Effects of azithromycin on asthma control, airway inflammation and bacterial colonisation in smokers with asthma: a randomised controlled trial

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Abstract

Smokers with asthma represent an important sub-group of asthmatics displaying both reduced response to inhaled and oral corticosteroids as well as demonstrating accelerated decline in lung function and increased use of health care services. Clinical and laboratory studies have suggested that macrolide antibiotics may exhibit anti-inflammatory properties in a variety of airways disease including asthma. The anti-inflammatory properties of macrolides have been recognised for almost 50 years. Indirect evidence from both pre-clinical and clinical studies suggests that the mechanism of action may be of particular benefit in smokers with asthma. A proof of concept study was designed to test the hypothesis that the macrolide antibiotic azithromycin improves measures of asthma control, airway inflammation and bacterial colonisation in smokers with asthma. Azithromycin was chosen for its convenience of once daily dosing and its oral tolerability in addition to its more limited interactions.

Seventy-seven adults with allergic asthma were recruited to a 12-week parallel group randomised controlled trial comparing the effects on asthma control, airway inflammation and bacterial colonisation of oral azithromycin 250 mg daily with matched placebo. The primary outcome measure was peak expiratory flow at the final study visit. Secondary outcome measures included spirometry, asthma control questionnaire [ACQ] score, asthma quality of life questionnaire [AQLQ], Leicester cough questionnaire [LCQ] score, provocation concentration to methacholine PC_{20}, and inflammatory markers: exhaled nitric oxide, sputum differential cell counts, sputum supernatant and serum inflammatory markers such as interleukin-1β [IL-1β], IL-2, -4, -5, -6, -10, TNF-α, IFN-γ, GM-CSF, Leukotriene B4, and high sensitivity C-reactive protein. Microbiological culture and PCR of sputum was also performed to assess for any changes associated with treatment.

At 12 weeks, the change in PEF at the final study visit, as compared with baseline, did not differ significantly between the azithromycin and placebo treatment groups [mean difference azithromycin-placebo -10.3L/min, 95% CI -47.1 to 26.4, p=0.58]. No statistically significant difference was observed between the azithromycin and placebo groups in each of the measures of spirometry, ACQ, AQLQ, LCQ, PC20, or evening PEF. The LCQ-psychological domain did reach statistical significance, [mean difference azithromycin-placebo -0.46, 95%CI -0.9 to 0.02 p=0.04], however this indicates a deterioration in the treatment group.

No change was seen in exhaled nitric oxide. The total cell counts recovered from sputum were similar following treatment with azithromycin compared to placebo. In addition, differential cell counts remained unchanged and lymphocyte proliferation assays did not demonstrate any statistically significant changes following 12 weeks of treatment with azithromycin when compared to placebo. There was no substantial difference in any of the measured sputum supernatant or plasma cytokines. Peripheral blood monocyte stimulation was performed, with supernatant being measured against a panel of cytokines. There was again no substantial difference in any of the measured panel of cytokines collected from the monocyte stimulation assays when the azithromycin group was compared to placebo.

There was no correlation between changes in ACQ, AQLQ, LCQ, PC20, sputum macrophage count, sputum neutrophil count, sputum eosinophil count, and PEF.

Adverse event rates were similar in patients taking azithromycin compared with placebo. A total of 4 patients were lost to follow up [1 in the azithromycin group, 3 in the placebo group]. One patient died of a cardiovascular cause. This occurred following completion of the study but within the pre-specified regulatory reporting period.

In conclusion there were no clinically important improvements in a range of clinical indices of asthma control, airway inflammation or bacterial colonisation following 12 weeks treatment with azithromycin when compared with placebo in smokers with asthma.

The lack of any evidence of clinical benefit of azithromycin in smokers with asthma is a new finding and extends the current knowledge base and evidence for the use of macrolides in asthma. There exists no firm evidence to suggest the widespread use of macrolides in asthma and the current study suggests that no benefit will be observed in the sub-group of asthmatics whom are current smokers.

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Declaration

I am the sole author of this thesis and have personally consulted all the references listed. I was solely responsible for the daily running of the project. I recruited the majority of the subjects with support and assistance from Dr Katie Gallacher and Dr Deborah Morrison [both General Practice and Primary Care, University of Glasgow]. I screened, obtained informed consent and performed randomisation of all the patients and the great majority of follow up visits. The remainder of follow up visits were occasionally performed by Jane Lafferty, Joyce Thomson and Maureen Brannigan [all employees of Asthma Research Unit, University of Glasgow]. Approach letters were generated and GP searches were performed by Yvonne McIlvenna and Janice Reid [both West Node SPCRN], and Drs Katie Gallacher and Deborah Morrison.

Pharmacy dispensing was provided by Maria Nguyen, Colin Rodden and Ann Watt. Pharmacy R&D supervision and regulatory oversight was provided by Dr Elizabeth Douglas. Lisa Jolly and Iona Donnelly [GBRC, University of Glasgow] prepared the sputum, supernatant and plasma Luminex as well as performing the cellular assays.

I performed all the data entry, checking and resolved all the data queries for the study. Analysis was performed by Nicola Greenlaw [Robertson Centre for Biostatistics, University of Glasgow].

Appendix 8 was written in collaboration with colleagues at the Dept of General Practice for submission as a manuscript for peer-review. I was involved in drafting this for important intellectual content and provided some of the data.

This thesis has not previously been submitted for a higher degree.

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It is clear to me that the project presented within this thesis would not have been possible without the valuable input from a number of my colleagues. Professor Neil Thomson supervised me throughout, with quiet steadiness, providing me with the guidance I needed when it was most required. His approach, and encouragement has focussed my own thinking, leaving me with knowledge and experience beyond the work presented here. Dr Rekha Chaudhuri provided daily advice, supervision and support in organisation. Dr Charlie McSharry supervised the laboratory assays and instructed me in statistical methods and laboratory techniques. Jane Lafferty, Joyce Thomson and Maureen Brannigan, my office colleagues and now friends; they filled in the gaps when I was stretched thin and provided support and coffee by the tankful! Lisa Jolly and Iona Donnelly, for their dedication in performing the laboratory assays. Professor Frances Mair, at the University Department of General Practice, who rallied the troops when recruitment was proving troublesome and encouraged as well as shamed GP's in to taking part. Dr Katie Gallacher and Dr Deborah Morrison, who helped with contacting potential recruits. Mrs Alison Rennie, Deputy Head of English, Duncanrig Secondary School, for giving her own time to proof read this thesis when I have stared at it too long to see the errors. To the anonymous subjects who participated in this trial; without their time and effort, it would not have been possible.

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My gratitude will never cease!

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Abbreviations

ACQ	Asthma Control Questionnaire	GINA	Global Initiative on Asthma
95% CI	95% confidence interval	GM-CSF	Granulocyte/Monocyte-Colony Stimulating Factor
A&E	Accident and Emergency	HDAC	Histone Deacetylase
ABPA	Allergic Bronchopulmonary Aspergillosis	GCP	International Conference on Harmonization of Good Clinical Practice
AE	Adverse Event	ICS	inhaled corticosteroid
ALT	Alanine Amino Transferase	IFN-γ	Interferon-γ
AP-1	Activator Protein 1	IgA	Immunoglobulin A
AQLQ	Asthma Quality of Life Questionnaire	IgE	Immunoglobulin E
AST	Aspartate Amino Transferase	IgG	Immunoglobulin G
BAL	Broncho-Alveolar-Lavage	IgM	Immunoglobulin M
C_{alv}	Alveolar Nitric Oxide Concentration	IL	Interleukin
C_{aw}	Airway Wall Concentration of Nitric Oxide	IL-1RA	IL-1-receptor antagonist
CCL	Chemokine with C-C N Terminal Motif	IL-2R	IL-2 receptor
CD	Cluster of Differentiation	IMP	Investigational Medicinal Product
CO	Carbon Monoxide	iNOS	Inducible Nitric Oxide Synthase
COPD	Chronic Obstructive Pulmonary Disease	IP-10	Interferon-inducible Protein of 10 kDa [aka CXCL10]
CRF	Case Report Form	IQR	Interquartile Range
CRP	C-Reactive Protein	IU	International Units
CXC	Chemokine with C-X-C N Terminal Motif	IκB	Inhibitor of κB
D_{aw}	Airway Wall Nitric Oxide Diffusion	J'aw	Maximal Airway Wall Nitric Oxide Flux
DNA	Deoxyribonucleic Acid	Jaw	Airway Wall Nitric Oxide Flux
DTT	Dithiothreitol	JNK	c-Jun N-terminal Kinase
EBC	exhaled breath condensate	kDa	Kilodalton
ECG	Electrocardiogram	Kg	Kilogram
ECP	Eosinophilic Cationic Protein	Kg/m^2	Kilograms per Metres Squared
ELISA	Enzyme Linked Immunosorbent Assay	L/min	Litres per Minute
eNOS	Endothelial Nitric Oxide Synthase	LABA	Long Acting Beta-2 Agonist
FEF_{25-75}	Forced Mid-Expiratory Flow Rate	LFT	Liver Function Test
FEF_{75}	Forced Expiratory Flow at 75% of FVC	Log	Logarithm
F_ENO	Fraction of Expired Nitric Oxide	LPS	Lipopolysaccharide
F_ENO_{50}	Exhaled Nitric Oxide Concentration at Flow Rate of 50ml/sec	LTB_4	Leukotriene B4
FEV_1	Forced Expiratory Volume in 1 second	mcg	Microgram
FVC	Forced Vital Capacity	MCP-1	Monocyte Chemotactic Protein 1

Mg	Magnesium	mg	Milligram
MID	Minimal Important Difference		
MIP-1α	Monocyte Inflammatory Protein 1α [aka CCL3]		
MIP-1β	Monocyte Inflammatory Protein 1β [aka CCL4]		
MPO	Myeloperoxidase		
mRNA	Messenger Ribonucleic Acid		
NFκB	Nuclear Factor Kappa B		
nNOS	Neuronal Nitric Oxide Synthase		
NO	Nitric Oxide		
PBMC	Peripheral Blood Borne Monocyte		
NSAID	Non-Steroidal Anti-Inflammatory Drug		
PC_{20}	Provocation Concentration of Methacholine causing a 20% fall in FEV_1		
PCR	Polymerase Chain Reaction		
PDE	Phosphodiesterase		
PEF	Peak Expiratory Flow		
PI3K	Phosphatidylinositol 3 Kinase		
pl/s	Picolitres Per Second		
pl/s/ppb	Picolitres per Second per Parts Per Billion		
ppb	parts per billion		
RNA	Ribonucleic Acid		
rRNA	Ribosomal Ribonucleic Acid		
SAE	Serious Adverse Event		
SD	Standard Deviation		
SOP	Standard Operating Procedure		
SUSAR	Suspected Unexpected Serious Adverse Reaction		
TGFβ	Transforming Growth Factor β		
T_H1	Type 1 Helper T Cell		
T_H17	IL-17 Positive T Helper Cells		
T_H2	Type 2 Helper T Cell		
TNFα	Tumour Necrosis Factor-α		
Treg	Regulatory T Lymphocyte		
ULN	Upper Limit of Normal		
V_E	Exhalation Flow Rate		
V_{NO}	Elimination Rate of Exhaled Nitric Oxide		
p38	p38 MAPK		

Publications arising from this project

Cameron EJ, Chaudhuri R, Mair FS, McSharry C, Greenlaw N, Weir CJ, Jolly L, Donnelly I, Gallagher K, Morrison D, Spears M, Anderson K, Thomson NC. Randomized controlled trial of azithromycin in smokers with asthma. *Eur Respir J, Accepted for publication.*

Cameron EJ, McSharry C, Chaudhuri R, Farrow, SK, Thomson NC. Long-term macrolide treatment of chronic inflammatory airway diseases: risks, benefits and future developments. *Clin Exp Allergy*. 2012 Sep;42[9]:1302-12

Morrison D, **Cameron EJ**, Wright GR, Thomson NC, Chaudhuri R, Mair FS. Recruitment Challenges in Primary Care Randomised Controlled Trials: Lessons From Three Asthma Trials. *Submitted for publication* - Appendix 8: Recruitment Challenges in Primary Care Randomised Controlled Trials: Lessons From Three Asthma Trials

Abstracts arising from this project

Morrison D, Cameron EJ, Braganza G, Thomson NC, Chaudhuri R, Mair FS: Understanding recruitment challenges in Randomised Controlled Trials [RCTs] in primary care: findings from two asthma trials. Presented at the Society of Academic Primary Care Physicians Annual Meeting 6-8th July 2011, University of Bristol.

Oral Presentations

Morrison D, **Cameron EJ**, Braganza G, Thomson NC, Chaudhuri R, Mair FS. **Managing the challenges of recruitment of patients with asthma to randomised controlled trials.** Poster [P205] discussion at the British Thoracic Society Winter Meeting 09/12/2011

Cameron EJ, Chaudhuri R, McSharry C, Greenlaw N, Weir CJ, Jolly L, Donnelly I, Gallagher K, Morrison D, Spears M, Anderson K, Mair FS, Thomson NC. **Effects of azithromycin on asthma control, airway inflammation and bacterial colonisation in smokers with asthma – a randomised controlled trial.** Poster discussion [27047] at the American Thoracic Society Conference, San Francisco 22/05/12

1. Introduction

1.1. Asthma

1.1.1. Definition

Asthma represents a diagnostic challenge, in many instances more difficult to prove than is generally appreciated. The diagnosis is ultimately a clinical one; there is no standard definition of the type of asthma, the frequency or severity of the symptoms nor the findings on clinical examination [1]. At the organ level, asthma is a chronic inflammatory condition of the airways in which many cells and cell mediators play a role. Whilst the clinical spectrum of disease is highly variable the single most consistent feature is the presence of airway inflammation [2]. In a not too dissimilar fashion we are now beginning to appreciate that this underlying airways inflammation is heterogeneous [3] and this may be informative of the reasons for the disease phenotype.

The diagnosis of asthma in adults is therefore based on the recognition of a pattern of symptoms in the absence of an alternative explanation. Even with a classical case, it is important to obtain objective evidence in support of the diagnosis. There is a considerable burden of treatment, and consequent cost in healthcare provision. The best confirmatory evidence is the objective demonstration of reversible airflow obstruction. Further supporting evidence can be obtained from other tests of airways responsiveness and inflammation.

The aims of asthma management are to control the disease and hence render the patient symptom free [1, 2] The mainstays of treatment are inhaled bronchodilators and inhaled corticosteroids [ICS]. The majority of patients can achieve these treatment goals with inhaled corticosteroid alone or additionally with other inhaled

or oral therapies, but there still exists a sub-population who remain symptomatic despite this.

1.1.2. Diagnosis

1.1.2.1. Clinical features

Central to the diagnosis is the description of symptoms such as wheeze, coughing, chest tightness, breathlessness and the finding of variable airflow obstruction on objective testing. There may be a diurnal or seasonal pattern of wheezing. Family history may be present.

These symptoms are frequently episodic in nature which is as much part of the clinical syndrome as it is a confounding factor to the physician when seeing the patient, particularly if the physical findings are absent.

1.1.2.2. Physical examination

The episodic nature of asthma means that no abnormal findings may be present. Commonly the most frequent finding when symptoms are present is polyphonic wheeze, present on auscultation of the chest. There exists the possibility that significant airflow limitation may be present with no apparent wheeze.

1.1.2.3. Objective testing

Whilst a diagnosis can be made on clinical grounds, this is not always the case, particularly when subjects present to the out-patient clinic with few symptoms. In such instances objective measurements of lung function and airway hyper-responsiveness are required, not only to support the diagnosis but also to give the clinician an impression of disease severity in terms of both airflow limitation and bronchial hyper-responsiveness.

Reduction in FEV_1 is not exclusively found in asthma and may be present in other diseases than those which cause airflow limitation. Therefore a more useful assessment is based on the ratio of FEV_1 to FVC. Normal spirometric measurements will yield a ratio of FEV_1/FVC of greater than 80% with values less than this suggestive of some form of airflow limitation [2, 4].

Reversibility testing [with nebulised β_2-agonists] is also utilised and is most useful in patients who are symptomatic with evidence of a reduced FEV_1, but not exclusively so. Various guidance exists, but it is generally accepted that improvements in FEV_1 of 12% with an absolute volume improvement of ≥200ml is required following treatment with nebulised bronchodilator or oral corticosteroids [2, 4].

Broncho-provocation testing is another method to test the responsiveness of the airways to various stimulants [e.g. methacholine, histamine or mannitol]. Standardised testing has been adopted with inhalation of aerosol for timed periods of tidal breathing or delivery of a pre-determined amount via a dosimeter [5]. When a reduction in FEV_1 of 20% is measured a positive result is determined [2, 5] and subsequently the concentration [PC_{20}] or dose [PD_{20}] or drug required to achieve this drop can be calculated. The calculated concentration or dose gives an overall impression as to the degree of airway hyper-responsiveness. These tests are sensitive for a diagnosis of asthma, but have limited specificity. This is because airway hyper-responsiveness has been described in patients with other respiratory diseases [2].

Peak expiratory flow diary measurements are extremely useful along with the documented presence of nocturnal/early morning symptoms. A diurnal variation of PEF of 20% or more is thought to be highly characteristic of asthma [2].

Table 1.1: Key diagnostic features of asthma

Symptoms	**Signs**
Episodic/variable	None [common]
Shortness of breath	Wheeze – bilateral, diffuse
Wheeze	Expiratory [± inspiratory]
Chest tightness	Tachypnoea
Cough	
Helpful additional information	
Personal and family history of asthma or atopy [eczema, allergic rhinitis]	
History of sensitivity to aspirin/NSAIDs or β blockers [including eye drops]	
Recognised triggers – pollen, dust, animals, exercise, viral infections	
Pattern and severity of symptoms and exacerbations	
Objective measurements	
>20% diurnal variation on ≥3 days in a week for two weeks on PEF diary or	
FEV_1 ≥ 12% [and 200ml] increase after short acting β2 agonist or	
FEV_1 ≥ 15% [and 200ml] increase after a trial of steroids or	
FEV_1 ≥ 15% [and 200ml] increase after a trial of steroids or	
FEV_1 ≥ 15% decrease after 15 minutes of vigorous exercise	
Bronchoprovocation testing positive	

1.1.2.4. Quality of life measures and asthma control

There are various ways to classify asthma control; however no classification has been universally accepted. Overall, asthma control consists of two domains; one is achieving day-to-day control [or current] asthma control, indicated by the absence of asthma symptoms, minimal reliever use, normal activity levels and lung function values close to normal. The second domain is to minimise future risk to patients by ensuring the absence of asthma exacerbations, the prevention of accelerated lung function decline over time and minimal side effects from medication.

The goals of asthma control [defined by GINA [2]] are:

- No daytime symptoms [maximum occurring twice or less/week]
- No limitation of activity
- No nocturnal symptoms/wakening
- No requirement for reliever/rescue medication [max twice or less/week]
- Normal lung function – PEF or FEV_1
- No exacerbations

The evidence demonstrates that despite these aspirations, large numbers of patients with asthma, 74% in a European study [6] are not fully controlled. Similar numbers are found in the United States [7].

1.1.2.4.1. Numeric measures of asthma control

The Asthma Control Questionnaire [8] was developed to assess asthma control in clinical trials and clinical practice. The ACQ has been validated against quality of life and physician global assessment [9]. The score is an arithmetic mean based on 7 questions marked on a 7-point scale [0-6], with a minimal important difference of 0.5 [10]. The optimal cut-point for "Well-controlled" using the Gaining Optimal Asthma Control [GOAL] [11] classification is less than or equal to 0.75, and a value of greater than or equal to 1.50 reflects "not Well-Controlled" asthma [11]. A copy of the ACQ can be found in Appendix 1.

1.1.2.4.2. Quality of life

Measuring health related quality of life [HRQOL] can add valuable information to better assess the impact of poor asthma control and/or its severity. HRQOL questionnaires were not intended to be used as endpoints in clinical trials but many studies now include and assessment of HRQOL [9].

Generic questionnaires exist, for example the Medical Outcomes Short Form-36, but this questionnaire was designed for use in chronic illness such as tiredness and lethargy hence the clinical utility is questionable in asthma [9]. Asthma specific questionnaires are therefore preferable and the Juniper-Asthma Quality of Life Questionnaire [AQLQ] [12] is frequently used. The AQLQ has 4 domains: physical, social, emotional and occupational. A copy of the AQLQ can be found in Appendix 2. Patients score their experiences during the last 2 weeks on a 7-point scale [1 = severe impairment to 7 = no impairment]. The overall score and the means for the different domains are calculated. The minimal important difference [MID] is reported to be 0.5 [13].

1.1.3. Global Burden of Asthma

Estimates report that 300 million people worldwide are affected by asthma [14]. Sadly, Scotland is the world leader in prevalence rates amongst children [35%] and also has a high proportion of affected adults [18%]. Across the UK, over 5 million people receive treatment for asthma [15]. The economic cost of asthma is considerable both in direct medical costs [hospital admissions and purchase of pharmaceuticals] and indirect medical costs [time off work and premature death]. It is also estimated that 1 in every 250 deaths worldwide is due to asthma, many being preventable and resultant due to long-term sub-optimal care and delay in seeking help during the terminal attack [14].

1.1.4. Pathogenesis

Asthma is an airways disease that involves airway inflammation and impaired airflow. It affects the conducting airways causing them to spontaneously contract too much, too easily, and in response to a wide range of exogenous and endogenous stimuli. The reduced airway calibre causes increased turbulence during breathing

resulting in the characteristic wheeze. The airways undergo structural and functional changes, leading to airway hyper-responsiveness. The inflammation of asthma is heterogeneous but generally consists of varying levels of acute and chronic inflammation, smooth muscle contraction, mucosal oedema, tissue remodelling and mucus hyper-secretion.

Most, but not all asthma is associated with atopy [the genetic predisposition to generate Immunoglobulin E against common environmental allergens]. This has led asthma to be recognised as an allergic disorder along with other atopic diseases. However, there are phenotypes of asthma that appear independent of atopy, for example late onset asthma or intrinsic asthma] [16].

1.1.4.1. Cells of the respiratory immune system

Airway inflammation in asthma is a multi-cellular process with the most striking feature being eosinophilic infiltration [17]. Other cells involved include neutrophils, $CD4^+$ T-lymphocytes and mast cells. It is important to note that eosinophilic infiltration is pathognomonic of asthma, and subtypes of asthma are also well described based on the relative absence of eosinophils and predominance on neutrophils [18, 19].

1.1.4.1.1. Eosinophils

Eosinophils are not only a prominent cell in the airway mucosa of asthmatics [20], but are also found in large numbers in the sputum and broncho-alveolar lavage fluid [17, 21]. Importantly there is a correlation between eosinophil counts in sputum and asthma exacerbations [22]. Eosinophils are recruited to inflammatory tissues in response to chemotactic and trophic cytokines such as eotaxin, Interleukin [IL]-5 and IL-8, with egress from the vascular tree being mediated by the cell surface expression

of CD11b/CD18 and VLA-4 on eosinophils and epithelial expression of ICAM-1 and VCAM-1 [23]. Eosinophils can secrete an array of cytokines [IL-2, IL-4, IL-6 and others] which, acting as pro-inflammatory mediators can promote T-cell proliferation and activation [24]. As an effector cell, eosinophils can release pre-formed lytic enzymes such as major basic protein, eosinophil peroxidise, and eosinophil cationic protein, as well as release potentially tissue damaging superoxide [25]. In addition to these pro-inflammatory roles, eosinophils also play a central role in airway remodelling, releasing growth factors and stimulating the production of extracellular matrix proteins [26, 27]. Eosinophils are sensitive to corticosteroid, a common treatment for asthma. Corticosteroids can induce apoptosis of eosinophils and also increase the tissue clearance of these apoptotic cells by resident macrophages [28].

1.1.4.1.2. T lymphocytes

T lymphocytes are present in the airways of both atopic and non-atopic asthmatic individuals [29, 30]. Immature naïve T-helper lymphocytes are classified as T_H0. In stable asthmatics these T_H0 cells can be found in the airways [31]. Stimulation in the correct cytokine environment and/or in the presence of antigen presenting cells [typically dendritic cells] will lead to final differentiation towards a specific functional activity [32]. T-lymphocyte functional subsets can be defined by their ability to produce characteristic cytokines. T-helper $CD4^+$ lymphocytes can be categorised as either as T_H1 or T_H2 – asthma is considered to be predominantly a T_H2 cell driven disease [29, 33]. $CD4^+$ T_H2 cells produce IL-4, IL-5 and IL-13 which drive an immune response that can initiate and maintain the key pathophysiological features of asthma [34].

Other lymphocyte subsets have received less attention in asthma, but their presence in the mucosa of asthmatics is well documented. Cytotoxic $CD8^+$ T-cells and innate

lymphocytes, or nuocytes are present in the mucosa of asthmatics. Together they can produce a range of cytokines [32]. The exact role that these lymphocytes play in airway inflammation is less clear and there still remains many uncertainties as to their relative roles, for example a subset of $CD8^+$ T lymphocytes with the γδ T-cell receptor has been found to be inhibitory to the allergic response but this is not the case with $CD8^+$ cells bearing the T-cell receptor [32, 35].

1.1.4.1.3. Macrophages

In chronic asthma macrophages are prominent cells in the airway mucosa and undoubtedly play an important role in disease pathogenesis [25]. The alveolar macrophage is the predominant immune effector cell, responsible for homeostatic removal of particles and apoptotic cells without inflammatory activation [36]. However, with the appropriate stimulation for example with bacterial endotoxin, macrophages can respond by producing inflammatory cytokines. In addition they can act as antigen presenting cells for primed T-lymphocytes [36], although this is much more efficiently done by lung dendritic cells. Thus macrophages may have two distinct phenotypes. M1 Macrophage [M] phenotype 1 [M1] are described as classically activated by their response to bacterial endotoxin. They secrete pro-inflammatory cytokines, chemokines, exhibit enhanced phagocytosis and have increased oxidative burst to kill phagocytosed organisms [37]. In contrast, macrophages stimulated with IL-4/IL-13 [M2 phenotype] display a distinct pattern of activation and play a role in directing T_H2 humoral and allergic responses, and the co-ordination of repair following an inflammatory reaction [38]. The precise role of the macrophage is likely to be complex in asthma, but they appear to preferentially infiltrate the mucosa in steroid refractory disease [25] and there is evidence of their corticosteroid resistance [39].

1.1.4.1.4. Neutrophils

Increasing use of induced sputum and broncho-alveolar lavage has revealed that some patients with asthma have a sputum neutrophilia in the absence of eosinophils [19, 40]. Neutrophils traffic to the airway mucosa in response to various chemokines the most potent being IL-8 [also known as CXCL8] [41]. Egress from the bloodstream is undertaken by the expression of adhesion molecules such as LFA-1 and Mac-1, binding to their ligand ICAM-1 on the surface of the endothelium [41].

Once at the site of inflammation, the neutrophil can act to recruit more neutrophils by the release of chemoattractants and pro-inflammatory cytokines or operate as an effector cell by release of potent oxidative enzymes. Examples of the main mediators released by activated neutrophils are summarised on Table 1.2: Mediators produced by neutrophils.

Table 1.2: Mediators produced by neutrophils

Mediator	Function
TNFα	Promotes bronchial hyper-responsiveness Activates epithelium
IL-8	Potent neutrophil chemo-attractant Neutrophil activator Down regulates IgE production
Reactive Oxygen Species	Cytotoxic to epithelium Promotes IL-8 release Promotes mucus hypersecretion
Myeloperoxidase	Produces HO-CL and cytotoxic to epithelium Activates mast cells
Matrix Metalloproteinase 9	Predominant MMP in asthmatic airways
Neutrophil elastase	Cytotoxic to epithelium Promotes mucus hypersecretion Promotes bronchial hyper-responsiveness
Lipid mediators – leukotrienes	Recruits neutrophils and monocytes to airway epithelium

Better understanding of this is of particular importance because chronic asthmatics with a predominant airway neutrophilia appear to be insensitive towards corticosteroids [40]. In keeping with this, evidence is now available that airway neutrophilia plays a role in the progression of persistent airflow limitation in asthma [42].

Tobacco smoking is also associated with an increased airway neutrophil proportion and, importantly, corticosteroid refractoriness in airways [43, 44] and systemically [45]. Corticosteroids appear to reduce neutrophil apoptosis and lead to prolonged survival [42] which could provide a possible explanation for steroid resistance in asthma. Conversely, eosinophils which become apoptotic in response to corticosteroids are removed from the inflamed airway leaving the neutrophil as a "substitute granulocyte" [46].

1.1.4.1.5. Mast Cells

The mast cell has long been associated with asthma. Of particular interest is the finding that in chronic asthma, mast cells are markedly increased in association with airway smooth muscle in both the large and small airways [47]. Mast cells possess the high affinity receptor for IgE – FcεRI, with binding and cross-linking of this receptor by allergen leading to mast cell activation [25]. Mast cells contain pre-formed inflammatory mediators in granules which are released following activation. These include histamine, tryptase, other proteases, most known cytokines including those associated with asthma pathogenesis e.g. IL-4, IL-5 and IL-13. In addition following activation mast cells can synthesise newly formed prostanoid mediators from arachidonic acid metabolites e.g. the powerful pharmacologically active molecules cysteinyl leukotrienes [LTC_4 and LTD_4] [48]. Mast cells are also a rich

source of matrix metalloproteinases [MMP3 and MMP9] which are involved in tissue remodelling.

1.1.4.2. Inflammatory mediators

There are multiple mediators of inflammation in asthma which are produced from a variety of sources, some listed above.

1.1.4.2.1. Cytokines

Cytokines are a descriptive category for small [glycol-]proteins whose main role is as signalling molecules between cells of the immune system, although their role extends to all biological functions. Their effects are multiple, pleiotropic and can exhibit redundancy. Cytokines are critical not only to mounting an inflammatory response but also in developing an appropriately measured response, with excessive inflammation causing harm and insufficient leading to failure to resolve the initiating inflammatory stimulus. Thus cytokines can be generally classified into two groups of pro-inflammatory and anti-inflammatory.

The cellular responses to cytokines are mediated by cell surface receptors. The receptors are made up of several sub-units. Several of these receptors share similar sub-units but elicit their specific effects by aggregating with either a unique co-receptor, or unique intracellular subunit. This also explains why certain families of cytokines will elicit similar downstream signalling events.

Cytokines can be produced by cells of the immune system as well as tissue stromal cells such as airway epithelium, smooth muscle cells, fibroblasts and endothelial cells [49]. Some examples of the variety of cytokines and their functions are listed in Table 1.3.

Table 1.3: Cytokines and their involvement in asthma

Cytokine	General function	Functional association with asthma
IL-1	Increase in epithelial and inflammatory cell adhesion molecules Activation of T-cell and epithelial cells	Neutrophil accumulation Eosinophil accumulation Promotes bronchial- hyper-responsiveness
IL-4	Growth, differentiation and activation of B-cells.	Potentiates IgE production and enhances IgE mediated responses
IL-5	Regulates most aspects of eosinophil behaviour – growth, maturation, differentiation, survival and activation,	Central role in the accumulation and activation of eosinophils in the lungs. Potent eosinophil chemoattractant
IL-6	Activates eosinophils and macrophages B and T cell growth factor	Increases IgE production
IL-8	T cell chemoattractant	Potent chemoattractant of neutrophils Down regulates IgE production Promotes eosinophils chemoattractant
IL-10	Reduces monocyte and macrophage activation Inhibits T_H1 cytokine production	Reduces IgE production Decreased eosinophils survival
TNF-α	Generalised activation of cells – epithelium, endothelium, monocytes, macrophages	Promotes bronchial hyper-responsiveness
IFN-γ	Activates endothelium and epithelium Activates fixed alveolar and circulating macrophages/monocytes	Reduces bronchial hyper-responsiveness Reduces IgE production
GM-CSF	Mast cell, macrophage, epithelial cell, eosinophil and neutrophil differentiation and activation	Neutrophil survival Promotes bronchial hyper-responsiveness
LTB_4	Neutrophil and monocyte chemoattractant and activator	Recruits neutrophils and monocytes in to airway

Probably the most important cytokines in asthmatic airways disease are T_H2 family of cytokines which include – IL-4, IL-5, IL-9, IL-13 and IL-25.

1.1.4.2.2. Chemokines

Chemokines are a subgroup of cytokines that have a specific function to act as attractants of inflammatory cells of the immune system. They are small, therefore can diffuse rapidly from sites of inflammation where they are synthesised in abundance.

Cells recognise a particular chemokine by its binding to a specific receptor, which then restructures the internal actin cytoskeleton towards this bound receptor and to moves up the concentration gradient of the chemokine leading to the site of inflammation. The chemokines are generally categorised according to a specific motif within their protein structure and this divides chemokines in to four groups – CXC, CC, C and CX3C. The two main groups are CXC [α chemokines] and CC [β chemokines] [49]. The membrane receptors for these proteins are 7-transmembrane G-protein coupled. Like cytokines, chemokines can influence the immune response by activation and differentiating different cell populations involved in allergic diseases, such as T_H1 and T_H2 cells. A selection of chemokines have relevant to the pathogenesis of asthma can be found in Table 1.4.

Table 1.4: Chemokines and their role in asthma

Chemokine	General/function	Functional association with asthma
IL-5	Potent eosinophil chemoattractant	Enhances eosinophil survival by preventing apoptosis
IL-8	Potent neutrophil chemoattractant	Significant role in neutrophilic asthma
IP-10	Monocyte/macrophage, T cell and NK cell chemoattractant	Increases adhesion molecules on surface of eosinophils for trafficking to airway epithelium
Eotaxin	Potent eosinophil chemoattractant. Leads to expression of α4 and β1 integrins for trafficking to inflammatory sites	Produced in high concentrations in the lungs of asthmatics
RANTES	Monocyte and T cell chemo-attractant	Produced in high concentrations in the lungs of asthmatics Potent eosinophils chemoattractant Enhances IgE production
MCP-1	Monocyte/macrophage chemoattractant and activating factor	Increase T cell production of IL-4
MCP-4	Monocyte/macrophage chemoattractant and activating factor	Increase T cell production of IL-4 Potent eosinophil chemoattractant
MIP-1α	Monocyte/macrophage chemoattractant and activating factor	Chemotactic for T cells and eosinophils
MIP-1β	Monocyte/macrophage chemoattractant and activating factor	Chemotactic for T cells and eosinophils

1.1.4.2.3. Apoptosis

Apoptosis or programmed cell death is a mechanism within the immune system for switching off inflammation. In essence it is a mechanism whereby a cell will die without releasing its potentially pro-inflammatory contents. The cell can then be "mopped-up" without inducing an inflammatory response. The apoptotic cell will first become senescent before displaying cell surface recognition markers e.g. Fas, which direct macrophages to engulf and remove the cell in a non-inflammatory manner. The perpetuation of inflammation is therefore due to a balance of cell survival and activity with apoptosis. Experimentally engineered Fas deficiency can lead to persistence of inflammation [50]. Both eosinophils and neutrophils, two of the predominant cell types in asthma both express Fas constitutively [51] and may be primed for rapid apoptosis by expression of an additional apoptotic ligand. Evidence exists in severe asthma that neutrophil apoptosis is dysregulated and neutrophil survival is enhanced [52].

Of interest to the present thesis is the finding that some macrolides can induce apoptosis in immune cells. Roxithromycin can promote apoptosis in sensitised lymphocytes [53], with additional evidence that clarithromycin, azithromycin and josamycin can do the same with peripheral blood lymphocytes *in vitro* [54]. There is also evidence of macrolides inducing apoptosis in neutrophils [55], as well as the described evidence for glucocorticoids inducing eosinophil apoptosis and delaying the neutrophil apoptotic programme [56, 57].

1.1.4.3. Acute inflammation in asthma

An acute inflammatory episode in asthma is characterised by an influx of inflammatory cells to the injured or infected tissue followed by the release of a self-amplifying network of pro-inflammatory mediators that perpetuate cell recruitment

and activation. At the same time there is a coordinated delayed production of anti-inflammatory cytokines that help to resolve the inflammation when the initiating stimulus is removed. Inflammation in asthma can be divided in to early and late phase [58] and can be prompted by a number of insults, from allergens, to viruses or pollutants. Following on from the acute inflammation we then observe airway remodelling and the process of tissue repair [59]

1.1.4.3.1. Early Phase and Late Phases

Early phase [acute] inflammation in atopic asthma is typified by the activation of cells bearing the high affinity IgE receptor – FcεRI. IgE is critical to the development of the early phase reaction with most IgE pre-bound to FcεRI on the surface of mast cells and basophils [60]. Cross-linking of these cell surface receptors with allergen or antigen leads to cellular activation and degranulation and release of pre-formed mediators such as histamine one of the main mediators of the early phase pharmacological reaction [58, 61]. The late phase inflammatory response occurs between a timeframe of 6-9 hours and involves contraction of smooth muscle cells within the airways and tissue oedema [58]. The delay is primarily due to the *de novo* synthesis of mediators and the recruitment and activation of eosinophils [58] with the ultimate consequence being the development of airflow obstruction.

1.1.4.4. Chronic inflammation in asthma

Chronic inflammation occurs when the normal homeostatic "stop-processes" of acute inflammation fail and the initial inflammatory response fails to resolve normally. Persistent asthma, where chronic inflammation is present can be seen as a disorder in which a dysregulation of each individual phase in the resolution process could be an important contributory factor to the chronicity [59]. All cells within the airway are involved, from infiltrating leukocytes to resident structural cells such as epithelial

cells, fibroblasts and smooth muscle cells, which all become activated and secrete an array of pro-inflammatory mediators [58, 59]

Chronic inflammation is accompanied by structural changes in the airways, such as sub-epithelial fibrosis and smooth muscle hyperplasia. Chronic inflammation and remodelling are thought to be two interdependent processes [59].

1.1.4.4.1. Remodelling

There is ample evidence that after inflammation, changes in the airway contribute significantly to the pathophysiology of asthma. The most obvious change is in the airway smooth muscle, which not only increases in amount due to hypertrophy and hyperplasia, but also spreads both up and down the airways [25]. In chronic asthma the airways become thickened, not only due to an increase in airway smooth muscle, but also as a consequence of the laying down of new extracellular matrix proteins including collagen fibres, and increased proliferation of micro-vessels along with vascular leakage and deposition of proteoglycans [25].

1.1.4.5. Systemic inflammation in asthma

The evidence for systemic inflammation in COPD is now well described; however there is less evidence for systemic inflammation in asthma is [62]. Asthma does not demonstrate the consistently abnormal systemic inflammatory responses that are now well defined in COPD but probably the most widely sustained biomarker is CRP [62]. Combination inhaled therapy has been shown to lower CRP in association with improved asthma control [63]. Studies with other biomarkers are perhaps not as clear-cut and so further investigation to identify a reliable biomarker is required.

1.2. Non-invasive methods of assessment of inflammation

1.2.1. Rationale for use of non-invasive methods

Whilst measurement of asthma outcome indices can be performed in the clinic what has become more apparent in recent years is the importance of the cellular changes that the asthmatic airway undergoes. Examining tissue at the cellular level poses a problem. Previously this was almost wholly based on autopsy specimens, and although useful, has the caveat that it is generally the endpoint changes that are seen and not the dynamic changes observed in life. Bronchoscopy is a safe procedure, particularly in those with normal lung function. Unfortunately in difficult asthma this is not always the case and has associated risk. In addition, it is an expensive investigation, utilising significant human resource as well as specialist equipment.

1.2.2. Induced sputum

Spontaneously produced sputum is widely used to assess bacterial carriage however, the vast majority of asthma patients do not regularly produce spontaneous sputum. The use of spontaneously produced sputum for studying airway cytology is not without caveats; there can be high proportions of necrotic cells or squamous cells from the oropharynx. Induced sputum is now a well validated technique to provide a representation of the cytology of the underlying inflammatory pathology within the airways [3, 64]. However, induced sputum requires specific training in order to obtain and identify and quantify the cells. This has significant cost implications and hence induced sputum is used mainly for clinical trials or research and debate surrounds its use out-with specialist centres [65].

1.2.3. Induced sputum methodology

1.2.3.1. Induction method

There are various differing protocols for the induction of sputum from study subjects. Commonly the subject is pre-treated with salbutamol, followed by inhalation of sterile saline nebulised via a high output nebuliser. Regular monitoring of patient symptoms and FEV_1 is also undertaken to ensure subject safety [64, 66]. There are some differences in the procedures, for example, different inhalation times and different saline concentrations. Our group currently uses 3 inhalation periods of 7 minutes with concentrations escalating from 3% to 4% then 5% for each period. After each period the lung function is measured and the subject continues with the next saline concentration if the lung function remains within set safety parameters [64, 66].

1.2.3.2. Sputum processing

Methods of sputum processing are also the subject of much discussion with some groups recommending the use of the whole sputum sample whilst others suggest that individual mucus plugs be separated from the rest of the expectorate fluid for analysis [67]. An international working group found that both methods were acceptable and that in keeping with good scientific practice a single method should be used for the duration of a clinical trial [67]. Our group has consistently used the "plug-method" and hence our experience base would favour its continued use for this study. Once selected the sputum plugs are added to a known volume of the reducing agent dithiothreitol to break down the disulphide rich mucin protein and to disperse the cells. These can now be counted by haemocytometer and cytocentrifuge specimens can be prepared on slides, stained and counted to provide a differential profile for the cytology. Specific parameters are utilised to determine sample quality. The current consensus is that a minimum of number of 400 non-squamous cells are

required for a representative cell count [67]. The count should consist of a total cell count, squamous cell count and a differential for non-squamous cells with samples being discarded when the leukocyte viability is less than 40% and/or the percentage of squamous cells is greater than 80% [68]. Expressing the results in terms of percentage of the non-squamous cells reduces the effect of dilution or sputum mucous plug volume and allows for good reproducibility [68, 69]

1.2.4. Induced sputum – Clinical Trials

Induced sputum analysis has been found to be highly reproducible in asthma [70]. Further studies have demonstrated that changes in cell counts respond appropriately to corticosteroid treatment and allergen challenge [64] and correlates with broncho-provocation and exhaled nitric oxide levels in adults [71].

1.2.4.1. Induced sputum – eosinophilia

The information generated by the cytology from induced sputum has contributed to the understanding that asthma is not just a single disease but can be sub-categorised based on the underlying type of inflammation. This sub-categorisation allows the clinician to define groups that may respond better to conventional treatment based on the predominant cell type. This advancement can lead to more informed decision making by the clinician. Sputum eosinophilia indicates better corticosteroid responsiveness [3, 72] and has been found to negatively correlate with FEV_1 [73]. Therefore induced sputum is widely used as a study endpoint in clinical trials. Treating subjects with sputum eosinophilia with corticosteroids to reduce the percentage to below a pre-set target has resulted in a greater improvement in asthma control relative to standard clinical measures [74]. Conversely the absence of sputum eosinophilia indicates that dose reduction of corticosteroid can be undertaken with relative safety [74].

1.2.4.2. Induced sputum – neutrophilia and paucicellular sputum

Sputum neutrophilia is associated with a reduced response to corticosteroid treatment in asthma. Sputum neutrophilia is also described in smokers with asthma [75] and in subjects with severe asthma [76-78]. Prospective clinical trials also demonstrate that sputum neutrophilia correlates with steroid resistant inflammation [40, 79].

In subjects with raised sputum neutrophil counts there is evidence of an inverse correlation with FEV_1 [73, 80] and irreversible airflow obstruction [80]. In some subjects with asthma an induced sputum profile is observed which has neither a raised eosinophil or neutrophil count. This group has been described as "paucicellular" and appears to indicate a milder form of asthma as it is associated with better asthma control [79].

1.2.4.3. Induced sputum – definition of eosinophilia and neutrophilia

Research studies using sputum profiles from healthy subjects have provided a definition for sputum eosinophilia. The current consensus utilises a cut-off value of >2% eosinophils to be greater than normal [30, 40, 79]. Defining sputum neutrophilia is not as straightforward given the finding that sputum neutrophil percentage rises with age [80, 81]. A pragmatic approach suggests that to define neutrophilia a sample must be >50% neutrophils. There is a clear need to better define this area with increasing study evidence.

1.2.4.4. Induced sputum – reproducibility of cytology

Induced sputum demonstrates good reproducibility with intraclass correlation co-efficients [ICC] for eosinophils of 0.85 and neutrophils of 0.57 [68] for whole sputum sampling. Selected sputum processing has been associated with ICCs of 0.63 for

eosinophils and 0.57 for neutrophils [82] in one study and 0.94 for eosinophils and 0.81 for neutrophils in another [83].

1.2.5. Sputum supernatant cytokines

The relative accessibility of sputum sampling in severe asthma has led to research interest in identifying and quantifying the soluble factors within induced sputum fluid. The large numbers of inflammatory cells is sputum is associated with a variety of cytokines and chemokines. As research progresses the significance of these cells and cytokines are being identified and better understood. The associated inflammatory pathways and mechanisms for the development of asthma are being elucidated in both human and animal models as well as *in vitro* systems.

Advanced analysis techniques with more specific antibodies and multiple testing systems [such as the Luminex® Multiplex System] has allowed for multiple testing concurrently from the same volume of sample, giving faster and more accurate results for panels of many cytokines.

1.2.5.1. Effect of sputum processing on supernatant cytokines

The technique to disperse sputum plugs utilises the reducing agent dithiothreitol [DTT] [64, 84-86]. This allows the cells and fluid in the sputum plugs to be separated by centrifugation. The caveat of this procedure unfortunately is that since DTT is a reducing agent, that reduces sulfhydryl [thiol] bonds it can disrupt the tertiary structure of protein cytokines thus modifying its immunoreactivity [85, 86] and measurements by immunoassay. Methods to overcome this include removal of the fluid phase from sputum plugs by centrifugation before addition of DTT, or reduction in the concentration of DTT, or addition of an oxidisation agent to neutralise this effect. The effect of DTT on individual assays is now being identified

and hence the effect can be controlled in the results [86] but despite this knowledge a consistent cytokine profile predictive of treatment response has yet to be identified.

1.2.6. Exhaled markers of inflammation – nitric oxide

Nitric oxide was first demonstrated to be produced in the airways in 1991 [87]. Its clinical utility was enhanced by the finding that it can be measured non-invasively in exhaled breath and that levels are high in asthma [88] and decrease after steroid treatment [89]. NO production from its precursor, L-arginine is mediated by nitric oxide synthase [NOS]. Three different forms of nitric oxide synthase iso-enzymes have been described in mammals:

- Endothelial NOS [eNOS or NOS1]
- Inducible NOS [iNOS or NOS2]
- Neuronal NOS [nNOS or NOS3]

All three exist in the human respiratory system [90]. Inducible-NOS is up-regulated in response to immunological and inflammatory stimulation and produces much larger amounts than either of the constitutively produced eNOS or nNOS. Where eNOS and nNOS responsd to increases in intracellular calcium concentrations, iNOS is calcium independent [91].

Orally exhaled NO can be measured in the low parts per billion [ppb] range [87] and this is in contrast to the high concentrations found in air sample from the nose and paranasal sinuses [88, 92, 93]. Although the mRNA expression levels for iNOS is very low in the normal peripheral airways, expression of this enzyme has been found in the central lower airways [94, 95]. The difference in NO output between the lower and upper airways can be attributed to more dense iNOS expression in the epithelium of the nasal airways.

Bronchial epithelial cells, airway smooth muscle cells, macrophages, neutrophils and alveolar cells all express iNOS and contribute to the production of NO. However, the majority of the production is provided by the bronchial epithelium and these other cells contribute very little [91, 96, 97]. NO has important functions in the respiratory system, including promotion of vascular and bronchial dilatation, medication of ciliary beat frequency, promoting mucus secretion and acting as a neurotransmitter for non-adrenergic, non-cholinergic neurons [98-101]. NO can also have a toxic effect in the lung where it is oxidised to peroxynitrite, a potent anti-microbial toxin which can also damage epithelium and is found in asthmatic airways after allergen exposure.

1.2.7. Nitric Oxide in asthma

The measurement of exhaled nitric oxide is now established as a method of monitoring asthma control. This arises from the observation that the concentration of nitric oxide is raised in exacerbations of asthma and decreases in response to steroid treatment and improved asthma control. Not only is it used in disease monitoring but it has also become an established endpoint in clinical trials assessing new therapies to reduce airway inflammation [9]. The fractional concentration of exhaled nitric oxide [FE_{NO}] measurements provide easily obtainable information on underlying disease activity when it is characterised by eosinophilic airway inflammation, but the positive and negative predictive values for eosinophilia are suboptimal [9]. This is best illustrated by the finding that a raised NO is observed in other inflammatory diseases such as liver cirrhosis, SLE, lung transplantation and COPD [102]. Nevertheless, measuring F_ENO in the clinic setting can help guide the physician in making more appropriate management decisions.

1.2.7.1. Employment in asthma control algorithms

The employment of F_ENO in asthma control has been investigated and explored, but there remains debate over its usefulness. Data is conflicting with some groups demonstrating the ability to reduce therapy successfully [103]. Other groups have shown very little benefit in using F_ENO to guide asthma management [104] and a much larger randomised, controlled trial demonstrated that the use of F_ENO as a measure of asthma control does not improve control or enable reduction in dose of inhaled corticosteroid [105]. Clearly measuring F_ENO in the clinic does not always add a great deal to decision making and results must be interpreted in the clinical context presented before the physician.

1.2.7.2. Reference ranges

Reference values for F_ENO are not yet fully established but the most recent consensus guidelines determine the range between 5ppb and 35ppb for adults and between 5ppb and 25 ppb for children. 97% of healthy individuals have levels of <35ppb; this drops to <22.4 if outliers and subjects with atopy are removed. Providing a single cut-off is difficult and detailed analysis of receiver operator curves is required in order to do so. In keeping with the overall uncertainty in reference ranges, a variety of cut-off values have been derived in order to determine the presence of underlying eosinophilia, ranging from >8.3ppb [250ml/s flow rate] [106] with sensitivity and specificity of 72% and 71% respectively with another giving a cut-off >42ppb at the more commonly and easily measure 50ml/s [107], sensitivity and specificity of 65% and 79%.

ATS/ERS guidelines suggest reference ranges are difficult to apply to asthmatics because in this population the F_ENO can be high even when the patient is asymptomatic and has good disease control [9]. It has been suggested that

comparisons for individual patients are best made against serial measurements compared with when the patient is clinically stable [9].

Clearly, debate exists in this area and perhaps larger population based studies are required to gain further insight.

1.2.7.3. Effects of cigarette smoking

F_ENO is lower in current smokers [108-110]. However, NO can rise acutely immediately following cigarette smoke exposure, most likely reflecting the nitric oxide within the cigarette [111] but this is a transient effect. Recent evidence demonstrated that not only is F_ENO reduced in the airways of chronic smokers but even following treatment with oral corticosteroid F_ENO measurements in asthmatic smokers respond as they do in non-smoking asthmatics [112]. Even passive smoking can lead to transient lowering of NO levels and so recommendations in smokers would be to remain abstinent from cigarettes for at least 1 hour before the test and to avoid smoke-filled environments during this time [9]. Another noted effect in smokers is the loss of association of F_ENO being an estimate of underlying eosinophilic inflammation [106].

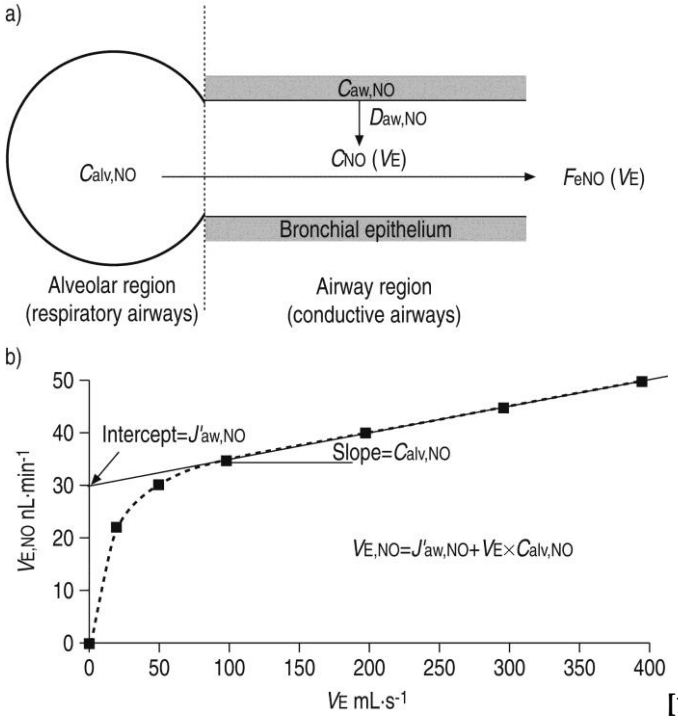
1.2.7.4. Extended flow Nitric Oxide analysis

Research in nitric oxide measurement demonstrated that asthmatic subjects have inflammation throughout the airway tree [113]. A proposed non-invasive means of measuring peripheral airways inflammation is to estimate alveolar NO concentration [C_{alv}], or the contribution from peripheral airways to exhaled NO. The estimation of C_{alv} is based on the measurement of NO at multiple exhalation flow rates [usually in the range of 100-300ml/s]. Exhaled NO follows an exponential curve, with lower F_ENO at higher exhalation flow rates, indicative of NO originating from two sources:

alveoli/small airways, where steady state is reached and a bronchial origin, where the NO diffuses from the airway wall. The estimation of alveolar NO is commonly based on simplified models of the airways' anatomy: a two compartment model of the airways where the conductive airways are a cylinder and an expandable alveolar region - Figure 1.1a [114]. Alveolar NO is thought to reflect inflammation of the smaller airways [115].

This two compartment model allows for the derivation of estimates for alveolar NO levels [C_{alv}, ppb], airway wall diffusion [D_{aw}, pl/s/ppb], airway wall nitric oxide flux [J_{aw}, pl/s] and airway wall NO concentration [C_{aw}, ppb] depending on the flow rates used and the regression model employed - Figure 1.1b. Plotting of the production of NO against a variety of flow rates, allows derivation of these parameters using linear and non-linear regression. The 2005 ATS/ERS guidelines recommended measuring F_ENO at a flow of 50ml/s [102].

Figure 1.1: a] Two compartment model of NO production b] NO output [VNO] as a function of VE in a healthy subject



[114]

1.3. Smokers with asthma

1.3.1. Prevalence of active smoking in asthma

In the United Kingdom in 2007, 22% of adults aged 16 and over smoked with a further 27% being ex-smokers. Whilst the percentage of active smokers has decreased in prevalence since 1996 [28%] the overall proportions have changed little [116]. These figures are similar to most westernised nations [117]. It is disappointing to find that smoking prevalence rates have changed little despite extensive public health measures. The UK smoke-free legislation has been well received by the public and greater than 95% compliance rates within premises have been demonstrated [118]. Whether or not this will lead to a long-term reduction in smoking prevalence remains to be seen.

In terms of visits to hospital emergency departments the impact of smoking in asthma is clearly demonstrated with the finding the prevalence of smoking is higher in asthmatics attending with an exacerbation [119, 120]; and in those asthmatics who die as a result of asthma [121, 122].

1.3.2. Reduced corticosteroid sensitivity in smokers with asthma

International guidelines in asthma management emphasise inhaled corticosteroids as the most effective anti-inflammatory therapy for chronic asthma [2]. The evidence for these guidelines is based on clinical studies performed predominantly in asthmatic patients whom have never smoked or are former smokers. Several studies have suggested that the efficacy of corticosteroids is attenuated in asthmatics who are active smokers [43, 123-125].

The earliest evidence for impaired response to inhaled corticosteroids was found in 1993. A study designed to identify factors which predicted response to inhaled

corticosteroids in obstructive airways disease [asthma and COPD] found that current smoking predicted an impaired FEV_1 response to inhaled beclometasone 800mcg q.d. at 3 months [123]. Although this study was a randomised controlled trial, this unexpected finding led to questioning over which group [or both] demonstrated this impairment, as the study was not designed to look for this.

Further to this, in a randomised placebo controlled crossover study of corticosteroid naïve adult asthmatics, high dose inhaled corticosteroid [1000mcg fluticasone per day] did not demonstrate any improvement in mean morning peak expiratory flow, mean FEV_1, methacholine bronchial reactivity [measured by PC_{20}] or sputum eosinophil counts in current smokers when compared with non-smokers [125].

This resistance to steroid is still present even when high doses [40mg] of oral prednisolone is used [43]. The efficacy of short term [2 weeks] of oral prednisolone, 40mg was assessed in a randomised controlled crossover trial in asthmatic smokers, ex-smokers and never smokers. All subjects had clinical asthma as evidenced by international standards. There was a significant improvement following oral prednisolone compared with placebo in FEV_1, morning PEF and asthma control score in asthmatic never-smokers, but no change in asthmatic smokers.

1.3.3. Asthma treatments

1.3.3.1. Pharmacological treatments

Medications for asthma can be classified in two ways either as controllers or relievers. Controllers are medications taken regularly and long-term to provide sustained control of airway inflammation and hence improved clinically relevant symptoms. Relievers are used as-required to deal with episodes of symptom deterioration. They generally act much more quickly to provide rapid relief of the clinical worsening.

Asthma treatments can be delivered in a variety of methods – inhaled, oral, or by injection. The prime method of drug delivery remains the inhaler. Inhalers contribute the majority of asthma treatment and inhaled corticosteroid is the mainstay of controller therapy in mild to moderate asthma [1, 2].

Relievers constitute a group of medications that require a rapid onset of action to reduce the immediate duration of symptoms. Short-acting-β_2-agonists [SABA's] fulfil this requirement and act principally on airway smooth muscle to promote relaxation, thus reducing airflow obstruction and hence relieving symptoms of wheeze and breathlessness. Longer acting derivatives of these drugs are also in clinical use, known as long- acting-β_2-agonists [LABA's]. They have the benefit of reduced frequency of dosing due to their longer duration of action but as a consequence of their altered pharmacology do not have the rapid onset necessary to provide immediate relief of symptoms. Short-acting-β_2-agonists do not reduce underlying airway inflammation [126] but the same is not true for LABA's [127, 128]. Combination devices are also available and confer a greater degree of benefit in terms of asthma control [129, 130], and due to the pharmacological properties of formoterol, can also be used as a single maintenance and reliever inhaler.

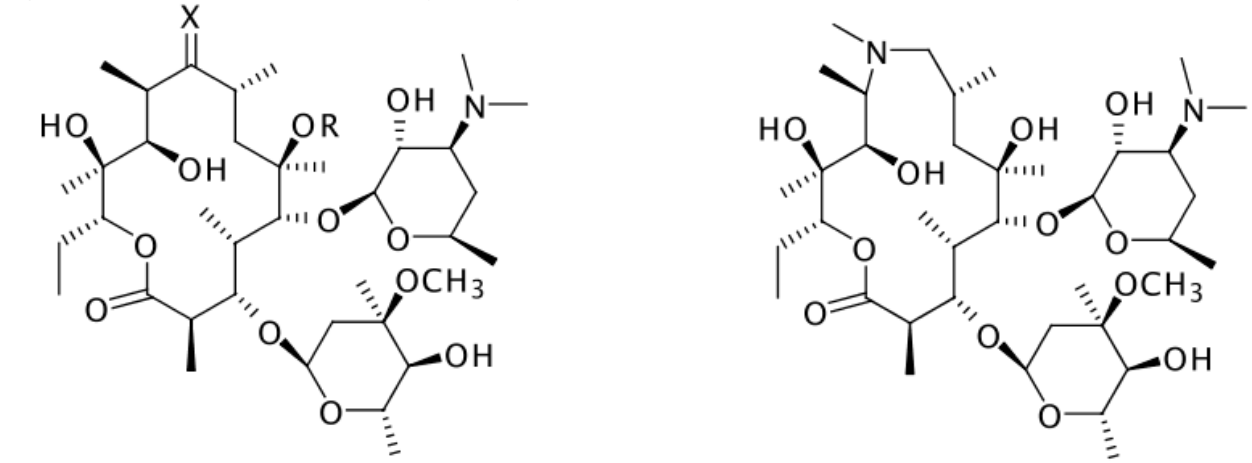
A less used form of inhaled therapy is anticholinergics. A meta-analysis found ipratropium bromide to have benefit, albeit modest when used in acute asthma [131]. Oral medication can also be prescribed for asthma in the form of leukotriene receptor antagonists, theophyllines or oral glucocorticocoids [1, 2]. Parenteral injections of the monoclonal antibody omalizumab [a recombinant humanised monoclonal antibody directed against IgE] has also been shown to reduce exacerbations in patients with raised IgE in allergic asthma [132].

1.4. Macrolides antibiotics and anti-inflammatory properties

1.4.1. Macrolide antibiotics

Erythromycin was the first macrolide antibiotic and has been used since 1952. This prototypical macrolide consists of a 14-membered macrocyclic lactone ring with two sugar moieties. Based on this, several other semi-synthetic macrolides have been developed, including clarithromycin; by substituting a methoxy group for the C-6 hydroxyl group of erythromycin [133], and azithromycin; by inserting a methyl-substituted nitrogen in place of a carbonyl-group in the aglycone ring, thus creating a 15-membered ring-structure [133], Figure 1.2. These structures confer better acid stability, greater bioavailability and a longer half-life; 3-5hrs and 40-68hrs respectively [133].

Figure 1.2: Chemical structure of Erythromycin and derivatives



Reproduced from [134] with permission

1.4.2. Evidence of efficacy in other respiratory disease

1.4.2.1. Diffuse panbronchiolitis

Diffuse panbronchiolitis [DPB] is characterised by chronic inflammation of the respiratory airways and is largely restricted to the Far East. In 1998 a large retrospective study of 498 patients demonstrated clear improvement in lung function and survival for patients treated with low dose erythromycin [135]. Macrolides are now guideline therapy for DPB with recommendation to commence immediately on diagnosis [136].

1.4.2.2. Cystic fibrosis

Cystic fibrosis [CF] has clinical and bacteriological similarities to DPB, and following the establishment of macrolides in DPB treatment, pilot clinical trials were undertaken in CF [137]. A meta-analysis of prolonged-use azithromycin in CF confirmed the improvement in lung function [138]. This evidence has led to macrolides being a guideline recommendation in CF.

1.4.2.3. Non-CF bronchiectasis

Five clinical studies [two randomised] investigated macrolides in non-CF bronchiectasis. The most reproducible finding was decreased sputum volume. This appears to be a class-effect with similar improvements following treatment with azithromycin [139], clarithromycin [140] and erythromycin [141]. These results were used to support recommendations for long-term macrolide treatment for patients with bronchiectasis *and* frequent exacerbations [142].

1.4.2.4. Post-transplant obliterative bronchiolitis

Bronchiolitis obliterans syndrome [BOS] is the leading cause of death following lung transplantation, with a progressive decline in FEV_1 with mortality rates from 25-50% [143]. Open-label studies involving small patient numbers have found improvement in FEV_1 following treatment with azithromycin; 250 mg for 12-36 weeks [144, 145]. The mechanism suggested was a reduction in airway neutrophilia and IL-8 [145].

1.4.2.5. COPD

Few clinical studies have investigated the efficacy of long-term macrolides in COPD. The largest randomised controlled trial in patients with COPD [n=1142] showed that 250mg daily azithromycin for 1 year reduced the frequency of exacerbations [hazard ratio for time to first exacerbation 0.72 [95% CI 0.63 to 0.84, $p<0.001$]] [146], as well as improved quality of life. The number-needed-to-treat was low at 2.86 and the benefit was apparent after only 40 days of treatment [146]. Interestingly, current smoking abrogated the benefit, and yielded the only positive hazard ratio in the subgroup analysis [146].

1.4.3. Efficacy in other inflammatory diseases – RA, IBD and skin disorders

The anti-inflammatory effects of macrolide antibiotics are not just restricted to disorders of the respiratory system. Whilst a significant amount of research has focused on respiratory disease, evidence exists that macrolides can have beneficial anti-inflammatory effects in other chronic inflammatory diseases such as psoriasis and rosacea [147], inflammatory bowel disease [148] and rheumatoid arthritis [148, 149]. Again, the beneficial effect is not just related to a single antibiotic but is seen across the class with both clarithromycin and roxithromycin demonstrating efficacy [149, 150].

1.5.Macrolides in asthma

Anti-inflammatory use of macrolides for asthma was first reported with troleandomycin in the 1960's [151]. Most recent studies have used newer macrolides clarithromycin, azithromycin and telithromycin.

Clarithromycin is the most widely studied macrolide in asthma. Trials with clarithromycin have demonstrated reduced requirement for prednisolone in oral corticosteroid dependant asthma [152] but without significant improvements in lung function, asthma quality of life or symptom scores [152]. Conversely another randomised placebo-controlled study reported improved asthma quality-of-life and symptoms scores [153]. A dose dependant improvement in bronchial hyper-responsiveness [BHR] has also been demonstrated [154]. Improvement in spirometry has been more difficult to achieve with only one RCT demonstrating a small rise in FEV_1. This occurred in a subgroup of subjects PCR-positive for *M. pneumoniae* or *C. pneumoniae.* The improvement in lung function was not found in the PCR negative subjects or when both groups were analysed together versus placebo [155]. Another clarithromycin study [stratified by PCR-positivity for *M. pneumoniae* or *C. pneumoniae* in bronchial biopsies] [156] showed no improvements in lung-function or asthma control questionnaire scores, however, there was an improvement in BHR in those who were PCR-negative or the cohort as a whole; PCR-positive patients did not show improvement. Finally, a recent open label study with clarithromycin administered for 3 weeks following an acute exacerbation, with 12 weeks follow up, demonstrated an improvement in symptom free days and a reduction in the duration of the exacerbation [157].

There are few clinical studies of azithromycin in asthma, with its advantage of once-daily dosing. One of the first pilot studies was terminated early due to treatment

failure [158]. Data analysis, from this study suggested that azithromycin was unlikely to have had an inhaled corticosteroid-sparing effect [158]. In contrast to this conclusion, two other trials using azithromycin showed improvement in BHR [159, 160], although they were limited in their size and design. Another azithromycin study has demonstrated improvements in symptom scores, and reduction in the use of rescue inhalations [161]. More recent reports have suggested a role in attenuating mucus hypersecretion [162]; and using azithromycin in non-eosinophilic [predominantly neutrophilic asthma] subtypes confers benefits in reducing exacerbation frequency [163].

The largest macrolide study is the "Telithromycin in Acute Exacerbations of Asthma" [TELICAST] study; a multicentre, double-blind, RCT evaluating the efficacy of telithromycin in acute exacerbations. Of the two specified outcome measures, there was significant reduction in symptom scores in the telithromycin group, but no treatment effect on morning PEF [164].

Taken together, the results of clinical trials of macrolides in chronic asthma suggest that treatment may improve symptoms and non-specific BHR, which may be independent of *M. pneumoniae* or *C. pneumoniae*, but at present there is little evidence of a sustainable beneficial effect on lung function. A Cochrane review holds the opinion that there is currently insufficient evidence to either support or refute the use of macrolides as anti-inflammatory therapy in patients with chronic asthma [165]. Future studies need to assess macrolide treatment in the management of refractory asthma as well as in acute asthma, perhaps in conjunction with assessment of changes in the microbial colonisation. This would control for the anti-inflammatory effects of either change in bacterial load or diversity.

1.5.1. Macrolides in smokers with asthma

The previously detailed discussion on the utilisation of macrolides in various clinical diseases [1.4.2], suggests that their anti-inflammatory properties may extend beyond those described. At present there are no fully reported studies looking exclusively at smokers with asthma being treated with a macrolide antibiotic.

There are several key factors in the phenotype of smokers with asthma which suggests macrolides may be of benefit:

- Smokers with asthma are commonly steroid insensitive [117].
- Smokers with asthma tend to have a non-eosinophilic [neutrophilic] phenotype [45, 117, 166].
- Neutrophils are recognised to be poorly responsive to corticosteroids [40].

Macrolides have been shown to inhibit migration, activation and the oxidative burst of neutrophils *ex-vivo*, *in vitro* and in experimental models [167] and therefore may be therapeutically important in asthma in smokers. Evidence also exists that treatment with erythromycin in mouse-smoking models leads to reduction in neutrophil and lymphocytes in BAL as well as decreases in TNFα [168].

1.5.2. Effect of macrolides on response to corticosteroid

Macrolides act synergistically with corticosteroids to suppress lymphocyte activation [169] with a possible mechanism for this being related to restoration of HDAC activity – HDAC activity is reduced in COPD and possibly a similar mechanism may exist in smokers with asthma [170]. Erythromycin can increase HDAC2 levels in vitro [171] suggesting a possible mechanism by which macrolides therapy may restore corticosteroid sensitivity and improve asthma control in smokers with asthma. Clarithromycin has no significant effect on prednisolone clearance or mean prednisolone plasma concentrations [172]. Although macrolide antibiotics are

inhibitors of CYP3A4 and can increase the plasma concentration of CYP3A4 substrates, which include fluticasone and budesonide [173], this mechanism is unlikely to contribute to the localized therapeutic effects of inhaled corticosteroids on the airways

1.5.3. Anti-bacterial effects of macrolides

The mechanism of anti-microbial function is thought to occur by binding to the bacterial 23SrRNA and inhibition of protein synthesis [174]. The bactericidal function may provide an additional indirect anti-inflammatory effect by the removal of microbes. The relationship between bacterial colonisation, airway inflammation and lung function has been described in COPD in which bacterial load correlated with higher sputum IL-8, and with a decline in FEV_1; these effects were all associated with a greater pack-year history of cigarette smoking [175]. In chronic bronchitis, sputum bacterial load correlated with sputum MPO, IL-8, LTB_4 levels, and albumin reflecting leakage from serum to sputum [176]. There is some limited evidence to suggest bronchial infection with atypical bacteria is likely to be associated with increased airway inflammation and possible thereby increase asthma severity; 15 out of 19 studies found an association between *C. pneumonia* and/or *M. pneumonia* infection in chronic stable asthma [177]. There is preliminary data to suggest that *C. pneumonia* titres may be increased in smokers both with and without asthma compared to non-smokers [178, 179]. Taken together the anti-bacterial effects of macrolide therapy may improve asthma control in smokers with asthma by reducing airway bacterial load due to *C. pneumonia* and *M. pneumonia* infection.

1.5.4. Mechanisms of anti-inflammatory action of macrolide antibiotics

The mechanism[s] of action of long-term macrolide treatment in chronic respiratory diseases is unresolved. Macrolides have anti-inflammatory properties that are independent from their antibacterial activity and which may be beneficial in reducing airway inflammation. Furthermore macrolides have potential additional beneficial properties including anti-viral activity and an ability to restore corticosteroid sensitivity.

A part of macrolide anti-inflammatory activity may be a consequence of the reduction in bacterial load afforded by the antibiotic effects [176], in addition to the anti-inflammatory activity that is distinct from its anti-bacterial activity [133]. The nature and mechanism of the anti-inflammatory activity has been reviewed [55]. The diversity of these activities suggests a variety of mechanisms affecting immune and tissue-derived cell function including cytokine production. There is no single immunomodulatory-axis through which macrolides exert their effect.

1.5.4.1. Macrolide effects on inflammatory cells

1.5.4.1.1. Neutrophils

Macrolide therapy can reduce neutrophil accumulation in the airway epithelium [145, 153, 160]. This may be associated with macrolides reducing the local production of CXCL-8 [IL-8]; a powerful chemotactic factor for neutrophils, as reported in DPB [145, 180, 181]. This mechanism was also suggested in a study using clarithromycin and azithromycin in refractory asthma leading to reduced airway CXCL-8 and neutrophils [153, 160] [Figure 4]. Azithromycin has also been shown to inhibit neutrophil accumulation in the airways of mice, possibly by affecting IL-17 downstream signals [182]. It must be stressed this was in a non-specific inflammatory

airways mouse-model, and may not necessarily reflect the inflammatory processes in the human [asthmatic] airway.

1.5.4.1.2. Monocyte and macrophages

Macrolides have anti-inflammatory effects on monocytes/macrophages. Classically-activated macrophage [M1] phenotypes, induced by stimulation with interferon [IFN]γ and bacterial lipopolysaccharide [LPS] are associated with microbicidal and cytotoxic function, and pro-inflammatory cytokine production [37]. Alternatively-activated [M2] phenotypes induced by IL-4/13 are associated with T_H2-type, immunosuppressive and remodelling responses. Azithromycin reduced the production of pro-inflammatory IL-1β, and tumour necrosis factor [TNF]α in mouse M1 macrophages [183] and polarised cells towards M2, with reduced pro-inflammatory IL-6 and IL-12, and increased anti-inflammatory IL-10 [184] [Figure 5]. *Ex-vivo* human monocytes treated with clarithromycin could effectively and significantly reduce LPS stimulated IL-8 production in a dose dependant manner [185]. The relevance to lung disease is that, alveolar macrophages become polarised during infection towards M1 [186] but their pro-inflammatory activity may be attenuated by macrolides e.g. by reduced production of TNFα, IL-1β and IL-8 [183], and polarisation towards anti-inflammatory M2 [184] characterised by IL-10 production and scavenger-receptor expression associated with clearing apoptotic cells [187]. Reduced apoptotic bronchial epithelial cells have also been observed in COPD patients receiving azithromycin [187].

1.5.4.1.3. Macrolide effects on cytokine production

Azithromycin has many anti-inflammatory effects [188] including down-regulation of production of pro-inflammatory mediators e.g. prostaglandin E2, nitric oxide and cytokines TNF-α, IL-8, IL-1α, growth-related oncogene [GRO]-α and soluble vascular

cell adhesion molecule [sVCAM]-1. Many of these are chemotactic, activation and survival factors for neutrophils.

IL-8 [CXCL8] is a potent chemo-attractant of neutrophils. A randomised double-blind placebo-controlled trial with clarithromycin in refractory asthma demonstrated a significant reduction in airway IL-8 levels and neutrophil numbers [153]. Subgroup analysis has shown that airway IL-8 protein and gene expression was predominantly reduced in the non-eosinophilic asthmatic patients. Similarly, azithromycin reduced airway IL-8 mRNA in patients with post-transplant BOS following 3 months treatment [145].

IL-1β is sufficient to induce neutrophil accumulation in the lung [189], GM-CSF is a neutrophil survival factor [190], and both are derived from macrophage and airway epithelial cells and are central to airway infectious inflammation [186]. In a murine model of LPS-induced pulmonary neutrophilia, azithromycin or clarithromycin were able to reduce airway neutrophilia with striking reductions of IL-1β and GM-CSF [191] .

The effects of azithromycin on cytokine inhibition are not just restricted to the above noted cytokines. More recently azithromycin has been found to inhibit the production of IL-5 in ex vivo CD4+ T cells from asthmatic children[192].

1.5.4.2. Molecular mechanisms of macrolide anti-inflammatory activity

Defining molecular mechanisms of anti-microbial macrolide function received the 2009 Nobel prize, yet the mechanisms of anti-inflammatory macrolide function remain unresolved, probably reflecting their complexity. Macrolides accumulate and persist inside leukocytes [133] suggesting effects on cell signalling. Azithromycin can inhibit mitogen-activated protein [MAP] kinases [193]; which regulate cellular

processes, e.g. gene expression, cell growth, proliferation, differentiation and survival in response to a variety of extracellular stimuli, especially cytokines including IL-8 and GM-CSF as mentioned above. Putative mechanisms e.g. with clarithromycin suggest altered DNA binding activity of transcription factors NF-kB and AP-1 [185, 194], and inhibition of synthesis and/or secretion of pro-inflammatory cytokines [55].

In summary, different macrolides can cause reductions in pro-inflammatory cytokines, suggesting there is a class effect inhibiting airway neutrophilia. Individuals with neutrophilic inflammation may therefore derive greatest benefit.

1.5.4.3. Macrolide effects on mucus production

Macrolides appear not to affect normal physiological secretion of mucus [55] but can reduce hypersecretion [195], possibly by inhibiting production of pro-inflammatory cytokines, e.g. TNFα, that stimulate mucin genes MUC5B and MUC5AC in airway goblet cells [196].

1.5.4.4. Macrolide microbicidal activity as a factor in immuno-modulation

Evidence against anti-microbial activity of macrolides accounting for their anti-inflammatory activity includes effective long-term treatment of DPB at sub-antibacterial concentrations, and efficacy when colonised with macrolide-resistant *P. aeruginosa* [197]. Mechanisms may include macrolide interference with microbial protein synthesis even below minimal antimicrobial concentration [198, 199].

1.5.4.5. Macrolide antiviral effects

Rhinoviruses [RV] cause ~60% of virus-induced asthma exacerbations. Macrolides appear to have inherent anti-viral properties, and induce anti-viral responses. Several

studies have demonstrated the beneficial effects of macrolides in experimental RV and influenza infections. The addition of a macrolide led to reduced virus titres, most likely as a consequence of inhibition of RV-induced up-regulation of ICAM-1 and also inhibition of pro-inflammatory cytokine production; ICAM-1 is the RV receptor on airway epithelium [200-202].

1.5.4.6. Corticosteroid-sparing effects

Macrolides may have corticosteroid-sparing effects, first shown with troleandomycin, but limited due to adverse effects [203]. Corticosteroid-sparing efficacy is limited to case reports [204] and small open-label pilot studies [169], which demonstrate improvement in clinical laboratory endpoints – enhanced sensitivity of lymphocytes to suppression by dexamethasone. Although macrolide antibiotics are inhibitors of CYP3A4 [133] and can increase the plasma concentration of CYP3A4 substrates, which include fluticasone and budesonide , this mechanism is unlikely to contribute to the localised therapeutic effects of inhaled corticosteroids on the airways of patients given macrolides.

Smokers with COPD have decreased histone deacetylase-2 [HDAC2] activity in alveolar macrophages, and this may lead to increased inflammatory gene expression and reduced sensitivity to corticosteroids [205] and a similar mechanism may occur in smokers with asthma [170]. Erythromycin can increase HDAC2 levels *in vitro* [171], suggesting a mechanism by which macrolides therapy may restore corticosteroid sensitivity and improve asthma control in smokers with asthma.

1.6. Summary

Given the preliminary data that macrolides have anti-inflammatory properties both *in vitro* and *ex vivo* and can improve asthma control in non-smokers with asthma we propose that the anti-inflammatory effects of macrolides manifest in these models can be achieved also in smokers with asthma, a major subgroup that are currently undertreated. Several large trials are underway to examine the clinical benefits of macrolide therapy in asthma. Presumably to avoid the potential confounding effects of cigarette smoking, studies are generally designed to recruit non-smokers [156] or predominantly non-smokers [206] and none of the studies have specifically targeted smokers with asthma.

There is now substantial evidence from pre-clinical and clinical studies of the efficacy of macrolides and in particular azithromycin. Smokers with asthma have a phenotype which, given laboratory endpoint data would suggest they represent a population of individuals who may experience benefit from macrolides. Macrolides are effective therapy in many other respiratory illnesses, but at present there is no firm evidence to advocate their use in asthma [165]. Smokers with asthma are an under-investigated patient group and bear a significant burden of disease, and stand to gain benefit in asthma control and quality of life if new treatments are efficacious.

2. Hypothesis

In a proof-of-concept clinical trial, we tested the hypothesis that macrolides [azithromycin] improve asthma control and reduce sputum neutrophil counts of smokers with chronic asthma.

2.1. Primary end-point

The primary endpoint to test the hypothesis was a change peak expiratory flow measurement measured at the study visits. It was expected that an improvement of 25L/min would be observed.

2.2. Secondary end-points

2.2.1. Clinical

It is expected that that other clinical indicators of asthma control will also demonstrate improvement. These secondary clinical endpoints will include

- Average of the last 7 days PEF measurements before each visit [from home recordings]
- Spirometry
- Airway responsiveness to methacholine
- Asthma control score
- Cough score
- Diary symptom scores
- Exacerbation rates

2.2.2. Inflammatory

In addition to the above measured endpoints it is expected there will be improvement in biomarkers of inflammation. Measurements will include:

- Sputum differential cell counts including sputum neutrophil count
- F_ENO and Alveolar NO
- Immunological tests in blood & sputum

3. Randomised controlled trial

Seventy-seven adult asthmatic smokers were recruited to the study. These subjects were recruited from hospital respiratory clinics, general practice database registries and from the database of the Asthma and COPD Research Centre, Gartnavel General Hospital. Potential subjects had their records scrutinised for suitability and were then contacted by letter from their GP or directly from the Research Centre. A second site was opened at Crosshouse Hospital, Ayrshire and subjects were recruited in the same way. Volunteers were then screened for eligibility, initially by telephone and subsequently at the research unit.

3.1. Regulatory approval

Clinical trial regulations within the EU require approval from a competent authority within the member state in combination with a favourable opinion from an appropriate ethics committee. In the United Kingdom this competent authority is the Medicines and Healthcare Regulatory Agency [MHRA]. Before recruitment to the trial could be undertaken a clinical trial application was made to the MHRA. At the same time ethical approval was obtained from the West Glasgow Ethics Committee 1. All subjects received an information sheet and attended for a discussion of the protocol prior to consent and enrolment.

3.2. Recruitment methods

Primary care was the main reservoir of patient subjects for recruitment to this clinical trial. Additionally, patients who had attended hospital clinics or had in-patient stays were also considered for recruitment. Finally the research unit had an active database of patients willing to take part in clinical trial research.

General practitioners were contacted by letter and asked to complete a form agreeing to participate in the study. Research co-ordinators from the Scottish Primary Care Research Network then visited the practice and performed a database search for prospective patient volunteers. A list of volunteers was then generated and screened by a member of the GP practice to ensure appropriateness for further contact. Letters were then sent from the GP to each individual patient asking them to complete a contact form and return in a reply paid envelope. Once a form was received, prospective patients were contacted by telephone and if found suitable, invited to attend for a screening visit.

Case note screening was also undertaken of patients whom had prior attendance at either respiratory out-patient clinics or in-patient wards. These patients were contacted directly by telephone.

The Asthma and COPD Research Centre has an active patient database of prospective patients. This was scrutinised and prospective patient volunteers were contacted directly by telephone.

In this clinical trial over 8000 invitation letters were sent from 80 GP practices in the West-of-Scotland. A summary of patient flow is found below – Figure 5.1: CONSORT Flow Diagram.

3.2.1. Search strategy

The GP practices in the West-of-Scotland utilise databases provided by "General Practice Administration System for Scotland" [GPASS], "In-practice systems Ltd" [INPS] "*Vision"* and more recently "Egton Medical Information Systems" [EMIS]. Each handles data in a slightly different manner and so searches had to be tailored in respect of each individual database. Each of these systems has its own shortcomings

but commonly, the accuracy of the database is dependent on the quality of the data entered. Some patients may not have their data fully updated on a regular basis and hence may not be completely accurate.

In performing searches on any database there is a compromise between capturing highly accurate data and excluding inappropriate records without excluding potential candidates. Hence, in the first instance the search aims to capture as broad a pool of potential participants as possible.

Search criteria used were:

- Age 18-70
- Diagnosis of asthma
- Current smoker

These had to be tailored for each individual database but remained essentially the same.

3.3. Subjects

3.3.1. Inclusion criteria

The main inclusion criteria for the study are detailed in Table 3.1.

Table 3.1: Main inclusion criteria

Diagnosis of asthma [207]: • Typical symptoms [episodic wheezing, chest tightness and/or dyspnoea] And either • reversible airflow obstruction [≥ 12% and 200 ml change in FEV_1 with nebulised salbutamol 2.5 mg] at any study visit prior to randomisation or • methacholine airway hyper-responsiveness [20% drop in FEV_1 at a concentration of methacholine ≤ 8 mg/ml].
Age range 18-70 years [subjects above the age of 60 should have had asthma symptoms starting before the age of 40]
Duration of asthma symptoms ≥ 1 year and on stable medication for 4 weeks
Able to maintain asthma without exacerbations - at BTS step 2 level [beclometasone dose of 400 mcg/day and salbutamol as required] during the run-in period of the study.
Able to wean off other asthma medication, other than inhaled corticosteroid and short acting bronchodilator in the two weeks prior to the screening visit.
No other medication for asthma other than the above following the screening visit.
Symptomatic, defined as an asthma control questionnaire score of ≥ 1.0 [range 0-6] prior to randomisation [208].
Smokers with asthma will be defined as current cigarette smokers who have a ≥ 5 pack year smoking history. Subjects should be smoking at least 5 cigarettes a day. If currently on 2-5 cigarettes a day, a previous 10 pack year smoking history would be required.
If female and able to conceive, willing to utilize medically acceptable forms of contraception. A pregnancy test will be performed in urine in all women of child bearing age and able to conceive.

3.3.2. Exclusion criteria

Main exclusion criteria are detailed in Table 3.2.

Table 3.2: Main exclusion criteria

Ex-smokers or never smokers
Planning to quit smoking during duration of trial
Patients with unstable asthma; defined as the presence of 1 or more of the following events in the month prior to randomisation [Emergency/'out of hours' visit of patients to the GP; GP visit to patient at home; A & E hospital attendance; hospital admission].
Patients with current epilepsy, psychosis or history of significant atrial or ventricular tachyarrhythmia.
Corrected QT-interval greater than 450msec in women and greater than 430msec in men on baseline electrocardiogram [ECG].
Low potassium levels [less than normal values for the laboratory]. If low potassium can be corrected, screening can continue with confirmation of normal levels prior to taking study medication.
Liver disease [alanine transaminase and/or aspartate transaminase levels 2 or more times the upper limit of normal].
Significant renal disease: creatinine or urea levels 2 or more times the upper limit of normal.
Any previous severe adverse reaction to macrolides.
Patients who are known to have specific IgE sensitivity or skin test positivity to grass pollen allergen and a history of worsening of asthma due to hay fever, will not be recruited from mid May to the end of July [grass allergen season in UK].
Upper or lower respiratory tract infection in the 4 weeks prior to randomisation. Run-in period can be prolonged in this situation to have 4 weeks with no respiratory infection prior to randomisation.
Weight < 45 kg.
Frequent asthma exacerbations [> 4] requiring oral corticosteroids in the year prior to randomisation.
Presence of active lung disease other than asthma, including bronchiectasis and vocal cord dysfunction.
Current or past diagnosis of allergic broncho-pulmonary aspergillosis [ABPA] in the past
Pregnancy and breast-feeding.
Mental impairment or language difficulties that makes informed consent not possible

In addition to the above exclusion criteria, consideration was required in patients who with medications known to interact with azithromycin – Table 3.3.

Table 3.3: Drugs known to interact with azithromycin

antimalarials
anti-psychotics, including Reboxitine and Quetiapine
antivirals, including Nelfinavir, Zidovudine, Didanosine
bromocriptine
carbergoline
clozapine
coumarin-type oral anticoagulants including warfarin
cyclosporin
ergot derivatives
itraconazole
midazolam
mizolastine
moxifloxacin
rifabutin
rifamycins
theophylline
vinblastine
Other immunosuppressants or chronic antibiotics e.g. methotrexate, azathioprine, tetracycline.

Certain medications did not exclude subjects from the trial but additional care needed to be taken with some of these. These concomitant medications are detailed in Table 3.4.

Table 3.4: Medications requiring additional consideration/monitoring

Antacids: In patients receiving antacids, azithromycin should be taken at least 1 hour before or 2 hours after the antacid.
Digoxin: Some macrolide antibiotics have been reported to impair the metabolism of digoxin [in the gut]. Therefore, in patients receiving concomitant azithromycin and digoxin the possibility of raised digoxin levels should be borne in mind and digoxin levels monitored.
Verapamil
Amiodarone
Simvastatin

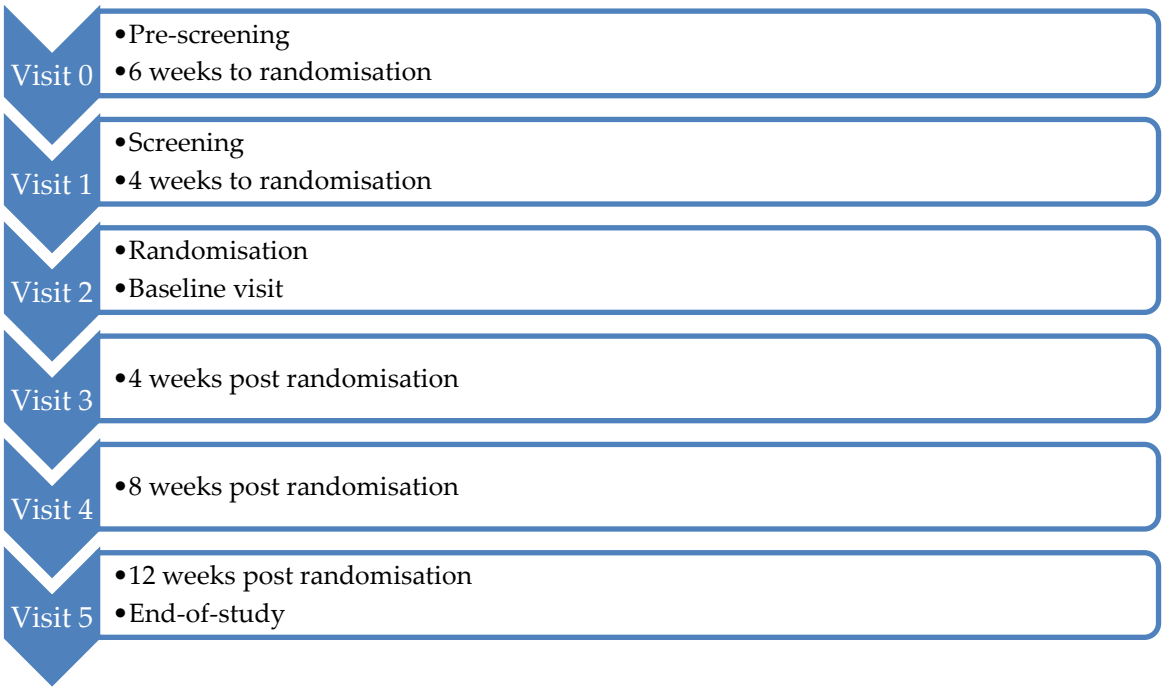
3.4.Study design

The study is a 12 week double-blind randomised controlled study [Figure 3.1]. Subjects who required weaning from high-dose combination inhalers underwent pre-screening 2 weeks before the screening visit to enable weaning. Weaning consisted of stopping the combination inhaler and providing an equivalent dose of inhaled corticosteroid in their now stopped combination inhaler.

Two weeks after this a screening visit was performed. The dose of inhaled corticosteroid was reduced further to a standardised dose [200mcg beclometasone equivalent twice daily]. Patients continued on this for a further four weeks. An optional study visit was available at two weeks post screening to ensure safety.

At 4 weeks post screening patients would undergo a randomisation visit over two separate days, up to 5 days apart. If all of the entrance criteria were achieved and no exclusion criteria existed the patient would be randomised to receive either active treatment or matched placebo. Randomisation was undertaken using an automated interactive voice response system [IVRS]. Patient visits then continued at 4, 8 and 12 weeks post randomisation.

Figure 3.1: Patient journey in the study



3.5. Data management and statistical analysis

All data for the trial was entered on to specially designed case report forms [CRF's]. These were designed in conjunction with the Robertson Centre for Biostatistics [RCB] at The University of Glasgow, with appropriate input from the study team. The CRFs were then sent to the RCB for data entry and generation of the research database. The quality of the data entry was assessed by monitors employed by the sponsor. Monitoring was performed at the study initiation, midpoint and shortly after completion of all patient follow up visits. Where areas of uncertainty were identified either by the monitors or the data managers a data query was generated. I was responsible for clarification of the data where required and corrected any errors where appropriate.

I performed the vast majority of CRF data entry for the study and was solely responsible for checking the entries and resolving data queries.

3.5.1. Power calculation

A sample size of 34 in each group will have 80% power to detect a difference in means of 25L/min in peak expiratory flow [PEF] change [primary endpoint], assuming a standard deviation of changes of 36L/min, using a two sample t-test with a 0.050 two-sided significance level [129]. Based on experience in our previous clinical studies we intended to recruit a total of at least 80 patients to ensure that 68 patients completed the study.

3.5.2. Analysis sets

3.5.2.1. Full Analysis Set [FAS]

The intention-to-treat principle implies that the primary analysis should include all randomised subjects. Compliance of this principle would necessitate complete follow-up of all randomised subjects for study outcomes. In practice this ideal may be difficult to achieve. The "full analysis set" is used to describe the analysis set which is as complete as possible and as close to the intention-to-treat ideal of including all randomised subjects. Preservation of the initial randomisation in analysis is important in preventing bias and in providing a secure foundation for statistical tests.

There are a limited number of circumstances that might lead to excluding randomised subjects from the full analysis set including the failure to satisfy major entry criteria [eligibility violations], the failure to take at least one dose of study medication and the lack of any data post randomisation. Violations of the protocol that occur after randomisation may have an impact on data and conclusions particularly if their occurrence is related to treatment assignment.

3.5.2.2. Per Protocol Set [PPS]

The "per protocol" set of subjects, sometimes described as the "valid cases", the "efficacy" sample or the "evaluable subjects" sample, defines a subset of the subjects in the full analysis set whom are more compliant with the protocol, and is characterised by criteria such as the following:

i. The completion of a certain pre-specified minimal exposure to the treatment regimen;
ii. The availability of the measurements of the primary variable[s];
iii. The absence of major protocol violations including the violation of entry criteria.

The precise reasons for excluding subjects from the per-protocol set should be fully defined and documented before breaking the blind in a manner appropriate to the circumstances of the specific trial. This would be a data set generated by the subset of subjects who complied with the protocol sufficiently to ensure that they would be likely to exhibit the effects of treatment, according to the underlying scientific model. Compliance covers such considerations as exposure to treatment, availability of measurements and absence of major protocol violations.

3.5.2.3. Safety Set

All randomised subjects who received at least one dose of randomised treatment. The primary safety data set will be used to determine the safety of the intervention.

3.5.2.4. Baseline data

Baseline characteristics will be summarised for each randomised treatment group separately and overall and compared informally for the FAS, and the PPS patients.

All baseline data will be obtained from the CRF's used during the baseline or screening/pre-screening visits.

3.5.3. Endpoints

3.5.3.1. General principles for statistical analysis

The treatment period lasted for 12 weeks. Categorical variables will be summarised with the number and proportion of subjects within each category. Continuous variables will be summarised using the mean, standard deviation [SD], or median and interquartile range; dependent on the distribution [normal or skewed], and minimum and maximum values.

Differences between treatment groups for the primary and secondary outcomes will be assessed using appropriate tests.

Analysis of covariance models, adjusting for the baseline data, will be used to compare the treatment groups after 12 weeks for both the primary outcomes and the secondary outcomes.

3.5.3.2. Treatment of ACQ scores [clinic and diary versions]

The standard version of the ACQ contains seven fields each scored on a seven point scale [0=good control, 6=poor control]. The overall score is the arithmetic mean of the seven responses. A copy of the clinic-ACQ can be found in Appendix 1.

Utilising a daily diary card to derive and ACQ differs from above. The morning PEF data is used as a surrogate of FEV_1, and converted to a percent-predicted value which is scored like FEV_1 on the standard version of the questionnaire. The parameters are the same as the standard ACQ. $Beta_2$-agonist puffs are totalled for the morning and

evening, to calculate the usage over 24hours. This total is then mapped to the relevant field within the standard ACQ to derive a numerical value to be used in the final calculation. Once this is done the mean of the seven fields can be calculated.

3.5.3.3. Treatment of AQLQ scores

This study utilised the standardised version of the Asthma Quality of Life Questionnaire [12]. This questionnaire allows the clinician to gain an overall measure of the problems those adults with asthma experience in their day to day lives. The questionnaire is divided in to 5 generic activities – Table 3.5. Each question is scored on a 7-point Likert scale and is completed independently by the patient with no external influence. Subjects are asked to answer the questions based upon their experiences over the past 2 weeks.

Table 3.5: Five generic activities of the AQLQ

Strenuous activities [such as hurrying, exercising, running up stairs, sports]
Moderate activities [such as walking, housework, gardening, shopping, climbing stairs]
Social activities [such as talking, playing with pets/children, visiting friends/relatives]
Work related activities* [tasks that you have to do at work]
Sleeping

*If you are not employed or self-employed, these should be tasks you have to do most days.

The questionnaire contains 12 items on symptoms, 11 items on activity limitations, 5 items on emotional functions, and 4 items concerning environmental stimuli. The minimal important difference is determined to be 0.5. Change of 1.0 represents a moderate change and change of >2.0 represents a large change [13, 209]. A copy of the AQLQ can be found in Appendix 2.

3.5.3.4. Treatment of LCQ scores

The Leicester Cough Questionnaire is a 19-item self-completed quality-of-life measure of chronic cough [210], consisting of 3 domains: physical [8 items], psychological [7 items] and social [4 items]. Each item has a response rating from 1 to 7, with the lowest value indicating the worst response to the question with the highest value indicating the best. Each domain score is an average of the items within, and the total score is the sum of each domain's average. A domain score will range from 1-7 with the total score ranging from 3-21.

A minimal change in the total score of 2.56 is felt to be significant of change in the severity of cough [210]. A copy of the LCQ can be found in Appendix 3.

3.5.3.5. Treatment of Diary Cards

The diary cards have space to collect up to five weeks of data. For the purposes of analysis we used the last seven days that were completed prior to each visit. A card was declared null and void if there were no morning peak flow data. The number of completed days in the seven-day period up to and including the last day of data was counted. At least three days of data for each variable had to be available separately for that variable to be considered evaluable. The means of seven days were calculated for each of the evaluable variables. A copy of the diary card can be found in Appendix 4.

As a general observation – the quality of diary entry was variable and despite extensive patient education, the completeness of data entry could in some instances be poor. Diary data was scrutinised to ensure accuracy of date and time entry – data recorded on the Piko-1 meter was given precedence over the data recorded on paper.

An ad-hoc report was generated for manual checking of data. Manual checking was then performed.

3.5.3.6. Treatment of asthma related events

The total number of these events [out of hours visits to GP, GP home visits, visits to accident and emergency, hospitalisations] was calculated across each of the visits in the treatment period [i.e. Weeks 4, 8 and 12]. The totals were also calculated separately for each of the four different types of event, and further categorized in terms of intensity, relationship with the study medications, treatment required and outcome.

3.5.4. Analysis techniques

3.5.4.1. Primary efficacy analysis

Analysis of covariance [ANCOVA] models were used to compare change in the primary endpoint [change in Peak Expiratory Flow] from baseline across the treatment groups at 12 weeks adjusting for the baseline peak expiratory flow. The treatment difference and corresponding 95% confidence interval will be presented and a p-value for the adjusted mean difference [azithromycin-placebo] will be reported. Due to the small number of anticipated missing data for the primary outcome at the last visit, the analysis will be run using both complete case analysis and analysis using multiple imputation methods.

3.5.4.2. Secondary efficacy analyses

The interaction between selected baseline, screening and pre-screening variables and treatment efficacy will be tested by adding the relevant main effect and the interaction terms to the ANCOVA model above. The potential interaction of each screening or baseline variable will be tested in a separate statistical model.

Continuous variables will be dichotomised according to whether the values are either lower than, or greater than, or equal to, the mean difference [IMP minus placebo] and will be presented alongside its 95% confidence interval; the p-value for the interaction term will be reported.

3.5.4.3. Exploratory analyses

Exploratory endpoints will be analysed in the same way as the secondary endpoints.

3.5.5. Software

All statistical analysis will be performed using SAS version 9.2 or later.

3.6. Patient safety

3.6.1. Drug interactions and side effects associated with azithromycin

Azithromycin is generally a very well tolerated antibiotic and does not have many clinically significant interactions. Whilst caution is advised when administering the drug in severe renal impairment, the protocol would exclude such patient. No dose adjustment is necessary in mild to moderate impairment, since azithromycin is metabolised in the liver and excreted in the bile

Side effects in association with azithromycin therapy are generally not serious with the commonest being gastro-intestinal [133] upset most likely relating to activity at the motilin receptor. The commonest side effects are diarrhoea; nausea; abdominal pain; headache; dizziness; anorexia; visual impairment; deafness; pruritis; rash; arthralgia; fatigue; changes in blood lymphocyte [decreased] and eosinophil [increased] counts.

Patients concurrently taking antacids, digoxin, verapamil, amiodarone and simvastatin were closely monitored. Concomitant use of antacids can lead to a reduction in peak serum concentrations, but not overall bioavailability. If the subject was on antacids then they were asked to delay taking these by one hour after administration of the IMP to ensure adequate oral absorption. Digoxin, verapamil, and amiodarone are all cardioactive drugs and whilst there is no significant pharmacological interaction, there is the theoretical potential for alteration in cardiac-QTc. In the majority, these drugs tend to be prescribed for tachyarrhythmias or additionally in the case of verapamil, hypertension. The potential for increased toxicity is more applicable to erythromycin and clarithromycin rather than azithromycin. History of atrial or ventricular tachyarrhythmia was an exclusion to the study and hence patients on either digoxin or amiodarone were generally excluded for another reason. Azithromycin is not known to interact with any of the statins but there is well documented interaction between simvastatin and erythromycin/clarithromycin and so it was felt prudent to also include this drug as one for special consideration.

3.6.2. Unused medication

Unused medication was returned to our local pharmacy where each pack's contents were first counted, logged then destroyed. Regulatory requirements ensure that a log is kept of all returned and destroyed investigational medicinal products.

3.6.3. Current anti-asthma medication

During the study patients required to be on standardised treatment containing a maximum of 200mcg beclometasone equivalent twice daily. Our protocol required each participant to be on either budesonide 200mcg, 1 inhalation twice daily, or Symbicort® 200/6, 1 inhalation twice daily. All other asthma medication was

discontinued in the run-in phase and patients were not allowed to restart any additional asthma medications during the study or increase the dose of inhaled therapy either.

Upon completion of the study, participants were either placed back on their usual medication if there was no perceived benefit from the study inhaler, or if evidence of ongoing symptoms were present a letter was written to their GP advising an escalation of therapy to the next appropriate step.

3.6.4. Adverse events [AE's]

An adverse event [AE] is defined by the UK Medicines for Human Use [Clinical Trials] Regulations 2004 [SI 2004/1031] as:

An exacerbation, or unexpected increase in the frequency or intensity of a pre-existing condition [other than asthma], including intermittent or episodic conditions. This could be significant or unexpected worsening or exacerbation of asthma, a suspected drug interaction or any clinically significant laboratory abnormality.

Adverse events are graded according to their severity as follows:

- **Mild:** Awareness of signs or symptoms, but easily tolerated. There is no loss of time from normal activities. Symptoms resolve easily with no medical treatment [other than short-acting bronchodilators]. Signs and symptoms are transient.
- **Moderate:** Discomfort severe enough to cause interference with the patient's usual activities. Symptomatic treatment is possible.

- **Severe:** Incapacitating with inability to do work or usual activities, signs and symptoms may be of a systemic or require medical intervention and/or treatment. Hospitalisation may be required.

A reasonably related event is one that is in the opinion of the investigator, possibly, probably or is definitely related to the study product.

3.6.4.1. Serious Adverse Events [SAE's]

A serious adverse event is defined as any adverse event or adverse reaction that:

- Results in death
- Is life threatening
- Requires hospitalisation or prolongation of existing hospitalisation
- Results in persistent or significant disability or incapacity
- Consists of a congenital anomaly or birth defect

For the purposes of the study the following events, although not classified as SAE's would also require reporting.

- Important adverse events/reactions that are not immediately life threatening or do not result in death **or** hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above
- Pregnancy

3.6.4.2. Serious Adverse Reaction

Any adverse reaction that is classed in nature as serious and which is not consistent with the information about the medicinal product in question, as set out in the summary of product characteristics [SmPC] or the Investigator's Brochure [IB]

3.6.4.3. Suspected Unexpected Serious Adverse Reaction [SUSAR's]

SUSAR's are serious adverse reactions related to an investigational medicinal product that are both serious and unexpected.

3.6.4.4. Method for reporting of Adverse Events

All adverse events [AE's] must be recorded, notified, assessed, reported, analysed and managed in accordance with the Medicines for Human Use [Clinical Trials] regulations 2004 [as amended]. All adverse events must be assessed for seriousness, causality, expectedness and severity. This assessment is the responsibility of the Chief Investigator. The Chief Investigator is required to inform the sponsor immediately [within 24hrs]. For all SAE's, SAR's and SUSAR's a generic [Robertson Centre of Biostatistics] Serious Adverse Event form was completed and a copy forwarded to the Pharmaco-vigilance Officer in the Research & Development Department, Greater Glasgow & Clyde Health Board.

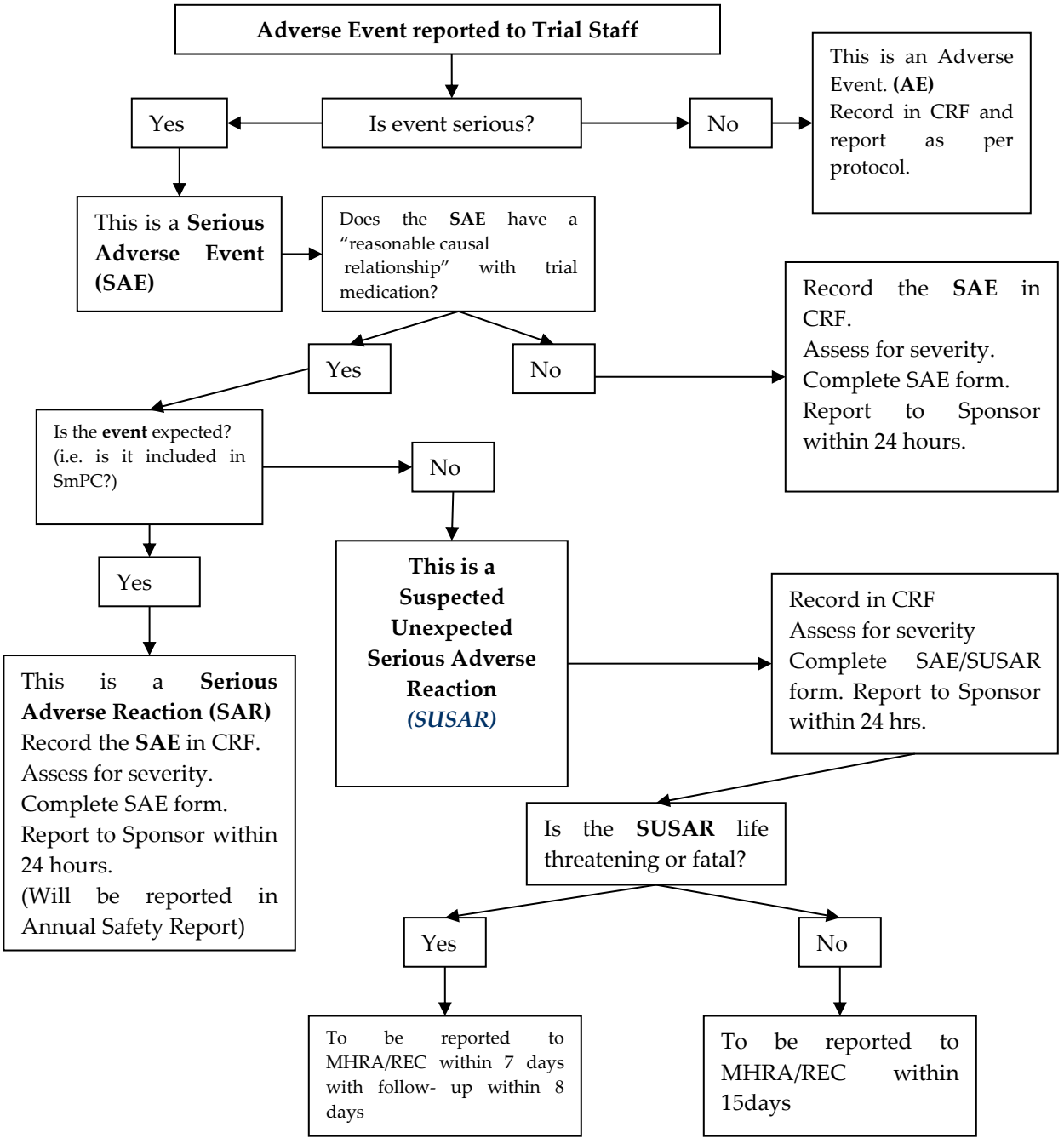
Serious Adverse Events: were collected in the CRF, an SAE form completed for each one, and sent to the pharmaco-vigilance office as above. Copies were then held in the site file and formed part of the Annual Safety Report which was sent to the MHRA, Ethics and the sponsor.

Serious Adverse Reactions [SAR's]: are reactions judged by the chief investigator to be related to the study drug, although listed in the protocol as expected drug reactions. These were collected in the CRF, an SAE form completed for each one, and sent to the pharmaco-vigilance office as above. All SAR's were held in the site file and formed part of the Annual Safety Report sent out as above.

Suspected Unexpected Serious Adverse Reactions: are reactions judged by the chief investigator to be related to the study drug, and are *unexpected* study drug reactions according to the protocol. If they occur, they are collected in the CRF and an SAE form completed for each one. SUSARs should be sent to the MHRA, ethics committee and the sponsor within 7 days for all fatal or life-threatening SUSARs and 15 days for all others.

SAE's that occur at any time after the inclusion of the subject in the study [defined as the time when the subject signs the informed consent] up to 30 days after the subject competed or discontinued the study will be reported.

Figure 3.2: Safety Flow Chart



3.6.5. Clinical trial obligations

In 2004, the introduction of EU directive 2001/20/EC [SI1031] meant that in order to conduct a clinical trial of a medicinal product, the following must be obtained:

3.6.5.1. Sponsorship

A sponsor is an individual, company, institution or organisation which takes responsibility for the initiation, management or financing of a clinical trial. The University of Glasgow and NHS Greater Glasgow & Clyde operate a joint Research & Development department. For the purposes of this clinical trial U.o.G. and NHS GG&C acted as co-sponsors.

3.6.5.2. Ethical approval

Ethical approval for clinical research is now centralised under the auspices of the National Research Ethics Service [NRES], part of the National Patient Safety Agency. The purpose of NRES is twofold:

- to protect the rights, safety, dignity and well being of research participants and
- to facilitate and promote ethical research that is of potential benefit to participants, science and society

Application is made using an electronic form and subsequent to this accompanying documents are submitted to an ethics committee for consideration. An ethics committee is an independent body consisting of health-care professionals and lay members. Their function is to provide an opinion about whether a trial clinical trial is ethically responsible. This opinion is offered before a clinical trial commences based on the following information which they should be provided with:

- Summary of the study and principal research question
- Study design and type of medicinal trial
- Scientific justification for the research
- Risks and ethical issues including patient selection and interventions
- Recruitment methods and confidentiality of subjects
- Research sponsor and other regulatory oversight

3.6.5.3. MHRA approval

Application to the MHRA is fully electronic. For the purposes of this clinical trial we were required to provide copies of the following documents in PDF format for review:

- Clinical trial application form with accompanying data in XML format
- Confirmation of EudraCT number
- Copy of favourable ethics opinion
- Copy of letter of authorisation from the sponsor
- Current version of clinical trial protocol
- Simplified investigation medicinal product dossier [Simplified-IMPD] for Azithromycin 250mg capsules
- Copy of the manufacturing authorization from Bilcare [GCS] Europe Limited ML[IMP]10284 – GMP documents
- Example of the IMP label in the national language

Formal application to the MHRA was made on the 23rd of March with clinical trial being authorised on the 24th April 2009

3.6.5.4. Amendments

Several amendments were made during the trial. Amendments can be classified as either substantial or non-substantial. Examples of changes require application for a substantial amendment would be:

i. change of the main objective of the clinical trial;
ii. change of primary or secondary endpoint
iii. changes to inclusion/exclusion criteria
iv. addition of a new site

This list is not exhaustive.

Examples of changes that are typically non-substantial

i. the addition/deletion of tertiary/exploratory endpoints
ii. minor clarifications of the protocol
iii. correction of typographical errors

This list is not exhaustive

3.6.5.5. Annual Safety Reports

Annual safety reports are compiled by the sponsor based upon the SAE's reported during the period of the trial. Copies of the annual safety report are circulated to the ethics committee, MHRA and the study team.

3.6.5.6. Other obligations

3.6.5.6.1. Trial registration

Clinical trial registration is a voluntary undertaking. However, the International Committee of Medical Journal Editors member journals now require, as a

consideration for publication, registration in a public trials registry. This policy applied to all clinical trials after 1st July 2005, and trial registration must be undertaken before the first patient is recruited. Clinical trial registration reduces the likelihood of selective reporting, more of a concern in commercially funded trials. The clinical trial discussed in this thesis commenced as a non-commercial study and latterly some of the secondary exploratory endpoints were only measured through some commercial funding.

There are various clinical trial registries. The macrolides in asthma study was registered at clinicaltrials.gov, an American based but international registry of clinical trials operated by the United States National Institutes of Health, National Library of Medicine.

The macrolides in asthma study was registered on the 26th February 2009 and was given registration number:

NCT00852579.

3.6.5.6.2. Confidentiality

Patient confidentiality is ensured by statute – Data protection act [1998] and all trial investigators had a responsibility to adhere to this act and ensure patient confidentiality was maintained throughout the study and thereafter during the archiving of data. Confidentiality is also covered by the Clinical Trials Directive 2001/20/EC "Good Clinical Practice" [GCP] guidelines.

3.6.5.6.3. Monitoring of the study

Monitoring of the study is the responsibility of the sponsor. An initiation visit was performed shortly after the first patient was screened, thereafter a mid-point visit and finally a close-out visit once the study has closed. Monitors from Research & Development attended the unit performing a 10% sample check of all CRF data entered and verified adherence to the protocol. The Principal Investigator and research team

3.7. Location of work

The clinical visits for this study were performed at two sites. The main site was The Asthma and COPD Research Centre, Gartnavel General Hospital, Glasgow with the secondary site being the pulmonary function lab at Crosshouse Hospital, Kilmarnock. Sputum and blood analysis were performed in the Graham Davies Building, University of Glasgow. Statistical analysis was performed in association with the Robertson Centre for Biostatistics, University of Glasgow.

4. Methods

4.1. Asthma control and quality of life

4.1.1. Assessment of asthma control

4.1.1.1. Asthma Control Questionnaire [ACQ]

The Junipers Asthma Control Questionnaire is a simple, reproducible, and sensitive questionnaire that was developed to allow a rapid assessment of asthma control in all severities of asthma and will demonstrate the impact of asthma treatment in interventions [208].

The score derived by the questionnaire is based on a series of seven questions. The first six questions cover symptoms the respondent has experienced in the past week. These include: night time wakening; limitation of normal daily activities; early morning wakening; dyspnoea and wheeze; and frequency of use of inhaled β_2 agonist. Each question is answered by the respondent selecting one choice from six. The severity of choices ranges from responses which signal no symptoms or none to a maximum severity for that particular symptom. The final question is completed by the clinic staff using the respondent's FEV_1 result from spirometry performed on the day of the assessment. A copy of this can be found in Appendix 1.

The ACQ is performed at each visit from the screening visit onwards. It was not performed if the subject underwent a pre-screening visit or optional safety visit during the run-in phase.

The respondents score is calculated by deriving the arithmetic mean of the 7 fields, with a score of 0 resulting if maximum asthma control is achieved and 6 if completely uncontrolled. Recent research suggests that a minimally significant difference of 0.5

[10] is clinically significant for altered control. A score of less than 0.75 is indicative of good asthma control, with a score above 1.5 indicating inadequate asthma control [211]. A copy of the ACQ can be found in Appendix 1.

4.1.1.2. Asthma Quality of Life Score [AQLQ]

Quality of life measurement was performed using the Juniper Asthma Quality of Life Questionnaire [12]. The AQLQ was recorded before and after treatment [baseline and 12 weeks respectively]. A copy of this is in Appendix 2.

4.1.1.3. Leicester Cough Questionnaire [LCQ]

Cough is not just a symptom exclusively related to asthma. The LCQ can be used generically to measure the response to treatment of any condition which might generate cough as a clinical symptom. The Leicester Cough Questionnaire is a self-completed, health related quality of life measure of chronic cough. It has a 19 items which are sub-divided in to 3 domains: physical; psychological and social. The minimally important clinical difference for the total score is 1.3, with each domain having an MICD of 0.2, physical; 0.2 social; and 0.8, psychological [212].

4.1.2. Diary card recordings

A validated diary card [213] was used to measure asthma symptoms, PEF recordings and inhaled beta$_2$-agonist use. PEF measurements were undertaken by patients at home using a PIKO-1 electronic peak flow meter [nSpire Health, Hertford UK]. The Piko-1 meter has been demonstrated to be comparable to the pneumotachograph [214]. On return visits the electronic diary was downloaded and analysed for exacerbations and measurement compliance [215]. The best of three measurements was recorded in the diary twice daily [am/pm] prior to treatment with salbutamol. A copy of the diary can be found in Appendix 4.

Home monitoring of peak flow in clinical trials is frequently undertaken for a variety of reasons. The most important of these is to alert the subject to the possibility of an exacerbation having developed. This is particularly useful if the subject's baseline inhaled therapy is being altered. In addition, it gives a more realistic view of the patient's clinical status than a single isolated measure of either FEV_1 or PEF in the clinic setting. This too is not without its caveats, as frequently patient effort can be reduced in the private setting of their home than when they are being prompted to perform maximal efforts by a member of the study team. Nonetheless, daily peak flow measurement provides a better impression of the diurnal variation in airway calibre and hence evidence of day-to-day fluctuation in the patient's clinical status [216].

4.2. Assessment of airway inflammation

4.2.1. Non-Invasive investigation

4.2.1.1. Induced sputum

Sputum induction was performed using a widely accepted method [64], and was performed following pre-treatment with 2.5mg nebulised salbutamol. Subjects were initially asked to inhale nebulised 3% saline [Stockport Pharmaceuticals, Stockport] using an ultrasonic handheld nebuliser [Sonix 2000, Medic Ltd, Harlow, Essex, UK]. Inhalation continued for a total of 7 minutes with the opportunity during this period for the subject to expectorate into a polypropylene container. After completion of this phase, time was allowed for further expectoration and spirometry checks. If lung function remained within safety parameters, further concentrations of 4 and 5% saline were administered, each for 7 minutes and with spirometry monitoring between doses.

The test was not performed if FEV_1 was less than 1L. If FEV_1 fell by 10-19% from post-salbutamol the concentration of saline was not increased. If FEV_1 fell by >20% or the patient felt unable to continue [e.g. due to nausea] the test was discontinued.

The sample was stored on ice until laboratory processing for cell counts and centrifugation to harvest the soluble phase could be performed. For optimum cytology this was always done within 2 hours [217]. Cell counts were performed and in addition sputum supernatant fluid was analysed for leukotriene [LT]B_4 and myeloperoxidase [MPO] using EIA [LTB_4 from R&D Systems, Abingdon, UK, MPO from Cambridge Bioscience, Cambridge, UK] and interleukin [IL]-1β, 2, 4, 5, 6, 8, 10, tumour necrosis factor [TNF]-α, granulocyte-macrophage colony stimulating factor [GM-CSF] and interferon [IFN]-γ using a Luminex microbead fluorescence kit [Biosource, Invitrogen, Paisley, UK].

4.2.1.2. Sputum sample preparation

Induced sputum samples were processed using a sputum-plug-selection method as previously described [218]. Mucus plugs within the expectorate were selected using forceps and placed in a pre-weighed tube and then re-weighed to estimate volume. Sputum plugs were then dispersed by addition of a 1:10 dilution dithiothreitol [DTT, Calbiochem, Merck Biosciences Ltd, Beeston] in phosphate-buffered saline [PBS, VWR International Ltd, Poole], the amount of DTT being 4x the weight of sputum. The volume was increased again by the addition of PBS, at 4x the original weight of sputum. The sample is then filtered through a nylon [nitex] mesh to remove clumps. After centrifucation at 1200rpm for 5 minutes the pellet is resuspended in 1ml of culture medium and a 20µl aliquot is removed and diluted 1:1 with 0.1% Trypan blue [Sigma-Aldrich, Gillingham]. A manual total cell count and a viability count were

then performed using a haemocytometer. Samples were assessed against quality indicators and were excluded if they failed to meet these – Table 4.1.

Table 4.1: Sputum quality indicators

Parameter	Threshold for exclusion
Cell viability	<40%
Total cells to be counted	<400
Proportion of squamous cells	>80%

4.2.1.2.1. Quality control for sputum cytokine analysis

The validity of sputum cytokine analysis is assessed using technique known as "spiking" [86]. This is a form of quality control where a known amount of mediator to unprocessed sputum, processing the sputum as usuaul and then measuring recovery by immunoassay. Unspiked sputum is simultaneously processed and assayed so that percentage recovery can be calculated. This is an expensive technique and hence is not possible to run spiking experiments for all the mediators in this study. We have done this work previously and have shown good levels of recovery [Appendix 7: Cytokine analysis quality control consistent with those described in other centres [86]

4.2.1.3. Differential Cell Counting

The cell pellet was resuspended at a concentration of 0.6 $x10^6$/ml in culture medium which was kept on ice. 75µl was pipette in to each cyto-funnel to obtain 7 x 10^4/ml and inserted in to a cytospin holder. 2 slides [VWR International Ltd, Poole] were prepared for cytospins using filter cards, funnels and cytoclips [Thermo Electron Corporation, Basingstoke] as per manufacturer's instructions. These were centrifuged at 450 rpm for 6 minutes [Shandon Cytospin 4 Centrifuge, Thermo Electron Corp.], and then air dried for 1-2 hours. Samples were then fixed in

methanol for 10 minutes. Fixed slides were then stained using Romanowsky staining – Rapi Diff II Stain Pack [Triangle Biomedical Sciences Ltd, Skelmersdale].

A differential cell count of greater than 400 inflammatory cells was performed. Squamous epithelial cells were counted in addition to this in order to estimate contamination from saliva.

4.2.1.4. Measurement of exhaled Nitric Oxide [eNO]

In this study F_ENO was measured using a Niox Flexflow Analyser [Aerocrine AB, Sundbybergsvagen 9, SE-171 73 Solna, Sweden]. This analyser meets joint ATS/ERS criteria for the measurement of on-line F_ENO [219]. F_ENO present in exhaled air is measured using a chemiluminescence technique where ozone generated within the analyser reacts with NO to produce NO_2. This reaction leads to a change in the energy state of the electrons from low energy to higher energy. The movement of the electrons between different orbitals [from high to low as the energy is released] emits electromagnetic radiation between the wavelengths of 600 and 3000nm. A linear relationship exists between Nitric Oxide present and the amount of light emitted and so once passed through a photomultiplier a derived value can be obtained for the F_ENO [220].

Measurements were performed to include extended flow rates [30, 50, 100, 150, 200, 250 & 300ml sec^{-1}]. The Niox-Flex has a published measuring range of 0-200 ppb, a detection limit of 1 ppb, a sampling frequency of 20Hz, a response time of <1.5 seconds and an accuracy of +/- 2.5 ppb for levels < 50 ppb and +/- 5% of values >50 ppb. Calibration was carried out every two weeks or as required when the machine instructed the user to do so.

Adherence to strict measurement techniques can prevent contamination of the exhaled air from the lungs. Nose clips should not be worn as these can affect closure of the soft palate. F_ENO is also best measured before other airways tests as repeated spirometric manoeuvres can lower the NO [221, 222]. Subjects received instruction on how to perform the test by the study doctor or nurse. Tests were then preformed following consensus guidelines [219]. Subjects are required to take a deep inhalation through the mouthpiece of the machine which is connected to a filter [scrubber] to ensure removal of any ambient nitric oxide. Exhalation continues without removal of the mouthpiece and the Niox-Flex will begin the measurement. A visual feedback mechanism is employed by the machine to inform the subject and aid provision of a constant flow rate and provide sufficient pressure above that required for closure of the velum, reducing nasal contamination of the exhaled breath. The Niox-Flex automatically calculates nitric oxide output [V_{NO}] and exhaled nitric oxide concentration, discarding measurements inconsistent with previous results at that flow rate and which did not demonstrate a plateau.

4.3. Asthma specific measurements

4.3.1. Spirometry [FEV_1, FVC and reversibility testing]

Baseline pre-bronchodilator spirometry was performed using a dry wedge spirometer [Vitalograph, Buckingham, UK], with the best of three measurements being recorded. All tests were performed following the appropriate recommendations in consensus guidelines [223]. Measurements were deemed acceptable if they were reproducible to within 5% or 0.15L. Spirometric measurements were performed before and after nebulised salbutamol [2.5mg]. Salbutamol was delivered via a compressed air nebuliser [Pari Boy, PARI, VA, USA] for 5 minutes, following suitable baseline recordings. Subjects then performed spirometry from 15mins post completion of the

salbutamol inhalation. Measurements were performed as before to the same standards and following consensus guidelines [223]. Reversible airflow obstruction was defined as an improvement in FEV_1 of ≤12% [and greater than 200ml in absolute volume] and was calculated using the formula:

$$\%\boldsymbol{reversibility} = \left[\frac{\boldsymbol{Post\ BD\ FEV_1 - Pre\ BD\ FEV_1}}{\boldsymbol{Pre\ BD\ FEV_1}}\right] \times \mathbf{100}$$

4.3.2. Asthma severity

4.3.2.1. GINA Severity Classification

Conventional assessments of asthma severity have combined assessments of symptoms, amounts of β_2-agonist used to treat symptoms and lung function [224]. Asthma classification based on severity criteria can aid decision making about management at the initial assessment of the patient. This is because asthma therapy involves a stepwise approach in which the level of therapy is increased as the severity of the asthma increases.

In this study, asthma severity was based not only on the classifications of GINA but also in combination with the level of treatment the patient was receiving at the time of screening. The subject was first questioned about their current asthma therapy.

Therapy was categorised as follows

- Intermittent – salbutamol alone
- Mild [low ICS or theophylline or LTA]
- Moderate [low/med ICS+LABA or high dose ICS or med ICS + theo/LTA/oral salbutamol]
- Severe [high ICS+LABA ± other tablets]

Following this, the level of symptoms the patient experience whilst receiving this treatment was explored. Categorising symptoms was based on sub-divisions in Table 4.2.

Table 4.2: Classification of Asthma Severity by Clinical Features

Step 1: Intermittent
Symptoms less than once a week Brief exacerbations Nocturnal symptoms not more than twice a month • FEV_1 or PEF ≥ 80 % predicted • PEF or FEV_1 variability < 20 %
Step 2: Mild persistent
Symptoms more than once a week but less than once a day Exacerbations may affect activity and sleep Nocturnal symptoms more than twice a month • FEV_1 or PEF ≥ 80 % predicted • FEV_1 or PEF variability 20-30%
Step 3: Moderate persistent
Symptoms daily Exacerbations may affect activity and sleep Nocturnal symptoms more than once a week Daily use of inhaled short-acting β_2-agonist • FEV_1 or PEF 60-80% predicted • PEF or FEV_1 variability >30%
Step 4: Severe persistent
Symptoms daily Frequent exacerbations Frequent nocturnal asthma symptoms • FEV_1 or PEF ≤60% predicted • PEF or FEV_1 variability >30%

If the patient's symptoms were controlled on their current therapy they remained within the symptom category in Table 4.2, above. If however their symptoms persisted despite their current treatment they would be moved up a category. Subjects already in the severe persistent group would remain in that group. A copy of the questionnaire can be found in Appendix 5. The GINA severity classification is now only recommended for research purposes [224].

4.3.2.2. GINA Asthma Control

There is the understanding that asthma severity involves not only the severity of the underlying disease but also its responsiveness to treatment, and that severity can be a variable feature of an individual patient's asthma but may change over time. GINA have developed a system for categorizing asthma control, although this is based on current opinion and has not been formally validated [224].

Table 4.3: Global Initiative for Asthma [GINA] definitions of asthma control

Characteristic	Controlled	Partly controlled	Uncontrolled
Daytime symptoms	None [twice or less/week]	More than twice per week	Three or more features of partly controlled asthma present in any week
Limitations of activities	None	Any	
Nocturnal symptoms/awakenings	None	Any	
Need for reliever/rescue treatment	None [twice of less per week]	More than twice per week	
Lung function PEF or FEV_1	Normal	<80% predicted or personal best [if known]	
Exacerbations	None	One or more per year	One in any week

[224]

4.3.3. Exacerbations of asthma and Adverse Events

Asthma is a heterogeneous disease and as a consequence asthma exacerbations can be heterogeneous in nature. The difficulty in providing an accurate and encompassing definition for asthma is mirrored in the difficulty that has arisen in providing an acceptable definition of exacerbation that has relevance for every patient. A joint ATS/ERS Task Force attempted to clarify this, and noted the difficulty in doing so [9]. It was observed that for severe exacerbations no two studies had the same definition [9]. Most studies regard severe exacerbation as those requiring either systemic corticosteroid or hospitalisation [including emergency department admission] [9] and moderate exacerbations as those leading to increasing clinical symptoms from baseline for 2 consecutive days [9]. Classification of moderate exacerbation is

unfortunately not based on any validated criteria. Monitoring PEFR is an important safety aspect for clinical studies and is generally a requirement for ethics. A PEFR drop of >30% is a useful measure for subjects to monitor and seems reasonable based on the Task Force guidance [9]. A mild exacerbation is even more difficult to define as this could simply be normal variation in the patient's symptoms and representative of loss of control as opposed to the development of an exacerbation episode. For the purposes of the study, exacerbations of asthma were defined by the criteria outlined in Table 4.4.

Table 4.4: Criteria for reporting asthma exacerbation

Severity	Criteria
Severe	Oral steroid use for at least 3 days [course separated by 1 week or more should be treated as separate exacerbations
	Hospitalisation because of asthma requiring oral steroids
	Emergency department visit because of asthma requiring oral steroids
Moderate	One or more of the following for 2 consecutive days
	Drop in peak flow>30% baseline value, or
	Night awakening due to asthma [more than the individual considers normal]
Mild	Increased asthma symptoms
	Drop in peak flow >20% baseline value

Table derived from [225]

4.3.4. Airways responsiveness – Methacholine hyper-reactivity

Airway responsiveness is an objective, well standardised measure of variable airflow limitation and is accepted as diagnostic of the condition. For patients with symptoms consistent with asthma but normal lung function a positive challenge test to methacholine may help establish the diagnosis with reasonable certainty [2, 226]. Methacholine PC_{20} is frequent employed within clinical trials as an endpoint. Broncho-provocation testing is performed using a calibrated nebuliser [Airlife® Sidestream high efficiency nebuliser] to supply an output of 0.13ml/min of solution per minute in serial doubling doses using the tidal breathing method in 2 minute

intervals. As a safety aspect, patients undergoing broncho-provocation testing were required to have a baseline FEV_1 >60% predicted. Patients were also required to abstain from using their normal asthma medication, anti-histamines and caffeine containing drinks as per recommendations [5] and Table 4.5. Female subjects were required to have a pregnancy test and methacholine broncho-provocation was only performed if not pregnant. Methacholine was provided by the Western Infirmary & Gartnavel General Hospital Sterile Production Unit and Stockport Pharmaceuticals, Stockport Hospital NHS trust.

Table 4.5: Factors that decrease bronchial responsiveness

Medication	Duration of withdrawal
Short acting β2 agonist	8 hours
Long acting β2 agonist	48 hours
Anti-histamines	72 hours
Caffeine containing food/drinks [chocolate/tea/coffee/soft drinks]	Day of study

Adapted from [5]

Baseline spirometry was performed on all subjects before receiving any form of bronchial challenge. Nebulised saline was then delivered for 2 minutes followed by measurement of FEV_1 at 30, 90 and 180 seconds. The highest post-saline FEV_1 is used to calculate the target drop of 20%. The subject begins by inhalation of 0.03125mg/ml of methacholine for 2 minutes [3ml of solution placed in nebuliser] followed by sequential measurement of FEV_1 as outlined. If the FEV_1 stays within 20% of baseline [highest post-saline], the test is continued by administering a sequence of doubling concentrations of methacholine – 0.0625mg/ml, 0.125mg/ml, 0.25mg/ml to 16mg/ml at which point the test is discontinued. The target drop is identified when the FEV_1 has dropped beneath the target on two measurements within a single phase. If the target

drop occurs before all concentrations have been administered the test is stopped. At this point the patient is nebulised with 2.5mg of salbutamol.

The provocation concentration of methacholine required to produce a drop in FEV_1 of 20% [PC_{20}] for each subject was calculated by interpolation.

Figure 4.1: Methacholine calculation

$$PC_{20} = antilog\left[\log C_1 + \frac{(\log C_2 - \log C_1)(20 - R_1)}{R_2 - R_1}\right]$$

C_1 = second to last methacholine concentration; C_2 = last methacholine calculation; R_1 = %age fall in FEV_1 after C1; R_2 = %age fall in FEV_1 after C_2 [227]

A provocation concentration of <8mg/ml in the context of the appropriate clinical history and good quality spirometric efforts was considered to confirm the diagnosis of asthma and eligibility for the clinical trial.

4.4.Measurement of atopy

Serum samples were taken from each patient and analysed. Total IgE was measured and in addition specific IgE for house dust mite, cat dander, grass pollen, aspergillus and specific IgG for aspergillus. Atopy was defined as having IgE antibody against any of the common inhalant allergens, house dust mite, cat dander or grass pollen. The assay is an automated fluorescent immunoassay [UniCAP 100, Pharmacia UK Ltd, Milton Keynes, UK]. Total IgE >120 kilo-International Units/L [kIU/L], and specific IgE>0.35 kA[arbitrary]U/L were considered positive. Grass pollen serology was reported as either positive or negative with no specific value reported.

Specific IgE antibodies within the subject's serum binds to the allergen or antigen of interest, which in turn is bound to a flexible cellulose matrix. This is then washed to remove any unbound antibody and a second detection antibody that recognises bound human IgE ie anti-IgE antibody-beta-galactosidase conjugate is then added. If the subject has specific IgE remaining this antibody conjugate will then bind. The activity of the bound enzyme is then measured by the addition of a colourless substrate which is metabolised to a fluorescent product [umbelliferone] and thus can be measured. The fluorescence is produced is directly proportional to the presence of IgE and can be quantified to a standard curve. The assay has a working range of 0.35 – 100 arbitrary units [kU/L].

4.5. Blood lymphocyte proliferative response in vitro

Peripheral blood mononuclear cells are readily separated by centrifugation [1800 rpm for 20min] on a Ficol gradient [lymphoprep, specific gravity SG1.088]. The mononuclear cell fraction which collects between the plasma ficol interface is harvested, washed with HBSS and cultured at 10^6cells/ml in-vitro in complete RMPI media, 10% autologous plasma, L-glutamine and with antibiotic [penicillin, streptomycin] cover for 3 days with various additives in a humidified 5% CO_2 atmosphere for 3 days.

To test a functional proliferation response the lymphocytes were cultured with the mitogen phytohaemagglutinin PHA at 8mg/ml. The lectin non-specifically binds and cross-links CD3 T-cell receptor thus activating the cell. This activation was measured by the incorporation of tritiated thymidine added 16 hours before harvesting automatically by washing on glass fibre filters then counting in a beta counter. The purpose of the assay was to assess the immune-competence in response to mitogen [as a surrogate for antigen] and to test if azithromycin had altered this response.

One important purpose of this assay is its potential value as an *in-vitro* means of exploring the mechanisms of glucocorticoid refractory immune responses. This was done by adding varying concentrations of the immune-suppressive dexamethasone to determine if there was any alteration in response to this exogenous steroid by azithromycin. Appropriate controls were included in all assays.

4.6. Other immunological tests in blood

Serum was analysed for C-Reactive Protein [CRP] using a high sensitivity [hs]-CRP assay by enzyme immune-assay [EIA] [R&D Systems] and IL-1β, 2, 4, 5, 6, 8, 10, TNF-α and GM-CSF [using a multiplex fluorescence bead kit, Biosource] and a Luminex platform [Biorad].

4.7. Measurement of renal and liver safety biomarkers in blood

Blood was taken to measure serum markers of renal and liver function at baseline, 8 and 12 weeks. Subjects with significantly abnormal renal function [urea or creatinine >2 times ULN] or abnormal liver function [ALT or AST >2 times ULN] at baseline were excluded from the study. Abnormal results during the study [8 weeks] led to repeat specimens being sent urgently in the first instance and if confirmed, a decision was made on continuance of the subject within the trial. Subjects with abnormal results at 12 weeks had these repeated in the first instance. In all cases clinical assessment would be made to define a cause and appropriate onward referrals carried out if necessary.

4.8. Virological analysis of induced sputum and blood

Virological analysis of the sputum was done using a panel of primers for upper airway viruses as well as for *M. pneumonia* [Superscript III Platinum One Step

Quantitative RT-PCR System, Invitrogen, with appropriate primer and probe mix[228, 229]] and *C. pneumonia* [Platinum Quantitative PCR Supermix, Invitrogen, with CP primer[230]]. Serological analysis was also performed for evidence of antibody response to each organism [CP, Medac GmbH, Hamburg, Germany; MP, Sekisui Virotech GmbH, Russelsheim, Germany].

Subjects would be categorised as carriers of *C pneumonia* if both IgA [IgA antibodies appear early in infection and then persist] and IgG [IgG antibodies develop within 2 to 3 weeks of infection] were positive. Confirmation of the presence of *M pneumonia* is dependent upon there being the development of IgM antibodies. Serological results would be supported by PCR.

4.9. Bacteriological analysis of induced sputum

Simple quantitative bacteriology is a crude and convenient method that is regarded as an acceptable technique and results from it are regularly published following peer-review [176, 231-234]. The technique does not guarantee to collect a sample free of contamination from elsewhere in the upper airway – the samples must traverse the oropharynx during expectoration and so there is the possibility of contamination with the flora or the oropharyngeal mucosa. There is no certainty that any growth truly reflects the bacterial burden as individual isolates of bacteria may not grow particularly well in culture. Identification of individual bacteria is not always possible as there could be overgrowth from non-pathogenic organisms leading to crowding on the plate. The count can sometimes only be estimated, with results reported within a range estimate. Finally it can be difficult to identify some organisms e.g. *S. pneumoniae* particularly if this is in low numbers given its similarity colonies of commensal α-streptococci.

0.2ml of homogenised sputum was delivered to the routine service bacteriology, Yorkhill Royal Hospital for Sick Children, Glasgow, in a sterile container. 10µl of the homogenate was inoculated onto blood agar+optochin disc, chocolate agar, CLED agar and Sabouraud agar and spread with a loop for discrete colonies. 100µl of the homogenate was then added to 5ml sterile saline and mixed, making a 1:100 dilution. 50µl of the 1:100 dilution was then added to 5ml sterile saline, giving a final dilution of 10^{-4}. A 20µl loop of the 10^{-4} dilution was inoculated onto a blood and chocolate agar and spread over the entire surface with the loop. This was followed by incubation of the blood and chocolate agars at 37C in 5% CO_2, the CLED and Sabouraud agars at 37C.

The number of colony forming units per gram of sputum was enumerated from the total number of colonies obtained and the dilution [10^{-4}] to give the total bacterial count for each sample expressed in colony forming units [cfu]/ml.

Table 4.6: Interpretation of the 10^{-4} dilution cultures

Number of colonies	Count per ml
0-2	10^6 cfu/ml
2-20	10^7 cfu/ml
20-200	10^8 cfu/ml
>200	10^9 cfu/ml

Only significant pathogens were to be counted and followed up with the appropriate identification and sensitivity. Normal respiratory flora was recorded as normal.

4.10. Equivalent steroid dose

Beclometasone dipropionate [BDP] and budesonide are approximately therapeutically equivalent in clinical practice, although there may be variations with different devices. Mometasone and fluticasone appear to provide equal clinical activity to BDP and budesonide at half the dosage [1, 2]. Subjects who screened, and were on high dose ICS or combination ICS/LABA, required weaning to the standard run-in dose of 200mcg budesonide±LABA. Relative potency of ICS compared to BDP/beclometasone can be found in Table 4.7

Table 4.7: Relative potency of inhaled corticosteroids

Inhaled corticosteroid	Relative potency
Budesonide	1
Beclometasone dipropionate	1
Fluticasone	2
QVAR®	2
Mometasone	2

4.11. Pre-specified and *post-hoc* analysis

All of the aforementioned outcome measures were included in the original protocol and analysed as a pre-specified outcome measure. Any additional measurement and analysis not included above was performed as a *post-hoc* analysis.

5. Clinical results

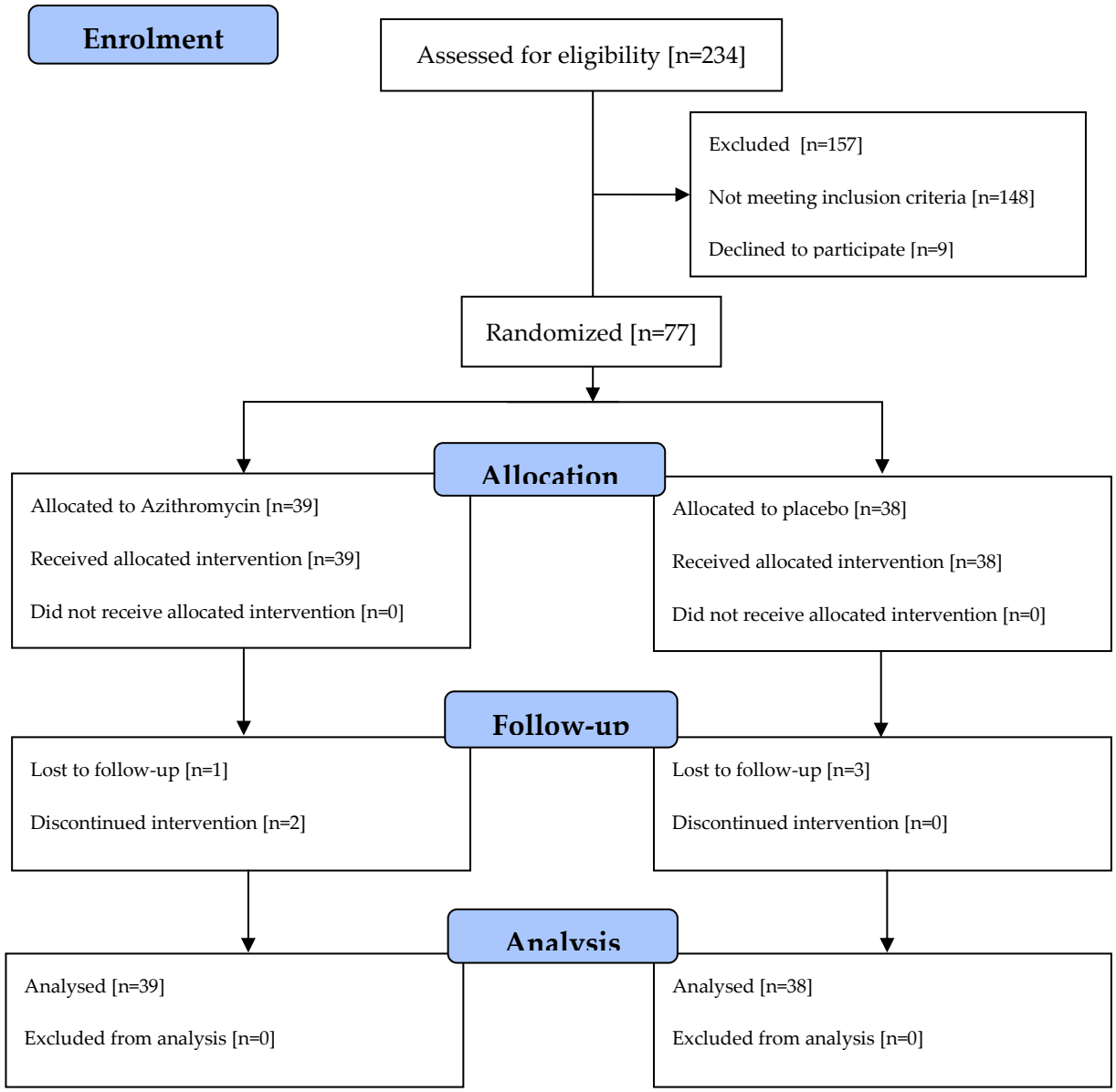
5.1. Introduction

A total of 80 GP practices were visited over the 2 year period from commencement of the study. 8398 approach letters were generated and mailed to prospective participants. 715 positive responses were received, in the form of a returned acceptance slip or a telephone call leading to 705 being further assessed for eligibility. Prospective participants were "pre-screened" at the point of invitation to attend for a screening appointment, with 347 not meeting the entrance criteria. 23 people were unwilling to take part following explanation of the study and 101 did-not-attend for their initial screening appointment.

5.2. Flow of participants

Of the 715 replies received, screening visits were arranged for 234 and 77 were randomised. Baseline demographic data and clinical characteristics of the patients are shown in Table 5.2, and the participant flow can be found in Figure 5.1.

Figure 5.1: CONSORT Flow Diagram



A complete breakdown of the reasons for screening failure can be found in Table 5.1: Breakdown of exclusions of screened patients from the study.

Table 5.1: Breakdown of exclusions of screened patients from the study

Reason for exclusion	Number of participants
Exacerbation of asthma	56
Did not attend for randomisation visit	37
ACQ<1	11
Withdrew consent	10
Prolonged QTc	7
No reversibility & methacholine negative	7
Unable to wean inhaler	4
Abnormal blood indices	4
Other	12
Total	**148**

5.3. Baseline characteristics

5.3.1. Demographics

Baseline demographic characteristics of the 77 subjects who underwent randomisation were similar between the two groups and are displayed in Table 5.2.

The mean ages were similar between the two groups. There were slight differences in men and women between the two groups; 21 females in the placebo group and 19 in the azithromycin group. There were 3 fewer men in the placebo group.

The duration of asthma was 24.6 years in the placebo group and 18.8 years in the azithromycin group. There was a slight difference in the mean number of pack years between the two groups with the azithromycin group having a greater number although the mean number of cigarettes was almost the same. The difference was not

of importance and was well over the minimum pack exposure [5 years] required for inclusion in the study.

For each of the specific measurements there were no significant differences in the baseline outcomes.

Table 5.2: Baseline demographics and subject characteristics

Variable	Placebo [n=39]†	Azithromycin [n=38]†
Age, years	42.84 [8.8]	46.44 [8.8]
Male sex, n [%]	17 [44.7%]	20 [51.3%]
Smoking history [pack years]	23.6 [15.8]	28.6 [16.4]
Duration of asthma symptoms, years	24.6 [12.6]	18.8 [12.5]
Atopic, n [%]	23 [60.1]	27 [69.2]
Total IgE [IU/ml] [Median [Q1, Q3]	103 [38, 291]	265 [48, 254]
Use of inhaled corticosteroid at screening, n [%]	31 [81.6%]	35 [89.7%]
Equivalent beclometasone dose at screening, µg	709 [564]	603 [457]
Use of LABA at randomisation, n [%]	18 [47.4%]	15 [38.5%]
Pre-bronchodilator FEV_1 [L]	2.54 [0.77]	2.43 [0.72]
Pre-bronchodilator FEV_1 % predicted	81 [16.8]	78.3 [16.4]
Post-bronchodilator FEV_1 % predicted	89.0 [15.1]	86.8 [15.2]
FEV_1 % reversibility	11.3 [9.8]	12.3 [10]
Geometric mean [range] PC_{20} methacholine [mg/ml]	1.06 [4.10]	1.07 [3.13]

Mean [SD] unless stated. †Number of randomized subjects with at least one post-baseline assessment of peak expiratory flow [PEF].

5.4. Clinical endpoint results

5.4.1. Primary outcome peak expiratory flow [PEF]

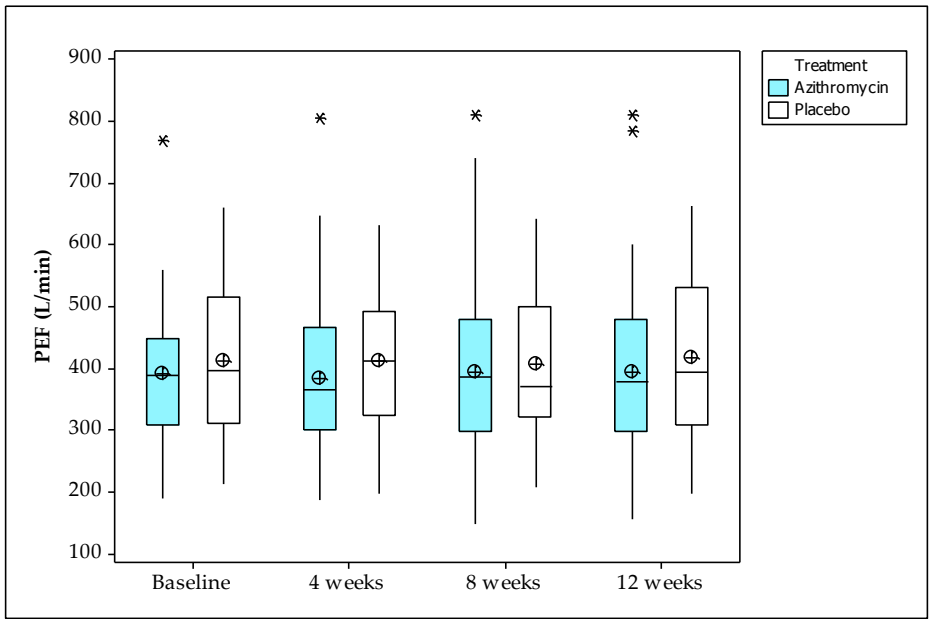
Baseline measurements between the two groups were well matched, the mean [SD] peak expiratory flow for all the randomised patients was 400.7 L/min [119.1] and when separated for the placebo group was 411.1 L/min [124.3] and the azithromycin group was 390.5 L/min [114.5].

The primary outcome was clinic visit PEF at 12 weeks. At 12 weeks, the mean difference in clinic visit PEF for azithromycin compared with placebo was not statistically different [mean difference -10.4 L/min 95% CI -47.1 to 26.4, p=0.58]. A detailed breakdown of these results can be found in Table 5.3. This table demonstrates there was no substantial difference between the azithromycin and placebo group at baseline and 12 weeks for the primary endpoint. The primary outcome results in Table 5.3 are presented graphically in Figure 5.2

Table 5.3: Change from baseline to 12 weeks in clinic visit PEF [L/min]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	400.7	119.1	38	411.1	124.3	39	390.5	114.5
Visit 5	71	405.3	140.2	35	416.7	122.7	36	394.7	156.3
Δ baseline to Visit 5		6.6	76.6		11.8	64.7		1.4	87.2
Mean Difference [95% CI]									-10.3 [-47.1 , 26.4], p=0.58

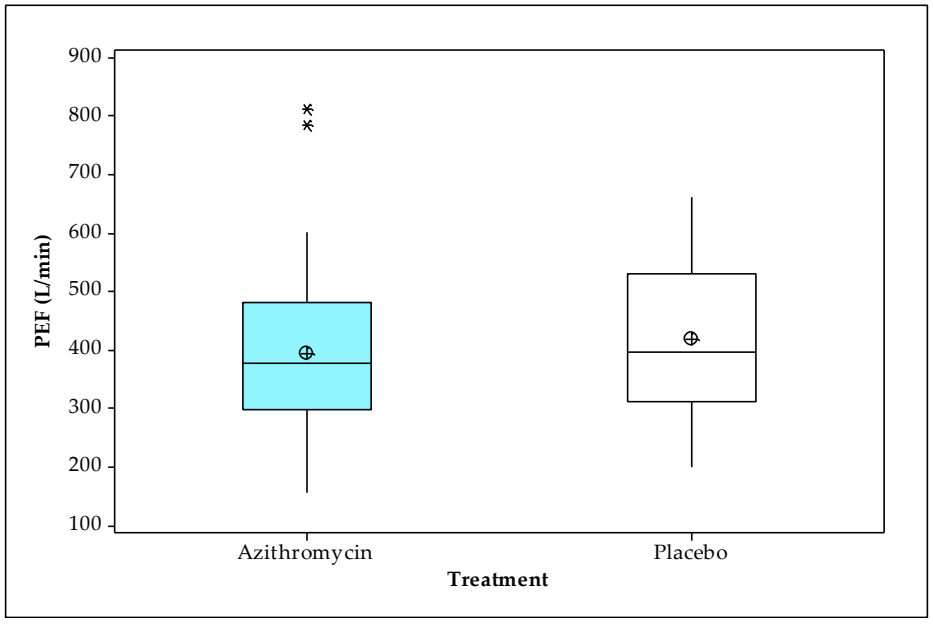
Figure 5.2: Box-whisker plot clinic visit PEF [L/min] by visit



No significant difference is seen between the treatment groups at 4 [p=0.75], 8 [p=0.94] or 12 weeks [p=0.58] Mean denoted by crossed-circle and outliers with asterisk.

The data collected at the final visit is displayed in an enlarged, more detailed form in Figure 5.3.

Figure 5.3: Box-whisker plot of clinic PEF [L/min] at 12 weeks by treatment group.



No significant difference is seen between the treatment groups [p=0.58] Mean denoted by crossed-circle and outliers with asterisk.

5.4.1.1. FEV_1

FEV_1 [pre and post-salbutamol] did not show any statistically significant difference after 12 weeks treatment with azithromycin. Pre-salbutamol FEV_1 treatment difference 0.03L [95%CI -0.08 to 0.14], p=0.62, with post-salbutamol FEV_1 treatment difference 0.04L [95%CI -0.08 to 0.17]. p=0.50. The baseline means [SD] for both pre and post-salbutamol FEV_1 measurements are displayed in Table 5.4, Table 5.5 and graphically in Figure 5.4.

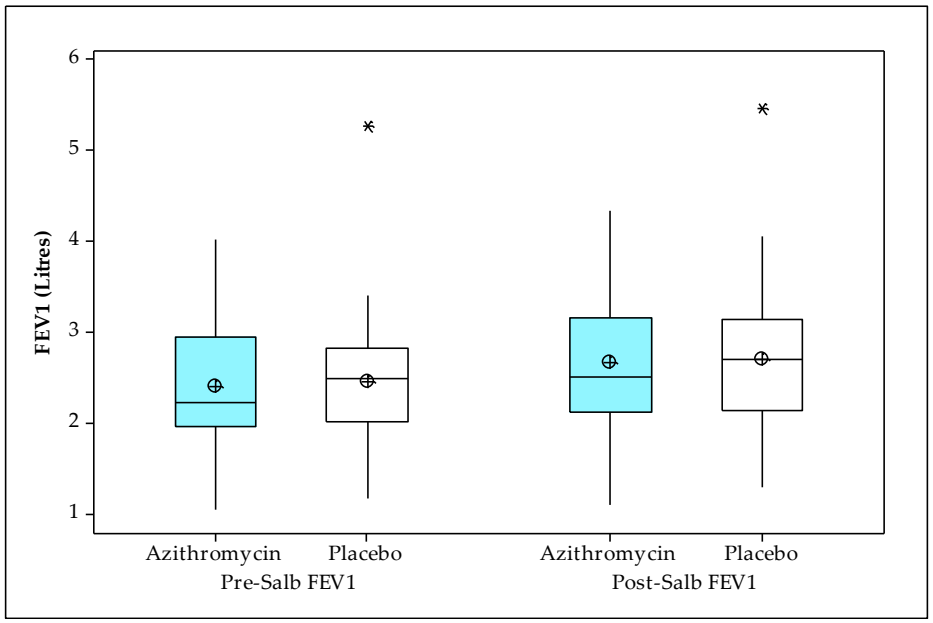
Table 5.4: Pre-salbutamol FEV_1 [L] at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	2.48	0.74	38	2.54	0.77	39	2.43	0.72
Visit 5	71	2.43	0.76	35	2.46	0.75	36	2.41	0.77
Δ baseline to Visit 5		-0.04	0.23		-0.06	0.24		-0.03	0.23
Mean Difference [95% CI]								0.03 [-0.08 , 0.14], p=0.62	

Table 5.5: Post-salbutamol FEV_1 [L] at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	2.74	0.76	38	2.8	0.8	39	2.68	0.73
Visit 5	71	2.66	0.73	35	2.73	0.72	36	2.59	0.74
Δ baseline to Visit 5		-0.07	0.26		-0.09	0.28		-0.04	0.25
Mean Difference [95% CI]								0.04 [-0.08 , 0.17], p=0.50	

Figure 5.4: Box-whisker plot of FEV_1 [L], [pre & post-salbutamol] at 12 weeks



No significant difference is seen between the treatment groups: Pre-salbutamol p=0.62; Post-salbutamol p=0.50. Mean denoted by crossed-circle and outliers with asterisk.

5.4.1.2. FVC

No effect was seen in either mean [SD] FVC either pre or post salbutamol following 12 weeks treatment with azithromycin. The mean difference in pre-salbutamol FVC was 0.01L [95%CI -0.13 to 0.15], p=0.89 with mean difference post-salbutamol FVC of 0.06L [95%CI -0.04 to 0.16], p=0.27. The details of these measurements are displayed in Table 5.6 and Table 5.7, with a graphical summary of the datasets in Figure 5.5.

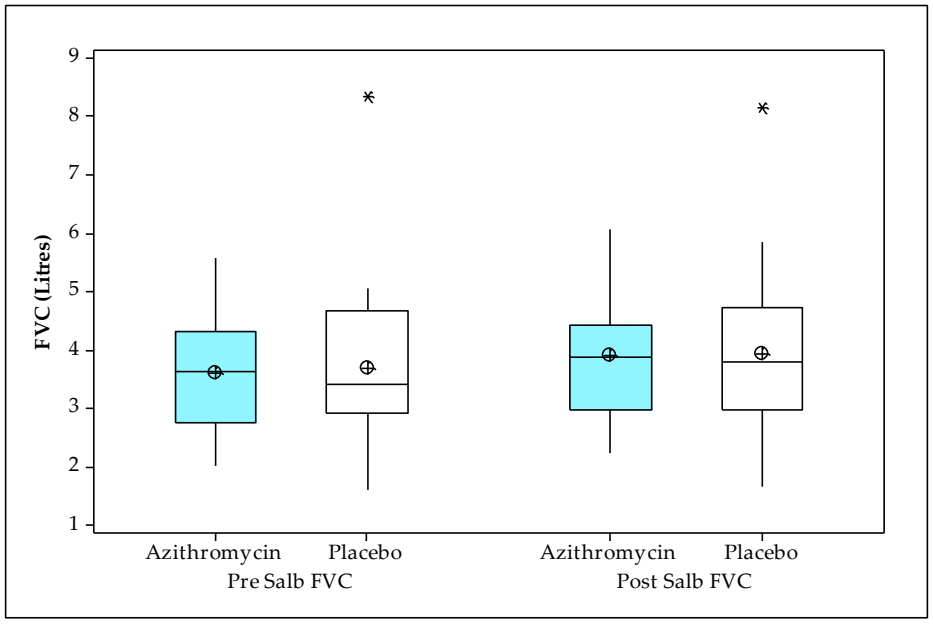
Table 5.6: Pre-salbutamol FVC [L] at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	3.65	1.06	38	3.72	1.16	39	3.58	0.97
Visit 5	71	3.64	1.12	35	3.68	1.26	36	3.6	0.98
Δ baseline to Visit 5		-0.02	0.29		-0.02	0.33		-0.02	0.24
Mean Difference [95% CI]									0.01 [-0.13 , 0.15], p=0.89

Table 5.7: Post-salbutamol FVC [L] at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	3.92	1.09	38	3.99	1.22	39	3.84	0.96
Visit 5	71	3.92	1.12	35	3.94	1.26	36	3.9	0.99
Δ baseline to Visit 5		-0.01	0.22		-0.04	0.25		0.02	0.18
Mean Difference [95% CI]									0.06 [-0.04 , 0.16], p=0.27

Figure 5.5: Box-whisker plot of FVC [L], [pre & post-salbutamol] at 12 weeks



No significant difference is seen between the treatment groups: Pre-salbutamol, p=0.89; Post-salbutamol p=0.27. Mean denoted by crossed-circle and outliers with asterisk.

5.4.1.3. FEF_{25-75}

No effect was seen in either mean [SD] FEF_{25-75} either pre or post salbutamol following 12 weeks treatment with azithromycin. The mean difference in pre-salbutamol FEF_{25-75} was 0.08L/sec [95%CI -0.07 to 0.24], p=0.28 with mean difference post-salbutamol FEF_{25-75} of -0.04L/sec [95%CI -0.21 to 0.12], p=0.60. The details of these measurements are displayed in Table 5.8 and Table 5.9, with a graphical summary of the datasets in Figure 5.6.

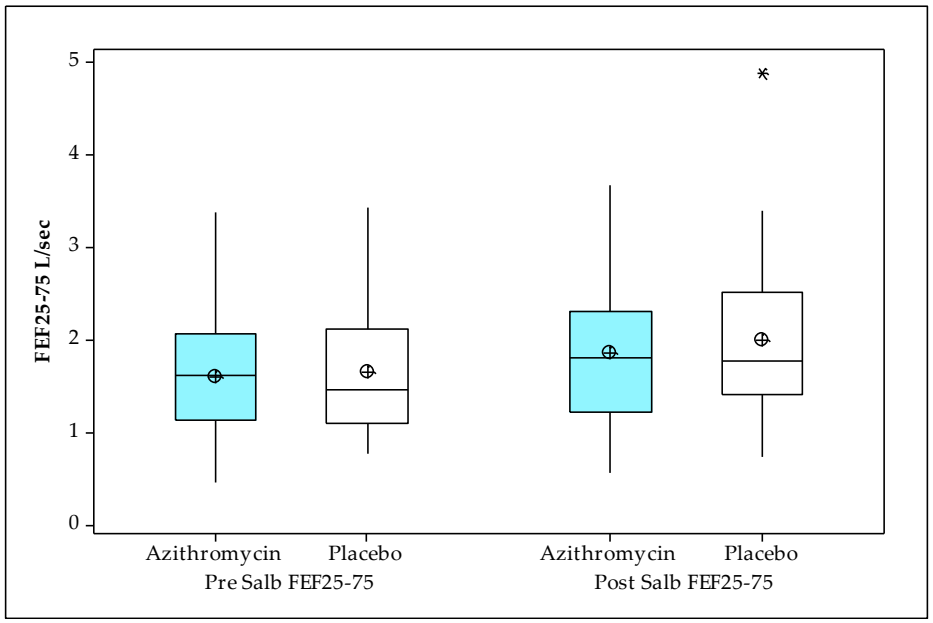
Table 5.8: Pre-salbutamol FEF_{25-75} [L/sec] at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	1.71	0.72	38	1.81	0.77	39	1.63	0.68
Visit 5	71	1.62	0.69	35	1.65	0.68	36	1.6	0.7
Δ baseline to Visit 5		-0.07	0.33		-0.11	0.36		-0.02	0.29
Mean Difference [95% CI]									0.08 [-0.07 , 0.24], p=0.28

Table 5.9: Post-salbutamol FEF_{25-75} [L/sec] at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	2.05	0.86	38	2.12	0.86	39	1.98	0.86
Visit 5	71	1.93	0.82	35	2.00	0.86	36	1.86	0.78
Δ baseline to Visit 5		-0.08	0.35		-0.06	0.44		-0.1	0.25
Mean Difference [95% CI]									-0.04 [-0.21 , 0.12], p=0.60

Figure 5.6: Box-whisker of FEF_{25-75} [L/sec], [pre & post-salbutamol] at 12 weeks



No significant difference is seen between the treatment groups: Pre-salbutamol, p=0.28; Post-salbutamol p=0.60. Mean denoted by crossed-circle and outliers with asterisk.

5.4.1.4. FEV_1/FVC ratio

No effect was seen in either mean [SD] FEV1/FVC ratio either pre or post salbutamol following 12 weeks treatment with azithromycin. The mean difference in pre-salbutamol FEV1/FVC ratio was -0.2% [95%CI -2.0 to 1.6], p=0.84 with mean difference post-salbutamol FEV1/FVC ratio of -0.1% [95%CI -1.6 to 1.4], p=0.86. The details of these measurements are displayed in Table 5.10 and Table 5.11, with a graphical summary of the datasets in Figure 5.7.

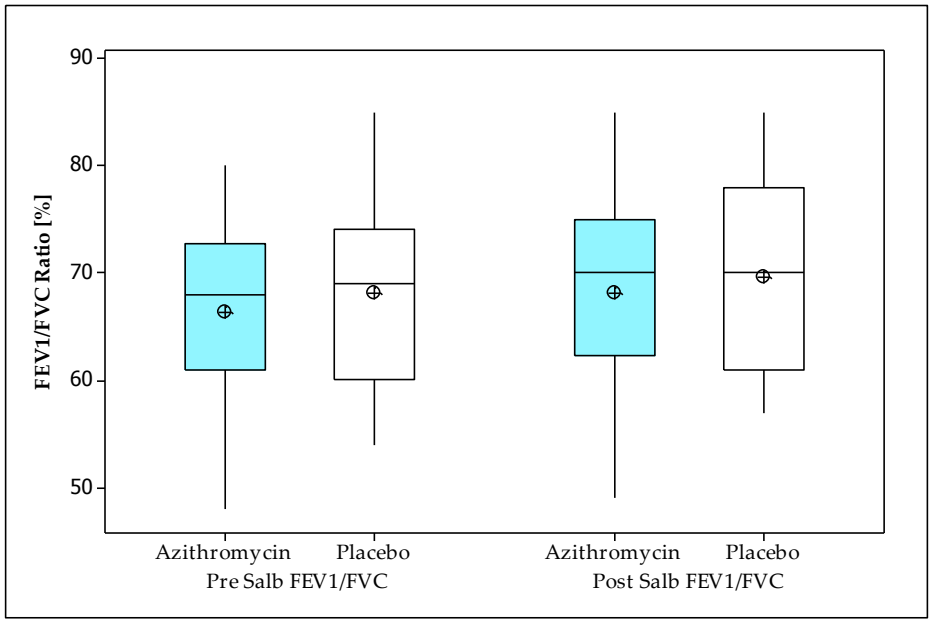
Table 5.10: Pre-salbutamol FEV_1/FVC ratio [%] at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	68.4	8.5	38	69.2	8.5	39	67.6	8.5
Visit 5	71	67.2	8.4	35	68.1	8.2	36	66.4	8.7
Δ baseline to Visit 5		-0.9	3.8		-0.8	4.0		-0.9	3.6
Mean Difference [95% CI]									-0.2 [-2.0 , 1.6], p=0.84

Table 5.11: Post-salbutamol FEV_1/FVC ratio [%] at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	70.5	8.6	38	71.1	8.6	39	69.9	8.7
Visit 5	71	68.9	8.6	35	69.6	8.5	36	68.1	8.8
Δ baseline to Visit 5		-1.3	3.1		-1.2	3.6		-1.3	2.6
Mean Difference [95% CI]									-0.1 [-1.6 , 1.4], p=0.86

Figure 5.7: Box-whisker of FEV_1/FVC ratio [%], [pre & post-salbutamol] at 12 weeks



No significant difference is seen between the treatment groups: Pre-salbutamol, p=0.84; Post-salbutamol p=0.86. Mean denoted by crossed-circle and outliers with asterisk.

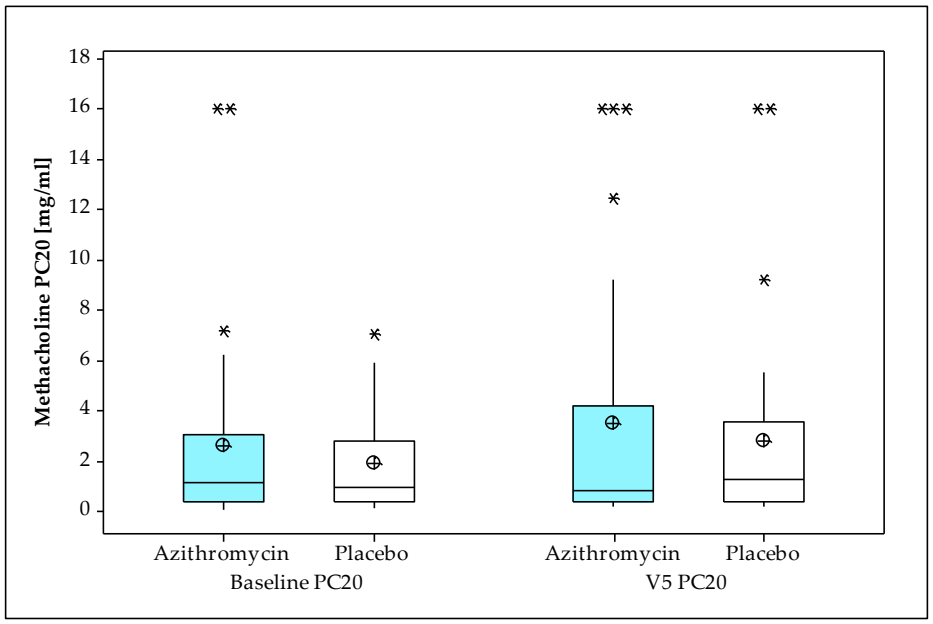
5.4.2. Methacholine responsiveness

No effect was seen on mean [SD] methacholine-PC_{20} following 12 weeks treatment with azithromycin. The mean difference was 0.08mg/ml [95%CI -1.8 to 1.97], p=0.93. The details of these measurements are displayed in Table 5.12, with a graphical summary of the dataset in Figure 5.8.

Table 5.12: Methacholine responsiveness PC_{20} [mg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	2.22	3.05	34	1.88	1.94	35	2.55	3.84
Visit 5	61	3.11	4.58	30	2.73	4.12	31	3.47	5.03
Δ baseline to Visit 5		0.87	3.6		0.86	3.63		0.87	3.63
Mean Difference [95% CI]								0.08 [-1.8 , 1.97], p=0.93	

Figure 5.8: Box-whisker plot of methacholine PC_{20} [mg/ml] responsiveness



No significant difference is seen between the treatment groups [p=0.93]
Mean denoted by crossed-circle and outliers with asterisk.

The geometric mean was calculated on the log transformed methacholine data in addition to the raw data, due to its distribution. This can be found in Table 5.13, but has not been represented graphically. Even with log-transformation there was no statistically significant difference [p=0.93], between the two groups.

Table 5.13: Log transformed methacholine responsiveness PC_{20}

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	0.06	1.28	34	0.07	1.14	35	0.06	1.41
Visit 5	61	0.19	1.4	30	0.19	1.29	31	0.2	1.52
Δ baseline to Visit 5		0.16	0.93		0.17	1.04		0.14	0.82
Mean Difference [95% CI]									-0.02 [-0.49 , 0.45], p=0.93

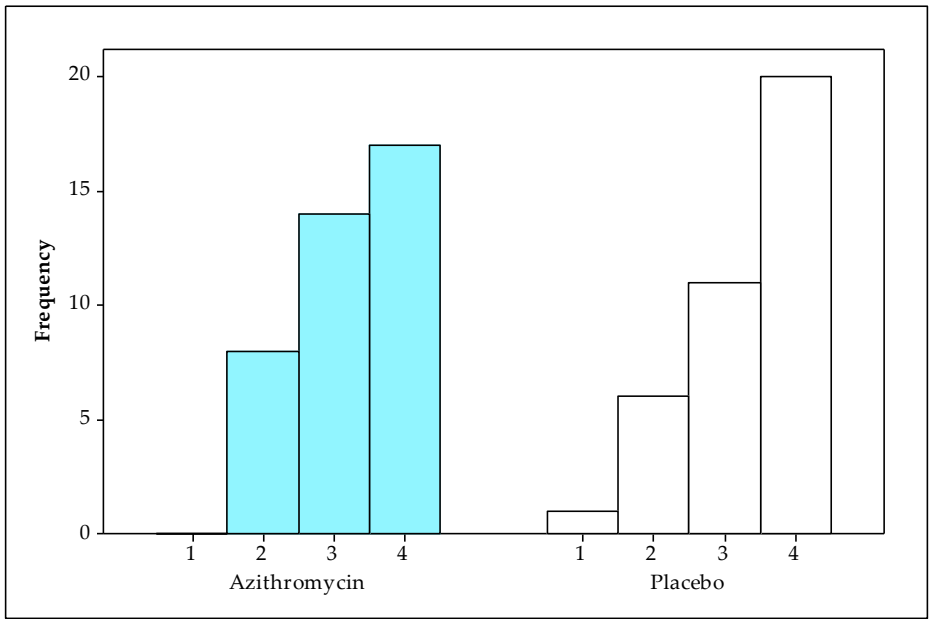
5.4.3. GINA Asthma Severity

Asthma severity was measured at baseline only using the GINA severity classification. This was similar across both groups. The majority of patients in both groups were classified in category 4 – Severe Persistent Asthma and overall the population was positively skewed. The results are detailed in Table 5.14 and graphically in Figure 5.9.

Table 5.14: GINA asthma severity category

Category	All subjects n=77	Placebo n=38	Azithromycin n=39
Intermittent Asthma	1 [1.3%]	1 [2.63%]	0 [0%]
Mild Persistent Asthma	14 [18.18%]	6 [15.79%]	8 [20.51%]
Moderate Persistent Asthma	26 [33.77%]	12 [31.58%]	14 [35.9%]
Severe Persistent Asthma	36 [46.75%]	19 [50%]	17 [43.59%]

Figure 5.9: Baseline GINA severity category divided by treatment group



Score: 1 – Intermittent; 2 – Mild persistent; 3 – Moderate persistent; 4 – Severe persistent.

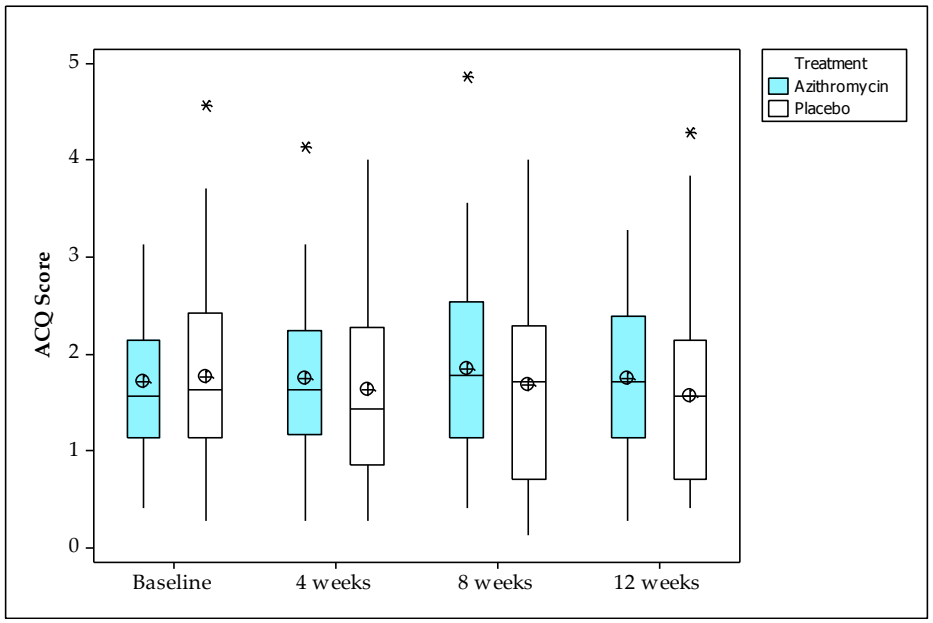
5.4.4. Asthma control score [ACQ]

No effect was seen on mean [SD] ACQ following 12 weeks treatment with azithromycin. The mean difference was 0.21 [95%CI -0.11 to 0.53], p=0.20. The details of these measurements are displayed in Table 5.15, with a graphical summary of the dataset in Figure 5.10.

Table 5.15: ACQ total score at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	1.74	0.81	38	1.76	0.88	39	1.73	0.74
Visit 5	71	1.67	0.89	35	1.58	0.96	36	1.75	0.83
Δ baseline to Visit 5		-0.09	0.72		-0.21	0.81		0.02	0.61
Mean Difference [95% CI]									0.21 [-0.11 , 0.53], p=0.20

Figure 5.10: Box-whisker plot of ACQ score by visit



No significant difference is seen between the treatment groups at 4 weeks [p=0.43], 8 weeks [p=0.23] and 12 weeks [p=0.20]. Mean denoted by crossed-circle and outliers with asterisk.

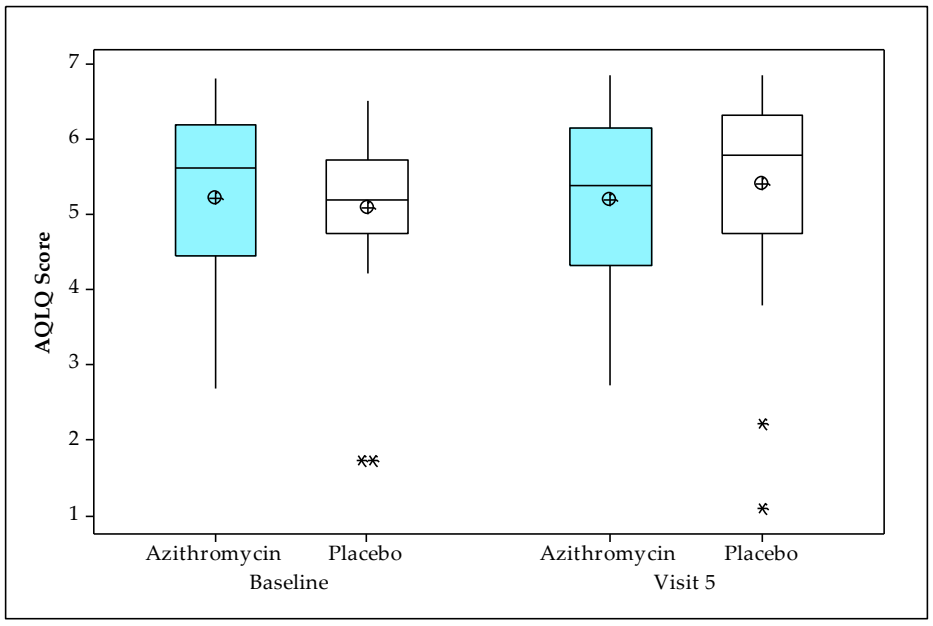
5.4.5. Asthma quality of life [AQLQ]

AQLQ was measured at baseline and 12 weeks. No effect was seen on mean [SD] AQLQ following 12 weeks treatment with azithromycin. The mean difference was -0.31 [95%CI -0.69 to 0.07], p=0.11. The details of these measurements are displayed in Table 5.16, with a graphical summary of the dataset in Figure 5.11.

Table 5.16: AQLQ total score at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	5.17	1.09	38	5.09	0.99	39	5.25	1.18
Visit 5	71	5.31	1.19	35	5.42	1.31	36	5.2	1.06
Δ baseline to Visit 5		0.2	0.83		0.37	0.97		0.04	0.65
Mean Difference [95% CI]									-0.31 [-0.69 , 0.07], p=0.11

Figure 5.11: Box-whisker plot of AQLQ score



No significant difference is seen between the treatment groups [p=0.11]
Mean denoted by crossed-circle and outliers with asterisk.

5.4.5.1. AQLQ Individual domain scores

Subdivision of the AQLQ in to its component domains did not demonstrate any difference between azithromycin and placebo following 12 weeks of treatment.

- AQLQ-*symptoms* domain had a mean difference -0.32 [95%CI -0.75 to 0.11], p=0.14

- AQLQ-*activity limitation* domain, mean difference -0.17 [95%CI -0.53 to 0.19] p=0.35

- AQLQ-*emotional function* domain almost reached a statistically significant difference but was in direction suggesting deterioration, mean difference -0.44 [95%CI -0.9 to 0.02] p=0.06

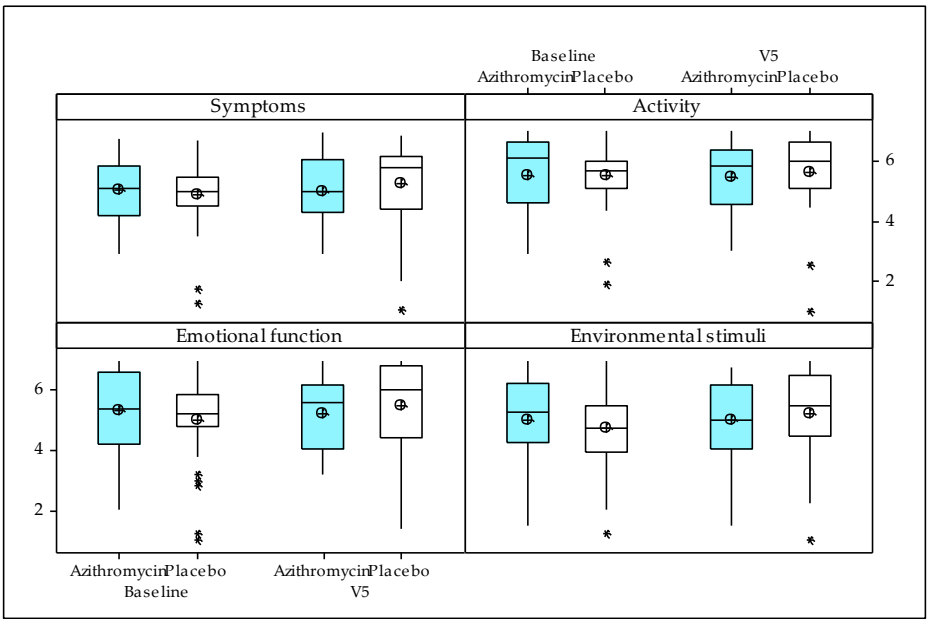
- AQLQ-*environmental stimuli* domain, mean difference -0.38 [95%CI -0.87 to 0.11], p=0.13.

These results are tabulated in Table 5.17, with the summary statistics being represented graphically in Figure 5.12.

Table 5.17: AQLQ by domain following 12 weeks

AQLQ domain	Placebo			Azithromycin			Treatment difference	95% CI	p value
	n	Mean	Standard Deviation	n	Mean	Standard Deviation			
Symptoms	35	5.26	1.37	36	5.01	1.08	-0.32	-0.75, 0.11	0.14
Activity limitation	35	5.64	1.27	36	5.48	1.21	-0.17	-0.53, 0.19	0.35
Emotional function	35	5.51	1.56	36	5.23	1.19	-0.44	-0.9, 0.02	0.06
Environmental stimuli	35	5.2	1.49	36	5.01	1.28	-0.38	-0.87 , 0.11	0.13

Figure 5.12: AQLQ individual domain scores at baseline and 12 weeks



No significant difference is seen between the treatment groups: Symptoms, p=0.14; Activity, p=0.35; Emotional function, p=0.06; Environmental Stimuli, p=0.13. Mean denoted by crossed-circle and outliers with asterisk.

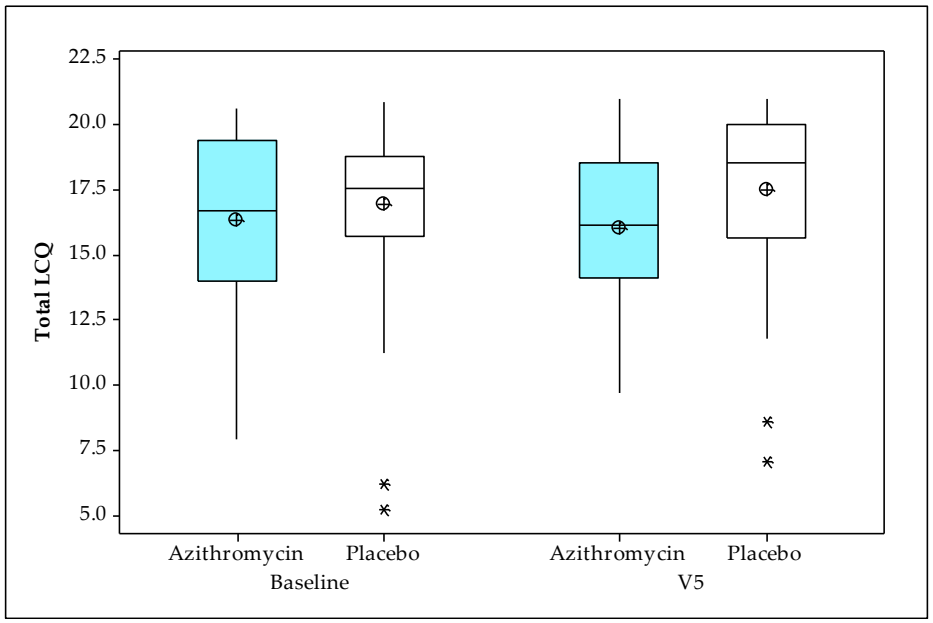
5.4.6. Leicester cough questionnaire [LCQ]

LCQ was measured at baseline and 12 weeks. No effect was seen on mean [SD] LCQ following 12 weeks treatment with azithromycin. The mean difference was -1.06 [95%CI -2.16 to 0.05], p=0.06. The details of these measurements are displayed in Table 5.18, with a graphical summary of the dataset in Figure 5.13.

Table 5.18: LCQ total score at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	16.60	3.50	38	16.90	3.49	39	16.31	3.53
Visit 5	71	16.75	3.36	35	17.51	3.55	36	16.01	3.02
Δ baseline to Visit 5		0.38	2.61		0.81	2.16		-0.03	2.96
Mean Difference [95% CI]									-1.06 [-2.16 , 0.05], p=0.06

Figure 5.13: LCQ score at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.06]
Mean denoted by crossed-circle and outliers with asterisk.

5.4.6.1. LCQ Individual domain scores

Subdivision of the LCQ in to its component domains did not demonstrate any difference between azithromycin and placebo following 12 weeks of treatment.

- LCQ-*physical* domain almost reached statistical significance with a mean difference -0.33 [95%CI -0.67 to 0.02], p=0.07

- LCQ-*psychological* domain did reach statistical significance, mean difference -0.46 [95%CI -0.9 to 0.02] p=0.04

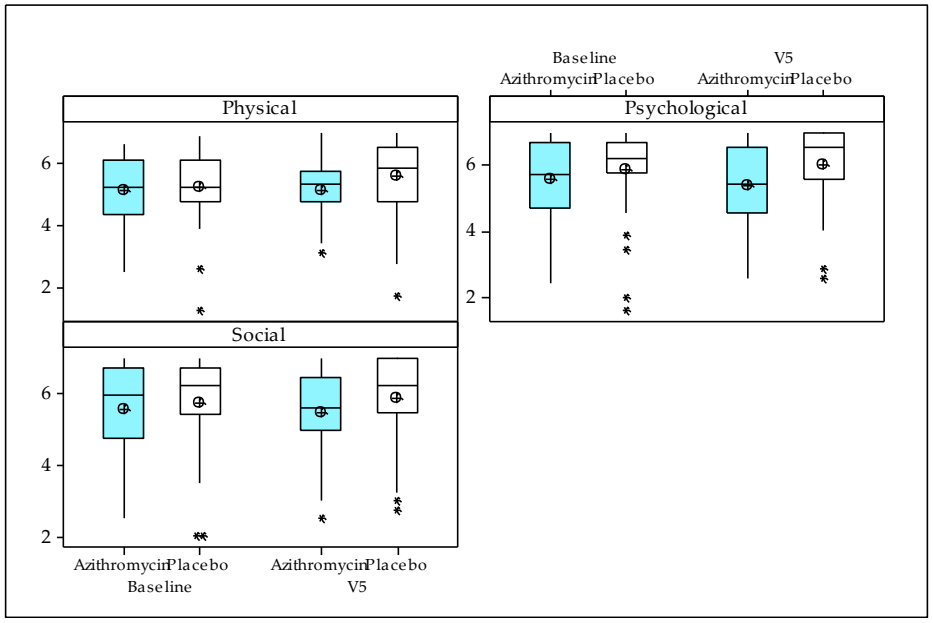
- LCQ-*social* domain mean difference -0.29 [95%CI -0.73 to 0.15] p=0.19

These results are tabulated in Table 5.19, with the summary statistics being represented graphically in Figure 5.14.

Table 5.19: LCQ individual domain scores

LCQ domain	Placebo			Azithromycin			Treatment difference	95% CI	p-value
	n	Mean	Standard Deviation	n	Mean	Standard Deviation			
Physical	35	5.58	1.21	36	5.16	0.89	-0.33	-0.67 , 0.02	0.07
Psychological	35	6.03	1.24	36	5.38	1.18	-0.46	-0.9 , -0.02	0.04
Social	35	5.89	1.25	36	5.47	1.26	-0.29	-0.73 , 0.15	0.19

Figure 5.14: LCQ individual domain scores divided by visit



No significant difference is seen between the treatment groups: Physical, p=0.07; Psychological, p=0.04; Social, p=0.19. Mean denoted by crossed-circle and outliers with asterisk.

5.5. Home diary card recordings

5.5.1. Evening PEF recordings

Daily PEF was performed in the evening by the study subjects and recorded electronically by the Piko-1 meter. This was downloaded at each study visit with the data for the previous seven days [minimum of three useable] recordings being analysed. There was no difference between the azithromycin or placebo group in either the evening PEF [L/min] at 4, 8 or 12 weeks [mean difference, 12 weeks, -4.5, 95%CI -36.3 to 27.4, p=0.78]. Details of the daily PEF recordings are summarised in Table 5.20.

Table 5.20: Evening PEF [L/min] recordings at baseline, 4, 8, and 12 weeks

Variable	Placebo											
PEF	Baseline			Week 4			Week 8			Week 12		
	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
Evening	38	378.3	120.9	34	388.0	115.0	31	392.6	118.2	34	386.8	117.6
	Azithromycin											
	Baseline			Week 4			Week 8			Week 12		
	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
Evening	39	363.7	120.0	34	366.4	140.9	32	367.7	145.4	36	373.3	155.9
	Treatment difference											
				Week 4			Week 8			Week 12		
				Δ	95%CI	p	Δ	95%CI	p	Δ	95%CI	p
				-1.0	-27.1, 25.2	0.94	-7.2	-36.41 , 22.03	0.62	-4.5	-36.3, 27.4	0.78

5.5.2. Symptom scores

Diary card symptom scores were assessed at each visit comprising: frequency of asthma symptoms; annoyance of asthma symptoms; activity and activity limitation. No effect was found at 4, 8 or 12 weeks treatment with azithromycin in any of these parameters. The results are summarised for all the questions at 12 weeks in Table 5.21. In the interests of clarity only 12 week endpoint data is shown.

Table 5.21: Diary card questions at 12 weeks

Diary card question	Placebo			Azithromycin			Treatment difference	95% CI	p value
	n	Mean	SD	n	Mean	SD			
Early morning wakening	30	0.23	0.65	31	0.21	0.33	0.07	-0.09, 0.23	0.38
Frequency of asthma symptoms	30	1.33	1.32	31	1.24	1.03	-0.11	-0.51, 0.29	0.58
Annoyance of asthma symptoms	32	1.2	1.3	31	1.21	1.13	0.04	-0.36, 0.44	0.85
Activity	31	2.07	1.33	31	1.85	1.37	-0.22	-0.65, 0.22	0.33
Activity limitation	31	1.2	1.34	31	1.22	1.32	0.01	-0.36, 0.38	0.96

5.5.3. Reliever inhaler use

Reliever inhaler use was recorded in the daily diary card. No effect was found at 4, 8 or 12 weeks treatment with azithromycin when compared to placebo. Mean difference at 4 weeks, -0.1 puffs per 24 hours, 95%CI -0.8 to 0.6, p=0.78; 8 weeks, 0.3 puffs per 24 hours, 95%CI -0.5 to 1.0, p=0.50 and 12 weeks -0.3 puffs per 24 hours, 95%CI -1.3 to 0.7, p=0.55. The results for the 12 week endpoint are displayed in Table 5.22.

Table 5.22: Reliever inhaler use [puffs per 24hours], [diary card] at 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	66	2.9	3.8	30	2.8	2.9	36	2.9	4.4
Visit 5	58	2.9	3.4	26	2.7	2.5	32	3.0	4.0
Δ baseline to Visit 5		0.1	2.0		0.3	1.2		-0.1	2.4
Mean Difference [95% CI]									-0.3 [-1.3 , 0.7], p=0.55

5.5.4. Clinical results: Summary

Changes in clinical outcomes after treatment with oral azithromycin are listed in Table 5.3 to Table 5.18 and Figure 5.2 to Figure 5.11. At 12 weeks the change in mean morning PEF [primary outcome], as compared with baseline, did not differ substantially between the azithromycin and placebo treatment groups [mean difference -10.3 L/min, 95% CI -47.1 to 26.4, p=0.58. Secondary outcome measures of mean morning PEF at 4 and 8 weeks were also not substantially different from their baseline between the azithromycin and placebo groups [mean difference at 4 weeks -4.2 L/min, 95% CI -30.6 to 22.2; 8 weeks 1.2 L/min 95% CI -28.1 to 30.5]. There were no improvements in any of the health-related quality-of-life-outcomes. The 7 point ACQ score did not differ substantially from baseline between the azithromycin and placebo groups at 12 weeks [0.21, 95% CI -0.11 to 0.53, p=0.20]. There was no substantial difference at either 4 or 8 weeks. AQLQ score did not differ substantially from baseline between the azithromycin and placebo groups at 12 weeks [mean difference -0.31 95% CI -0.69 to 0.07]. In addition when addressing each domain of the AQLQ separately, there were no substantial differences between the two groups [Table 5.17]. The LCQ score did not differ substantially from baseline between the azithromycin and placebo groups at 12 weeks [mean difference -1.06, 95% CI -2.16 to 0.05, p=0.06]. When addressing each of the domains separately both the social and physical domains had non-significant differences but the LCQ-psychological domain did, with a mean difference between the two groups of -0.46, 95% CI -0.9 to -0.02, p=0.04. The minimal clinically important difference for the psychological domain is 0.8 [212], and whilst there is statistical significance in this domain, this might not reflect true clinical improvement across the groups and so needs to be interpreted with caution. There was no difference in either pre- or post-salbutamol FEV_1 at 4, 8 or 12 weeks between the two groups. The PC_{20} of methacholine was measured at

baseline and 12 weeks with no differences observed between the azithromycin or placebo groups 0.08mg/ml, 95% CI -1.8 to 1.97, p=0.93.

6. Laboratory endpoint results

6.1. Induced sputum analysis

Sputum induction was performed at baseline and 12 weeks following standard operating procedure. Safety aspects precluded some subjects from performing this test and some subjects were unable to expectorate. In addition, some subjects were able to produce a sample at baseline only. Subjects who did not produce a sample at baseline were not asked to do so at the 12 week visit.

6.1.1.1. Sputum quality indicators

Median total filtrate volume was 33.5ml [IQR 15.4, 66.5] at baseline and 37.6ml [IQR 13.1, 57.7] at 12 weeks. This was similar between the two groups. Additionally, indicators of sputum quality such as total cells recovered and viability were also similar across the groups. These results are detailed in Table 6.1.

Table 6.1: Sputum quality indicators at baseline [Visit 2] and 12 weeks [Visit 5]

Variable	Visit	Total			Placebo			Azithromycin		
		n	Median	IQR	n	Median	IQR	n	Median	IQR
Filtrate Vol. [ml]	2	71	33.5	15.4, 66.5	34	24.6	14.4, 57.6	37	45.5	17.1, 78.9
	5	65	37.6	13.1, 57.7	32	36.3	12.3, 55.1	33	38.2	16.2, 63.2
Absolute number of cells per slide	2	69	559	486, 743	33	565	492, 753	36	540	480, 724
	5	59	624	520, 760	29	585	509, 767	30	628	524, 745
Total cell count [x10⁶/ml]	2	69	0.76	0.40, 1.45	33	0.56	0.34, 1.25	36	0.92	0.52, 1.66
	5	61	0.77	0.50, 0.97	29	0.73	0.56, 0.97	32	0.8	0.37, 1.03
Total viable cells [x10⁶/ml]	2	69	0.39	0.20, 0.81	33	0.24	0.17, 0.65	36	0.46	0.24, 0.96
	5	61	0.38	0.19, 0.61	29	0.42	0.20, 0.62	32	0.37	0.18, 0.62
Viability [%]	2	69	53.0	44.5, 64.5	33	51.0	43.0, 63.0	36	56.5	46.5, 64.8
	5	61	55.0	40.0, 60.0	29	55.0	36.0, 67.0	32	54.5	42.0, 64.8

The lower number of samples between visits represents samples discarded due to inadequate quality.

6.1.2. Sputum cell differential

6.1.2.1. Neutrophils

No significant difference was seen in either total neutrophil cell count [mean difference 19.2 x10^4 cells 95% CI -24.2 to 62.6, p=0.38] or the proportion of neutrophils [mean difference 3.0% 95% CI -5.9 to 11.8, p=0.50] within the sample when the azithromycin group was compared to the placebo group at 12 weeks. These results are detailed in Table 6.2 and Table 6.3 and displayed graphically in Figure 6.1 and Figure 6.2.

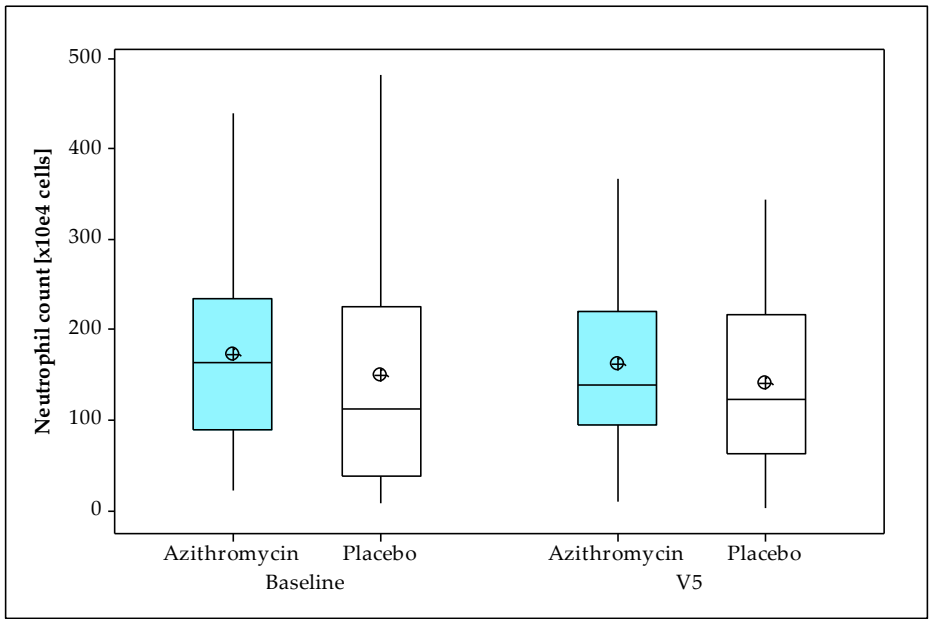
Table 6.2: Absolute neutrophil cell count [x10^4 cells] at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	161.7	113.0	33	149.9	123.3	36	172.5	103.3
Visit 5	59	151.5	96.1	29	140.7	95.7	30	161.8	96.9
Δ baseline to Visit 5		-4.6	98.9		-9.3	93.6		-0.1	105.2
Mean Difference [95% CI]									19.2 [-24.2 , 62.6], p=0.38

Table 6.3: Proportion neutrophils [%] at baseline and 12 weeks

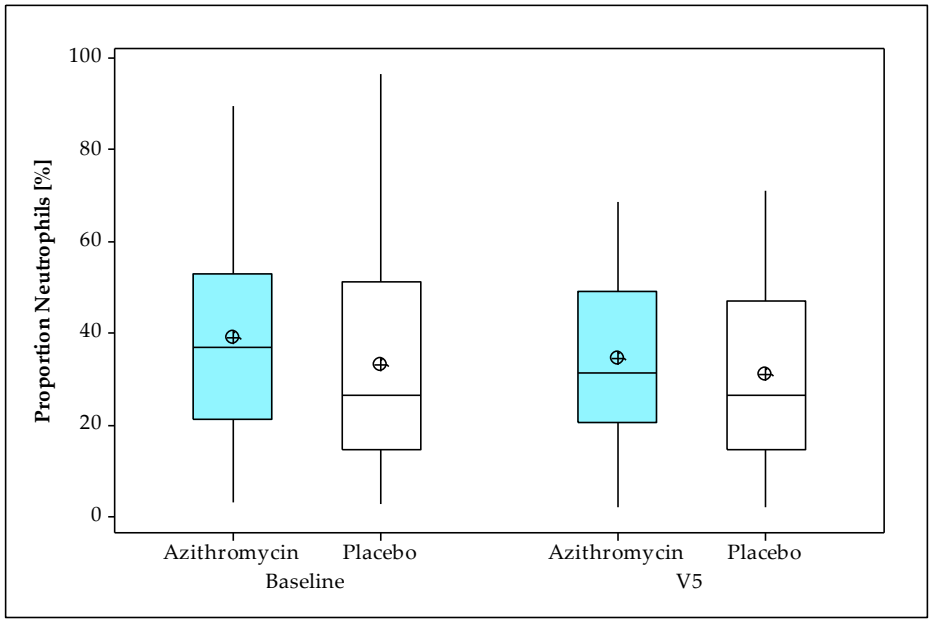
Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	36.2	23.4	33	33.1	24.3	36	39.0	22.5
Visit 5	59	32.7	19.1	29	31.0	19.9	30	34.4	18.6
Δ baseline to Visit 5	57	-2.4	20.9	28	-2.6	20.0	29	-2.2	22.1
Mean Difference [95% CI]									3.0 [-5.9 , 11.8], p=0.50

Figure 6.1: Absolute neutrophil count [x10^4 cells] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.38]
Mean denoted by crossed-circle and outliers with asterisk.

Figure 6.2: Proportion neutrophils [%] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.50]
Mean denoted by crossed-circle and outliers with asterisk.

6.1.2.2. Eosinophils

No significant difference was seen in either total eosinophil cell count [mean difference 1.0 x104 cells 95% CI -0.5 to 2.0, p=0.89] or the proportion of eosinophils [mean difference -0.4% 95% CI -1.7 to 1.0, p=0.50] within the sample when the azithromycin group was compared to the placebo group at 12 weeks. These results are detailed in Table 6.4 and Table 6.5 and graphically in Figure 6.3 and Figure 6.4.

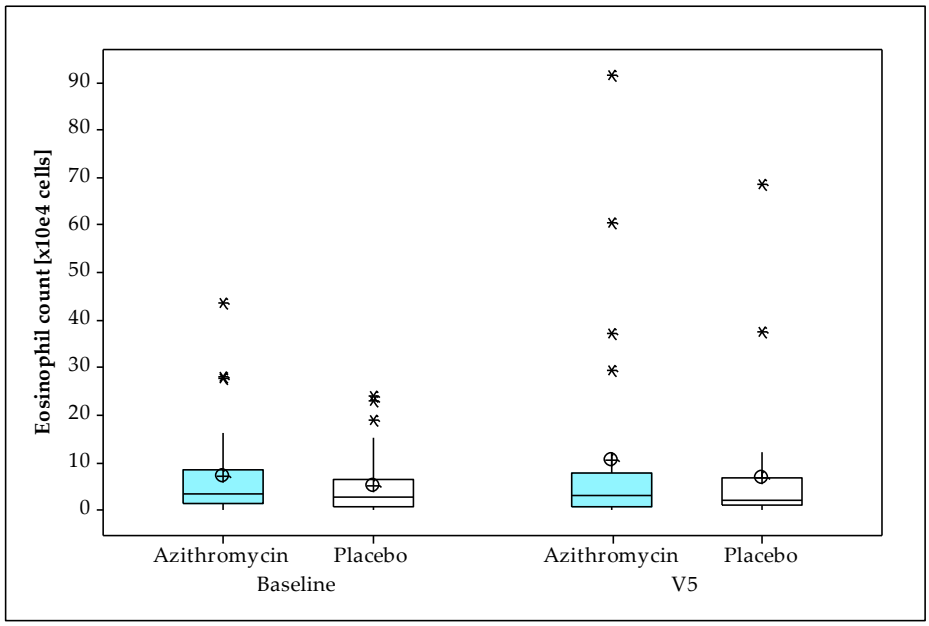
Table 6.4: Absolute eosinophil cell count [x10^4 cells] at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	6.0	8.1	33	4.9	6.4	36	6.9	9.3
Visit 5	59	8.6	17.3	29	6.8	13.9	30	10.3	20.1
Δ baseline to Visit 5		3.11	16.5		2.1	12.0		4.1	20.0
Mean Difference [95% CI]									1.0 [0.5 , 2.0], p=0.89

Table 6.5: Proportion eosinophils [%] at baseline and 12 weeks

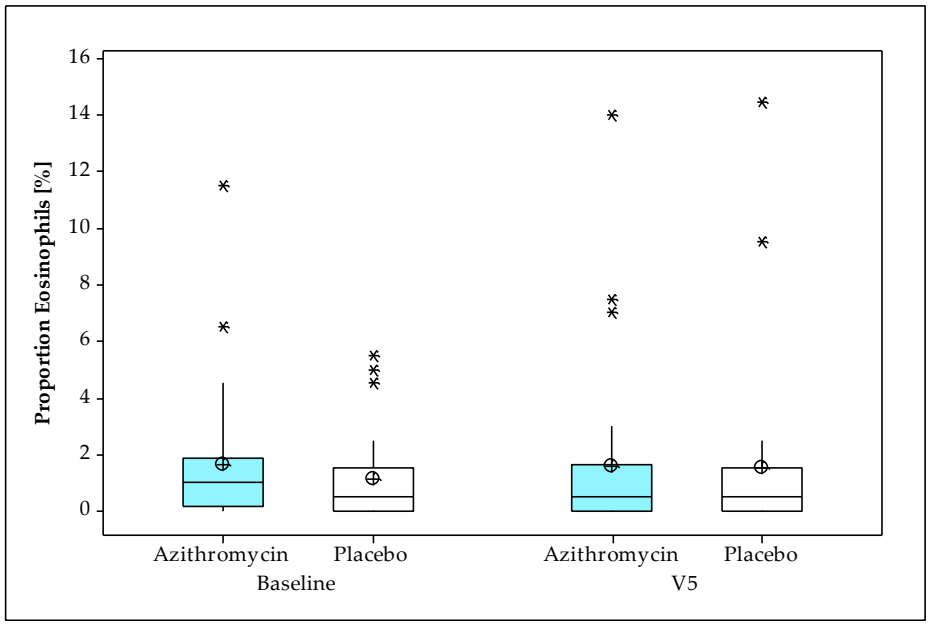
Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	1.4	1.9	33	1.1	1.5	36	1.6	2.3
Visit 5	59	1.6	3.0	29	1.5	3.1	30	1.6	3.0
Δ baseline to Visit 5	57	0.3	2.5	28	0.5	2.6	29	0.1	2.4
Mean Difference [95% CI]									-0.4 [-1.7 , 1.0], p=0.55

Figure 6.3: Absolute eosinophil count [x10^4 cells] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.89]
Mean denoted by crossed-circle and outliers with asterisk.

Figure 6.4: Proportion eosinophils [%] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.55]
Mean denoted by crossed-circle and outliers with asterisk.

6.1.2.3. Macrophages

No significant difference was seen in either total macrophage cell count [mean difference -1.2 x10^4 cells 95%CI -41.2 to 38.8, p =0.95] or the proportion of macrophages [mean difference 0.8% 95% CI -7.3 to 9.0, p=0.84] within the sample when the azithromycin group was compared to the placebo group at 12 weeks. These results are detailed in Table 6.6 and Table 6.7 and displayed graphically in Figure 6.5 and Figure 6.6.

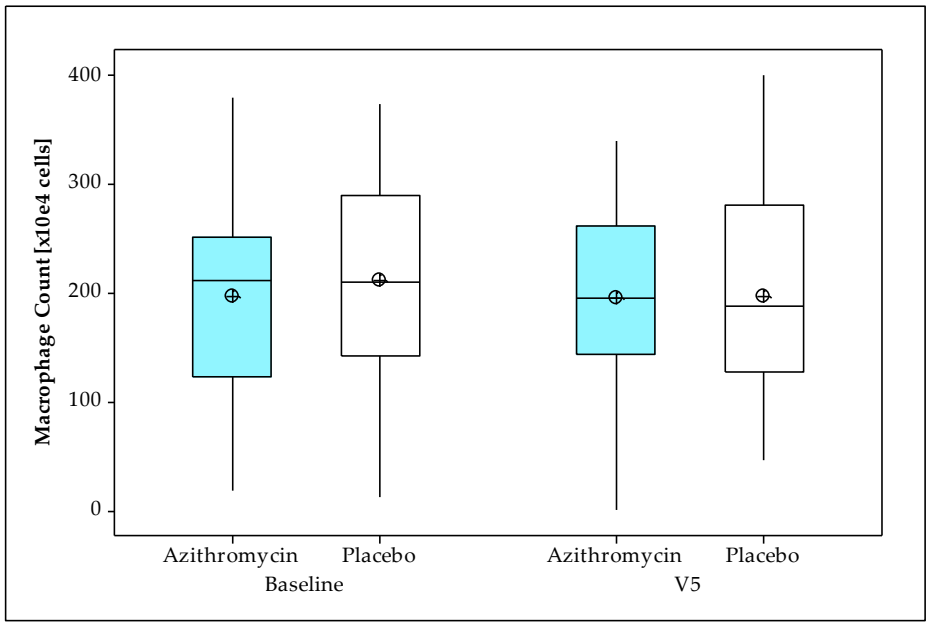
Table 6.6: Absolute macrophage cell [x10^4 cells] count at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	204.3	94.5	33	211.4	99.6	36	197.7	90.5
Visit 5	59	196.1	87.2	29	197.1	92.5	30	195	83.3
Δ baseline to Visit 5		-11.3	85.1		-13.0	98.0		-9.7	72.1
Mean Difference [95% CI]									-1.2 [-41.1 , 38.8], p=0.95

Table 6.7: Proportion macrophages [%] at baseline and 12 weeks

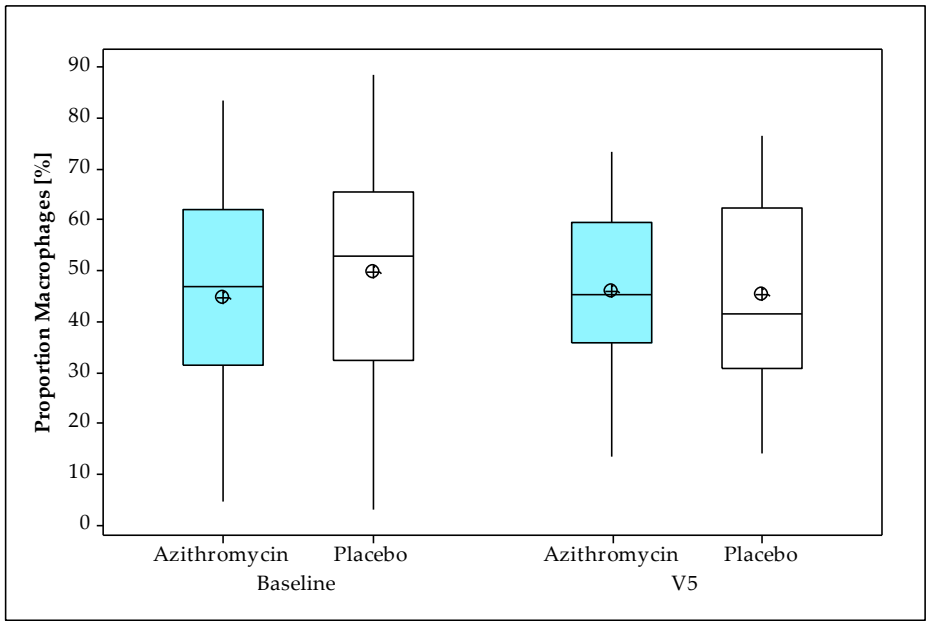
Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	47.1	21.5	33	49.7	22.9	36	44.7	20.2
Visit 5	59	45.6	17.0	29	45.3	18.7	30	45.8	15.5
Δ baseline to Visit 5	57	-2.0	19.5	28	-3.5	19.8	29	-0.7	19.4
Mean Difference [95% CI]									0.8 [-7.3 , 9.0], p=0.84

Figure 6.5: Absolute macrophage count [x10^4 cells] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.95]
Mean denoted by crossed-circle and outliers with asterisk.

Figure 6.6: Proportion macrophages [%] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.84]
Mean denoted by crossed-circle and outliers with asterisk.

6.1.2.4. Lymphocytes

No significant difference was seen in either total lymphocyte cell count [p=1.00] or the proportion of lymphocyte [mean difference -0.4% 95% CI -1.8 to 1.0, p=0.55] within the sample when the azithromycin group was compared to the placebo group at 12 weeks. These results are detailed in Table 6.8 and Table 6.9 and graphically in Figure 6.7. The total lymphocyte count was analysed using a non-parametric test of association due to the low absolute counts and minimal observed differences. The proportion of percentage lymphocytes has not been displayed graphically due to the influence of outliers [compressing the boxplot]. The absolute lymphocyte count is displayed in Figure 6.7.

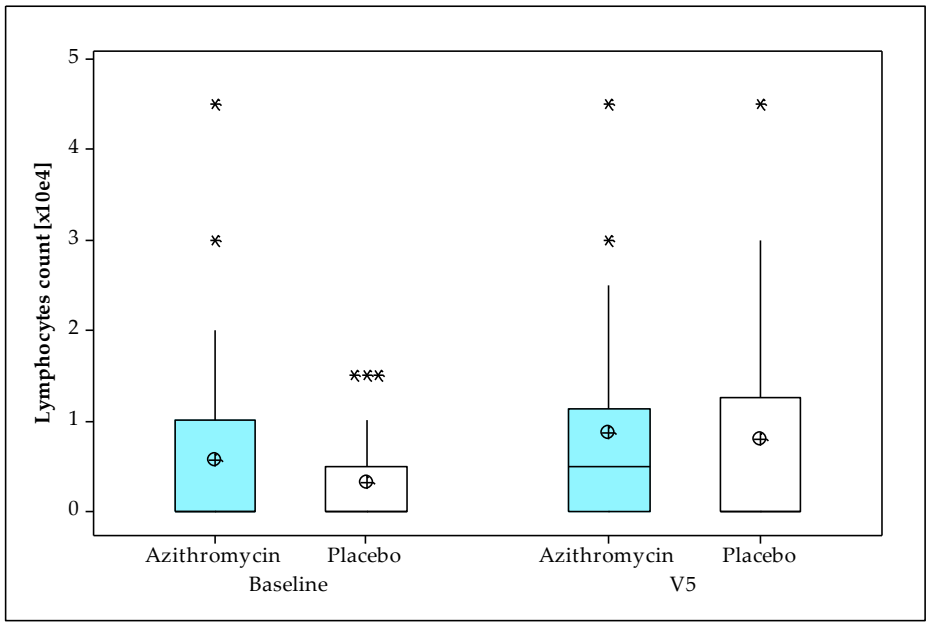
Table 6.8: Absolute lymphocyte count [x10^4 cells] at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	0.6	0.7	33	0.5	0.4	36	0.7	0.9
Visit 5	59	0.9	1.0	29	0.9	1.1	30	1.0	1.0
Δ baseline to Visit 5		0.3	1.2		0.4	1.1		0.2	1.4
Mean Difference [95% CI]									p=1.00

Table 6.9: Proportion lymphocytes [%] at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	1.4	1.9	33	1.1	1.5	36	1.6	2.3
Visit 5	59	1.6	3	29	1.5	3.1	30	1.6	3.0
Δ baseline to Visit 5	57	0.3	2.5	28	0.5	2.6	29	0.1	2.4
Mean Difference [95% CI]									-0.4 [-1.7 , 1.0], p=0.55

Figure 6.7: Absolute lymphocyte count [x10⁴ cells] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=1.00]
Mean denoted by crossed-circle and outliers with asterisk.

6.1.2.5. Bronchial epithelial cells [BEC's]

No significant difference was seen in either total broncho-epithelial cell count [mean difference 1.0 x10⁴ cells 95% CI -0.6 to 1.7, p=0.97] or the proportion of broncho-epithelial cells [BEC's] [mean difference -8.1% 95% CI -25.5 to 9.3, p=0.35] within the sample when the azithromycin group was compared to the placebo group at 12 weeks. These results are detailed in Table 6.10 and Table 6.11 and graphically in Figure 6.8 and Figure 6.9.

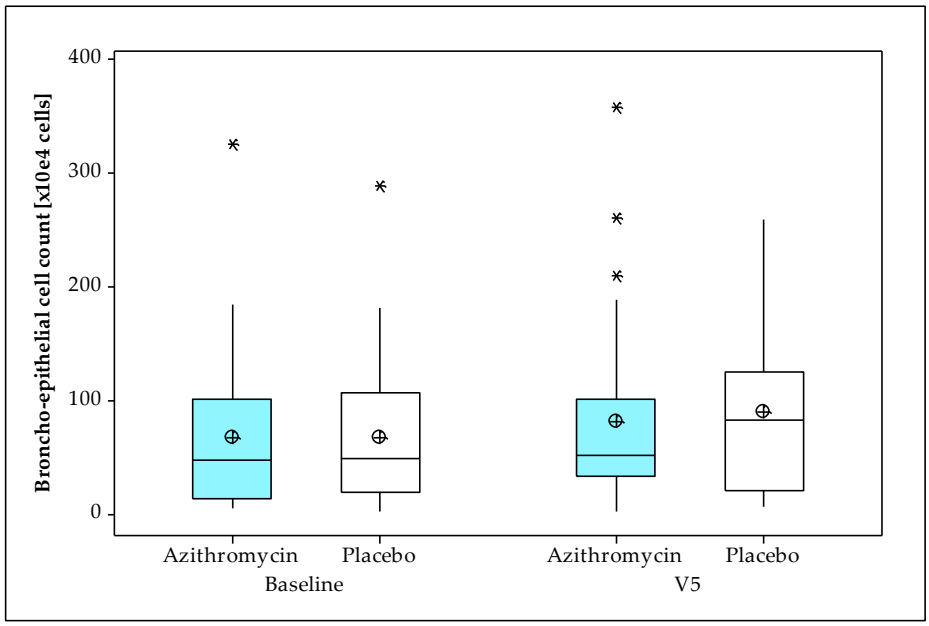
Table 6.10: Absolute bronchial epithelial cell count [x10^4 cells] at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	66.9	65.7	33	67.4	64.4	36	66.4	67.7
Visit 5	59	85.0	80.9	29	89.3	81.4	30	80.9	81.6
Δ baseline to Visit 5		15.6	66.4		20.8	65.4		10.6	68.1
Mean Difference [95% CI]									1.0 [0.6 , 1.7], p=0.97

Table 6.11: Proportion bronchial epithelial cells [%] at baseline and 12 weeks

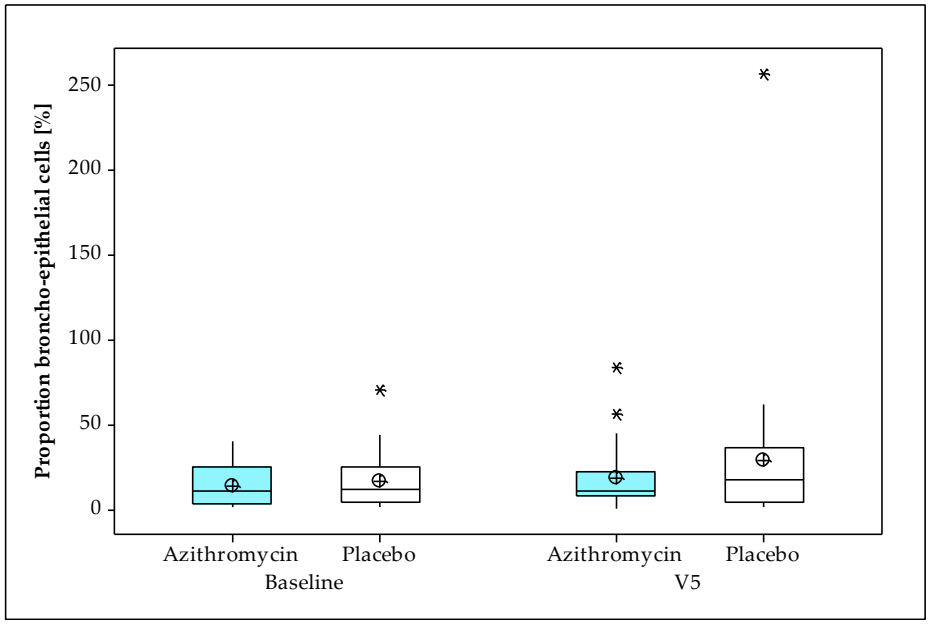
Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	69	15.0	13.8	33	16.2	15.9	36	13.9	11.6
Visit 5	59	23.4	36.1	29	28.7	47.9	30	18.3	18.5
Δ baseline to Visit 5	57	8.0	32.4	28	12.3	42.3	29	3.7	18.4
Mean Difference [95% CI]									-8.1 [-25.5 , 9.3], p=0.35

Figure 6.8: Absolute bronchial epithelial cell count [x10⁴ cells] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.97]
Mean denoted by crossed-circle and outliers with asterisk.

Figure 6.9: Proportion bronchial epithelial cells [%] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.35]
Mean denoted by crossed-circle and outliers with asterisk.

6.2. Plasma cytokines

6.2.1. IL-1β

There was no significant difference in plasma IL-1β in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.49 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.12.

Table 6.12: Change from baseline to 12 weeks in plasma IL-1β [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	9.7	24.4	20	13.7	34.5	20	5.6	0
Visit 5	40	9.1	21.3	20	12.6	30.1	20	5.6	0
Δ baseline to Visit 5	39	-0.6	3.2	20	-1.1	4.4	19	0	0
Test of association									p=0.49

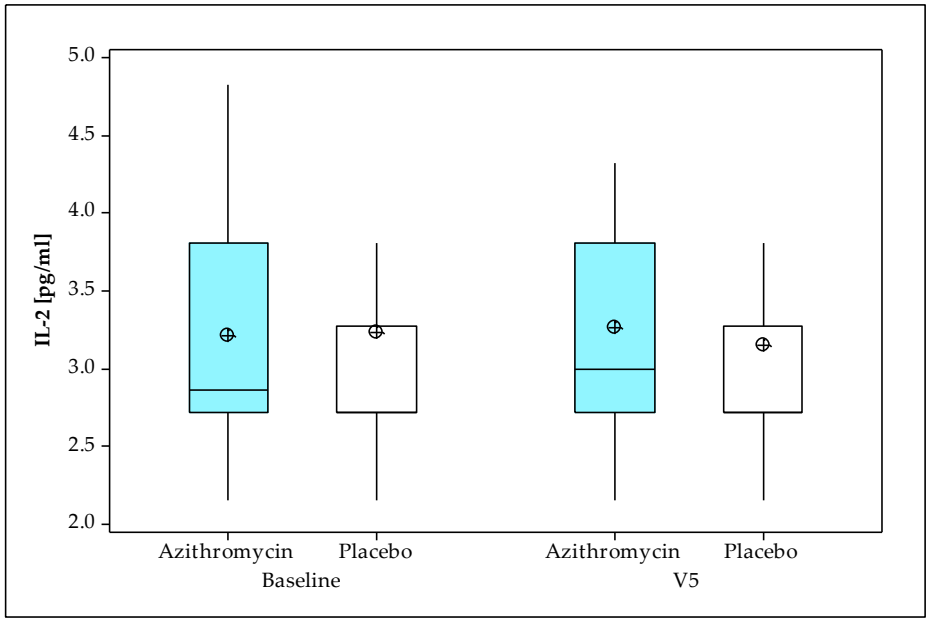
6.2.2. IL-2

There was no significant difference in plasma IL-2 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.16 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.13 and graphically in Figure 6.10.

Table 6.13: Change from baseline to 12 weeks in plasma IL-2 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	5.3	13.2	20	7.4	18.6	20	3.2	0.8
Visit 5	40	4.7	9.6	20	6.2	13.5	20	3.3	0.9
Δ baseline to Visit 5	39	-0.6	3.7	20	-1.2	5.1	19	0.1	0.8
Test of association									p=0.16

Figure 6.10: IL-2 [pg/ml] at baseline and 12 weeks



Outliers not shown: Baseline Placebo Group 86.10; V5 Placebo Group 63.11
No significant difference is seen between the treatment groups [p=0.16]
Mean denoted by crossed-circle.

6.2.3. IL-4

There was no significant difference in plasma IL-4 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.39 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.14.

Table 6.14: Change from baseline to 12 weeks in plasma IL-4 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	17.0	54.5	20	25.8	77.1	20	8.2	1.2
Visit 5	40	15.0	42.1	20	21.7	59.5	20	8.4	1.1
Δ baseline to Visit 5	39	-2.0	12.6	20	-4.1	17.5	19	0.2	1.4
Test of association									p=0.39

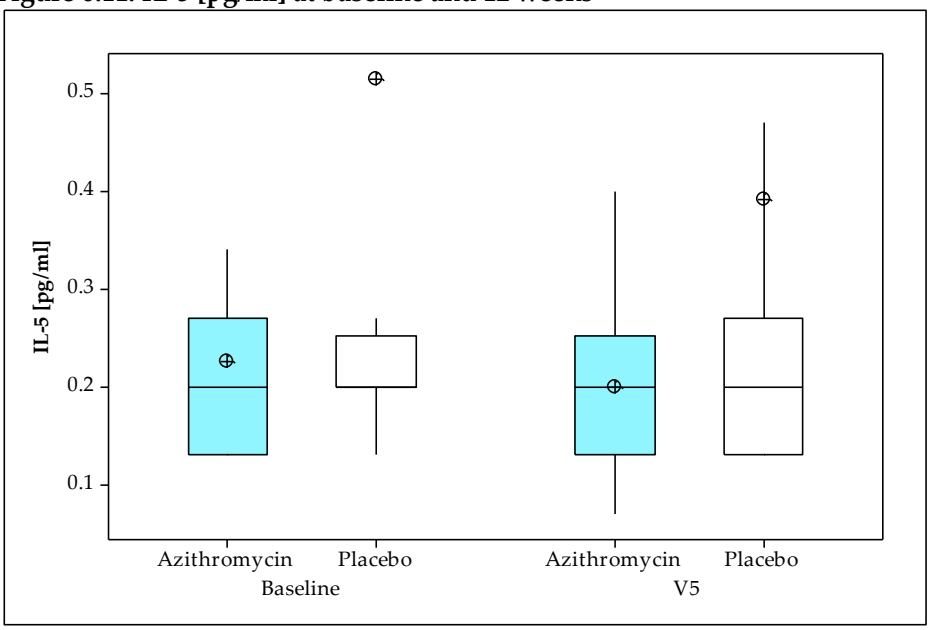
6.2.4. IL-5

There was no significant difference in plasma IL-5 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=1.00 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.15 and graphically in Figure 6.11.

Table 6.15: Change from baseline to 12 weeks in plasma IL-5 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	0.4	0.7	20	0.5	1.0	20	0.2	0.1
Visit 5	40	0.3	0.5	20	0.4	0.7	20	0.2	0.1
Δ baseline to Visit 5		-0.1	0.4		-0.1	0.5		0.0	0.1
Test of association									p=1.00

Figure 6.11: IL-5 [pg/ml] at baseline and 12 weeks



Outliers not shown: Baseline Placebo Group 3.54; 3.07; V5 Placebo Group 1.21
No significant difference is seen between the treatment groups [p=1.00]
Mean denoted by crossed-circle.

6.2.5. IL-6

There was no significant difference in plasma IL-6 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.14 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.16.

Table 6.16: Change from baseline to 12 weeks in plasma IL-6 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	22.5	131.6	20	43.0	186.1	20	1.9	3.6
Visit 5	40	7.1	36.2	20	13.1	51.1	20	1.0	2.1
Δ baseline to Visit 5	39	-15.9	96.7	20	-29.9	135.1	19	-1.3	4.1
Test of association									p=0.14

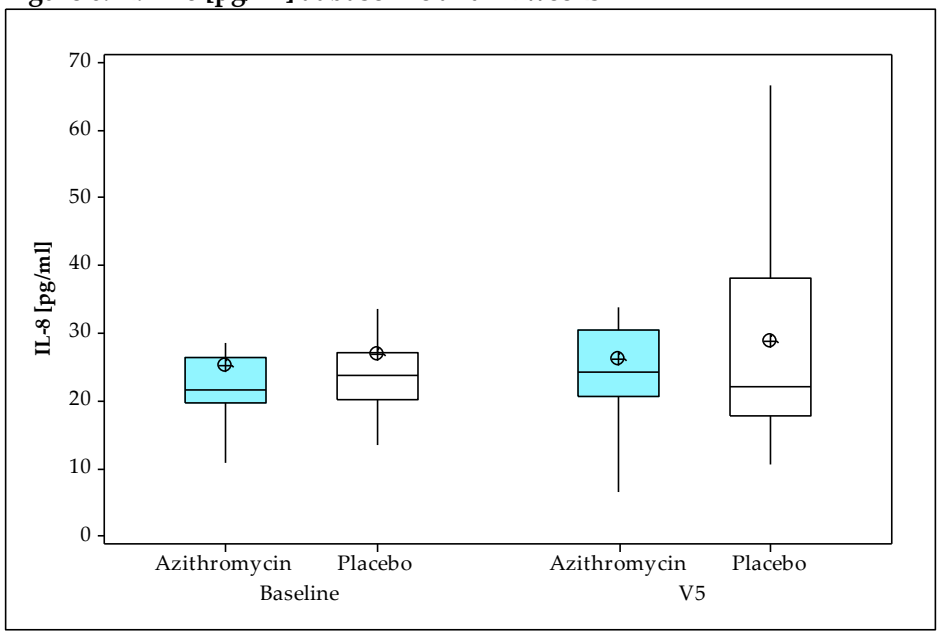
6.2.6. IL-8

There was no significant difference in plasma IL-8 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.35 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.17 and graphically in Figure 6.12.

Table 6.17: Change from baseline to 12 weeks in plasma IL-8 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	39	142.3	403.6	20	213.1	543.8	19	67.7	142.6
Visit 5	40	151.5	439.7	20	204.7	581.1	20	98.3	230.5
Δ baseline to Visit 5		12.5	633.5		-8.4	837.7		35.7	296.6
Mean Difference [95% CI]									p=0.35

Figure 6.12: IL-8 [pg/ml] at baseline and 12 weeks



Outliers not shown: Baseline Azithromycin – 616; 243; Baseline Placebo – 2162;1314; 328; V5 Azithromycin – 933; 562; V5 Placebo – 2437; 1138
No significant difference is seen between the treatment groups [p=0.35]
Mean denoted by crossed-circle.

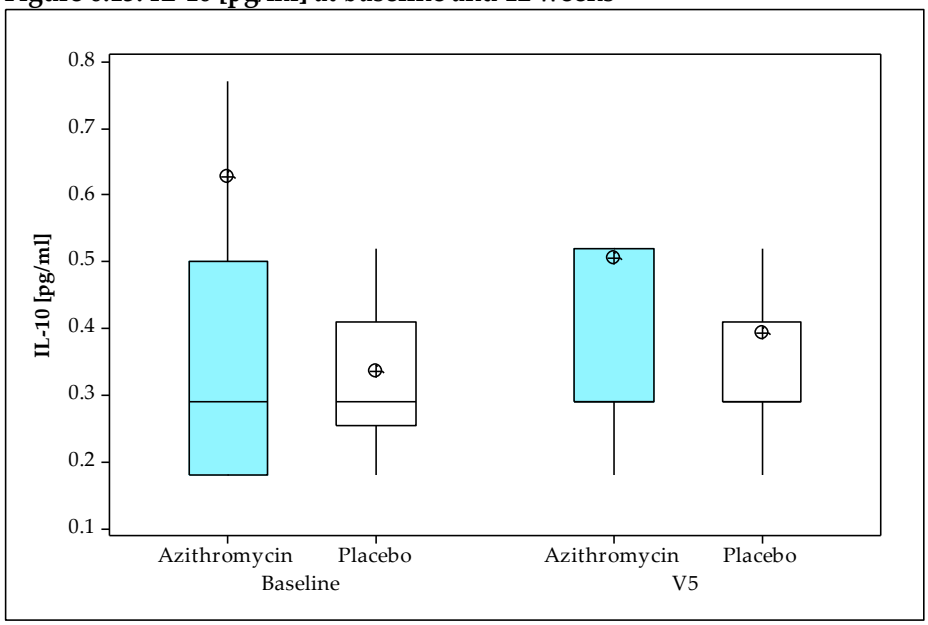
6.2.7. IL-10

There was no significant difference in plasma IL-10 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.16 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.18 and graphically in Figure 6.13.

Table 6.18: Change from baseline to 12 weeks in plasma IL-10 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	38	0.5	0.7	20	0.3	0.2	18	0.6	1.0
Visit 5	40	0.5	0.4	20	0.4	0.2	20	0.5	0.5
Δ baseline to Visit 5	37	0.0	0.6	20	0.1	0.1	17	-0.1	0.8
Test of association									p=0.16

Figure 6.13: IL-10 [pg/ml] at baseline and 12 weeks



Outliers not shown: Baseline Azithromycin 1.43; 1.03; 0.77; Baseline Placebo 0.77; V5 Azithromycin 1.03; 1.99; 1.16; 0.9; V5 Placebo 0.90; 0.77; 0.77
No significant difference is seen between the treatment groups [p=0.16]
Mean denoted by crossed-circle.

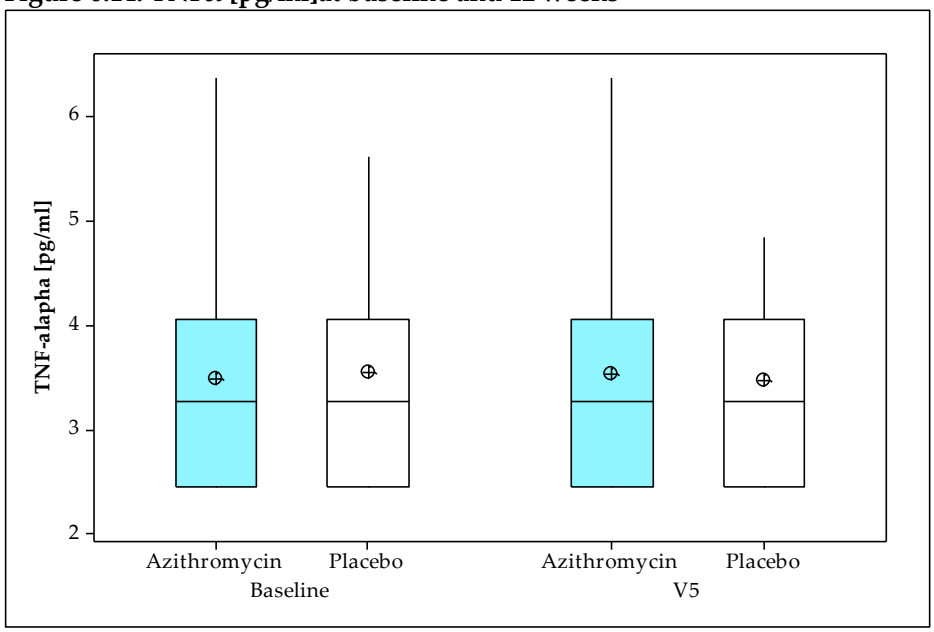
6.2.8. TNFα

There was no significant difference in plasma TNFα in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.39 [Pearson's Chi-squared test]. Mean [SD] for each and mean change is displayed in Table 6.19 and graphically in Figure 6.14.

Table 6.19: Change from baseline to 12 weeks in plasma TNFα [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	9.1	35.5	20	14.7	50.2	20	3.5	1.1
Visit 5	40	8.2	29.9	20	12.9	42.3	20	3.5	1.0
Δ baseline to Visit 5	39	-0.9	5.7	20	-1.8	7.9	19	0.1	0.8
Test of association									p=0.39

Figure 6.14: TNFα [pg/ml]at baseline and 12 weeks



Outliers not shown: Baseline Placebo 228.01; 7.12 V5 placebo 192.52
No significant difference is seen between the treatment groups [p=0.39]
Mean denoted by crossed-circle.

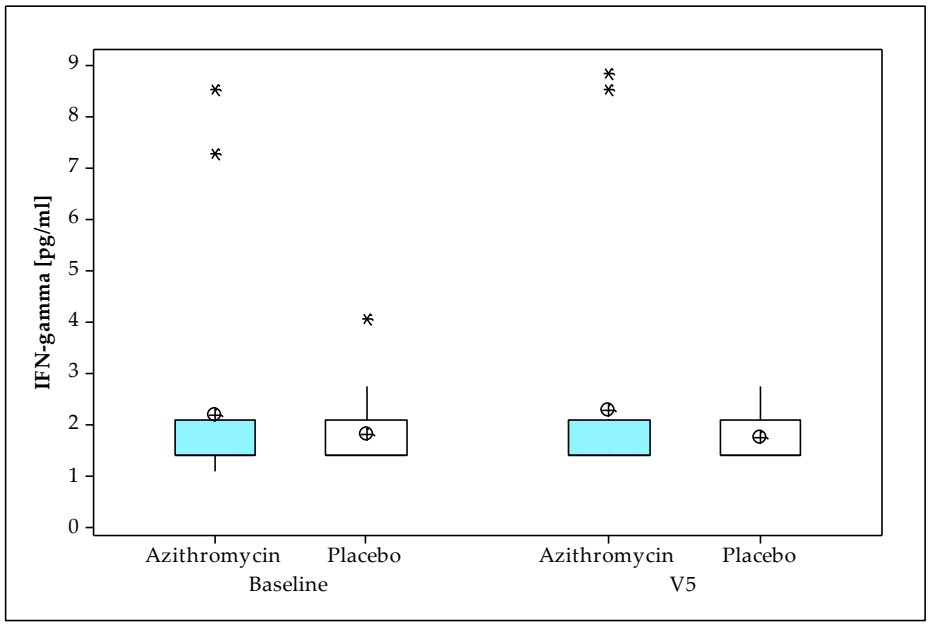
6.2.9. IFNγ

There was no significant difference in plasma IFNγ in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.25 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.20 and graphically in Figure 6.15.

Table 6.20: Change from baseline to 12 weeks in plasma IFNγ [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	39	2.0	1.5	20	1.8	0.7	19	2.1	2.0
Visit 5	40	2.0	1.6	20	1.8	0.5	20	2.3	2.2
Δ baseline to Visit 5	38	0.0	0.4	20	-0.1	0.4	18	0.1	0.3
Test of association									p=0.25

Figure 6.15: IFNγ [pg/ml] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.25]
Mean denoted by crossed-circle and outliers with asterisk.

6.2.10. GM-CSF

There was no significant difference in plasma GM-CSF in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.79 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.21. When outliers were removed and the data looked at in detail, the vast majority measures were 1.4 pg/ml. Hence, there was little value in showing this graphically.

Table 6.21: Change from baseline to 12 weeks in plasma GM-CSF [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	39	4.2	11.5	20	2.0	2.7	19	6.5	16.1
Visit 5	40	1.5	0.3	20	1.5	0.3	20	1.5	0.3
Δ baseline to Visit 5	38	-2.8	11.7	20	-0.5	2.8	18	-5.3	16.6
Test of association									p=0.79

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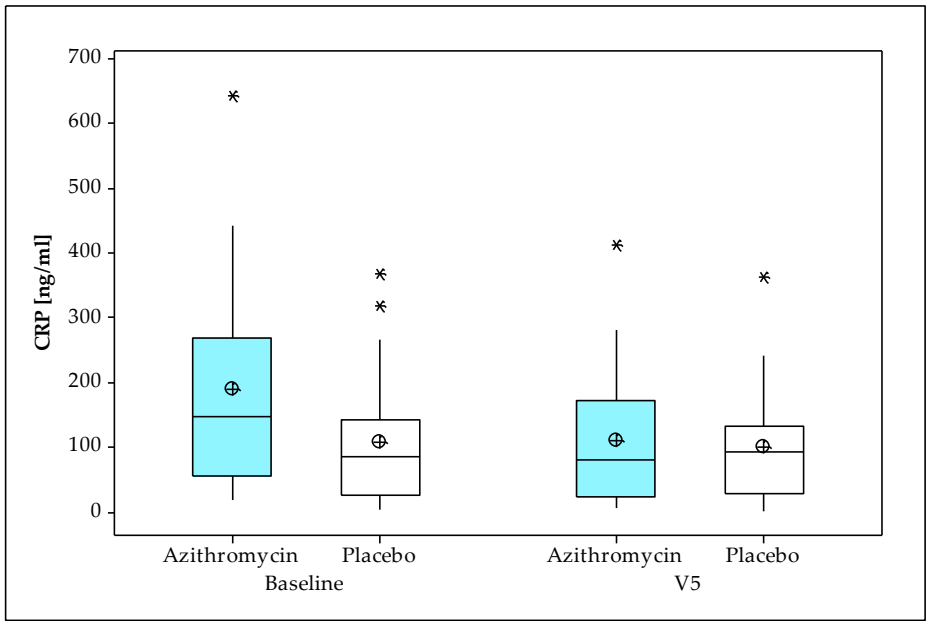
6.2.11. C-reactive protein

There was no significant difference in plasma CRP in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.08 [Fisher's exact test]. In this instance there was sufficient change to allow for calculation of a mean difference, -35.2 ng/ml 95%CI -84.5 to 14.2, p=0.16. Both statistical methods support this difference as not significant. Mean [SD] for each and mean change is displayed in Table 6.22 and graphically in Figure 6.16.

Table 6.22: Change from baseline to 12 weeks in plasma CRP [ng/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	148.8	140.4	20	108.3	102.2	20	189.4	163.0
Visit 5	40	105.0	99.2	20	99.9	90.8	20	110.1	109.2
Δ baseline to Visit 5	39	-50.0	119.2	20	-8.4	58.5	19	-93.7	149.9
Mean Difference [95% CI]									-35.2 [-84.5 , 14.2], p=0.16

Figure 6.16: CRP [ng/ml] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.16]
Mean denoted by crossed-circle and outliers with asterisk.

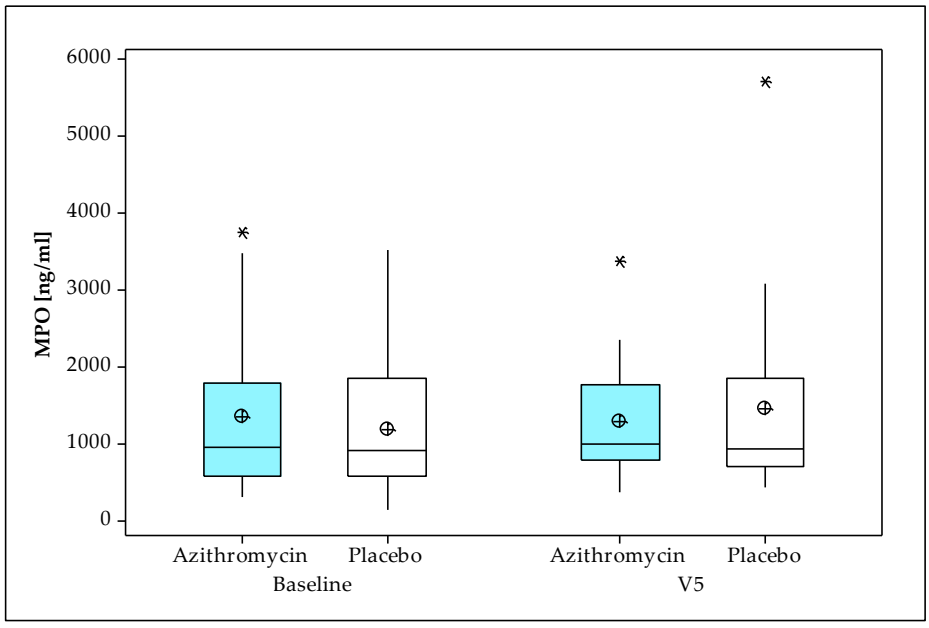
6.2.12. MPO

There was no significant difference in plasma MPO in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.52 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.23 and graphically in Figure 6.17

Table 6.23: Change from baseline to 12 weeks in plasma MPO [ng/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	1267.0	933.3	20	1178.7	836.0	20	1355.3	1035.7
Visit 5	40	1379.4	1031.1	20	1464.1	1260.0	20	1294.8	761.3
Δ baseline to Visit 5		124	1009.5		285.4	824.23		-46.0	1743.8
Test of association									p=0.52

Figure 6.17: Plasma MPO [ng/ml] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.]
Mean denoted by crossed-circle and outliers with asterisk.

6.3. Sputum Cytokines

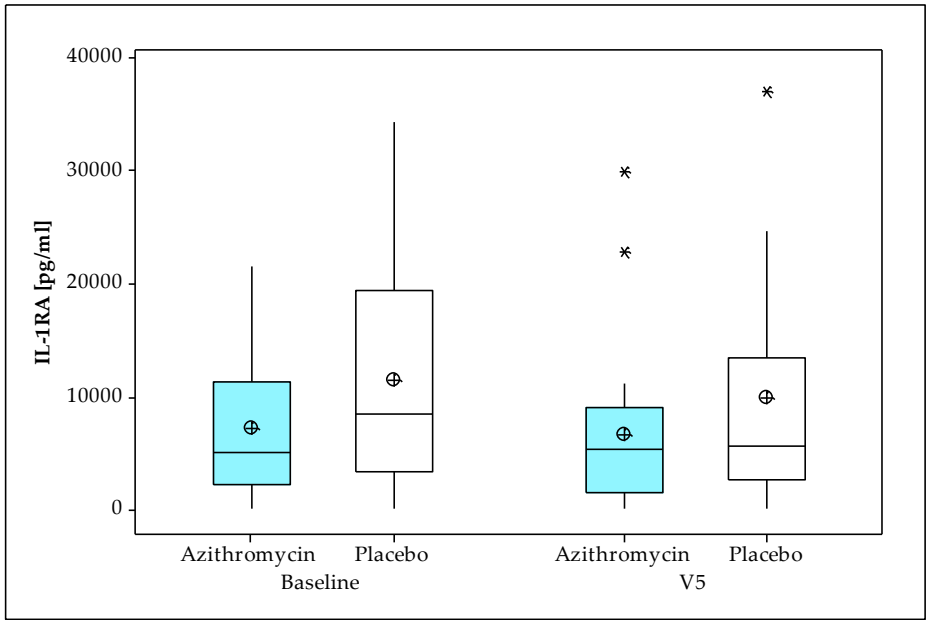
6.3.1. IL-1Rα

There was no significant difference in plasma IL-1Rα in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.75 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.24 and graphically in Figure 6.18.

Table 6.24: Change from baseline to 12 weeks in sputum supernatant IL-1Rα [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	9466.66	9268.4	21	11479.9	10811.9	19	7241.5	6803.2
Visit 5	40	8371.1	8964.7	21	9921.7	9814.8	19	6657.2	7822.3
Δ baseline to Visit 5		-1095.6	7277.8		-1558.2	8944.0		-584.3	5035.0
Test of association									p=0.75

Figure 6.18: Sputum IL-1Rα [pg/ml] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.75]
Mean denoted by crossed-circle and outliers with asterisk.

6.3.2. IL-1β

There was no significant difference in sputum IL-1β in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was not possible because there were too few patients with a change value between visits to allow for formal testing. Mean [SD] for each and mean change is displayed in Table 6.25 but the test of association has not been done. We would therefore regard this assay as having no change as a result of the azithromycin.

Table 6.25: Change from baseline to 12 weeks in sputum supernatant IL-1β [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	1.6	0.7	21	1.7	1.0	19	1.5	0
Visit 5	40	2.0	2.3	21	2.5	3.2	19	1.5	0
Δ baseline to Visit 5	40	0.4	1.7	21	0.8	2.3	19	0	0
Test of association									N/D

6.3.3. IL-2

There was no significant difference in sputum IL-2 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was not possible because there were too few patients with a change value between visits to allow for formal testing. Mean [SD] for each and mean change is displayed in Table 6.26 but the test of association has not been done. We would therefore regard this assay as having no change as a result of the azithromycin.

Table 6.26: Change from baseline to 12 weeks in sputum supernatant IL-2 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	5.9	0	21	5.9	0	19	5.9	0
Visit 5	40	5.9	0	21	5.9	0	19	5.9	0
Δ baseline to Visit 5	40	0	0	21	0	0	19	0	0
Test of association									N/D

6.3.4. IL-4

There was no significant difference in sputum IL-4 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was not possible because there were too few patients with a change value between visits to allow for formal testing. Mean [SD] for each and mean change is displayed in Table 6.27 but the test of association has not been done. We would therefore regard this assay as having no change as a result of the azithromycin.

Table 6.27: Change from baseline to 12 weeks in sputum supernatant IL-4 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	19.3	0	21	19.3	0	19	19.3	0
Visit 5	40	19.3	0	21	19.3	0	19	19.3	0
Δ baseline to Visit 5	40	0	0	21	0	0	19	0	0
Test of association									N/D

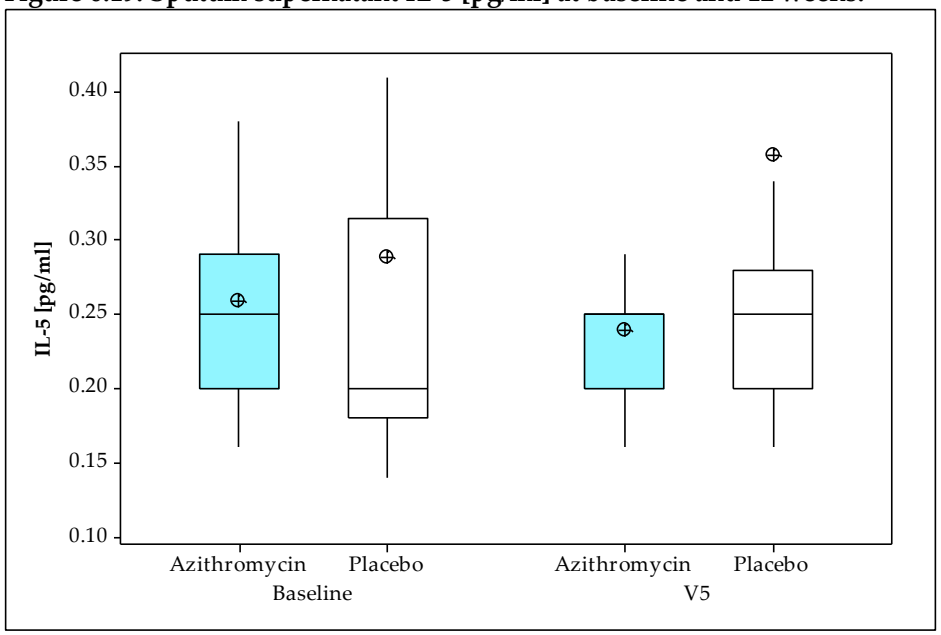
6.3.5. IL-5

There was no significant difference in sputum IL-5 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.62 [Pearson's Chi-Squared Test]. Mean [SD] for each and mean change is displayed in Table 6.28 and graphically in Figure 6.19.

Table 6.28: Change from baseline to 12 weeks in sputum supernatant IL-5 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	0.3	0.2	21	0.3	0.3	19	0.3	0.1
Visit 5	40	0.3	0.3	21	0.4	0.5	19	0.2	0.1
Δ baseline to Visit 5	40	0.0	0.4	21	0.1	0.5	19	0.0	0.1
Test of association									p=0.62

Figure 6.19: Sputum supernatant IL-5 [pg/ml] at baseline and 12 weeks.



Outliers not shown: Baseline Azithromycin 0.62; Baseline Placebo 1.33; V5 Azithromycin 0.34; 0.45; V5 Placebo 2.34; 0.70; 0.43
No significant difference is seen between the treatment groups [p=0.62]
Mean denoted by crossed-circle.

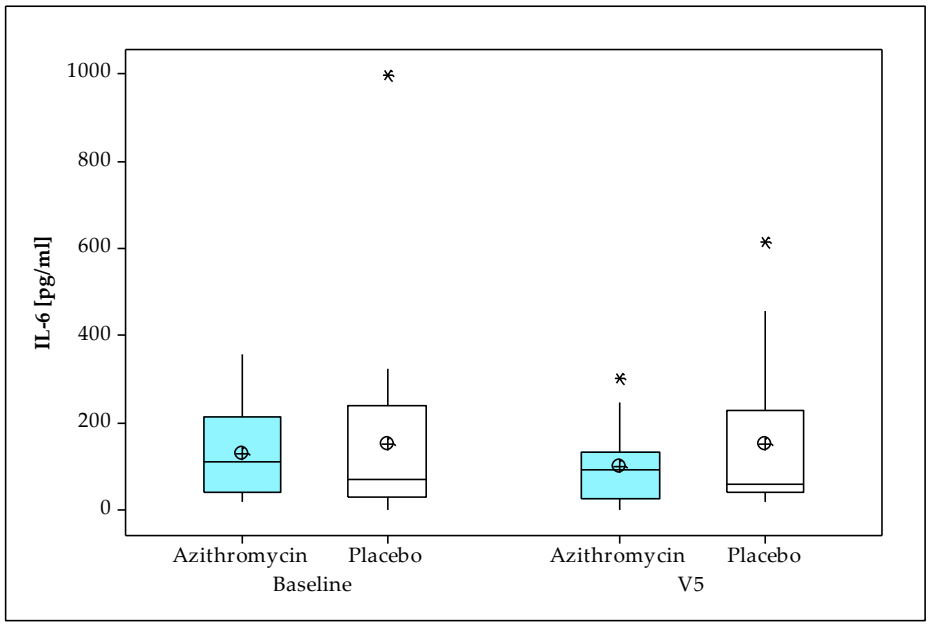
6.3.6. IL-6

There was no significant difference in sputum IL-6 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.14 [Fisher's exact test]. In this instance there was sufficient change to allow for calculation of a mean difference, -40.1 95%CI -90.6 to 10.4, p=0.12. Both statistical methods support this difference as not significant. Mean [SD] for each and mean change is displayed in Table 6.29 and graphically in Figure 6.20.

Table 6.29: Change from baseline to 12 weeks in sputum supernatant IL-6 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	140.9	171.8	21	151.5	220.0	19	129.0	99.6
Visit 5	40	125.8	131.3	21	151.3	161.0	19	97.7	83.6
Δ baseline to Visit 5	40	-15.0	103.7	21	-0.3	118.6	19	-31.4	84.3
Mean Difference [95% CI]									-40.1 [-90.6 , 10.4], p=0.12

Figure 6.20: Sputum supernatant IL-6 [pg/ml] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.12]
Mean denoted by crossed-circle and outliers with asterisk.

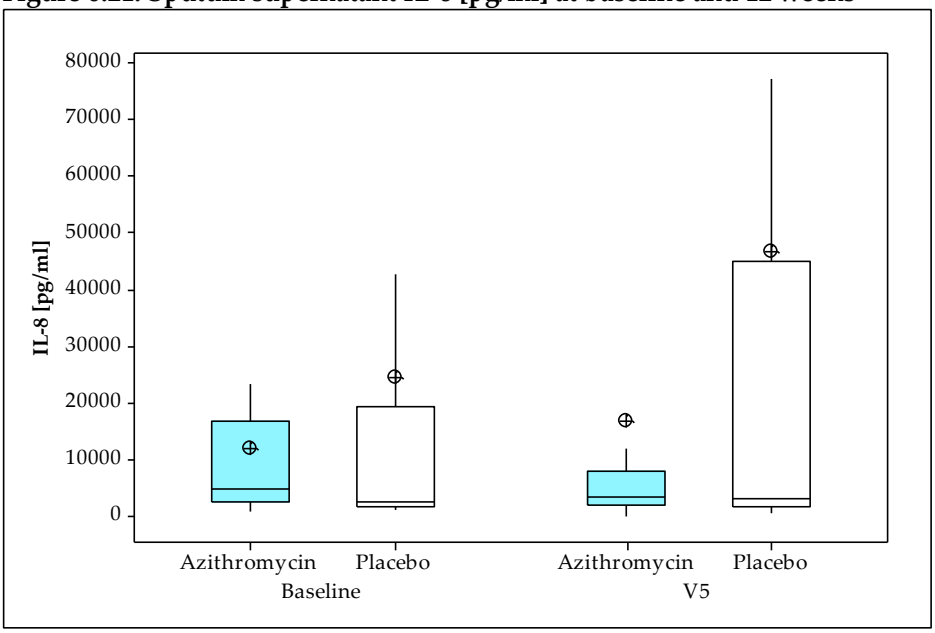
6.3.7. IL-8

There was no significant difference in sputum IL- in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.34 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in and graphically in Table 6.30 and graphically in Figure 6.21.

Table 6.30: Change from baseline to 12 weeks in sputum supernatant IL-8 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	18481.9	42434.4	21	24491.5	56067.3	19	11839.7	17886.9
Visit 5	40	32474.1	72387.6	21	46795.1	85443.2	19	16645.7	52324.3
Δ baseline to Visit 5		13992.2	62861.2		22303.6	71555.0		4806.0	51989.9
Test of association									p=0.34

Figure 6.21: Sputum supernatant IL-8 [pg/ml] at baseline and 12 weeks



Outliers not shown: Baseline Azithromycin 14441; Baseline Placebo 154800; 232200; V5 Azithromycin 232200; V5 Placebo 232200; 232200;154800; 77145
No significant difference is seen between the treatment groups [p=0.34]
Mean denoted by crossed-circle.

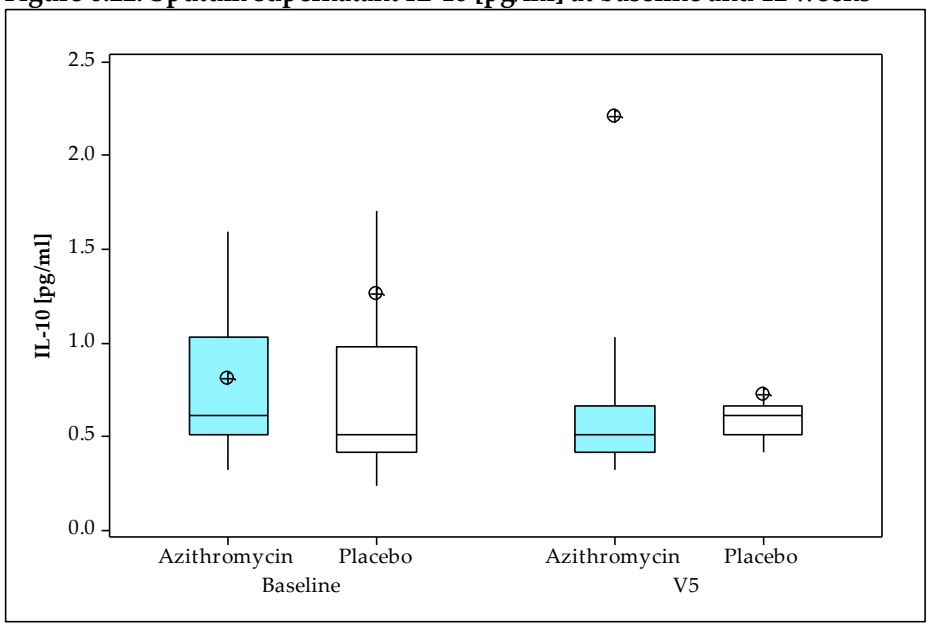
6.3.8. IL-10

There was no significant difference in sputum IL-10 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.92 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.31 and graphically in Figure 6.22.

Table 6.31: Change from baseline to 12 weeks in sputum supernatant IL-10 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	1.1	1.8	21	1.3	2.4	19	0.8	0.6
Visit 5	40	1.4	4.7	21	0.7	0.4	19	2.2	6.9
Δ baseline to Visit 5	40	0.4	5.0	21	-0.5	2.2	19	1.4	6.9
Test of association									p=0.92

Figure 6.22: Sputum supernatant IL-10 [pg/ml] at baseline and 12 weeks



Outliers not shown: Baseline Azithromycin 2.59; Baseline Placebo 11.31; 3.17; V5 Azithromycin 30.51; V5 Placebo 1.82; 1.53; 1.47; 1.14
No significant difference is seen between the treatment groups [p=0.92]
Mean denoted by crossed-circle.

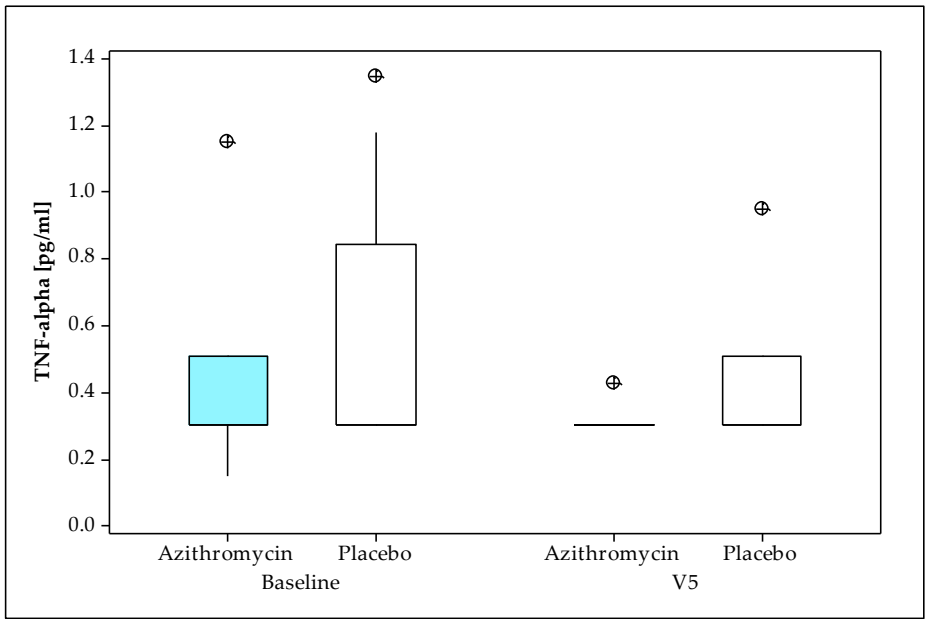
6.3.9. TNFα

There was no significant difference in sputum TNFα in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was not possible because there were too few patients with a change value between visits to allow for formal testing. Mean [SD] for each and mean change is displayed in Table 6.32 and graphically in Figure 6.23 but the test of association has not been done. We would therefore regard this assay as having no change as a result of the azithromycin.

Table 6.32: Change from baseline to 12 weeks in sputum supernatant TNFα [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	1.3	2.7	21	1.4	3.0	19	1.2	2.3
Visit 5	40	0.7	1.3	21	1.0	1.8	19	0.4	0.4
Δ baseline to Visit 5	40	-0.6	2.8	21	-0.4	3.3	19	-0.7	2.3
Test of association									N/D

Figure 6.23: Sputum supernatant TNFα [pg/ml] at baseline and 12 weeks



Outliers not shown: Baseline Azithromycin 8.92; 5.77; 1.18; 1.18; Baseline Placebo 13.92; 3.84; 2.99; V5 Azithromycin 2.11; 0.51; 0.51; 0.51; V5 Placebo 8.40; 2.40; 1.80; 1.18
No significant difference is seen between the treatment groups [p=N/D]
Mean denoted by crossed-circle.

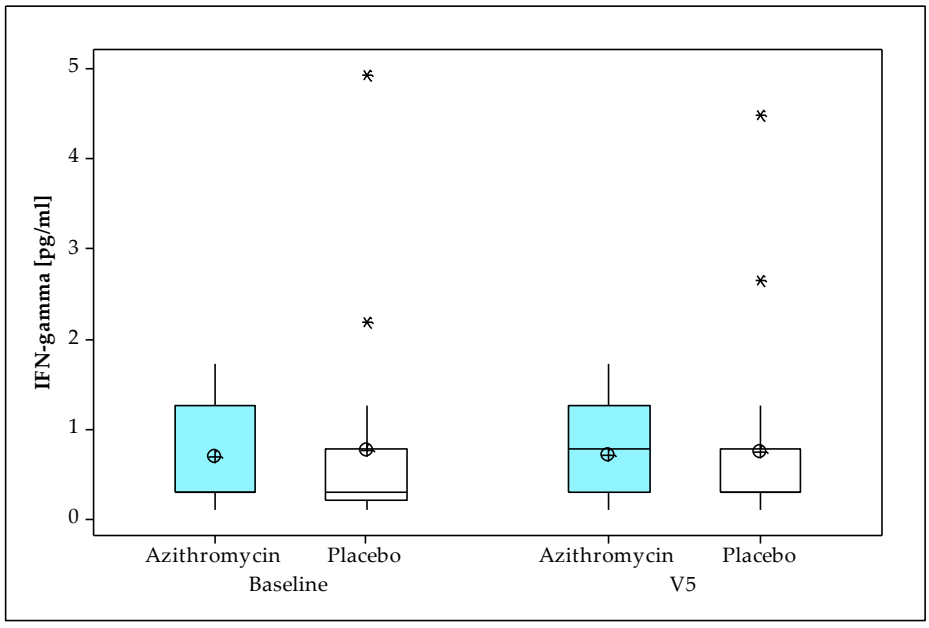
6.3.10. IFNγ

There was no significant difference in sputum IFNγ in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.72 [Pearson's Chi-Squared]. Mean [SD] for each and mean change is displayed in Table 6.33 and graphically in Figure 6.24.

Table 6.33: Change from baseline to 12 weeks in sputum supernatant IFNγ [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	0.7	0.9	21	0.8	1.1	19	0.7	0.5
Visit 5	40	0.7	0.8	21	0.7	1.0	19	0.7	0.5
Δ baseline to Visit 5	40	0	0.7	21	0.0	0.7	19	0.0	0.6
Test of association									p=0.72

Figure 6.24: Sputum supernatant IFNγ [pg/ml] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.72]
Mean denoted by crossed-circle and outliers with asterisk.

6.3.11. GM-CSF

There was no significant difference in sputum GM-CSF in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was not possible because there were too few patients with a change value between visits to allow for formal testing. Mean [SD] for each and mean change is displayed in Table 6.34 but the test of association has not been done. We would therefore regard this assay as having no change as a result of the azithromycin.

Table 6.34: Change from baseline to 12 weeks in sputum supernatant GM-CSF [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	0.2	0.6	21	0.3	0.8	19	0.1	0.1
Visit 5	40	0.1	0.0	21	0.1	0.0	19	0.1	0.0
Δ baseline to Visit 5	40	-0.1	0.6	21	-0.2	0.8	19	0.0	0.1
Test of association									N/D

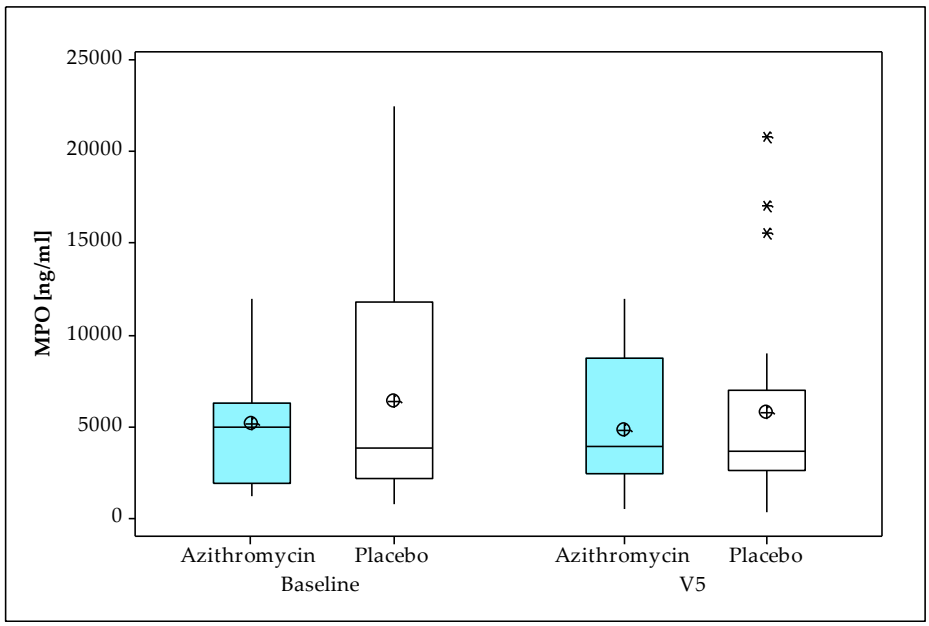
6.3.12. MPO

There was no significant difference in sputum MPO in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.76 [Fisher's exact test]. In this instance there was sufficient change to allow for calculation of a mean difference, -255.3 95%CI -2812.3 to 2301.8, p=0.84. Both statistical methods support this difference as not significant. Mean [SD] for each and mean change is displayed in Table 6.35 and graphically in Figure 6.25.

Table 6.35: Change from baseline to 12 weeks in sputum supernatant MPO [ng/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	5772.5	4655.7	21	6367.5	5658.1	19	5114.9	3243.9
Visit 5	40	5276.5	4629.8	21	5724.5	5529.6	19	4781.3	3460.7
Δ baseline to Visit 5	40	-496.0	4375.1	21	-643.1	5363.3	19	-333.5	3075.6
Test of association								-255.3 [-2812.3 , 2301.8], p=0.84	

Figure 6.25: Sputum supernatant MPO [ng/ml] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.84]
Mean denoted by crossed-circle and outliers with asterisk.

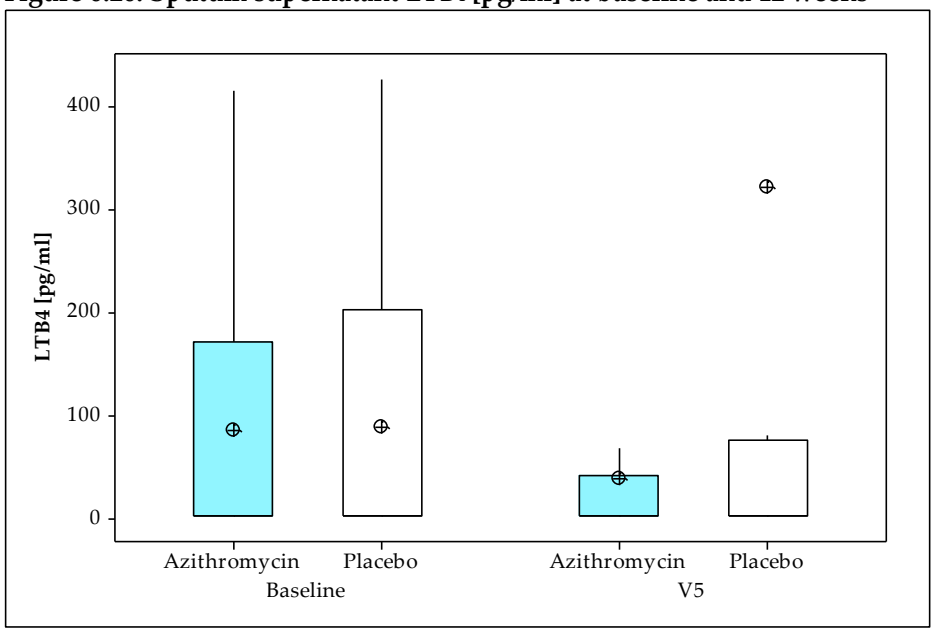
6.3.13. LTB_4

There was no significant difference in sputum LTB_4 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.84 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.36 and graphically in Figure 6.26.

Table 6.36: Change from baseline to 12 weeks in sputum supernatant LTB_4 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	88.0	134.8	21	90.0	132.4	19	85.9	141.1
Visit 5	40	187.7	883.6	21	322.6	1214.1	19	38.6	95.1
Δ baseline to Visit 5	40	99.7	846.1	21	232.6	1155.8	19	-47.2	153.3
Test of association									p=0.84

Figure 6.26: Sputum supernatant LTB_4 [pg/ml] at baseline and 12 weeks



Outliers not shown: V5 Azithromycin 410.72; 116.65; V5 Placebo 5596.66; 445.16; 308.73; 196.65
No significant difference is seen between the treatment groups [p=0.84]
Mean denoted by crossed-circle.

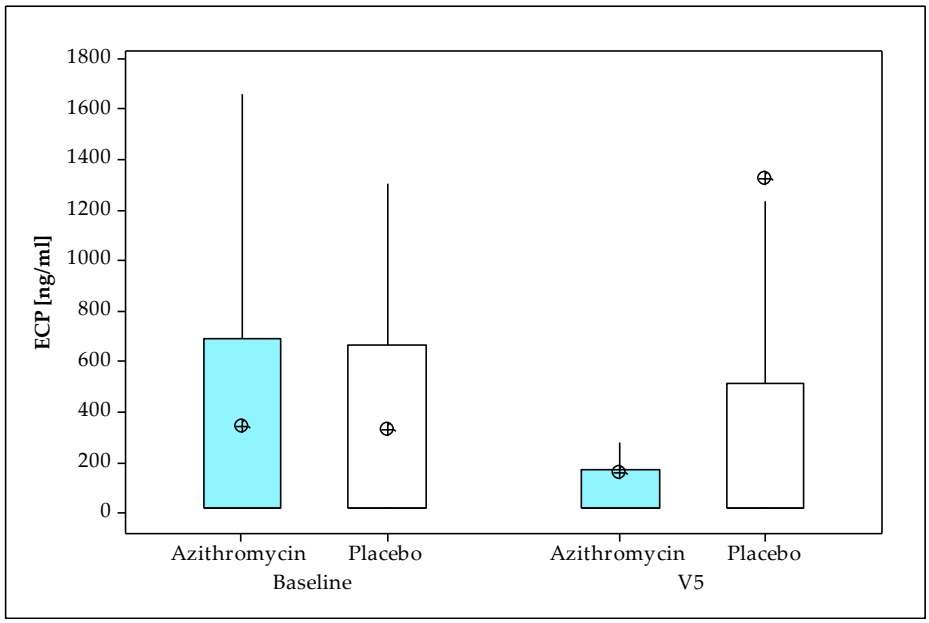
6.3.14. ECP

There was no significant difference in sputum ECP in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was not possible because there were too few patients with a change value between visits to allow for formal testing. Mean [SD] for each and mean change is displayed in Table 6.37 and graphically Figure 6.27 in but the test of association has not been done. We would therefore regard this assay as having no change as a result of the azithromycin.

Table 6.37: Change from baseline to 12 weeks in sputum supernatant ECP [ng/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	334.9	538.7	21	327.2	528.4	19	343.4	564.3
Visit 5	40	768.0	3532.4	21	1323.1	4849.8	19	154.5	380.3
Δ baseline to Visit 5	40	433.1	3380.0	21	995.9	4608.6	19	-188.9	613.1
Test of association									N/D

Figure 6.27: Sputum supernatant ECP [ng/ml] at baseline and 12 weeks



Outliers not shown: Baseline Placebo 1706.20; V5 Azithromycin 1642.90; 507.30; V5 Placebo 22386.00; 1780.60

No significant difference is seen between the treatment groups [p=N/D]

Mean denoted by crossed-circle and.

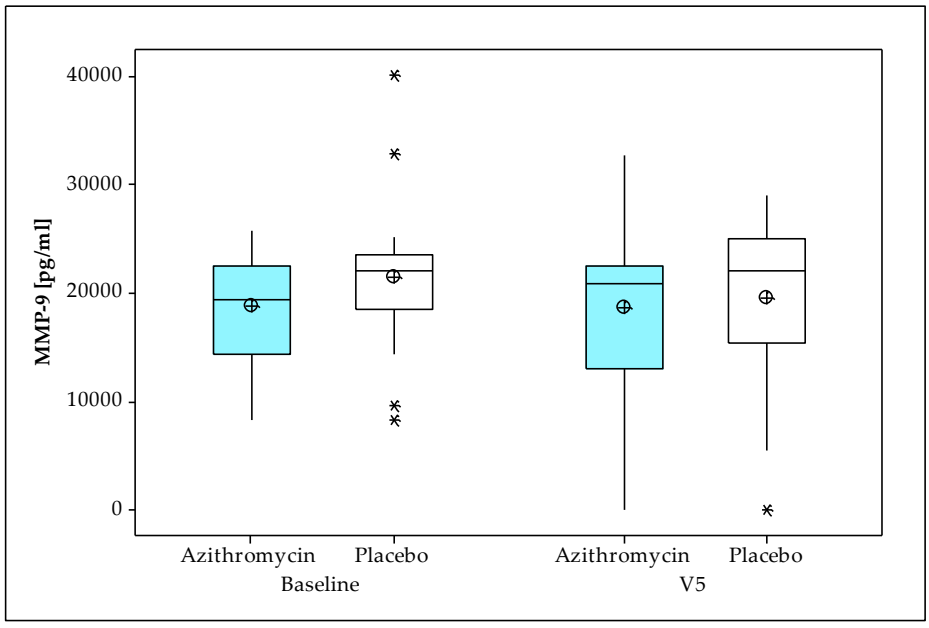
6.3.15. MMP-9

There was no significant difference in sputum MMP-9 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.75 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.38 and graphically in Figure 6.28.

Table 6.38: Change from baseline to 12 weeks in sputum supernatant MMP-9 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	20194.5	6159.3	21	21481.1	6767.6	19	18772.4	5219.5
Visit 5	40	19156.9	7552.4	21	19617.2	7910.0	19	18648.1	7317.3
Δ baseline to Visit 5		-1037.6	7347.4		-1863.8	7351.8		-124.3	7431.9
Test of association									p=0.75

Figure 6.28: Sputum supernatant MMP-9 [pg/ml] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.]
Mean denoted by crossed-circle and outliers with asterisk.

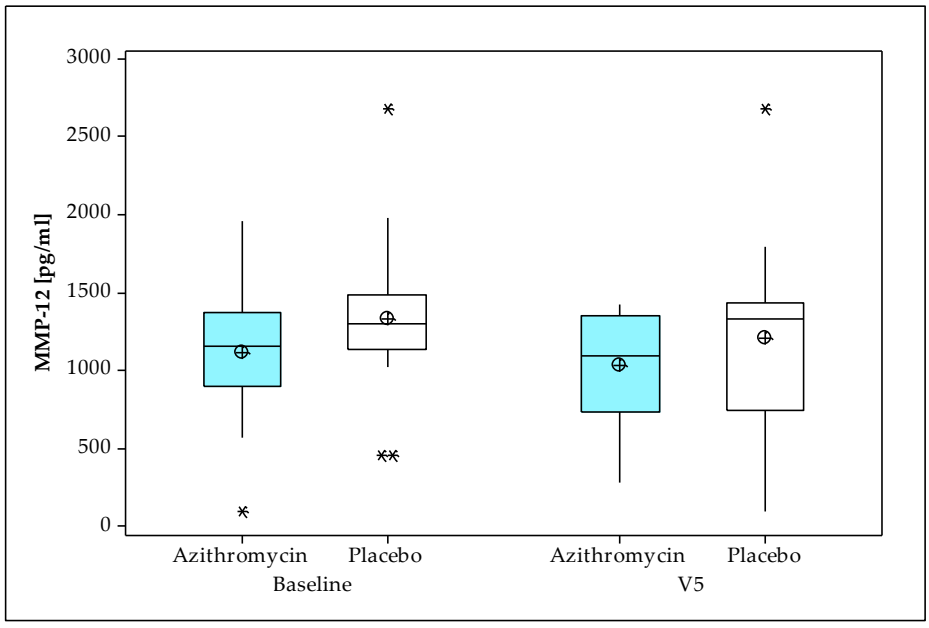
6.3.16. MMP-12

There was no significant difference in sputum MMP-12 in the azithromycin treated group after 12 weeks when compared to placebo. The test of association between treatment group and the selected variable was non-significant p=0.52 [Fisher's exact test]. Mean [SD] for each and mean change is displayed in Table 6.39 and graphically in Figure 6.29.

Table 6.39: Change from baseline to 12 weeks in sputum supernatant MMP-12 [pg/ml]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	40	1227.1	459.1	21	1332.1	477.9	19	1111.1	419.3
Visit 5	40	1124.7	483.5	21	1210.9	565.6	19	1029.5	364.5
Δ baseline to Visit 5		-102.4	424.0		-121.2	517.9		-81.6	301.0
Test of association]									p=0.52

Figure 6.29: Sputum supernatant MMP-12 [pg/ml] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.52]
Mean denoted by crossed-circle and outliers with asterisk.

6.4.Monocyte response to LPS stimulation

Blood monocytes from each treatment group were stimulated with LPS, either alone or in combination with varying concentrations of dexamethasone [10^{-6}, 10^{-8} and 10^{-10} mmol/L]. Appropriate controls were used. Cell culture supernatant was then collected and assayed for a panel of cytokines. This was intended not only to determine if there was any change in behaviour of these cells but to further examine if azithromycin had altered the cells to be respond differently to corticosteroid. Samples of each treatment group were assayed similar to above [n=20, each group]. The full panel of cytokines measured is detailed in Table 6.40.

Table 6.40: Cytokines measured during monocyte response to LPS

IL-1β
IL-2
IL-5
IL-6
IL-8
IL-10
TNFα
GM-CSF

Following the assays detailed above there was no evidence of any change in the behaviour of the monocytes to stimulation when the two groups were compared. Statistical tests of association were performed and no p values were found to be below or near 0.05, in any of the test conditions. The data is extensive, consistently negative and was felt of little value to include in any further detail.

6.5.Lymphocyte proliferation

No significant difference was seen in lymphocyte proliferation between baseline and 12 weeks following treatment with azithromycin. Lymphocyte proliferation assays were carried out with PHA mitogen, and PHA with varying concentrations of dexamethasone [10^{-6}, 10^{-8} and 10^{-10} mmol/L]. Appropriate controls were used. This would also help in determining if any sensitisation to corticosteroid had developed following treatment with azithromycin. Only the proliferation assays for the control group [no stimulation] and PHA alone have been displayed – Table 6.41 and Table 6.42. In the control group there was no significant difference in proliferation as measured by counts/min/cell of incorporated radioactive tracer [Fishers exact test, p=1.00] when the azithromycin group was compared to the placebo group at 12 weeks. Similarly there was no change in proliferation between the treatment and placebo groups when the cells were stimulated with PHA [Fishers exact test, p=1.00].

Table 6.41: Change from baseline to 12 weeks in proliferation [cpm/cell] – control

Variable	Total			Placebo			Azithromycin		
Control	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	14	77.7	35.5	7	67.6	31.3	7	87.9	38.8
Visit 5	14	77.0	48.8	7	78.4	52.5	7	75.6	48.9
Δ baseline to Visit 5		-0.7	69.1		10.8	59.1		-12.3	80.8
Test of association									p=1.00

Counts/min/cell

Table 6.42: Change from baseline to 12 weeks in proliferation [cpm/cell] - PHA alone

Variable	Total			Placebo			Azithromycin		
PHA alone	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	14	6978.2	7956.0	7	10769.7	7898.0	7	3186.7	6420.7
Visit 5	14	6714.5	12103.7	7	9480.5	16435.2	7	3948.5	5426.8
Δ baseline to Visit 5		-263.7	13597.2		-1289.2	17381.0		761.8	9799.4
Test of association									p=1.00

Counts/min/cell

In order to assess if there was any influence of the azithromycin in the steroid response during stimulation of lymphocytes, an interaction analysis was performed. This did not demonstrate any evidence of sensitisation of the lymphocytes by the azithromycin and hence there was no measureable change in responsiveness to mitogen. Details of the statistical analysis can be found in Table 6.43.

Table 6.43: Interaction analysis
Change from baseline to 12 weeks in lymphocyte proliferation [cpm/cell] with dexamethasone concentration

Dose	Estimate and 95% CI	p-value
Nothing [control]	-	0.52
Dexamethasone 10^{-10}	7850 [-5880, 21580]	0.25
Dexamethasone 10^{-8}	7075 [-7006, 21155]	0.31
Dexamethasone 10^{-6}	821 [-13267, 14908]	0.91

6.6. Exhaled gases

6.6.1. F_ENO_{50}

At 12 weeks, the mean difference in F_ENO_{50} for azithromycin compared with placebo was statistically no different [mean difference -0.9ppb 95%CI -5.3 to 3.4, p=0.67]. A

detailed breakdown of these results can be found in Table 6.44. Mean [SD] for each and mean change is displayed in and graphically in

Table 6.44: Change from baseline to 12 weeks in Exhaled Nitric Oxide at 50ml/s flow rate [ppb]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	13.5	16.2	38	15.6	21.2	39	11.5	8.9
Visit 5	71	13.6	15.3	35	16.2	20.1	36	11	7.9
Δ baseline to Visit 5	71	0.3	9.1	35	0.8	9.4	36	-0.2	9
Mean Difference [95% CI]									-0.9 [-5.3 , 3.4], p=0.67

6.6.2. Alveolar Nitric Oxide

Alveolar nitric oxide concentrations can be derived using multiple methods[235], utilising both linear and non-linear methods, both providing slightly differing results. No normal ranges are currently available for smokers with asthma and no consensus yet exists on which derivation method should be used as both have their advantages. For the purposes of our study, we have provided an analysis using both methods. The concentration alveolar NO [C_{alv}] increased by a statistically significant amount in the azithromycin group when compared to the placebo group, mean difference 1.3ppb 95% CI 0.3 to 2.3, p=0.01. This statistical difference was not observed when utilising then non-linear method, mean difference 0.7ppb 95% CI -0.86 to 2.26, p=0.38. These results are detailed in Table 6.45 and Table 6.46.

Table 6.45: Change from baseline to 12 weeks in Alveolar Nitric Oxide [ppb], [linear regression method]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	62	1.9	1.4	31	2.1	1.6	31	1.7	1.1
Visit 5	62	2.2	1.4	31	1.7	1.6	31	2.6	1.1
Δ baseline to Visit 5	53	0.4	1.9	28	-0.2	1.8	25	1.1	1.7
Mean Difference [95% CI]									1.3 [0.3 , 2.3], p=0.01

Table 6.46: Change from baseline to 12 weeks in Alveolar Nitric Oxide [ppb], [non-linear regression method]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	1.7	2.9	38	1.9	3.0	39	1.5	2.8
Visit 5	70	1.3	1.4	34	1.2	1.5	36	1.4	1.2
Δ baseline to Visit 5	70	-0.4	3.3	34	-0.7	3.4	36	-0.1	3.1
Mean Difference [95% CI]									0.7 [-0.9 , 2.3], p=0.38

6.6.3. Flux of nitric oxide

Flux of nitric oxide concentrations can also be derived using multiple methods [235, 236], utilising both linear [+/- correction] and non-linear methods, all providing slightly differing results. No normal ranges are currently available for smokers with asthma and no consensus yet exists on which derivation method should be used. For the purposes of our study, we have provided an analysed using the linear regression method with and without correction. The flux of NO [J'_{aw}] decreased with a tendency towards statistical significance when the azithromycin group was compared to the

placebo group, mean difference -304.6 pl/s 95% CI -622.1 to 12.8, p=0.06, detailed in Table 6.47. When the analysis was performed using the correction method, the p value tended further from significance [p=0.09]. The detailed results have not been tabulated as they do not add anything beyond the data in Table 6.47.

Table 6.47: Change from baseline to 12 weeks in Flux of Nitric Oxide [pl/s], [linear regression method]

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	74	714.1	880.8	36	822.9	1159.7	38	611.0	485.8
Visit 5	66	656.3	895.1	32	855.2	1158.1	34	469.1	493.7
Δ baseline to Visit 5		5.04	652.9		164.4	721.9		-140.3	554.5
Mean Difference [95% CI]									-304.6 [-622.1 , 12.8], p=0.06

6.7. Inflammatory markers results – summary

In summary, there was no substantial difference in every measure of inflammation measured in sputum and plasma, and whilst there was a measured difference in alveolar NO when this was analysed using a separate method, significance was not found. Overall, it is reasonable to say that there was no consistent improvement in measures of inflammation following 12 weeks treatment with azithromycin when compared to placebo. It is difficult to account for the changes observed in alveolar NO, but having no other supporting improvements leaves this finding isolated and difficult to interpret with any certainty.

6.8. Correlations

6.8.1. ACQ, AQLQ and LCQ

There was no correlation between efficacy on the primary outcome and effect of treatment on the change in ACQ, AQLQ or LCQ, Table 6.48. None of the individual AQLQ or individual LCQ domains demonstrated any correlation [data not shown]. p-values were not calculated but confidence intervals have been provided.

Table 6.48: Pearson's correlation coefficient for relationship between efficacy on the primary outcome and effect of treatment on ACQ, AQLQ and LCQ

Variable	Number of observations	Pearson's *r*	Approximate 95% CI
ACQ	71	-0.14	-0.37, 0.1
AQLQ	71	-0.01	-0.24, 0.23
LCQ	71	-0.01	-0.25, 0.23

6.8.2. Methacholine PC_{20}

There was no correlation between efficacy on the primary outcome and effect of treatment on the change in methacholine PC_{20}, Table 6.49. As before, p-values were not calculated but confidence intervals have been provided.

Table 6.49: Pearson's correlation coefficient for relationship between efficacy on the primary outcome and effect of treatment on Methacholine PC_{20} [mg/ml]

Variable	Number of observations	Pearson's *r*	Approximate 95% CI
Methacholine PC_{20}	61	-0.15	-0.39, 0.11

6.8.3. Percentage Cell Counts

There was no correlation between efficacy on the primary outcome and effect of treatment on the change in percentage neutrophils, eosinophils and macrophages, Table 6.50. As before, p-values were not calculated but confidence intervals have been provided.

Table 6.50: Pearson's correlation coefficient for relationship between efficacy on the primary outcome and effect of treatment on percentage cell counts

Variable	Number of observations	Pearson's *r*	Approximate 95% CI
Change in % Neutrophils	57	0.03	-0.2
Change in % Eosinophils	57	-0.06	-0.3, 0.2
Change in % Macrophages	57	-0.08	-0.3, 0.2

6.9. Correlations summary

No correlation was seen between the primary endpoint and various indices of asthma control and markers of airway inflammation. Multiple other comparisons were derived but none of these were found to have any correlation, data not shown. In the interests of brevity only a selection of relevant measures were detailed.

6.10. Bacteriology & Virology

6.10.1. Bacterial Colony Counts

Bacterial culture and colony counts were performed on every subject who provided sputum plugs with sufficient volume. Culture and colony counts were performed on a total of 55 subjects at baseline [placebo n=26; azithromycin n=29] and 52 subjects at 12 weeks [placebo n=24; azithromycin n=28]. Only subjects with results before and after treatment could be analysed for comparison and this is detailed in Table 6.51. There were very few positive cultures and colony counts provided data that changed very little from baseline to 12 weeks between the two groups. Therefore Fisher's exact test of association was performed and found to be non-significant, p=0.33. This suggests that treatment was not associated with any change in bacterial colony counts.

Table 6.51: Changes in bacterial colony counts from baseline to 12 weeks

Variable	n	Decreasing	No change	Increasing
Placebo	22	7 [31.8%]	11 [50%]	4 [18.2%]
Azithromycin	25	9 [36%]	14 [56%]	2 [8%]
Fishers exact test of association				p=0.33

6.10.2. Serological measurements

6.10.2.1. Mycoplasma antibody status

Of the 71 subjects who completed the study all had serum tested at baseline and 12 weeks for the presence of antibodies to *M pneumonia*. All subjects [n=77, 100%] tested negative for these antibodies at baseline. Of the subjects who completed the study [n=71] none tested positive. Two subjects [one in each group] had equivocal results. This was not significant [p=0.98]. The results are summarised in Table 6.52. In

support of these findings, no subject tested positive for *M pneumonia* by PCR of sputum.

Table 6.52: Mycoplasma antibody status at baseline and 12 weeks

Parameter	Total[n=71]	Placebo[n=35]	Oral Azithromycin [n=36]
Negative to Positive	0 [0%]	0 [0%]	0 [0%]
Equivocal to Positive	0 [0%]	0 [0%]	0 [0%]
Negative to Equivocal	2 [2.8%]	1 [2.9%]	1 [2.8%]
No change	**69 [97.2%]**	**34 [97.1%]**	**35 [97.2%]**
Equivocal to Negative	0 [0%]	0 [0%]	0 [0%]
Positive to Equivocal	0 [0%]	0 [0%]	0 [0%]
Positive to Negative	0 [0%]	0 [0%]	0 [0%]
Treatment effect estimate for Oral Azithromycin – Placebo [95%CI]			1.0 [0.1 , 16.2] p=0.98

6.10.2.2. Chlamydia antibody status

In chlamydial infection the subject first develops mucosal IgA and then subsequently IgG. Infection is confirmed if both are present when tested or when IgG is present in high concentrations. At baseline none of the 77 randomised subjects were positive for either IgG. Three subjects tested positive for IgA but by 12 weeks, none of the 71 subjects who completed the study tested positive for IgG and the same number tested positive to IgA. IgG testing is detailed in Table 6.53. This was deemed non-significant [p=0.96] for *C. pneumonia* IgG. Formal assessment of *C. pneumonia* IgA could not be undertaken as there were too few change values, Table 6.54. This result should be regarded as non-significant. Sputum PCR testing was also performed and was negative amongst all subjects at baseline and 12 weeks.

Table 6.53: *Chlamydia pneumoniae* antibody status [IgG] at baseline and 12 weeks

Parameter	Total [n=61]	Placebo [n=30]	Oral Azithromycin [n=31]
Negative to Positive	0 [0%]	0 [0%]	0 [0%]
Equivocal to Positive	0 [0%]	0 [0%]	0 [0%]
Negative to Equivocal	2 [3.3%]	1 [3.5%]	1 [3.2%]
No change	**56 [93.3%]**	**27 [93.1%]**	**29 [93.6%]**
Equivocal to Negative	2 [3.3%]	1 [3.5%]	1 [3.2%]
Positive to Equivocal	0 [0%]	0 [0%]	0 [0%]
Positive to Negative	0 [0%]	0 [0%]	0 [0%]
Treatment effect estimate for Oral Azithromycin – Placebo [95%CI]			0.9 [0.1 , 15.6] p=0.96

Table 6.54: *Chlamydia pneumoniae* antibody status [IgA] at baseline and 12 weeks

Parameter	Total [n=71]	Placebo [n=35]	Oral Azithromycin [n=36]
Negative to Positive	0 [0%]	0 [0%]	0 [0%]
Equivocal to Positive	0 [0%]	0 [0%]	0 [0%]
Negative to Equivocal	2 [2.8%]	0 [0%]	2 [5.6%]
No change	**68 [95.8%]**	**34 [97.1%]**	**34 [94.4%]**
Equivocal to Negative	1 [1.4%]	1 [2.9%]	0 [0%]
Positive to Equivocal	0 [0%]	0 [0%]	0 [0%]
Positive to Negative	0 [0%]	0 [0%]	0 [0%]
Treatment effect estimate for Oral Azithromycin – Placebo			N/D

6.10.2.3. Virological PCR status

No formal analysis was undertaken for these results due to the very low numbers of positivity found during the study. PCR was performed on a panel of upper airway respiratory viruses and also *M. pneumoniae*. The panel of viruses tested can be found in Table 6.55.

Table 6.55: Panel for upper airway respiratory PCR analysis

Influenza A and B
Adenovirus
Respiratory Syncytial Virus
Parainfluenza 1, 2, 3 and 4
Rhinovirus
Metapneumovirus
C pneumoniae
M pneumoniae

Of the 77 randomised patients only 5 subjects produced a positive result. All of these results were for rhinovirus, and only one patient produced a positive result at both baseline and 12 weeks [both rhinovirus].

6.10.3. Summary of bacteriology and virology results.

Bacterial colony counts did not demonstrate any treatment difference between the placebo and azithromycin groups. Whilst unique organisms could be cultured in some individual subjects, resistance assays were not undertaken. PCR for *M. pneumoniae* and *C. pneumoniae* were all negative at both baseline and 12 weeks. This was supported by there being no substantial difference in serological positivity for both these organisms as measured by IgA and IgG for *C. pneumonia*, and IgM, *M. pneumonia*. The only positive result from viral screening was for rhinovirus, which was found in 6 sputum samples across 5 subjects, 1 subject was positive at baseline and 12 weeks.

6.11. Haematological outcomes

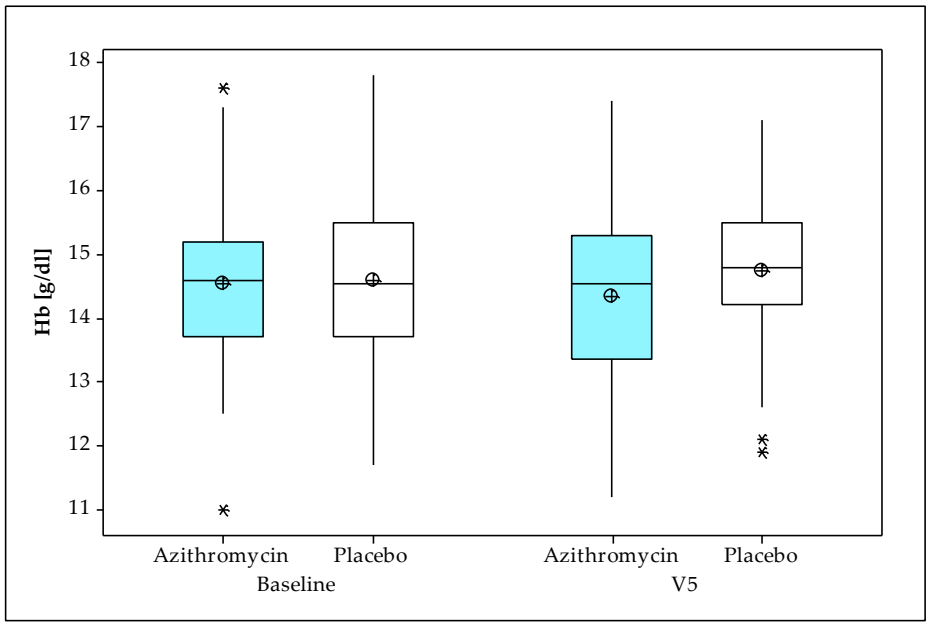
6.11.1. Haemoglobin concentration

Unexpectedly, following 12 weeks of treatment there was found to be a reduction in the mean haemoglobin concentration in the treatment group, by a small but statistically significant amount, mean difference -0.4g/dl 95%CI -0.8 to -0.1, p=0.02. Detailed results are displayed in Table 6.56 and are displayed graphically in Figure 6.30. This still falls within the normal range expected for an adult of male is 13 to 18 and adult female 11.5 to 16.5.

Table 6.56: Haemoglobin concentration [g/dl] at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	14.6	1.3	38	14.6	1.3	39	14.5	1.3
Visit 5	71	14.5	1.3	35	14.7	1.3	36	14.3	1.3
Δ baseline to Visit 5		0.0	0.8		0.2	0.7		-0.2	0.9
Mean Difference [95% CI]									-0.4 [-0.8 , -0.1], p=0.02

Figure 6.30: Haemoglobin concentration [g/dl] at 12 weeks



No significant difference is seen between the treatment groups [p=0.02]
Mean denoted by crossed-circle and outliers with asterisk.

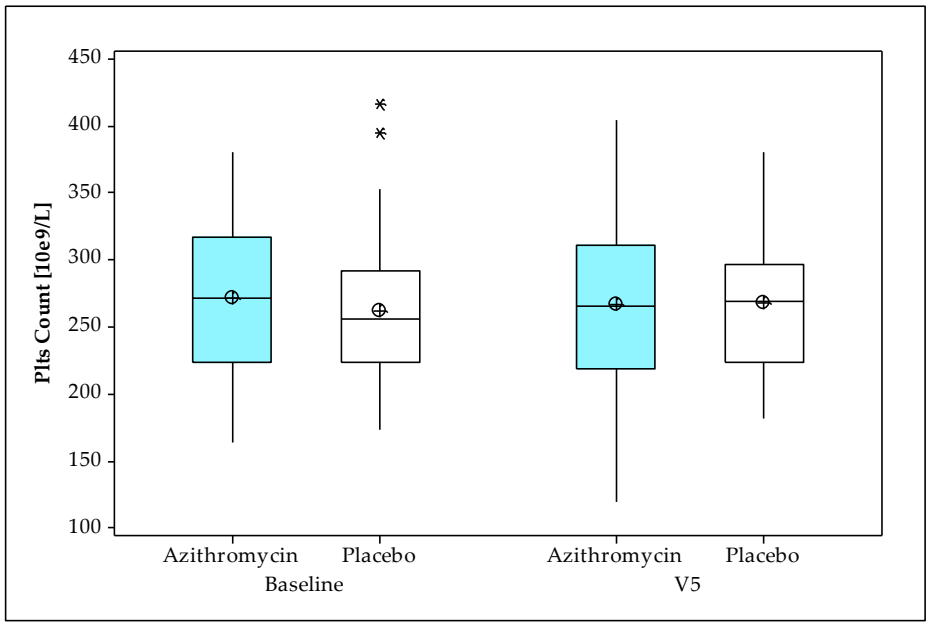
6.11.2. Platelet count

No effect was seen in either mean [SD] platelet count following 12 weeks treatment with azithromycin. The mean difference in was -6.06 $x10^9$/L [95%CI -19.88 to 7.77], p=0.38. The details of these measurements are displayed in Table 6.57, with a graphical summary of the datasets in Figure 6.31. The normal range for platelet concentration is 150 to 400 $x10^9$/L

Table 6.57: Platelet count [$x10^9$/L]at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	266.9	57.8	38	261.7	55.3	39	271.9	60.4
Visit 5	71	267.0	58.6	35	267.7	51.9	36	266.3	65.2
Δ baseline to Visit 5		-3.0	29.7		0.3	27.1		-6.3	32.0
Mean Difference [95% CI]									-6.1 [-19.9 , 7.8], p=0.39

Figure 6.31: Platelet concentration [x10⁹/L] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.39]
Mean denoted by crossed-circle and outliers with asterisk.

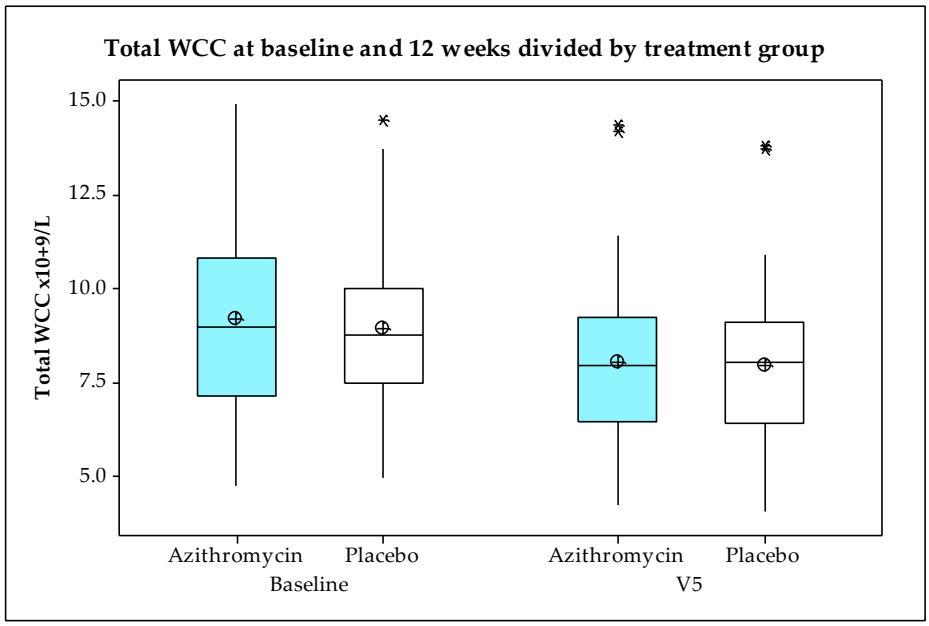
6.11.3. White cell count

No effect was seen in either mean [SD] platelet count following 12 weeks treatment with azithromycin. The mean difference in was 0.15 x10⁹/L [95%CI -0.66 to 0.96], p=0.71. The details of these measurements are displayed in Table 6.58, with a graphical summary of the datasets in Figure 6.32. The normal range for total white cell count is 4 to 11 x10⁹/L.

Table 6.58: Total white cell count [x10⁹/L] at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	9.1	2.4	38	9.0	2.2	39	9.2	2.5
Visit 5	71	8.2	2.0	35	8.1	1.9	36	8.3	2.2
Δ baseline to Visit 5		-0.96	2.1		-1.0	2.1		-0.9	2.2
Mean Difference [95% CI]									0.2 [-0.7 , 1.0], p=0.71

Figure 6.32: Total WCC [x10⁹/L] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.71]
Mean denoted by crossed-circle and outliers with asterisk.

6.11.4. Biochemical outcomes

6.11.5. Electrolytes and Creatinine

Serum electrolytes were measured at baseline and 12 weeks with none of them demonstrating any substantial difference when the azithromycin was compared to placebo. In the interests of succinctness only the mean differences have been summarised and can be found in Table 6.59.

Table 6.59: Summary mean differences for serum biochemistry following 12 weeks treatment

Parameter	Mean difference	95% CI	Significance
Sodium	0.2	-0.6 , 1.0	p=0.59
Potassium	0.0	-0.1 , 0.2	p=0.70
Chloride	-0.2	-1.2 , 0.8	p=0.68
Creatinine	-0.5	-3.5 , 2.6	p=0.76

6.11.6. Liver function tests

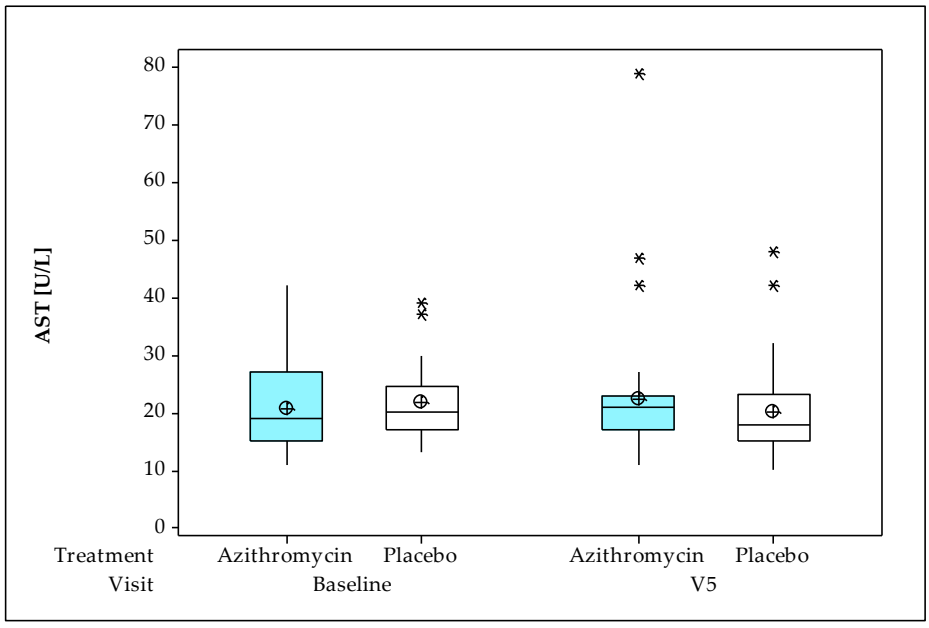
6.11.6.1. Aspartate aminotransferase

There was no substantial difference in the level of serum aspartate aminotransferase following 12 weeks treatment with azithromycin when compared to placebo. The mean difference was 2.2 U/L 95%CI -1.9 to 6.3, p=0.28. The normal range was anything below 40 U/L. Detailed results can be found in Table 6.60 with a graphical representation of the data set displayed in Figure 6.33.

Table 6.60: Serum AST [U/L] concentration at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	21.3	6.5	38	21.8	5.9	39	20.7	7.1
Visit 5	69	21.3	10.3	34	20.2	8.2	35	22.4	12.0
Δ baseline to Visit 5		-0.4	8.5		-1.6	6.6		0.7	10.0
Mean Difference [95% CI]									2.2 [-1.9 , 6.3], p=0.28

Figure 6.33: Serum AST [U/L] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.28]
Mean denoted by crossed-circle and outliers with asterisk.

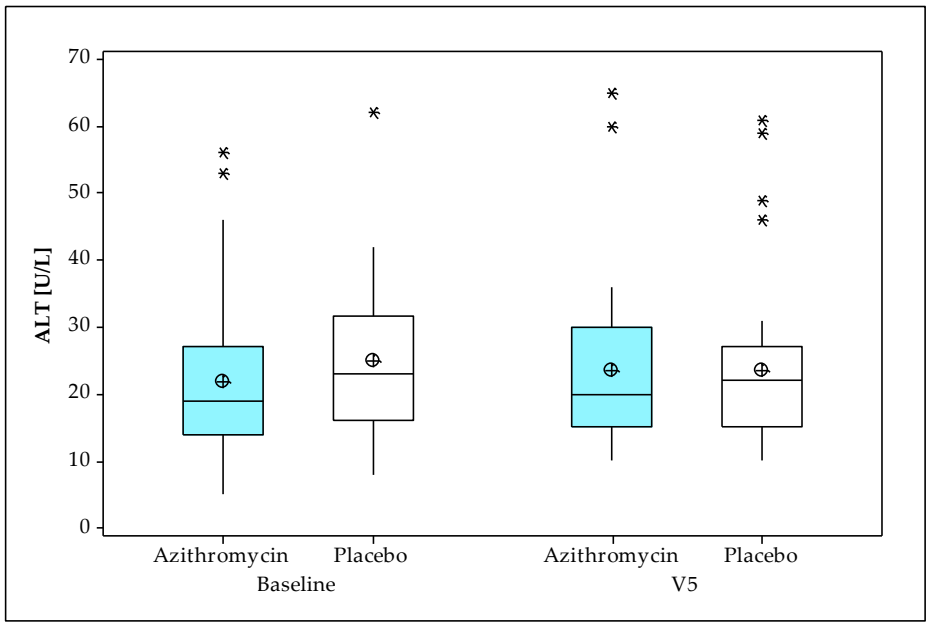
6.11.6.2. Alanine aminotransferase

There was no substantial difference in the level of serum alanine aminotransferase following 12 weeks treatment with azithromycin when compared to placebo. The mean difference was 1.39 U/L 95%CI -2.67 to 5.45, p=0.50. The normal range was anything below 50 U/L. Detailed results can be found in Table 6.61 with a graphical representation of the data set displayed in Figure 6.34.

Table 6.61: Serum ALT [U/L] concentration at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	23.3	11.61	38	24.87	11.46	39	21.77	11.71
Visit 5	70	23.53	12.38	35	23.51	12.65	35	23.54	12.28
Δ baseline to Visit 5		-0.61	8.74		-1.49	10.75		0.26	6.16
Mean Difference [95% CI]								1.39 [-2.67 , 5.45], p=0.50	

Figure 6.34: Serum ALT [U/L] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.50]
Mean denoted by crossed-circle and outliers with asterisk.

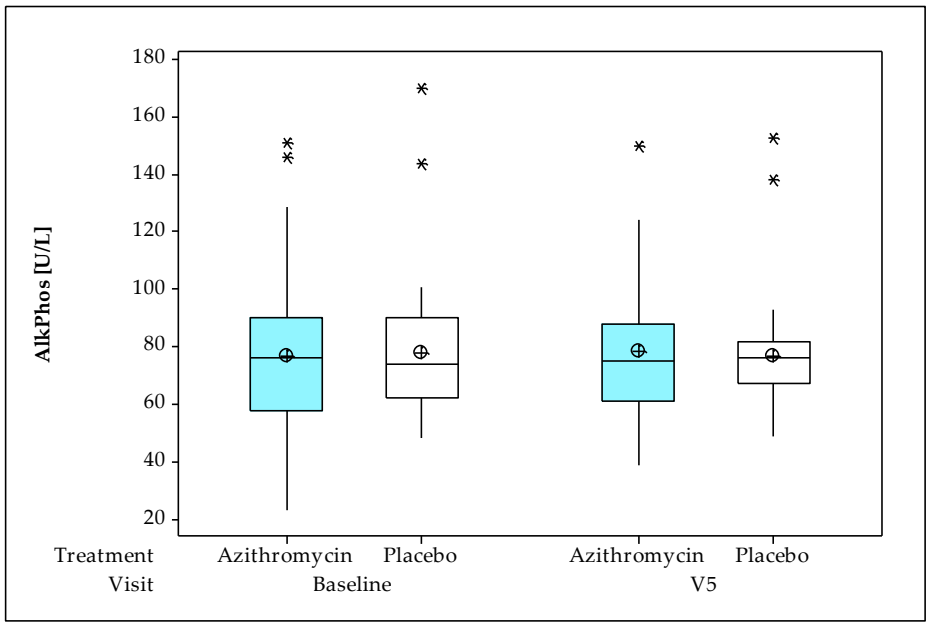
6.11.6.3. Serum Alkaline phosphatase

There was no substantial difference in the level of serum alkaline phosphatase following 12 weeks treatment with azithromycin when compared to placebo. The mean difference was 2.68 U/L 95%CI -2.46 to 7.83, p=0.30. The normal range was anything below 40 to 150U/L. Detailed results can be found in Table 6.62 with a graphical representation of the data set displayed in Figure 6.35.

Table 6.62: Serum Alkaline Phosphatase [U/L] concentration at baseline and 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	77.36	24.82	38	78.11	23.35	39	76.64	26.46
Visit 5	70	77.64	21.22	35	76.97	20.21	35	78.31	22.47
Δ baseline to Visit 5		-1.27	12.65		-2.86	8.58		0.31	15.67
Mean Difference [95% CI]									2.68 [-2.46 , 7.83], p=0.30

Figure 6.35: Serum Alkaline Phosphatase [U/L] at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.30]
Mean denoted by crossed-circle and outliers with asterisk.

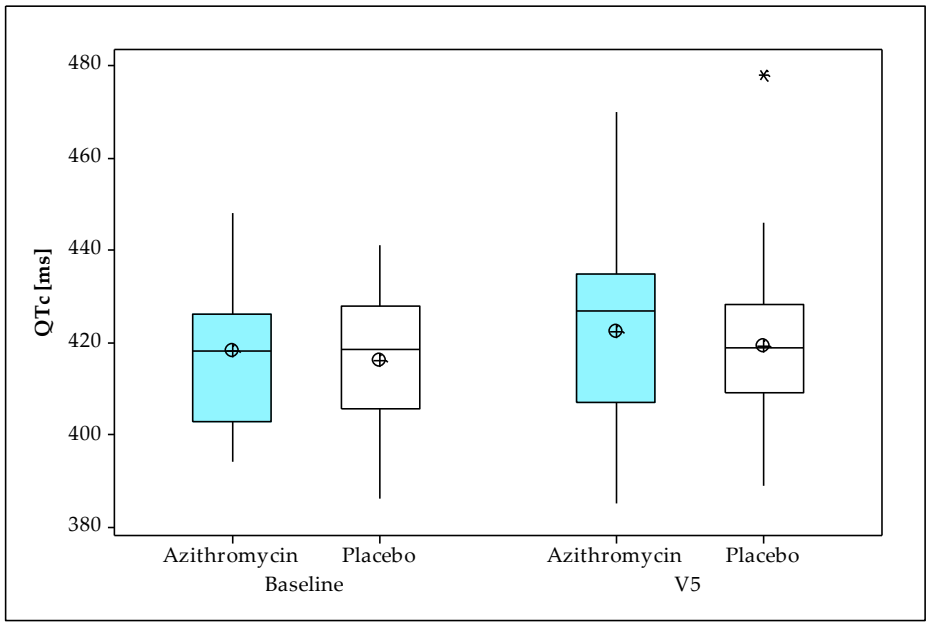
6.11.7. Electrocardiography

An electrocardiogram was recorded at baseline then again at the final study visit. Subjects were screen failed if the corrected-QT-interval was greater than 430ms in men or 450ms in women. There was no substantial change in the mean corrected-QT-interval between the azithromycin and placebo group following 12 weeks of treatment. The mean difference was 2.8ms 95%CI -5.08 to 10.68, p=0.48. The results are detailed in Table 6.63, and depicted graphically in Figure 6.36.

Table 6.63: Change in QTc interval [ms] from baseline to 12 weeks

Variable	Total			Placebo			Azithromycin		
	n	Mean	Standard Deviation	n	Mean	Standard Deviation	n	Mean	Standard Deviation
Baseline	77	417.01	15.09	38	415.95	14.75	39	418.05	15.55
Visit 5	69	420.72	18.86	34	419.12	18.3	35	422.29	19.53
Δ baseline to Visit 5		3.38	17.05		2.06	19.65		4.66	14.25
Mean Difference [95% CI]									2.8 [-5.08 , 10.68], p=0.48

Figure 6.36: QTc Interval [ms] measured at baseline and 12 weeks



No significant difference is seen between the treatment groups [p=0.48]
Mean denoted by crossed-circle and outliers with asterisk.

When the raw data is reviewed a total of 5 patients had prolongation of QTc, 4 in the azithromycin group and 1 in the placebo. In the treatment group 3 of these subjects had only slight prolongation of the QTc of <10ms above the upper limit of normal [ULN]. One subject in each group had QTc prolongation of >20ms above ULN. Only 3 people had QTc intervals above 450ms [all were female].

7. Regulatory reporting

7.1. Patients who did not complete the trial

7.1.1. Loss to follow up

Four patients were lost to follow-up after randomisation. Two subjects did not attend any further visits following randomisation with another subject attending only at the four week visit and the remaining subject attending at 8 weeks before non-attendance. Contact was attempted by telephone in all cases and messages left on several occasions to encourage attendance. One subject did re-contact the unit but this was out-with the timeframe for completion of all their study visits and they could not be incorporated back in to the trial. When made aware of this they informed the study team that they would not re-attend for a safety visit and returned all of the study materials by mail.

7.1.2. Patient withdrawals

Only one patient withdrew consent following randomisation. This was due in part to a relapse of alcohol abuse [reported as an SAE] and the patient deciding that treatment of the addiction took precedence and they could no longer commit to daily PEF monitoring, compliance with medication or attendance for study visits.

One patient was withdrawn due to an unrelated ailment requiring the prescription of a long-term antibiotic. This was an exclusion criterion for the study and it was felt treatment of the condition [hidradenitis suppurativa] should not be deferred.

7.2. Adverse events

No suspected unexpected serious adverse events [SUSARS] occurred during the reporting period of the study. There were more adverse events in the azithromycin group [52 events occurring in 34 subjects] than the placebo group [events in 23 subjects], and hence a single subject may account for multiple AE's. The breakdown of adverse events, [excluding asthma related and SAE's can be found in Table 7.1].

Four serious adverse events occurred – 3 during the study and 1 three weeks after the final visit. All 4 were in the azithromycin group. One was constipation, the second angina, and the third a relapse of alcoholism. The fatal SAE was recorded as myocardial infarction and occurred in a patient with known coronary artery disease, several cardiovascular risk factors and under regular cardiology follow-up. The event occurred three weeks after completion of the trial medication. The study team were not made aware of this until several months had passed and they were informed by a family member attending the unit for a different study. Despite the delay in the study team becoming aware of the death, an SAE was reported within the regulatory timeframe. The study protocol stated that AE's and SAE's required reporting up to 4 weeks following completion of study medication. Hence this event was reported as an SAE. There was no evidence to suggest this was an asthma related death or a death related to any adverse drug effect.

Table 7.1: Unique adverse events excluding asthma related and serious adverse events

Event type	Placebo	Azithromycin
Alcohol related	0	2
Ankle swelling	0	1
Assault	0	1
Back pain	6	2
Cellulitis	1	3
Common cold	1	5
Conjunctivitis	0	1
Constipation	0	1
Cough	1	1
Dental related	2	0
Dermatitis	2	1
Diarrhoea	3	4
Dyspepsia	1	1
Epistaxis	0	1
Gastroenteritis	0	7
Haemorrhoids	2	0
Headache	1	2
Hypothyroidism	1	0
Increasing breathlessness	0	1
Knee pain	0	2
Leg cramps	0	1
Malaise	2	1
Non-specific viral illness	2	0
Oral candidiasis	1	1
Otitis/vertigo	7	0
Psychiatric related	2	4
Recurrence of herpes virus	0	1
Respiratory tract infection	5	5
Sinus infection	2	0
Urinary Tract Infection	1	0
Vomiting	0	2
Total number of adverse events	42	52

QTc was recorded in every patient as an entrance criterion and again at 8 and 12 weeks as a safety measure. There was no significant difference between the two groups at 12 weeks and in particular was not abnormally prolonged in the subject

who died. Of the 3 adverse events during the study only one [relapse of alcoholism] led to withdrawal from the study. Gastrointestinal upset was uncommon with only 7 reported events, 5 in the azithromycin group and 2 in the placebo. Only 2 subjects reported diarrhoea in the azithromycin group.

7.2.1. Exacerbations rates

Recording the frequency of exacerbation in the randomised patients helps provide us with a crude impression of any difference between the treatment groups. In this study we recorded exacerbation severity as mild, moderate or severe based on the criteria discussed in section 4.3.3, Table 4.4, p107. Across both groups there were exacerbations throughout the study, reassuringly none were severe. There were an apparent higher number of exacerbations in the azithromycin group 12 [30.8%] subjects vs. 3 [7.9%] subjects in the placebo group. The study was not powered to assess differences in exacerbation frequency, but when the available data is analysed using Fisher's exact test there is no difference between the two groups or their severity of exacerbations, p=1.00]. The results for exacerbations can be found in Table 7.2.

Table 7.2: Severity of asthma exacerbations by treatment group

Asthma exacerbation category	Total [n=77]	Placebo [n=38]	Azithromycin [n=39]
Mild	10 [13.0%]	2 [5.3%]	8 [20.5%]
Moderate	5 [6.5%]	1 [2.6%]	4 [10.3%]
Severe	Nil	Nil	Nil
Total for all categories	15 [19.5%]	3 [7.9%]	12 [30.8%]

7.2.2. Asthma related adverse events

In addition to the recording of exacerbation frequency, other asthma related adverse events were also captured at each study visit. These included GP attendance

[unscheduled or home visit], A&E visit or hospital admission and treatment with oral steroids. There were only 3 asthma related events in total, all in the azithromycin group. Although this was never formally tested, it probably did not represent any statistically significant difference between the two groups. The results can be found in Table 7.3.

Table 7.3: Asthma related adverse events

	Total [n=77]	Placebo [n=38]	Azithromycin [n=39]
Oral steroid treatment	0	0	0
Unscheduled GP appointment	3 [3.9%]	0	3 [7.7%]
GP home visit	0	0	0
A&E visit	0	0	0
Hospital admission	0	0	0
Total asthma related adverse events	3 [3.9%]	0	3 [7.7%]

7.2.3. Possible drug related adverse events

The frequency of possible drug related adverse events were low with a total of 3 [7.9%] in the placebo group and 6 [15.4%] in the azithromycin group. The summary product characteristics of azithromycin report GI related side effects as the most common side effect found in approximately 1:10 patient treatments, this would include diarrhoea. Constipation is expected less frequently and is rarely seen, although there were 2 reports of this in the treatment group. The details of the reported possible drug related adverse effects can be found in Table 7.4.

Table 7.4: Possible adverse drug reactions

Event	Placebo n=38 Number of events [% total]	Azithromycin n=39 Number of events [%total]
Any adverse event	3 [7.9%]	6 [15.4%]
Constipation	0	2 [5.1%]
Diarrhoea	0	2 [5.1%]
Oral candidiasis		1 [2.6%]
Fatigue	1 [2.6%]	0
Back pain	1 [2.6%]	0
Cough	0	1 [2.6%]
Sore throat	1 [2.6%]	0

7.2.3.1. Haemoglobin concentration

Unexpectedly the haemoglobin concentration was found to have dropped in the treatment group. The cause for this was not clear. Azithromycin is not a drug recognised to cause alterations in the haemoglobin concentration. Three factors need to be considered in the interpretation of this drop.

Firstly, the drop was very small, only 0.4g/dl. Most automated analysers have will measure with a standard deviation of 0.4, which could considerably influence this result and hence it may not have fallen to a level that would be considered statistically significant. It could be argued therefore that the result is within the laboratory error of the assay and is not clinically significant.

Secondly, the drop as well as being small is still well within the normal range for both men and women and so would cause no physical symptoms in the study subjects.

Finally, in a study such as this, the result could simply be due to multiple testing.

Overall, whilst a statistically significant result was detected this is irrelevant due to the possibility of the confounding factors detailed above and hence the finding should be dismissed.

8. Discussion

8.1. Principal findings

This randomised controlled study examined the effects on lung function asthma control, airway inflammation and bacterial colonisation of azithromycin 250mg daily with matched placebo in adult smokers with asthma. The hypothesis was that azithromycin improves lung function, asthma control and airway inflammation in smokers with asthma. We found that azithromycin has no effect after 12 weeks treatment in a range of clinical indices, markers of inflammation or bacterial colonisation in smokers with chronic asthma. Immunological investigation did not demonstrate any changes which could be satisfactorily attributable to treatment with azithromycin.

8.1.1. Clinical effects

The lack of any clinical benefits of azithromycin in smokers with asthma cannot be compared with any other study. This was the first study utilising azithromycin, or any macrolide antibiotic, in smokers with asthma. It provides clear evidence that 12 weeks of azithromycin provides no benefit in smokers with asthma. Our study extends the findings by Albert et al [146] in COPD where current smokers did not derive any benefit in exacerbation frequency [primary outcome] when given azithromycin daily for one year.

8.1.1.1. Effects on lung function

The study presented here clearly demonstrates that 12 weeks treatment with 250mg azithromycin makes no difference to any of the lung function parameters measured in our patient group. There was no statistical difference in the primary outcome measure; PEF or any of the secondary outcomes of FEV_1 or FVC. In addition, all of

the derived measures e.g. % predicted or response to nebulised salbutamol was unchanged. Multiple studies of macrolides in asthma have been performed, and to date only one of these has demonstrated improvement in lung function[154]. Whilst this improvement was deemed statistically significant it was interpreted that the improvement of 3% had no clinical significance. Therefore our study remained consistent with the published literature.

Airways responsiveness measured by methacholine PC_{20} was unchanged in this study. This is different from many of the previously published findings in non-smoking asthmatics which demonstrate consistent evidence that macrolides, when given to non-smoking asthmatics have beneficial effects on bronchial hyper-responsiveness [156, 159, 160, 237]. No clear explanation can be given to account for the lack of response to treatment in our study and this is explored further in section 8.6.1.

8.1.1.2. Effects on symptom scores

Symptom scores were recorded in the patient diary and at clinic visits. The validated diary card recorded a modified ACQ. At each clinic visit the ACQ was recorded separately and at visits 2 and 5 two quality of life questionnaires, the LCQ and AQLQ were also recorded. Following the 12 week treatment period, none of the recorded questionnaires demonstrated any improvement. When the AQLQ and LCQ were broken down in to their individual domains, it was only the LCQ-psychological domain that demonstrated any change of statistical significance. This was an adverse change, with the true relevance unlikely to reflect any possibility that treating patients with azithromycin caused any deterioration in this measure. It could simply be explained by multiple testing leading to a significant result.

Only one study with azithromycin has demonstrated an improvement in AQLQ [238], but this was in non-smokers with asthma and required 6 months treatment. Other measures such as ACQ and EuroQOL 5D did not demonstrate any improvements[238] and so it is difficult to draw any firm conclusions of improvement following treatment. The published literature of clinical studies utilising other macrolides has failed to demonstrate any consistent improvements in patient reported symptom or quality of life scores. There is only one recent study by Koutsoubari et al [157] where there was clear evidence of improved symptom scores. The criticism of this study is that it was open-label and hence open to bias.

8.1.2. Effects on inflammatory outcomes

8.1.2.1. Serum measurements

8.1.2.1.1. Serum cytokine measurement

A wide-ranging panel of serum biomarkers were measured at baseline and 12 weeks. None of these demonstrated any statistically significant difference following 12 weeks treatment with azithromycin. We were uncertain if any serum biomarker response would be observed but from previous *in-vitro* work we would have expected to see some response in cytokine production[239]. There are a number of reports of macrolides affecting *in-vitro* analysis of cytokine production; one demonstrating up-regulation of IL-10 when dendritic cells are stimulated with LPS following pre-incubation with azithromycin [240], and another demonstrating reduced production of the pro-inflammatory cytokines IL-1β and TNFα by macrophages, following pre-incubation with azithromycin and subsequent stimulation with LPS [183]. In *ex-vivo* studies of CD4+ T-helper cells, [from asthmatic children] incubation of the cells with azithromycin led to a decreased production of IL-5 following stimulation [192]. The only study looking directly at serum levels *ex-vivo* following treatment with

azithromycin was in normal volunteers demonstrated a reduction in serum IL-6 [188]. Taken together, we might have expected to find minor some changes in cytokine concentration; at least in IL-6 in the current study. This was not the case, but the current study differs from those above. We obtained serum samples directly from subjects treated with azithromycin, and hence our samples give a more representative illustration of the response to treatment. We measured *in-vivo* production and response of cytokines in relation to treatment with oral azithromycin. There was no signal response, and this was consistent with the lack of any other clinical effects.

The hs-CRP measurement demonstrated a reduction of 93.7 ng/ml [SD 149.9] in the treatment group with the placebo group only having a reduction of 8.4 ng/ml [SD 58.5]. The mean difference and subsequent statistical analysis found this change to have a p=0.16. This is approaching statistical significance. Using this as pilot data, a power calculation can be performed [using G Power 3.1.7, Franz Faul, Universität Kiel, Germany]. A sample size of 33 in each group will have 80% power to detect a difference-in-means of 35.2 ng/ml in the C-reactive protein concentration [CRP], assuming a standard deviation of changes of 120 pg/ml, using a two sample t-test with a 0.050 two-sided significance level. More than 33 subjects in each group completed the trial. Resource implications prevented the purchase of kits to analyse all the serum specimens, and it is possible this result may have reached statistical significance. CRP is a non-specific marker of inflammation and is frequently elevated in bacterial infection. It is entirely possible that this was a real finding related to the anti-microbial activity of azithromycin. The low sample size means this result needs to be interpreted with caution.

8.1.2.1.2. Blood differential cell counts

There was no published literature [pre-study] to suggest an alteration in blood differential cell counts following treatment with azithromycin. Only the data for the total white cell and platelet count were presented, with no other differential cell absolute counts or relative proportions demonstrating any change following 12 weeks treatment.

8.1.2.2. Sputum measurements

8.1.2.2.1. Cellular response

There is much evidence to support the likelihood of changes to sputum differential cell count following treatment with azithromycin based on studies in murine models [182, 191, 241, 242]. These demonstrated a reduction in a number of inflammatory cell types, and non-asthma inflammation models [145, 243] demonstrating consistent evidence of a reduction in BAL fluid neutrophilia. This was also observed in experimental asthma models [160]. We expected therefore to see some alterations in the inflammatory cell differential counts from induced sputum, but this was not observed. We had a dataset comprising 59 of the 77 randomised subjects providing sputum specimens at baseline and at trial completion. The study was not powered for changes in induced sputum cell counts but with these numbers, there was convincing statistical evidence that azithromycin does not alter the inflammatory cell profile of induced sputum in smokers with asthma following 12 weeks treatment. The apparent lack of response could be accounted for by the potent inflammatory stimulus introduced by active cigarette smoking. The study controlled for evidence of active infection – either viral or bacterial and there was no evidence of any change in this at baseline or 12 weeks. There were no obvious external factors accounting for the lack of sputum cytology response.

8.1.2.2.2. Sputum mediator response

A similar panel of sputum mediators [*cf* serum] were assayed taking in to account previous experience of mediators expected to be below detection threshold in sputum fluid. Again there is a sufficient evidence from animal work [182, 191, 240, 241], other airways disease [145, 244] and in asthma related studies [192] to expect changes in concentration of soluble mediators when azithromycin is administered. Sputum mediator profiles and concentrations, in particular chemokines will indirectly reflect the overall inflammatory cell infiltrate, for example sputum fluid IL-8 was found to correlate with sputum neutrophilia [145]. In keeping with the lack of change observed in induced sputum differential cell counts, the administration of azithromycin was not associated with any significant change in concentrations of any of the sputum mediators measured. This lack of cytokine response to azithromycin appears similar to that detailed above for sputum inflammatory cells. There were a few minor limiting factors. Firstly, only 20 of the available samples in each treatment group were assayed as a pilot study, and although the data was statistically convincing it was still possible that measuring all the samples may have changed the statistical analysis. This number of sputum fluids were analysed due to funding constraints for the purchase of assay kits. Secondly, the mediator analysis was a secondary endpoint and the study was not powered for changes in sputum cytokine levels.

8.2. Summary of background to study

Macrolides have anti-inflammatory properties both *in vitro* [185], in experimental animal models [241] and in non-smokers with asthma, that demonstrate they can restore corticosteroid sensitivity *in vitro* and *ex vivo* [192] and can improve asthma control in non-smokers with asthma [154, 159, 160]. We proposed that the anti-

inflammatory effects of macrolides demonstrated in these models could also be achieved in smokers with asthma, a major subgroup that are currently undertreated. Several licensed drugs and new drugs under development might be of benefit for these patients [117], but none have yet been evaluated specifically in smokers with asthma. This group of patients is often excluded from clinical trials, especially studies involving new therapies. The clinical trial presented here was to determine whether macrolide treatment can improve asthma control of smokers with asthma. The long-term goal of this research was to improve the quality of life of smokers with asthma.

8.3. Comparison with other clinical studies of macrolides in asthma

Comparing the findings from this current study with the literature is difficult. Other studies with azithromycin have demonstrated no or mixed responses. Our study supports the findings of Strunk et al [158] where no benefit was found. Other investigators have demonstrated improvements in symptom scores [161] or other measures of asthma control such as bronchial hyper-responsiveness [159, 160]. Whilst the above studies have statistically significant outcomes, the clinical evidence is weak – the study by Hahn et al [161] did not find any improvement in AQLQ and the reported positive outcome used was in an unvalidated symptom score. The studies by Ekici [159] and Piacientini [160] were both in small studies n=11 and n=16 respectively. Our study was of a longer duration than the above three studies and used a daily dose of azithromycin [250mg], giving a greater accumulated treatment dose, and to a larger group of participants [n=71]. A recent study [n=109] with azithromycin [250mg thrice weekly] given for 6 months to never or ex-smokers with frequent exacerbations of asthma did not demonstrate any statistically significant differences in the primary endpoint – exacerbation frequency [163]. A subgroup analysis was performed on patients with non-eosinophilic asthma and found a statistically significant lower rate of exacerbations [163]. Aside from this, the study

did not demonstrate any benefits in measures of lung function, quality of life scores or asthma control when comparing treatment to placebo.

Our findings were consistent with those in a large [n=1142] study by Albert et al[146] in patients with COPD demonstrating no clinical benefits of azithromycin in current smokers. Our study extends the current evidence that azithromycin should not be given to current smokers with asthma.

Reviewing the wider use of macrolides in asthma most studies have used clarithromycin with a few using roxithromycin. The most consistent and reproducible finding has been improvement in bronchial hyper-responsiveness [154, 156, 237, 245]. The azithromycin-related studies detailed above also demonstrated improvements in bronchial hyper-responsiveness.

Sputum and serum cytokine concentrations were measured in the current study and have been measured in others. Clarithromycin has been shown to reduce sputum and blood eosinophil counts [237] as well as sputum cytokine levels [153, 237] as has roxithromycin[245]. The presented study found no significant reduction in either sputum cell counts or sputum or serum cytokine levels. Administration of clarithromycin was associated with an improvement in the dose response of lymphocytes to dexamethasone *in-vitro* [169] and clinically, reduced the steroid dose requirement in steroid dependant asthma [152]. Our study also looked at lymphocyte proliferation responses *in-vitro* and found no change in the inhibitory effects of exogenous dexamethasone after azithromycin. No other study utilising azithromycin has looked specifically at this effect. Our study group was representative of those previously found to have corticosteroid insensitivity [117] and the *in-vitro* assays were intended to identify if steroid sensitivity had been restored. There was no statistical evidence for change in steroid sensitivity [Table 6.43].

Only two studies have demonstrated an improvement in FEV_1 following treatment, both using clarithromycin[154, 155], one of which [Kostadima et al], although demonstrating statistical significance, did not correspond to a clinically significant improvement.

Measures of asthma control or quality of life have been variably studied using different macrolides. The findings lack any consistency with contradictory outcomes between studies of clarithromycin [152, 153, 157] and within studies of azithromycin [161, 163]. Our study found no improvements in any of the symptom score or quality of life outcomes.

8.4. Pre-study hypothesised mechanism of action of macrolides in smokers with asthma

Current evidence has suggested a number of mechanisms whereby macrolide antibiotics may exert their anti-inflammatory effects [55]. The laboratory assays for the present clinical study were chosen prospectively to identify potential mechanisms. The lack of any clinical efficacy means there is no mechanism of action per se, but this does not exclude the possibility that azithromycin may have had some effect on biomarkers, for example, in sputum differential cell counts; cytokine expression in serum or sputum; inflammatory cell behaviour; or exhaled gases without any measurable effects in any clinical outcomes.

In summary, there were no changes in any of the laboratory biomarker outcomes. Whilst there was an isolated statistical change in airway wall flux of NO [J_{aw}] and in the concentration alveolar NO [C_{alv}], the former was an adverse change. These conflicting findings suggest that no firm conclusion can be made on the effect of azithromycin on airway inflammation in asthma as assessed by extended flow

measurements of nitric oxide. This is unlikely to be reflective of any effect by the study drug and may just be a chance finding.

Macrolides may have corticosteroid-sparing effects, first shown with troleandomycin, but the use of this macrolide has been limited due to adverse effects [203]. Corticosteroid-sparing efficacy is limited to case reports [204] and small open-label pilot studies [169], which demonstrate improvement in laboratory biomarkers, for example there was an enhanced sensitivity of lymphocyte proliferations to suppression by dexamethasone *in-vitro*. Although macrolide antibiotics are inhibitors of CYP3A4 [133] and can increase the plasma concentration of CYP3A4 substrates, which include fluticasone and budesonide, this mechanism is unlikely to contribute to the localised therapeutic effects of inhaled corticosteroids on the airways of patients given macrolides.

Smokers with COPD have decreased histone deacetylase-2 [HDAC2] activity in alveolar macrophages, and this may lead to increased inflammatory gene expression and reduced sensitivity to corticosteroids [205] and a similar mechanism may occur in smokers with asthma [170]. Erythromycin can increase HDAC2 levels *in vitro* [171], suggesting a mechanism by which macrolides therapy may restore corticosteroid sensitivity and improve asthma control in smokers with asthma.

The functional effect of azithromycin on cytokine production has been assessed by many investigators. What is clear is that macrolides as a family can alter the production of cytokines both *in-vivo* and *in-vitro*. Azithromycin has many anti-inflammatory effects [188] including down-regulation of production of pro-inflammatory mediators e.g. prostaglandin E2, nitric oxide and cytokines TNF-α, IL-8, IL-1β, growth-related oncogene [GRO]-α and soluble vascular cell adhesion molecule [sVCAM]-1. Many of these are chemotactic, activation and survival factors

for neutrophils. IL-1β is sufficient to induce neutrophil accumulation in the lung [189], GM-CSF is a neutrophil survival factor [190], and both are derived from macrophage and airway epithelial cells and are central to the homeostatic response to airway infectious inflammation [186]. In a murine model of LPS-induced pulmonary neutrophilia, azithromycin or clarithromycin were able to reduce airway neutrophilia with striking reductions of IL-1β and GM-CSF [191]. In a murine CF cell model azithromycin reduced production of IL-1β, CCL2 and TNFα [183] in harvested alveolar macrophages *in-vitro*.

Clarithromycin, when given to subjects with refractory asthma, was associated with a significant reduction in airway IL-8 levels and neutrophil numbers [153]. Subgroup analysis showed that airway IL-8 protein and gene expression was predominantly reduced in the non-eosinophilic asthmatic patients. Similarly, azithromycin reduced airway *IL-8-mRNA* in patients with post-transplant bronchiolitis obliterans syndrome [BOS] following 3 months treatment [145]. In a mouse model of allergic asthma pre-treatment with azithromycin led to attenuation of IL-5 and IL-13 production in response to allergen in BAL fluid, and also reduction in the chemokines CCL2, CCL3 and CCL4 [241]. These studies were replicated in patients. T_H2 lymphocytes from blood samples from from asthmatic children, when treated with azithromycin and then stimulated *in-vitro*, demonstrated a reduction in the production of IL-5 compared to untreated cells [192]. Putative mechanisms of clarithromycin function suggest altered DNA binding activity of the transcription factors NF-kB and AP-1[185, 194], causing inhibition of synthesis and/or secretion of these pro-inflammatory cytokines [55].

Cellular changes in response to treatment with macrolides have also been observed. Azithromycin causes increased phagocytosis by alveolar macrophages of apoptotic

bronchial epithelial cells and neutrophils in patients with COPD [246]. Classically-activated macrophage [M1] phenotypes, induced by stimulation with interferon [IFN]γ and bacterial lipopolysaccharide [LPS] are associated with microbicidal and cytotoxic function, and pro-inflammatory cytokine production [37]. Alternatively-activated [M2] phenotypes induced by IL-4/13 are associated with Th2-type, immunosuppressive and remodelling responses. Azithromycin reduced the production of pro-inflammatory IL-1β, and tumour necrosis factor [TNF]α in mouse M1 macrophages [183] and polarised cells towards M2, with reduced pro-inflammatory IL-12 and IL-6, and increased anti-inflammatory IL-10 [184].

A component of macrolide anti-inflammatory activity may be a consequence of the reduction in bacterial load afforded by the antibiotic effects [176] distinct from its anti-inflammatory activity [133]. This concept may be valid when macrolides are given to patients with Mycoplasma or Chlamydia infection, but does not explain why submicrobicidal concentrations of azithromycin can attenuate the production of IL-8 and GM-CSF from bronchial epithelial cells in response to LPS [247]. Again, the concept of reducing bacterial burden falters when azithromycin is given to patients with CF who are chronically colonised by *P. aeruginosa* [248]; macrolides do not have significant anti-pseudomonal activity, and so the improvement in inflammation cannot be attributed to an antibacterial effect.

8.5. Generic issues relevant to clinical trials

8.5.1. General issues in proof of concept studies

Proof of concept studies are used in a variety of ways to answer research questions [249] including feasibility, safety or drug efficacy in the present study. Other considerations need to be reviewed before a proof of concept study can be performed, including:

- *process* – recruitment and retention rates;
- *resources* – assessing time and resource problems
- *management* – challenges of the participating centres in managing the study

This study was intended to answer a scientific question. This was accomplished and in order to do this we had to overcome problems in the *process, resource* and *management*.

Recruitment is a common problem in clinical studies, and this was observed here. This was successfully addressed [discussed in section 8.5.3], but doing so required additional resources in terms of staff numbers, availability and training, and an extension to the study. This challenge was also met with success, partly by utilising the assistance of GP colleagues to contact GP practices and patients directly, but also in the opening of a second recruitment site. This required additional management in order to ensure adequate training at the second site, the appropriate availability of resources [equipment and office space] and the correct maintenance of regulatory paperwork. Other unexpected delays were also encountered – the manufacturer of the IMP initially offered placebo with an 8 month shelf life and although this was finally resolved once the medication did arrive it was embargoed due to poor and damaged labelling. These delays led to a change in the plan for analyses of the laboratory specimens. It had been my intention to complete all the clinical work in the first 2 years and spend the third year analysing the stored plasma and sputum specimens. Unfortunately due to the delays detailed above, the majority of the third year was spent on patient visits and data collection, with less time available for bench work. The difficulties faced with recruitment have been detailed further in Appendix

8 and formed the basis for the writing of a manuscript looking at the patient flow in three studies performed at the Asthma Research Unit [250, 251]

The study is of considerable scientific importance. There is an unmet need for treatment in a group of patients where the current mainstay treatment; corticosteroids lacks efficacy and azithromycin represents a strong candidate drug [based on sound scientific evidence] that might confer benefit by restoring scientific sensitivity. This was a small study by comparison to large Phase III investigations. As such any findings need to be balanced by careful interpretation of methods by which it was conducted, and hence the presumed validity of its findings. We recruited a very well defined cohort which was well balanced in baseline characteristics of placebo and active groups. The study design was a randomised, placebo controlled, double blind study gives additional confidence in the results. The appropriate regulatory, safety requirements and reporting were followed and our sponsor monitored the study closely to ensure compliance.

Whilst there is a considerable burden of administration in running a clinical trial it is strictly necessary to ensure both patient safety and scientific integrity.

8.5.2. Regulatory issues: pre-study problems

The introduction of the EU clinical trial directive in 2005 had wide ranging impacts on the performance of clinical trials and research within the UK almost as soon as it was introduced[252, 253]. Of concern is the long term impact on academic research within the UK. Observational evidence has found there to be a 5.3% average annual diminution of clinical drug trials in the UK, with the decrease being caused by a decline in academic trial activities[254]. The layers of bureaucracy have increased and are now significant burdens to researchers. This and an over-riding fear that

even minor breach of the regulations could lead to site closure or even the order to cease all research related activities by the sponsor cause significant anxiety and inhibition from pursuing innovative research. Every stage of the current study was delayed due to the requirements and time required to obtain approvals from the MHRA, ethics and sponsor. In particular there is no difference in the sponsor monitoring requirements between low or high risk studies. Our study utilised an already licensed medicinal product with almost 20 years post marketing safety surveillance, yet there was no proportionality to the risk assessment or the delay this incurred.

The clinical trial directive is now about to undergo its first major revision since its introduction[255] and clearly we would hope that any changes will be beneficial to hypothesis testing studies such as the one presented here.

8.5.3. Generic recruitment issues

As with many clinical studies, recruitment of randomised subjects proved extremely challenging. A number of problems were encountered. Following the delays in ethical and R&D approval, the next difficulty to overcome was recruitment of GP practices to allow patient searches to be performed. Reasons to decline participation included workload, lack of remuneration, and scepticism over the scientific merit of the study or implications for practice should it prove positive. The patient responses rate based on our experience of previous studies was felt to be acceptable, but unfortunately these potential subjects were not readily converted in to randomised patients. Previous studies had a randomisation rate of far in excess of ours, with over 1 in 2 of those screened being randomised [251]. At the time of our recruitment this had dropped to just above 1 in 3 [Figure 5.1]. Our rate of recruitment and the targets required are displayed in Figure 8.1. Patients were generally very keen to be

involved and only a small number refused consent at the initial study visit. The problem in randomisation rate was recognised early and several steps were taken to remediate this:

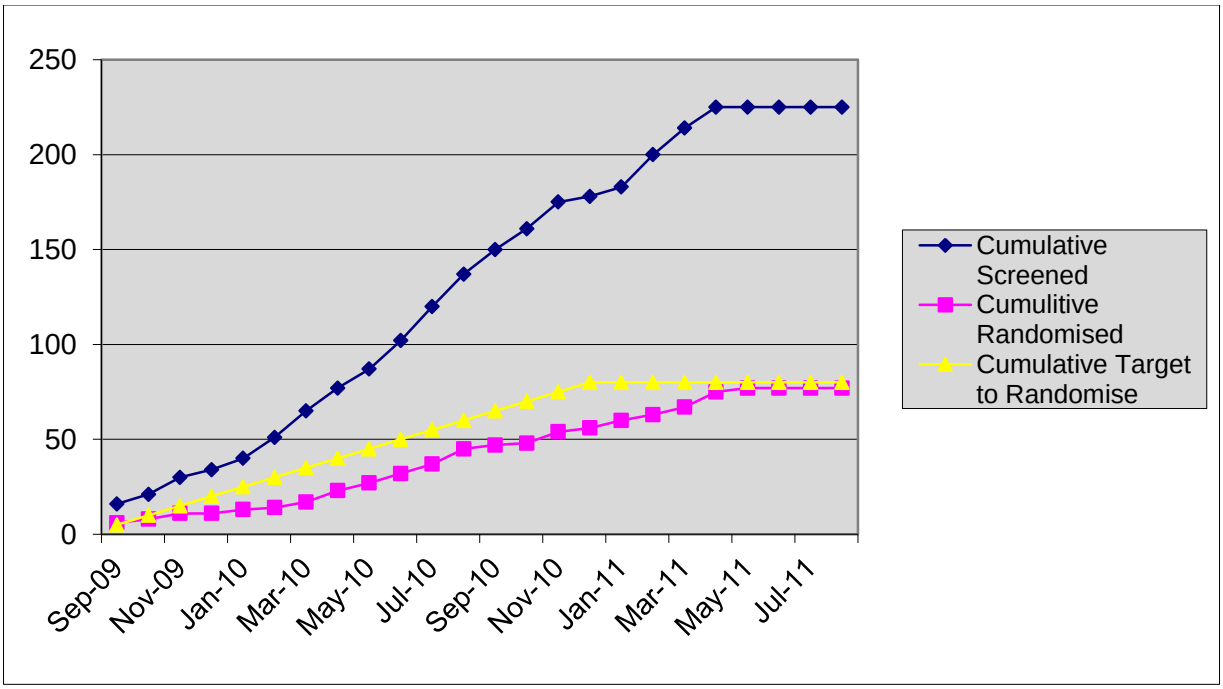
- The protocol was changed to allow patients to remain on combination inhaler and reduce the requirement to wean and reduce the likelihood of loss of control during the run-in period

- A second site was opened

- Additional support was made available to telephone and book suitable patients for screening

The protocol amendment to change the run in medication and open the second site required a further application to ethics, R&D and the MHRA for approval. Again there was delay in each of these steps. Whilst changing the study run-in medication led to a significant increase in the randomised patients, opening a second site did not. Nevertheless all these efforts had the desired effect and the randomisation target was achieved and hence the study became adequately powered.

Figure 8.1: Cumulative recruitment by month during the study



8.6. Strengths and limitations of the study

8.6.1. Factors potentially contributing to lack of clinical efficacy

8.6.1.1. Patient population

A Canadian cross-sectional population study of asthma prevalence and severity reported that 78.9% of patients could be described as having mild to moderate asthma [256] according to the Canadian Asthma Consensus guidelines, which compares well to the patients recruited to the current study. This is therefore an appropriate patient group to study. If milder patients are treated with combination inhalers and additional leukotriene receptor antagonists then findings in this group will become less generalizable to the wider asthma clinical population.

Baseline measures of our study group demonstrated that they had relatively mild asthma with FEV_1 >75% predicted and low measures of airway inflammation [PC_{20} <2.22mg/ml with mean percentage eosinophils of 1.38% in induced sputum cytology].

These are important features in patients recruited to this type of trial as clinical stability is essential during the run-in period and is required to ensure progress through to randomisation. Screening a more severe patient group could have limited the recruitment further and excluded them from the study. All of our patients remained symptomatic as evidenced by the baseline mean ACQ of 1.74 when grouping all subjects together. This allowed the opportunity to improve the asthma control in our group.

Smokers with asthma tend to represent a population of asthmatics with an airway neutrophilia. Evidence suggests that airways with neutrophilic inflammation have a better response to azithromycin [145] but unfortunately our population was neither neutrophilic or eosinophilic [6.1.2]. Severe asthmatics tend to be neutrophilic and the failure to identify a positive response to azithromycin in the present study could be due to our asthma study population being clinically too mild to identify a signal.

8.6.1.2. Macrolide used

Azithromycin is a derived compound of erythromycin[133] and its prolonged half-life of 40 hours allows it a convenient once daily dosing regimen. Antibiotic efficacy of the various macrolides and spectrum of activity are subtly different but in terms of antimicrobial efficacy there are no direct comparison studies between different macrolides. The anti-inflammatory properties of macrolides have never been directly compared in efficacy studies and probably never will. The initial studies in obliterative bronchiolitis utilised erythromycin, but this has now been replaced by azithromycin with no reported decrement in survival [257]. Hence our choice of macrolide was based the convenience of administration and safety profile. Azithromycin benefits by not having the propensity for GI side effects of erythromycin and also lacks the significant interactions of either erythromycin or

clarithromycin. Despite the beneficial interaction profile, large numbers of prospective subjects were not invited for screening visits at the point of telephone pre-screening because of the presence of interacting medications.

8.6.1.3. Dose

We administered a dose of 250mg once daily azithromycin. This is based on the available published evidence in asthma related studies and studies in other airway disease. The majority of other studies utilised intermittent dosing – twice or thrice weekly of azithromycin. This was common in studies of post-transplant bronchiolitis obliterans syndrome where 250mg was give three times per week [144, 258]. Studies in cystic fibrosis used weight dependant dosing of 250 or 500 mg daily [137] or three times per week [259]. Similar regimens have been used in non-CF bronchiectasis with 500mg administered twice weekly[139].

Asthma related studies have utilised azithromycin in an even wider dosing interval, with positive outcomes. The study by Hahn et al administered 600mg thrice weekly for the first week then at weekly intervals[161]. Other azithromycin-asthma studies have administered 250mg twice weekly[159] or 10mg/kg in children for 3 consecutive days per week over an 8 week period[160].

The pharmacological properties of azithromycin lend it to an intermittent dosing regimen. It has a half-life of 40 hours and accumulates within the cytoplasm of inflammatory cells, having high tissue concentrations relative to serum[133]. It was therefore appropriate for us to use a dose of 250mg. Daily dosing allowed the subjects to follow a regular routine and hence improved compliance. We hypothesised that 250mg once-daily would be an adequate dose to generate a positive response in our subjects. Higher doses [500mg] have been used and it is

possible that repeating our study with this increased dose may lead to a positive response.

8.6.1.4. Duration of treatment

The duration of the study drug treatment was 12 weeks. Treatment regimens in similar studies of 6-16 weeks with clarithromycin [152, 153, 155] have been used with success in non-smoking asthmatics. Longer treatment durations have been used in studies where the primary endpoint is improvement in exacerbation frequency [138, 146] or in post-transplant BOS where the endpoint is reduced evidence of disease progression. Within the cohort of patients with BOS, the response was noted after 12 weeks treatment[258]. Very short treatments have been used in acute-asthma studies of 10 days[164, 169] and 3 weeks[157]. This evidence suggests that shorter durations of treatment of 6-8 weeks are adequate to obtain positive outcomes in asthma studies with azithromycin [159-161]. It was therefore appropriate for us to choose a 12 week treatment duration. We were utilising daily dosing, which many studies did not, and hence we believed this would be sufficient to produce a response in our patient group. Our study, although negative, was performed using a dose and duration of treatment of known efficacy in other airway diseases. The negative response is unlikely to be due to inadequate dosing [we demonstrated compliance >95%] or treatment length.

8.6.1.5. Outcome measures

8.6.1.5.1. Efficacy

Only two outcome measures demonstrated a statistically significant difference following 12 weeks of treatment with azithromycin.

The LCQ when divided in to its individual domains found the psychological domain to deteriorate over the 12 weeks. When the domains were combined the total-LCQ score had dropped almost reaching a statistically significant difference p=0.06. The absolute difference was -1.06. If this had dropped further and reached significance it would still have been well within the minimal clinical important difference of 2.56, therefore any deterioration observed within this study would have had no clinical significance.

Measurements of alveolar nitric oxide [C_{alv}] demonstrated an increase which was statistically significant p=0.01, with airway wall flux of nitric oxide [J_{aw}] demonstrating an increase with a trend towards statistical significance, p=0.06. These results need interpreted with caution. The C_{alv} concentration demonstrated a small but statistically significant increase when calculated using linear-regression but when calculated using a non-linear-regression method, the same measurements did not produce a statistically significant result, p=0.38 [see section 6.6.2]. The clinical interpretation of these derived measurements has not been validated and hence normal ranges for of C_{alv} and J_{aw} have not been defined in non-smokers or smokers with asthma. It is difficult therefore to determine a minimal important difference. It is unlikely that these changes represented any change to underlying airway inflammation.

8.6.1.5.2. Immunological outcomes

The immunological measurements showed uniformly no significant effect of azithromycin treatment. None of the serum or sputum biomarkers demonstrated any change. This is consistent with the negative clinical and physiological findings of the study. We measured a large panel of pro-inflammatory and anti-inflammatory cytokines as well as sputum cytology and blood monocyte and lymphocyte activity

and airway wall inflammation. Funding for the biomarker aspect of the study limited the measurements within our study group to 20 in each treatment arm for the soluble biomarkers, although almost an entire dataset was available for the inflammatory sputum cell counts. It was anticipated that measuring this pragmatic sample sub-group would give representative results for the entire group. The study was not powered for changes in inflammatory endpoints but it was felt that sample measurement would at least provide pilot data for future power analysis.

8.6.1.5.3. Exacerbations

Reporting of exacerbation frequency is given as an annualised rate and hence studies to assess this tend to be of much longer duration e.g. 6-12 months[130, 238, 260-262]. The present study comprised a treatment period of 12 weeks. This is insufficient to accurately comment on any change in exacerbation frequency. Whilst exacerbations were recorded as part of the adverse event reporting requirements this was not analysed to assess any change. To do so would require a study of several hundred to several thousand patients, with considerable resource implications. Other studies have looked at exacerbation rates in non-smokers with asthma[238] and found no improvement in the treatment group with azithromycin. This was a small study n=109 and did not provide any supporting evidence for their sample size calculation and hence the possibility exists, the sample was too small to detect any difference at 6-months. We are therefore unable to provide any more comment than to say there was no difference in exacerbation rates between the two groups in our study, p=1.00.

8.6.1.6. Power of the study

The original power calculations indicated a sample size of 68 would give an 80% power to detect a difference in means of 25L/min in peak expiratory flow [PEF] [primary endpoint], assuming a standard deviation of 36L/min, using a two sample

t-test with a 0.050 two-sided significance level[129]. We planned to recruit a total of 80 subjects, with the final number being 77. This ensured that we met the requirement of at least 68 subjects completing [71 completed]. Fewer patients provided sputum samples thus limiting the potential of the trial to find significant differences between the groups. Secondary and exploratory endpoints were intended as "exploratory and hypothesis generating", rather than definitive.

8.6.1.7. Compliance

Drug compliance across the two groups was greater than 90%. Unfortunately there is no readily available method to corroborate medicine use as in the case of statins where the lipid profile will alter and can be easily measured in a hospital biochemistry lab. To establish compliance we simply asked patients to record drug consumption on a daily basis in their diary card and confirmed this with tablet counts. Overall compliance was probably helped by the azithromycin being well tolerated.

8.7.Patient Safety

Oral erythromycin prolongs cardiac repolarisation and is associated with case reports of torsades de pointes. A large retrospective epidemiological study reported that the risk of sudden death from cardiac causes among patients currently using erythromycin was twice as high as those not using any antibiotic and the adjusted rate of sudden death from cardiac causes was five times as high among those who concurrently used CYP3A inhibitors along with erythromycin [263], although this adverse effects is rare [55]. Recent work has also identified the possibility that azithromycin may also lead to an increase in cardiovascular death [264]; this study was a retrospective observational study and it could not be confirmed that there is a causal link between the azithromycin and increase in mortality. However there is

good evidence of survival benefit in prescribing macrolides in severe community acquired pneumonia [265], and this appears to contradict the findings of Ray et al [264].

Our study had one cardiovascular death, but this occurred three weeks after the last dose of IMP. History of cardiovascular disease was not a direct exclusion to the study, but past history of atrial or ventricular tachyarrhythmia was. Subjects had to have a normal QTc for entry criterion and therefore we selected a study population that may have been less likely to develop cardiovascular complications, furthermore there was no statistical difference in QTc following 12 weeks of treatment. Whilst these concerns may be valid it does not explain why patients with severe community acquired pneumonia have an overall survival advantage when placed on macrolides. The study presented here was never designed to test safety but we can say that the study drug did not lead to any increase in QTc and hence our study patients should not have been at any greater risk of arrhythmias or possible adverse cardiovascular events. This is supported in a longer duration study [6-months] where no adverse cardiovascular events were reported[238].

8.8. Implications for future research

8.8.1. Conclusions & future directions

This randomized controlled study tested the hypothesis that azithromycin when added to regular inhaled corticosteroids could improve lung function, asthma control, airways inflammation and bacterial colonization in adult smokers with asthma. We found that there were no clinically important changes in both the primary endpoint, morning PEF, and in a range of clinical indices of current asthma control and measures of airway inflammation after 12 weeks of treatment. This is the first study to test the efficacy of the macrolide antibiotic azithromycin in smokers with asthma. Smokers with asthma are an understudied population of patients that do not respond well to steroids. These current medications are being used on the basis of limited evidence because there has been general exclusion of smokers from RCT's. The randomised controlled study presented here provides clear evidence that the macrolide antibiotic azithromycin administered daily at a dose of 250mg confers no additional benefit over usual therapy.

8.8.2. Overview of alternative treatment management of smokers with asthma

The evidence for the effectiveness of smoking cessation in smokers with asthma is rather limited[266-268]. When smokers are monitored during the quitting process there are improvements in asthma control and lung function at 6 weeks[268]. These subjects were not monitored for long enough to determine if there was a recovery of airway sensitivity to corticosteroids, however longer periods of cessation [>1yr] in a separate study appears to confer a degree of restoration of corticosteroid sensitivity[43]. There is evidence that smoking cessation is associated with a reduction in airway inflammation; with lower sputum neutrophil counts 6 weeks

after smoking cessation compared with smokers with asthma[268]. Every effort should be made to encourage and support smokers in their attempts to quit. To this end patients should be offered drug-treatment such as nicotine replacement therapy, nicotine receptor antagonist varenicline or the antidepressant buproprion[269].

Inhaled β_2 adrenoreceptor agonists have a central role in the management of asthma but currently there are no studies that have specifically examined the effect of cigarette smoking on the β_2 adrenoreceptor and its behaviour and expression *in vivo* in the context of asthma. With the development of newer ultra-long acting β_2 agonists such as indacaterol, there now exists the possibility to examine the effects of this newer drug class in smokers with asthma. The current license for this drug is currently only for COPD in the UK. Studies are underway for its use in asthma [NCT00529529, NCT01609478] but these all exclude current smokers.

There is ample clinical evidence for a reduced therapeutic response to inhaled corticosteroids in smokers with asthma [270] but this may not apply to all smokers with asthma [271]. In addition administration of inhaled corticosteroid for a long duration might have beneficial effects on the rate of decline in lung function[272] that is not apparent in heavy smokers [272]. In all likelihood, in the knowledge that current smokers with asthma have more severe symptoms and following international guidelines, it would be more common to see patients placed on combination inhalers.

Combination therapy with an inhaled corticosteroid and LABA is central to the management of chronic asthma [2]. A *post hoc* analysis of data collected from the GOAL trial looking specifically at smokers with asthma found reduced exacerbation rates with combination therapy compared to ICS [inhaled fluticasone] alone[273]. However the level of control was still inferior to that achieved by never smokers with

asthma using the same combination. This has been supported by a prospective study comparing the combination inhaled therapy of salmeterol/fluticasone versus a double dose of the fluticasone component in smokers with asthma. The group on combination therapy had significant reductions in airway hyper-responsiveness and improved airway calibre[274]. Taken together it could be suggested that international guidelines should include specific guidance on smokers with asthma being treated with combination inhalers earlier as well as advising on smoking cessation.

Leukotriene receptor antagonists [LTRA] are currently included in guideline recommendations for patients not controlled on regular inhaled therapies[2]. An initial proof-of-concept study utilising the LTRA montelukast found there to be an increase morning PEF in smokers with mild asthma but not in non-smokers with mild asthma [275]. This observation was extended by the findings of another larger study looking specifically at smokers with asthma where treatment with montelukast led to increased asthma control [276]; so far, this study has only been reported in abstract form.

The once daily selective anti-muscarinic tiotropium has become well established in its use for COPD since the publication of the UPLIFT trial[277]. At present, no trials have been carried out in smoking subjects with asthma, although one study has been performed in subjects with features of both asthma and COPD. This demonstrated that tiotropium improved in lung function and reduced reliever use [278]. Unfortunately this study did not report the response in current smokers [approximately 40-45% of the randomised subjects] compared with ex-smokers. The high proportion of current smokers and the fact that there was a positive

improvement suggests that there may be a response in this patient group. Further investigation is clearly warranted to clarify this in smokers with asthma.

Theophylline is currently recommended as an add-in therapy in subjects with asthma who are not controlled on regular inhaler therapy [2], with the exact mechanism being poorly understood and current smoking leads to enhanced clearance of theophylline due to induction of CYP1A2. The standard dose is thought to act principally as a bronchodilator through cyclic AMP phosphodiesterases [279]. Theophylline has a number of immunomodulatory properties that include a reduction in the proliferative response of lymphocytes to mitogens, a reduced IL-5 mediated eosinophils survival and eosinophilic cationic protein production and an increase in IL-10 by peripheral blood mononuclear cells[280]. Specific studies have been performed in smokers with asthma and this is detailed in Section 8.8.5.

Omalizumab is a monoclonal anti-IgE immunoglobulin and has demonstrated efficacy in improving asthma control and reducing the frequency of exacerbations in allergic asthma[132, 281]. Unfortunately these major studies excluded current smokers and ex-smokers. Of note, smokers tend to have a reduced responses to common allergens, with evidence of a reduction in Th2 responses[282]. Nevertheless elevated serum total IgE in this group suggests a role for anti-IgE therapy. Due to the cost implications of treatment it would be highly beneficial to have sound investigational evidence looking specifically at the effect of omalizumab in smokers with asthma.

8.8.3. Non-smokers with asthma

There is now a considerable evidence base for all of the aforementioned class of medicines to be used in non-smokers with asthma. This study looked at the potential

beneficial effects of the macrolide antibiotic azithromycin. There continues to be no consensus on whether macrolide antibiotics can be recommended for general use in patients with poorly controlled asthma.

8.8.4. Non-antibacterial macrolides

Recent development of chemically modified non-antibiotic macrolides that maintain anti-inflammatory properties may overcome the risk of contributing to emerging microbial drug resistance. EM703 is a 12-membered-ring derivative of erythromycin that is devoid of anti-bacterial actions but exhibits anti-inflammatory properties. For example, EM703 inhibits transforming growth factor-β induced proliferation, transcription of type I collagen and collagen production of both human and murine lung fibroblasts [283, 284] and NFκB activation and IL-8 production in human bronchial epithelial cells [285]. In addition, EM703 can increase HDAC2 levels in vitro [171], suggesting a possible mechanism by which this compound may restore corticosteroid sensitivity in COPD and in smokers with asthma [170, 205]. There is evidence in the patent literature of novel compounds that are claimed to demonstrate dissociation of anti-inflammatory from antibiotic effects. A derivative of the macrolide azithromycin [CYS0073] has been developed that has anti-inflammatory actions in the absence of any bactericidal properties [148]. This candidate molecule has been found to reduce inflammation in animal models of inflammatory bowel disease and rheumatoid arthritis [148]. If similar anti-inflammatory effects are found in experimental models of airway inflammation then this molecule could be developed as a potential therapy for chronic inflammatory airway diseases in man.

Macrolide antibiotics have the ability to accumulate in inflammatory cells including neutrophils, monocytes and alveolar macrophage as well as epithelial cells [133]. Due to this property, non-antibiotic macrolides have been developed that are conjugated

with a second, pharmacologically active drug that has anti-inflammatory properties and as such, these compounds are used to transport the active moiety into the cell and thereby increase local anti-inflammatory drug action. Using this property macrolides have been developed that act as a drug carrier for an active compound, such as a corticosteroid[286], cyclo-oxygenase or 5-lipoxygenase inhibitor[287], which is covalently bound to the macrolide. Macrolide conjugates have been patented that include pyrrolizine and indolizine compounds that act as inhibitors of 5-lipooxygenase and cyclooxygenase[287] or that contain coumarins[288]. Macrolide conjugate compounds that include a corticosteroid can bind to the glucocorticoid receptor, accumulate in cells and can inhibit T cell proliferation, IL-2 production and ovalbumin-induced airway inflammation in mice [286]. In addition the intracellular localisation of the active compound such as a corticosteroid limits the likelihood of systemic adverse effects and ensures a localised anti-inflammatory action of the active compound. There are no published reports on the clinical efficacy and anti-inflammatory effects of non-bacterial macrolides in the treatment of airway diseases.

8.8.5. Other treatments for smokers with asthma

Standard asthma therapies are licensed from data obtained in non-smokers with asthma, and in addition, smokers with asthma have a different type of inflammation compared with non-smokers with asthma. Meeting the needs of this patient group with new or novel therapies could revolutionise the long term prospects in morbidity and mortality as well as social and health economic benefits of reduced burden on healthcare and fewer lost days to illness in the workplace.

Recent work has suggested alternatives to standard forms of smoking cessation therapy. A phase I/II trial for a nicotine vaccine in humans has confirmed safety and immunogenicity [289]. This novel approach aims reduce the pleasurable surge of

nicotine sought by the [ex-]smoker. If successful this could increase smoking cessation and subsequent relapse rates. A monoclonal antibody has also been developed separately to reach the same goal [290]. It remains to be seen in larger Phase III trials if both these approaches will be effective.

Another approach is to restore corticosteroid sensitivity. Smokers have reduced HDAC activity [117] and so it would follow that if HDAC activity could be restored there could potentially be a restoration of corticosteroid response. Pre-clinical and pilot studies in smokers with asthma have shown that low-dose oral theophylline can restore HDAC activity [291] with a measurable therapeutic response of improved lung function in [292]. Given the suggested benefit further larger trials need to be considered.

The ability for low-dose theophylline to successfully increase HDAC activity has led to greater scrutiny of the underlying mechanism. This has led to the discovery that theophylline inhibits phosphotidylinostol-3 kinsases [PI3K] specifically subtype p110-δ [293]. Successful pilot studies with a theophylline-related PI3K-inhibitor compound have been performed *ex-vivo* in peripheral blood mononuclear cells harvested from patients with COPD, demonstrating a restoration in steroid sensitivity [294].

Theophylline is a non-specific phosphodiesterase inhibitor but its bronchodilator effect is thought to arise from inhibition of the subtype phosphodiesterase-4. Two specific inhibitors of this enzyme have been developed – cilomilast and roflumilast. Of these, only roflumilast has shown significant benefit in non-smoking asthmatics [295]. There are currently no studies investigating the potential benefits in smokers with asthma, but it is now likely given the findings in the pilot studies that further trials may be carried out.

The thiazolindinedione rosiglitazone belongs to a family of drugs which act on the nuclear hormone receptor peroxisome proliferator-activated receptor [PPAR]-γ. A proof-of-concept study was performed with rosiglitazone in a group of smokers with asthma and demonstrated a bronchodilator effect after 4 weeks of treatment [296]. Concern exists over the side-effect profile of this class of medicines and so it remains to be seen if further steps can be taken to make them a clinically relevant class of drugs for asthma.

Statins have pleiotropic anti-inflammatory actions. Large retrospective observational studies suggest there is a beneficial effect when given to asthmatics [297] and that they can slow the decline in lung function in current or ex-smokers independent of the presence of underlying lung disease [298]. With particular relevance to the current study there is prospective randomised controlled trial data which found there is improvement in airway inflammation and quality of life when atorvastatin is given to adult asthmatics [299] and in smokers with asthma [251]. Some of the evidence for statin use can be contradictory with other studies finding no benefit [300]. Large high quality studies will be required to determine if clear efficacy exists.

Oxidative stress is thought to play a role in the pathogenesis of asthma [301] and COPD [302]. Cigarette smoke is the leading cause of COPD in the developed world thought to be caused by the amount of oxidative compounds per inhalation. Patients with COPD on the widely prescribed mucolytic [and anti-oxidant] drug carbocisteine have been shown to have reduced frequency of exacerbations [303]. The likelihood of extreme oxidative stress in the airways of smokers with asthma makes this an area of worthwhile investigation.

Smokers with asthma form a large proportion of patients; roughly 40% in our experience and these do not respond well to treatment. Understanding the

mechanism may provide scientific rational for new treatments that would also benefit ex-smokers and possibly smokers with other inflammatory disease that may be treatment refractory.

In conclusion; we have shown that azithromycin is unlikely to improve asthma control in smokers with asthma and at present cannot be recommended for use in this group.

9. References

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10. Appendices

Appendix 1: Asthma Control Questionnaire

Macrolide Asthma Study

Protocol AR010, Version 1, 7th January 2009

Visit 2, Baseline
Asthma Control Questionnaire A
Page 1

Version 1.0

Screening No. ____ 01 Initials ____ 02 Date of Visit ____ D D M M Y Y Y Y 03

Please circle the number of the response that best describes how you have been during the **past week**

			Office Use
1	On average, during the past week, how often were you **woken by your asthma** during the night?	0 Never 1 Hardly ever 2 A few times 3 Several times 4 Many times 5 A great many times 6 Unable to sleep because of asthma	04
2	On average, during the past week, how **bad were your asthma symptoms when you woke** up in the morning?	0 No symptoms 1 Very mild symptoms 2 Mild symptoms 3 Moderate symptoms 4 Quite severe symptoms 5 Severe symptoms 6 Very severe symptoms	05
3	In general, during the past week, how **limited were you in your activities** because of your asthma?	0 Not limited at all 1 Very slightly limited 2 Slightly limited 3 Moderately limited 4 Very limited 5 Extremely limited 6 Totally limited	06
4	In general, during the past week, how much **shortness of breath** did you experience because of your asthma?	0 None 1 A very little 2 A little 3 A moderate amount 4 Quite a lot 5 A great deal 6 A very great deal	07
5	In general, during the past week, how much of the time did you **wheeze?**	0 Not at all 1 Hardly any time at all 2 A little of the time 3 A moderate amount of the time 4 A lot of the time 5 Most of the time 6 All the time	08
6	On average, during the past week, how many **puffs of short-acting bronchodilator** (eg Ventolin) have you used each day?	0 None 1 1-2 puffs most days 2 3-4 puffs most days 3 5-8 puffs most days 4 9-12 puffs most days 5 13-16 puffs most days 6 More than 16 puffs most days	09

Macrolide Asthma Study

Protocol AR010, Version 1, 7th January 2009

Visit 2, Baseline
Asthma Control Questionnaire B
Page 2

Version 1.0

Screening No. 01 Initials 02 Date of Visit D D M M Y Y Y Y 03

To be completed by a member of the Research Team

FEV_1 prebronchodilator	> 95 % predicted	0
☐.☐☐ L 04	95-90%	1
FEV_1 predicted	89-80%	2
☐.☐☐ L 05	79-70%	3
FEV_1 % predicted	69-60%	4
☐☐☐ % 06	59-50%	5
	<50% predicted	6 07

[Record actual values in the cells and score the FEV1 % predicted in the last column]

TOTAL SCORE	08

SCORE / 7	09

Appendix 2: Asthma Quality of Life Questionnaire

Macrolide Asthma Study

Protocol AR010, Version 1, 7th January 2009

Visit 2, Baseline
Asthma Quality of Life Questionnaire 1
Page 6

Version 1.0

Screening No. ____ 01 Initials ____ 02 Date of Visit D D M M Y Y Y Y 03

Please complete all questions by circling the number that best describes how you have been during the last 2 weeks as a result of your asthma.

HOW LIMITED HAVE YOU BEEN **DURING THE LAST 2 WEEKS** IN THESE ACTIVITIES **AS A RESULT OF YOUR ASTHMA**?

	Totally Limited	Extremely Limited	Very Limited	Moderate Limitation	Some Limitation	A Little Limitation	Not at all Limited
1. STRENUOUS ACTIVITIES (such as hurrying, exercising, running up stairs, sports)	1	2	3	4	5	6	7 04
2. MODERATE ACTIVITIES (such as walking, housework, gardening, shopping, climbing stairs)	1	2	3	4	5	6	7 05
3. SOCIAL ACTIVITIES (such as talking, playing with pets/children, visiting friends/relatives)	1	2	3	4	5	6	7 06
4. WORK-RELATED ACTIVITIES (tasks you have to do at work*)	1	2	3	4	5	6	7 07
* *If you are not employed or self-employed, these should be tasks you have to do most days.*							
5. SLEEPING	1	2	3	4	5	6	7 08

HOW MUCH **DISCOMFORT OR DISTRESS** HAVE YOU FELT **DURING THE LAST 2 WEEKS**?

	A Very Great Deal	A Great Deal	A Good Deal	Moderate Amount	Some	Very Little	None
6. How much discomfort or distress have you felt over the last 2 weeks as a result of CHEST TIGHTNESS?	1	2	3	4	5	6	7 09

IN GENERAL, **HOW MUCH OF THE TIME DURING THE LAST 2 WEEKS** DID YOU:

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	Hardly Any of the Time	None of the Time
7. Feel CONCERNED ABOUT HAVING ASTHMA?	1	2	3	4	5	6	7 10
8. Feel SHORT OF BREATH as a result of your asthma?	1	2	3	4	5	6	7 11
9. Experience asthma symptoms as a RESULT OF BEING EXPOSED TO CIGARETTE SMOKE	1	2	3	4	5	6	7 12
10. Experience a WHEEZE in your chest?	1	2	3	4	5	6	7 13
11. Feel you had to AVOID A SITUATION OR ENVIRONMENT BECAUSE OF CIGARETTE SMOKE?	1	2	3	4	5	6	7 14

Macrolide Asthma Study

Protocol AR010, Version 1, 7th January 2009

Visit 2, Baseline
Asthma Quality of Life Questionnaire 2
Page 7

Version 1.0

Screening No. ____ 01 Initials ____ 02 Date of Visit ____ D D M M Y Y Y Y 03

HOW MUCH **DISCOMFORT OR DISTRESS** HAVE YOU FELT **DURING THE LAST 2 WEEKS**?

	A Very Great Deal	A Great Deal	A Good Deal	Moderate Amount	Some	Very Little	None
12. How much discomfort or distress have you felt over the last 2 weeks as a result of COUGHING?	1	2	3	4	5	6	7 04

IN GENERAL, **HOW MUCH OF THE TIME DURING THE LAST 2 WEEKS** DID YOU:

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	Hardly Any of the Time	None of the Time
13. Feel FRUSTRATED as a result of your asthma?	1	2	3	4	5	6	7 05
14. Experience a feeling of CHEST HEAVINESS?	1	2	3	4	5	6	7 06
15. Feel CONCERNED ABOUT THE NEED TO USE MEDICATION for your asthma?	1	2	3	4	5	6	7 07
16. Feel the need to CLEAR YOUR THROAT?	1	2	3	4	5	6	7 08
17. Experience asthma symptoms as a RESULT OF BEING EXPOSED TO DUST?	1	2	3	4	5	6	7 09
18. Experience DIFFICULTY BREATHING OUT as a result of your asthma?	1	2	3	4	5	6	7 10
19. Feel you had to AVOID A SITUATION OR ENVIRONMENT BECAUSE OF DUST?	1	2	3	4	5	6	7 11
20. WAKE UP IN THE MORNING WITH ASTHMA SYMPTOMS?	1	2	3	4	5	6	7 12
21. Feel AFRAID OF NOT HAVING YOUR ASTHMA MEDICATION AVAILABLE?	1	2	3	4	5	6	7 13
22. Feel bothered by HEAVY BREATHING?	1	2	3	4	5	6	7 14
23. Experience asthma symptoms as a RESULT OF THE WEATHER OR AIR POLLUTION OUTSIDE?	1	2	3	4	5	6	7 15
24. Were you WOKEN AT NIGHT by your asthma?	1	2	3	4	5	6	7 16
25. AVOID OR LIMIT GOING OUTSIDE BECAUSE OF THE WEATHER OR AIR POLLUTION?	1	2	3	4	5	6	7 17

Macrolide Asthma Study

Protocol AR010, Version 1, 7th January 2009

Visit 2, Baseline
Asthma Quality of Life Questionnaire 3
Page 8

Version 1.0

Screening No. ____ 01 Initials ____ 02 Date of Visit ____ D D M M Y Y Y Y 03

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	Hardly Any of the Time	None of the Time
26. Experience asthma symptoms as a RESULT OF BEING EXPOSED TO STRONG SMELLS OR PERFUME?	1	2	3	4	5	6	7 04
27. Feel AFRAID OF GETTING OUT OF BREATH?	1	2	3	4	5	6	7 05
28. Feel you had to AVOID A SITUATION OR ENVIRONMENT BECAUSE OF STRONG SMELLS OR PERFUME?	1	2	3	4	5	6	7 06
29. Has your asthma INTERFERED WITH GETTING A GOOD NIGHT'S SLEEP?	1	2	3	4	5	6	7 07
30. Have a feeling of FIGHTING FOR AIR?	1	2	3	4	5	6	7 08

HOW LIMITED HAVE YOU BEEN **DURING THE LAST 2 WEEKS**?

	Totally Limited	Extremely Limited	Very Limited	Moderate Limitation	Some Limitation	A Little Limitation	Not at all Limited
31. Think of the OVERALL RANGE OF ACTIVITIES that you would have liked to have done during the last 2 weeks? How much has your range of activities been limited by your asthma?	1	2	3	4	5	6	7 09
32. Overall, among ALL THE ACTIVITIES that you have done during the last 2 weeks, how limited have you been by your asthma?	1	2	3	4	5	6	7 10

DOMAIN CODE:

SYMPTOMS: 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 29, 30
ACTIVITY LIMITATION: 1, 2, 3, 4, 5, 11, 19, 25, 28, 31, 32
EMOTIONAL FUNCTION: 7, 13, 15, 21, 27
ENVIRONMENTAL STIMULI: 9, 17, 23, 26

For office use only:

Form checked for completeness ☐

Appendix 3: Leicester Cough Questionnaire

Macrolide Asthma Study

Protocol AR010, Version 1, 7th January 2009

Visit 2, Baseline
Leicester Cough Questionnaire 1
Page 10

Version 1.0

Screening No. 01 Initials 02 Date of Visit D D M M Y Y Y Y 03

This questionnaire is designed to assess the impact of cough on various aspects of your life. Read each question carefully and answer by CIRCLING the response that best applies to you. Please answer ALL questions, as honestly as you can.

1. In the last 2 weeks, have you had chest or stomach pains as a result of your cough?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

04

2. In the last 2 weeks, have you been bothered by sputum (phlegm) production when you cough?

1	2	3	4	5	6	7
Every time	Most times	Several times	Some times	Occasionally	Rarely	Never

05

3. In the last 2 weeks, have you been tired because of your cough?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

06

4. In the last 2 weeks, have you felt in control of your cough?

1	2	3	4	5	6	7
None of the time	Hardly any of the time	A little of the time	Some of the time	A good bit of the time	Most of the time	All of the time

07

5. How often during the last 2 weeks have you felt embarrassed by your coughing?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

08

6. In the last 2 weeks, my cough has made me feel anxious

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

09

7. In the last 2 weeks, my cough has interfered with my job, or other daily tasks

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

10

8. In the last 2 weeks, I felt that my cough interfered with the overall enjoyment of my life

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

11

9. In the last 2 weeks, exposure to paints or fumes has made me cough

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

12

10. In the last 2 weeks, has your cough disturbed your sleep?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

13

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Macrolide Asthma Study

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Visit 2, Baseline
Leicester Cough Questionnaire 2
Page 11

Version 1.0

Screening No. [01] Initials [02] Date of Visit [03] D D M M Y Y Y Y

11. In the last 2 weeks, how many times a day have you had coughing bouts?

1	2	3	4	5	6	7
All of the time (continuously)	Most times during the day	Several times during the day	Some times during the day	Occasionally through the day	Rarely	None

04

12. In the last 2 weeks, my cough has made me feel frustrated

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

05

13. In the last 2 weeks, my cough has made me feel fed up

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

06

14. In the last 2 weeks, have you suffered from a hoarse voice as a result of your cough?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

07

15. In the last 2 weeks, have you had a lot of energy?

1	2	3	4	5	6	7
None of the time	Hardly any of the time	A little of the time	Some of the time	A good bit of the time	Most of the time	All of the time

08

16. In the last 2 weeks, have you worried that your cough may indicate serious illness?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

09

17. In the last 2 weeks, have you been concerned that other people think something is wrong with you, because of your cough?

1	2	3	4	5	6	7
All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

10

18. In the last 2 weeks, my cough has interrupted conversation or telephone calls

1	2	3	4	5	6	7
Every time	Most times	A good bit of the time	Some of the time	A little of the time	Hardly any of the time	None of the time

11

19. In the last 2 weeks, i feel that my cough has annoyed my partner, family or friends

1	2	3	4	5	6	7
Every time I cough	Most times when I cough	Several times when I cough	Some times when I cough	Occasionally when I cough	Rarely	Never

12

Thank you for completing this questionnaire.

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Appendix 4: Patient Diary Card

Subject number_____ Initials: ____ ____ ____								
	Date/Month DD/MM/YY							
MORNING	Peak flow [best of 3]							
	Was blue [reliever] inhaler used in the 4 hours before peak flow testing?	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No
	Did you wake up with asthma symptoms? No = 0; Once = 1 More than once = 2 Awake "all night" =3	0 1 2 3	0 1 2 3	0 1 2 3	0 1 2 3	0 1 2 3	0 1 2 3	0 1 2 3
EVENING	Night peak flow [best of 3]							
	Was blue inhaler taken within 4 hours of night peak flow ?	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No	Yes No
	1.How often did you experience asthma symptoms today? 0 → 6 0= none of the time 6 = all of the time	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6
	2. How much did your asthma symptoms bother you today? 0 → 6 0= not at all bothered 6 = severely bothered.	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6
	3. How much activity could you do today? 0 → 6 0= more than usual activity 6= less than usual activity.	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6
	4. How often did your asthma affect your activities today? 0 → 6 0=none of the time 6=all of the time.	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6	0 1 2 3 4 5 6
	5. How many puffs of the blue inhaler did you use today?							
	6. Have you taken your study tablets today?	Yes No N/A	Yes No N/A	Yes No N/A	Yes No N/A	Yes No N/A	Yes No N/A	Yes No N/A

Appendix 5: GINA Severity Classification

Macrolide Asthma Study

Protocol AR010, Version 1, 7th January 2009

Visit 1, Screening
GINA Severity Classification
Page 6

Version 1.0

Screening No. ____ (01) Initials ____ (02) Date of Visit D D M M Y Y Y Y (03)

Done at pre-screening? Yes ☐ 1 No ☐ 2 (04)

A. GINA SEVERITY CLASSIFICATION

1. Symptoms
< once a week ☐ 1 > once a week, but not daily ☐ 2
daily ☐ 3 (05)

2. Nocturnal symptoms
Not > twice a month ☐ 1 > twice a month ☐ 2
at least once a week ☐ 3 (06)

3. Exacerbations
brief ☐ 1 affect activity and sleep ☐ 2
frequent ☐ 3 (07)

4. Inhaled corticosteroid daily dose, mcg
None ☐ 1
Low Dose ☐ 2
Medium Dose ☐ 3
High Dose ☐ 4 (08)

Low dose: beclometasone 200-500, budesonide 200-400, fluticasone 100-250, ciclesonide 80-160, mometasone 200-400
Medium dose: beclometasone > 500-1000, budesonide > 400-800, fluticasone > 250-500, ciclesonide > 160-320, mometasone > 400-800
High dose: beclometasone > 1000, budesonide > 800, fluticasone > 500, ciclesonide > 320, mometasone > 800

5. LABA ☐ 1 Theophylline ☐ 2 LTA ☐ 3 Oral salbutamol ☐ 4 (09)

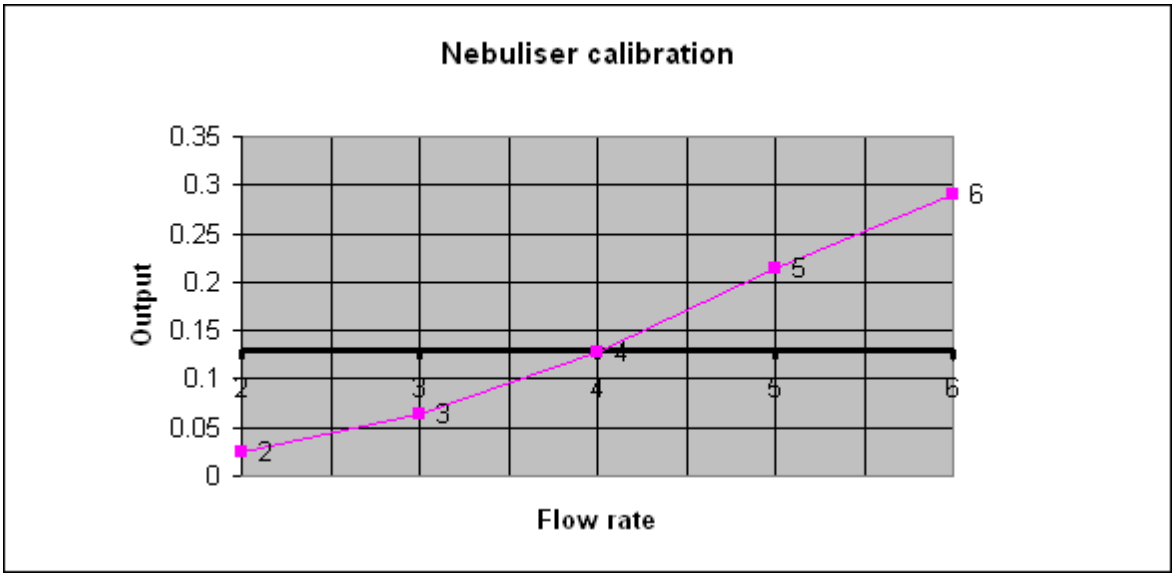
6. GINA Asthma Rx Category
1. Intermittent (salb alone) ☐ 1
2. Mild (low ICS or theo or LTA) ☐ 2
3. Moderate (low/medium ICS+LABA or high dose ICS or medium ICS+theo/LTA/oral salb) ☐ 3
4. Severe (high ICS+LABA +/- other tablets) ☐ 4 (10)

7. FEV_1 % predicted
≤60 ☐ 1 61-80 ☐ 2 ≥80 ☐ 3 (11)

8. GINA Severity Classification
1. Intermittent Asthma ☐ 1
2. Mild Persistent Asthma ☐ 2
3. Moderate Persistent Asthma ☐ 3
4. Severe Persistent Asthma ☐ 4 (12)

Appendix 6: Nebuliser calibration - methacholine

Flow	Preweight [g]	Postweight[g]	Difference in weight	Average Output ml	Output /min
2	35.38	35.327	0.053		
	35.334	35.29	0.044	0.0485	0.02425
3	35.335	35.205	0.13		
	35.368	35.24	0.128	0.129	0.0645
4	35.438	35.178	0.26		
	35.389	35.137	0.252	0.256	0.128
5	35.456	35.04	0.416		
	35.464	35.026	0.438	0.427	0.2135
6	35.483	34.948	0.535		
	35.551	34.923	0.628	0.5815	0.29075
	Average				
2	0.02425				
3	0.0645				
4	0.128				
5	0.2135				
6	0.29075				

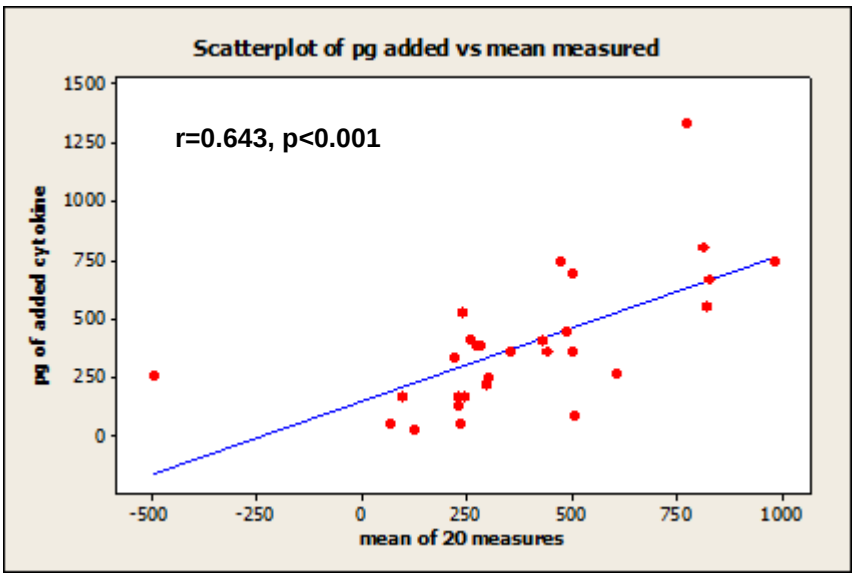


Appendix 7: Cytokine analysis quality control

Spiking of selected sputum samples with a single amount of each of the cytokines.

	Amount added (pg)	Amount recovered (pg)	
		Mean	S.D.
IL-1	444	488.2	235.4
IL-2	333	218.7	111.8
IL-4	417	260.6	110.7
IL-5	667	827	455
IL-6	133	227.2	91.8
IL-8	256	493	1240
IL-10	267	604.5	236.8
TNF	167	245.9	108.6
INFα	250	301.7	118.6
IFNγ	694	500.3	225.1
GM-CSF	750	979.3	354.2
RANTES	56	234.9	99.6

Graph below is a comparison of the amounts added vs mean amount recovered in table above



Appendix 8: Recruitment Challenges in Primary Care Randomised Controlled Trials: Lessons From Three Asthma Trials

Recruitment Challenges in Primary Care Randomised Controlled Trials: Lessons From Three Asthma Trials.

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Keywords: asthma, randomised controlled trial, recruitment, primary care

Abbreviations: ACQ – asthma control questionnaire; DNA – did not attend; ECG – electrocardiograph; FEV_1 – Forced expiratory volume in 1 second; ICS – Inhaled corticosteroids; SABA – short acting beta agonist; LABA – long acting beta agonist; PEF – Peak flow

Word Count: 2590

Abstract

Introduction:

There is a paucity of information regarding the challenges faced by those recruiting to randomised controlled trials (RCTs) from primary care. Here, we describe three primary care RCTs involving adults with asthma to illustrate the difficulties faced and highlight potential strategies to improve recruitment.

Methods:

We reviewed the participant flow charts and trial documentation relating to three trials in order to establish recruitment details with a particular focus on Trial 3. Trial 1 examined the effect of improved home ventilation on asthma control and house dust mite allergen levels. Trial 2 examined whether short-term treatment with atorvastatin improves lung function, asthma control and quality of life in smokers with asthma. Trial 3 examined whether short-term treatment with azithromycin improves lung function, asthma control and quality of life in smokers with asthma.

Results:

Recruitment rates across the three trials ranged from: 19% [68/444] to 40% [63/157] of practices invited; and 9% [715/8398] to 16% [820/4986] for patients. Strategies to improve recruitment included: employment of additional staff; direct telephone calls to patients; protocol amendments to broaden participation rates; addition of a second recruiting site.

Conclusion:

Achieving completion targets in primary care based RCTs is labour intensive. Close monitoring of recruitment targets is essential to identify problems early and allow remedial action. Recruitment can be improved with increased administrative support, closer partnership with primary care and direct contact with potential recruits. Openly acknowledging and addressing the challenges of recruitment will mean fewer trials are underpowered providing better returns for grant awarding bodies.

Word Count: 250

Introduction

Recruitment to randomized controlled trials [RCTs] is acknowledged to be challenging [1]. Less than a third of clinical trials successfully recruit 100% of their target, with 45% recruiting less than 80% of target. Consequently, over half of all clinical trials request an extension for recruitment [2]. Failure to achieve recruitment targets adds expense and logistical difficulties. Crucially this may result in a study being underpowered and unable to confidently answer its original research question. There is a paucity of information regarding the challenges faced by investigators from primary care [3] and how this can be overcome. A recent literature review has highlighted the need for research in this sphere, focusing particularly on the development of a repository of evidence based techniques and methods to aid those conducting primary care based RCTs [4]. Few recruitment methods employed by researchers in primary care are evidence based [5]. A Cochrane review quantified the effects of strategies to improve the recruitment of participants to RCTs [6]. Interventions such as telephone reminders to non-responders, use of opt-out rather than opt-in procedures for contacting potential trial participants, and open design were the most effective [6]. However, the latter two suggestions may be impractical for certain RCTs. It is noteworthy that this review intended to compare primary versus secondary care studies, but could not, because of the small number of eligible primary care studies. In this paper, we describe three separate primary care based RCTs featuring participants with asthma. These three RCTs illustrate the scale of recruitment difficulties. We focus particularly on the most recent trial [Trial 3], which, by learning from the preceding two studies, reached targets for completion. We aim to highlight the strategies employed to overcome recruitment challenges and discuss how these can be implemented to RCTs in general.

Methodology

We scrutinized three trials undertaken by the University of Glasgow Asthma Research Unit at Gartnavel General Hospital, Glasgow in collaboration with General Practice and Primary Care, University of Glasgow [7-9]. These three trials recruited predominantly from primary care. We also examined documents [protocols; ethics applications; amendments; patient databases and response rate records; CONSORT reports; subjects screening logs; reasons for screen-failure] detailing methods of recruitment throughout the lives of the trials, in order to identify potential strategies for improving future trial recruitment, focusing attention the most recent trial [9].

The first trial assessed the use of ventilation units in dwelling houses to establish if improved ventilation impacted on asthma control and house dust mite allergen levels [7]. Patients in this trial were contacted using a combination of media advertising, and personal phone calls to family practitioners by a respiratory physician.

The second trial examined whether short term treatment with atorvastatin improves lung function, asthma control, and quality of life in people with asthma who smoke [8]. The third trial asked the same question but tested azithromycin. A primary care research network, the Scottish Primary Care Research Network [SPCRN] supported Trials 2 and 3. Both of these trials used similar methods to contact potential patients; family practitioners were contacted by mail in the first instance. If practices agreed to participate a researcher or staff member from SPCRN would arrange to visit the practice to identify study eligible patients and send out study invites.

The focus of this paper is the most recent RCT [Trial 3] [9], in addition, details from the other two trials are provided to illustrate that recruitment challenges were generic and not specific to any one trial, trial medications, or specific patient groups.

During each trial, recruitment rates were monitored with details of the patient response rate, screening attendance, screen fail rate [with categorization of cause], randomization rate and completion rate circulated between all members of the study team on a bi-weekly basis.

The inclusion/exclusion criteria, recruitment methodology, and summary of protocol for each trial are detailed in Table 1.

Results

Recruitment

Recruitment results for all three studies are detailed in Figure 1 and Table 2. These show the low rates of participation by both family practices, ranging from 12-40%, and patients, ranging from 9-16% across the three trials. Trial 3, had a particularly low ratio of patients invited, to patients randomized (0.013%). A similar pattern was found in Trial 2. Only Trial 3 had an extension to the timeframe of the study to facilitate patient recruitment. There was a six-month lead time from employment of the clinical research fellow, until final approval was given to commence screening, with the study closing to new patients exactly 24-months later.

Administrative Support

A key reason for success in achieving the target number to complete in Trial 3 was the early recognition that randomization rates were lower than expected [Figure 1]. In January 2010, an additional staff member was seconded to the study one day per week to facilitate recruitment. Figure 1 illustrates the positive effect this had on screening and subsequent randomization numbers [vertical arrows]. By November 2010, this secondment ended, with a negative impact on recruitment rates thereafter. A new staff member was seconded from January 2011, and once more an increase in the screening and randomization rate can be observed.

Participation by Family Practices

Family practitioners were asked to permit access to computer registries and screen the list of potential patients to ensure unsuitable patients [e.g. terminally ill] were excluded. Although this limited input was clearly specified on the study information leaflets, and the subsequent practice workload being minimized by the study team, the majority of family physicians cited lack of time and current workload as the reason for non- participation. This problem was partially overcome by having a family practitioner seconded to the study telephoning practices that had responded negatively, and discuss the trial directly with family physicians. Non-responding practices were also contacted and practitioners were reassured about the minimal volume of work involved. Consequently the pool of practices agreeing to participate was increased by a further 10, to a total of 84.

Patient participation

This poor response rate had been identified in previous studies within our unit [10] and elsewhere [11] and in anticipation of this, ethical approval had been sought and granted to permit follow up phone calls to non-responders. Although labour intensive, this strategy did improve recruitment [Figure 1 – arrows]. Pre-emptory ethical approval reduced the lead time in commencement of this method, hence maximizing the time available to reach the recruitment targets.

Increasing the patient pool

A second site was opened, extending the geographical reach of the study. The original study approval was for one health board area within Scotland [Greater Glasgow and Clyde], with a patient population of 1.2 million. The study recruitment area was extended to include additional catchment areas, still recruiting predominantly from primary care, providing an additional pool of 367,000 patients. This extension was not as helpful as originally envisaged. There were delays in getting additional ethical and research governance approvals, and fewer practices than expected agreed to participate, often citing the large distances which patients would have to travel as a the main barrier. These limitations led to only eight patients being screened, and none being randomized.

Protocol amendments

The original trial protocol required patients be maintained on short- acting-β_2-agonists and low-dose [400mcg daily] inhaled beclometasone or equivalent. Weaning and withdrawal of long-acting β_2-agonist [LABA] had a negative impact on recruitment in two ways. Many patients experienced an apparent deterioration in asthma control, precluding them from subsequent randomization and a proportion of patients declined participation as they preferred not to interfere with their medication regimes. The study protocol was amended during the trial to allow patients to remain on their LABA.

Peak expiratory flow [PEF] technique is effort dependent and this was identified as a cause of high screen fail rates during the run-in phase. A baseline PEF reading was set at the initial screening visit, based on the results during that visit. Patients monitored their PEF daily at home twice daily for 4 weeks. Providing there was no evidence of exacerbation at the baseline visit the patient could then be randomized. Patients were advised that if their PEF fell below a threshold they should contact the research team. Less than 36% [20/56] of the patients who screen failed due to falling PEF contacted the research team, the rest continued for the screening period with levels below their alert level. A proportion of these patients had no clinical deterioration in asthma control, with the drop in PEF being accounted for due to poor technique when performing measurements unsupervised at home. This phenomenon led to numerous patient exclusions. This issue was difficult to address, with increasing tuition on PEF technique appearing to make little difference when patients returned home to perform recordings unsupervised.

Discussion

This report suggests that there are two specific areas presenting challenges to investigators recruiting from primary care: firstly encouraging family practice involvement, and secondly encouraging patient participation Recruiting from primary rather than secondary care is generally viewed as more

challenging, but there is little in the way of formal comparisons [4, 12, 13]. The CONSORT statement does not require provision of such information [14] and hence the full extent of recruitment difficulties are rendered invisible when trials are reported. Primary care involvement in recruitment varies and results in difficulty establishing the magnitude of the challenges faced by recruitment in primary versus secondary care. This can range from as little as allowing research teams to access primary care databases, allowing mailings to specific patient populations, or formally recruiting, consenting and even undertaking the intervention. Our findings suggest several key areas for investigators to consider when planning RCTs, both in relation to improving partnership with primary care, anticipating recruitment challenges, and regarding disease specific issues, in this case asthma. Several studies suggest that minimizing physician workload is crucial to maximizing participation and retention in clinical trials [4, 15-18]. However, despite efforts to emphasize the minimal work this study entailed, the majority of family physicians suggested workload was the reason for not participating. Finding the right balance in the practice-information-sheet regarding workload seems equally as important as the patient-information-sheets. Personal phone calls to family physicians in trials 1 and 3 did aid recruitment, and been found elsewhere [19]. In addition the peer-to-peer approach was also beneficial in our studies as in others [19]. Financial incentives have been advocated by some, but there is conflicting evidence of its effectiveness. Studies, with much larger financial incentives than our trial still reported poor recruitment [16, 20], whilst others found benefits [21]. Generally, the literature regarding physician attitudes to financial payments suggests that this has little bearing on the decision to participate [5, 22-24]. However, more recent studies suggest that financial payment may actually be more important than previously recognized [16, 17, 20, 25]. Again, this information is rarely reported, making it difficult to draw firm conclusions on this issue. In Trial 3, approximately half the patients who responded positively to the initial mailing were excluded. Telephoning patients is a useful recruitment method with known positive effects on recruitment [21] but in Trial 3 particularly, a significant amount of time was lost telephoning patients who were not suitable. This raises the question of whether the search undertaken in the practices could have been more specific and excluded patients on certain medications or with insufficient symptoms. This strategy could be considered for future studies; however there are risks that patients who would be eligible could be missed.

Importance of patient participation rates

Patient participation rates were low across all three trials, ranging from 9-16%. There appears to be no available data on what 'average' participation rates are, and often trials do not specifically volunteer this information. Patient participation rates to family physician mailings range from as low as 7% [26], to as high as 55% [20]. The authors from the latter trial felt that their high participation rate reflected specific targeting of individuals with known cardiovascular disease in a secondary prevention intervention.

Anticipating difficulties

Close monitoring of recruitment rates allowed early identification of slow recruitment rates and was crucial in alerting the investigators to institute changes in order to achieve target randomization in trial 3, albeit with the a 6 months extension. A range of strategies, recommended in the general literature on RCT recruitment, were used to maximize success. Recruitment difficulties were anticipated, and strategies were already in place, to be utilized as soon as rates were identified as unsatisfactory. Importantly, a significant amount of time and resources were invested in adding a second recruitment site in this study, which yielded limited benefits. It has been suggested previously that increasing eligibility of participants to the available recruitment site is more effective than the inclusion of additional sites, which our experience would corroborate [18]. Also, flexibility of the funder to allow protocol changes, and importantly the time to allow protocol changes to take effect was critical in our

study, and elsewhere in the literature [27]. Dedicated enrollers were utilized as an adjunct with significant impact. This has been identified elsewhere as an important factor in recruitment [28].

Trial specific issues

Specific issues were identified which contributed to the problems with recruitment. The number of patients who screen-failed [67% of 234] due to a deterioration in asthma control was a major hurdle to achieving the randomization target [Table 3]. This was in part ameliorated by the change in protocol to allow continuation of LABA. The loss of these patients who had undergone a lengthy screening visit represented a significant waste of investigator time, and consideration of how to minimize this is important for future trials.

Limitations

While this paper addresses an important issue for primary care investigators, it has a number of limitations. The trials described all focused on one disease area. Whilst the findings resonate with the limited literature on primary care trials, it will be important to explore whether primary care trials in other disease areas encounter the same difficulties. Secondly, the trials described were all conducted in one country, Scotland, and it is possible that recruitment may be less difficult in other nations. However, our findings are consistent with the current evidence that recruitment to randomized controlled trials is difficult, with targets often missed, leading to underpowered trials, or requiring extension of recruitment phases and additional funds. The issues are present in countries other than the UK [2, 12, 15, 29]. In addition, we presented the details of three trials to demonstrate that the difficulties described in the most recent were evident with different research personnel, and also in non-drug trials [7, 8]. Finally, this paper provides a retrospective account of the recruitment challenges faced. Ideally future studies would be prospective and might use qualitative methods to help increase understanding of the difficulties faced.

Conclusion

Achieving the completion target in primary care based RCTs is extremely labour intensive. Close partnership working between research units and primary care practitioners, increased access to primary care patient databases and direct contact with potential recruits is beneficial. Close monitoring of recruitment targets is essential in order to identify problems early, and allow remedial action to be taken. Based on our experience we recommend obtaining ethical approval for strategies to be deployed in the face of recruitment difficulties during the initial ethics application, rather than applying for amendments once difficulties arise, a strategy which proved successful in this latter trial. Increased transparency about the magnitude of recruitment difficulties is required to allow more realistic estimates of recruitment timescales and costs. This is unlikely to happen unless the CONSORT statement is extended to require reporting of such information. There are increasing calls for recruitment information to be provided on public registers of clinical trials such as ClinicalTrials.gov [30]. For primary care asthma trials, consideration should be given to methods to minimize "false" deterioration of patients during the run-in period due to poor PEF technique.

The bulk of health care is delivered within primary care, and thus recruitment from primary care for RCTs is essential. If we are to achieve recruitment targets, meet end points and provide a better return for grant awarding bodies, it is essential for more primary care trials to meet recruitment targets.

Conflicts of Interest: The authors declare that they have no conflicts of interest in relation to this article.

Author contribution:

Conception and design of this study [DM, FSM]; Trial 1 [NCT, RC]; Trials 2 & 3 [NCT, RC, FSM]
Analysis and interpretation: DM, EJC, FSM, NCT, RC, GW.
Drafting the manuscript for important intellectual content: DM, EJC, FSM, NCT, RC, GW.

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Figure and Table Legends

Figure 1: Cumulative recruitment for Trial 3.

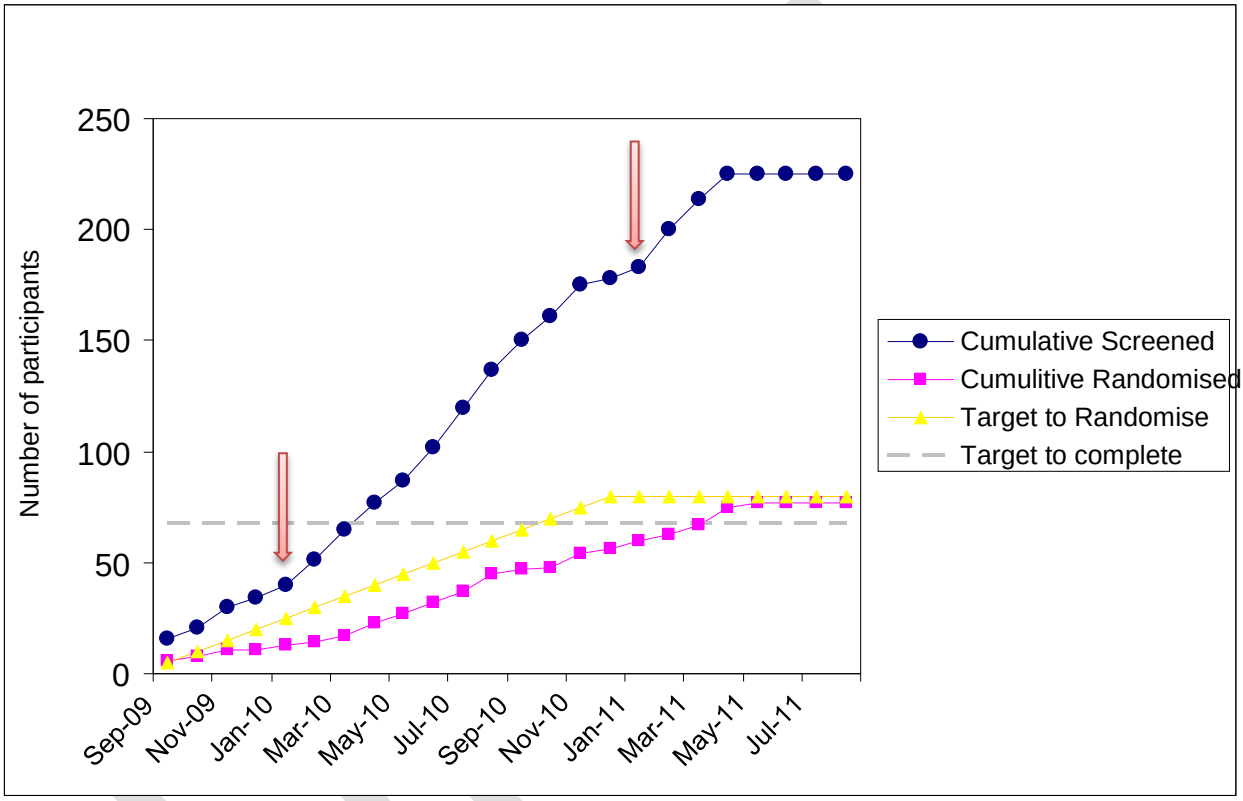

Table 1. Description of featured trials

	Trial 1 [2009] [7]:	Trial 2 [2011] [8]:	Trial 3 [Completed September 2011]:
Title	Effect of improved home ventilation on asthma control and house dust mite allergen levels	Effects of short-term treatment with atorvastatin in smokers with asthma - a randomised controlled trial	Effect of macrolides on asthma control, airway inflammation and bacterial colonization in smokers with asthma.
Inclusion Criteria	Age 16 to 60 years asthma symptoms for > 1 year on regular ICS daily symptoms reversible airflow obstruction minimum FEV_1 > 50% predicted at baseline no exacerbations in the preceding month	aged 18 to 60 years asthma symptoms for > 1 year current smokers reversible airflow obstruction	aged 18 to 70 years asthma symptoms for > 1 year current smokers reversible airflow obstruction maintain asthma without exacerbations on beclometasone 400mcg/day & SABA as required, for 4 weeks pre-screening, requiring weaning off other asthma medications e.g. LABA
Exclusion Criteria	were likely to move house had a pet that provoked symptoms	already on a statin, or a medication which could interact with statins asthma exacerbation within the preceding 6 weeks	ex-smokers or those who planned to quit unstable asthma respiratory infection within the preceding 4 weeks allergy to, or on medication with interactions with, azithromycin
Recruitment methods	Participants were recruited from family practices and hospital clinics	Participants were recruited from family practices, hospital clinics and research databases	Participants were recruited from family practices, hospital clinics and research databases.
GP Practice reimbursement	Practice time reimbursed at hourly rate £39 for GP, £15.53 for practice nurse, £12 for practice manager	£25 per practice	£101 per practice
Protocol	Baseline screening visit, with diary of preceding 2 weeks PEF readings Randomized to receive ventilation unit or placebo unit Attended for follow up visits at 3, 6, 9 & 12 months (measuring PEF twice daily for preceding 2 weeks)	weaned off ICS and LABA, for 2 weeks prior to randomization randomized to atorvastatin or placebo for 8 weeks, with ICS commenced in both groups at week 4 Short acting β_2 agonists allowed throughout minimum of 4 further visits required for completion of the study	attended for clinical screening, informed consent & issuing of diary & electronic PEF meter to record their PEF daily Four weeks later diary and PEF readings examined to ensure no exacerbations Randomized to 12 weeks of azithromycin or placebo 5 visits over the 12 week period

Table 2. Recruitment figures for featured RCTs

	Trial 1	Trial 2	Trial 3
Target to Randomize	128	80	80
Target to Complete	n/a	68	68
Total number of GP practices invited	157	438	444
Positive GP practice responses (% of invited)	63 (40%)	54 (12%)	84 (19%) §
Total number of patients invited	4986	2839	8398
Positive responses received from patients (% of invited)	820 (16%)	331 (12%)	715 (9%)
Assessed for Eligibility	820	286	705
Excluded by telephone /pre visit screening	338	111	370
Not meeting eligibility criteria	n/a	n/a	347
Unwilling/unable to consent	n/a	n/a	23
DNA initial appointment	n/a	44	101
Attended initial appointment	482	131	234
Consented	482	129	225
Screen failed	319 ‡	53	148
Randomised (% of those consented)	119 (25%)	71 (55%)	77 (34%)
Completed	100	60	71

§ includes 4 practices in responding post recruitment closure; 80 practices visited. 10 practices agreed during post-invitation phone call

‡ includes participants whose houses were not suitable for installation of the ventilation system

n/a not available/applicable

Table 3. Reasons for screen-failure in Trial 3

Reason	**Trial 3**
	n
Exacerbation of asthma	56
DNA Visit 2	37
Not symptomatic enough (ACQ<1)	11
Withdrew	10
Prolonged QTc on ECG	7
No reversibility/methacholine negative	7
Unable to wean off asthma medications	4
Abnormal blood tests	4
Other	12
Total	**148**