AE/SAE: Adverse events/ Serious adverse events

Procedures	**Visit 1 (randomization)**	**Visit 2**	**Visit 3**	**Visit 4**	**Unscheduled visit**
Time	0 weeks	4 weeks	8 weeks	12 weeks	As needed
Consent	X				
Medical History	X				
Inclusion criteria check	X				
FeNO	X	X	X	X	X
Spirometry	X	X	X	X	X
ACQ	X	X	X	X	X
LCQ	X	X	X	X	X
MRC	X	X	X	X	X
Venepuncture	X				
Inhaler technique training	X				
AE/SAE's	X	X	X	X	X
Adherence check		X	X	X	X
Dispense prescription	X				
Collect study inhaler				X	

4.13 RESULTS

A total of 4700 patients were contacted. Of these, 236 were screened and 180 were entered into the study and were randomized to receive either budesonide or placebo. During the study, 46 participants failed to complete all visits, 23 from both the active and placebo groups. In total 134 patients completed all study visits and were included in the analysis (See **Figure 4.2**).

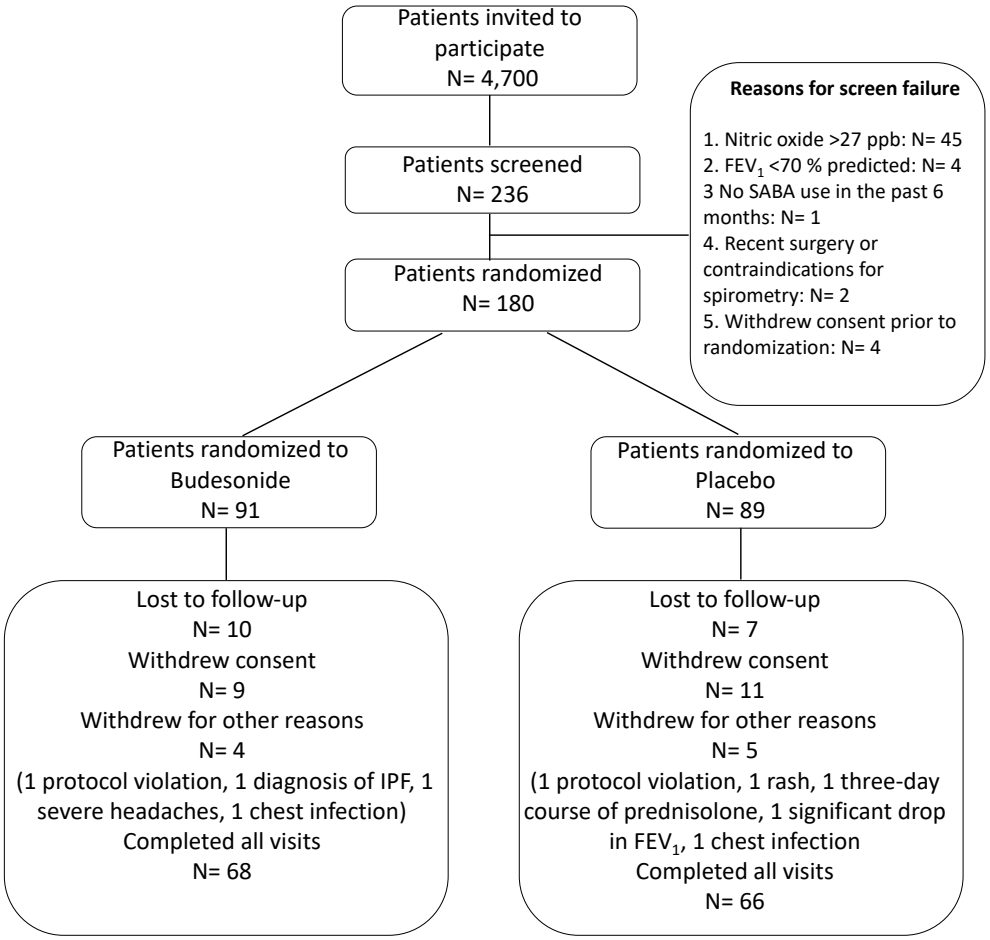


Figure 4.2 Consort diagram

Approximately 4700 patients were invited to take part in the study. Of those 236 were screened and 180 were randomized, 91 to the budesonide group and 89 to the placebo group. A total of 46 patients who did not complete all study visits, 23 from each group.

4.13.1 BASELINE DEMOGRAPHICS

The baseline demographics and clinical characteristics for all 180 patients who were randomized and completed visit 1 are presented below (**Table 4.2**). Patients were well matched between the groups at baseline. The majority of patients were female, 70 % and 74 %, non-smokers 78 % and 80 %, and Caucasian 88 % and 88 % in the budesonide and placebo groups respectively. There was considerable variability in the length of time from when asthma was diagnosed to entering the study (visit 1). However, this variability was similar in both groups (**Table 4.3**). All measures of lung function, inflammation, presenting symptoms and questionnaires were similar between groups.

Table 4.2 Baseline demographics

Results are given in mean and standard deviation where the data was normally distributed. In cases where the data was not normally distributed the median and interquartile range are given. BMI: body mass index, SOB: shortness of breath, ACQ: Juniper asthma control questionnaire, LCQ: Leicester cough questionnaire, MRC: Medical research council, FEV_1: forced expiratory volume over one second, FVC: Forced vital capacity, FeNO: Fractional exhaled nitric oxide, ppb: parts per billion

Variable	Budesonide n=91	Placebo n=89
Age	42.49 (18.76)	45.38 (18.31)
BMI	28.19 (23.53-33.81)	26.67 (23.27- 31.62)
Duration of asthma (months)	1.30 (0.20-84)	2.00 (0.20-89)
Sex- Female (%)	64.00 (70%)	66.00 (74%)
Ethnicity (%)		
Caucasian	80 (88%)	78 (88%)
Asian	2 (2%)	3 (3%)
Black	2 (2%)	5 (6%)
Other	7 (8%)	3 (3%)
Smoking Status		
Never	71.00 (78%)	72.00(80%)
Current	20.00 (21%)	17.00 (20%)
Presenting Symptoms		
Cough	74.00 (83%)	69.00 (80%)
Wheeze	58.00 (65%)	62.00 (69%)
SOB	65.00 (73%)	69.00 (80%)
Questionnaires		
ACQ6	1.50 (0.83-2.33)	1.16 (0.75-1.83)
ACQ7	1.43 (0.85-2.14)	1.42 (0.78-1.92)
LCQ	17.00 (12.12-20.10)	17.64 (13.90-19.99)
MRC	2.00 (2.00-2.00)	2.00 (1.00-2.00)
Lung Function		
FEV_1 (L)	2.76 (2.22-3.33)	2.53 (2.10-3.05)
FEV_1 % predicted	92.65 (83.43-98.32)	88.28 (78.64-103.86)
FVC (L)	3.65 (2.96-4.32)	3.33 (2.94-3.94)
FEV_1/FVC	79.00 (74-86)	78.00 (71-82)
FeNO (ppb)	16.06 (6.21)	16.65 (6.71)
Blood Eosinophils (cells x10^9/L)	0.16 (0.10-0.24)	0.20 (0.10-0.30)

Table 4.3 Length of time from asthma diagnosis to visit 1

The number of patients in each group and the length of time they had been diagnosed with asthma prior to entering the study.

Time from asthma diagnosis to visit 1	Budesonide	Placebo
0-30 days	45	39
31-180 days	5	7
181-365 days	3	1
366- 1825 days (5 years)	13	13
>5 years	25	29

4.13.2 PRIMARY OUTCOME

4.13.2.1 ACQ7

A total of 53 participants, 29 (42 %) in the budesonide and 24 (36 %) in the placebo group had an improvement in ACQ7 of ≥0.5 following 12 weeks of treatment (p=0.4). The budesonide group demonstrated a greater decrease in ACQ7 score than the placebo group (0.53 vs 0.29). The between group mean difference in ACQ7 was 0.25 and the 90 % CI around this difference was -0.004 to 0.49 which fell within the equivalence interval of -0.5 to 0.5, confirming equivalence (p=<0.0001, p=0.0462) (**Figure 4.3** and **4.4**).

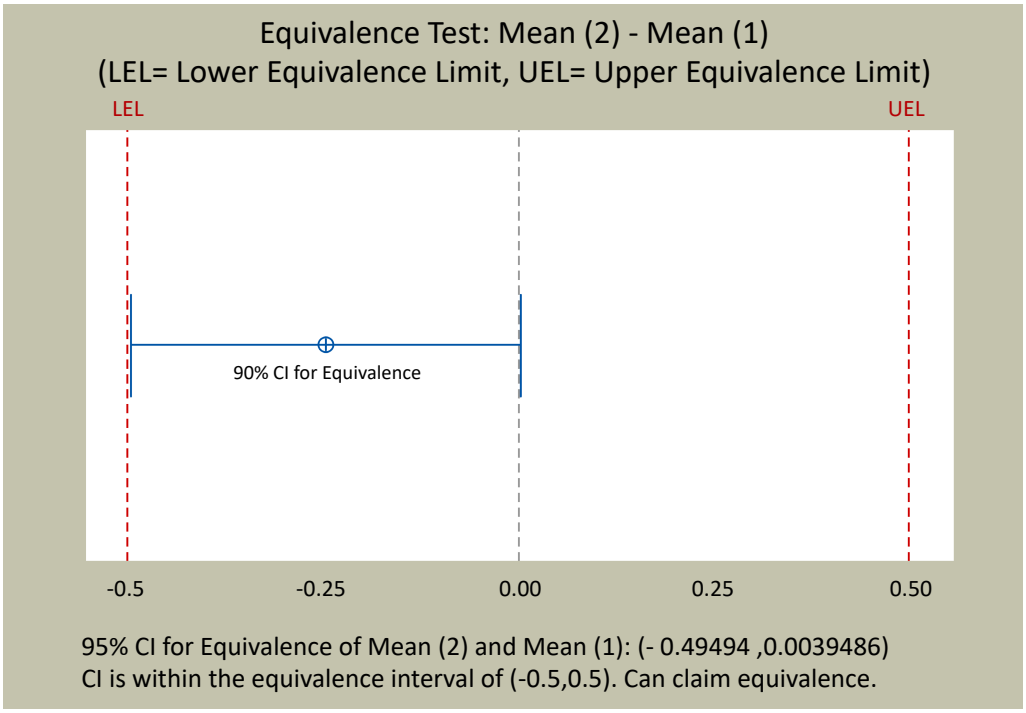


Figure 4.3 Two, one-sided equivalence test (TOST) ACQ7

The 90 % confidence interval (-0.004 and 0.49) for equivalence of the means was within the equivalence interval (-0.5 to 0.5) which confirmed that the difference in ACQ7 scores was equivalent between the budesonide and placebo groups.

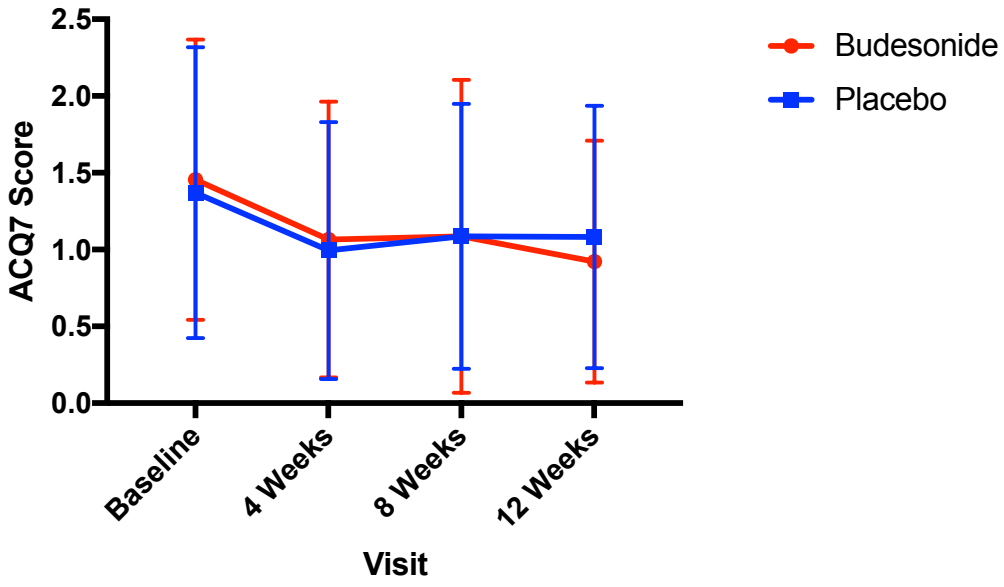


Figure 4.4 Asthma Control Questionnaire (ACQ7) Mean ACQ7 scores of the budesonide and placebo groups are sown at each visit. Decreasing scores represent an improvement in control. Error bars represent the standard deviation.

4.13.3 SECONDARY OUTCOMES

The number of responders at each visit according to each secondary outcome is summarized in **Table 4.4**.

4.13.3.1 LCQ

Upon completion of the study, a total of 57 participants 27 (40 %) and 30 (45 %) in the budesonide and placebo groups respectively had a significant improvement in LCQ of ≥1.3 (p=0.501). There was a greater increase in mean LCQ scores in the placebo group than the budesonide group (1.97 vs 1.85). The between group mean difference in LCQ was 0.12 and the 90 % CI around this difference was (-1.06 and 1.31) which fell just outside the equivalence interval of -1.3 to 1.3, therefore equivalence could not be claimed (p=0.0235, p=0.0501) (**Figure 4.5**). In general, there was a slight increase in LCQ score from baseline to the final visit (12 weeks); the mean increased from 16.60 to 18.45 in the budesonide group and 15.87 to 17.84 in the placebo group (See **Figure 4.6**).

4.13.3.2 MRC

Following 12 weeks of treatment 41 participants, 22 (32 %) in the budesonide and 19 (30 %) in the placebo group had an improvement in MRC score (p=0.654) (**Table 4.4**). MRC scores were similar between groups. Equivalence could not be determined as there is no accepted minimal level of clinical significance. There was a downward trend in both groups over the course of the study with a slightly greater decrease in the mean values in the budesonide group than the placebo group (0.29 vs 0.23). (**Figure 4.7**).

4.13.3.3 FEV_1

At the end of the study, 13 (10 %) participants had a significant improvement in FEV_1 of ≥200 mL, four of them also had a ≥12 % increase in FEV_1 % predicted. Of the 13 patients, nine (13 %) were in the budesonide group and four (6 %) were in the placebo group (p=0.161) (**Table 4.4**). Mean FEV_1 values were largely unchanged throughout the study. The between group mean difference in FEV_1 was 0.01 and the 90 % CI around this difference was -0.06, 0.06) which fell within the equivalence interval of -0.2 to 0.2, confirming equivalence (p=<0.0001, p=<0.0001) (**Figure 4.8**). Values were slightly higher in the budesonide group, mean 2.90 L at baseline and 2.92 at 12 weeks than in the placebo group, 2.69 L at baseline and 12 weeks (**Figure 4.9**).

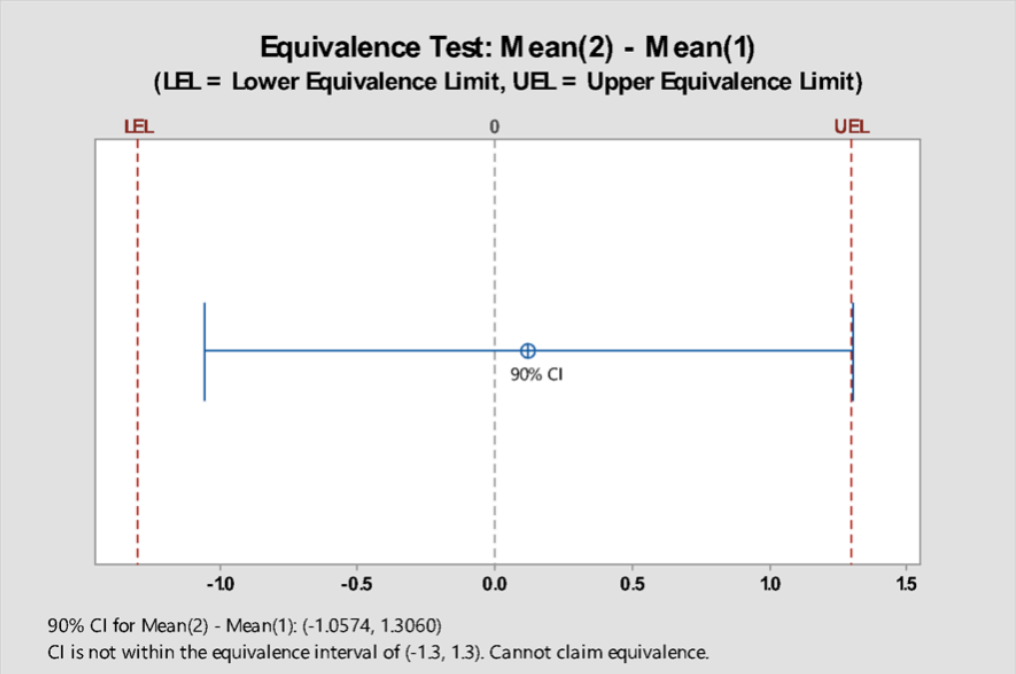


Figure 4.5 Two, one-sided equivalence test (TOST) LCQ

The 90 % confidence interval (-1.06 and 1.31) for equivalence of the means was just outside the equivalence interval (-1.3 to 1.3) which failed to confirm that the difference in LCQ scores was equivalent between the budesonide and placebo groups.

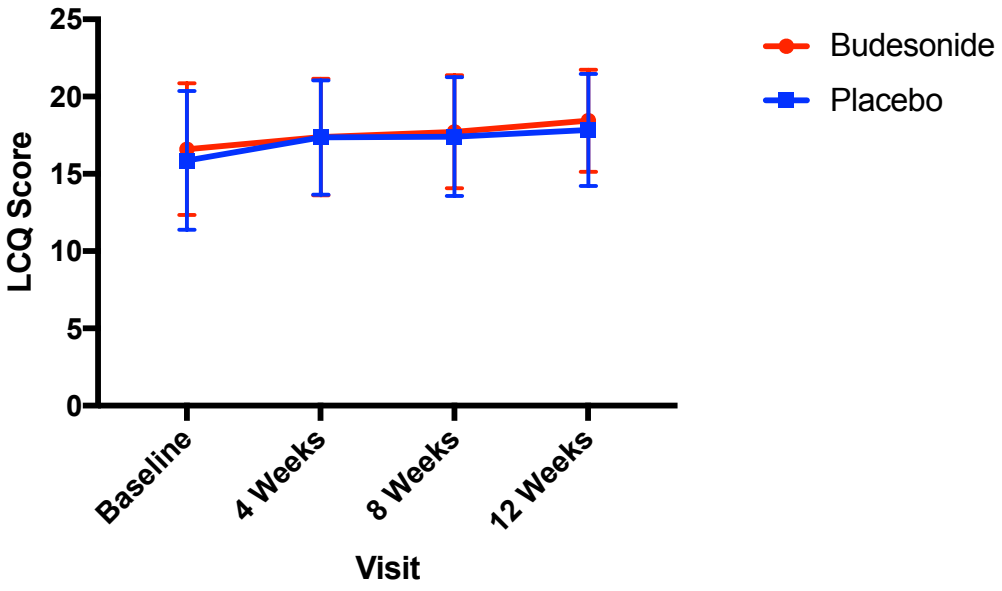


Figure 4.6 Leicester cough questionnaire (LCQ)

Mean LCQ scores in the budesonide and placebo groups are shown at each visit. Increasing LCQ scored represent an improvement in cough. Error bars represent standard deviation.

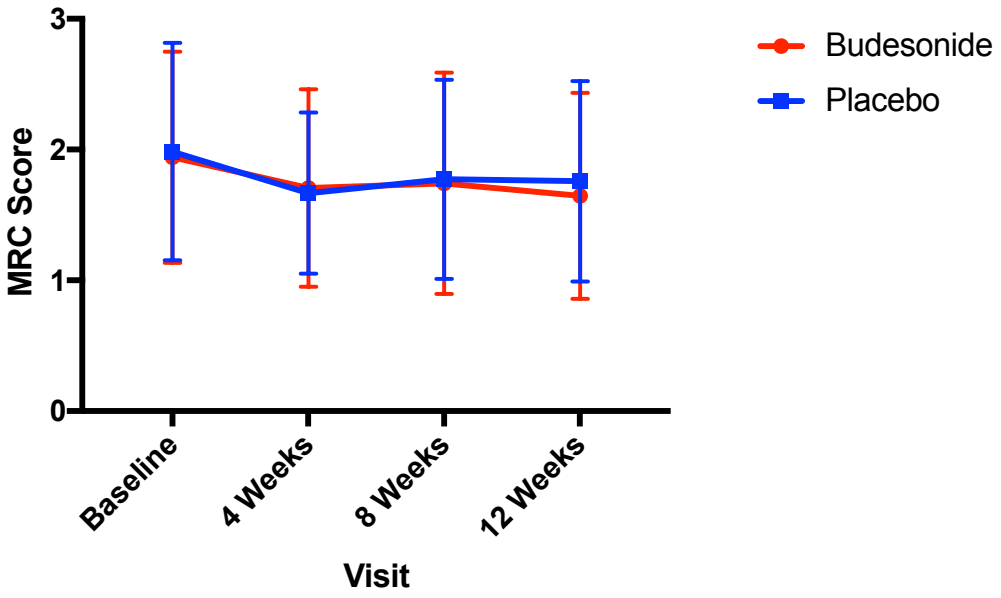


Figure 4.7 Medical research council dyspnea scale (MRC)

Mean MRC scores in the budesonide and placebo groups are shown at each visit. Error bars represent standard deviation.

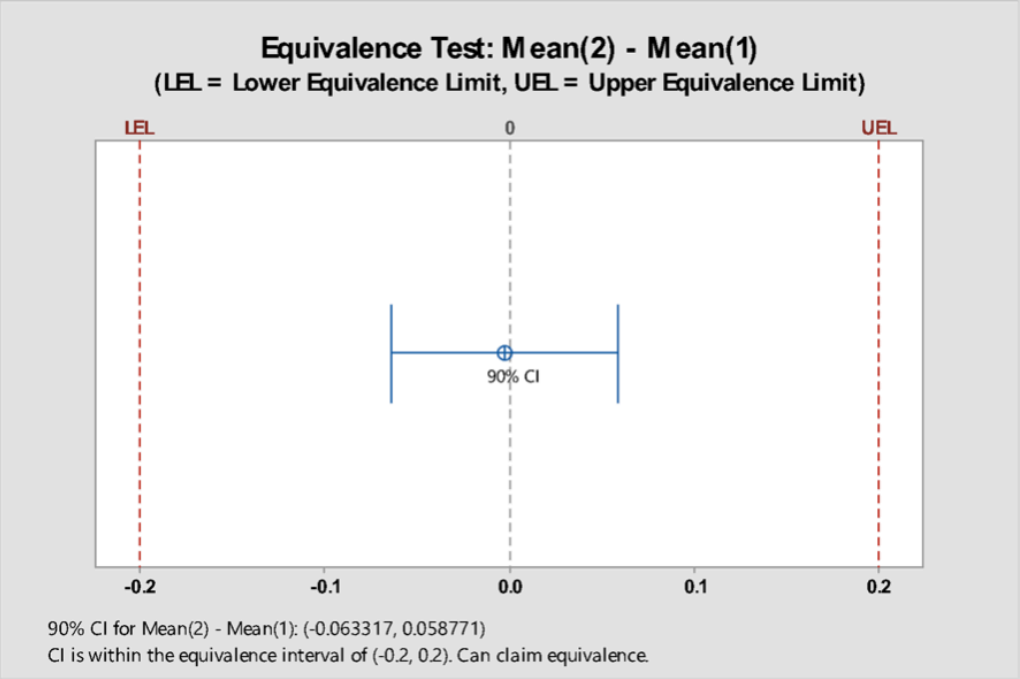


Figure 4.8 Two, one-sided equivalence test (TOST) FEV_1

The 90 % confidence interval (-0.06, 0.06) for equivalence of the means was within the equivalence interval (-0.2 to 0.2) which confirmed that the difference in FEV_1 was equivalent between the budesonide and placebo groups.

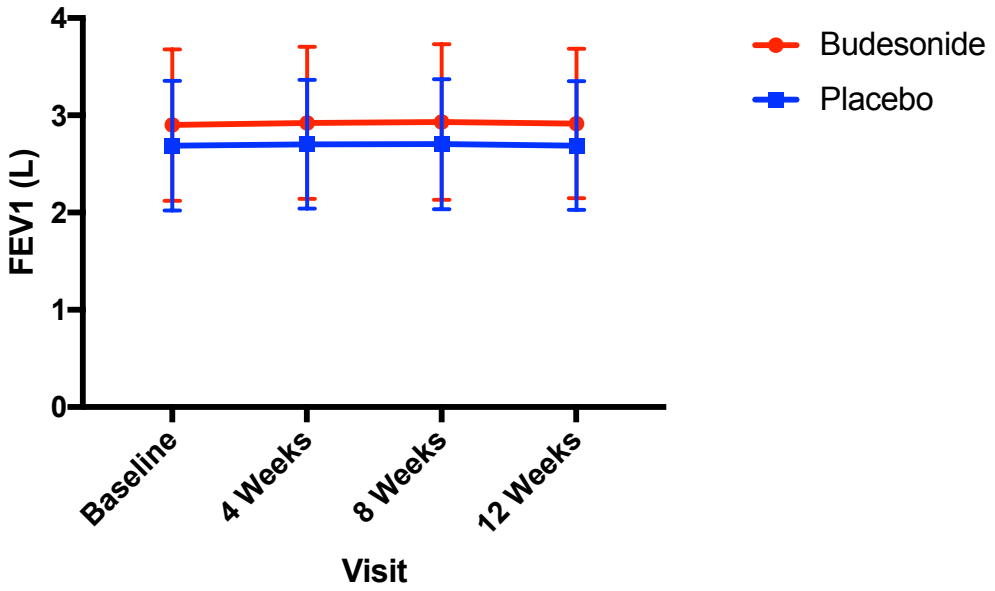


Figure 4.9 Forced expiratory volume over one second (FEV_1)

Mean FEV_1 scores in the budesonide and placebo groups are shown at each visit. Error bars represent standard deviation.

Table 4.4 Secondary outcome treatment responders

The number of LCQ, MRC and FEV_1 responders in the budesonide and placebo groups are given as well as the χ^2 p-values for each study visit. LCQ: Leicester Cough Questionnaire, MRC: Medical research council dyspnea scale, FEV_1: Forced expiratory volume over one second

	LCQ Score (≥1.3)			MRC Score (decrease)			FEV_1 (≥200 mL or ≥12 %)		
Visit	**Budesonide**	**Placebo**	**p-value**	**Budesonide**	**Placebo**	**p-value**	**Budesonide**	**Placebo**	**p-value**
4 Weeks	28	27	0.975	18	21	0.496	6	8	0.533
8 Weeks	25	29	0.397	21	20	0.942	6	8	0.533
12 Weeks	27	30	0.501	22	19	0.654	9	4	0.161

4.13.4 FRACTIONAL EXHALED NITRIC OXIDE LEVELS

4.13.4.1 Baseline FeNO levels and response to treatment

Baseline FeNO levels were divided into tertiles (low; ≤5-12 ppb, medium; 13-20 ppb and high; 21-27ppb) for the active and placebo groups to determine if there was a relationship between baseline FeNO levels and response to treatment (ACQ7). The number of responders in the low and medium tertiles were not statistically different between the active and placebo groups. In the budesonide group, seven out of 29 (24.1%) total responders vs nine of 24 (37.5%) total responders in the placebo group (χ^2 p=0.1509) were in the low tertile and eight (27.6%) in the budesonide group vs 11 (45.8%) in the placebo group (χ^2 p=0.8149) were in the medium tertile. A significant difference was noted in the number of responders in the high FeNO tertile between the active and placebo groups (14 vs four; χ^2 p=0.0043). These results are represented in **Figure 4.10**.

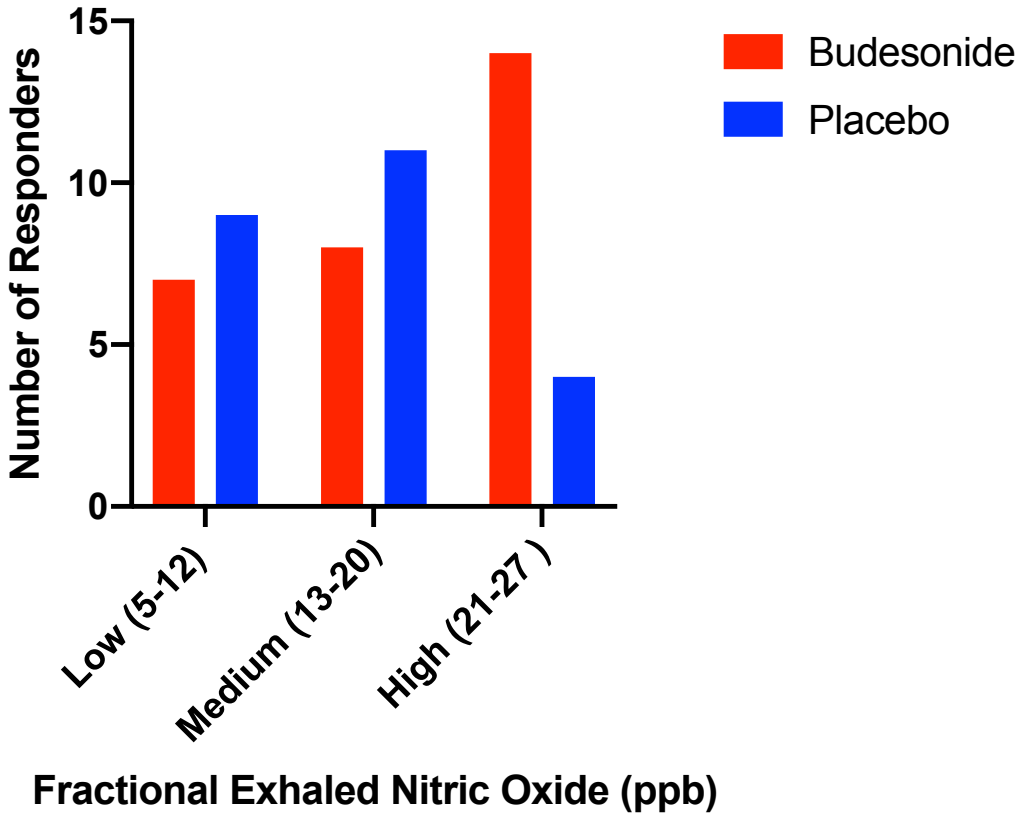


Figure 4.10 Baseline fractional exhaled nitric oxide (FeNO) divided into subgroups

Baseline FeNO levels were divided into low, medium and high tertiles. Of the 29 responders in the budesonide group seven were in the low FeNO tertile compared with nine of 24 responders in the placebo group (χ^2 p=0.1509). In the medium tertile there were eight responders from the budesonide group and 11 from the placebo group (χ^2 p=0.8149) and in the high tertile there were 14 responders from the budesonide group compared with four in the placebo group (χ^2 p=0.0043).

4.13.4.2 Changes in FeNO levels

FeNO levels in the budesonide and placebo groups increased throughout the study. After four weeks nine (13%) participants in the active and four (6%) in the placebo group had FeNO levels above 27 ppb. This increased to 10 participants in each group (15% active and placebo) by the final visit. At baseline the median (SD) was 17 ppb (6.39) and 15 ppb (6.00) in the budesonide and placebo groups respectively. At the end of the study, the median (SD) and maximal values were 16.75 ppb (9.90) and 66.5 ppb in the budesonide group

and 17.5 ppb (11.27) and 73 ppb in the placebo group. The highest recorded value was 74 ppb in the placebo group at the eight-week visit.

These results presented graphically in **Figure 4.10** and summarized in **Table 4.5**. Of the 10 participants who had FeNO levels >33 after 12 weeks (4 budesonide, 6 placebo) only one participant in the budesonide group and two in the placebo group responded to treatment in terms of ACQ7 and none had an improvement in FEV_1 of either ≥200 mL or ≥12 %.

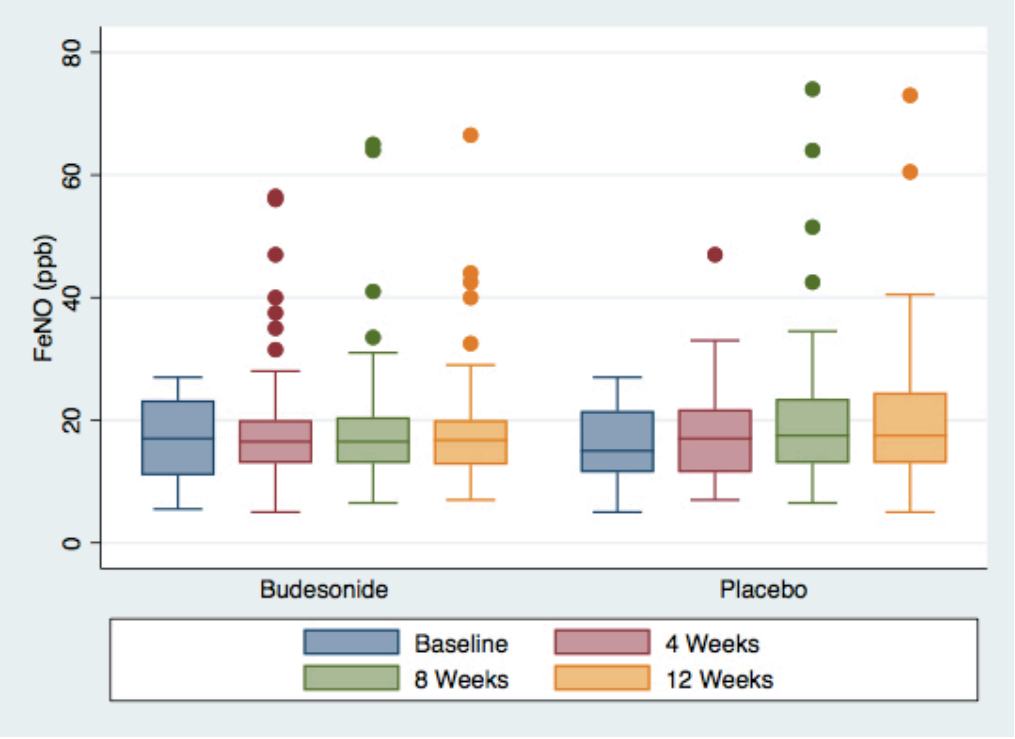


Figure 4.11 Fractional exhaled nitric oxide (FeNO) levels

FeNO (median and IQR) levels are shown for the budesonide and placebo groups at each study visit.

Table 4.5 Fractional exhaled nitric oxide (FeNO) levels

Median FeNO levels with standard deviation (SD) are given for each study visit as well as the minimum and maximum reading for each group. Values are given in parts per billion (ppb).

	Budesonide				Placebo			
Visit	**Observations**	**Median (SD)**	**Minimum**	**Maximum**	**Observations**	**Median (SD)**	**Minimum**	**Maximum**
Baseline	68	17.00 (6.39)	5.5	27	66	15.00 (6.00)	5	27
4 Weeks	68	16.50 (10.30)	5	56.5	64	17.00 (7.31)	7	47
8 Weeks	66	16.50 (10.54)	6.5	65	65	17.50 (12.23)	6.5	74
12 Weeks	68	16.75 (9.90)	7	66.5	66	17.50 (11.27)	5	73

4.13.5 BASELINE FACTORS THAT PREDICT RESPONSE TO TREATMENT

When baseline features were considered independently, age (odds ratio (OR) 0.97; 95% CI, 0.95 to 0.99; p=0.006) LCQ (0.87; 95% CI, 0.81 to 0.96; p=0.002), ACQ7 (3.74; 95% CI, 2.21 to 6.36; p=<0.001) and ACQ6 (3.75; 95% CI, 2.24 to 6.25; p<0.001) scores were found to be significant predictors of response of treatment (**Table 4.6**). ACQ6 was analyzed in addition to the ACQ7 in order to evaluate subjective responses only. Blood eosinophils were borderline significant (OR: 17.45; 95% CI, 0.92 to 331.92; p=0.057). These individual factors were then used to generate a logistical regression model. Although blood eosinophils bordered on significance this was excluded from the model as including it would have reduced the number of observations from 134 down to 92. ACQ7 and FEV_1 were not included as ACQ6 with FEV_1 % predicted considered separately provided the same information broken down into more detail. ACQ6 was broken down into categories of control (0-0.75: well controlled, 0.76-1.5: moderately controlled, >1.5: poorly controlled) to provide additional detail.

When considered together age (OR 0.96; 95% CI, 0.93 to 0.99; p=0.011) was the only significant predictor of response. Poorly controlled ACQ6 score just missed significance (OR: 5.19, p=0.050) (**Table 4.7**).

To generate the final model, LCQ and MRC were removed as each questionnaire only addressed one symptom and neither were

significant predictors of response when combined with other factors. As the ACQ6 encompassed a range of symptoms and the final score could be broken down into categories this along with FEV_1 % predicted were included in the final model. Smoking status was not a predictor of response but was included as subjects were randomized based on smoking. Treatment intervention and patient demographics; age, sex and BMI were also included.

When all factors were combined in the final model age, and a poorly controlled ACQ6 score (OR: 0.96, 95% CI, 0.93 to 0.99; p=0.008; OR:11, 95% CI, 2.76 to 43.82; p=0.001 respectively) were predictors of response to treatment (**Table 4.8**).

Table 4.6 Baseline factors to predict response to treatment: considered independently

When baseline factors were considered independently age, LCQ, ACQ6 and ACQ7 scores were predictive of response to treatment. OR: odds ratio, BMI: Body Mass Index, MRC: Medical Research Council Dyspnea Scale, LCQ: Leicester Cough Questionnaire, ACQ: Asthma Control Questionnaire, FEV_1: Forced Expiratory Volume over one second.

	N (%)	OR	95% CI	p-value
Treatment intervention				
Active	66 (49%)	1		
Placebo	68 (51%)	0.76	0.38-1.53	0.457
Age	134 (100%)	0.97	0.95-0.99	**0.006**
Gender				
Female	99 (74%)	1		
Male	35 (26%)	0.73	0.33-1.65	0.459
BMI	110 (82%)	1.03	0.98-1.10	0.226
Smoking status				
Non-smoker	111 (83%)	1		
Smoker	23 (17%)	2.3	0.93-5.74	0.072
Blood eosinophils	92 (69%)	17.45	0.92-331.92	0.057
MRC	134 (100)			
1	36 (27%)	1		
2	76 (57%)	2.3	0.95-5.55	0.063
3	15 (11%)	3.42	0.96-12.14	0.056
4	5 (4%)	4.49	0.64-31.37	0.129
5	2 (1%)	1		
LCQ	134 (100%)	0.87	0.81-0.96	**0.002**
ACQ7	134 (100%)	3.74	2.21-6.36	**<0.001**
ACQ6	134 (100%)	3.75	2.24-6.25	**<0.001**
FEV_1	134 (100%)	1.37	0.85-2.21	0.203
FEV_1 % predicted	134 (100%)	1	0.98-1.02	0.916

Table 4.7 Baseline factors to predict response to treatment: regression model

When baseline factors were incorporated into a model age, an overweight BMI and poorly controlled ACQ score were predictive of response to treatment. *: adjusted odds ratio, OR: odds ratio, BMI: Body Mass Index, MRC: Medical Research Council Dyspnea Scale, LCQ: Leicester Cough Questionnaire, ACQ: Asthma Control Questionnaire, FEV1: Forced Expiratory Volume over one second.

	N (%)	OR *	95% CI	p-value
Treatment intervention				
Active	66 (49%)	1		
Placebo	68 (51%)	1.08	0.41-2.83	0.874
Age	134 (100%)	0.96	0.93-0.99	**0.011**
Gender				
Female	99 (74%)	1		
Male	35 (26%)	1.88	0.56-6.28	0.305
BMI	110 (82%)	1.05	0.97-1.15	0.214
Smoking status				
Non-smoker	111 (83%)	1		
Smoker	23 (17%)	0.84	0.20-3.46	0.807
MRC				
1	36 (27%)	1		
2	76 (57%)	0.94	0.27-3.24	0.924
3	15 (11%)	1.65	0.25-10.73	0.6
4	5 (4%)	2.48	0.14-44.85	0.538
5	2 (1%)	1		n/a
LCQ	134 (100%)	0.89	0.77-1.01	0.074
ACQ6				
Well controlled: 0-0.75	35 (26%)	1		
Moderately controlled: 0.76-1.5	51 (38%)	1.73	0.44-6.79	0.434
Poorly controlled >1.5	48 (36%)	5.19	1.00-26.87	0.05
FEV_1 % predicted	134(100%)	1	0.96-1.03	0.98

Table 4.8 Baseline factors to predict response to treatment: final model

In the final model age, overweight BMI and poorly controlled ACQ scores were predictive of response to treatment. *: adjusted odds ratio, OR: odds ratio, BMI: Body Mass Index, ACQ: Asthma Control Questionnaire, FEV_1: Forced Expiratory Volume over one second.

	N (%)	OR *	95% CI	p-value
Treatment intervention				
Active	66 (49%)	1		
Placebo	68 (51%)	1.31	0.52-3.32	0.564
Age	134 (100%)	0.96	0.93-0.99	**0.008**
Gender				
Female	99 (74%)	1		
Male	35 (26%)	1.89	0.60-6.01	0.279
BMI	110 (82%)	1.06	0.98-1.15	0.157
Smoking status				
Non-smoker	111 (83%)	1		
Smoker	23 (17%)	1.20	0.33-4.39	0.779
ACQ6				
Well controlled: 0 0.75	35 (26%)	1		
Moderately controlled: 0.76-1.5	51 (38%)	2.22	0.60-8.22	0.235
Poorly controlled >1.5	48 (36%)	11	2.76-43.82	**0.001**
FEV_1 % predicted	134 (100%)	1	0.97-1.04	0.708

4.13.6 BASELINE SERUM CYTOKINE LEVELS

Cytokine profiles were obtained from 42 patients, 18 (43 %) in the budesonide and 24 (57 %) in the placebo group. The cytokine concentrations are given in **Table 4.9 and 4.10** and are presented graphically in **Figure 4.11**. Cytokine profiles were similar between groups and between responders and non-responders. High concentrations of Th2 cytokines were not predictive of response to treatment with an ICS.

IL-13 was of detectable levels in 12 (67%) of patients in the placebo group (mean 9878.5 pg/ mL) and 19 (79%) of the budesonide group (mean 7854.9 pg/ mL). IL-13 and TSLP were the most frequently detected cytokines regardless of group or response.

Table 4.9 Budesonide group- cytokine profile raw data

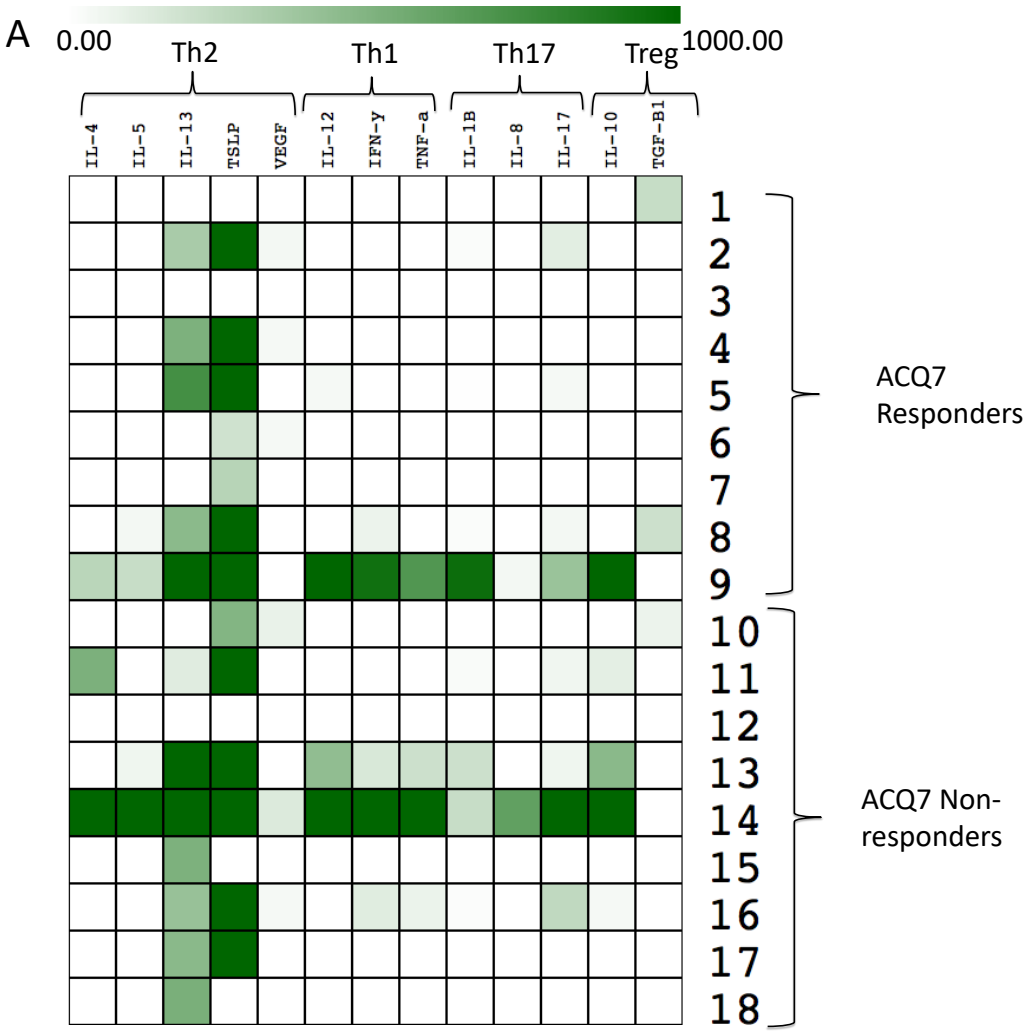
Thirteen cytokines including Th2, Th1, Th17 and Treg cytokines were analyzed at baseline.

	Th2 Cytokines (pg/mL)					Th1 Cytokines (pg/mL)			Th17 Cytokines (pg/mL)			Treg Cytokines (pg/mL)		Responder (Y/N)
Subject	IL-4	IL-5	IL-13	TSLP	VEGF	IL-12	IFN-γ	TNF-α	IL-1β	IL-8	IL-17	IL-10	TGF-β1	
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	226.7	Y
2	0.0	0.0	337.7	5577.3	44.9	0.0	0.0	0.0	22.6	0.0	111.6	0.0	0.0	Y
3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Y
4	0.0	0.0	510.3	1161.6	36.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Y
5	0.0	0.0	735.8	1805.3	0.0	38.8	0.0	0.0	5.3	0.0	35.6	0.0	0.0	Y
6	0.0	0.0	0.0	191.3	38.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Y
7	0.0	0.0	0.0	283.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Y
8	0.0	48.5	453.7	2286.9	0.0	0.0	76.9	0.0	20.5	0.0	48.5	0.0	203.4	Y
9	270.5	220.3	21168.3	30894.1	0.0	2136.1	938.5	684.8	950.4	43.6	394.6	1473.7	0.0	Y
10	0.0	0.0	0.0	490.6	88.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	76.1	N
11	512.0	0.0	123.0	3780.9	0.0	0.0	0.0	0.0	25.4	0.0	58.0	102.1	0.0	N
12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
13	0.0	65.8	1389.6	3188.6	0.0	429.1	156.2	196.5	202.0	0.0	65.9	459.4	0.0	N
14	51572.5	21376.9	151197.1	84697.3	138.9	39762.6	22928.3	13231.8	223.0	623.6	2982.8	29843.1	0.0	N
15	0.0	0.0	509.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
16	0.0	0.0	407.3	10842.6	35.8	0.0	118.2	81.9	17.3	0.0	243.9	35.3	0.0	N
17	0.0	0.0	459.5	2815.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
18	0.0	0.0	521.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N

Table 4.10 Placebo group- cytokine profile raw data

Thirteen cytokines including Th2, Th1, Th17 and Treg cytokines were analyzed at baseline.

	Th2 (pg/mL)					Th1 (pg/mL)			Th17 (pg/mL)			Treg (pg/mL)		Response (Y/N)
Subject	IL-4	IL-5	IL-13	TSLP	VEGF	IL-12	IFN-y	TNF-a	IL-1B	IL-8	IL-17	IL-10	TGF-B1	
1	65.2	0.0	723.3	2306.0	39.8	101.2	100.4	0.0	31.1	0.0	612.0	0.0	0.0	Y
2	0.0	0.0	0.0	0.0	108.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Y
3	310.7	57.9	73455.7	80248.8	7864.5	10450.8	1587.6	1715.1	4760.6	67.4	525.3	1860.9	0.0	Y
4	0.0	0.0	285.1	49716.9	0.0	41.6	0.0	0.0	38.9	0.0	0.0	31.4	0.0	Y
5	0.0	0.0	522.6	44.3	0.0	0.0	0.0	0.0	0.0	301.3	0.0	0.0	0.0	Y
6	88.6	102.8	2791.6	663.2	0.0	169.2	0.0	71.0	39.6	0.0	76.6	409.9	0.0	Y
7	0.0	0.0	506.4	179.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Y
8	191.2	168.4	68734.7	340536.6	1463.4	6999.9	5182.3	2187.9	2816.7	146.3	2756.5	3121.7	0.0	Y
9	0.0	0.0	339.0	878.0	0.0	0.0	123.3	0.0	0.0	0.0	68.8	0.0	55.4	N
10	0.0	0.0	0.0	3426.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	39.7	N
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
12	0.0	132.9	2535.0	10287.7	0.0	1044.0	679.9	262.7	262.6	0.0	173.8	76.0	228.4	N
13	0.0	0.0	524.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
14	0.0	0.0	195.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
15	0.0	123.1	520.4	1962.8	77.3	128.4	121.3	114.6	188.5	0.0	65.2	707.9	0.0	N
16	74.1	0.0	2071.3	25991.1	332.3	3369.1	278.6	1038.6	314.2	33.2	637.4	1335.7	0.0	N
17	0.0	0.0	495.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
18	0.0	0.0	478.1	1299.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
19	0.0	0.0	288.4	4130.0	0.0	74.4	0.0	0.0	0.0	0.0	26.4	0.0	0.0	N
20	0.0	0.0	495.2	102.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
21	0.0	0.0	476.3	1000.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
22	8248.2	1453.6	33077.8	60730.0	454.6	4817.8	4445.8	1633.0	2890.0	115.4	594.2	909.9	0.0	N
23	0.0	0.0	0.0	708.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	N


A
0.00
1000.00
Th2
Th1
Th17
Treg
IL-4
IL-5
IL-13
TSLP
VEGF
IL-12
IFN-γ
TNF-a
IL-1B
IL-8
IL-17
IL-10
TGF-B1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
ACQ7 Responders
ACQ7 Non-responders

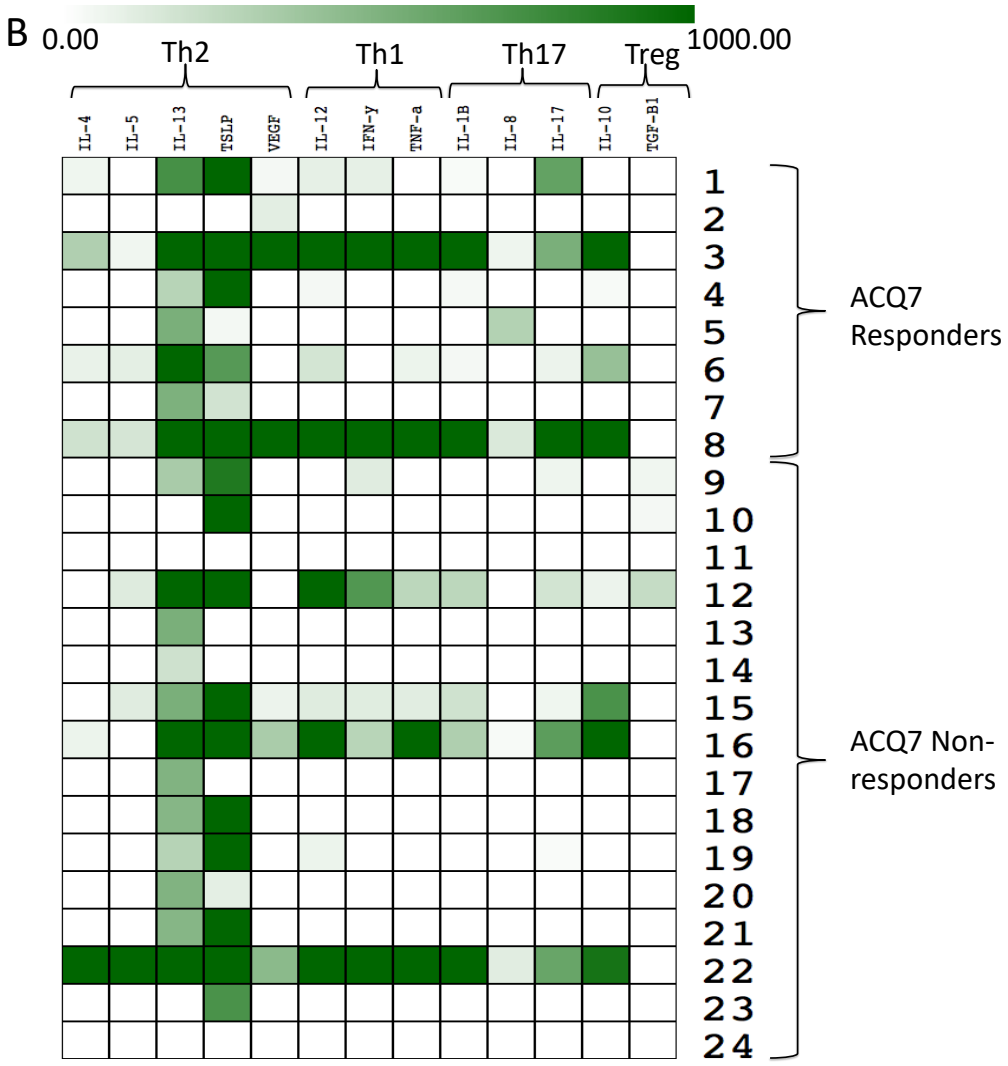


Figure 4.12 Cytokine profile heat map

Baseline cytokine concentrations for the budesonide **A**. and placebo **B**. are represented on a scale from 0-1000 pg/mL where 0 pg/mL is white and the darkest green is 1000 pg/mL.

4.14 DISCUSSION

The results of this randomized, double-blind, placebo-controlled study indicate that initiating ICS treatment in patients with symptoms suggestive of asthma who have a FeNO ≤27 ppb is unlikely to be beneficial in the short-term in terms of decreasing symptoms or improving lung function. Age and a poorly controlled ACQ6 score at baseline were associated with treatment response.

4.14.1 USING FENO TO PREDICT RESPONSE TO CORTICOSTEROIDS

Inhaled corticosteroids are the cornerstone of asthma management. They are recommended when patients remain symptomatic despite treatment with a SABA and they are increased stepwise until control is achieved (9). As there is no definite test to diagnose asthma or determine which treatments will be beneficial the decision to initiate or increase corticosteroids is typically driven by symptoms. This is not ideal as symptoms are non-specific and do not reflect the type or severity of inflammation in the airways. Several studies have indicated that lung function (FEV_1 and AHR) does not improve with ICS treatment in 25-35 % of patients with asthma (296, 297).

Numerous studies have evaluated the utility of FeNO to predict response to corticosteroids. Smith and colleagues determined that regardless of the patient's diagnosis, a FeNO level >47 ppb had a greater response to ICS than patients with low FeNO levels (<15 ppb) (292). Hahn and colleagues determined patients with chronic cough and a FeNO of >35 ppb were more likely to respond to an ICS than patients with a FENO <35 ppb (298). Martin et al determined that

patients with symptoms suggestive of asthma and FeNO > 33 ppb were more likely to respond to an ICS (284). Therefore, although the cut points for high, moderate and low FeNO levels vary between studies, it has been well documented that FeNO >50 ppb is predictive of a positive response to an ICS (9). To our knowledge, this is the first study to demonstrate that low levels (≤27 ppb) are predictive of a lack of response in the short-term, which helps to identify a phenotype of patients in whom initiating corticosteroid treatment may not be beneficial.

FeNO is non-invasive, easy to perform, requires minimal effort and provides immediate results. Therefore, it is an ideal biomarker to be used in primary care to facilitate treatment decisions. This study demonstrated that the between group mean difference in ACQ7 fell within the equivalence interval of -0.5 to 0.5, (p=<0.0001, p=0.0462) confirming that initiating treatment with an ICS in steroid naïve patients with symptoms suggestive of asthma does not produce a symptomatic improvement. Equally, the between group mean difference in FEV_1 was 0.01 which also fell within the equivalence margin. Together these results suggest that FeNO ≤27 ppb at baseline is a good predictor of a lack of response to initiating ICS treatment in patients with suspected asthma both subjectively and objectively in the short-term (12 weeks). This finding is in agreement with a previous in-house study performed by Martin and colleagues who determined that baseline FeNO levels ≤27 ppb had a negative predictive value of 93 % to starting ICS treatment (284). The decrease in ACQ scores noted here is consistent with previous studies

that have investigated the effect of initiating ICS treatment in patients with mild asthma (299-301). Post hoc analysis looked at FeNO levels at baseline divided into tertiles (low; ≤5-12 ppb, medium; 13-20 ppb and high; 21-27ppb) and found a significantly greater number of responders in the budesonide group than the placebo group in the high FeNO tertile (14 vs four; χ^2 p=0.0043). This finding could suggest that a cut point of ≤27 ppb may be too high and further work should be conducted to evaluate lower cut points.

Age and ACQ6 (0.97; 95% CI, 0.95 to 0.99; p=0.006 and 3.75; 95% CI, 2.24 to 6.25; p<0.001 respectively) scores were significant baseline predictors of response. Cough alone, as evaluated by the LCQ, was a predictor (0.87; 95% CI, 0.81 to 0.96; p=0.002) but was not significant when combined with the ACQ which encompassed multiple symptoms. However, response was defined as a decrease in ACQ so those with high baseline values could be biased towards a positive response.

Interestingly mean IL-13 levels were significantly elevated in both groups. A previous study by Shehata and colleagues performed ELISAs on serum from patients with mild asthma and found the mean concentration of IL-13 to be 63.6 pg/ mL (302). This value is much lower than the mean values in this study; 9878.5 and 7854.9 pg/ mL in the budesonide and placebo groups respectively. As FeNO is thought to reflect IL-13 it is interesting that a group of patients with low FeNO would have elevated levels of IL-13.

4.14.2 STUDY LIMITATIONS

This study had several limitations that need to be addressed.

Firstly, due to the study design, it was difficult to transfer blood samples taken from participants seen in primary care back to the lab for processing in a timely manner and as such serum samples were obtained from only 53 patients, 42 of which completed the study. Due to such limited numbers it was not possible to demonstrate a relationship between high levels of Th2 cytokines (IL,4, IL-5, IL-13, TSLP and VEGF) and response to initiating treatment with an ICS. It is possible that with more samples a relationship could have been observed.

When considered independently blood eosinophils were a borderline predictor of response (OR: 17.45; 95% CI, 0.92 to 331.92; p=0.057). However, this had to be excluded from the regression model as including it would have reduced the number of observations down to 92. It is possible that if more samples had been obtained this could have been a significant predictive factor.

Second, although participants were asked about compliance at each visit there was no formal measure of treatment compliance. Corticosteroids are known to suppress FeNO and this has previously been used to assess compliance (303). In this study, we noted an increase in FeNO levels. This increase could suggest poor adherence in some participants, although FeNO levels in the placebo group had a greater increase than in the budesonide group. Non-compliance could be a reason some participants failed to respond. However, it is more likely that the increase seen here is due to regression to the mean as the study population was at the bottom end of the possible values at baseline.

Third, participants recruited into the study had symptoms suggestive of asthma as opposed to confirmed asthma. As such, we cannot say that patients with confirmed asthma and a low FeNO will not respond to ICS but rather that patients with symptoms suggestive of asthma and a low FENO are unlikely to receive a subjective or objective response in the short-term. For some patients this will be because they do not have asthma (or airway inflammation) and an alternative diagnosis for their symptoms should be considered, whilst others may have asthma and may still require ICS treatment to prevent subsequent exacerbations. However, even if this is true it is likely to be difficult to persuade patients to take regular ICSs when they do not benefit symptomatically in the short-term. Alternative approaches such as a combined SABA or LABA with an ICS used as required should be evaluated for these patients. Further studies powered for exacerbations are therefore required before ICS can be withheld in all patients with a low FeNO.

Fourth, our definition of response to initiating treatment with an ICS was based on ACQ scores. As the definition of treatment response varies between studies it can be difficult to compare findings between studies.

Finally, FeNO is affected by many individual factors such as age, height and gender (ex. males tend to have higher levels) as well as external factors such as nasal polyps, cigarette smoking, nitrate-rich foods (e.x spinach), allergens, RV infections, exercise and air pollution (287). As such, FeNO readings taken at one point in time

may not accurately reflect inflammation in the airways and should be interpreted with caution (9).

4.14.3 CONCLUSIONS

In conclusion, the results of this study suggest that FeNO levels ≤27 ppb can be used to identify patients with suspected asthma who will not benefit from starting treatment with an ICS in the short-term.

CHAPTER 5

AN EVALUATION OF INCREASED DOSES OF CORTICOSTEROID IN POORLY CONTROLLED ASTHMA

5.1 BACKGROUND INFORMATION

Chronic airway inflammation is a primary characteristic in the pathology of asthma. Inhaled corticosteroids are the basis of asthma management with the aim of reducing inflammation and thereby, improving lung function, reducing symptoms and the risk of future exacerbations (304). These effects are especially pronounced in patients with T2 high asthma (46) as the effects of corticosteroids are thought to be mediated by triggering eosinophils to undergo apoptosis and therefore specifically reduce eosinophilic inflammation (305). It has been well documented that initiating ICS treatment reduces sputum (87) and blood eosinophil (46) levels while decreasing the dose or stopping treatment increases eosinophil levels (306). However, studies have shown that 25-35 % of patients remain inadequately controlled and have persistently abnormal lung function and intermittent exacerbations despite treatment with ICSs (296). Currently, treatment guidelines advocate for increasing the dose of ICSs with increasing disease severity regardless of the patient's phenotype or endotype (9). As therapies have become more targeted there has been increased focus placed on identifying biomarkers that will reliably predict response to each treatment. Even in patients with T2 high asthma (elevated FeNO, sputum and blood eosinophils) there is a subgroup of patients that are unresponsive (307). As such, a common question in clinical practice is whether prescribing an increased dose of corticosteroid would actually be of benefit. To answer this, patients are often prescribed a month of high dose ICS or a two-week course of oral corticosteroids (OCS) to see if their

condition improves. However, what constitutes a positive response to more corticosteroid is poorly defined. As there are serious side effects associated with corticosteroids use (ie. suppression of the hypothalamic-pituitary-adrenal-axis, glaucoma, fluid retention, osteoporosis and weight gain) (48) it is important to be sure the patient will benefit before increasing their dose. In corticosteroid naïve patients previous studies have shown that elevated baseline sputum eosinophils, urinary bromotyrosine and increased FeNO are useful biomarkers in predicting a positive response to initiating corticosteroid treatment (308). However, it is unknown if there are phenotypic characteristics or identifiable biomarkers of patients currently taking ICSs that can reliably identify those who will respond from those who will not respond to an *increased* dose of corticosteroid.

5.2 AIMS

Aim 1: To describe the pattern of response to an increased dose of corticosteroid in terms of symptoms (ACQ), lung function (spirometry and FOT) and inflammation (FeNO, blood eosinophils, sputum cell count and cytokines levels in serum, plasma and sputum supernatant).

Aim 2: To propose criteria from each clinical outcome to categorize the patients into responders and non-responders based on each outcome.

Aim 3: To describe phenotypic characteristics of responders and non-responders for each outcome.

Aim 4: To determine whether a specific phenotype can reliably identify responders from non-responders.

5.3 STUDY DESIGN

A single center observational study was conducted at NCH.

5.4 ETHICAL APPROVAL

This study was sponsored by the University of Nottingham and obtained approved from the South East Coast- Surrey Research Ethics Committee (REC) and Health Research Authority (HRA). All participants completed a consent form and the study was conducted in accordance with GCP principles.

5.5 STUDY SETTING AND PARTICIPANT RECRUITMENT

Patients were recruited from severe asthma and respiratory clinics in NCH. All patients had a diagnosis of severe asthma confirmed by a severe asthma consultant.

5.5.1 INCLUSION CRITERIA

The inclusion criteria for participants were:

1. Aged 16 years or over
2. Ability to give informed consent
3. A diagnosis of moderate-to-severe asthma being treated at step three or above of the British Thoracic Society (BTS) guidelines
4. Evidence of inadequate control and being given an increased dose of either inhaled or oral corticosteroids as part of their clinical evaluation

5.5.2 EXCLUSION CRITERIA

The exclusion criteria for the participants were:

1. The presence of any significant co-existing respiratory conditions which would interfere with the aims of the study
2. Inability to undergo more than one of the investigations required

5.6 STUDY INTERVENTION

As part of routine care patients were prescribed either a two-week course of OCS or a four-week increase of their currently prescribed ICS.

5.7 OUTCOME MEASURES

5.7.1 PRIMARY OUTCOME

The primary outcome was to describe the features of responders and non-responders to an increased dose of corticosteroid. This was achieved by describing the pattern of response in terms of symptoms (ACQ7), lung function (spirometry, FOT) and inflammation (FeNO, blood and sputum eosinophils and cytokine levels in serum, plasma and sputum supernatant).

5.7.2 SECONDARY OUTCOME

The secondary outcome was to determine if cytokine levels in sputum supernatant, blood serum and plasma were linked with response to an increased dose of corticosteroid.

5.8 POWER CALCULATION

This was an exploratory study making it impossible to conduct a formal power calculation. The goal was to recruit as many participants as possible over a 12-month period, aiming for approximately 50.

5.9 ANALYSIS

Participant information was recorded in their case report form (CRF) and then entered and stored into Microsoft Excel.

Eight-point standard curves were produced to interpolate individual cytokine levels. A cubic spline fit using 1000 points was used to find the best fit for each standard curve. This analysis was performed using GraphPad Prism 7. Heat maps were then created in Multi Experiment Viewer (MeV_4_8) to visualize the results.

Histograms and paired t-tests were conducted in STATA/SE 15.1. Histograms as well as line graphs (Graphpad Prism 7) were created to visualize the responses to treatment and paired t-tests were used to determine a significant difference in each outcome with 95 % CI. The significance level for all tests was $p < 0.05$.

5.10 PROTOCOL

Participants were recruited from secondary care asthma clinics in NCH. Participants were approached if they had been prescribed a trial of increased inhaled or oral corticosteroid as part of their clinical care. All potential participants were provided with a PIS and were allowed time to read over it and ask any questions with respect to the study and/or their participation. If they were willing to take part, all participants signed a consent form.

During the first visit, a medical and asthma-specific history were taken. Participants completed the ACQ7 prior to any lung function tests. Baseline FeNO and FOT were performed first followed by spirometry. Venepuncture and, where possible, sputum collection were also conducted.

Participants receiving OCS were seen for their second visit after two weeks and participants receiving an increased dose of ICS were seen after four weeks.

During visit two, only the procedures that were successfully performed during visit one were repeated (for example if the participant was unable to produce sputum during visit one this was not reattempted during visit two).

Following completion of the study, a member of the study team advised participants on how to continue their daily care.

A minimum of one year after completing the study a review of the participant's clinical notes was conducted to determine the long-term impact of their participation in the study on their care.

An overview of the information collected during the study is presented in **Table 5.1**.

Table 5.1 Overview of the information obtained as part of the participants routine care and study specific information

Participants medical history, body mass index, spirometry, and nitric oxide are done as part of routine clinical care. To enter the study, they also had to be prescribed an increase in corticosteroid. As part of the study participants additionally had their small airway function measured, venipuncture, completed symptom questionnaires and where possible performed sputum induction. All variable measured for the study as well as spirometry and nitric oxide were measured at both visits. BMI: body mass index, FeNO: Fractional exhaled nitric oxide, ACQ: Asthma Control Questionnaire

Variable	Measured in routine clinical practice	Measured for study
BMI	x	
History	x	
Spirometry	x	
Small airway		x
FeNO	x	
Blood		x
Induced sputum		x
ACQ		x

5.11 RESULTS

From February 2017 to July 2018 a total of 13 patients with severe poorly controlled asthma who were prescribed an increased dose of corticosteroid (OCS n=9, ICS n=4) were enrolled in the study. All participants completed both study visits. One participant contracted a respiratory viral infection during the study and was started on a course of antibiotics (separate from the study) and as such their cytokine levels were not remeasured during the second visit.

5.11.1 BASELINE DEMOGRAPHICS

All 13 participants who were enrolled in the study completed both visits and were included in the analysis. The baseline demographics (**Table 5.2)** and clinical features (**Table 5.3**) are presented below. Oral corticosteroids were prescribed to nine (69 %) participants, and four (31 %) were prescribed an increase in their ICS. There were slightly more females (7; 54 %) and all participants but one were Caucasian (1; black). Participants ranged from 29 to 82 years of age (mean 58 years). There were no current smokers, eight (62 %) were ex-smokers with a mean pack year of 17.9. The mean duration of asthma was 20.3 years.

One participant reported an ACQ7 score in the moderately controlled zone (ACQ7 score 1.28), all other participants were poorly controlled (mean ACQ7 score 3.4). FEV_1 % predicted ranged from 28-82 % (mean 61.8 %) and FEV_1/FVC ratio ranged from 42 to 75 (mean 58.2). Blood eosinophils ranged from 0.0-1.8 cells $x10^9$/L (mean 0.6 cells $x10^9$/L). The mean small airway resistance (R5-20) was 1.8 Hz

and reactance (AX) 37.5 Hz. The mean large airways resistance (R20) was 3.5 Hz and overall resistance (R5) was 5.3 Hz. One participant was unable to perform FeNO. The remaining 12 ranged from 5-84 ppb (mean 38.8 ppb).

Four (31 %) participants were able to give a sputum sample at baseline. The remaining participants either did not meet the criteria for induction, were unable to produce a sample following induction or declined to participate in the procedure. Venepuncture was performed on all participants at both visits for an FBC and all but one participant at visit two also had cytokines measured.

Table 5.2 Baseline demographics

Thirteen patients were enrolled in the study and completed both study visits. BMI- Body mass index.

Subject	Treatment	Ethnicity	Sex	Age	BMI	Smoking status	Pack Years	Duration of asthma (years)
1	OCS	Caucasian	F	65	33.7	Never	0	17
2	OCS	Caucasian	M	61	33.5	Ex	53	7
3	ICS	Caucasian	M	41	36.5	Never	0	41
4	OCS	Caucasian	F	82	32.4	Ex	43	78
5	OCS	Caucasian	F	51	30.1	Ex	17	3
6	OCS	Caucasian	M	47	29.1	Ex	28	19
7	ICS	Caucasian	F	63	31.2	Never	0	0.17
8	ICS	Caucasian	F	63	36.4	Never	0	7
9	OCS	Caucasian	M	70	27.7	Ex	50	7
10	OCS	Caucasian	F	29	20.4	Never	0	27
11	OCS	Black	M	81	29.7	Ex	3	17
12	ICS	Caucasian	M	41	29.4	Ex	22	20
13	OCS	Caucasian	F	57	18.1	Ex	17	0.08
Mean				57.8	29.9		17.9	20.3
Standard deviation				15.7	5.5		20.1	21.5

Table 5.3 Baseline clinical features

Baseline symptoms (ACQ7) lung function (FEV_1, FEV_1 % predicted, FEV1/FVC, R5, R20, R5-20, AX) and inflammation (blood eosinophils, FeNO) for all patients are given as well as the mean and standard deviation (SD). ACQ: Asthma Control Questionnaire, FeNO: Fractional exhaled nitric oxide, FEV_1: Forced expiratory volume over one second, FVC: Full vital capacity, R: Resistance, AX: reactance.

Participant	ACQ7	Blood Eosinophil (cells x10^9/L)	FeNO (ppb)	FEV_1 (L)	FEV_1 % Predicted	FEV_1/FVC (L)	R5 (Hz)	R20 (Hz)	R5-20 (Hz)	AX (Hz)
1	3.40	0.5	75	1.68	81	69	0.65	0.39	0.26	2.78
2	4.60	1.8	84	0.90	28	42	0.84	0.36	0.48	9.81
3	2.70	0.4	20	1.56	40	46	8.31	4.45	3.86	64.52
4	3.85	1.2	5	0.83	54	49	9.11	5.45	3.66	86.10
5	2.40	0.7	35	2.23	76	62	6.33	3.61	2.72	73.60
6	3.40	0.4	15	2.95	82	70	4.44	3.32	1.12	18.23
7	1.28	0.2	36	1.68	73	55	6.19	4.90	1.29	19.53
8	3.57	0.4	N/A	1.54	75	75	8.06	5.09	2.98	91.04
9	4.30	0.6	64	0.88	35	42	8.10	5.35	2.75	56.44
10	2.83	0.4	5	1.50	47	52	6.65	4.98	1.68	31.82
11	2.85	0.1	40	1.30	58	72	4.42	2.98	1.44	15.98
12	4.67	0.0	64	2.82	77	55	2.84	2.21	0.63	15.38
13	3.85	0.6	23	2.47	78	68	2.53	2.35	0.18	2.65
Mean (SD)	3.4 (1.0)	0.6 (0.5)	38.8 (27.1)	1.7 (0.7)	61.8 (19.2)	58.2 (11.7)	5.3 (2.9)	3.5 (1.8)	1.8 (1.3)	37.5 (32.2)

5.11.2 PRIMARY OUTCOME

Histograms were created to visualize the effects of an increased dose of corticosteroid on ACQ7, blood eosinophils, FeNO, FEV_1, R5, R20, R5-20 and AX. The histograms were used to determine a cut-off value to define "response" for each criteria with the exception of ACQ7 where a decrease of 0.5 has been well established as the response threshold (260).

There was a significant difference in ACQ7 (p=0.036) from visit one to visit two with a mean decrease of 0.65 points. Seven (54 %) participants had a decrease of ≥0.5 which represented a clinically significant subjective response to an increased dose of corticosteroid (**Figure 5.1**).

There was a significant difference in blood eosinophils (p=0.03) from visit one to visit two. At baseline three participants (23 %) had a blood eosinophil count ≤ 0.2 compared with six (46 %) participants by visit two. The mean difference was a decrease of 0.29 cells $x10^9$/L. Nine (70 %) participants had a decrease in blood eosinophils, two (15 %) had an increase and two (15 %) had no change following treatment. Based on the histogram of response participants were considered to be responders if there was a decrease of ≥ 0.1. Based on this cut point, nine (70 %) participants responded to the increased dose of corticosteroid in terms of blood eosinophils. (**Figure 5.2**).

There was a significant difference in FeNO (P=0.01) from visit one to visit two. The mean change was a decrease of 17 ppb. One participant (8 %) was not able to perform FeNO (unable to achieve the required technique), eight (62 %) had a decrease in FeNO. At visit one, four

participants (31 %) had FeNO levels >50 ppb, by visit two all levels were <50 ppb. Based on the histogram of the changes in FeNO, participants were considered to have responded if there was a decrease of >10 ppb following treatment. Based on this cut point six (46 %) participants were responders in terms of FeNO to an increased dose of corticosteroid (**Figure 5.3**).

The mean change in FEV_1 was 0.21 L (p=0.98) from visit one to visit two. All participants had an increase in FEV_1 which ranged from 0.01-1.1 L. Based on the histogram of changes in FEV_1 there was no clear distinction between those who had responded and those who had not. Therefore, the commonly utilized threshold of an increase of ≥200 mL (0.2 L) was used to distinguish responders from non-responders. Based on this cut point three (23 %) participants had a significant improvement in FEV_1 in response to treatment (**Figure 5.4**).

The mean change in R5 was -0.62 (-10.54 %) (p=0.057) from visit one to visit two. Nine (70 %) participants had a decrease in R5 following treatment. Due to the wide range of values at the baseline visit, response to all FOT parameters was determined based on the percent change as opposed to the change in real values. Based on the histogram of changes in R5 participants were considered to have responded if there was a decrease of >30 %. Based on this cut point two (15 %) participants responded to the increased dose of corticosteroid in terms of total airway resistance (R5) (**Figure 5.5**).

The mean change in R20 was -0.11 (-2.62 %) (p=0.73) from visit one to visit two. Eight participants (62 %) had a decrease from visit one to visit two. Based on the histogram of change participants were

considered to have responded if they had a decrease of >10 %. Based on this cut point three (23 %) participants responded to the increased dose of corticosteroid in terms of large airway resistance (R20) (**Figure 5.6**).

The mean change in R5-20 was -0.51 (-27.77 %) (p=1.00) from visit one to visit two. Ten (77 %) participants R5-20 decreased following treatment. Based on the histogram of change participants were considered to have responded if there was a decrease of >50 %. Based on this cut point three (23 %) participants responded to the increased dose of corticosteroid in terms of small airway resistance (R5-20) (**Figure 5.7**).

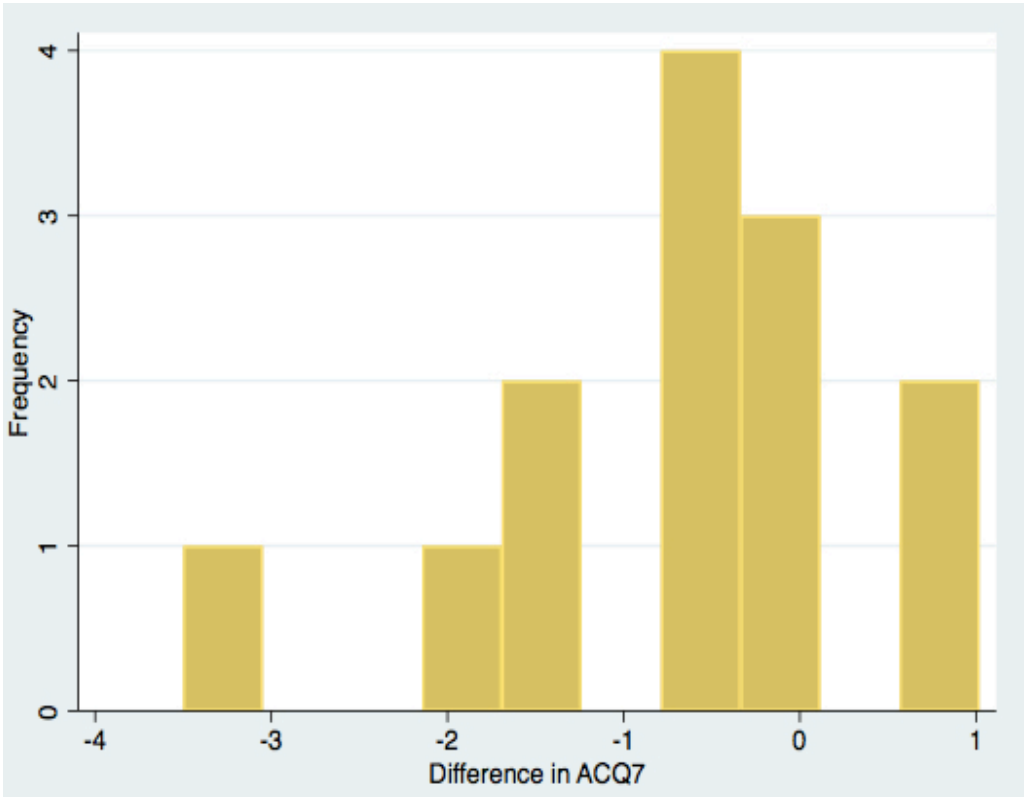
The mean change in AX was -11.0 (-19.62 %) (p=0.073) from visit one to visit two. Nine (70 %) participants had a decrease following treatment. Based on the histogram of change participants were considered to have responded to treatment if they had a decrease of >10 %. Based on this cut point nine (70 %) participants responded to the increased dose of corticosteroid in terms of small airway reactance (**Figure 5.8**).

Based on the response cut points determined above one participant (8 %) was considered to be a "responder" based on every criterion measured and one (8 %) was considered to be a "non-responder" based on every criterion. The remaining 11 participants had mixed responses. These results are summarized in **Table 5.4**.

Of the 13 participants enrolled in the study only four (31 %) were able to give a sputum sample at visit 1, three of which had eosinophils levels above 3 % and therefore were considered to be eosinophilic.

At visit two only two participants were able to give a sample, both had decreased eosinophilic levels, one sample remained above 3 %. Sputum neutrophil levels increased from visit 1 to visit 2. These results are summarized in **Table 5.5**.

A



B

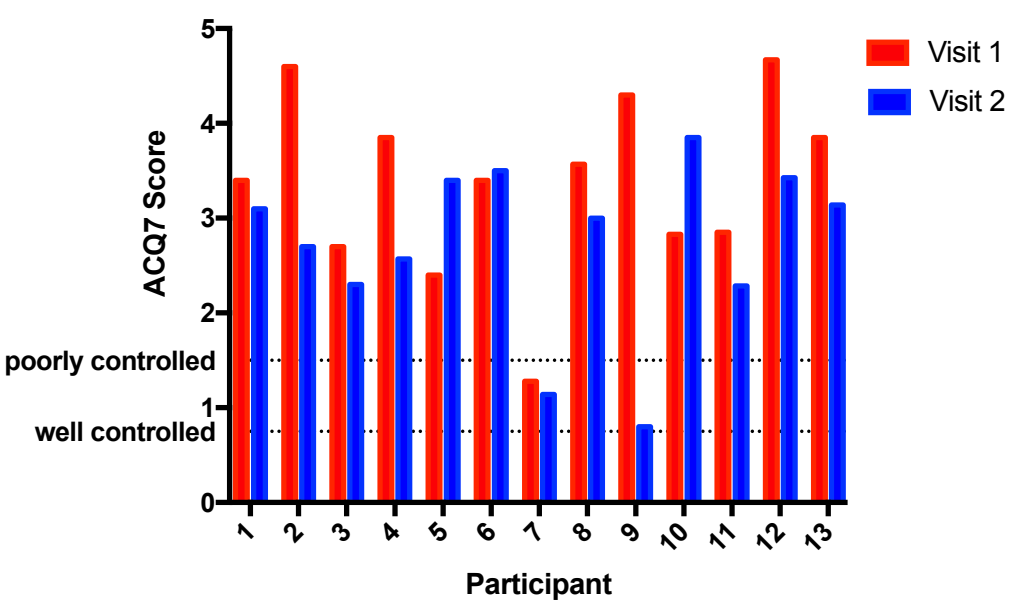
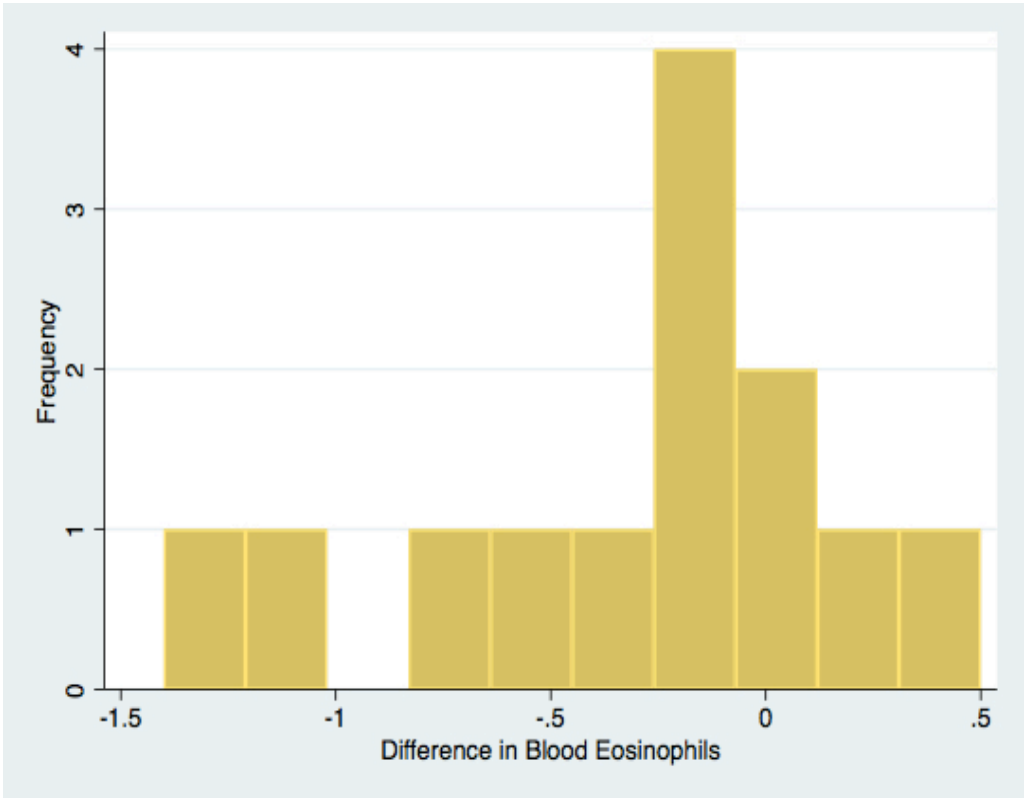


Figure 5.1 Asthma control questionnaire (ACQ) 7

The response to an increased dose of corticosteroid with respect to ACQ 7 from visit 1 to visit 2 is represented as a histogram **A**. and the individual differences **B**. Patients were considered responders if their score decreased by ≥0.5 from visit 1 to visit 2.

A



B

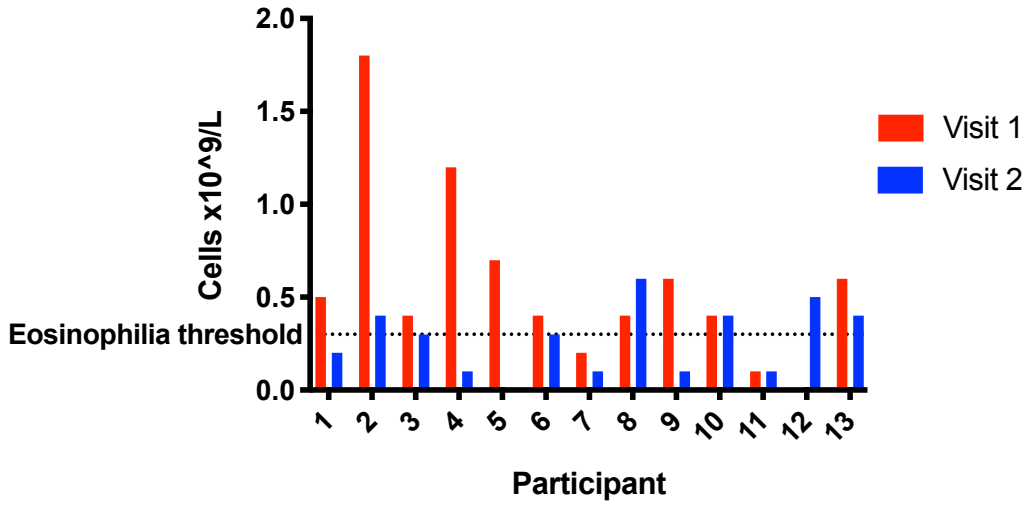
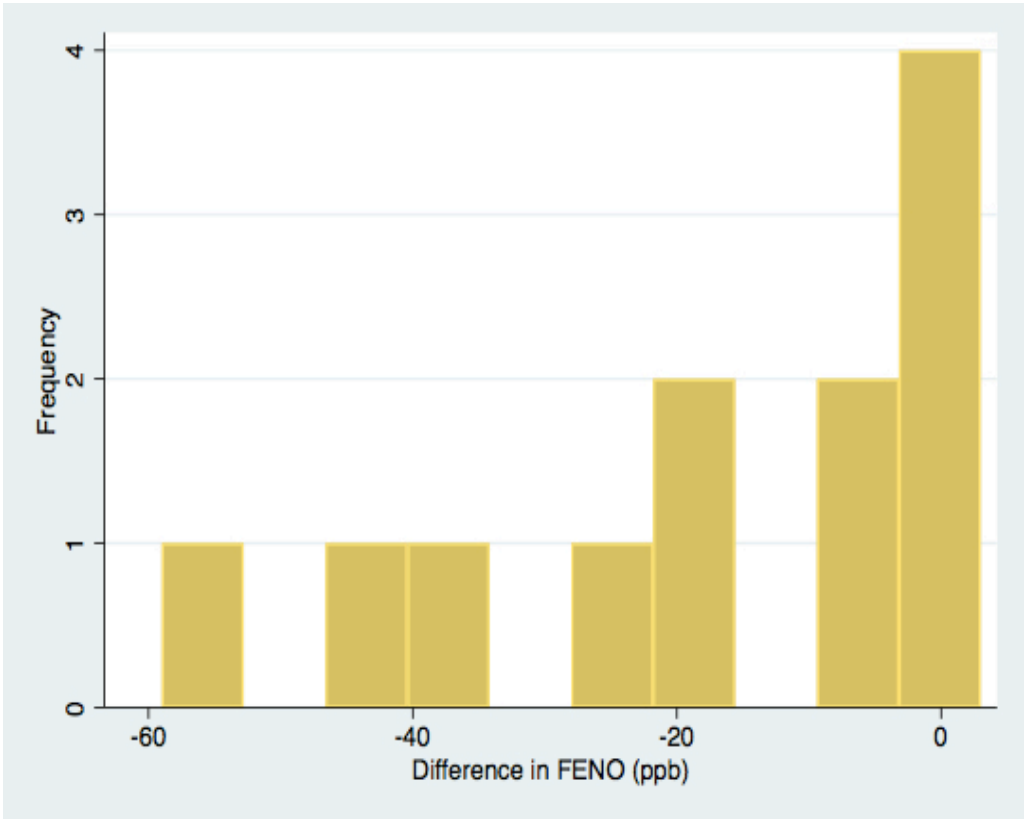


Figure 5.2 Blood eosinophils

The response to an increased dose of corticosteroid with respect to blood eosinophils from visit 1 to visit 2 is represented as a histogram **A**. and the individual differences **B**. Patients were considered responders if their levels decreased by at least 0.1 from visit 1 to visit 2.

A



B

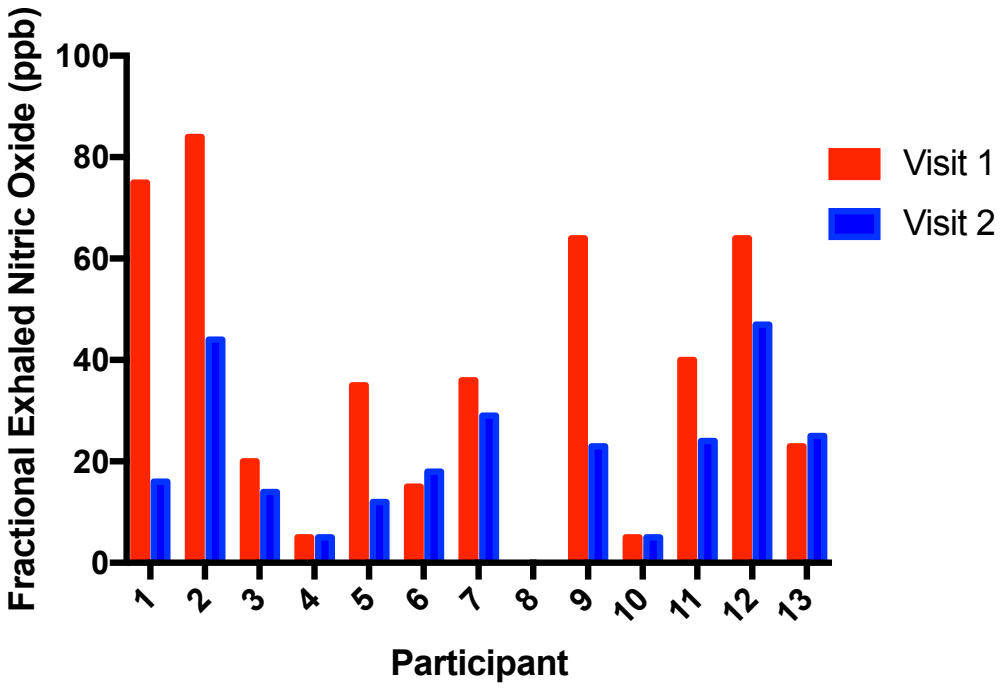
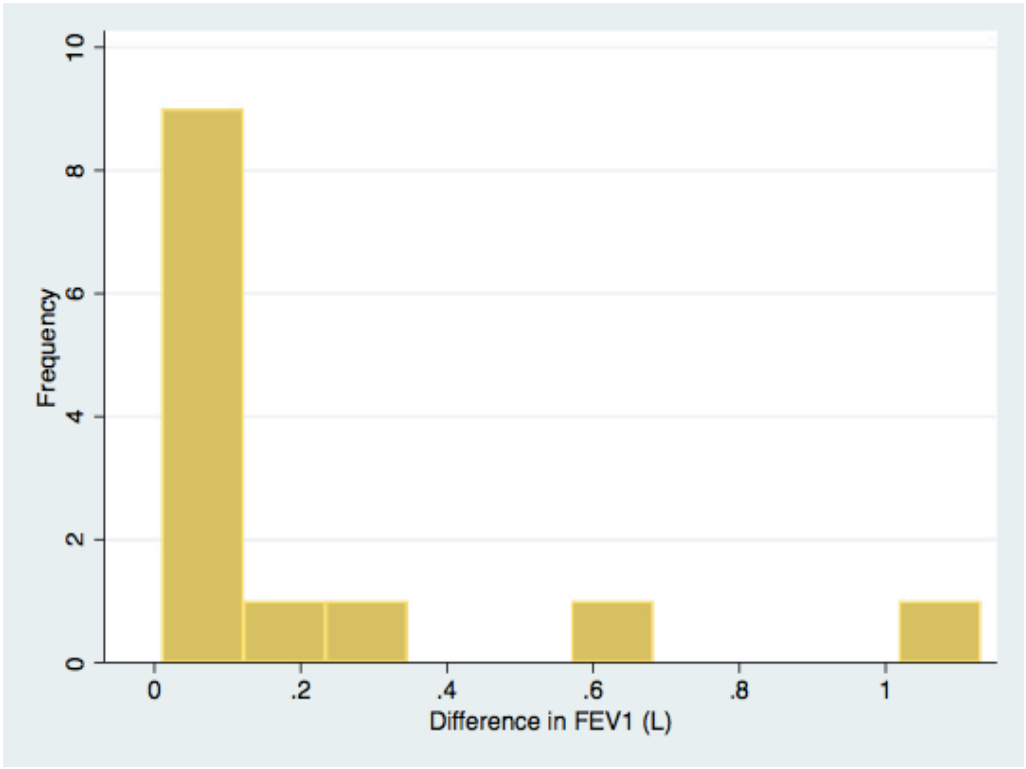


Figure 5.3 Fractional exhaled nitric oxide (FeNO)

The response to an increased dose of corticosteroid with respect to FeNO from visit 1 to visit 2 is represented as a histogram **A**. and the individual differences **B**. Patients were considered responders if their levels decreased by at least 10 ppb from visit 1 to visit 2. One patient was unable to perform FeNO.

A



B

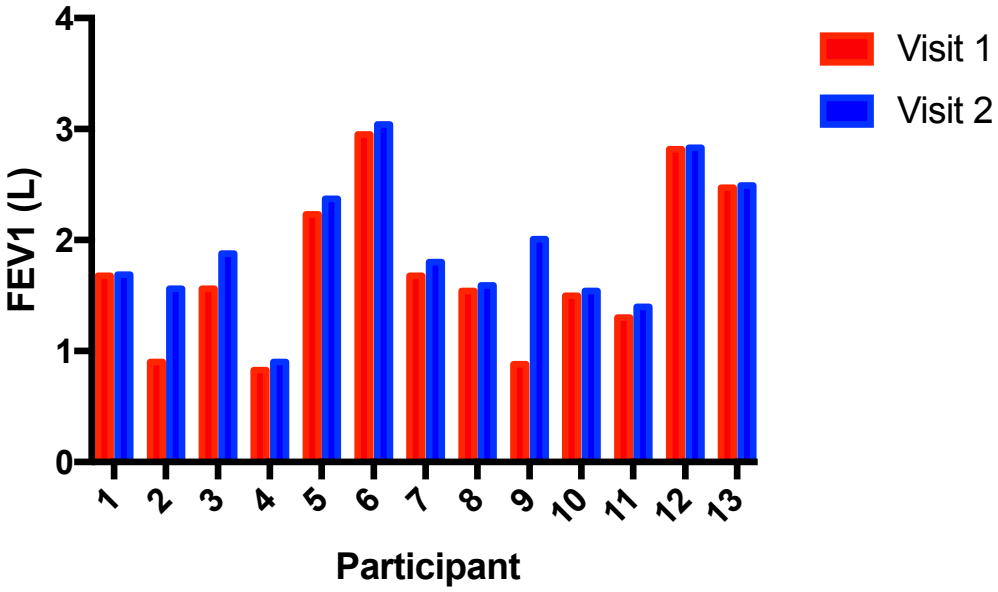
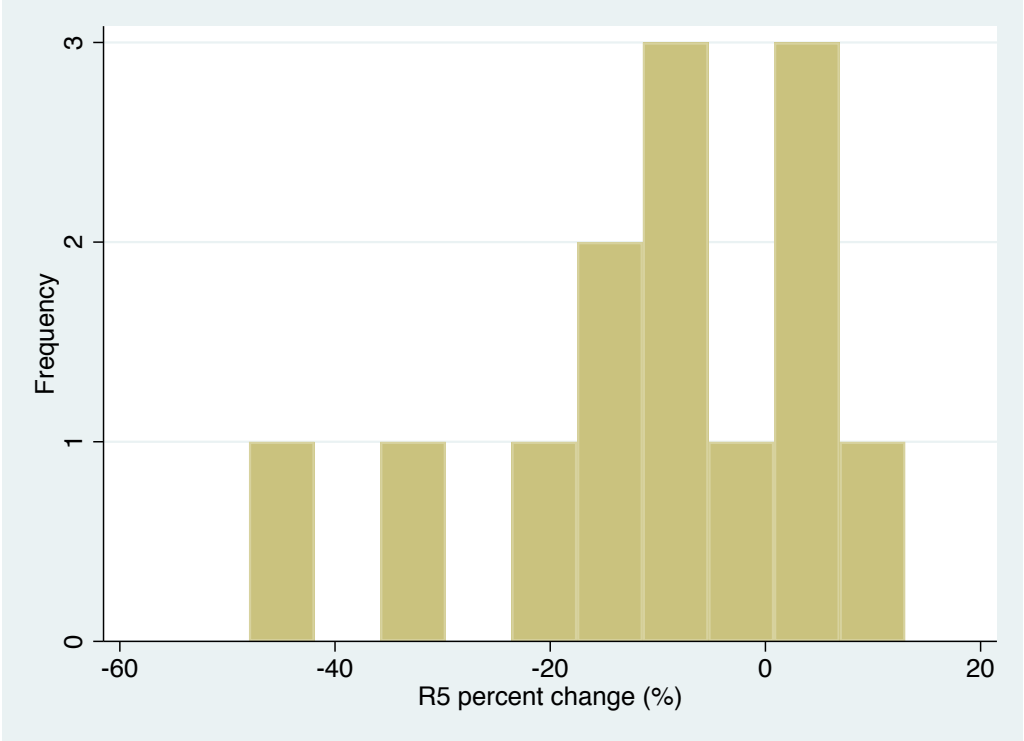


Figure 5.4 Forced expiratory volume over one second (FEV_1)

The response to an increased dose of corticosteroid with respect to FEV_1 from visit 1 to visit 2 is represented as a histogram **A**. and the individual differences **B**. Patients were considered responders if their levels decreased by at least 200 mL (0.2 L) from visit 1 to visit 2.

A



B

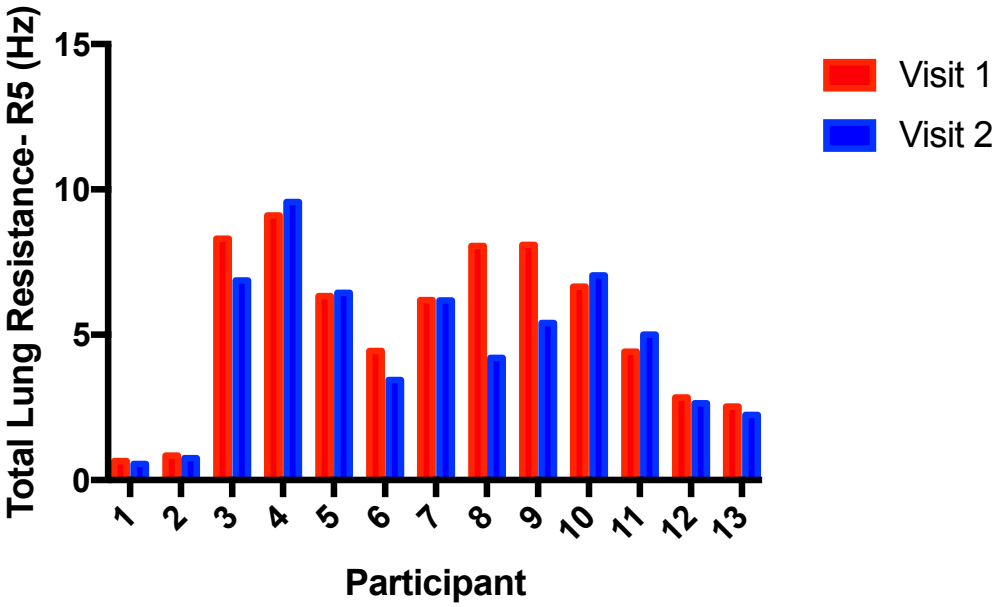
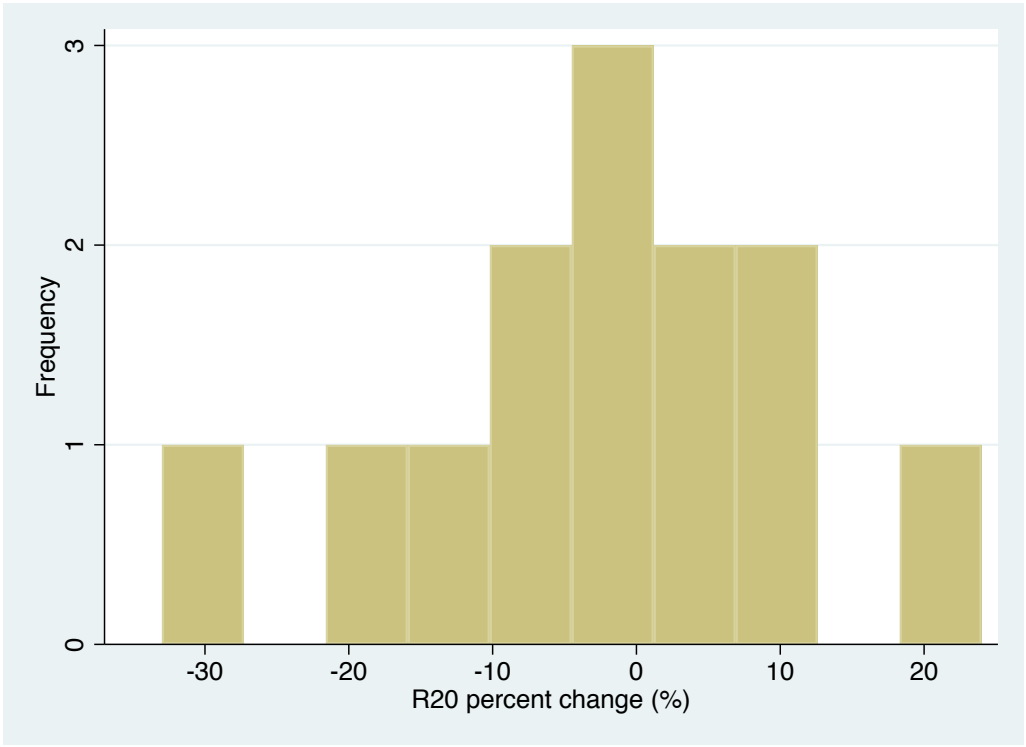


Figure 5.5 Total airway resistance at 5 Hz (R5)

Response to an increased dose of corticosteroid with respect to R5 from visit 1 to visit 2 is represented as a histogram for the percentage change **A**. and the individual differences of obtained values **B**. Patients were considered responders if their levels decreased by at least 30 % from visit 1 to visit 2.

A



B

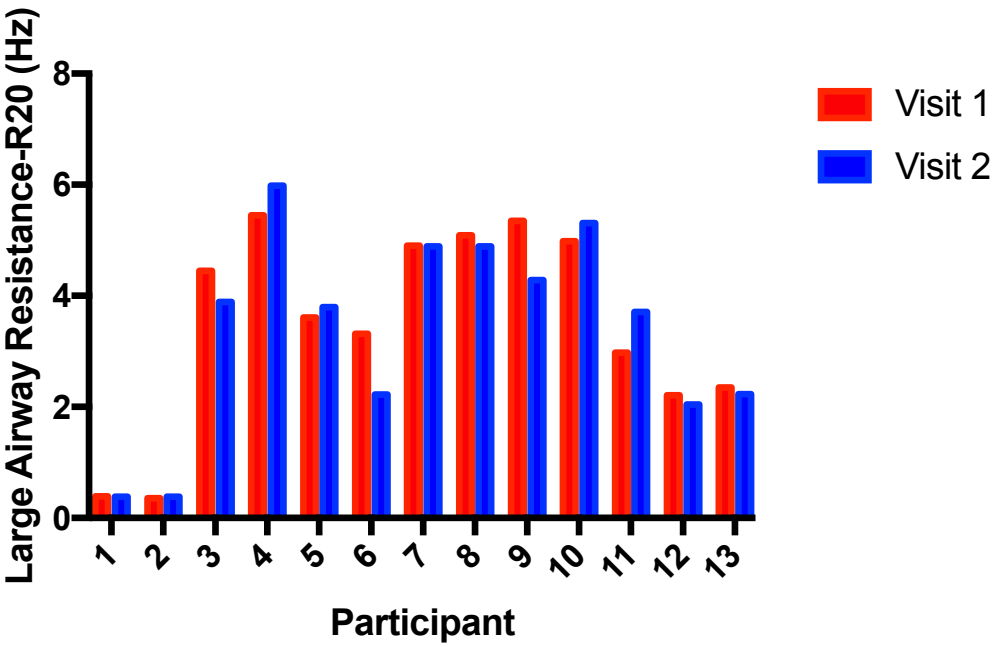
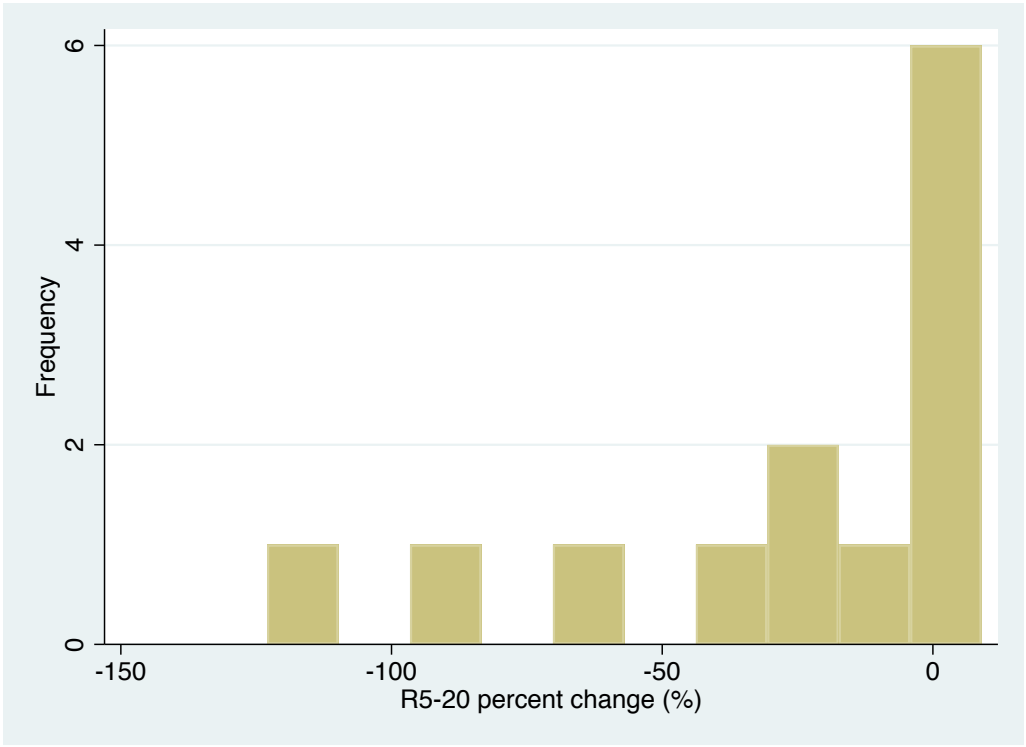


Figure 5.6 Large airway resistance at 20 Hz (R20)

The response to an increased dose of corticosteroid with respect to R20 from visit 1 to visit 2 is represented as a histogram for the percentage change **A**. and the individual differences of obtained values **B**. Patients were considered responders if their levels decreased by >10 % from visit 1 to visit 2.

A



B

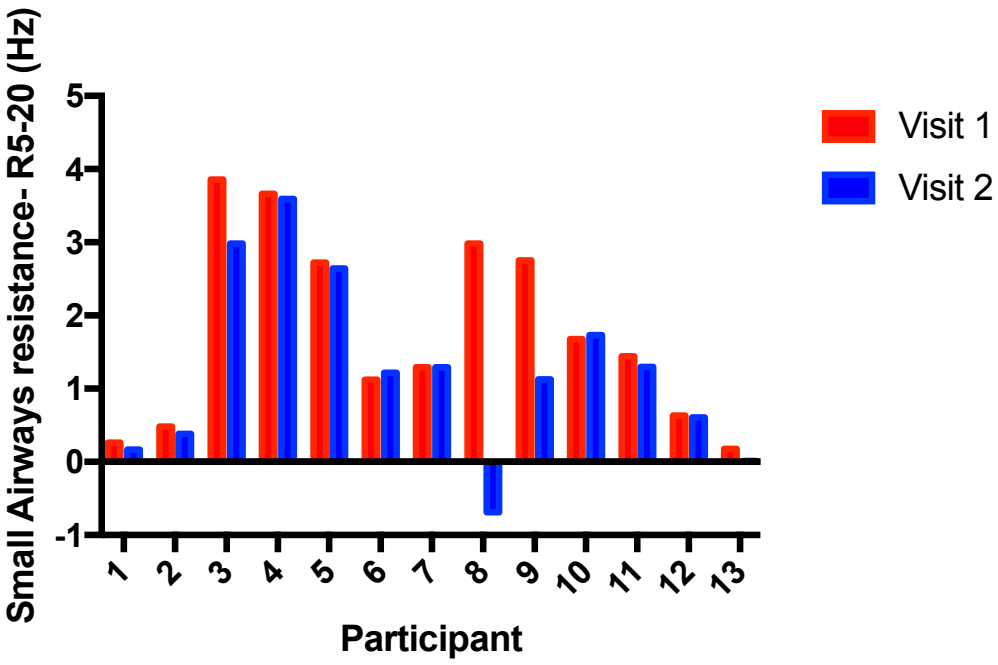
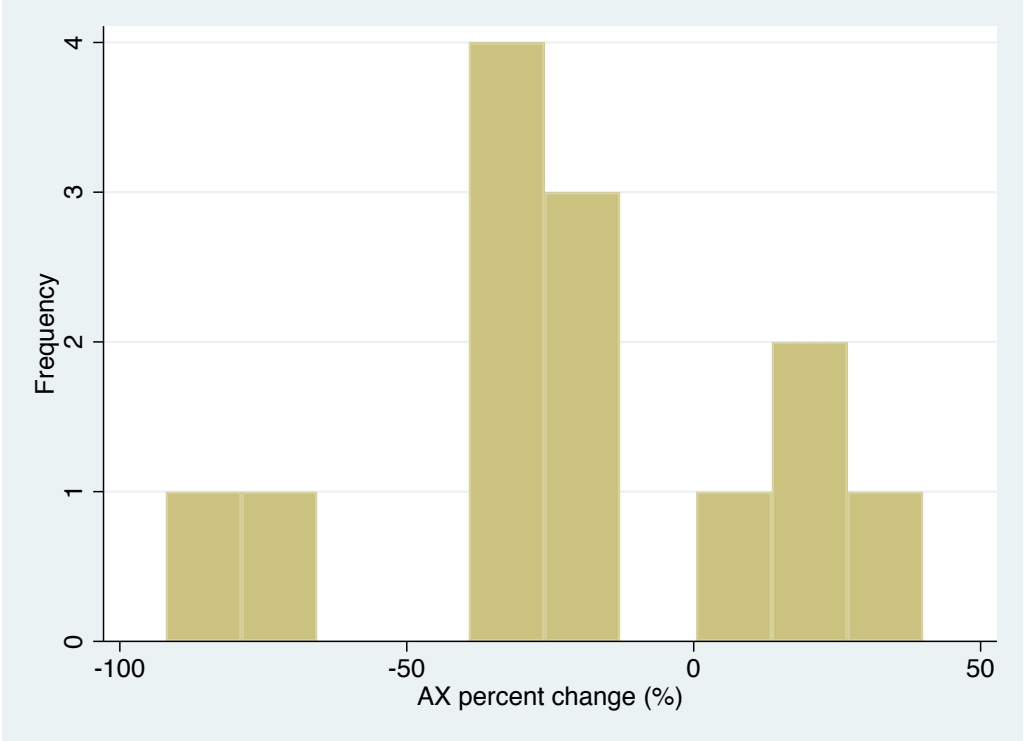


Figure 5.7 Small airway resistance at 5-20 Hz (R5-20)

The response to an increased dose of corticosteroid with respect to R5-20 from visit 1 to visit 2 is represented as a histogram for the percentage change **A**. and the individual differences of obtained values **B**. Patients were considered responders if their levels decreased by at least 50% from visit 1 to visit 2.

A



B

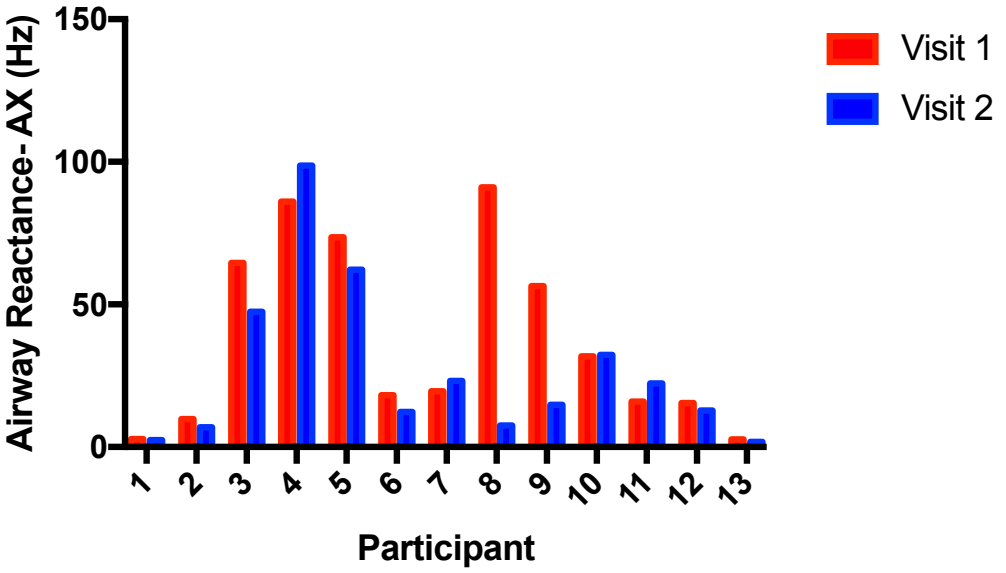


Figure 5.8 Airway reactance (AX)

The response to an increased dose of corticosteroid with respect to AX from visit 1 to visit 2 is represented as a histogram for the percentage change **A**. and the individual differences of obtained values **B**. Patients were considered responders if their levels decreased by at least 10 % from visit 1 to visit 2.

Table 5.4 Treatment response

The changes in each criterion are given as well as the overall mean and standard deviation (SD). Responders for each criterion based on the response thresholds and determined by the histograms are highlighted in yellow. ACQ- Asthma Control Questionnaire, FeNO- Fractional exhaled nitric oxide, FEV_1- Forced expiratory volume over one second, FVC- Full vital capacity, R- Resistance, AX- reactance.

Response Threshold	**≥-0.5**	**≥-0.1**	**>-10**	**≥0.2**		**≥-30**		**>-10**		**≥-50**		**≥-10**
Participant	**ACQ7**	**Blood Eosinophils (cells x10^9/L)**	**FeNO (ppb)**	**FEV1 (L)**	**R5 (Hz)**	**R5 % change**	**R20 (Hz)**	**R20 % change**	**R5-20 (Hz)**	**R5-20 % change**	**AX (Hz)**	**AX % change**
1	-0.30	-0.3	-59	0.01	-0.09	-14	-0.01	-3	-0.09	-35	-0.37	-13
2	-1.90	-1.4	-40	0.66	-0.08	-10	0.02	6	-0.10	-21	-2.87	-29
3	-0.40	-0.1	-6	0.32	-1.44	-17	-0.56	-13	-0.88	-23	-17.13	-27
4	-1.28	-1.1	0	0.07	0.46	5	0.53	10	-0.07	-2	12.57	15
5	1.00	-0.7	-23	0.14	0.11	2	0.19	5	-0.08	-3	-11.41	-16
6	0.10	-0.1	3	0.09	-1.00	-23	-1.10	-33	0.10	9	-5.87	-32
7	-0.14	-0.1	-7	0.12	-0.01	0	-0.01	0	0.00	0	3.67	19
8	-0.57	0.2	N/A	0.05	-3.86	-48	-0.20	-4	-3.67	-123	-83.46	-92
9	-3.50	-0.5	-41	1.13	-2.69	-33	-1.07	-20	-1.62	-59	-41.60	-74
10	1.02	0.0	0	0.04	0.39	6	0.33	7	0.05	3	0.44	1
11	-0.56	0.0	-16	0.10	0.59	13	0.73	24	-0.14	-10	6.39	40
12	-1.24	0.5	-17	0.01	-0.19	-7	-0.17	-8	-0.02	-3	-2.60	-17
13	-0.71	-0.2	2	0.02	-0.29	-11	-0.12	-5	-0.17	-94	-0.80	-30
Mean	-0.65	-0.29	-17	0.21	-0.62	-10.54	-0.11	-2.62	-0.51	-27.77	-11	-19.62
(SD)	1.19	0.52	20.15	0.33	1.32	17	0.54	14.34	1.06	40.65	25.51	35.75

Table 5.5 Sputum cell count

Four participants were able to give a sputum sample at visit 1, two of which were able to give a second sample at visit 2. Eosinophils decreased from visit 1 to visit 2 whereas there was an increase in neutrophils.

	Eosinophils (%)		Neutrophils (%)	
Participant	Visit 1	Visit 2	Visit 1	Visit 2
1	9.25	2.74	80.00	85.32
5	10.25	N/A	78.50	N/A
9	84.75	4.50	11.75	56.00
10	0.00	N/A	99.50	N/A

5.11.2.1 Inhaled vs. oral corticosteroids

In order to determine the effect that the route of delivery had on response to treatment ACQ7, blood eosinophils, FeNO and small airway FOT parameters were compared between those who had an increase in their ICS (N=4) vs OCS (N=9).

Mean ACQ7 scores decreased by a clinically significant amount in both the ICS and OCS treated patients (0.59 vs 0.68 points; p=0.9024) (**Figure 5.9**). There was a significant difference in the change in blood eosinophil levels in response to treatment between ICS and OCS. Blood eosinophils decreased by a mean of 0.48 cells $x10^9$/L in the OCS treated participants whereas there was a mean increase of 0.13 cells $x10^9$/L in the ICS treated participants (p=0.0483) (**Figure 5.10**). Mean FeNO levels decreased in both OCS and ICS (N=3 as one participant was unable to achieve the required technique) however, there was a greater decrease in the OCS treated participants (19.3 vs

10.0 ppb; p=0.5134) (**Figure 5.11**). Small airway parameters measured by FOT decreased with both treatments however, the decrease was greater in the ICS group than the OCS group. Small airway resistance (R5-20) decreased by 1.14 vs 0.24 Hz (p=0.1635) (**Figure 5.12**) and the area of reactance (AX) decreased by 24.88 vs 4.84 Hz respectively (p=0.2035) (**Figure 5.13**).

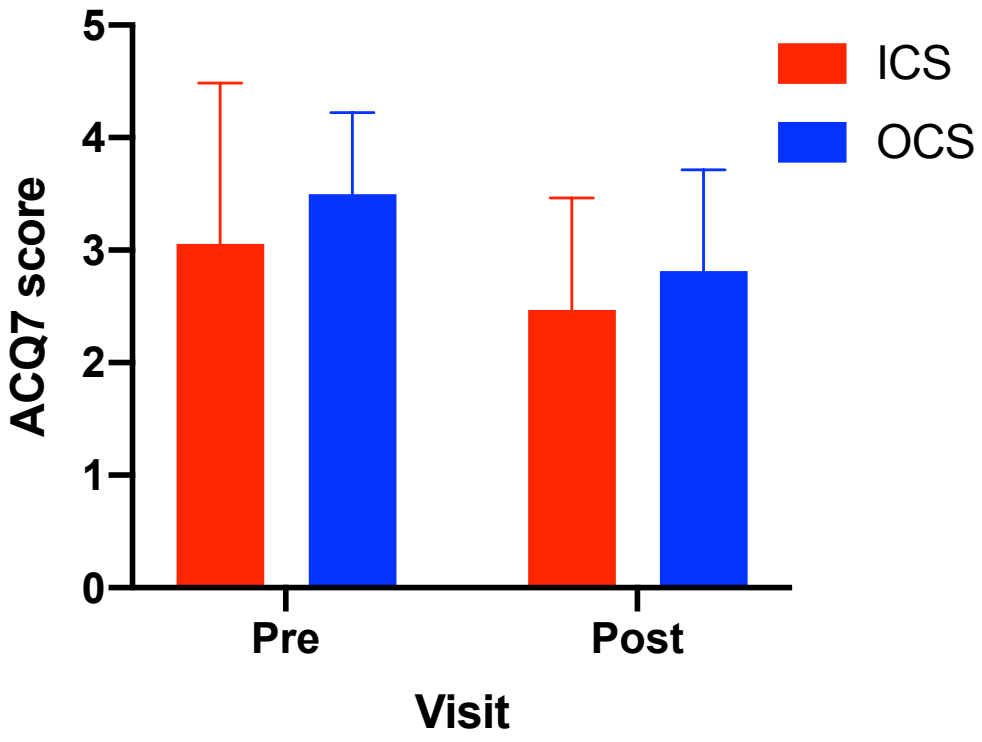


Figure 5.9 Asthma control questionnaire 7 (ACQ7)

ACQ7 scores decreased by a clinically significant amount in both the ICS (N=4) and OCS (N=9) treated participants. There was no statistically significant difference between the two groups (p=0.9024). Error bars represent the standard deviation.

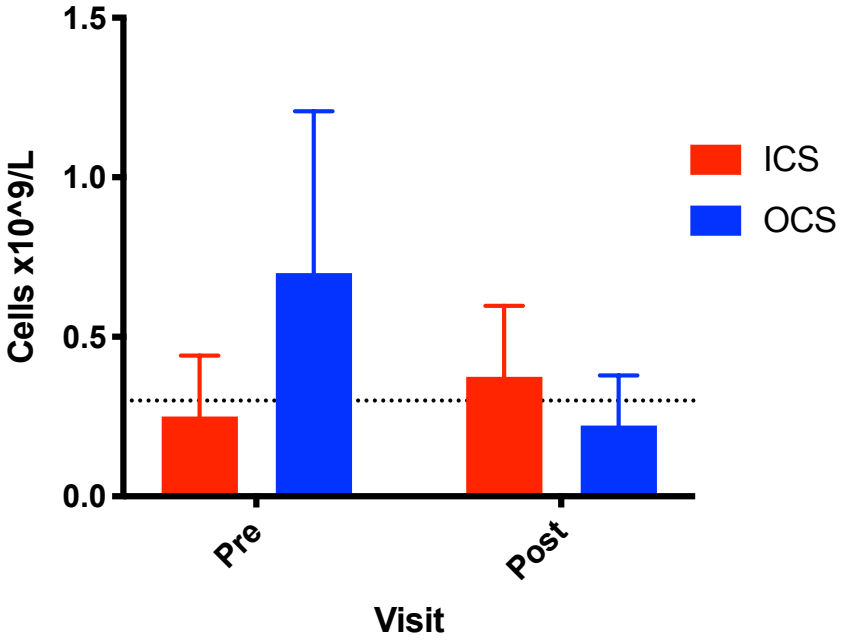


Figure 5.10 Blood Eosinophils

Mean blood eosinophils increased in participants treated with an increase in ICS (N=4) whereas there was a mean decrease in the OCS treated participants (N=9). The difference between the ICS and OCS treated participants was statistically significant (p=0.0483). The dashed line is at 0.3 cells $x10^9$/L which is the threshold of clinically elevated eosinophil levels. Error bars represent the standard deviation.

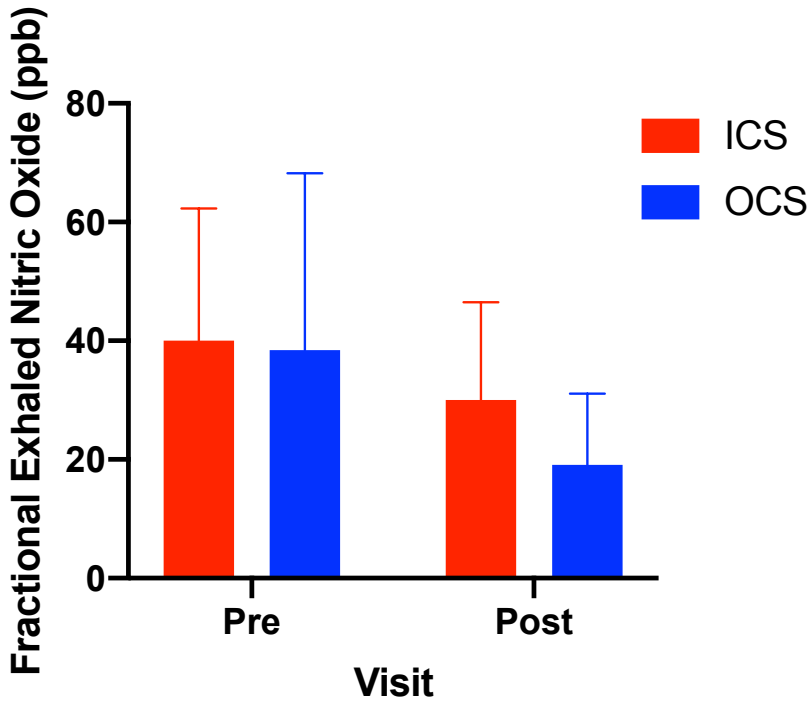


Figure 5.11 Fractional exhaled nitric oxide (FeNO)

Mean FeNO levels decreased in both ICS (N=3) and OCS (N=9) treated participants. The difference between the ICS and OCS treated participants was not statistically significant (p=0.5134). Error bars represent the standard deviation.

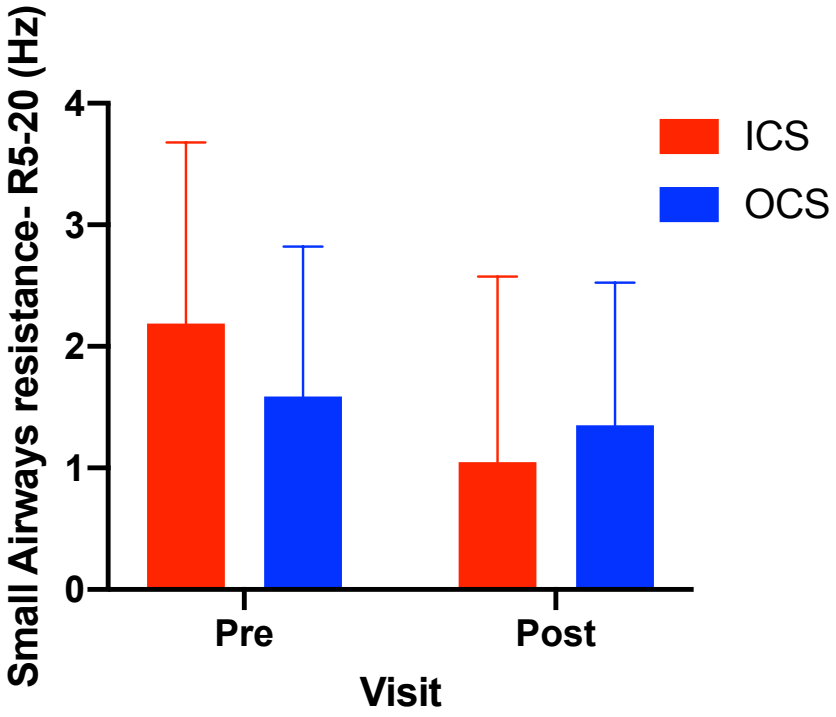


Figure 5.12 Small airway resistance (R5-20 Hz)

Mean R5-20 values decreased in response to treatment in both the ICS (N=4) and OCS (N=9) treated participants. The difference between the ICS and OCS treated participants was not statistically significant (p=0.1635). Error bars represent the standard deviation.

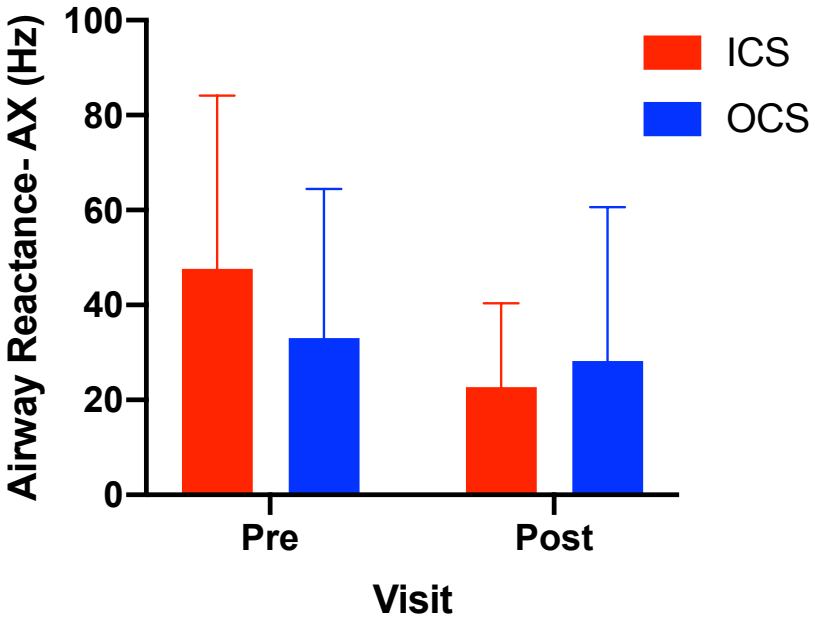


Figure 5.13 Airway reactance (AX)

Mean airway reactance values decreased in response to treatment in both the ICS (N=4) and OCS (N=9) treated participants. The difference between the ICS and OCS treated participants was not statistically significant (p=0.2035). Error bars represent the standard deviation.

5.11.2.2 Defining response to an increased dose of corticosteroid

Based on the observed changes following an increased dose of treatment it was determined that in order to be classified as a "responder" the participant must have demonstrated a significant subjective improvement (ACQ7 increase ≥0.5 points) plus either a significant clinical improvement in large airways (FEV_1 increase of ≥0.2 L) or small airway resistance (R5-20 decrease of >50 %). These criteria were selected to define response as a subjective improvement (ACQ7) reflects how the participant felt, FEV_1 is the standardized measurement of lung function and R5-20 reflects the region of the lungs not assessed by spirometry. A decrease in blood eosinophils or FeNO was not considered with respect to response as this change is considered to be a consequence of corticosteroid treatment rather than related to an improvement in the participant's condition.

5.11.2.3 Identifying responders to an increased dose of corticosteroid

Based on the criteria detailed above four participants (31 %) were classified as responders. Participants 2 and 9 demonstrated a significant subjective and large airway response (ACQ7 and FEV_1) while participants 8, 9 and 13 demonstrated a significant subjective and small airway response (ACQ and R5-20).

5.11.2.4 Baseline characteristics of responders to an increased dose of corticosteroid

Responders were all Caucasian, 50 % female and 75 % ex-smokers. Their mean age was 62.8 and they had been diagnosed with asthma

for a mean of 5.3 years. Three participants (75 %) were taking Fostair 100/6 two puffs twice daily as their maintenance ICS. The baseline and clinical features for the responders are summarized in **Table 5.6** and **5.7**.

Table 5.6 Baseline characteristics of the responders

BMI- Body mass index, OCS- oral corticosteroids, ICS- inhaled corticosteroids, M-male, F- female.

Participant	Treatment	Ethnicity	Sex	Age	BMI	Smoking status	Pack Years	Duration of asthma (years)	Current Asthma Treatment
2	OCS	Caucasian	M	61	33.5	Ex	53	7	Fostair 100/6, Carbocisteine 375mg, Uniphylline continus 200 mg, Ventolin, Ipratropium bromide 20 μg
8	ICS	Caucasian	F	63	36.4	Never	0	7	Fostair 100/6, Ventolin, Montelukast 10 mg, Azithromycin 250 mg, Carbocisteine 375 mg
9	OCS	Caucasian	M	70	27.7	Ex	50	7	Fostair 100/6, Ventolin, Montelukast 10 mg, Azithromycin 250 mg
13	OCS	Caucasian	F	57	18.1	Ex	17	0.08	Symbicort 200/6, Ventolin, Braltus 10 mcg
Mean				**62.8**	**28.9**		**30**	**5.3**	
Standard Deviation				**5.4**	**8.1**		**25.8**	**3.5**	

Table 5.7 Baseline clinical characteristics

Baseline clinical characteristics are given for the **A** responders and **B** non-responders. ACQ: Asthma Control Questionnaire, FeNO: Fractional exhaled nitric oxide, FEV_1: Forced expiratory volume over one second, FVC: Full vital capacity, R: Resistance, AX: reactance.

A

Participant	ACQ7	Blood Eosinophil (cells x10^9/L)	FeNO (ppb)	FEV_1 (L)	FEV_1 % Predicted	FEV_1/FVC (L)	R5 (Hz)	R20 (Hz)	R5-20 (Hz)	AX (Hz)
2	4.6	1.8	84	0.9	28	42	0.8	0.4	0.5	9.8
8	3.6	0.4	N/A	1.5	75	75	8.1	5.1	3.0	91.0
9	4.3	0.6	64	0.9	35	42	8.1	5.4	2.8	56.4
13	3.9	0.6	23	2.5	78	68	2.5	2.4	0.2	2.7
Mean	**4.1**	**0.9**	**57.0**	**1.4**	**54.0**	**56.8**	**4.9**	**3.3**	**1.6**	**40.0**
Standard Deviation	**0.5**	**0.6**	**31.1**	**0.7**	**26.2**	**17.3**	**3.8**	**2.4**	**1.5**	**41.6**

B

Participant	ACQ7	Blood Eosinophil (cells x10^9/L)	FeNO (ppb)	FEV_1 (L)	FEV_1 % Predicted	FEV_1/FVC (L)	R5 (Hz)	R20 (Hz)	R5-20 (Hz)	AX (Hz)
1	3.4	0.5	75	1.7	81	69	0.6	0.4	0.3	2.8
3	2.7	0.4	20	1.6	40	46	8.3	4.4	3.9	64.5
4	3.9	1.2	5	0.8	54	49	9.1	5.4	3.7	86.1
5	2.4	0.7	35	2.2	76	62	6.3	3.6	2.7	73.6
6	3.4	0.4	15	2.9	82	70	4.4	3.3	1.1	18.2
7	1.3	0.2	36	1.7	73	55	6.2	4.9	1.3	19.5
10	2.8	0.4	5	1.5	47	52	6.6	5.0	1.7	31.8
11	2.9	0.1	40	1.3	58	72	4.4	3.0	1.4	16.0
12	4.7	0	64	2.8	77	55	2.8	2.2	0.6	15.4
Mean	**3.0**	**0.4**	**32.8**	**1.8**	**65.3**	**58.9**	**5.4**	**3.6**	**1.8**	**36.4**
Standard Deviation	**0.9**	**0.4**	**24.6**	**0.7**	**15.8**	**9.7**	**2.6**	**1.6**	**1.3**	**30.1**

5.11.3 LONG TERM IMPACT

Of the four participants who responded to the increased dose of corticosteroids all had their maintenance dose of ICS increased as a result of their positive response. Two went on to receive an additional method of treatment, one started treatment with a biologic (omalizumab) and the other started azithromycin. All four participants are better controlled now than they were before entry into the study. Of the nine non-responders, two had their ICS dose increased as part of the study. Both participants remained on this higher dose despite the lack of response during the study. One exhibited long-term benefit and has improved lung function and reduced hospital visits the other participants' condition is unchanged. Seven non-responders were given a course of OCS as part of the study. Five of their treatments remained the same and there were no long-term changes to their conditions. Two participants were started on additional treatments. One participant started a biologic, mepolizumab, and is currently doing well. The other started treatment with azithromycin and has seen a dramatic improvement in lung function and asthma control.

5.11.4 SECONDARY OUTCOMES

5.11.4.1 Cytokine levels in blood serum and plasma

All participants had cytokine levels measured at visit one and all participants except one had their levels measured again at visit two. Of the 13 cytokines measured eight (IFN-γ, TNF-α, IL-17, IL-12, IL-4, TGF-β1, IL-10 and IL-5) were only of detectable levels in the serum of one participant (participant 8). This participant had detectable

levels of all but one cytokine (TGF-β1) at visit one and all were detectable at visit two. For this participant, the concentration of every cytokine with the exception of VEGF (which decreased very slightly from 86.5 to 48 pg/ mL) increased from visit one to visit two. TSLP was the most detected cytokine; detectable levels in eight participants at visit one and seven at visit two (**Table 5.8** and **Figure 5.14**).

Plasma samples were obtained from 11 participants at visit one and visit two. Of the 13 cytokines measured 8 (IFN-γ, TNF-α, IL-17, IL-12, IL-4, IL-10, IL-5 and IL-8) were only of detectable levels in the plasma of one participant (participant 8). TGF-β1 and VEGF were not detectable in any of the participants. Again, TSLP was the most detected cytokine; eight participants at visit one and seven at visit two (**Table 5.9** and **Figure 5.15**). For participant 8 the majority of cytokines increased from visit one to visit two except for IL-12, IL-1β, and IL-5.

5.11.4.2 Cytokine levels in sputum supernatant

A sputum sample was obtained from four participants (31 %) at visit 1, of these participants two of them also produced a sample at visit 2. Of the 13 cytokines measured seven (IFN-γ, IL-17, IL-12, IL-4, TGF-β1, IL-10 and IL-5) were not able to be detected in any samples. IL-8 was detected in all samples. Of the six cytokines that were detectable three of them were detected in more PBS than DTT samples (**Table 5.10** and **Figure 5.16**).

Table 5.8 Serum cytokine concentrations

Cytokine concentration (pg/mL) in blood serum are given for all 13 participants at visit 1 and 12 participants at visit 2. * Mean values are of detectable samples only

Participant	IL-4	IL-5	IL-13	TSLP	VEGF	IL-12	IFN-γ	TNF-α	IL-1β	IL-8	IL-17	IL-10	TGF-β1
1 V1	0	0	0	65.3	69.3	0	0	0	0	0	0	0	0
1 V2	0	0	0	97.2	106.1	0	0	0	0	0	0	0	0
2 V1	0	0	696.6	560.5	0	0	0	0	9.3	33.9	0	0	0
2 V2	0	0	716.8	667.9	0	0	0	0	19.1	0	0	0	0
3 V1	0	0	0	500	46	0	0	0	0	0	0	0	0
3 V2	0	0	0	954.7	0	0	0	0	0	0	0	0	0
4 V1	0	0	0	56.3	0	0	0	0	0	0	0	0	0
4 V2	0	0	0	0	0	0	0	0	0	0	0	0	0
5 V1	0	0	0	0	0	0	0	0	0	0	0	0	0
5 V2	0	0	0	0	0	0	0	0	0	0	0	0	0
6 V1	0	0	0	0	0	0	0	0	0	0	0	0	0
6 V2	0	0	0	0	0	0	0	0	0	0	0	0	0
7 V1	0	0	0	108.7	0	0	0	0	0	0	0	0	0
7 V2	0	0	0	150.1	0	0	0	0	0	0	0	0	0
8 V1	364.3	364.8	51025.2	21451	86.5	3234.7	2783.2	1181.9	4905.2	74	674.6	3633	0
8 V2	645.8	568.7	118334.5	29115.8	48	4778	3720.6	2184	10320.4	113.6	815.1	8544.8	199
9 V1	0	0	0	884.3	0	0	0	0	0	0	0	0	0
9 V2	0	0	0	489.6	0	0	0	0	0	0	0	0	0
10 V1	0	0	0	0	0	0	0	0	0	0	0	0	0
11 V1	0	0	0	305.5	0	0	0	0	8.4	0	0	0	0
11 V2	0	0	0	233.5	0	0	0	0	0	0	0	0	0
12 V1	0	0	0	0	0	0	0	0	0	0	0	0	0
12 V2	0	0	0	0	0	0	0	0	0	0	0	0	0
13 V1	0	0	524.2	0	39.9	0	0	0	0	0	0	0	0
13 V2	0	0	314.1	0	36.1	0	0	0	0	0	0	0	0
Detectable samples	2	2	6	15	7	2	2	2	5	3	2	2	1
Mean Visit 1*	364.3	364.8	17415.3	2991.5	39.9	3234.7	2783.2	1181.9	1641	54	674.6	3633	n/a
Mean Visit 2*	645.8	568.7	39788.5	4529.8	36.1	4778	3720.6	2184	5169.8	113.6	815.1	8544.8	199

Table 5.9 Plasma cytokine concentrations

Cytokine concentration (pg/mL) in blood plasma are given for all 13 participants at visit 1 and 12 participants at visit 2. * Mean values are of detectable samples only

Participant	IFN-γ	TNF-α	IL-17	IL-12	IL-4	IL-1β	TGF-β1	IL-10	IL-5	IL-8	VEGF	TSLP	IL-13
1 V1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	77.1	0.0
1 V2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	77.1	0.0
2 V1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	402.8	288.6
2 V2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	323.3	258.5
3 V1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1557.0	0.0
3 V2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1497.0	0.0
4 V1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4 V2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5 V1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5 V2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6 V1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6 V2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7 V1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	69.0	0.0
7 V2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	93.0	0.0
8 V1	3090.4	1295.1	358.9	1400.3	60714.8	1216.5	0.0	3673.8	734.5	247.4	0.0	11097.5	12364.0
8 V2	4681.8	2358.8	472.9	1316.6	89442.0	687.3	0.0	5105.5	536.4	434.3	0.0	12104.9	18641.6
9 V1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	635.2	0.0
9 V2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	770.3	0.0
10 V1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	30.7	0.0
11 V1	0.0	0.0	0.0	0.0	0.0	8.3	0.0	0.0	0.0	0.0	0.0	645.4	0.0
11 V2	0.0	0.0	0.0	0.0	0.0	9.5	0.0	0.0	0.0	0.0	0.0	642.0	235.2
13 V2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Detectable samples	2	2	2	2	2	4	0	2	2	2	0	15	5
Mean visit 1*	3090.4	1295.1	359.9	1400.3	60715.0	612.4	n/a	3674.0	734.5	247.4	n/a	1981.3	6326.3
Mean visit 2*	4682.0	2359.0	473.0	1316.6	89442.0	348.4	n/a	5105.0	536.4	434.3	n/a	2215.4	6378.5

Table 5.10 Sputum supernatant cytokine concentrations

Cytokine concentrations (pg/mL) in DTT and PBS are given for four patients at visit 1 and two patients at visit 2.

Participant	IFN-γ	TNF-α	IL-17	IL-12	IL-4	IL-1β	TGF-β1	IL-10	IL-5	IL-8	VEGF	TSLP	IL-13
1 V1 DTT	0.0	0.0	0.0	0.0	0.0	51.4	0.0	0.0	0.0	76.2	0.0	0.0	342.8
1 V1 PBS	0.0	24.1	0.0	0.0	0.0	1118.5	0.0	0.0	0.0	8405.1	143.5	75.1	0.0
1 V2 DTT	0.0	0.0	0.0	0.0	0.0	20.6	0.0	0.0	0.0	145.0	0.0	0.0	0.0
1 V2 PBS	0.0	0.0	0.0	0.0	0.0	173.0	0.0	0.0	0.0	5917.2	157.3	0.0	0.0
5 V1 DTT	0.0	0.0	0.0	0.0	0.0	5.4	0.0	0.0	0.0	2477.9	43.6	0.0	0.0
5 V1 PBS	0.0	0.0	0.0	0.0	0.0	5.9	0.0	0.0	0.0	2065.2	130.2	0.0	0.0
9 V1 DTT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	560.7	0.0	0.0	0.0
9 V1 PBS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	691.5	79.1	676.4	0.0
9 V2 DTT	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	579.4	0.0	0.0	0.0
9 V2 PBS	0.0	0.0	0.0	0.0	0.0	6.4	0.0	0.0	0.0	1699.9	136.0	148.0	0.0
10 V1 DTT	0.0	0.0	0.0	0.0	0.0	76.1	0.0	0.0	0.0	30698.5	0.0	0.0	0.0
10 V1 PBS	0.0	0.0	0.0	0.0	0.0	184.3	0.0	0.0	0.0	42841.1	163.2	0.0	0.0
Detectable samples (DTT)	n/a	0	n/a	n/a	n/a	4	n/a	n/a	n/a	6	1	0	1
Detectable samples (PBS)	n/a	1	n/a	n/a	n/a	5	n/a	n/a	n/a	6	6	3	0

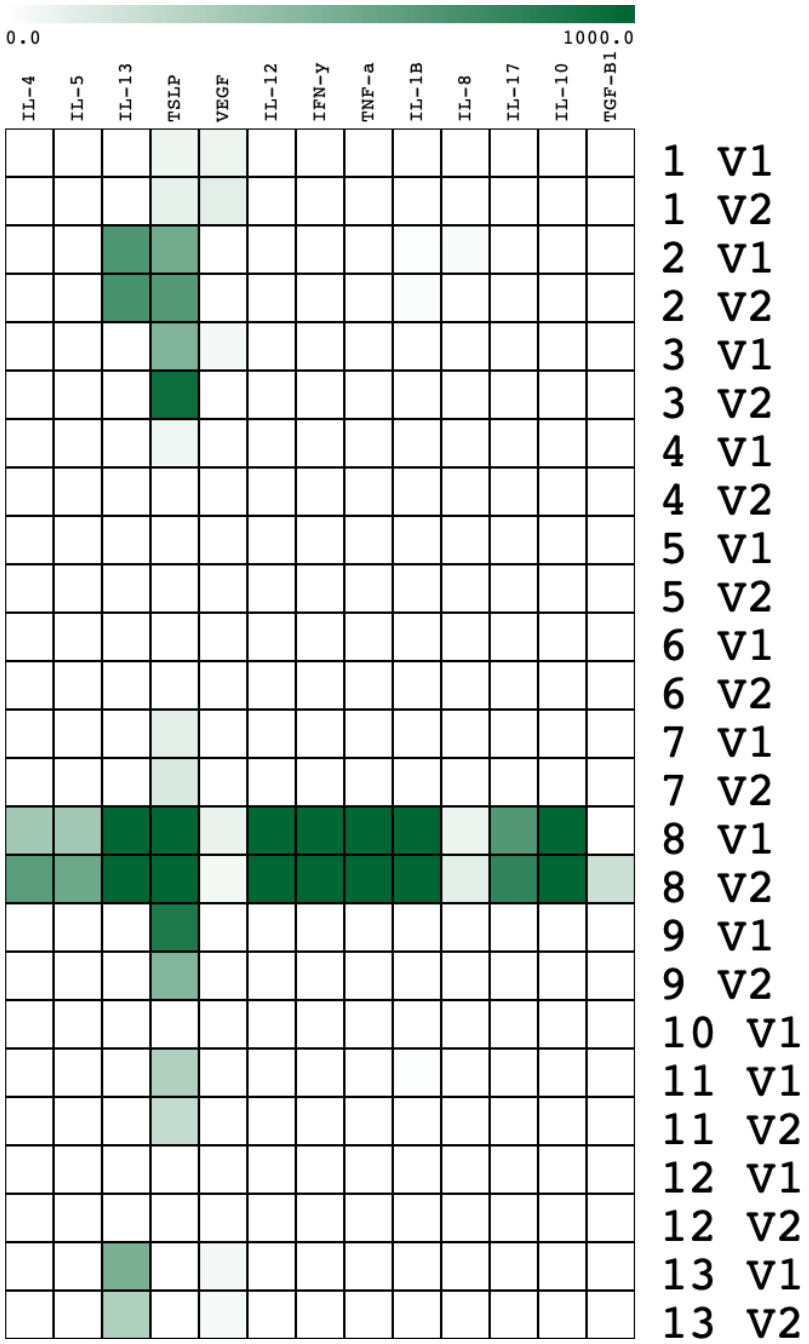


Figure 5.14 Serum cytokine profiles

Serum cytokine concentrations are represented for both visits on a scale from 0-1000 pg/mL where 0 pg/mL is white and the darkest green is 1000 pg/mL.

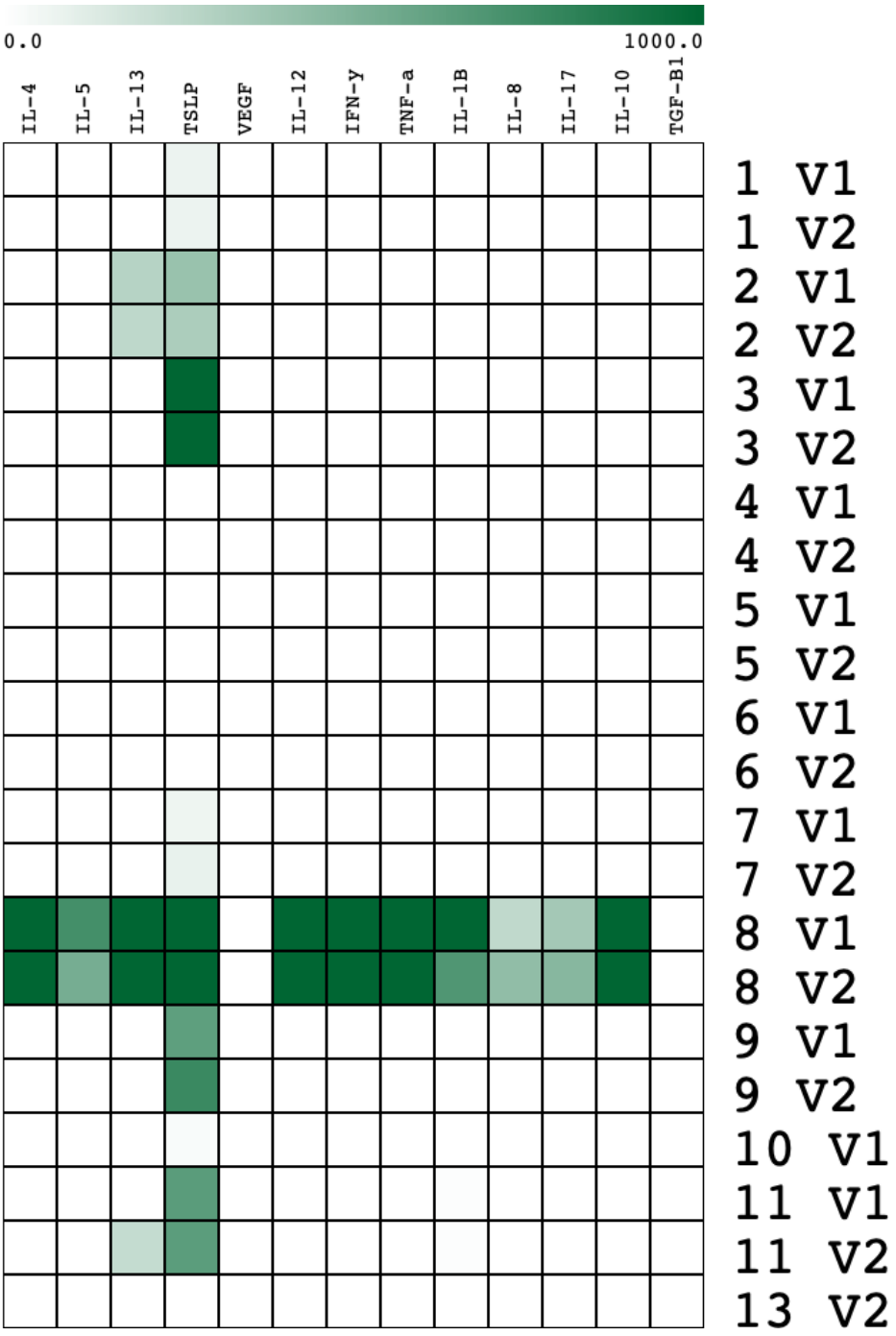


Figure 5.15 Plasma cytokine profiles

Plasma cytokine concentrations are represented for both visits on a scale from 0-1000 pg/mL where 0 pg/mL is white and the darkest green is 1000 pg/mL.

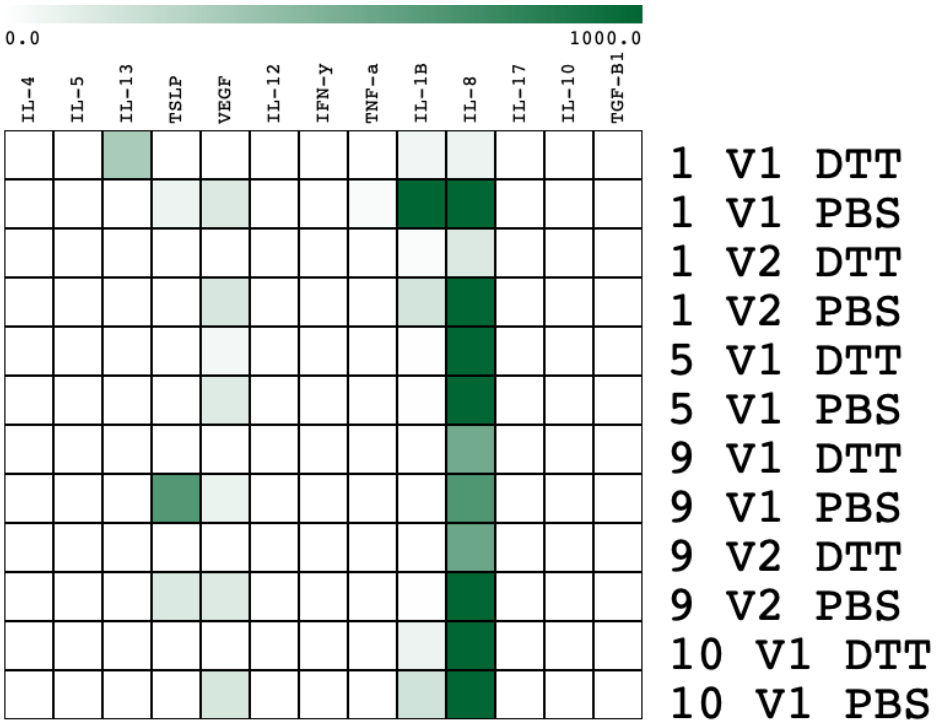


Figure 5.16 Sputum cytokine profiles

Cytokine concentrations in sputum supernatant are represented for both visits on a scale from 0-1000 pg/mL where 0 pg/mL is white and the darkest green is 1000 pg/mL.

5.12 DISCUSSION

Multiple predictive markers have been established for initiating treatment with an ICS (FEV_1/FVC, blood and sputum eosinophils, FeNO, and SABA reversibility) (296). However, very little has been done to establish markers of response to increased doses of corticosteroid. This study sought to define cut points of response to an increased dose of either oral or inhaled corticosteroid and to identify features that could distinguish the responders from the non-responders. The results of this study have identified four responders (31 %), two based on subjective (ACQ7) and large airway (FEV_1) improvements and three based on subjective (ACQ7) and small airway resistance improvements (R5-20) (one participant was a responder in both categories). Small airway resistance (R5-20) was chosen as the response criteria as opposed to reactance (AX) as several participants demonstrated an improvement in AX without an improvement in other areas and as such, we did not feel that AX accurately reflected a clinical change. FEV_1 is a well-established measure of lung function and is commonly used to assess response to treatment. However, FEV_1 only reflects the large airways. If FEV_1 had been the only measure of lung function assessed half of the responders would have been missed. This finding suggests that including a measure of small airway function in the clinical assessment of lung function and response to treatments may be beneficial.

Of the measured clinical features ACQ7, blood eosinophils and FeNO were significantly decreased following treatment. Few studies have

investigated the effects of adding OCS or increasing the dose of ICS however, these findings are consistent with a study by Oishi and colleagues who added oral prednisolone to patients (n=20) with persistent eosinophilia (FeNO ≥40 ppb and blood eosinophils ≥3 %) despite treatment with an ICS. They showed that 10 patients (50 %) responded to treatment, which they defined as both ≥20 % reduction in FeNO and blood eosinophil levels. They found that suppressing both FeNO and blood eosinophils was necessary to see an improvement in asthma control as measured with the ACQ (309). Response to treatment, in terms of ACQ7, FeNO, blood eosinophils, R5-20 and AX, was subdivided into those participants who had been prescribed an increase in their ICS vs OCS. The only significant difference in response between ICS and OCS was seen in blood eosinophils where the participants who were prescribed an increase in their ICS (N=4) exhibited a mean increase of 0.13 cells $x10^9$/L whereas there was a mean decrease of 0.48 cells $x10^9$/L in the OCS treated participants (N=9) (p=0.0483). This discrepancy could suggest a lack of adherence or poor inhaler technique in the ICS treated participants as eosinophil levels would be expected to decrease with corticosteroid treatment. Alternatively, this could simply reflect the difference in systemic corticosteroids versus local corticosteroids over a relatively short observation period with very low participation numbers. FOT measures of the small airways decreased in both the ICS and OCS treated participants, however, there was a greater decrease in those treated with an ICS. While the difference between the ICS and OCS treated participants did not reach statistical significance, it was

counterintuitive to find that the ICS treated participants displayed a greater decrease than those treated with OCSs as ICSs are not expected to be able to reach the small airways due to their relatively large particle size. However, as there were only four participants treated with an ICS further investigation is needed to determine if this is a consistent finding following an increase in ICS treatment.

Due to the limited number of participants in each responder category, it was difficult to determine baseline features that could predict response to an increased dose of corticosteroid. Interestingly 75 % of the responders were taking Fostair (beclomethasone/ formoterol) as their maintenance ICS. Although the beclomethasone in Fostair is claimed to be twice as potent as standard beclomethasone due to small particle size, Fostair 200 mcg twice daily is still low dose. As such, our main finding is that patients on low dose ICS are more likely to respond to a higher dose compared with those already on a moderate-to-high dose. (**Table 1.1**) (9).

It was interesting that only one participant had detectable levels of the majority of cytokines measured. As such, it appears that a severe clinical phenotype is not reflected in elevated levels of peripheral inflammatory cytokines. In contrast to this, a previous study found that corticosteroid resistance was linked with high levels of IL-17A and IFN-γ secreted by PBMC (310). Another study found that sputum T2 cytokine gene expression levels were higher in patients with asthma than in healthy controls and that these levels remained elevated despite treatment with an ICS (311).

5.12.1 MECHANISMS OF CORTICOSTEROID RESISTANCE

The majority of patients, 69 %, were not considered to have responded to the higher dose of corticosteroid. There are several possible pathologic mechanisms that could contribute to corticosteroid resistance.

The first is genetics. Microarray analysis of peripheral blood mononuclear cells (PBMCs) from patients with corticosteroid sensitive and resistant asthma revealed 11 genes that differed between the groups (312). In patients with asthma, functional polymorphisms of the gene glucocorticoid-induced transcript 1 (*GLCCI1*) have been associated with reduced responsiveness to ICS treatment and is linked with reduced inflammatory cell apoptosis (313). These studies along with the observation that resistance is more common in families suggests that it may be possible to develop a genetic test to determine response to corticosteroid treatment (314).

Second, The GR-β isoform which acts as a dominant negative inhibitor by competing with GR-α for binding sites on GREs, is induced by pro-inflammatory cytokines such as TNF-α, IL-1, IL-13 as well as IL-2 and IL-4 together (195). Some studies have reported increased levels of GR-β in steroid-resistant asthma patients, although this finding is controversial (315).

Third, modifications in histone acetylation can prevent gene transcription. The GR recruits and activates transcriptional co-activator proteins in order to alter gene expression with is mediated by changing chromatin structure via histone modifications (183).

Transcription co-activators such as CREB binding protein (CBP) have intrinsic HAT activity which increases gene transcription whereas hypoacetylation by histone deacetylases (HDACs) reduces gene transcription (184). In steroid responsive individuals GCs trigger acetylation however, a subset of steroid resistant patients have demonstrated normal GR nuclear translocation following GC binding but deficient acetylation which prevented gene transcription (173, 316). At low doses, theophylline inhibits PI3K which restores HDAC2 activity which improves overall response to the GCs both *in vitro* and *in vivo* and thus could be a potential treatment to combat resistance (317).

Fourth, in steroid responsive individuals GCs initiate the release of the regulatory cytokine, IL-10, from CD4 T cells which causes an increase in GR expression, however, IL-10 is underproduced in steroid resistance individuals (315). In these patients adding vitamin D3 to dexamethasone has been shown to enable CD4 cells to produce normal levels of IL-10. This suggests that adding vitamin D3 to the regime of steroid resistant individuals may help restore sensitivity (183).

Finally, Cigarette smoke is an oxidative stressor that affects the GRs ability to translocate into the nucleus. As such asthma patients who are current or ex-smokers are likely to be more steroid resistant than nonsmokers. Treatment with anti-oxidants and NO synthase inhibitors are possible options to restore sensitivity in these patients (318).

5.12.2 STUDY LIMITATIONS

This study has several limitations which need to be addressed.

First, the small sample number makes it very difficult to interpret results. Also, the limited number of patients were split between an increase in ICS and starting OCS treatment which could have affected the findings of the study. However, when response was separated into ICS and OCS the only statistically significant difference seen was in blood eosinophil levels.

Second, adherence was not strictly assessed. Participants were asked about their adherence and all claimed to have been adherent. As they were taking the increased treatment for a short period of time and were followed-up as part of the study it is likely that this is true. In support of this, FeNO and blood eosinophil levels tended to decrease as would be expected with an increased corticosteroid dose.

Third, blood eosinophil levels in the NCH pathology department are only recorded to one decimal point and as such smaller changes would have been missed.

Fourth, this study was not placebo-controlled. This was done for ethical reasons as it would have been unethical to give patients with symptoms requiring additional treatment a placebo. As there was no control group, we can't be sure that some of the clinical benefit seen following treatment was not due to placebo effect and therefore the response noted here may be slightly inflated. However, as placebo wasn't used it does make this study more relatable to routine clinical care.

Finally, sputum samples were not able to be obtained in the majority of participants (due to insufficient lung function, decline to be induced or inability to produce). As sputum cell counts reflect the local inflammation in the lungs directly this would have been a useful reflection of treatment.

5.12.3 SUMMARY

In conclusion, responses to an increased dose of corticosteroid were on a spectrum where one participant responded completely, one not at all and the majority of responses fell in the middle. The baseline features suggest that those participants who were on a relatively low daily dose of ICS were more likely to respond to the increased dose than those already on a high maintenance dose.

CHAPTER 6

AN EVALUATION OF RESPONSE TO TREATMENT WITH MEPOLIZUMAB IN PATIENTS WITH SEVERE ASTHMA

6.1 BACKGROUND INFORMATION

Asthma is a complex heterogeneous disease of the airways. Therefore, patients with different clinical and pathological characteristics respond differently to the available treatments. The majority of asthma patients are able to achieve control using conventional therapies, typically ICSs and bronchodilators, however, approximately 5-10 % of patients are considered to have severe disease and remain poorly controlled despite high-dose treatment (319). These patients have the highest risk of exacerbating which highlights the need for additional treatment options for these patients (320).

Patients who continue to have troublesome asthma despite high dose ICS treatment and have elevated eosinophil levels in their lung tissue, blood or sputum are considered to have severe eosinophilic asthma (SEA) (321). While eosinophils play an important role in the innate immune system fighting off infections and allergens (322), higher than normal concentrations have been linked with AHR and airway narrowing as their activation causes granular basic proteins and pro-inflammatory mediators to be secreted. This in turn results in inflammation, smooth muscle contraction and increased mucus production which damages the bronchial epithelium (323). As such, a major focus has been placed on targeting eosinophilic inflammation by antagonizing cytokines and/or their receptors that act on eosinophils. Many cytokines interact with eosinophils however IL-5 is the most relevant as it is involved with their development, maturation, recruitment, activation, survival and

proliferation (82, 242, 324). In addition to eosinophils, IL-5 is produced from CD4 Th2 cells, ILC2s, mast cells and basophils. IL-5 interacts with eosinophils, stimulates B cells to differentiate into antibody-producing plasma cells and upregulates the proliferation of eosinophil precursors (9, 324).

Mepolizumab (Nucala™) is a humanized IgG1/κ monoclonal antibody against IL-5 (82). It was the first biologic drug synthesized against IL-5 and is now approved for patients with SEA as an add-on treatment at step 5 of the GINA guidelines (9, 321). Mepolizumab inhibits the actions of IL-5 by binding to circulating IL-5 with high affinity and specificity (K_{on} 1.32-1.83 x 10^5, K_{off} 1.48-2.14 x 10^{-5}) preventing it from binding to its receptors on the surface of basophils, B cell precursors and eosinophils thereby preventing its downstream effects (322, 325). When mepolizumab was first trialed in asthma patients it did not improve lung function, reduce symptoms or exacerbation rates even though it was effective at reducing blood and sputum eosinophil levels (245, 326, 327). Following these reports, two studies determined that when mepolizumab was given to patients with persistent sputum eosinophilia (>3 %) and a history of exacerbations the rate of exacerbations was significantly reduced, the time to the first exacerbation was increased and patients were able to reduce their dose of oral corticosteroids (93, 328). This work highlighted the importance of utilizing biomarkers to ensure treatments are given to the most appropriate recipients. Since then, mepolizumab has been given to patients based on elevated blood

eosinophil levels and frequent exacerbations which has consistently resulted in reduced rates of exacerbations, a reduction in the maintenance dose of oral corticosteroids without negatively affecting lung function or symptoms and in some studies, it has also improved lung function (FEV_1) and symptoms (ACQ5) (208, 322, 329-331).

Currently, the National Institute for Health and Care Excellence (NICE) guidelines recommend mepolizumab as an add-on therapy only if the patient has had a blood eosinophil count of ≥300 cells/μL in the previous 12 months, ≥4 exacerbations requiring oral corticosteroids in the previous 12 month and/or requires continuous daily treatment with oral corticosteroids (≥5 mg/day of prednisolone or equivalent) for the past six months. According to these guidelines, a positive response to treatment can only be determined after 12 months and is based on a clinically significant reduction in exacerbations and/or oral corticosteroid dose without compromising asthma control (332). A "clinically significant reduction" is commonly interpreted as ≥50% and as such this was the response threshold utilized in this audit. However, the current criteria as recommended by NICE are not 100 % effective as some patients who meet these requirements do not respond to mepolizumab. There is a significant cost associated with treatment and it requires a considerable time commitment from the patient as it is administered every four weeks by injection at a specialty clinic. As such, it would be beneficial to be able to identify the patients who will respond to treatment prior to one year and ideally before treatment is started.

The primary goals of this audit were to determine if there are any clinical or biological features that could be used to distinguish between the responders and non-responders prior to one year.

6.2 AIMS OF THE AUDIT

The primary aim of the audit was to describe the features of the responders and non-responders to treatment with mepolizumab and determine if response could be predicted prior to completing one year of treatment.

To accomplish this phenotypic characteristic were described at baseline, one month, three months, six months and one year of treatment.

The secondary aim was to describe the pattern of response to mepolizumab in terms of symptoms (ACQ, ACT), lung function (spirometry, FOT) and inflammation (blood eosinophils, FeNO, systemic cytokine levels).

6.3 STUDY DESIGN

An observational audit was performed in conjunction with an NHS clinic and an observational study performed by GlaxoSmithKline (GSK) entitled: A multinational, single-arm, observational study to evaluate the real-world effectiveness and pattern of use of mepolizumab in patients with severe eosinophilic asthma (204710). The NHS covered the cost of the mepolizumab injections, GSK provided the funds for the study and

the audit was financed with funds held within the Department of Respiratory Medicine at NCH.

6.4 ETHICAL APPROVAL

Blood samples obtained for research as part of the audit were done so through the Nottingham Health Science Biobank. All participants were provided with a PIS from the biobank and provided written consent. All other procedures were conducted as part of clinical care and did not require consent.

6.5 STUDY SETTING AND PARTICIPANT RECRUITMENT

Severe asthma patients from across Nottinghamshire, Derbyshire and Lincolnshire who were recommended by their clinician for a severe asthma assessment were individually discussed at a multidisciplinary team (MDT) meeting held at NCH on a monthly basis. If the MDT recommended a one year trial of mepolizumab, they were automatically included in the observational audit if they met the criteria detailed below and were offered additional blood analysis with written consent for blood storage.

6.5.1 INCLUSION CRITERIA

The inclusion criteria for the audit were:

1. Diagnosis of severe asthma
2. Approved for treatment with mepolizumab

6.5.2 EXCLUSION CRITERIA

The exclusion criteria for the audit were:

1. Inability to perform more than two of the audit requirements
2. Failure to attend more than one monthly injection

6.6 STUDY INTERVENTION

All participants received a 100 mg subcutaneous injection of mepolizumab every four weeks for one year.

6.7 OUTCOME MEASURES

6.7.1 PRIMARY OUTCOME

The primary outcome was to:

A. describe the pattern of response to treatment over one year and to

B. identify the patients who did and did not demonstrate a clinical response of a ≥50 % reduction in the number of exacerbations from the previous year and/or a ≥50 % reduction in their maintenance dose of oral corticosteroid from baseline.

6.7.2 SECONDARY OUTCOMES

The secondary outcomes were to describe the phenotypic characteristics at baseline, one month, three months, six months and one year to determine if it was possible to use these characteristics to distinguish between the responders and non-responders prior to one year.

6.8 SAMPLE SIZE

As this was an observational audit a formal power calculation could not be performed. Every patient that was approved by the MDT and consented to treatment was given mepolizumab. All patients that met the inclusion and exclusion criteria were included in the audit. We aimed to have as many of these patients as possible consent to have blood stored in the biobank.

6.9 PROTOCOL

Patients who started treatment between May 17th, 2017 and January 16th, 2019 were included in the audit. Serum samples were analyzed for patient visits conducted between May 17th, 2017 and August 2nd, 2018. Patients from the NHS mepolizumab clinic were provided with a PIS from the biobank. They were provided with time to thoroughly read through the information and ask questions. Following this, anyone who agreed to blood storage completed the consent form.

At each visit, venepuncture was performed followed by the mepolizumab injection. After the injection, they were required to remain in the unit for two hours following the first four injections. During this time, they completed symptom questionnaires (ACQ, ACT), performed FeNO and FOT followed by spirometry.

Mepolizumab injections were given every four weeks in the NRRU in NCH. Audit procedures were performed only during visits one (baseline), two

(one month), four (three months), seven (six months) and thirteen (one year).

After one year of treatment, the MDT met again to determine if the patient had responded to the treatment. If they responded treatment was continued and if not, treatment was to be terminated. An overview of the information collected is presented in **Table 6.1**.

Table 6.1 Audit schedule of events

ACQ- Asthma Control Questionnaire; ACT: Asthma Control Test; FeNO: Fractional Exhaled Nitric Oxide; OCS: Oral Corticosteroid; * Weight was re-recorded throughout the year on a per patient basis if required;

Procedure	Baseline	1 month	3 months	6 months	1 year
Medical history	X				
Exacerbation history (past 12 months)	X				
Height	X				
Weight*	X				
Medications list	X				
Venepuncture	X	X	X	X	X
ACQ (5-7)	X	X	X	X	X
ACT	X	X	X	X	X
Spirometry	X	X	X	X	X
Oscillometry	X	X	X	X	X
FeNO	X	X	X	X	X
OCS dose check	X	X	X	X	X
Exacerbation check	X	X	X	X	X

6.10 ANALYSIS

Step 1. Identify responders and non-responders at 12 months based on a ≥50 % reduction in the number of exacerbations and/or maintenance dose of OCS.

Step 2. Determine if the patients who were labeled as responders after 12 months could have been identified earlier (i.e. at baseline or after one, three or six months of treatment)

Step 3. Identify baseline characteristics and/or early signs of response that could distinguish the responders from the non-responders.

All versions of the ACQ were analyzed. The ACQ7 was answered whenever possible as this version is the most accurate reflection of asthma control (260). However, some patients did not want to or were unable to perform spirometry at every visit. Therefore, the observation numbers of this version tended to be the lowest. The ACQ 6 was included as this version also includes a question regarding rescue bronchodilator use and has still been shown to be a reliable measure of asthma control. The ACQ 5 was included as a purely subjective measure of asthma control and had the highest number of responses (260).

T-tests (STATA / SE 15.1) were conducted in order to determine if there was a significant difference between responders and non-responders at each visit. The significance level was set at $p<0.05$ with 95% confidence intervals. Paired T-tests were used to analyze treatment effect whereas two-sample t-tests were used to compare the responders and non-responders.

GraphPad Prism V7 was used to produce eight-point standard curves which were used to interpolate individual cytokine levels. A cubic spline fit using 1000 points was used to find the best fit for each standard curve. Heat maps were then created in Multi Experiment Viewer (MeV_4_8) to visualize the results.

6.11 RESULTS

Between May 2017 and January 2019, 51 patients were started on mepolizumab and met the inclusion criteria for the audit. Of these patients, three were lost to follow up (two following the first injection and one after the second injection), one patient had an allergic reaction and became pregnant and was therefore stopped after the first injection. Forty-seven patients completed six months of treatment. Between six months and one year, two patients withdrew themselves from treatment as they felt they were not benefitting, and one patient was withdrawn based on a joint decision between themselves and their clinician also because they felt they were not benefitting. Twenty-three patients completed the 12 months of treatment by January 2019 (**Figure 6.1**).

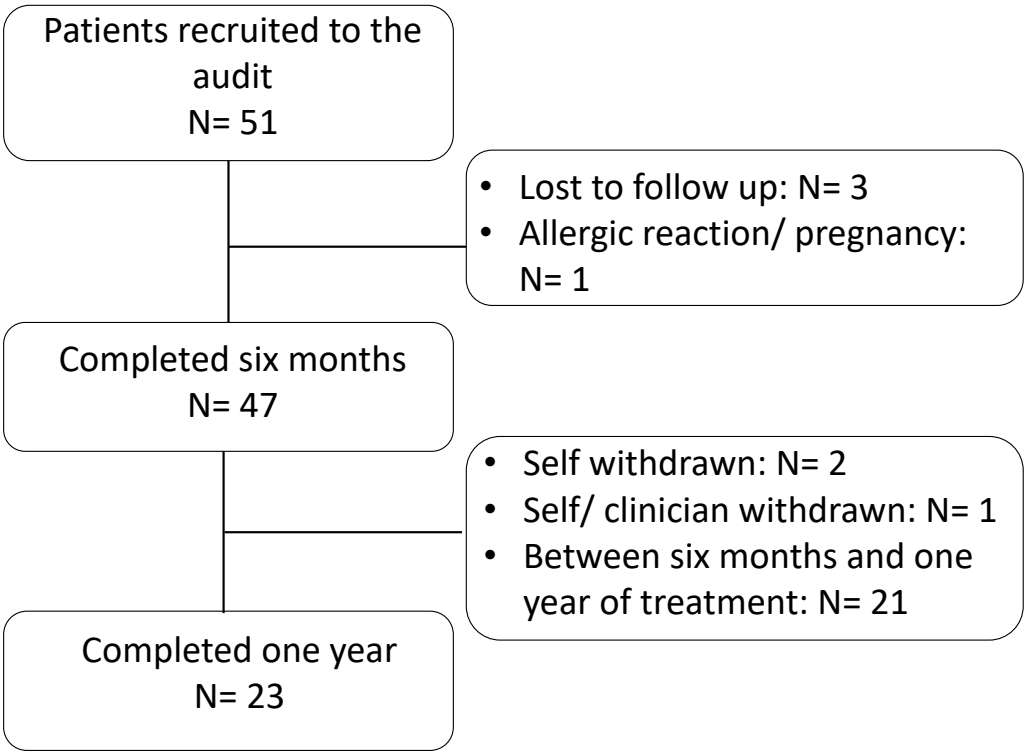


Figure 6.1 Consort diagram

Fifty-one patients were recruited to the audit. Of these 47 completed six months of treatment and 23 completed one year of treatment between May 2017 and January 2019.

6.11.1 BASELINE DEMOGRAPHICS

The baseline demographics and clinical characteristics for all 47 patients who completed six months of treatment are summarized below (**Table 6.2** and **6.3**). The majority of patients were female (60 %), never smokers (62 %), Caucasian (94 %) and were classified as obese (61.7%, mean BMI 34.2 **Figure 6.2**). In addition to severe asthma, the majority of patients also had rhinitis (82 %), GORD (62 %) and a positive skin prick test (55 %). The mean age at diagnosis was 24.3 years and the mean duration of diagnosis prior to starting mepolizumab was 23.6 years.

There was considerable variability between patients with respect to the clinical characteristics measured, this can be visualized in **Table 6.4** and **Figure 6.3**. Patients tended to have elevated blood eosinophils (mean 0.42 cells $x10^9$/L) and FeNO (mean 50.8 ppb). According to the ACQ5-7 the majority of patients had poorly controlled asthma (mean 3.43, 3.38, 3.45 respectively). The mean FEV_1 was 1.99 L (63.84 % predicted) and the mean FEV_1/FVC was 0.61. The mean oscillometry values were: R5; 5.95 Hz, R20; 3.97 Hz, R5-20; 1.98 Hz and AX; 42.76 Hz. During the previous 12 months, 45 (96 %) had experienced an exacerbation (mean 7.28 /year) and 28 (60 %) were taking a daily maintenance dose of OCS (mean 10.40 mg/ day).

Table 6.2 Baseline patient demographics

Demographic	Observations	Number (%)
Sex (female)	47	28 (60)
Ethnicity	47	
Caucasian		44 (94)
Asian		3 (6)
Smoking status	47	
Never		29 (62)
Ex		16 (34)
Current		2 (4)
Rhinitis	45	37 (82)
GORD	45	28 (62)
Positive skin prick test	44	24 (55)

Table 6.3 Baseline patient characteristics

BMI: Body Mass Index

Demographic	Observations	Mean	Standard Deviation	Range
Age (yrs)	47	48.0	12.1	21-70
BMI	47	34.2	7.8	20.50-54.59
Age at diagnosis (yrs)	46	24.3	19.7	1-60
Duration of asthma diagnosis (yrs)	46	23.6	15.5	2-55
pack years	18	19.9	16.5	2.5-60

Table 6.4 Baseline clinical characteristics

FeNO: Fractional Exhaled Nitric Oxide, ACQ: Asthma Control Questionnaire, ACT: Asthma Control Test, FEV_1: Forced Expiratory Volume Over one Second, PEF: Peak Expiratory Flow, FEV_1/FVC: Forced Expiratory Volume Over one Second/ Forced Vital Capacity, R: Resistance, AX: Airway Reactance, OCS: Oral Corticosteroid, *: One patient was receiving 80 mg monthly triamcinolone injections in addition to OCS

Clinical Characteristic	Observations	Mean	Standard deviation	Range
Blood Eosinophils (cells x10^9/L)	45	0.42	0.33	0-1.80
FeNO (ppb)	44	50.80	32.50	7-121.00
Questionnaires				
ACQ5	47	3.43	1.25	0.20-5.20
ACQ6	46	3.38	1.26	0.17-5.17
ACQ7	39	3.45	1.17	0.43-5
ACT	46	10.70	4.71	5.0-24.0
Spirometry				
FEV_1 (L)	40	1.99	0.65	0.79-3.54
FEV_1 % predicted	40	63.84	19.10	29.61-103.47
FEV_1/FVC	40	0.61	0.15	0.30-0.85
PEF (L/ min)	40	346.50	119.97	150.00-586.00
Oscillometry				
R5 (Hz)	46	5.95	2.10	2.38-11.85
R20 (Hz)	46	3.97	1.16	2.02-7.24
R5-20 (Hz)	46	1.98	1.36	0.10 - 4.81
AX (Hz)	46	42.76	38.45	3.05-165.65
Exacerbation in the previous 12 months	45	7.28	5.00	0.00 - 30.00
Maintenance dose of OCS* (mg/day)	28	10.40	12.23	0.00 - 50.00

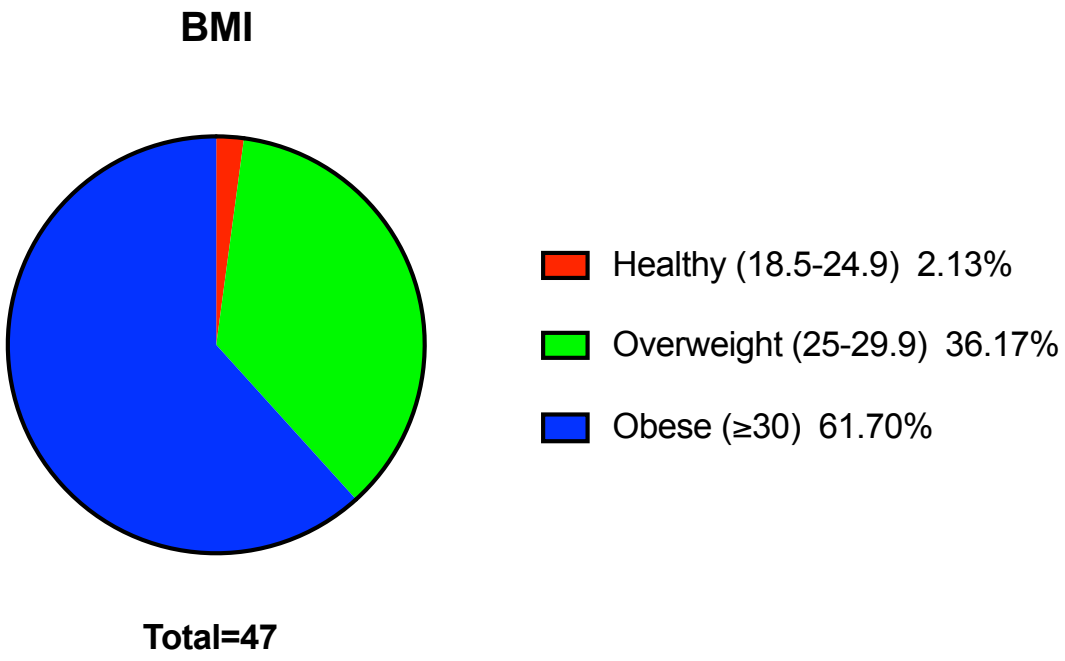
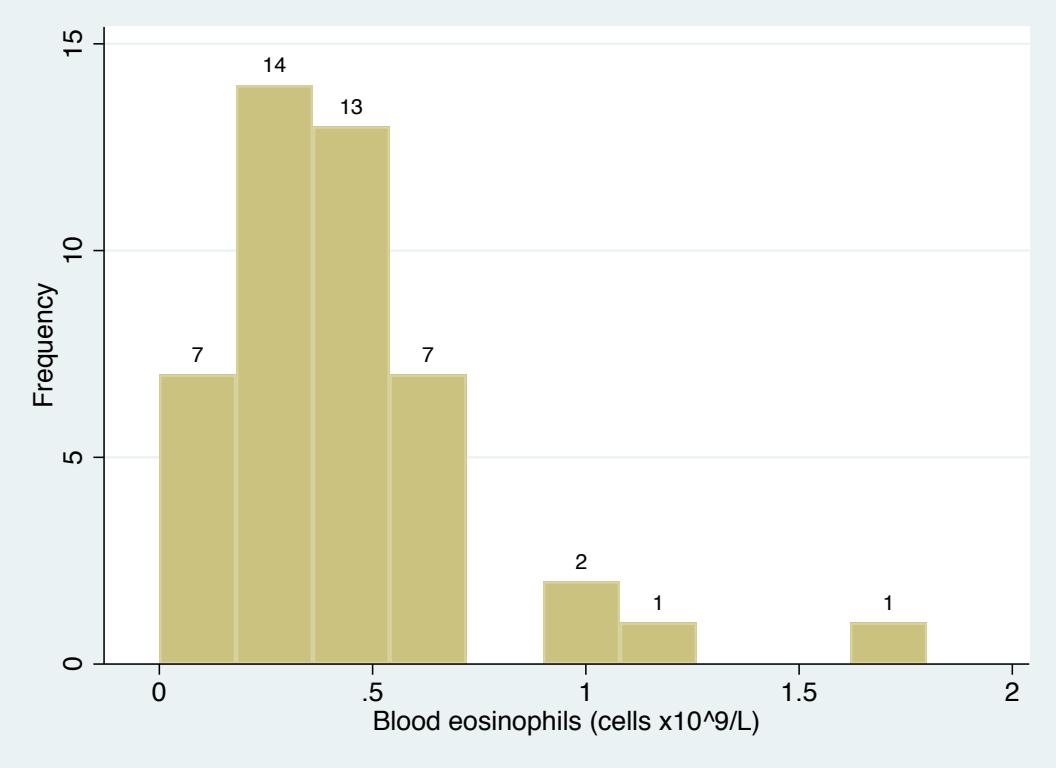


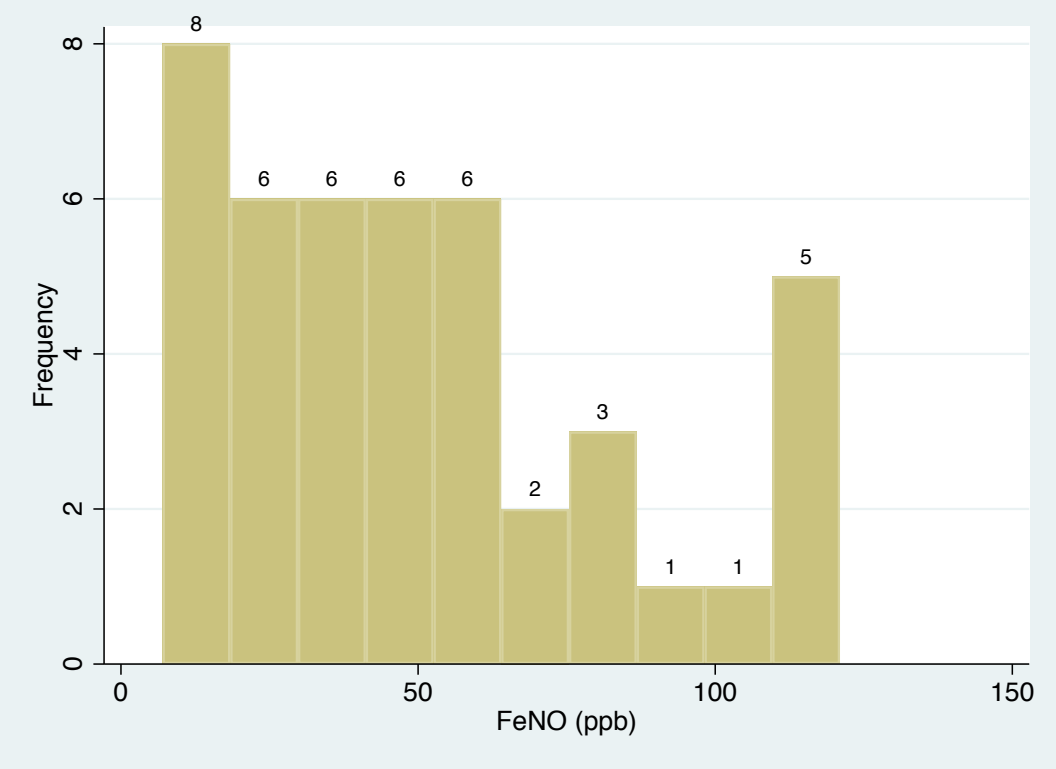
Figure 6.2 Body mass index (BMI) at baseline

At baseline none of the patients were underweight (BMI <18.5), one patient (2.13 %) was of a healthy weight (BMI 18.5-24.9), 17 patients (36.17 %) were overweight (BMI 25-29.9) and the majority of patients (29; 61.70 %) were obese (BMI ≥30).

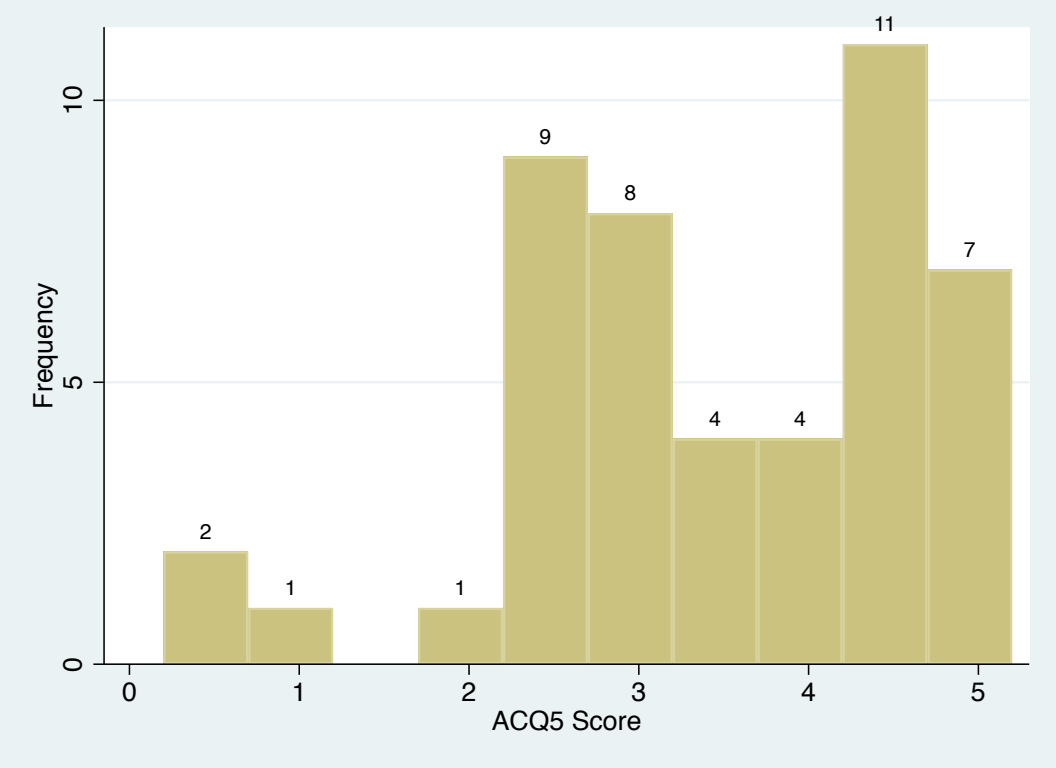
A


Frequency
0
5
10
15
7
14
13
7
2
1
1
0
.5
1
1.5
2
Blood eosinophils (cells x10^9/L)

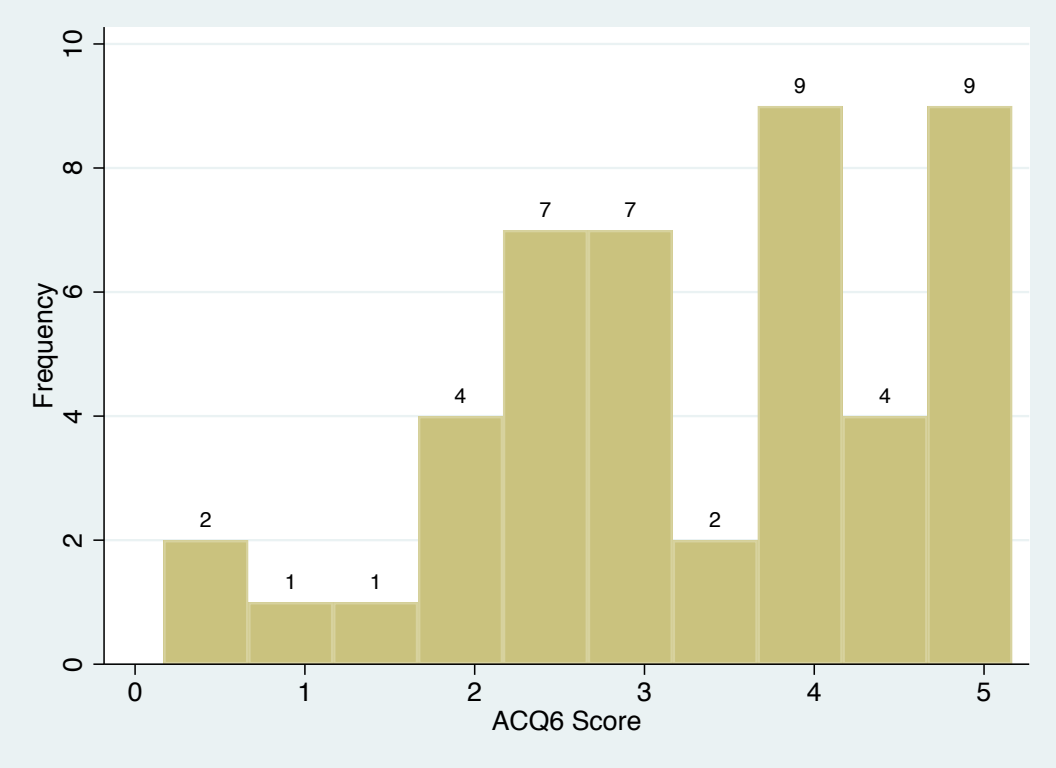
B


Frequency
0
2
4
6
8
8
6
6
6
6
2
3
1
1
5
0
50
100
150
FeNO (ppb)

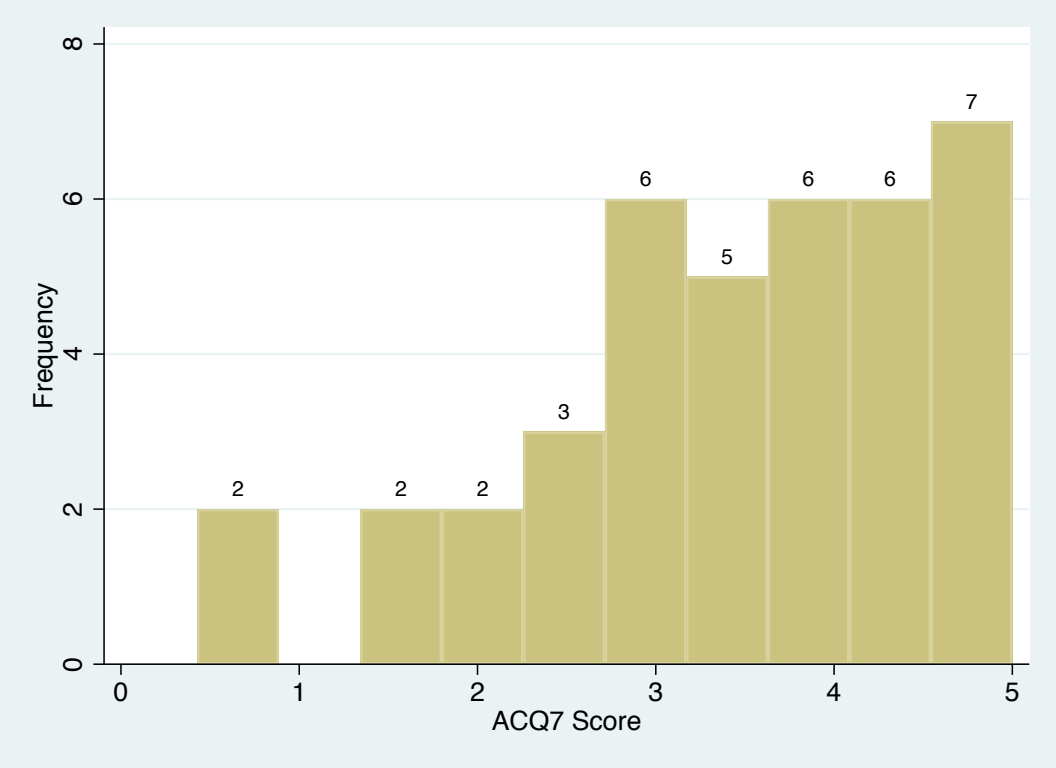
C


Frequency
0
5
10
2
1
1
9
8
4
4
11
7
0
1
2
3
4
5
ACQ5 Score

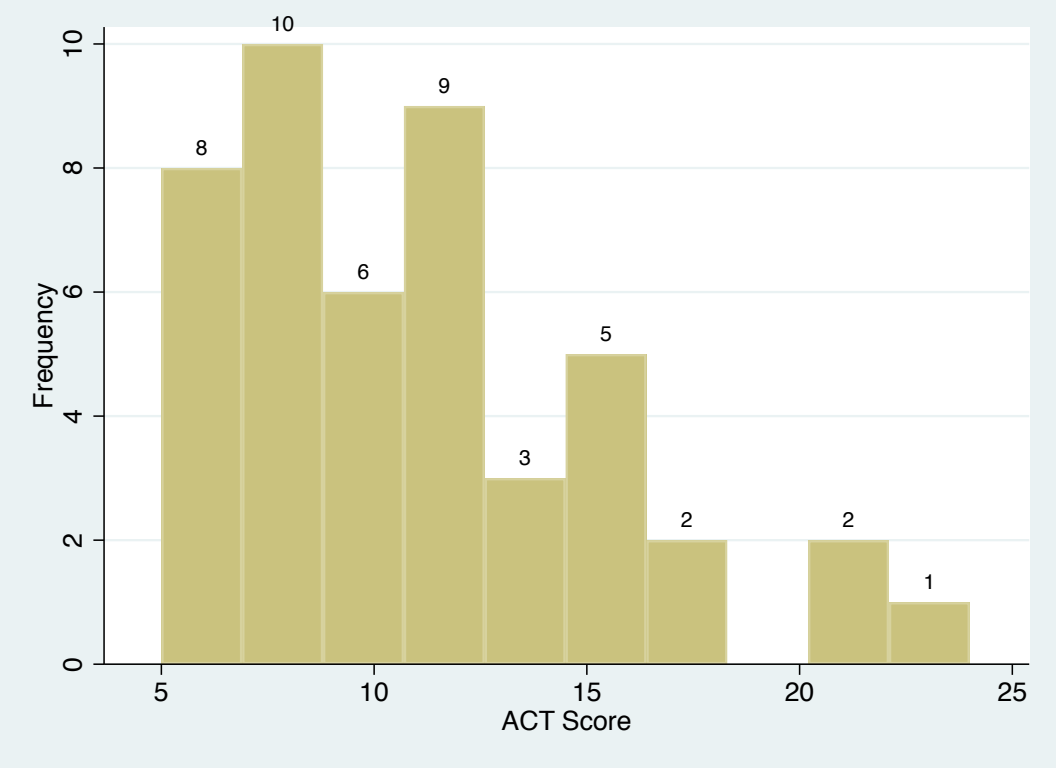
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Frequency
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2
4
6
8
10
2
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1
4
7
7
2
9
4
9
0
1
2
3
4
5
ACQ6 Score

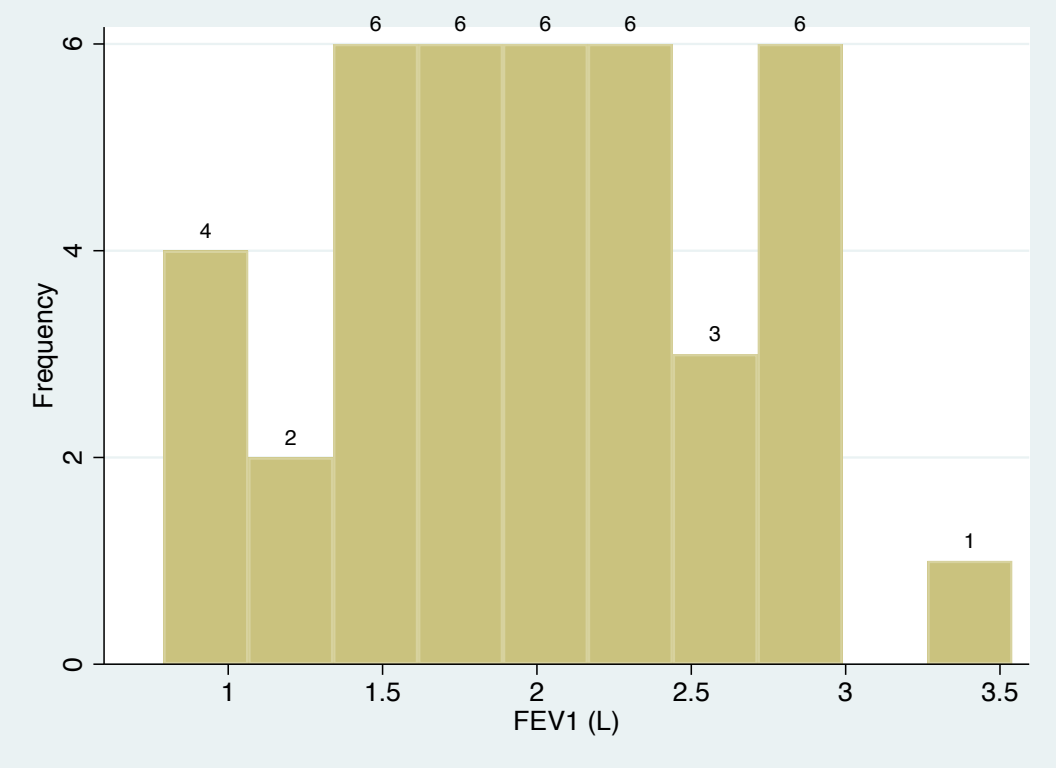
E


Frequency
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2
4
6
8
2
2
2
3
6
5
6
6
7
0
1
2
3
4
5
ACQ7 Score

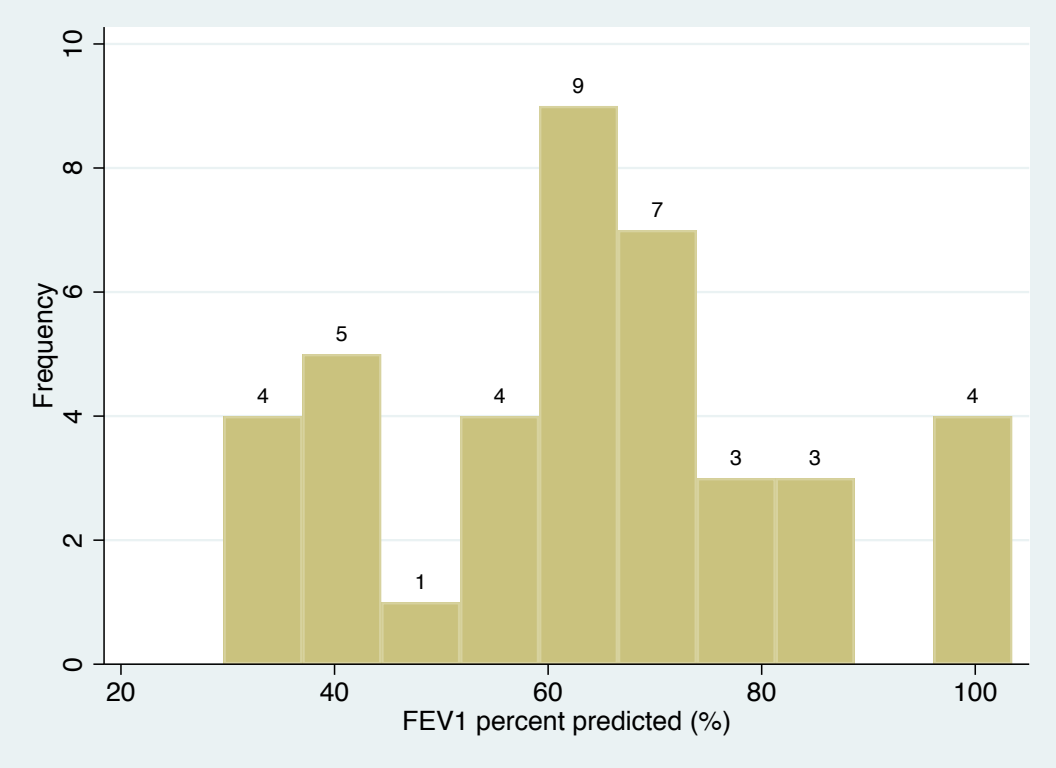
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Frequency
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2
2
1
5
10
15
20
25
ACT Score

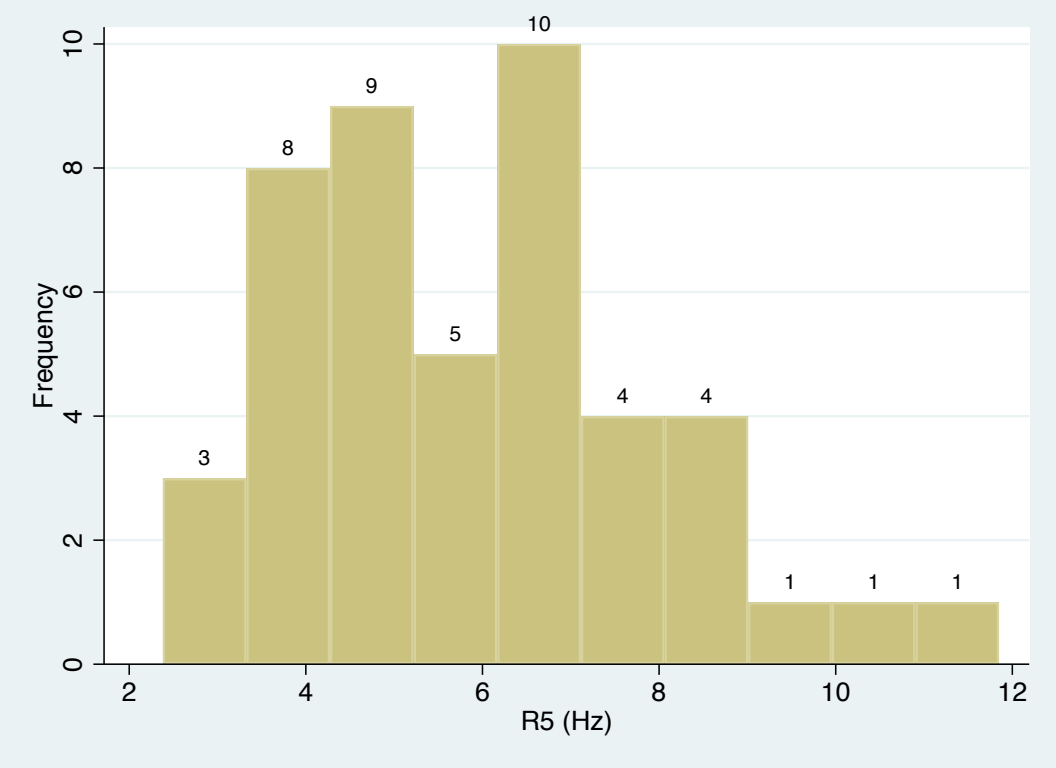
G


Frequency
0
2
4
6
4
2
6
6
6
6
3
6
1
1
1.5
2
2.5
3
3.5
FEV1 (L)

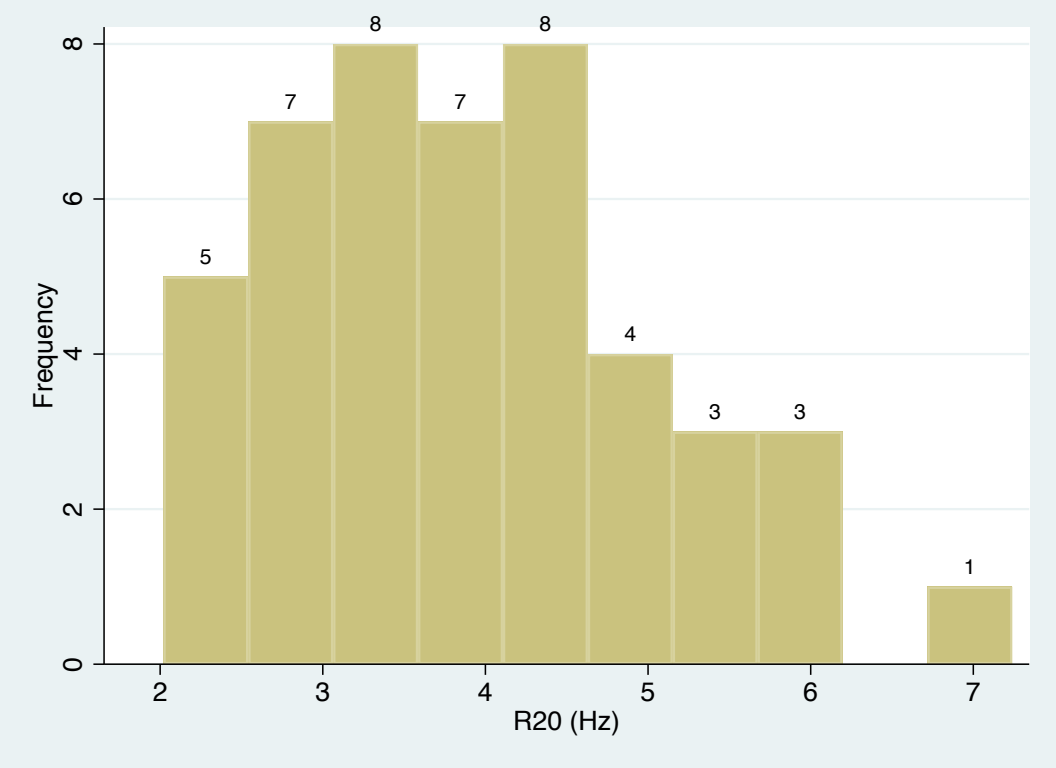
H


Frequency
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2
4
6
8
10
4
5
1
4
9
7
3
3
4
20
40
60
80
100
FEV1 percent predicted (%)

I


Frequency
0
2
4
6
8
10
2
4
6
8
10
12
R5 (Hz)
3
8
9
5
10
4
4
1
1
1

J


Frequency
0
2
4
6
8
2
3
4
5
6
7
R20 (Hz)
5
7
8
7
8
4
3
3
1

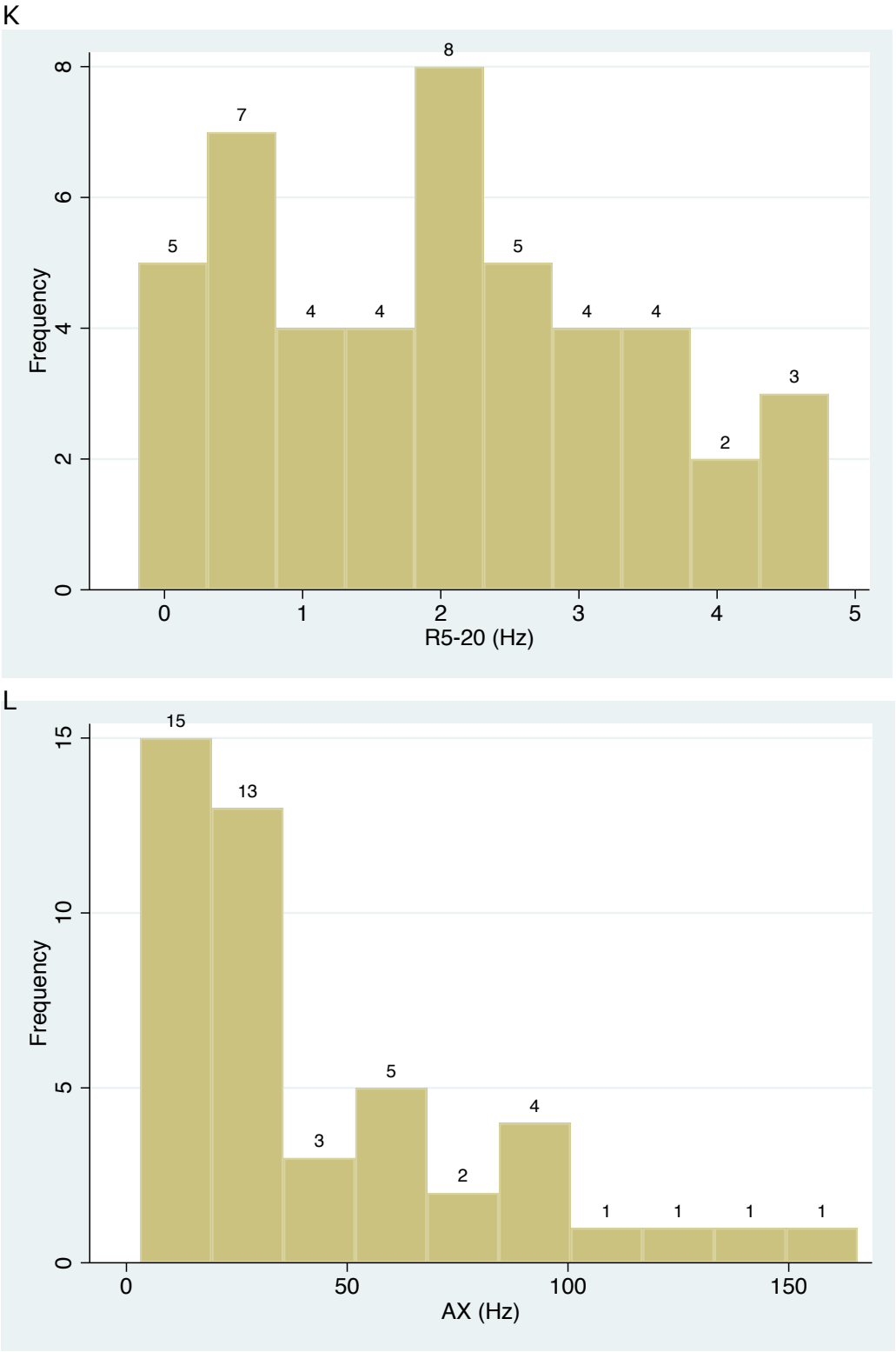


Figure 6.3 Distribution of clinical characteristics at baseline

Histograms denote the baseline distribution of **A**. blood eosinophils, **B**. Fractional exhaled nitric oxide (FeNO), **C**. ACQ5, **D**. ACQ6, **E**. ACQ7, **F**. ACT, **G**. FEV_1, **H**. FEV_1 percent predicted **I**. Resistance at 5 Hz (R5), **J**. Resistance at 20 Hz (R20), **K**. Resistance at 5-20 Hz (R5-20) and, **L**. Small airway reactance (AX).

6.11.2 PRIMARY OUTCOME

Twenty-three patients completed 12 months of treatment. Additionally, three patients stopped treatment after 6 months (but before 12 months) as they and/or their physician decided they were not responding. As such these three patients were included in the treatment response analysis as non-responders making a total of 26 patients.

6.11.2.1 Response to 12 months of treatment

In the 26 participants who completed the 12 months of treatment, blood eosinophils decreased significantly from a mean of 0.45 cells $x10^9$/L at baseline to 0.11 cells $x10^9$/L at one month (decrease of 0.34 cells $x10^9$/L, p=0.0007). This decrease was sustained throughout the 12 months of treatment. Mean blood eosinophil levels in response to treatment are summarized in **Figure 6.4**.

Mean FeNO values increased very slightly throughout the study from 49 ppb at baseline to 54 ppb at 1 month (p=0.3844) and finally to 56 ppb at 12 months (p=0.4956). Mean FeNO levels in response to treatment are summarized in **Figure 6.5**.

At baseline, only one patient reported well-controlled symptoms according to all versions of the ACQ, all other patients reported poorly controlled asthma. Mean scores for the ACQ5, 6 and 7 decreased by a clinically significant amount from baseline to one month; a mean decrease of 0.79 points p=0.0105; 0.57 points p=0.1428; and 0.50 points p=0.2522 respectively. By six months and through to 12 months more patients were well or moderately controlled, however, the majority

of patients still reported poorly controlled symptoms. Following 12 months of treatment, the highest percentage of poorly controlled asthma was reported in the ACQ7 while the ACQ6 reported the lowest (73.91 vs 56.52%). At 12 months ACQ5,6 and 7 decreased by a clinically and statistically significant amount (mean of 1.43 points $p<0.0001$; 1.38 points $p<0.0001$ and 1.24 points $p=0.0001$ respectively). This is represented in **Figures 6.6** and **6.7**. ACT increased from baseline to one month (mean 2.0 points $p= 0.0668$) and continued to increase throughout the trial. At 12 months the mean ACT score increased significantly by 5.8 points ($p=<0.0001$). ACT responses to treatment are summarized in **Figure 6.8**.

There were no clinically significant improvements in spirometry following treatment. FEV_1 remained consistent from a mean of 1.87 L at baseline to 1.86 L at one month and 1.88 L at 12 months (an increase from baseline of 0.01 L $p=0.7025$). The pattern of FEV_1 in response to treatment is summarized in **Figure 6.9**.

R5, R20, R5-20 and AX remained relatively constant from baseline throughout the study, with high levels of variability between patients. The distribution of resistance and reactance values in response to treatment are summarized in **Figure 6.10**.

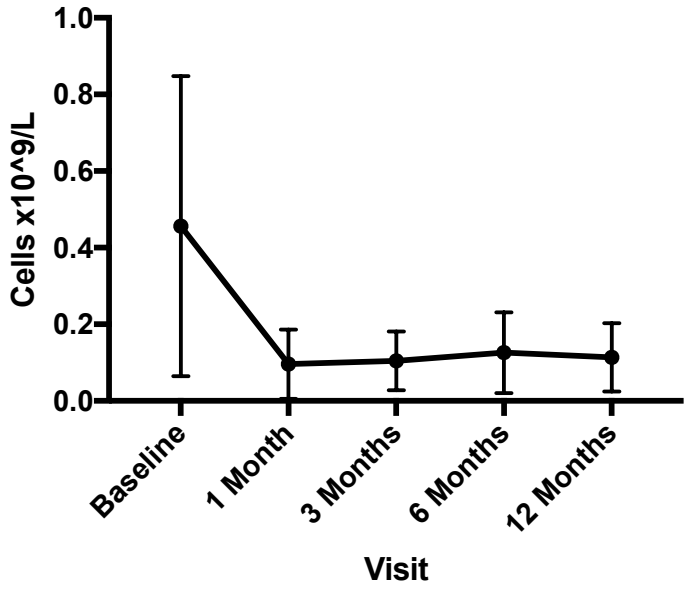


Figure 6.4 Blood eosinophils

Mean values are given for each visit. Error bars represent the standard deviation.

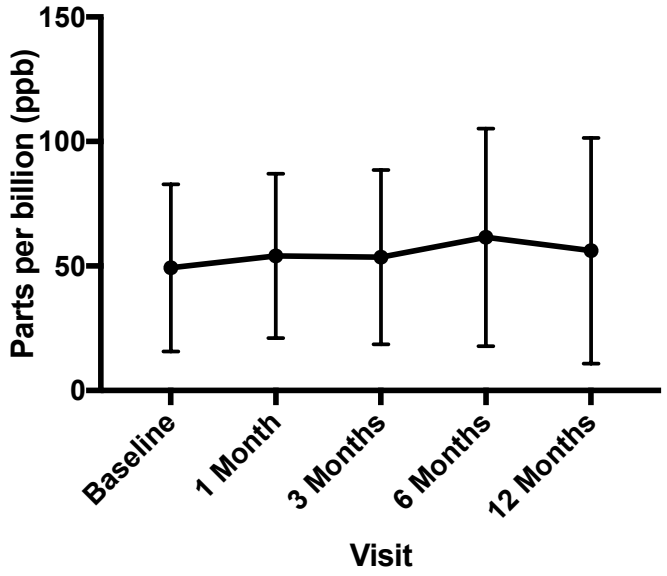


Figure 6.5 Fractional exhaled nitric oxide (FeNO)

Mean values are given for each visit. Error bars represent the standard deviation.

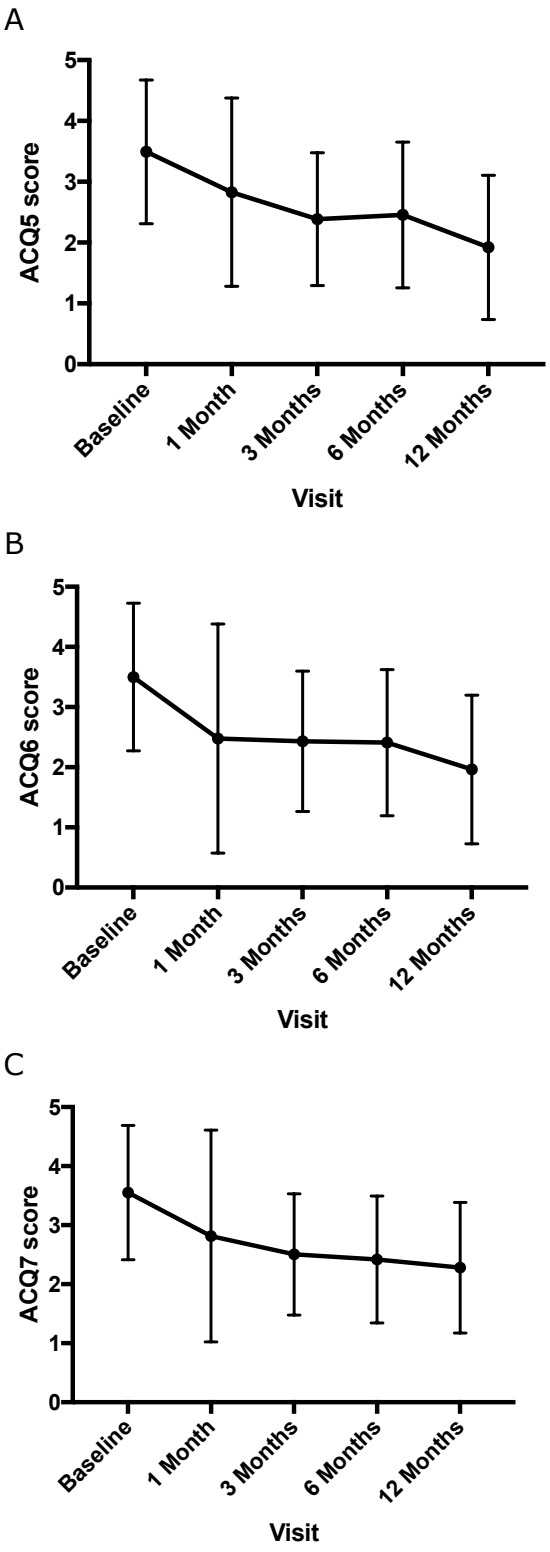


Figure 6.6 Asthma control questionnaire (ACQ)

Mean values are given for each visit. Decreasing scores represent increasing control. A decrease of ≥0.5 is considered to be clinically significant. Error bars represent the standard deviation.

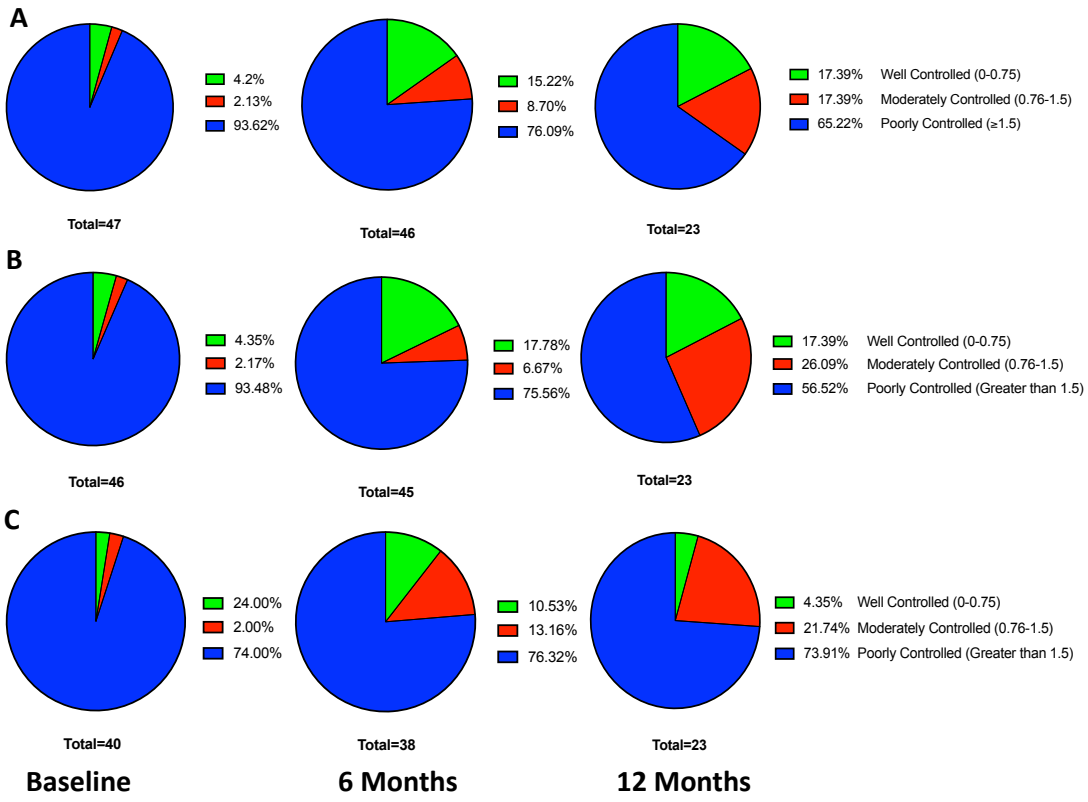


Figure 6.7 Asthma control questionnaire (ACQ) levels of control

The percentage of patients who were either well controlled, moderately controlled or poorly controlled according to **A**. ACQ5, **B**. ACQ6, **C**. ACQ7 are shown at baseline, six months and 12 months.

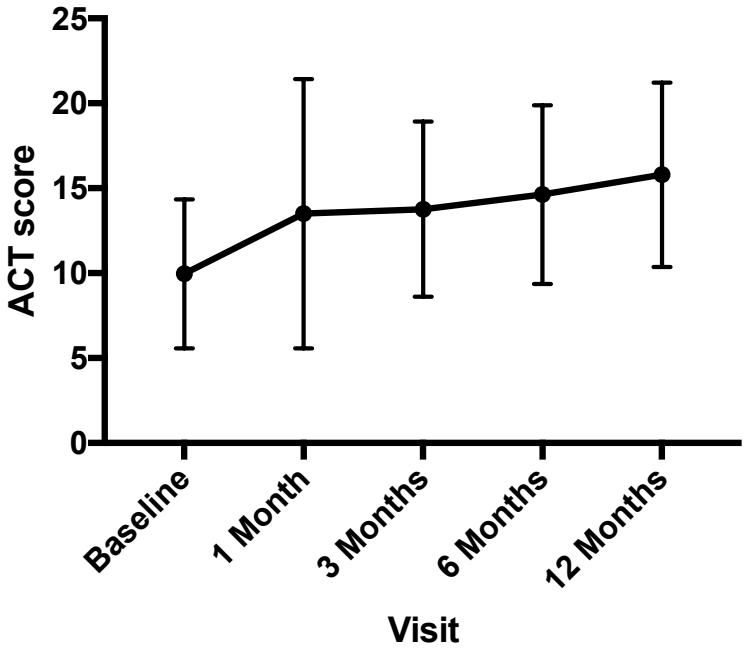


Figure 6.8 Asthma control test (ACT)

Mean values are given for each visit. Increasing scores represent increasing control. An increase of 3 points is considered clinically significant. Error bars represent the standard deviation.

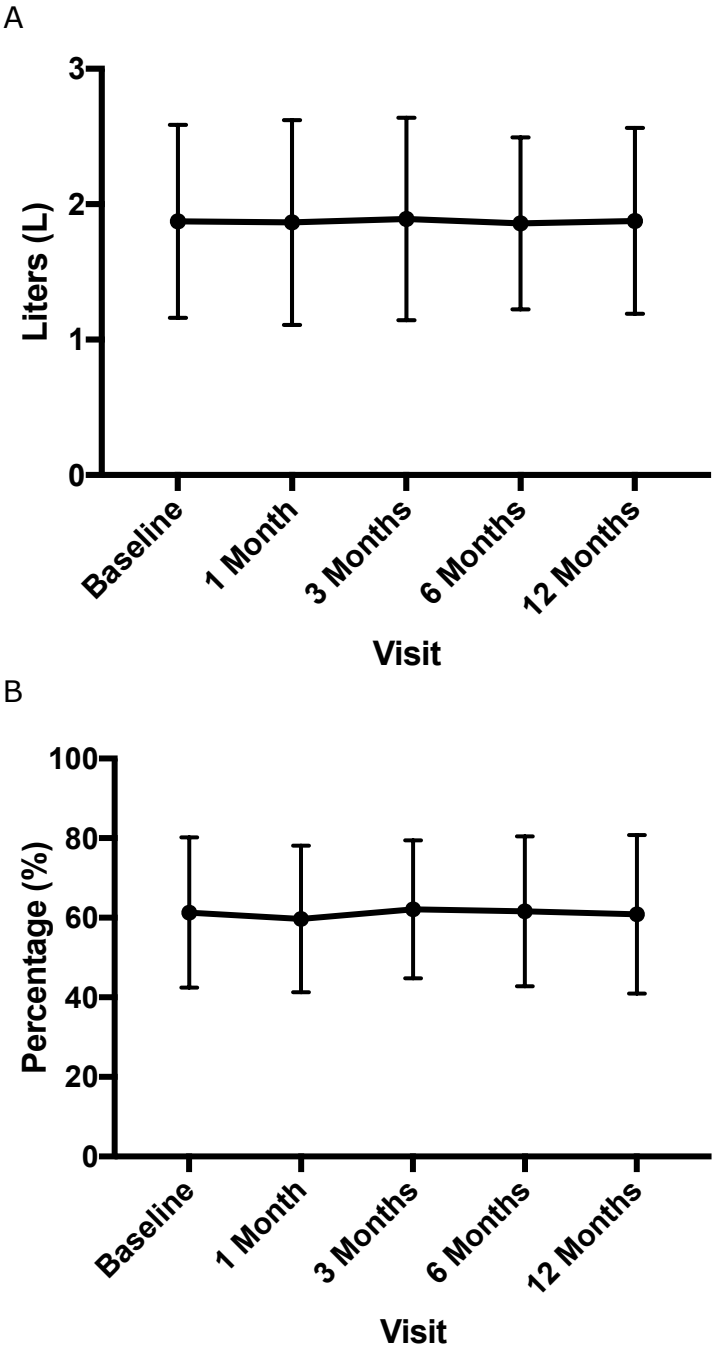
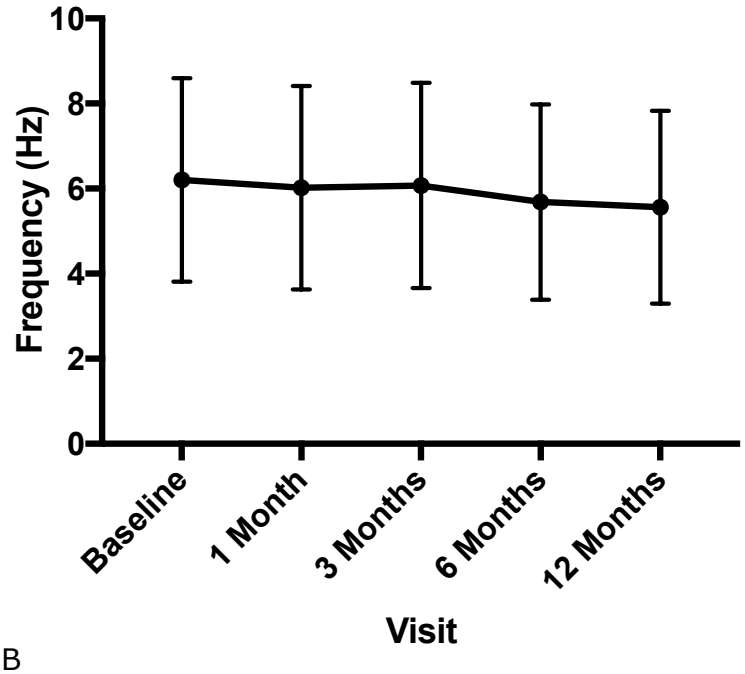


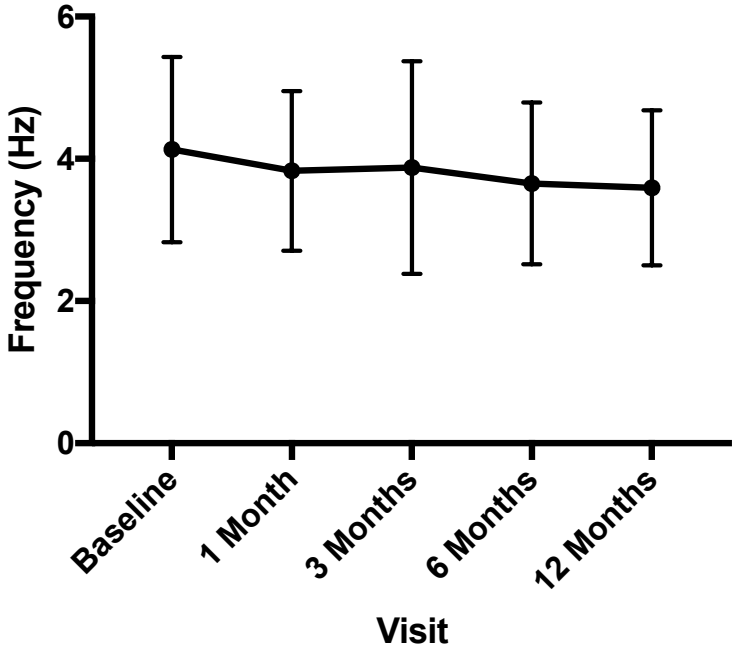
Figure 6.9 Forced expiratory volume over one second (FEV_1)

The distribution of **A**. FEV_1 and **B**. FEV_1 percent predicted are shown for each visit. A decrease of 200 mL and/or 20 % is considered to be clinically significant. Mean values are given for each visit. Error bars represent the standard deviation.

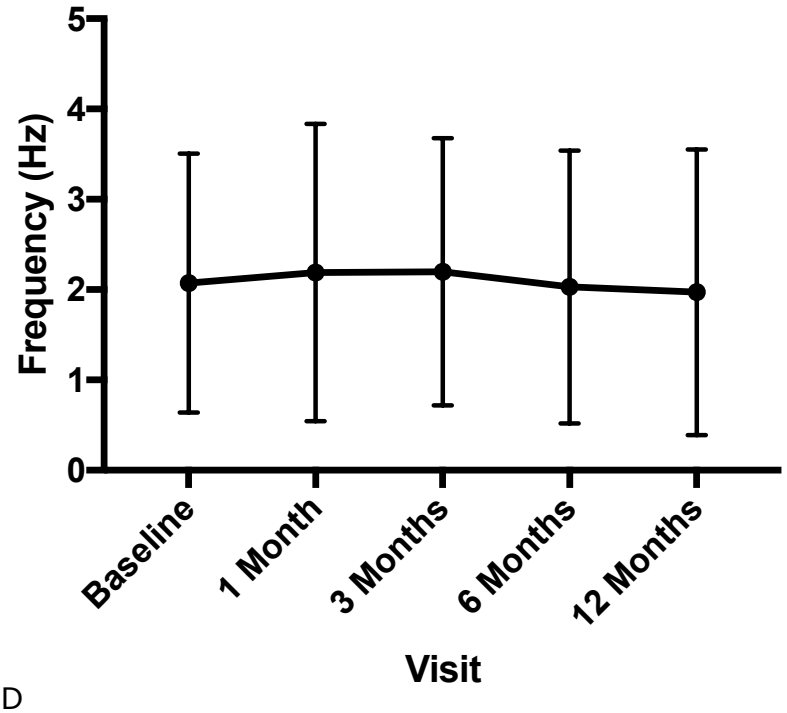
A


10
8
6
4
2
0
Frequency (Hz)
Baseline
1 Month
3 Months
6 Months
12 Months
Visit

B


6
4
2
0
Frequency (Hz)
Baseline
1 Month
3 Months
6 Months
12 Months
Visit

C



D

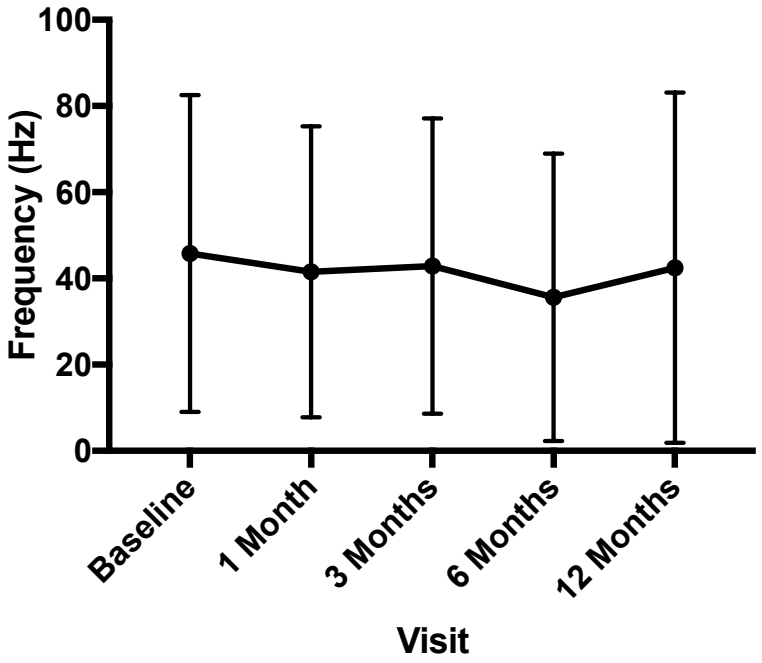


Figure 6.10 Forced Oscillation Technique (FOT)

The distribution of **A**. R5, **B**. R20, **C**. R5-20, **D**. AX are shown for each visit. Mean values are given for each visit. Error bars represent the standard deviation.

6.11.2.2 Responders vs non-responders

Of the 26 patients who completed 12 months of treatment, 16 (61.5 %) were considered responders based on either a 50 % reduction in exacerbations or OCS from the previous 12 months. Seven (44 %) were responders based only on a reduction in maintenance OCS, six (38 %) were responders based only on a reduction in exacerbations and three (23 %) patients responded based on both criteria (**Table 6.5**).

Table 6.5 Summary of responders to treatment with mepolizumab

* Patient was taking 80mg monthly triamcinolone injections at baseline which were completely stopped by the end of the audit in addition to the 20 mg/day reduction in oral corticosteroid. E= exacerbation, S= steroid, B= both.

	Maintenance OCS			Exacerbation number			
Patient	**Previous 12 months**	**1 Year Mepolizumab**	**Percentage decrease (%)**	**Previous 12 months**	**1 Year Mepolizumab**	**Percentage Decrease (%)**	**Type of Response (S/E/B)**
Responder							
1	0	0	0	6	1	83	E
2	0	0	0	12	4	67	E
*3	50	30	40	2	2	0	S*
4	30	12	60	0	1	-100	S
5	10	0	100	0	0	0	S
6	16	16	0	8	2	75	E
7	10	5	50	6	1	83	B
8	15	5	67	2	4	-200	S
9	5	0	100	6	2	67	B
10	15	0	100	3	2	33	S
11	5	0	100	12	9	25	S
12	5	0	100	1	0	100	S
13	15	0	100	6	0	100	B
14	0	0	0	4	0	100	E
15	0	0	0	8	4	50	E
16	20	25	-25%	10	4	60	E
Mean	**12.3**	**5.8**	**65**	**5.4**	**2.3**	**46**	
Non-Responder							
17	0	0	0	7	4	43	n/a
18	0	0	0	12	7	42	n/a
19	0	0	0	5	3	40	n/a
20	2.5	2.5	0	12	7	42	n/a
21	20	30	-50	12	7	42	n/a
22	0	0	0	3	5	-33	n/a
23	0	0	0	10	7	30	n/a
24	20	n/a	n/a	7	n/a	n/a	n/a
25	20	n/a	n/a	10	n/a	n/a	n/a
26	40	n/a	n/a	12	n/a	n/a	n/a
Mean	**10.3**	**16.3**	**-50.0**	**8.7**	**5.7**	**24**	n/a

6.11.3 SECONDARY OUTCOMES

6.11.3.1 Baseline features of responders and non-responders

At baseline, the responders and non-responders were of similar age (mean 46.9 and 43.8, p=0.5592) the majority of patients (100 % and 80 %) were Caucasian and never smokers (69 % and 60 %) respectively. There was a high prevalence of rhinitis and /or GORD in both groups. There was a statistically significant difference in the age at diagnosis. Responders were significantly older than non-responders (24.6 vs 11.3 years p=0.048). Responders also had significantly fewer exacerbations during the 12 months prior to starting treatment (mean 5.4 vs 9, p=0.0231) and were taking a slightly higher maintenance OCS dose (mean 12.3 mg vs 10.3 p=0.7160) (**Table 6.6**).

At baseline, responders had clinically better scores on the ACQ 5,6 and ACT, though the difference in ACT score was the only variable to reach statistical significance (3.2 vs 4.0, p=0.0830; 3.2 vs 4.0, p=0.0896; 11.5 vs 7.5, p=0.0203 respectively). The responders did have a better ACQ7 score than the non-responders though this difference just missed clinical significance; 3.4 vs 3.8, a difference of 0.4 (p=0.4137). FeNO was higher in the non-responders at baseline (59.8 vs 44.0 ppb) though this difference was not statistically significant (p=0.2888). Airway reactance was higher in the responders than non-responders (49.1 vs 40.5 Hz) though this difference also did not reach significance (p=0.5722). All other clinical features were very similar between

responders and non-responders at baseline. These results are summarized in **Tables 6.7**.

Table 6.6 Mean baseline demographics of responders and non-responders

BMI: Body Mass Index; OCS: Oral Corticosteroids

Variable	Responders N(%)	Non-Responders N(%)	P-value
Sex			
Female	12 (75%)	5 (50%)	
Age (yrs)	46.9	43.8	0.5592
Age at Diagnosis (yrs)	24.6	11.3	**0.0480**
Years with asthma (yrs)	21.9	32.5	0.0800
Ethnicity			
Caucasian	16 (100%)	8 (80%)	
Asian	0	2 (20%)	
Smoking status			
Never	11 (69%)	6 60%)	
Ex	5 (31%)	2 (20%)	
Current	0 (0%)	2 (20%)	
BMI	36.9	30.9	0.1007
Comorbidities			
Rhinitis	10 (63%)	7 (70%)	
GORD	7 (44%)	5 (50%)	
Patients with an exacerbation in the previous 12 months	14 (88%)	10 (100%)	
Mean number of exacerbations	5.4	9.0	**0.0231**
Patients taking a maintenance dose OCS	12 (75%)	5 (50%)	
Mean daily dose (mg/day)	12.3	10.3	0.7160

Table 6.7 Mean baseline clinical characteristics of responders and non-responders

FeNO: Fractional exhaled nitric oxide, ACQ: Asthma Control Questionnaire, ACT: Asthma Control Test, FEV_1: Forced expiratory volume over one second, R: Resistance, AX: Airway reactance.

Clinical Characteristic	Responders Mean (n=16)	Non-responders Mean (n=10)	P value
Blood Eosinophils (cells x 10^9/L)	0.4	0.5	0.6828
FeNO (ppb)	44.0	59.8	0.2888
Questionnaires			
ACQ5	3.2	4.0	0.0830
ACQ6	3.2	4.0	0.0896
ACQ7	3.4	3.8	0.4137
ACT	11.5	7.5	**0.0203**
Spirometry			
FEV_1 (L)	1.8	1.9	0.6141
FEV_1 % predicted	61.6	61.1	0.9623
Oscillometry			
R5 (Hz)	6.3	6.0	0.7027
R20 (Hz)	4.2	4.0	0.7199
R5-20 (Hz)	2.1	2.0	0.7534
AX (Hz)	49.1	40.5	0.5722

6.11.3.2 Response to treatment

Following 12 months of treatment, blood eosinophils decreased in both the responders and non-responders to a mean of 0.1 cells x10^9/L at 12 months (p=0.6614). FeNO increased in both groups though the increase was greater in the non-responders than the responders (59.8 to 79.5 vs 44.0 to 47.4 ppb; p=0.1421). The responders reported ACQ symptom scores that were clinically and statistically significantly better than the non-responders. The mean 12 month ACQ scores were: ACQ5 1.5 vs 3.0

(p=0.0036); ACQ6 1.5 vs 3.1 (p=0.0012), ACQ7 1.8 vs 3.3 (p=0.0007) for the responders and non-responders respectively. The responders also reported significantly better controlled asthma according to the ACT than non-responders at 12 months; 18.1 vs 11.1 (p=0.0012).

Lung function in terms of FEV_1 improved very slightly in the responders (mean increase 0.1 L, 6.6%) while the non-responders decreased slightly (mean decrease -0.1 L, -6.4%; p=0.5028). The difference between responders and non-responders in oscillometry parameters never reached statistical significance however, there was a mean decrease in all oscillometry values in the responders whereas in the non-responders only R5 and R20 decreased, R5-20 remained the same and AX increased. The changes from baseline to 12 months of treatment are shown in **Table 6.8** and are detailed in **Figures 6.11-6.21**

Table 6.8 Individual responses at baseline and 12 months of treatment

	Blood Eosinophil (cells x10^9/L)			FeNO (ppb)			ACQ5			ACQ6		
Patient	**Baseline**	**12 Months**	**Difference**	**Baseline**	**12 Months**	**Difference**	**Baseline**	**12 Months**	**Difference**	**Baseline**	**12 Months**	**Difference**
Responders												
1	1.8	0.2	**-1.6**	48	37	**-11**	3.4	1.6	**-1.8**	3.3	1.5	**-1.8**
2	0.7	0.1	**-0.6**	83	170	**87**	4.2	2.2	**-2**	4.3	2.2	**-2.2**
3	0.1	0	**-0.1**	26	19	**-7**	4.8	1.8	**-3**	4.8	1.7	**-3.2**
4	0.1	0	**-0.1**	17	54	**37**	4.6	2.6	**-2**	4.8	2.7	**-2.2**
5	0	0	**0**	23	14	**-9**	2.6	1.2	**-1.4**	2.5	1.0	**-1.5**
6	0.2	0.1	**-0.1**	7	18	**11**	2.6	1.4	**-1.2**	2.5	1.3	**-1.2**
7	0.4	0.1	**-0.3**	30	45	**15**	2.8	1.8	**-1**	2.3	1.5	**-0.8**
8	0.4	0.3	**-0.1**	68	89	**21**	2.8	0.8	**-2**	3.2	0.8	**-2.3**
9	0.3	0.1	**-0.2**	24	21	**-3**	2.8	2.6	**-0.2**	2.7	2.8	**0.2**
10	0.4	0.2	**-0.2**	89	42	**-47**	2.2	1.6	**-0.6**	2.0	1.5	**-0.5**
11	0.5	0	**-0.5**	117	34	**-83**	5	2	**-3**	5.0	2.0	**-3.0**
12	0.3			23	64	**41**	0.2	0	**-0.2**	0.2	0.0	**-0.2**
13	0.3	0.2	**-0.1**	32	83	**51**	2.4	0.6	**-1.8**	2.2	0.7	**-1.5**
14	0.6	0.1	**-0.5**	39	25	**-14**	2.2	0.2	**-2**	2.3	0.3	**-2.0**
15	0.6			61	15	**-46**	3.6	0.4	**-3.2**	3.8	0.5	**-3.3**
16	0.2	0.1	**-0.1**	17	28	**11**	4.6	2.8	**-0.2**	4.8	2.8	**0.0**
Mean	**0.4**	**0.1**	**-0.3**	**44.0**	**47.4**	**7.5**	**3.2**	**1.5**	**-1.6**	**3.2**	**1.5**	**-1.6**
Non-responders												
17	1.2		**-0.1**	77	154	**77**	2.8	2.6	**-1.8**	2.8	3.2	**-2.0**
18	0.9	0.1	**-0.8**	75			5.2	1.8	**-3.4**	5.0	1.7	**-3.3**
19				101	84	**-17**	3.2	3	**-0.2**	3.2	3.0	**-0.2**
20	0.3	0.1	**-0.2**	36	106	**70**	4	3.2	**-0.8**	4.0	3.2	**-0.8**
21	0.2	0.1	**-0.1**	114	99	**-15**	2.8	1.2	**-1.6**	2.8	1.7	**-1.2**
22	0.7	0	**-0.7**	20	19	**-1**	4.4	4.6	**0.2**	4.2	4.7	**0.5**
23	0.5	0.3	**-0.2**	46	15	**-31**	3.8	4.2	**0.4**	3.8	4.5	**0.7**
24	0.4	0.2	**-0.2**				5.2	4.6		5.2		
25	0.2	0.1	**-0.1**				4			4		
26	0.1	0.1	**0**	9			4.6	4.6	**0**	4.8	4.8	**0**
Mean	**0.5**	**0.1**	**-0.3**	**59.8**	**79.5**	**2.8**	**4.0**	**3.0**	**-0.9**	**4.0**	**3.1**	**-0.8**
P-Value	0.6828	0.6614	0.7311	0.2888	0.1421	0.6160	0.0830	**0.0036**	0.1542	0.0896	**0.0012**	0.1334

	ACQ7			ACT			FEV_1 (L)			FEV_1 % predicted		
Patient	**Baseline**	**12 Months**	**Difference**	**Baseline**	**12 Months**	**Difference**	**Baseline**	**12 Months**	**Difference**	**Baseline**	**12 Months**	**Difference**
Responders												
1	3.4	1.7	**-1.7**	10	16	**6**	1.75	2.06	**0.31**	62.9	74.4	**11.4**
2	4.4	2.1	**-2.3**	7	13	**6**	1.83	2.69	**0.86**	59.4	87.6	**28.2**
3	5.0	1.7	**-3.3**	7	16	**9**	0.98	2.76	**1.78**	29.6	83.9	**54.3**
4	5.0	3.0	**-2.0**	5	13	**8**	1.45	1.73	**0.28**	41.3	49.7	**8.4**
5	2.1	1.0	**-1.1**	12	22	**10**	2.75	2.69	**-0.06**	96.8	95.4	**-1.4**
6		1.9	**1.9**	15	19	**4**		1.74			50.6	
7		2.0		12	21	**9**		1.92			51.2	
8	3.0	1.1	**-1.9**	9	19	**10**	2.85	2.41	**-0.44**	84.8	71.7	**-13.1**
9	3.1	3.3	**0.1**	15	11	**-4**	1.08	0.86	**-0.22**	44.1	35.5	**-8.5**
10	2.3	2.1	**-0.1**	12	20	**8**	1.98	1.32	**-0.66**	69.2	46.3	**-22.9**
11	4.7	2.0	**-2.7**	5	15	**10**	2.37	2.93	**0.56**	70.7	88.3	**17.5**
12	0.4	0.6	**0.1**	24	24	**0**	1.83	1.4	**-0.43**	80.3	62.2	**-18.0**
13	2.4	0.9	**-1.6**	15	24	**9**	2.29	2.74	**0.45**	65.8	79.4	**13.6**
14	2.9	1.1	**-1.7**	16	24	**8**	1.14	1.25	**0.11**	40.6	45.1	**4.6**
15	4.0	1.3	**-2.7**	9	21	**12**	1.48	1.26	**-0.22**	50.5	43.6	**-6.9**
16	4.7	3.1	**-1.6**	11	12	**1**	1.60	2.31	**-0.29**	64.5	53.5	**25.0**
Mean	**3.4**	**1.8**	**-1.4**	**11.5**	**18.1**	**6.6**	**1.8**	**1.9**	**0.1**	**61.6**	**63.7**	**6.6**
Non-responders												
17	3.4	3.0	**-0.4**	9	11	**2**	1.57	2.21	**0.64**	60.6	85.7	**-11.0**
18	5.0	2.3	**-2.7**	6	20	**14**	0.89	0.72	**-0.17**	53.3	43.6	**-9.7**
19	3.6	3.4	**-0.1**	9	12	**3**	1.88	1.73	**-0.15**	44.1	40.7	**-3.4**
20	4.0	3.3	**-0.7**	7	13	**6**	2.33	2.18	**-0.15**	66.6	63.0	**-3.6**
21	2.9	2.3	**-0.6**	11			3.54	2.07	**-1.47**	75.0	44.2	**-30.8**
22	4.4	4.9	**0.4**	7	7	**0**	0.83	0.69	**-0.14**	30.6	25.7	**-4.9**
23	3.9	4.3	**0.4**	7	9	**2**	1.92	2.49	**0.57**	60.0	78.5	**18.5**
24				5	9	**4**						
25	3.4			8			2.86			98.3		
26				6	8	**2**						
Mean	**3.8**	**3.3**	**-0.5**	**7.5**	**11.1**	**4.1**	**1.9**	**1.7**	**-0.1**	**61.1**	**54.5**	**-6.4**
P-Value	0.4137	**0.0007**	0.1632	**0.0203**	**0.0012**	0.1972	0.6141	0.5028	0.3868	0.9623	0.3229	0.1565

	R5 (Hz)			R20 (Hz)			R5-20 (Hz)			AX (Hz)		
Patient	Baseline	12 Months	Difference	Baseline	12 Months	Difference	Baseline	12 Months	Difference	Baseline	12 Months	Difference
Responders												
1	4.3	4.7	**0.4**	3.9	3.8	**-0.1**	0.4	0.9	**0.5**	11	12	**1**
2	6.8	3.2	**-3.6**	5.1	3.1	**-2.0**	1.7	0.1	**-1.6**	28	3	**-25**
3	7.1	5.8	**-1.3**	3.7	4.2	**0.5**	3.3	1.6	**-1.7**	70	80	**10**
4	8.0	5.6	**-2.4**	5.6	3.8	**-1.9**	2.4	1.8	**-0.6**	64	34	**-30**
5	5.2	4.9	**-0.3**	3.9	3.8	**-0.1**	1.3	1.1	**-0.2**	43	7	**-36**
6	8.6	9.6	**1.0**	5.4	4.8	**-0.7**	3.1	4.8	**1.7**	136	94	**-42**
7	5.4	2.6	**-2.8**	3.3	2.5	**-0.8**	2.1	0.1	**-2.0**	21	3	**-18**
8	2.6	3.2	**0.6**	2.6	2.7	**0.1**	0.0	0.5	**0.5**	8	11	**3**
9	11.9	9.4	**-2.5**	7.2	6.8	**-0.4**	4.6	2.5	**-2.1**	95	118	**23**
10	3.4	6.1	**2.7**	3.1	3.2	**0.2**	0.3	2.9	**2.6**	5	40	**35**
11	9.6	5.4	**-4.3**	4.8	3.1	**-1.7**	4.8	2.2	**-2.6**	105	26	**-79**
12	3.5	3.4	**-0.1**	3.0	2.4	**-0.5**	0.5	1.0	**0.4**	12	16	**4**
13	7.7	4.3	**-3.4**	4.5	3.3	**-1.1**	3.2	1.0	**-2.2**	59	20	**-39**
14	3.8	4.6	**0.8**	3.0	2.5	**-0.6**	0.8	2.1	**1.3**	41	32	**-9**
15	6.7	7.0	**0.3**	4.4	4.5	**0.1**	2.3	2.5	**0.2**	34	46	**12**
16	7.2	10.8	**3.6**	3.7	4.6	**0.9**	3.5	6.2	**2.7**	55	151	**97**
Mean	**6.3**	**5.7**	**-0.7**	**4.2**	**3.7**	**-0.5**	**2.1**	**2.0**	**-0.2**	**49.1**	**43.3**	**-5.8**
Non-responders												
17	8.7	5.0	**-3.8**	6.1	4.9	**-1.2**	2.6	0.1	**-2.6**	35	5	**-30**
18	2.4	3.6	**1.2**	2.0	2.6	**0.5**	0.4	1.0	**0.7**	3	20	**16**
19	6.5	8.0	**1.6**	4.5	3.7	**-0.8**	1.9	4.3	**2.4**	19	76	**57**
20	4.4	4.4	**0.0**	2.5	2.6	**0.0**	1.8	1.8	**0.0**	23	21	**-2**
21	4.1	4.3	**0.2**	2.3	2.4	**0.1**	1.8	1.9	**0.1**	17	45	**29**
22	10.0	8.4	**-1.6**	5.8	4.6	**-1.1**	4.2	3.7	**-0.5**	91	96	**5**
23	4.8	3.9	**-0.9**	3.5	2.8	**-0.8**	1.3	1.2	**-0.1**	31	22	**-9**
24	6.7			4.6			2.1			92.6		
25	5.2			5.4			-0.2			3.1		
26	6.9			3.3			3.6			89.9		
Mean	**6.0**	**5.4**	**-0.5**	**4.0**	**3.4**	**-0.5**	**2.0**	**2.0**	**0**	**40.5**	**40.7**	**9.4**
P-Value	0.7027	0.7900	0.8141	0.7199	0.5322	0.9239	0.7534	0.9601	0.7993	0.5722	0.8937	0.3706

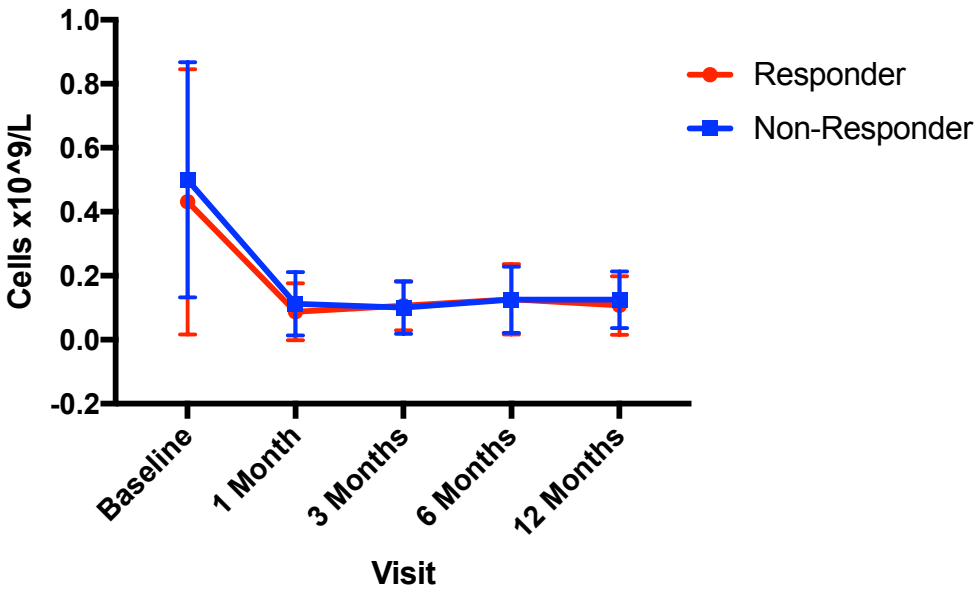


Figure 6.11 Blood eosinophils

Mean blood eosinophils levels are shown for each visit. Error bars represent the standard deviation.

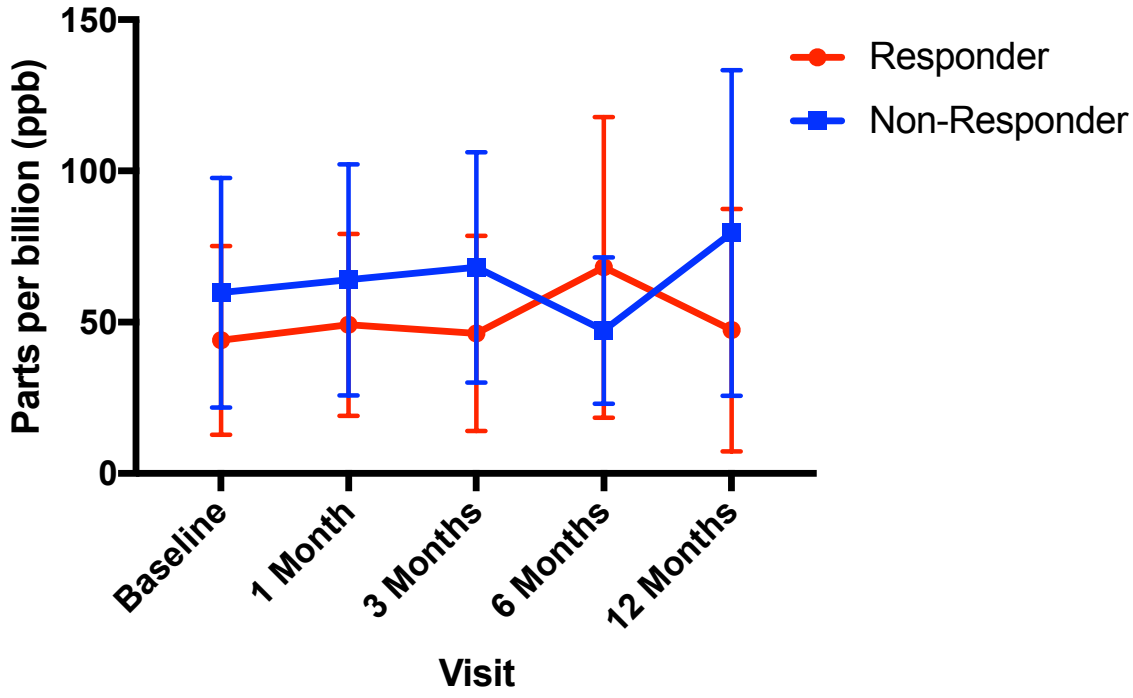


Figure 6.12 Fractional exhaled nitric oxide (FeNO)

Mean FeNO levels are shown for each visit. Error bars represent the standard deviation.

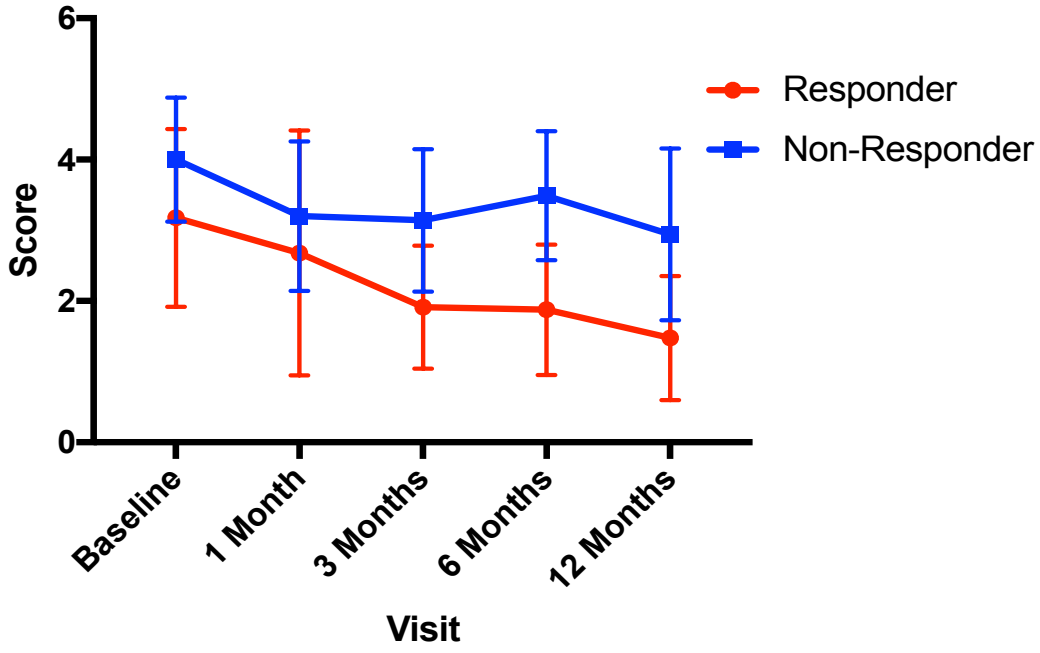


Figure 6.13 Asthma control questionnaire (ACQ) 5

Mean ACQ5 scores are shown for each visit. Error bars represent the standard deviation.

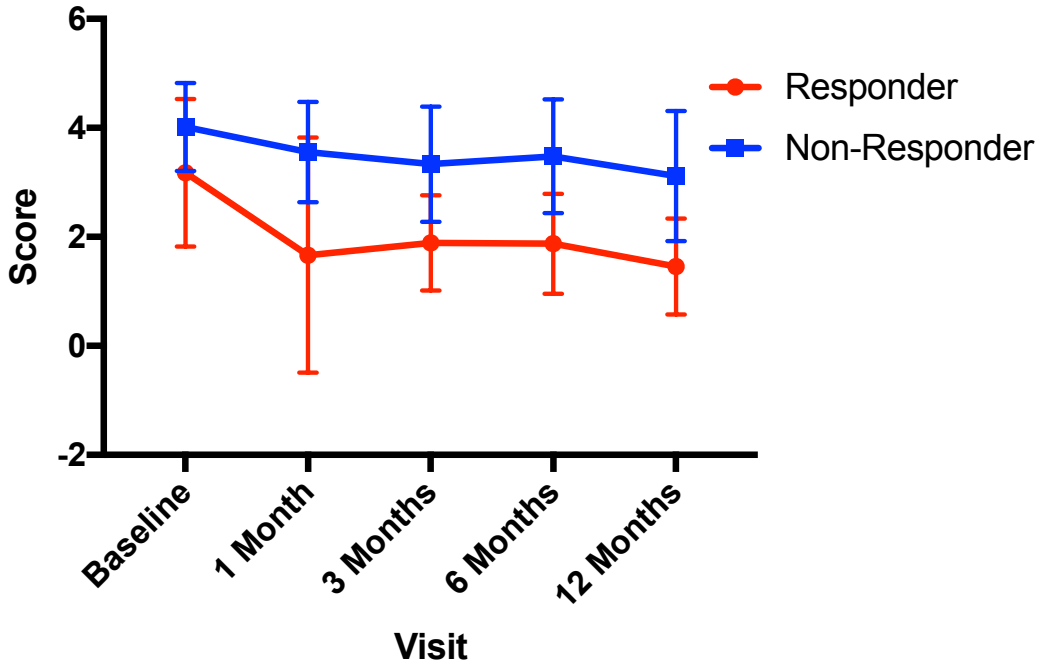


Figure 6.14 Asthma control questionnaire (ACQ) 6

Mean ACQ6 scores are shown for each visit. Error bars represent the standard deviation.

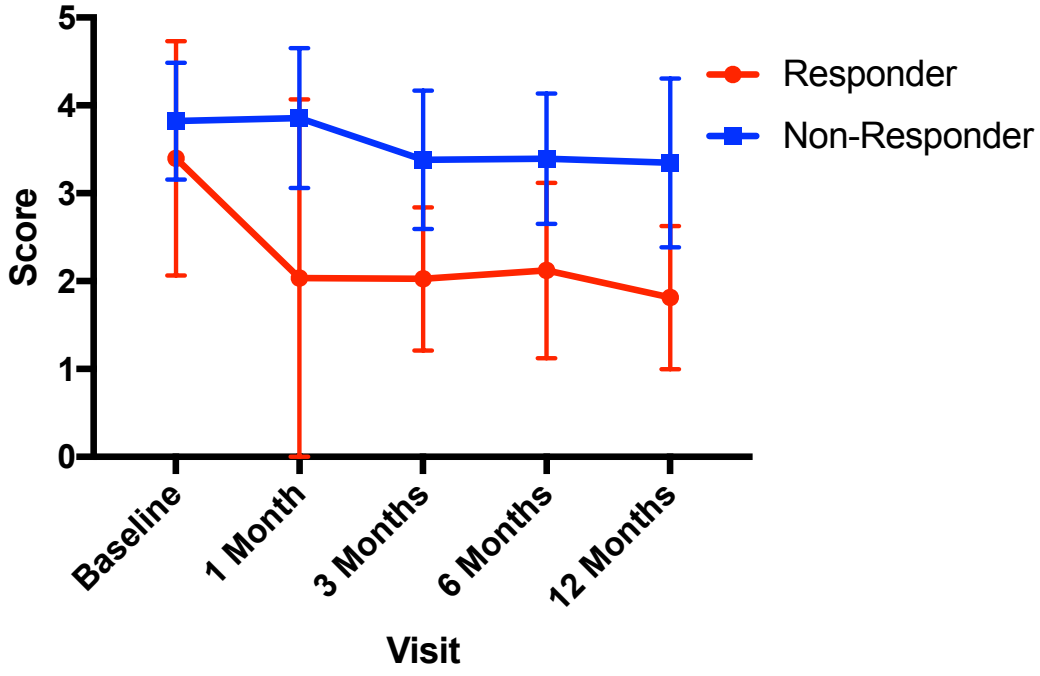


Figure 6.15 Asthma control questionnaire (ACQ) 7

Mean ACQ7 scores are shown for each visit. Error bars represent the standard deviation.

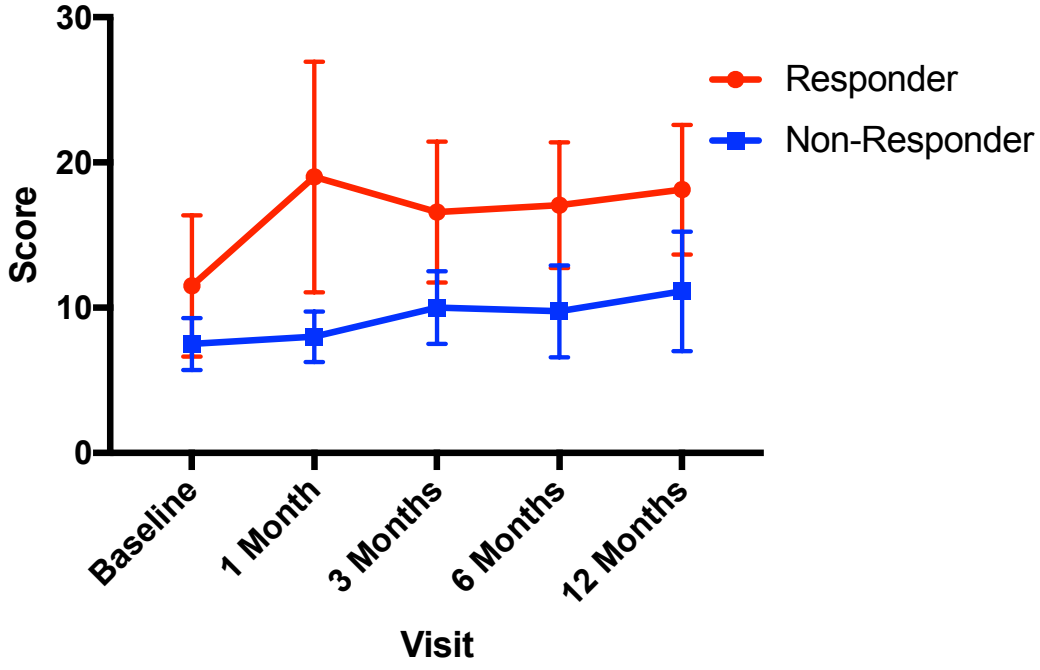


Figure 6.16 Asthma control test (ACT)

Mean ACT scores are shown for each visit. Error bars represent the standard deviation.

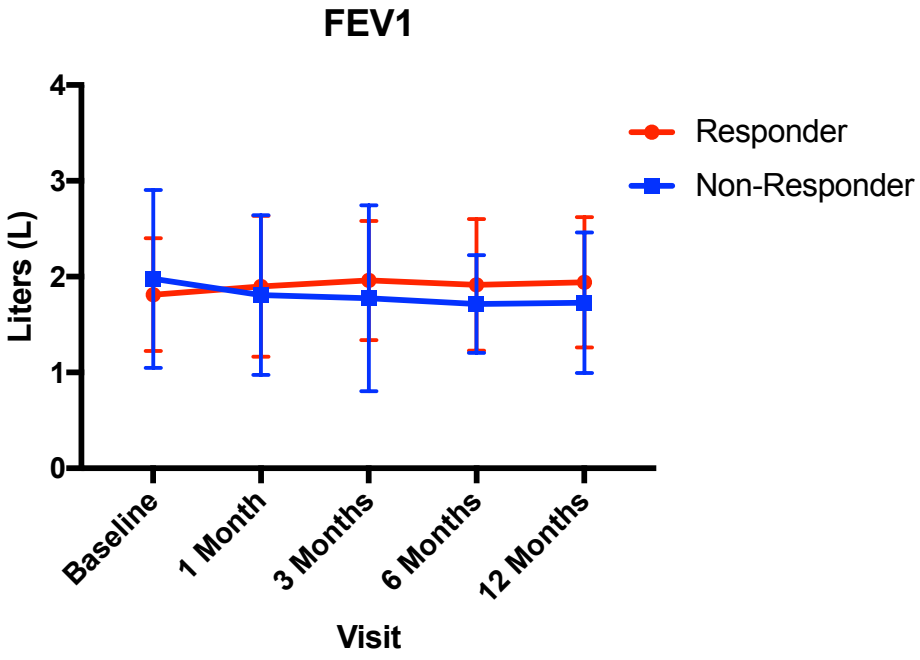


Figure 6.17 Forced expiratory volume over one second (FEV_1)

Mean FEV_1 values are shown for each visit. Error bars represent the standard deviation.

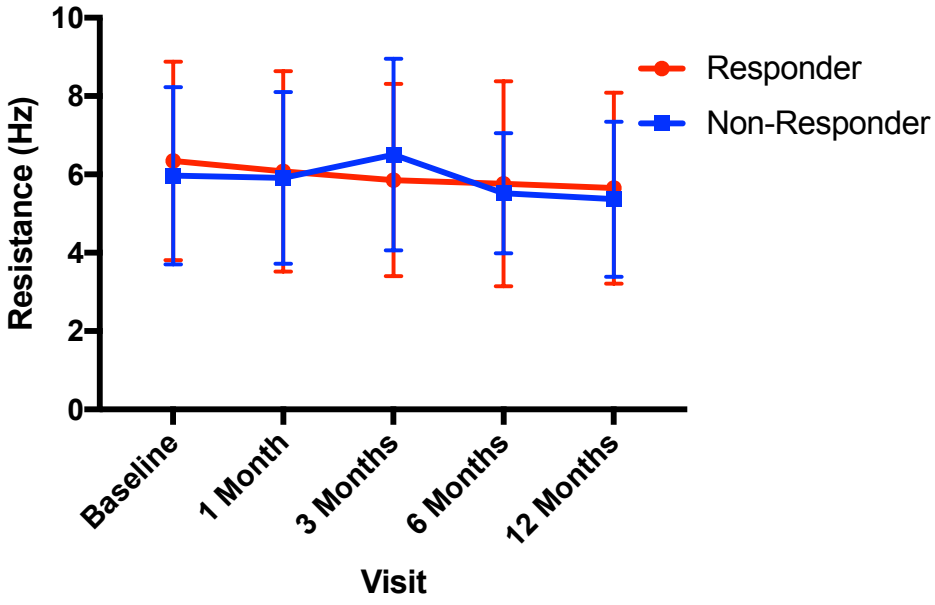


Figure 6.18 Total airway resistance (R5)

Mean R5 scores are shown for each visit. Error bars represent the standard deviation.

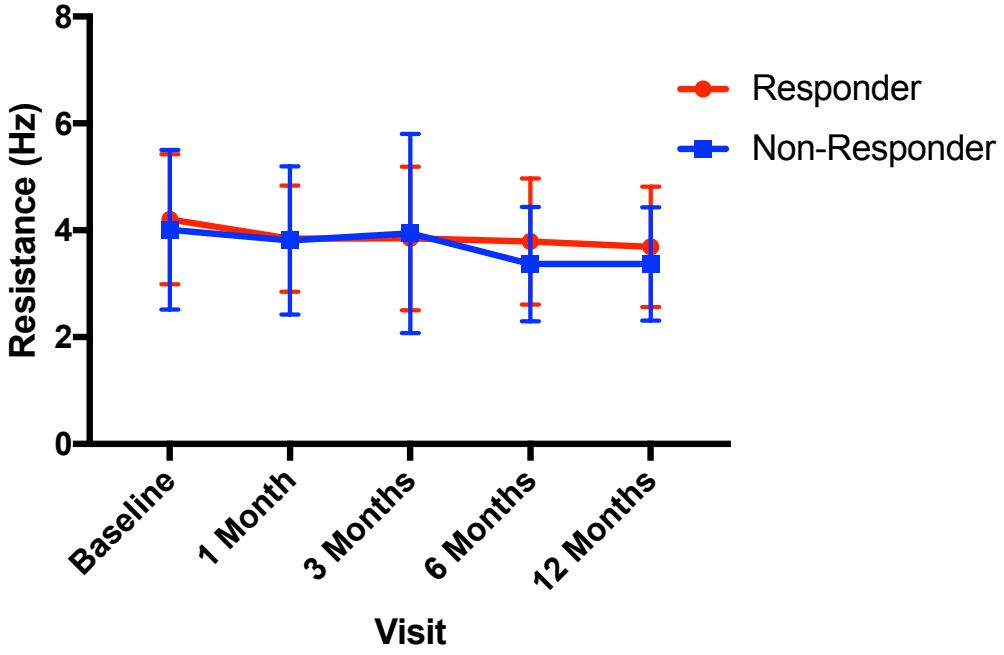


Figure 6.19 Large airway resistance (R20)

Mean R20 scores are shown for each visit. Error bars represent the standard deviation.

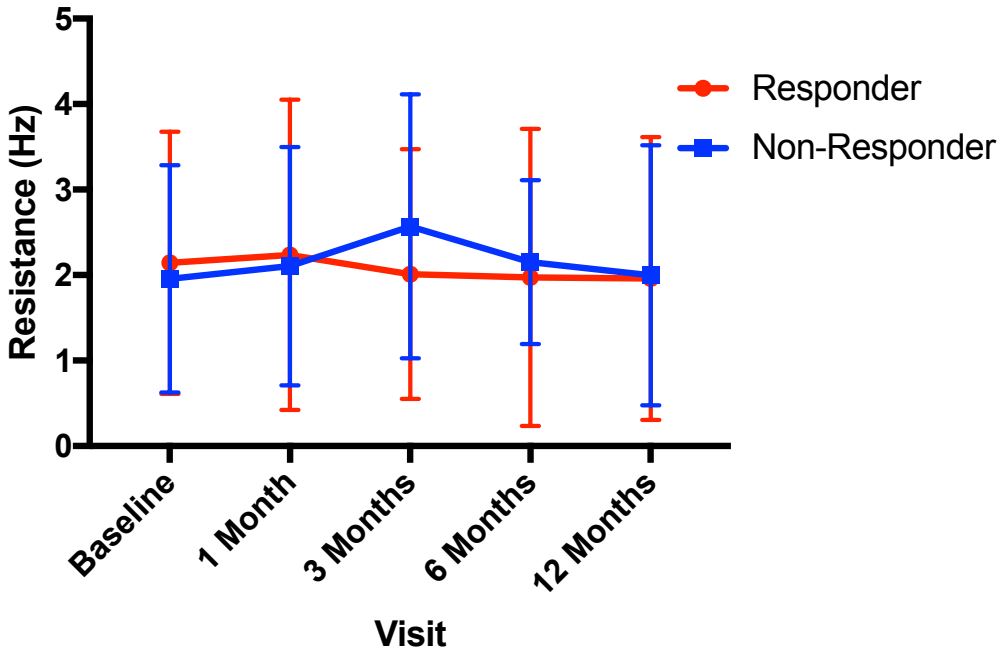


Figure 6.20 Small airway resistance (R5-20)

Mean R5-20 scores are shown for each visit. Error bars represent the standard deviation.

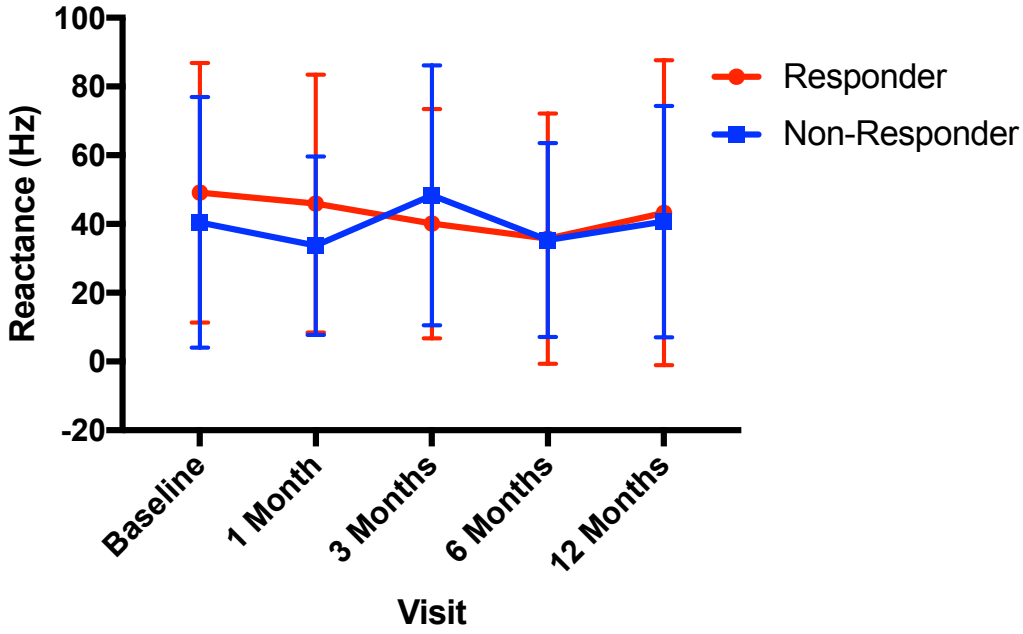


Figure 6.21 Airway reactance (AX)

Mean AX scores are shown for each visit. Error bars represent the standard deviation.

6.11.3.3 Features of early response

At baseline, the only clinical characteristic that was statistically significantly different between the responders and non-responders was ACT score (p=0.0203). ACQ 5 and 6 scores were clinically better in the responders than non-responders, 3.2 vs 4.0 for both questionnaires. These features along with FEV_1 and AX, which showed clinical differences between the groups at 12 months, were analyzed at each study visit in order to describe the response over the course of the year and determine when the difference between the groups became significant.

ACQ scores tended to decrease through the 12 months of treatment in both response groups however, this decrease was greater in the responders than non-responders which first reached statistical

significance following three months of treatment (ACQ5, p=0.0030; ACQ6, p=0.0015, ACQ7, p=0.0047). These results are summarized in **Table 6.9**. Observation numbers for the ACT were very low at the one month visit, however, the difference between the responders and non-responders was clinically significant, 19.0 vs 8.0 respectively (p=0.0789). During every other visit, ACT scores were clinically and statistically significantly better in the responders than non-responders. These results are summarized in **Table 6.10**.

Mean FEV_1 increased slightly throughout the 12 months of treatment in the responders whereas there was a slight decrease in the non-responders. For the responders, the mean difference from baseline was clinically significant (0.2 L) after one month which decreased to 0.1 L after 12 months whereas the non-responders had a mean decrease of 0.1 L at 12 months. The difference between responders and non-responders never reached statistical significance. These results are summarized in **Table 6.11**.

The mean difference in AX score decreased in the responder group at every visit whereas the non-responders decreased slightly from baseline to the first visit and then increased throughout the remainder of the observation period. The largest difference between the responders and non-responders was seen at 3 months where the responders decreased by a mean of 9 Hz from baseline and the non-responders increased by a mean of 9.7 Hz (18.7 Hz difference, p=0.1660). These results are summarized in **Table 6.12**

Table 6.9 Individual responses to the asthma control questionnaire (ACQ) at each visit

A. ACQ5, **B**. ACQ6, **C**. ACQ7

A.	ACQ5								
Patient	**Baseline**	**1 Month**	**Difference**	**3 Months**	**Difference**	**6 Months**	**Difference**	**12 Months**	**Difference**
1	3.4	2.0	**-1.4**	2.4	**-1.0**	3.2	**-0.2**	1.6	**-1.8**
2	4.2	4.0	**-0.2**	1.2	**-3.0**	2.6	**-1.6**	2.2	**-2.0**
3	4.8	4.6	**-0.2**	3.4	**-1.4**	2.2	**-2.6**	1.8	**-3.0**
4	4.6	3.4	**-1.2**	2.2	**-2.4**	2.4	**-2.2**	2.6	**-2.0**
5	2.6	2.0	**-0.6**	2.8	**0.2**	2.4	**-0.2**	1.2	**-1.4**
6	2.6			1.2	**-1.4**	1.8	**-0.8**	1.4	**-1.2**
7	2.8			2.0	**-0.8**	2.8	**0.0**	1.8	**-1.0**
8	2.8			1.8	**-1.0**	1.4	**-1.4**	0.8	**-2.0**
9	2.8			3.2	**0.4**	3.0	**0.2**	2.6	**-0.2**
10	2.2			1.8	**-0.4**	1.6	**-0.6**	1.6	**-0.6**
11	5.0			2.4	**-2.6**	1.6	**-3.4**	2.0	**-3.0**
12	0.2	0.0	**-0.2**	0.0	**-0.2**	0.0	**-0.2**	0.0	**-0.2**
13	2.4	1.0	**-1.4**	1.2	**-1.2**	0.8	**-1.6**	0.6	**-1.8**
14	2.2	0.8	**-1.4**	1.0	**-1.2**	0.4	**-1.8**	0.2	**-2.0**
15	3.6	4.6	**1.0**	2.2	**-1.4**	1.4	**-2.2**	0.4	**-3.2**
16	4.6	4.4	**-0.2**	1.8	**-2.8**	2.4	**-2.2**	2.8	**-1.8**
Mean	**3.2**	**2.7**	**-0.6**	**1.9**	**-1.3**	**1.9**	**-1.3**	**1.5**	**-1.7**
Non-responders									
17	2.8			3.2	**0.4**	3.4	**0.6**	2.6	**-0.2**
18	5.2	2.2	**-3.0**	2.4	**-2.8**	2.8	**-2.4**	1.8	**-3.4**
19	3.2			2.8	**-0.4**	2.2	**-1.0**	3.0	**-0.2**
20	4.0			2.8	**-1.2**	4.0	**0.0**	3.2	**-0.8**
21	2.8			2.8	**0.0**	3.2	**0.4**	1.2	**-1.6**
22	4.4	2.6	**-1.8**	2.2	**-2.2**	2.4	**-2.0**	4.6	**0.2**
23	3.8	3.4	**-0.4**	4.4	**0.6**	4.2	**0.4**	4.2	**0.4**
24	5.2			3.6	**-1.6**	4.6	**-0.6**	4.6	**-0.6**
25	4.0			2.0	**-2.0**				
26	4.6	4.6	**0.0**	5.2	**0.6**	4.6	**0.0**	4.6	**0.0**
Mean	**4.0**	**3.2**	**-1.3**	**3.1**	**-0.9**	**3.5**	**-0.5**	**3.0**	**-0.7**
P-value	0.0830	0.5910	0.2301	**0.0030**	0.3805	**0.0003**	0.0923	**0.0036**	**0.0254**

B.	ACQ6								
Patient	**Baseline**	**1 Month**	**Difference**	**3 Months**	**Difference**	**6 Months**	**Difference**	**12 Months**	**Difference**
1	3.3			2.2	**-1.2**	3.2	**-0.2**	1.5	**-1.8**
2	4.3			1.3	**-3.0**	2.7	**-1.7**	2.2	**-2.2**
3	4.8			3.3	**-1.5**	2.2	**-2.7**	1.7	**-3.2**
4	4.8			2.5	**-2.3**	2.7	**-2.2**	2.7	**-2.2**
5	2.5			2.5	**0.0**	2.3	**-0.2**	1.0	**-1.5**
6	2.5			1.2	**-1.3**	1.7	**-0.8**	1.3	**-1.2**
7	2.3			1.8	**-0.5**	2.5	**0.2**	1.5	**-0.8**
8	3.2			1.8	**-1.3**	1.3	**-1.8**	0.8	**-2.3**
9	2.7			3.2	**0.5**	2.8	**0.2**	2.8	**0.2**
10	2.0			1.7	**-0.3**	1.5	**-0.5**	1.5	**-0.5**
11	5.0			2.3	**-2.7**	1.7	**-3.3**	2.0	**-3.0**
12	0.2	0.0	**-0.2**	0.0	**-0.2**	0.0	**-0.2**	0.0	**-0.2**
13	2.2	1.0	**-1.2**	1.2	**-1.0**	0.7	**-1.5**	0.7	**-1.5**
14	2.3	0.8	**-1.5**	1.0	**-1.3**	0.5	**-1.8**	0.3	**-2.0**
15	3.8	4.8	**1.0**	2.3	**-1.5**	1.7	**-2.2**	0.5	**-3.3**
16	4.8					2.7	**-2.2**	2.8	**-2.0**
Mean	**3.1**	**1.7**	**-0.5**	**1.9**	**-1.0**	**1.9**	**-1.4**	**1.5**	**-1.8**
Non-responders									
17	3.2			3.5	**0.3**	3.8	**0.7**	3.2	**0.0**
18	5.0					2.7	**-2.3**	1.7	**-3.3**
19	3.2			2.8	**-0.3**	2.2	**-1.0**	3.0	**-0.2**
20	4.0			2.8	**-1.2**			3.2	**-0.8**
21	2.8			2.8	**0.0**	3.2	**0.3**	1.7	**-1.2**
22	4.2	2.7	**-1.5**	2.2	**-2.0**	2.3	**-1.8**	4.7	**0.5**
23	3.8	3.5	**-0.3**	4.7	**0.8**	4.2	**0.3**	4.5	**0.7**
24	5.2			3.8	**-1.3**	4.7	**-0.5**	4.7	**-0.5**
25	4.0			2.2	**-1.8**				
26	4.8	4.5	**-0.3**	5.2	**0.3**	4.8	**0.0**	4.8	**0.0**
Mean	**4.0**	**3.6**	**-0.7**	**3.3**	**-0.6**	**3.5**	**-0.5**	**3.1**	**-0.5**
P-value	0.0896	0.2207	0.7755	**0.0015**	0.1703	**0.0008**	0.1183	**0.0012**	**0.0158**

C.	ACQ7								
Patient	**Baseline**	**1 Month**	**Difference**	**3 Months**	**Difference**	**6 Months**	**Difference**	**12 Months**	**Difference**
1	3.4			2.3	**-1.1**	3.4	**0.0**	1.7	**-1.7**
2	4.4			1.6	**-2.9**	2.9	**-1.6**	2.1	**-2.3**
3	5.0							1.7	**-3.3**
4	5.0					3.1	**-1.9**	3.0	**-2.0**
5	2.1			2.4	**0.3**	2.1	**0.0**	1.0	**-1.1**
6								1.9	**1.9**
7								2.0	**2.0**
8	3.0			1.9	**-1.1**	1.6	**-1.4**	1.1	**-1.9**
9	3.1			3.6	**0.4**	3.3	**0.1**	3.3	**0.1**
10	2.3			1.9	**-0.4**	2.0	**-0.3**	2.1	**-0.1**
11	4.7			2.3	**-2.4**	1.4	**-3.3**	2.0	**-2.7**
12	0.4	0.4	**0.0**	0.4	**0.0**	0.3	**-0.1**	0.6	**0.1**
13	2.4	1.1	**-1.3**	1.4	**-1.0**	0.9	**-1.6**	0.9	**-1.6**
14	2.9	1.6	**-1.3**	1.7	**-1.1**	1.3	**-1.6**	1.1	**-1.7**
15	4.0	5.0	**1.0**	2.9	**-1.1**	2.3	**-1.7**	1.3	**-2.7**
16	4.7					3.0	**-1.7**	3.1	**-1.6**
Mean	**3.4**	**2.0**	**-0.4**	**2.0**	**-1.0**	**2.1**	**-1.2**	**1.8**	**-1.2**
Non-responders									
17	3.4			3.6	**0.1**	3.7	**0.3**	3.0	**-0.4**
18	5.0							2.3	**-2.7**
19	3.6			3.3	**-0.3**	2.7	**-0.9**	3.4	**-0.1**
20	4.0			3.0	**-1.0**			3.3	**-0.7**
21	2.9			2.9	**0.0**			2.3	**-0.6**
22	4.4	3.1	**-1.3**	2.7	**-1.7**	2.9	**-1.6**	4.9	**0.4**
23	3.9	3.7	**-0.1**	4.9	**1.0**	4.3	**0.4**	4.3	**0.4**
24									
25	3.4								
26		4.7							
Mean	**3.8**	**3.9**	**-0.7**	**3.4**	**-0.3**	**3.4**	**-0.4**	**3.3**	**-0.5**
P-value	0.4137	0.2085	0.7593	**0.0047**	0.2340	**0.0335**	0.2369	**0.0007**	0.3403

Table 6.10 Individual responses to the asthma control test (ACT) at each visit

	ACT								
Patient	**Baseline**	**1 Month**	**Difference**	**3 Months**	**Difference**	**6 Months**	**Difference**	**12 Months**	**Difference**
1	10.0			14.0	**4.0**	10.0	**0.0**	16.0	**6.0**
2	7.0			18.0	**11.0**	14.0	**7.0**	13.0	**6.0**
3	7.0					13.0	**6.0**	16.0	**9.0**
4	5.0					15.0	**10.0**	13.0	**8.0**
5	12.0					16.0	**4.0**	22.0	**10.0**
6	15.0			18.0	**3.0**	18.0	**3.0**	19.0	**4.0**
7	12.0			14.0	**2.0**	16.0	**4.0**	21.0	**9.0**
8	9.0			13.0	**4.0**	17.0	**8.0**	19.0	**10.0**
9	15.0			8.0	**-7.0**	13.0	**-2.0**	11.0	**-4.0**
10	12.0			18.0	**6.0**	18.0	**6.0**	20.0	**8.0**
11	5.0			12.0	**7.0**	18.0	**13.0**	15.0	**10.0**
12	24.0	25.0	**1.0**	25.0	**1.0**	25.0	**1.0**	24.0	**0.0**
13	15.0			22.0	**7.0**	25.0	**10.0**	24.0	**9.0**
14	16.0	22.0	**6.0**	22.0	**6.0**	24.0	**8.0**	24.0	**8.0**
15	9.0	10.0	**1.0**	15.0	**6.0**	16.0	**7.0**	21.0	**12.0**
16	11.0					15.0	**4.0**	12.0	**1.0**
Mean	**11.5**	**19.0**	**2.7**	**16.6**	**4.2**	**17.1**	**5.6**	**18.1**	**6.6**
Non-responders									
17	9.0			8.0	**-1.0**	9.0	**0.0**	11.0	**2.0**
18	6.0					9.0	**3.0**	20.0	**14.0**
19	9.0			12.0	**3.0**	17.0	**8.0**	12.0	**3.0**
20	7.0			10.0	**3.0**			13.0	**6.0**
21	11.0			11.0	**0.0**	7.0	**-4.0**		
22	7.0	9.0	**2.0**	12.0	**5.0**	11.0	**4.0**	7.0	**0.0**
23	7.0	9.0	**2.0**	8.0	**1.0**	8.0	**1.0**	9.0	**2.0**
24	5.0			9.0	**4.0**	9.0	**4.0**	9.0	**4.0**
25	8.0			14.0	**6.0**				
26	6.0	6.0	**0.0**	6.0	**0.0**	8.0	**2.0**	8.0	**2.0**
Mean	**7.5**	**8.0**	**1.3**	**10.0**	**2.3**	**9.8**	**2.3**	**11.1**	**4.1**
P-value	0.0203	0.0789	0.4989	**0.0015**	0.2765	**0.0003**	0.0571	**0.0012**	0.1972

Table 6.11 Individual responses to forced expiratory volume over one second (FEV_1) at each visit

	FEV1								
Patient	**Baseline**	**1 Month**	**Difference**	**3 Months**	**Difference**	**6 Months**	**Difference**	**12 Months**	**Difference**
1	1.8	1.9	**0.1**	2.0	**0.2**	1.6	**-0.1**	2.1	**0.3**
2	1.8	2.1	**0.3**	2.4	**0.5**	2.0	**0.2**	2.7	**0.9**
3	1.0	0.9	**-0.1**					2.8	**1.8**
4	1.5	1.7	**0.2**			1.6	**0.1**	1.7	**0.3**
5	2.8			2.3	**-0.5**	2.6	**-0.1**	2.7	**-0.1**
6								1.7	
7								1.9	
8	2.9	3.2	**0.3**	2.7	**-0.1**	2.5	**-0.3**	2.4	**-0.4**
9	1.1	1.0	**-0.1**	1.0	**-0.1**	1.0	**-0.1**	0.9	**-0.2**
10	2.0	2.2	**0.2**	2.0	**0.0**	1.6	**-0.4**	1.3	**-0.7**
11	2.4	2.9	**0.6**	2.8	**0.4**	3.2	**0.8**	2.9	**0.6**
12	1.8	1.8	**0.0**	1.8	**-0.1**	1.8	**0.0**	1.4	**-0.4**
13	2.3	2.9	**0.6**	2.6	**0.4**	2.9	**0.7**	2.7	**0.5**
14	1.1	1.2	**0.1**	1.4	**0.2**	1.3	**0.1**	1.3	**0.1**
15	1.5	1.4	**-0.1**	1.1	**-0.4**	1.4	**-0.1**	1.3	**-0.2**
16	1.6	1.5	**-0.1**	1.5	**-0.1**	1.4	**-0.2**	1.3	**-0.3**
Mean	**1.8**	**1.9**	**0.2**	**2.0**	**0.0**	**1.9**	**0.0**	**1.9**	**0.1**
Non-responders									
17	1.6	1.4	**-0.2**	1.9	**0.3**	2.0	**0.5**	2.2	**0.6**
18	0.9	0.9	**0.0**	0.8	**-0.1**			0.7	**-0.2**
19	1.9	1.7	**-0.2**	1.6	**-0.3**	2.0	**0.1**	1.7	**-0.2**
20	2.3	2.4	**0.1**	2.1	**-0.2**	1.9	**-0.4**	2.2	**-0.2**
21	3.5	3.5	**-0.1**	3.7	**0.2**			2.1	**-1.5**
22	0.8	1.0	**0.1**	1.0	**0.2**	0.8	**0.0**	0.7	**-0.1**
23	1.9	1.7	**-0.2**	1.3	**-0.6**	1.9	**-0.1**	2.5	**0.6**
24									
25	2.9								
26		1.9							
Mean	**1.9**	**1.8**	**-0.1**	**1.8**	**-0.1**	**1.7**	**0.0**	**1.7**	**-0.1**
P-value	0.6141	0.7958	0.0398	0.6156	0.4981	0.5692	0.8877	0.5028	0.1741

Table 6.12 Individual responses to airway reactance (AX) at each visit

Patient	AX								
	Baseline	1 Month	Difference	3 Months	Difference	6 Months	Difference	12 Months	Difference
Responders									
1	11.1	10.1	**-1.0**	18.1	**7.0**	21.3	**10.2**	12.2	**1.1**
2	28.5	108.2	**79.7**	4.0	**-24.5**	5.0	**-23.5**	3.0	**-25.5**
3	69.8	82.6	**12.8**	30.6	**-39.2**			79.5	**9.7**
4	63.7	59.4	**-4.3**	62.3	**-1.4**	56.5	**-7.2**	33.6	**-30.1**
5	42.9	6.8	**-36.2**	13.5	**-29.4**	12.0	**-30.9**	6.7	**-36.2**
6	135.5	84.6	**-51.0**	71.6	**-64.0**	87.0	**-48.5**	93.9	**-41.6**
7	21.1	5.4	**-15.7**	1.9	**-19.2**	5.5	**-15.5**	3.0	**-18.0**
8	7.8	9.0	**1.3**	11.9	**4.1**	9.4	**1.7**	11.0	**3.2**
9	94.7	87.7	**-7.0**	103.5	**8.8**	98.2	**3.5**	117.7	**23.0**
10	4.8	3.9	**-0.9**	18.5	**13.8**	16.5	**11.8**	39.7	**35.0**
11	104.5	41.2	**-63.3**	38.4	**-66.1**	22.6	**-81.9**	25.7	**-78.8**
12	12.5	10.6	**-1.9**	11.9	**-0.6**	9.5	**-3.0**	16.3	**3.8**
13	59.4	32.6	**-26.7**	44.5	**-14.9**	12.9	**-46.4**	20.3	**-39.1**
14	40.9	37.5	**-3.4**	33.2	**-7.7**	19.4	**-21.5**	32.2	**-8.7**
15	33.8	53.0	**19.2**	79.2	**45.4**	46.1	**12.4**	46.0	**12.2**
16	54.8	103.3	**48.5**	98.8	**43.9**	114.1	**59.3**	151.4	**96.6**
Mean	**49.1**	**46.0**	**-3.1**	**40.1**	**-9.0**	**35.7**	**-12.0**	**43.3**	**-5.8**
Non-responders									
17	35.0	56.6	**21.6**	19.9	**-15.1**	17.3	**-17.7**	4.9	**-30.2**
18	3.4	22.6	**19.2**	36.7	**33.3**	21.6	**18.2**	19.7	**16.3**
19	19.1	23.5	**4.4**	29.4	**10.3**	20.9	**1.8**	75.7	**56.6**
20	23.1	22.1	**-0.9**	23.4	**0.3**	30.3	**7.2**	21.2	**-1.9**
21	16.8	18.8	**2.1**	12.4	**-4.4**	19.1	**2.4**	45.4	**28.7**
22	91.3	81.5	**-9.8**	63.7	**-27.6**	96.2	**4.9**	96.3	**5.0**
23	30.8	60.3	**29.5**	77.6	**46.8**	42.0	**11.2**	21.9	**-8.9**
24	92.6								
25	3.1	3.9	**0.9**						
26	89.9	13.9	**-76.0**	123.7	**33.9**				
Mean	**40.5**	**33.7**	**-1.0**	**48.3**	**9.7**	**35.3**	**4.0**	**40.7**	**9.4**
P-value	0.5722	0.3940	0.8803	0.5900	0.1660	0.9798	0.2319	0.8937	0.3705

6.11.4 SERUM CYTOKINE LEVELS

Baseline serum samples were obtained from 45 patients at baseline. The results can be visualized in **Figure 6.22.** Three patients did not have detectable levels of any of the 13 cytokines measured and one patient had detectable levels of every cytokine. TSLP was the most detected cytokine (31 samples, 69 %) while TGF-β1 was the least detected cytokine (3 samples, 7 %). Interestingly IL-5 was only detectable in 11 samples (24 %) which ranged from 24.8- 10029.6 pg/ mL, median 406.3 pg/ mL. These results are summarized in **Table 6.13**.

Following 12 months of treatment, nine patients who had their baseline cytokines measured had them remeasured at every visit. Eight patients (89 %) were classified as responders, one (11 %) was a non-responder. The concentrations of all cytokines, including IL-5, remained relatively constant throughout the course of treatment. The concentrations of IL-4, IL-12, IFN-γ, IL-1β, TNF-α, IL-8, IL-10, and TGF-β1 were generally higher in the responders than in the non-responders, the remaining cytokines were similar between groups. The change in cytokines throughout treatment is represented in **Figure 6.23**.

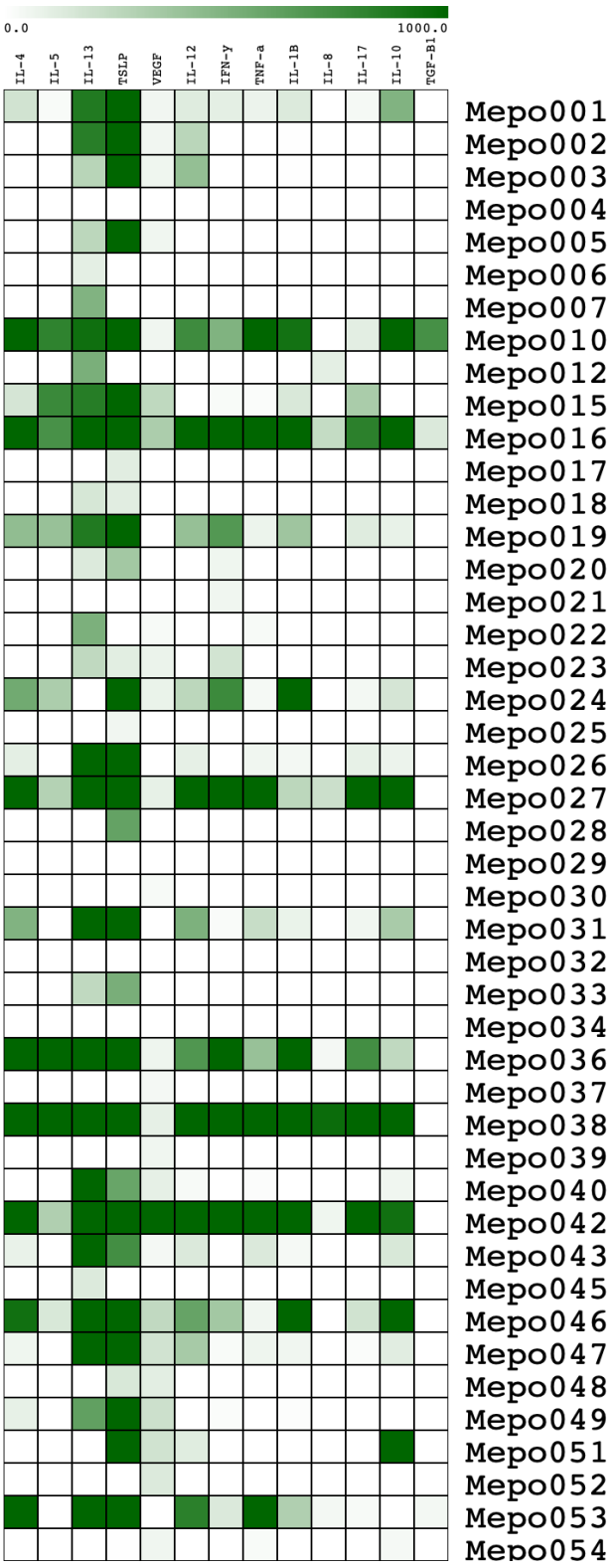


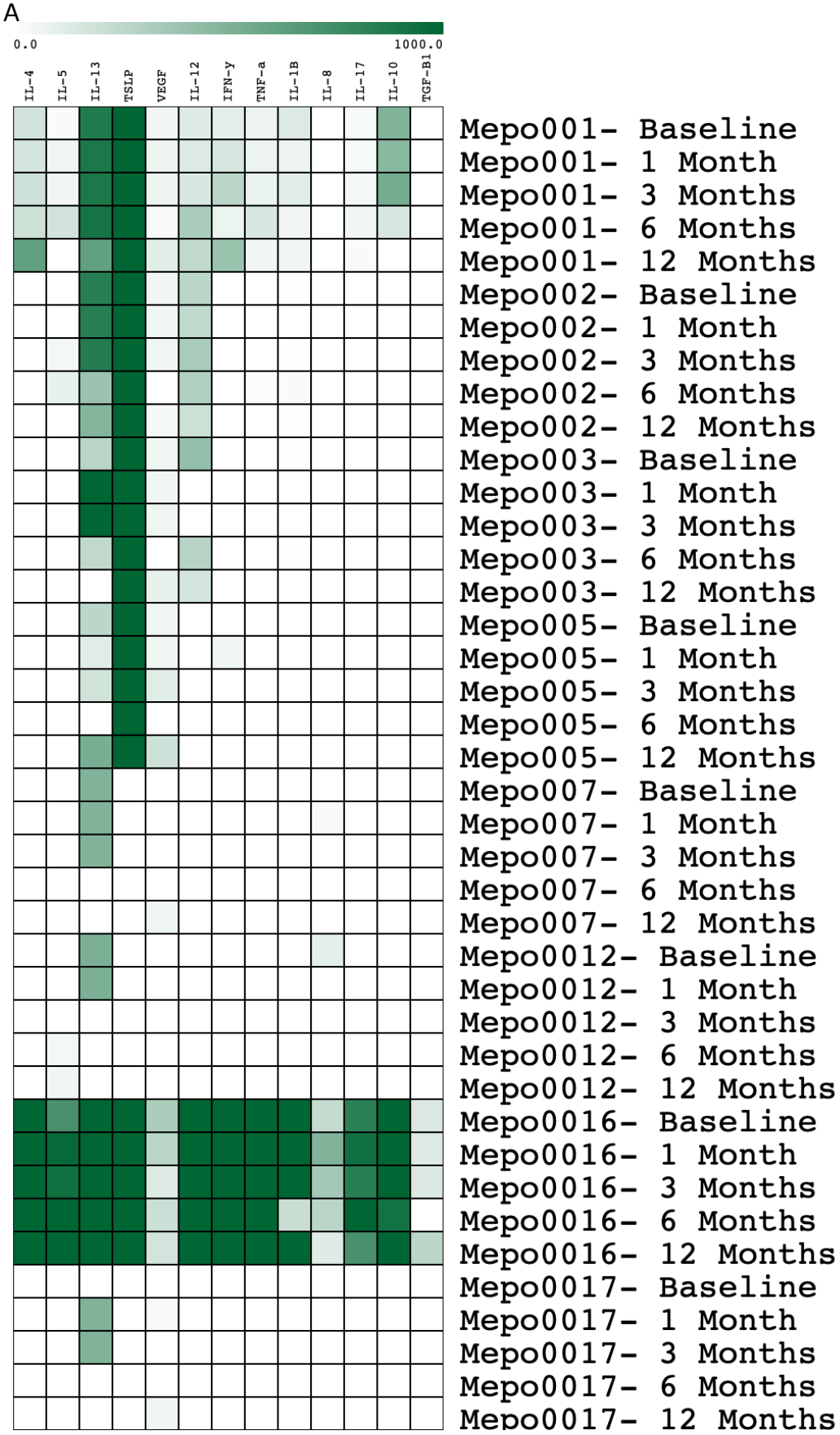
Figure 6.22 Concentrations at Baseline

Concentrations are represented on a heat map where 0.0 pg/ mL is represented as white and the colour darkens as the concentration increases to a maximum of 1000.0 pg/ mL.

Table 6.13 Summary of serum cytokine concentrations at baseline

	Detectable Samples	Median (pg/ mL)	Range (pg/ mL)
IL-4	17	55.4	66.4-28902.6
IL-5	11	406.3	28.4-10029.6
IL-13	29	870.8	103.4-228604.9
TSLP	31	3012	7.3-144905.2
VEGF	26	80.8	33.2-30610.7
IL-12	19	417.1	34.9-20872.0
IFN-y	18	267.8	20.2-25526.6
TNF-a	19	76.2	17.0-17317.0
IL-1B	21	149.7	3.0-17719.5
IL-8	7	103.2	39.7-959.9
IL-17	15	127.9	23.5-5609.9
IL-10	17	340.3	38.7-15357.9
TGF-B1	3	141.7	50.7-721.8

A


0.0
1000.0
IL-4
IL-5
IL-13
TSLP
VEGF
IL-12
IFN-γ
TNF-α
IL-1B
IL-8
IL-17
IL-10
TGF-B1
Mepo001- Baseline
Mepo001- 1 Month
Mepo001- 3 Months
Mepo001- 6 Months
Mepo001- 12 Months
Mepo002- Baseline
Mepo002- 1 Month
Mepo002- 3 Months
Mepo002- 6 Months
Mepo002- 12 Months
Mepo003- Baseline
Mepo003- 1 Month
Mepo003- 3 Months
Mepo003- 6 Months
Mepo003- 12 Months
Mepo005- Baseline
Mepo005- 1 Month
Mepo005- 3 Months
Mepo005- 6 Months
Mepo005- 12 Months
Mepo007- Baseline
Mepo007- 1 Month
Mepo007- 3 Months
Mepo007- 6 Months
Mepo007- 12 Months
Mepo0012- Baseline
Mepo0012- 1 Month
Mepo0012- 3 Months
Mepo0012- 6 Months
Mepo0012- 12 Months
Mepo0016- Baseline
Mepo0016- 1 Month
Mepo0016- 3 Months
Mepo0016- 6 Months
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Mepo0017- 6 Months
Mepo0017- 12 Months

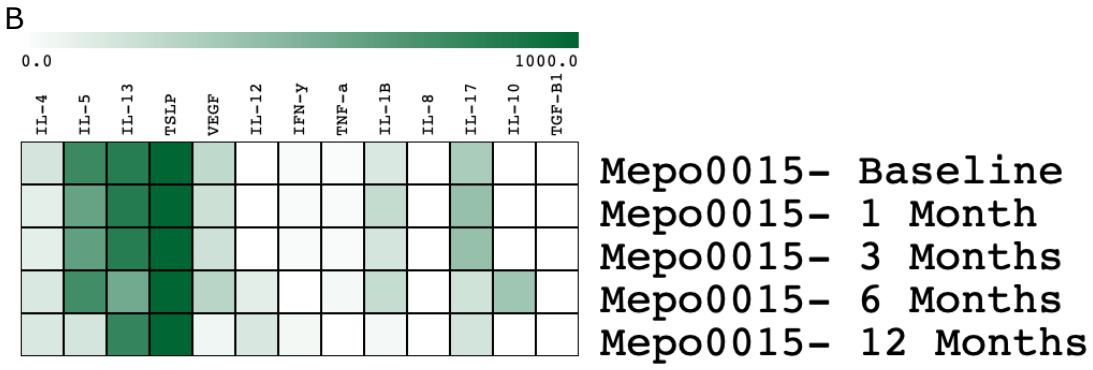


Figure 6.23 Serum Cytokine concentrations over 12 months

Serum cytokine concentrations are represented on a scale from 0-1000 pg/mL where 0 pg/mL is white and the darkest green is 1000 pg/mL.**A**. Responders, **B**. Non-responders.

6.11.4.1 Mepolizumab assay validation

To confirm that mepolizumab binds exclusively to IL-5 a 384 well ELISA was conducted using a capture IL-5 antibody and IL-5 standard with mepolizumab as the detection antibody. Results confirmed that mepolizumab does bind IL-5 with approximately 2.5x the absorbance reading of any other cytokine (**Figure 6.24**).

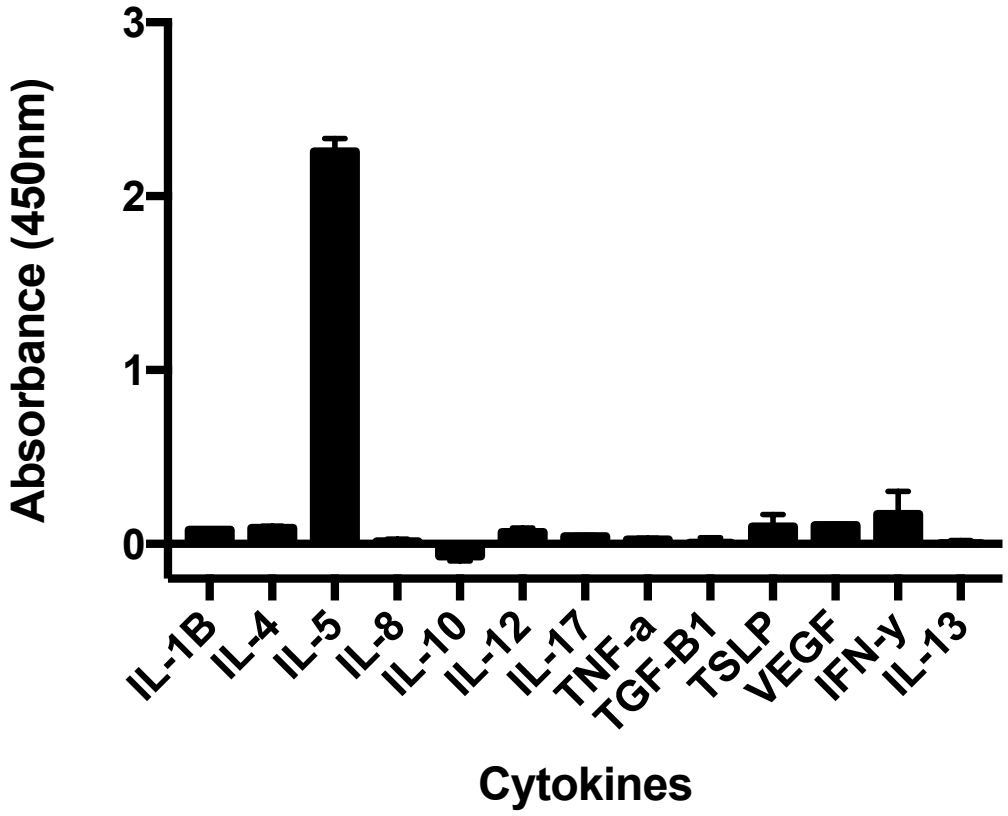


Figure 6.24 Mepolizumab enzyme-linked immunosorbent assay

Mepolizumab was used as the capture antibody to demonstrate specific binding to IL-5.

6.12 DISCUSSION

Mepolizumab is the first anti-IL-5 monoclonal antibody approved to treat severe exacerbation-prone eosinophilic asthma. Currently, treatment success is determined following 12 months of treatment based on a clinically significant reduction in the number of exacerbations from the previous 12 months and/or a reduction in the dose of maintenance OCS, which for the purposes of this audit was determined to be ≥50%. Based on these guidelines this audit demonstrated that 61.5 % of patients were responsive to mepolizumab.

Consistent with previous studies we showed that blood eosinophils were reduced after just one injection (93, 245, 328, 333). We also demonstrated that symptom scores (ACQ and ACT) were improved by a clinically significant amount from baseline to one month which either remained constant or continued to improve throughout the study. The occurrence of a symptomatic improvement is inconsistent in the literature (93, 208, 328). By analyzing all versions of the ACQ we were able to demonstrate that the percentage of patients who felt their symptoms were well controlled following treatment was higher in the ACQ5 and 6 than the ACQ7. This finding is consistent with the lack of symmetry between symptoms and lung function. Completing all versions of the ACQ was useful to identify those who exclusively report a subjective improvement from those who also have an objective improvement (FEV_1).

Previous studies have not demonstrated that mepolizumab significantly improves lung function (323, 326, 334). Here we demonstrated that there was an increase in FEV_1 after one month in the responders. The greatest individual improvement was an increase of 1.8 L by 12 months (0.98 L to 2.76 L). The non-responders experienced a mean decrease of 0.1 L from baseline to 12 months.

To our knowledge, this is the first study to investigate the effects of mepolizumab on FOT. We determined that all measured parameters of resistance and reactance were improved in the responders whereas only total airway and large airway resistance were improved in the non-responders after 12 months. In particular, there was a large difference in reactance (AX) between groups. At baseline, reactance was higher in the responders than non-responders (mean 49.1 Hz vs 40.5 Hz) however, by the 12 months visit the responders decreased by a mean of 5.8 Hz while the non-responders increased by a mean of 9.4 Hz.

One of the aims of the audit was to identify factors that could predict response to treatment prior to 12 months. Previous studies have consistently found that elevated blood eosinophils at baseline and frequent exacerbations are predictive features of a positive response to mepolizumab (93, 208, 330). However, in this audit the predictive factors we identified were that responders were diagnosed at a significantly older age (24.6 vs 11.3 years of age, p=0.0480), experienced significantly fewer exacerbations in the 12 months prior to initiating treatment (5.4 vs 9.0, p=0.0231) and had clinically and

statistically significantly better ACT scores (11.5 vs 7.5, p=0.0203) than the non-responders. The responders also had clinically better ACQ5 and 6 scores than the non-responders. However, the responders were taking a slightly higher dose of OCS at baseline which may account for why they had fewer exacerbations during the year prior to starting treatment than the non-responders. As such, the most useful predictive features we identified were older age at diagnosis, a slightly higher dose of OCS and a better ACT score.

All symptom questionnaire scores improved following treatment regardless of whether they were classified as responders or not however, the improvement was greater in the responders. The difference between groups became statistically significant at three months. There was a trend for improved lung function in the responders based on FEV_1 and small airway reactance (AX) which was also greatest after three months, however, this trend was not strong enough to suggest that stopping treatment early in patients who do not show these trends would be advisable.

Interestingly, at baseline only 11 patients (24 %) had detectable levels of IL-5. Baseline IL-5 levels were not correlated with baseline blood eosinophil levels (results presented in **Chapter 3**). Based on a study by Alexander and colleagues the physiologic serum concentration of IL-5 in patients with severe asthma is approximately 150 pg/ mL (335). In this audit, the median detectable concentration was higher than this at baseline (406.3 pg/ mL) and remained relatively stable throughout the

study. It is difficult to determine if having a high level of IL-5 at baseline was related to response as only three of the 11 patients completed 12 months of treatment and had their levels measured at every visit (two responders, one non-responder). However, a study by Stein and colleagues determined that response to mepolizumab was not related to baseline plasma IL-5 concentrations. They found that plasma IL-5 concentrations increased with treatment which they speculated may be due to long-lived mepolizumab/IL-5 complexes (242). This finding was not replicated here as serum IL-5 levels remained relatively constant.

6.12.1 LIMITATIONS

This audit has several limitations that need to be addressed.

First, due to the nature of the audit and the severity of the disease, there are several gaps in data collection where patients did not want to or were unable to perform certain tests at various time points which makes it difficult to accurately measure the changes throughout the year.

Second, again due to disease severity, it was not possible to obtain sputum samples which would have been useful in providing information regarding inflammatory cells and cytokines in the local lung environment. However, sputum cell counts are not performed routinely due to the need for specialized processing equipment and training. Therefore, not including this in our audit may make the findings more applicable to routine clinical care. While it would have been interesting to determine if sputum eosinophilia was a baseline or early marker of response, a report by Yancey and colleagues suggests that this may not

have been the case. They demonstrated that blood eosinophils were a better predictor and correlated better with clinical outcomes than sputum eosinophils (197).

Third, as patients were enrolled into the audit and started taking mepolizumab at different times throughout the year, exacerbation prone times (ex. winter or allergy season) occurred at different visits which may have contributed to some clinical variation at different time points.

Fourth, relatively few patients completed the full year of treatment and even less had cytokine measurements at one year which made it difficult to find baseline predictors or trends resulting from treatment.

Fifth, as this was an audit performed on an NHS clinic it was not possible to include a control group. As such, we cannot conclude that some of the benefit seen here was not due to being part of a study and therefore some of the findings may be slightly inflated.

Finally, the study population was primarily comprised of obese patients. As such, it may be difficult to determine what proportion of their symptoms and poor lung function is due to their weight and how much can actually be attributed to asthma. Mepolizumab is not dosed per kilogram so it could be possible that some patients did not receive an adequate dose to see a response. However, this seems unlikely as the responders had a higher BMI at baseline than the non-responders (36.9 vs 30.9) and dosing and route of administration have previously been studied extensively and determined that dosing per kg was not necessary (245, 319, 322).

6.12.2 CONCLUSION

In conclusion, mepolizumab was effective at reducing exacerbations and allowing patients to reduce their maintenance dose of OCS without compromising lung function or control in 61.5 % of patients. Additionally, treatment resulted in reduced blood eosinophil counts and improved asthma symptom scores. The predictive factors identified here were: an older age at diagnosis, fewer exacerbations during the previous year, a slightly higher daily dose of OCS, statically significantly better ACT and clinically better ACQ5 and 6 scores. While scores improved with treatment in both groups, this increase was much greater in the responders than the non-responders. These findings may be useful to start distinguishing between the responders from the non-responders prior to one year.

CHAPTER 7

DISCUSSION

Asthma is a complex heterogeneous disease of the airways, meaning that there are multiple contributing components not of which are present in every patient or at every point in time. This underlines the importance of identifying reliable biomarkers and the need to implement precision medicine consisting of treatments that can be targeted to individuals, maximizing clinical outcomes and minimizing unnecessary treatment side effects. Despite the well-acknowledged heterogeneity in asthma, the majority of patients are treated the same way. This is because pharmacological management is largely administered on the basis of severity which is dictated by symptoms rather than according to individual factors and phenotypes or endotypes. Phenotypes really only being to direct treatment when patients are classified as "severe" at which point there are a few biologic options available depending on the patient's phenotype.

The work presented in this thesis further supports the utilization of biomarkers and baseline or early predictive traits to identify those patients who are most likely to respond to specific treatments. However, there are clearly still some challenges to using biomarkers and further work is needed to identify highly specific and sensitive predictive biomarkers for asthma treatments.

The first study presented, "Does a low exhaled nitric oxide level exclude a clinical benefit from inhaled corticosteroids in suspected asthma" demonstrated that in steroid-naive patients a low FeNO (≤27 ppb) can be used to identify patients who are unlikely to benefit from an ICS in

the short-term. Further work needs to be conducted to determine if this trend persists over the long-term and particularity whether these patients are still at risk of experiencing exacerbations. Regardless, the lack of a symptomatic response at three months is likely to impair adherence with low dose ICS so this could be an ideal group to consider for treatment with an as required ICS/LABA inhaler to prevent exacerbations. This finding has the potential to minimize unnecessary negative treatment side effects and reduce prescription costs by identifying a group of patients who are unlikely to benefit before treatment is started.

Post hoc analysis found there were significantly more responders in the high FeNO tertile (21-27 ppb) in the budesonide group (n=14) than in the placebo group (n=4) and further work is needed to evaluate if a lower FeNO cut point would be beneficial

The second study presented, "An evaluation of increased doses of corticosteroid in poorly controlled asthma" demonstrated that there is considerable variability in responses to increased doses of corticosteroid. Increasing the dose is a common practice for patients who are poorly controlled. However, as this work demonstrated, simply increasing the dose is not always effective and it subjects the patients to an increased risk of experiencing adverse treatment effects. The main finding was that patients on low dose ICS at baseline were more likely to respond to an increased dose than those already on a medium or high dose. This study involved very few participants (n=13) and was not placebo controlled.

As such, we cannot conclude that all of the benefit observed was due to treatment alone. Future studies involving more patients and a control group would be beneficial to be able to reliably identify baseline features that could predict a positive response to increased corticosteroid treatment. Additionally, these studies should evaluate the long-term effects (ie. exacerbation prevention vs increased potential for adverse side-effects) as well as effects on the small airways. This region is poorly described in the literature but is likely to be responsive to corticosteroids, as was seen in this study by an improvement in FOT R5-20 readings but not necessarily in spirometry FEV_1.

The final piece of work, an audit entitled "An evaluation of response to treatment with mepolizumab in patients with severe asthma" demonstrated considerable variability in treatment response to mepolizumab even though all patients fulfilled the NICE criteria to receive mepolizumab i.e. severe, eosinophilic, on high-dose corticosteroids and exacerbation prone. Patients were required to meet certain clinical (≥4 exacerbations in the previous year and/or a minimum of 5 mg/day of oral corticosteroid for the past six months) and phenotypic criteria (blood eosinophils of ≥300 cells/μL). However, despite these strict requirements, only 16 patients (61.5 %) responded to treatment. Therefore, these requirements are not specific enough to identify only those who will benefit. As mepolizumab is very costly (£840 per injection before the NHS discount is applied) and time-consuming for the patients it is crucial to identify those who are most likely to benefit

as early as possible (248). This audit revealed that the responders were older at diagnosis, had fewer exacerbations in the previous year, and reported clinically better ACQ 5, 6 scores and significantly better ACT scores than the non-responders. The responders were taking a slightly higher dose of OCS at baseline which may be why they experienced fewer exacerbations in the year leading up to the audit than the non-responders. As such, the most helpful baseline factors identified here were age at diagnosis, a high daily OCS dose and ACT score. This is in contrast to previous studies which have consistently shown that elevated baseline blood eosinophil levels and more frequent exacerbations were predictive of a positive response to treatment (93, 208, 330).

Following treatment, there was a general improvement in symptom scores. However, this improvement was greater in the responders than non-responders. FEV_1 and AX were also improved in the responders whereas there was either a decrease or no change in the non-responders. Due to the nature of the observational audit, it was not possible to include a control group and as such, some of the observed results may not be due to treatment alone.

Additional studies need to be conducted to identify baseline features that are better able to predict treatment outcomes. For example, determining sputum cells counts would be ideal to provide valuable insights into the local inflammation which may be different from systemic inflammation reflected in an FBC.

In conclusion, asthma is a highly heterogeneous disease, presenting and responding to treatments differently in each patient. The work performed in this thesis supports the idea that the identification of predictive biomarkers is essential to optimize asthma treatments and progress care towards personalized medicine. Personalizing care will reduce unnecessary trial prescription costs, minimize adverse side-effects and improve overall patient care and quality of life.

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APPENDICES

A: Consent Form

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Nottingham Asthma Centre

CONSENT FORM
Final version 1.0: 12/08/2016

Title of Study: An evaluation of increased doses of steroid in poorly controlled asthma

IRAS Project ID: 207761

Name of Researcher: Dr. Tim Harrison

Name of Participant: **Please initial box**

1. I confirm that I have read and understand the information sheet version number 1.0 dated 12/08/2016 for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐
3. I understand that relevant sections of my medical notes and data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐
4. I understand and agree that a blood, urine and sputum sample will be taken for analysis of biomarkers. ☐
5. Consent for storage and use in possible future research (Optional)
   I agree that the samples I have given and the information gathered about me can be stored by the University of Nottingham at the Clinical Sciences Building, for possible use in future studies. I understand that some of these studies may be carried out by researchers other than the current team who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and I will not be identified in anyway. ☐
6. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

3 copies: 1 for participant, 1 for the project notes and 1 for the medical notes

B: Participant Information Sheet





Participant Information Sheet
Final version 1.0: 03/10/2016
IRAS Project ID: 207761

Title of Study: An evaluation of increased doses of steroid in poorly controlled asthma

Name of Researcher(s):Dr. Tim Harrison

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. Talk to others about the study if you wish. Ask us if there is anything that is not clear.

What is the purpose of the study?
Most people with asthma take an inhaled steroid to control their symptoms. A common question in clinical practice is whether more steroid would be of benefit? To determine this patients are often prescribed a month of high dose inhaled steroids or a two-week course of oral steroids to document steroid responsiveness. The purpose of this study is to describe the features of responders and non-responders to an increased dose of steroid and establish if it is possible to identify patients who will and will not respond to increased doses of steroids in terms of clinical measurements and biomarkers. In addition this project will provide an educational project towards a PhD.

Why have I been invited?
You are being invited to take part because your consultant has determined that your asthma is inadequately controlled and you have been prescribed an increased dose of steroids. We are inviting 50 participants like you to take part.

Do I have to take part?

It is up to you to decide whether or not to take part, your decision will not affect your medical care in any way. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This would not affect your legal rights.

What will happen to me if I take part?
Before you start the increased dose of steroids and on your last day of the increased dose (either two or four weeks later) you will come into the clinic where you will fill out a questionnaire and give a blood, sputum and urine sample for analysis. Three breathing tests will be performed. Spirometry involves filling your lungs and then blowing as hard and as long as you can into a machine to evaluate lung function. Fractional exhaled nitric oxide test involves a long slow exhaled breath into a machine which will determine airway inflammation. Oscillometry involves breathing into a machine that measures the functionality of your small airways. The visits are likely to last 1-2 hours.

Expenses and payments
You will not be paid to participate in the study but, we will refund your travelling expenses if you have to make extra visits to the hospital.

What are the possible disadvantages and risks of taking part?
Some of the breathing tests can be a little uncomfortable. However, the tests being conducted in this study are standard tests used in clinical practice and will be stopped immediately if you find them unpleasant.

What are the possible benefits of taking part?
We cannot promise the study will help you but the information we get from this study may help improve treatment guidelines and assessment of response to steroid treatment.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you remain unhappy and wish to complain formally, you can do this by contacting Patient Advice and Liaison Services free from a UK landline: 0800 183 0204; from a mobile or abroad 0115 924 9924 ext 6512 or 62301; email pals@nuh.nhs.uk; text message 07812 270 003; or by post at NUH NHS Trust, c/o PALS. Freepost, NEA 14614, Nottingham NG& 1BR

Will my taking part in the study be kept confidential?
We will follow ethical and legal practice and all information about you will be handled in confidence.

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept strictly confidential, stored in a secure and locked office, and on a password protected database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it.

Your personal data (address, telephone number) will be kept for one year after the end of the study so that we are able to contact you about the findings of the study. All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team will have access to your personal data.

What will happen if I don't want to carry on with the study?
Your participation is voluntary and you are free to withdraw at any time, without giving any reason, and without your legal rights being affected. If you withdraw then the information collected so far cannot be erased and this information may still be used in the project analysis.

What will happen to any samples I give?
We would also like to seek your consent so that any remaining samples may be stored and used in possible future research – this is optional (please indicate you agree to this on the consent form). The samples will be stored with a code unique to you and securely at the University of Nottingham under the University's Human Tissue Research Licence (no 12265).
Some of these future studies may be carried out by researchers other than current team running this study including researchers working for commercial companies. Any samples or data used will be anonymised, and you will not be identified in anyway. If you do not agree to this any remaining samples will be disposed of in accordance with the Human Tissue Authority's codes of practice.

What will happen to the results of the research study?
The results of the research will form part of a PhD thesis and will be presented at a scientific meeting and hopefully published in a medical journal. You will not be identified in the results. All findings will be made available to you.

Who is organising and funding the research?
This research is being organised by the University of Nottingham and is being funded from internal sources.

Who has reviewed the study?
All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by University of Nottingham Research Ethics Committee.

Further information and contact details

Co-investigator
Lissa Sutherland

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C: REC Favourable Opinion

South East Coast - Surrey Research Ethics Committee
Whitefriars
Level 3, Block B
Lewins Mead
Bristol
BS1 2NT

Telephone: (020) 71048053

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

06 October 2016 – Revised 25 November 2016

Professor Tim Harrison
Department of Respiratory Medicine
University of Nottingham
Nottingham NG5 1PB

Dear Professor Harrison

Study title:	**An evaluation of increased doses of steroid in poorly controlled asthma**
REC reference:	**16/LO/1828**
Protocol number:	**16075**
IRAS project ID:	**207761**

Thank you for your documents sent on 04 October 2016, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Mr Raj Khullar, nrescommittee.secoast-surrey@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management

permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" above).

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
IRAS Application Form [IRAS_Form_04102016]		04 October 2016
Participant consent form [Consent]	1.0	30 August 2016
Participant information sheet (PIS) [PIS]	1.0	03 October 2016
Research protocol or project proposal [Protocol]	1.0	30 August 2016
Summary CV for Chief Investigator (CI) [CI CV]		17 August 2016
Summary CV for student [Student CV]	1.0	30 August 2016
Validated questionnaire [Asthma Control Questionnaire]		30 August 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

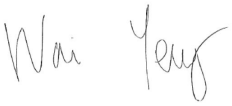
You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

16/LO/1828	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely



PP - Dr Mark Atkins
Chair

Email: nrescommittee.secoast-surrey@nhs.net

Enclosures: *"After ethical review – guidance for researchers"*

Copy to: *Ms Angela Shone*
Alexandra Zeros, Nottingham University Hospitals NHS Trust