Lipoxin A$_4$ on neutrophil reprogramming in bronchiectasis

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This thesis is dedicated to my husband Punit for his love, encouragement and immense patience and to my parents Rita and Alok who have supported me all the way.

CONTENTS

DECLARATION

This thesis describes work undertaken in the University of Edinburgh's Centre for Inflammation Research, Queen's Medical Research Institute and the Department of Respiratory Medicine, Royal Infirmary of Edinburgh from September 2013 to August 2016. The work described in this thesis has been my own and the writing of this thesis has been entirely my own undertaking. This work has not previously been submitted for a higher degree or other professional qualification.

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ABSTRACT

INTRODUCTION

Bronchiectasis is a common chronic debilitating respiratory condition. Patients suffer daily cough, excess sputum production and recurrent chest infections because of inflamed and permanently damaged airways. The pathogenesis of bronchiectasis is poorly understood. Pulmonary pathology shows excess neutrophilic airways inflammation, but despite this over two thirds of patients are chronically infected with potential pathogenic microorganisms. The acute inflammatory response is a protective mechanism that is evolved to eliminate invading organisms and should ideally be self-limiting and lead to complete resolution. The driver for persistent neutrophilic airway inflammation in bronchiectasis is unknown, but infection is considered to play a major role.

AIMS

The main aims of this thesis were to: (i) Characterize neutrophils in the serum and airways in bronchiectasis in the stable state and during exacerbations; (ii) Cohort study to establish if LXA_4 deficiency correlates with disease severity (iii) Characterize lipids in bronchiectasis airways and peripheral blood to establish the correlation of LXA_4 to disease severity; (iv) To investigate a potential mechanism for low levels of LXA_4 in bronchiectasis, lipoxin biosynthetic genes expression will be measured; (v) Assess the anti-inflammatory and pro resolution effect of LXA_4 on neutrophils and monocyte derived macrophages from healthy volunteers; (vi) Assess the anti-inflammatory and pro resolution effect of LXA_4 on neutrophils during exacerbations in bronchiectasis and community acquired pneumonia.

Methods

(I) To establish the serum neutrophil subtype in stable state and following antibiotic treatment in patients with bronchiectasis, the following studies were done.

Inclusion criteria: Patients aged 18-80 were recruited. All had an established radiological diagnosis of bronchiectasis (CT of the chest). Patients had clinically significant bronchiectasis, aetiology being either idiopathic or post infection.

Exclusion Criteria: current smokers or ex-smokers of less than 1 year; >20 pack year history; cystic fibrosis; active allergic bronchopulmonary aspergillosis; active

tuberculosis; poorly controlled asthma; severe COPD requiring nebulised bronchodilators or long term oxygen therapy; patients on aspirin or leukotriene inhibitors, pregnancy or breast feeding, active malignancy.

A. 6 patients with mild bronchiectasis, 6 patients with severe bronchiectasis and 6 healthy volunteers were recruited. Serum and airways neutrophils were subsequently isolated. Neutrophil apoptosis, CD11b and CD62L expression, myeloperoxidase release, superoxide generation, phagocytosis and killing of GFP labeled bacteria were assessed.

B. To compare serum with airways neutrophils function, bacterial phagocytosis and killing of GFP labeled bacteria was done, with both serum and airways neutrophils. Samples were obtained from the above group of patients.

C. To establish neutrophil function following antibiotic treatment, 6 bronchiectasis patients at the beginning (day1) and the end (day14) of intravenous antibiotic therapy for an exacerbation were studied. As a control group, 6 community acquired pneumonia patients at the beginning (day1) and the end (day 5) of intravenous antibiotic therapy for infection were studied. Induced sputum and peripheral blood was taken at day1 and 5, where able. Phagocytosis and killing of GFP labeled bacteria was assessed and the two groups compared.

(II) To address if lipoxin A_4 deficiency correlates with disease severity, a cohort study was done in bronchiectasis patients. 169 patients were recruited and followed up for 1 year. Assessments done were Bronchiectasis severity index, systemic inflammatory markers (white cell count, ESR and c-reactive protein), Forced Expired Volume in 1sec, Forced Vital Capacity and its ratio, antibiotic courses in 1 year, hospital admissions in 1 year, sputum microbiology, quality of life assessments by Leicester Cough Questionnaire and St. Georges Respiratory Questionnaire, interleukin 8, myeloperoxidase, neutrophil elastase and leukotriene B_4 (from sputum).

(III) To assess effect of lipoxin on disease severity, 6 healthy volunteers, 10 patients with mild disease, 15 with moderate and 9 with severe disease were recruited. Disease severity was calculated as per the bronchiectasis severity index. All participants had

60mls of blood taken and underwent a bronchoscopy. Two segments of the lungs were washed out from bronchiectasis patients, an area affected by bronchiectasis and an area unaffected by bronchiectasis. This led to patients acting as their own internal control. Serum and airways neutrophils (from both segments) were subsequently isolated.

Assessments done were systemic inflammatory markers (white cell count, ESR and c-reactive protein), serum lipoxin A_4 and the cathelicidin LL-37, Forced Expired Volume in 1sec, Forced Vital Capacity and its ratio, transfer factor for carbon monoxide, antibiotic courses in 1 year, hospital admissions in 1 year and sputum microbiology. Phagocytosis and bacterial killing were assessed by both serum and airways neutrophils. From bronchoalveolar lavage fluid (BALF), I measured myeloperoxidase and neutrophil elastase. For both serum and BALF, lipidomics were obtained.

(IV) To address the impact of anatomic compartment, gene expression was measured in from endobronchial brushings from the same cohort of bronchiectasis patients and controls as above, where samples were available. qPCR was performed for the following eicosanoid biosynthetic genes- 5 Lipoxygenase (LOX), 15 LO-A, 15LO-B and leukotriene (LT) A_4 hydrolase.

(V) To assess the anti inflammatory and pro resolution effect of LXA_4 on neutrophils and monocyte derived macrophages from healthy volunteers, freshly isolated PMN will be treated with LXA_4 or vehicle control. Spontaneous apoptosis was measured. fMLF and cytochalasin B was added and the inflammatory response assessed measuring myeloperoxidase (MPO), free neutrophil elastase (NE), CD11b, CD18 and CD62L. Human monocytes and PMNs were isolated from bronchiectasis patients. Following differentiation, LXA_4 treated or control adherent, washed MDMs will be incubated with apoptotic stained PMNs. Efferocytosis was analyzed by flow cytometry.

(VI) To establish the effect of Lipoxin A_4 on neutrophil function following antibiotic treatment, the same study group used to evaluate aim 1 was taken. As a control group, 6 community acquired pneumonia patients at the beginning (day1) and the end (day 5) of oral or intravenous antibiotic therapy for infection were studied. Induced sputum

and peripheral blood was taken at day1 and 5, where able.

Phagocytosis and killing of GFP labeled bacteria and the effect of Lipoxin A_4 was assessed and the two groups compared. Serum and sputum lipidomics were obtained in bronchiectasis exacerbations on day 1 and day 14. Serum lipidomics was obtained in pneumonia on day 1 and day 5.

RESULTS

(I) Neutrophil sub type study (Studied on healthy volunteers/ mild/ severe bronchiectasis)

Peripheral blood neutrophils from bronchiectasis patients showed that there was significantly more viable neutrophils in mild and severe bronchiectasis compared to healthy volunteers, $p=0.002$ and $p=0.005$ respectively. In addition, there was significantly less apoptotic neutrophils in mild and severe bronchiectasis compared to healthy volunteers, $p=0.0003$ and $p<0.0001$ respectively. There was a significantly higher level of CD11b in the mild ($p=0.01$) and severe bronchiectasis ($p=0.01$) compared to healthy volunteers. There was more CD62L shedding ($p=0.02$) and myeloperoxidase release ($p=0.04$) in bronchiectasis compared to healthy volunteers. There was lesser phagocytosis in mild ($p=0.04$) and severe ($p=0.03$) bronchiectasis compared to healthy volunteers. This led to lesser bacterial killing in mild ($p=0.04$) and severe ($p=0.0004$) bronchiectasis compared to healthy volunteers.

On comparison of serum to airways neutrophils, peripheral blood neutrophils had higher phagocytic capacity ($p<0.0001$ for both mild and severe) and bacterial killing ($p=0.02$ for both mild and severe) than airways neutrophils.

During exacerbations, there was significantly higher phagocytosis ($p=0.02$) and killing at the end of exacerbation ($p=0.03$).

Similarly, in community-acquired pneumonia, there was significantly higher phagocytosis ($p=0.03$) and killing at the end of exacerbation ($p=0.03$). When I compared bronchiectasis exacerbations with pneumonia, I found although there was no difference in phagocytosis, there was significantly higher bacterial killing in pneumonia than bronchiectasis, both at the start ($p<0.0001$) and at the end ($p<0.0001$) of infection.

(II) Longitudinal cohort study in bronchiectasis

In patients sufficient in serum LXA_4 (>135 pg/ml) they had lesser antibiotic courses for an exacerbation (p=0.02), lesser levels of LTB_4 (p<0.0001) and myeloperoxidase (p<0.0001), over the course of a year compared to lipoxin deficient patients.

(III) Bronchoscopy study (Studied on healthy volunteers/ mild/ severe bronchiectasis)

In serum, there was significantly higher levels of Lipoxin A_4 detected in healthy volunteers compared to bronchiectasis patients, p=0.04.

In peripheral blood neutrophils, there was a statistically significant improvement in phagocytosis by LXA_4 in mild, moderate and severe bronchiectasis groups, p=0.01, p<0.0001 and p=0.01 respectively. Additionally, there was a statistically significant improvement in bacterial killing by LXA_4 in mild, moderate and severe bronchiectasis groups, p=0.04, p=0.03 and p=0.01 respectively, in a dose dependent manner.

In airways neutrophils, in the unaffected segments, there was no significant improvement in phagocytosis with LXA_4 100nM in any of the groups. In the affected segments, there was a statistically significant improvement in phagocytosis by LXA_4 100nM in the mild, moderate and severe bronchiectasis groups by LXA_4 100nM, p=0.01, p=0.02 and p=0.04 respectively.

In the unaffected segments, there was a statistically significant improvement in bacterial killing with LXA_4 100nM in the severe group of patients only; p=0.02. In the affected segments, there was a statistically significant improvement in killing by LXA_4 in the mild, moderate and severe bronchiectasis groups by LXA_4 100nM, p=0.02, p=0.0005 and p=0.04 respectively.

There were significantly higher levels of myeloperoxidase (p=0.002) and neutrophil elastase (p=0.005) detected in bronchoalveolar lavage fluid from severe patients.

Serum lipidomics showed that there were significantly higher levels of PGE2, 15 HETE and LTB_4 in patients with moderate-severe disease compared to healthy controls, p=0.03, p=0.03 and p=0.02 respectively. BALF lipidomics showed that there were significantly higher levels of PGE2, 5HETE and 15 HETE in patients with moderate-severe disease compared to patients with mild disease and healthy volunteers, p<0.0001, p=0.004 and p=0.005 respectively. There were significantly higher levels of 9HODE and LTB_4 in moderate-severe patients compared to mild patients and healthy volunteers, p=0.04 and p<0.0001 respectively.

(IV) Gene expression (Studied on mild and moderate- severe bronchiectasis)

The delta Ct values for 5-LOX (p=0.01) and 15-LO-B (p=0.01) were both significantly increased in subjects with moderate-severe bronchiectasis compared with subjects with mild bronchiectasis. Delta Ct value of LTA_4 hydrolase was significantly decreased in moderate-severe bronchiectasis compared to mild bronchiectasis (p=0.007), indicating increased expression of in moderate-severe bronchiectasis. LTA_4 hydrolase was the most abundant RNA (lowest DCt) in bronchial brushings.

(V) *In vitro* studies of lipoxin on neutrophil function (Studied on healthy volunteers/ mild/ severe bronchiectasis)

Lipoxin was unable to modulate spontaneous apoptosis in any of the three groups; p=0.4, p=0.5 and p=0.4 in healthy, mild and severe bronchiectasis, respectively. There was a significant reduction in upregulation of CD11b by LXA_4 in a dose dependent manner in healthy volunteers, mild bronchiectasis and severe bronchiectasis patients; p= 0.005, p=0.008 and p=0.01 respectively. There was a significant improvement in shedding of CD62L by LXA_4 in a dose dependent manner in healthy volunteers, mild bronchiectasis and severe bronchiectasis patients; p=0.01, p=0.03 and p=0.04 respectively. There was a significant reduction in myeloperoxidase release by LXA_4 in healthy volunteers, mild bronchiectasis and severe bronchiectasis patients; p= 0.02, p=0.04 and p=0.02 respectively. There was significant reduction of superoxide release by LXA_4 in healthy volunteers (p=0.004) and severe bronchiectasis (p=0.03) but not on mild bronchiectasis (p=0.1). Lastly, on assessing bacterial phagocytosis and killing, there was a significant increase in phagocytosis pre treatment with LXA_4, led to a dose dependent increase in phagocytosis in healthy volunteers (p=0.0001), mild bronchiectasis (p<0.0001) and severe bronchiectasis (p=0.03). Similarly, Lipoxin A_4 led to a dose dependent increase in killing of GFP PAO1 in healthy volunteers (p=0.001), mild bronchiectasis (p=0.04) and severe bronchiectasis (p=0.01).

(VI) Exacerbations

In peripheral blood neutrophils, LXA_4 was able to significantly improve bacterial phagocytosis (start p=0.03, end p=0.004) and killing (start p=0.01, end p=0.007) (blood neutrophils) both at the start and end of pneumonia exacerbations.

In airways neutrophils, LXA_4 was did not improve phagocytosis but improved bacterial killing at the start (p=0.02) and end (p=0.01) of exacerbation in bronchiectasis.

In community acquired pneumonia, LXA_4 was able to significantly improve bacterial phagocytosis (start p=0.01, end p=0.03) and killing (start p=0.04, end p=0.01) both at the start and end of pneumonia.

CONCLUSION

(i) Peripheral blood neutrophils are reprogrammed and persist longer in bronchiectasis. These reprogrammed neutrophils are the key contributors to the ongoing persistence of inflammation in bronchiectasis.

(ii) There is a dysregulation of lipid mediators in the serum as well as in the airways of bronchiectasis patients. Lipoxin is able to override the pro survival signals in these reprogrammed neutrophils, stabilize and improve neutrophil function.

THESIS

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INTRODUCTION

1.1. BRONCHIECTASIS

"This affection of the bronchia is always produced by chronic catarrh, or by some other disease attended by long, violent, and often repeated fits of coughing."
R.T.H. Laennec, 1819.

1.1.1. DEFINITION AND EPIDEMIOLOGY

Bronchiectasis is a common chronic debilitating respiratory condition. First described by Laennec in 1819, then by Osler in the late 1800s, and then further detailed by Reid in 1950s, bronchiectasis has since undergone significant changes in prevalence, etiology, diagnosis and management (Reid 1950). Patients suffer daily cough, excess sputum production and recurrent chest infections because of inflamed and permanently damaged airways (Bilton 2008). Pulmonary pathology shows excess neutrophilic airways inflammation, but despite this over two thirds of patients are chronically infected with potential pathogenic microorganisms (Angrill 2002).

Reid defined bronchiectasis as permanent dilatation of the airways, a term that has stayed with the disease for more than 60 years. Bronchiectasis can be characterised by the pathologic or radiographic appearances. Cylindrical or tubular bronchiectasis is characterised by dilated airways; varicose bronchiectasis (so named because its appearance is similar to that of varicose veins) is characterized by focal constrictive areas along the dilated airways that result from defects in the bronchial wall; and saccular or cystic bronchiectasis is characterized by progressive dilatation of the airways, which leads to formation of large cysts or saccules and is always indicative of the most severe form of bronchiectasis (figure 1) (Reid 1950).
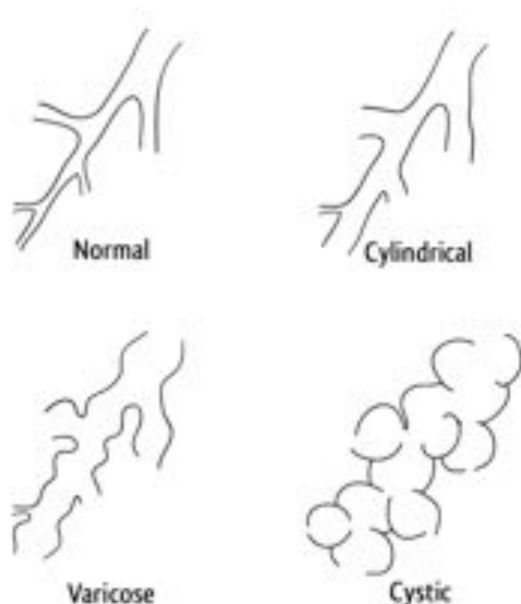
Figure 1. Bronchial dilatation in bronchiectasis as defined by Reid *et al*.

Quint and colleagues, used the Clinical Practice Research Datalink for participants registered between January 2004 and December 2013, to determine incidence, prevalence and mortality associated with bronchiectasis in the UK and investigate changes over time. The incidence and point prevalence of bronchiectasis increased yearly during the study period. Across all age groups, they found that the incidence in women increased from 21.2 per 100000 person-years in 2004 to 35.2 per 100000 person-years in 2013 and in men from 18.2 per 100000 person-years in 2004 to 26.9 per 100000 person-years in 2013. The point prevalence in women increased from 350.5 per 100000 in 2004 to 566.1 per 100000 in 2013 and the numbers in men from 301.2 per 100000 in 2004 to 485.5 per 100000 in 2013. Comparing mortality rates in women and men with bronchiectasis in England and Wales (n=11862) with mortality rates in the general population, data showed that in women the age-adjusted mortality rate for the bronchiectasis population was 1437.7 per 100000 and for the general population 635.9 per 100000. In men, the age-adjusted mortality rate for the bronchiectasis population was 1914.6 per 100000 and for the general population 895.2 per 100000. Bronchiectasis is surprisingly common and is increasing in incidence and prevalence in the UK, particularly in older age groups. Bronchiectasis is associated with a markedly increased mortality (Navaratnam et al 2016).

1.1.2. PATHOPHYSIOLOGY

The pathogenesis of bronchiectasis is poorly understood. Pulmonary pathology shows excess neutrophilic airways inflammation (Angrill 2001), but despite this over two thirds of patients are chronically infected with potential pathogenic organisms (Angrill 2002). The affected areas may show a variety of changes, including transmural airways inflammation, oedema, scarring, and ulceration. Patients suffer daily cough, daily sputum production and recurrent chest infections, leading to an inflammatory disease condition. The acute inflammatory response is a protective mechanism that is evolved to eliminate invading organisms and should ideally be self-limiting and lead to complete resolution (Serhan 2007, Mantovani et al 2011, Medzhitov 2010). However there is failure of resolution of inflammation in bronchiectasis, leading to irreversible damage and dilatation of the bronchial airways with loss of mucociliary function (Cole 1984). It is assumed there are intrinsic abnormalities of the innate and adaptive immune systems that predispose to impaired clearance of respiratory pathogens and exaggerated inflammatory responses secondary to abnormal immune regulation. Perhaps, repeated micro-aspiration of upper airway organisms during recurrent respiratory infections when mucociliary clearance is temporarily compromised may help establish lower airway infection. The driver for persistent neutrophilic airway inflammation in bronchiectasis is unknown, but infection is considered to play a major role (Angrill 2001). This is easy to understand as part of a 'vicious circle' which was first described by Cole and colleagues and all of our current management strategies are directed towards breaking this circle (Cole 1984).

1.1.3. VICIOUS CIRCLE

Described by PJ Cole in 1984 (Cole 1984), there is a 'vicious circle' (figure 2) of airways inflammation and bacterial infection in bronchiectasis. The lung is continuously exposed to inhaled pathogen and it is the primary and secondary defense of the lung that maintains sterility of the lung. The excessive neutrophilic airways inflammation leads to damage of the bronchial wall and paradoxically promotes more airways inflammation and bacterial infection creating a vicious cycle (Barker 2002, Cole 1984). During natural resolution, polymorphonuclear neutrophils are required for antimicrobial defense (Medzhitov 2010), but these cells must then apoptose and are removed from the inflammatory site by macrophages (Henneke and Golenbock 2004, Rossi et 2006, Dinarello 2012). Is this natural resolution impaired in bronchiectasis? There are no studies exploring the resolution mechanism in bronchiectasis or perhaps the lack of it, in the literature to date.
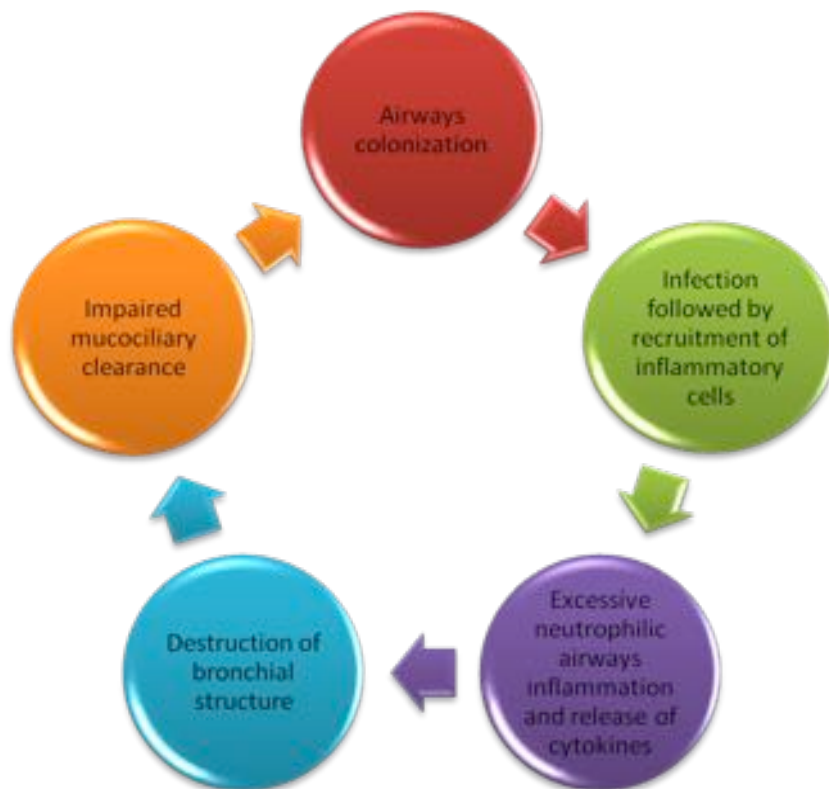
Figure 2. Vicious circle in the bronchiectatic airways as described by Cole.

1.1.4. AETIOLOGY

Although there are many different disease processes, bronchiectasis is the final pathological end point. All the different etiologies ultimately lead to destruction of the airway epithelium, damage of the mucociliary clearance thereby leading to persistent infection and inflammation. Part of the assessment of patients with bronchiectasis involves identifying where possible the primary insult and allowing specific management. Identifying an underlying cause may direct appropriate management. Examples include the commencement of immunoglobulin replacement therapy in patients with common variable immune deficiency and considering oral corticosteroid treatment in those with allergic bronchopulmonary aspergillosis. A significant proportion of adults have bronchiectasis secondary to previous pneumonia or other lower respiratory tract infections but often no cause is identified (Pasteur MC et al BTS bronchiectasis guidelines 2010). All other causes are much less frequent. Causes are summarized in table 1.

Etiology	Prevalence (%)	History/ Signs	Investigation	Expected findings
Idiopathic	53		Diagnosis of exclusion	
Post infection	29	History of previous infection (e.g. pneumonia; whooping cough; measles; TB);		
Immune defect	8		Immunoglobulin G, A, M and protein electrophoresis. IgG subclasses and antibody response to vaccines (especially *Pneumococcal* and *Tetanus*).	Decreased values of immunoglobulins, subclasses or functional antibody deficiency.
Allergic bronchopulmonary aspergillosis (ABPA)	7	History of asthma and fleeting infiltrates or	Full blood count; Total IgE, IgE to aspergillus, Aspergillus	Raised eosinophils, total IgE and IgE specific to aspergillus.

		proximal bronchiectasis on CXR or CT chest	precipitins; 24 hour sputum for aspergillus; HRCT	CXR or HRCT evidence of fleeting infiltrates or proximal bronchiectasis.
Aspiration/ Gastro oesophageal reflux (GORD)	4	History consistent with aspiration or reflux	Bronchoscopy if indicated (e.g. foreign body aspiration): oesophageal manometry	Foreign body/ mucus plugging in bronchus
Rheumatoid Arthritis (RA)	3	History of RA	Autoimmune screen (Anti CCP)	Positive screen
Cystic Fibrosis (CF)	3	Age <40 years, mal-absorption, male infertility; diabetes	CF cytogenetics (CFTR gene mutations); Sweat test	CFTR mutations Positive sweat test: chloride>60mmol/l
Ciliary Dysfunction	1.5	History of situs inversus/ productive cough/ deafness/ infertility	Ciliary motility assessment by inhalation of colloid albumin tagged with 99Tc	Radioactivity in the lungs is measured.

Ulcerative Colitis	<1	History of chronic productive cough, mal-absorption; diarrhoea; weight loss; joint pain; faecal calprotectin	Gastro-intestinal investigations (colonoscopy and GI referral if not already done)	Biopsy suggestive of inflammatory bowel disease.
Congenital	<1		N/A	

Table 1. Etiology, clinical findings and relevant investigations in bronchiectasis. HRCT= high resolution computed tomography; CFTR= cystic fibrosis transmembrane conductance regulator. (BTS bronchiectasis guidelines 2010)

1.1.4.1 Post infectious

Lower respiratory tract infections are associated with the etiology of bronchiectasis and most common is bacterial pneumonia. Pertussis, pulmonary tuberculosis, mycoplasma and viral pneumonia (particularly adenoviruses and measles but also influenza and respiratory syncytial viruses) have, in addition, all been linked directly to permanent lung damage and bronchiectasis (Pasteur et al 2000). Post-tuberculous bronchiectasis is often segmental or lobar in the area primarily affected (Scala et al 2000). In studies investigating the aetiology of bronchiectasis in those over the age of 50, infection was identified as the etiology in more than a third of the cases (Lonni et al 2015).

1.1.4.2. *Mycobacterium tuberculosis* and non-tuberculous mycobacteria

Bronchiectasis may result from pulmonary *Mycobacterium tuberculosis* infection, with the incidence reflecting the prevalence of tuberculosis in the population. It is also increasingly recognised that environmental mycobacteria are associated with localised or widespread bronchiectasis. Environmental mycobacteria have been isolated in 2% and 10% of random sputum specimens from patients with bronchiectasis, but the clinical significance is unclear (Fowler et al 2006). Patients with Mycobacterium avium complex (MAC) infection may develop bronchiectasis over years. Middle-aged or elderly women are particularly prone to MAC infection and this is known as Lady Windermere disease which particularly affects the middle lobe or the lingula (Reich and Johnson 1992). However, isolation of an environmental mycobacterial species should not necessarily be interpreted as pathogenic. Persistent isolation (colonisation) may occur without any change in clinical status. Careful follow-up is mandatory because colonisation can change to infection. Patients should be reassessed if there is a change in clinical features, change in sputum conventional microbial culture, there is a rapid decline in spirometry, or if there are characteristic HRCT changes (exudative 'tree-in-bud' bronchiolitis, mucus plugging, cavitating nodules, rapid progression of bronchiectasis or indeed cavitatory disease; (figure 3)). The species isolated will also influence the likelihood of infection (*M avium complex, M kansasii, M malmoense*), for example mycobacteria such as *M gordonae* maybe just a contaminant (Pasteur MC et al BTS bronchiectasis guidelines 2010).
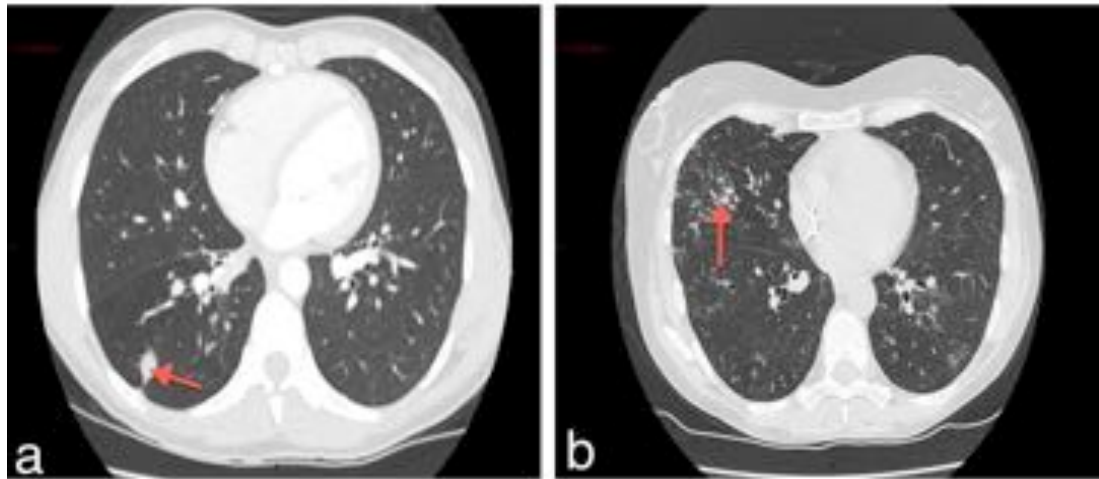
Figure 3a. HRCT showing tuberculous granuloma in bronchiectasis (as indicated by arrow). 3b. HRCT showing 'tree in bud' appearances in bronchiectasis (as indicated by arrow).

1.2. EXACERBATIONS

Definition of an exacerbation in bronchiectasis

The British Thoracic Society guideline (figure 4) recommends antibiotics for exacerbations that present with an acute deterioration (usually over several days) with worsening local symptoms (cough, increased sputum volume or change of viscosity, increased sputum purulence with or without increasing wheeze, breathlessness, haemoptysis) and/or systemic upset.
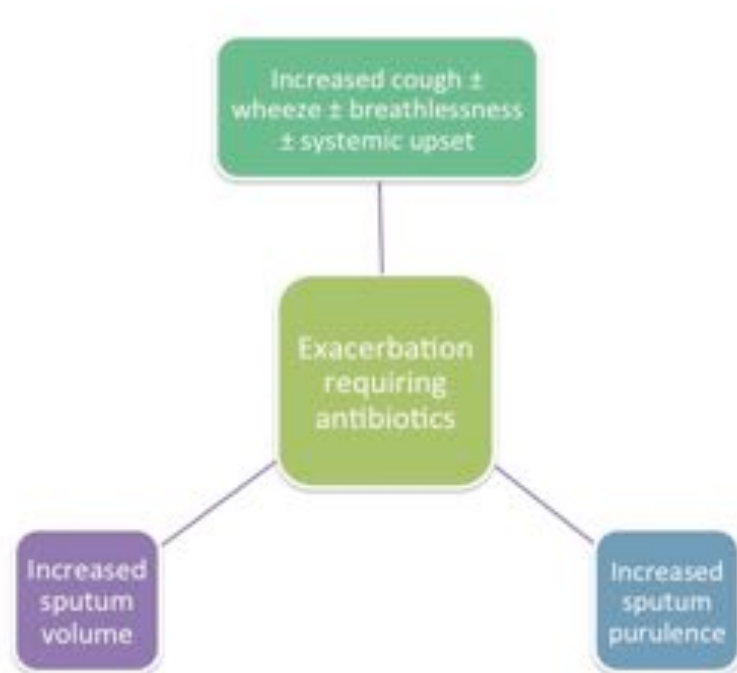
Figure 4. Exacerbations requiring antibiotics, as defined by the BTS guidelines, 2010.

1.3. SPUTUM MICROBIOLOGY AND COLONIZATION

In bronchiectasis, the normally sterile airways are chronically colonised with pathogenic organisms in about 70% of patients (Angrill 2002). In adults, definitions of colonization have included at least three isolates of an organism over a period of at least 3 months; and at least two isolates 3 months apart over 1 year (Pasteur MC et al BTS bronchiectasis guidelines 2010).

Common gram-positive organisms colonising the airways include *Streptococcus pneumoniae* and *Staphylococcus aureus* (both methicillin-sensitive and methicillin-resistant). Gram-negative pathogens include non-capsulated nontypable *Haemophilus influenzae*, *Moraxella catarrhalis*, *Klebsiella*, *Enterobacter* and non-mucoid and mucoid *Pseudomonas aeruginosa (*Angrill et al 2002*)*. The type of bacterial infection is important in the determination of the patient's prognosis. Studies have shown that patients colonised with *Pseudomonas aeruginosa* usually have worse symptoms, worse health-related quality of life and accelerated decline in forced expiratory volume over one second (FEV_1) (Martinez-Garcia et al 2007).

Recently, Finch and colleagues collected data from 21 observational studies that included a total of 3683 patients, comparing patient with *P. aeruginosa* to those without it. They concluded that *P. aeruginosa* was associated with a three fold increased risk of death and an increase in hospital admissions and exacerbations in adult bronchiectasis (Finch et al 2015).

The frequency of occurrence of the microorganisms is shown in figure 5 (Chalmers et al 2012).
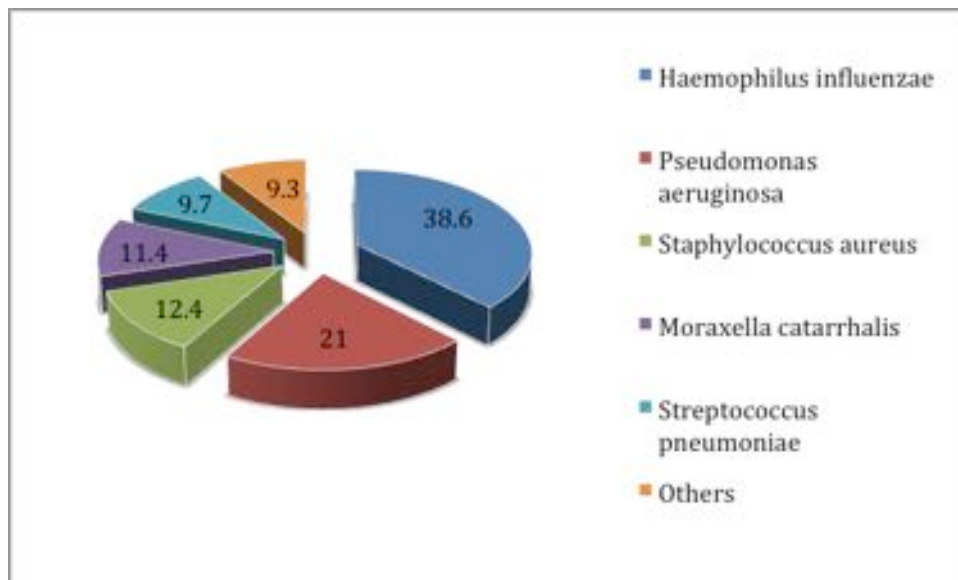
Figure 5. Summary of common pathogens isolated from bronchiectasis patients.

1.3.1. *Haemophilus influenzae*

In bronchiectasis, 39% of the patients are colonized with *Haemophilus influenzae* (Chalmers *et al* 2012). It is a small (1 μm X 0.3 μm), non-motile, pleomorphic, gram-negative coccobacillus. There are both capsulated and non-capsulated (also known as nontypable) strains. It has now been recognized that NTHi (nontypable *Haemophilus influenzae*) is a major cause of respiratory infection, which tends to be chronic and recurrent and includes sinusitis, otitis media, tonsillitis, pneumonia and chronic bronchitis and systemic infection (Murphy 2001). In the context of infection, it has the capacity to live intracellularly especially in macrophages (Craig et al 2002). In a study done by King *et al*, they confirmed that NTHi was the most commonly isolated *H influenzae* in bronchiectasis (King et al 2003).

There is no widely used effective vaccine for NTHi. There have been problems of antigenic variability and finding a suitable animal model. Most work has focused generally upon enhancing humoral immunity. Developing a vaccine to enhance T lymphocyte responses to NTHi may be important (Williams et al 1988).

H influenzae is the most common pathogen isolated from sputum of bronchiectasis patients. Most bronchiectasis patients with chronic NTHi infection are able to mount a highly effective humoral immune response that prevents systemic infection. *H influenzae* die within 3 hours hence to maximize the chances of isolating *H influenzae,* sputum specimens should reach the microbiology laboratory within 3h. This should be done routinely prior to starting antibiotic therapy for exacerbations. The choice of antibiotic and the duration of antibiotic therapy for treating exacerbations with *H influenzae* infection are summarized in table 2 and long-term antibiotic treatment in Table 3.

1.3.2. *Pseudomonas aeruginosa*

P. aeruginosa is a gram-negative bacillus (non capsulate and non sporing) that affects particularly the lower respiratory tract (Banerjee and Stableforth 2000). Without any treatment, *P. aeruginosa* infection persists in spite of the recruitment of the host's defence mechanisms and leads to decreased respiratory function (Dudley et al 2008).

In bronchiectasis, chronic infection with *P. aeruginosa* is observed in 24-33% (Pasteur et al 2000) patients. Early aggressive antibiotic treatment is recommended and an attempt should be made to eradicate it (Pasteur MC et al BTS bronchiectasis guidelines 2010). The BTS guidelines recommends that an attempt to eradicate *P. aeruginosa* should be made with high dose oral ciprofloxacin 750mg BD, for 2weeks (Pasteur MC et al BTS bronchiectasis guidelines 2010). Failing this, it is recommended to administer intravenous or nebulised anti pseudomonal antibiotic for 2-4weeks (Pasteur MC et al BTS bronchiectasis guidelines 2010). However, careful monitoring of renal function and side effects of high doses of antibiotics should be considered and treatment should be based on a balance of benefits outweighing risks. In a recent single blind randomised trial by Orriols and colleagues, they showed that 3 months of nebulised tobramycin following a short course of intravenous antibiotics may prevent bronchial infection with *P. aeruginosa* and has a favourable clinical impact on bronchiectasis. At the end of the study 54.5% of the patients were free of *P. aeruginosa* in the tobramycin group and 29.4% in the placebo group. The numbers of exacerbations, hospital admissions and days of hospitalisation were lower in the tobramycin than in the placebo group (Orriols et al 2015).

Chronic *P. aeruginosa* infection is associated with poorer lung function, decreased quality of life and frequent hospital admissions (Evans et al 1996, Wilson et al 1997). Martinez and colleagues (Martinez-Garcia et al 2007) conducted a study in 67 patients with stable bronchiectasis and followed them up for 2 years, conducting spirometry, microbiological analysis of sputum and recorded number of exacerbations over the study period. They detected chronic colonization with *P. aeruginosa* was an independent predictor of forced expiratory volume in 1-second (FEV_1) decline [odds ratio (OR), 30.4; 95% CI, 3.8-39.4; p=0.005].

1.3.3. Other potential pathogenic microorganisms (PPM)

The other PPMs of significance in bronchiectasis are *S. pneumoniae*, *M. catarrhalis*, enteric gram-negative organisms like *E Coli* and *Klebsiella* and *S. aureus* (both methicillin sensitive and methicillin resistant).

1.3.3.1. *Streptococcus pneumoniae*

In bronchiectasis, *S. pneumoniae* is detected in about 1-22% of patients (Angrill et al 2002). It is grown on blood agar. *S. pneumoniae* are lancet-shaped, gram-positive, facultative anaerobic bacteria with over 90 known serotypes, of which 23 are included in the pneumococcal vaccine Pneumovax ®. Most *S. pneumoniae* serotypes have been shown to cause disease, but only a minority of serotypes produces the majority of pneumococcal infections. Pneumococci are common inhabitants of the respiratory tract and may be isolated from the nasopharynx of 5-70% of adults, depending on the population and setting. *S. pneumoniae* is the most common cause of community acquired pneumonia and bacterial meningitis. In bronchiectasis, *S. pneumoniae* is frequently isolated in sputum cultures, varying between 1-22%.

S. pneumoniae infections are estimated to cause 500,000 cases of pneumonia, 55,000 cases of bacteraemia, and 6,000 cases of meningitis annually in the United States (Williams et al 1988). The clinical course of *S. pneumoniae* infections is affected by a number of factors including the site and severity of infection, the underlying health of the patient, and the adequacy of antimicrobial therapy. Therefore, it is not surprising that estimates of mortality are reported to range from <1% to >50% (Williams et al 1988). The choice of antibiotic and the duration of antibiotic therapy for treating exacerbations with *S. pneumoniae* infection in bronchiectasis are summarized in table 2 and long-term antibiotic treatment in Table 3.

1.3.3.2. *Moraxella catarrhalis*

It is a gram-negative, aerobic, oxidase-positive diplococcus. Studies have demonstrated that *M. catarrhalis* is isolated in 1-20% of patients with bronchiectasis. Its persistence within the respiratory tract is most likely aided by its capacity to invade respiratory epithelial cells, inactivate complement and form biofilms (Murphy et al 2009). *M. catarrhalis* is acquired early in life and is a prominent colonizer of the upper respiratory tract in infants and children, before decreasing substantially during the adult years. Antibiotic recommended for acute exacerbations is summarized in Table 2 and long-term antibiotic treatment for *M. catarrhalis* is summarized in Table 3.

1.3.3.3. *Staphylococcus aureus*

It is a facultative anaerobic gram-positive coccus. Staphylococci are nonmotile, non–spore-forming, and catalase-positive bacteria. The cell wall contains peptidoglycan and teichoic acid. Most strains of *S. aureus* are sensitive to the more commonly used antibiotics. Those that are sensitive to methicillin are termed methicillin-sensitive *S. aureus* (MSSA). Methicillin-resistant *S. aureus* (MRSA) and MSSA only differ in their degree of antibiotic resistance. Over the last 20 years, community and hospital infections with *S. aureus* have risen-in particular with MRSA and more recently vancomycin resistant *S. aureus*. Antibiotic recommended for acute exacerbations is summarized in Table 2 and long-term antibiotic treatment in Table 3.

1.3.3.4. Inflammatory response due to other bacteria (*Klebsiella, Enterobacteriaceae*)

Angrill and colleagues showed that bronchial colonization by PPMs is common in patients with bronchiectasis in a stable clinical situation and in more than 60% of these patients the distal airways were colonized. They also concluded that sputum culture is an adequate tool for the evaluation of bronchial colonization in bronchiectasis and that early diagnosis of bronchiectasis (before the age of 14), evidence of varicose-cystic bronchiectasis and a FEV_1 of <80% predicted are risk factors for the presence of PPMs in the airways (Angrill et al 2001). Additionally, in a further study done by the same group, they performed bronchoalveolar lavage (BAL) in 49 patients with bronchiectasis and 9 controls and found that in patients

with clinically stable bronchiectasis: (*1*) there is an active neutrophilic inflammatory response in the airways that is present in patients with sterile bronchi but is exaggerated in patients with PPMs colonizing the airways; (*2*) colonizing bacteria act as an inflammatory stimulus with greater bacterial load producing greater inflammatory response; (*3*) the bronchial inflammatory response was compartmentalized and could not be accurately evaluated in blood samples (Angrill et al 2002). Chalmers *et al*, investigated this further in interventional and observational studies in bronchiectasis. They studied the relationship between bacterial load and airway and systemic inflammation was investigated in 385 stable patients, 15 stable patients treated with intravenous antibiotics, and 34 patients with an exacerbation of bronchiectasis treated with intravenous antibiotics. Long-term antibiotic therapy was investigated using samples from a 12-month controlled trial of nebulized gentamicin. They found that in stable patients, there was a direct relationship between airway bacterial load and markers of airway inflammation ($P < 0.0001$ for all analyses). High bacterial loads were associated with higher serum intercellular adhesion molecule-1 (ICAM-1), E-selectin, and vascular cell adhesion molecule-1 (VCAM-1) ($P < 0.05$ above bacterial load $\geq 1 \times 10^7$ cfu/ml). In stable patients, there was a direct relationship between bacterial load and the risk of subsequent exacerbations ($P < 0.0001$) and severe exacerbations ($P = 0.02$). They concluded that short- and long-term antibiotic treatments were associated with reductions in bacterial load, airways, and systemic inflammation (Chalmers et al 2012). Antibiotic recommended for acute exacerbations is summarized in Table 2.

Organism	Recommended first line treatment	Recommended second line treatment	Length of treatment
Streptococcus pneumoniae	Amoxicillin 500mg tds (po)	Clarithromycin 500mg bd (po)*	7-14 days
Haemophilus influenzae, β-lactamase negative	Amoxicillin 500mg tds (po)	Doxycycline 100mg bd (po) Or Clarithromycin 500mg bd* (po) Or Ciprofloxacin 500 mg* bd (po) Or Ceftriaxone 2G od* (IV) or 1g bd if administering as iv bolus.	7-14 days
Haemophilus influenzae, β-lactamase positive	Co-amoxiclav 625mg TDS (po)	Doxycycline 100mg bd (po) Or Clarithromycin 500mg bd* (po) Or Ciprofloxacin 500mg bd* (po) Or Ceftriaxone 2G od* (IV) or 1g bd if administering as iv bolus.	7-14 days
Moraxella catarrhalis	Co-amoxiclav 625mg TDS (po)	Doxycycline 100mg bd (po) Or	7-14 days

		Clarithromycin 500mg bd* (po) Or Ciprofloxacin 500mg bd* (po) Or Ceftriaxone 2G od* (IV) or 1g bd if administering as iv bolus.	
Staphylococcus aureus (MSSA)	Flucloxacillin 500mg QDS (po)	Clarithromycin 500mg BD (po)* Doxycycline 100mg BD or Co-amoxiclav 625mg TDS	7-14 days
Staphylococcus aureus (MRSA) **Oral preparations**	Rifampicin 300mg bd (po) + Trimethoprim 200mg bd (po)	Rifampicin 300mg bd (po)+ Doxycycline 200mg od (po) Third line Linezolid 600mg bd (po)	14 days 14 days
Staphylococcus aureus (MRSA) **Intravenous preparations**	Vancomycin* or Teicoplanin* 400mg od (iv)	Linezolid 600mg bd (po)	14 days
Coliforms e.g. Klebsiella, Enterobacter	Oral Ciprofloxacin 500mg bd* (po)	Intravenous Ceftriaxone 2G od or 1g bd if administering as iv bolus.	7-14 days
Pseudomonas aeruginosa	Oral Ciprofloxacin 500mg bd* (po)	Monotherapy Intravenous Ceftazidime 2G tds* Or	14 days

	(750mg bd in more severe infections)	Tazobactam/Piperacillin 4.5G tds* Or Meropenem 2G tds* Or Aztreonam 2G tds* Combination therapy: The above can be combined with gentamicin* (refer to BNF) or colistin 1-2 MU tds* (under 60 kg, 50 000-75 000 units/kg daily separated into 3 divided doses) OR oral ciprofloxacin 500mg BD*.	39

Table 2. Choice of antibiotic and duration of treatment. BTS bronchiectasis guidelines 2010. *dose needs adjusted in renal impairment

1.4. DO LONG-TERM ORAL ANTIBIOTICS INFLUENCE OUTCOME?

The BTS bronchiectasis guidelines 2010 recommend long-term antibiotics in:

1. Patients having ≥ 3 exacerbations per year requiring antibiotic therapy or patients with fewer exacerbations causing significant morbidity should be considered for long-term antibiotics.

2. In the first instance, high doses should not be used, to minimize side effects.

3. The antibiotic regimen should be determined by sputum microbiology when clinically stable

4. Long-term quinolones should not be used until further studies are available.

5. Macrolides may have disease-modifying activity and preliminary data suggest the need for a large randomised controlled trial. There have been subsequently 3 RCTs that have been conducted with macrolides in bronchiectasis and all three have shown that long-term macrolide treatment leads to decrease in

exacerbation frequency (Wong et al 2012, Serisier et al 2013, Altenburg et al 2013). These trials are discussed in details in section 1.14.1.

Organism	Recommended first line treatment	Recommended second line treatment
Streptococcus pneumoniae	Amoxicillin 250 mg bd	Clarithromycin 250 mg bd
Haemophilus influenzae, β-lactamase negative	Amoxicillin 250 mg bd	Clarithromycin 250 mg bd
Haemophilus influenzae, β-lactamase positive	Co-amoxiclav 375 mg tds	Clarithromycin 250 mg bd
Moraxella catarrhalis	Co-amoxiclav 375 mg tds	Clarithromycin 250 mg bd
Staphylococcus aureus (MSSA)	Flucloxacillin 500 mg bd	Clarithromycin 250 mg bd

Table. 3. Long term antibiotic treatment as recommended by the BTS.

1.5. DIAGNOSIS

1.5.1. RADIOLOGY

1.5.1.1. Chest X ray

The chest X-ray is usually normal unless patients have severe bronchiectasis. However, a normal chest x ray does not rule out bronchiectasis. Digital acquisition devices are capable of producing x-rays with improved visualisation of, for example, bronchiectatic airways behind the heart, with the added potential of radiation dose reduction (Young et al 1991). However, unless disease is severe, the radiographic signs of bronchiectasis are usually inconspicuous. Characteristic chest X-ray appearances include crowding of bronchi, parallel line opacities (tram lines) caused by thickened dilated bronchi, ring opacities or cystic spaces as large as 2 cm in diameter resulting from cystic bronchiectasis, sometimes with air-fluid levels, and oligaemia as a result of a reduction in pulmonary artery perfusion (figure 6). In terms of specificity, the chest x-ray of a patient with COPD showing bronchial wall thickening (tramline and ring shadows) and large volume lungs may be erroneously interpreted as indicating bronchiectasis. In summary, although a baseline chest x ray is recommended in all patients with bronchiectasis, they are not routinely indicated for follow up of patients with no change in symptoms (Pasteur MC et al BTS bronchiectasis guidelines 2010). Additionally, there is very little correlation during an exacerbation of bronchiectasis and chest x ray changes.

Figure 6. Cystic bronchiectasis- as indicated by arrows.

1.5.1.2. High Resolution CT Scan

The current gold standard for diagnosing bronchiectasis is high resolution CT (HRCT) of the chest. The appropriate high resolution CT study is a non contrast study with the use of 1.0-1.5mm window every 1cm with acquisition times of one second, reconstructed with the use of a high spatial frequency algorithm during full inspiration (Munro et al 1990). Bronchiectasis is diagnosed when there is bronchial wall dilatation, defined as the internal lumen of the bronchus being greater than that of the accompanying pulmonary artery, described as the 'signet' ring sign (figure 7). Characteristic features include, lack of tapering toward the periphery producing cylindrical bronchiectasis (figure 8), varicose constrictions along the airway (figure 9) and ballooned cysts at the end of a bronchus (figure 10). Cylindrical bronchiectasis is by far the most common bronchiectasis identified on CT. The usefulness of categorising bronchiectasis into cylindrical, varicose or cystic subtypes is limited, but cystic bronchiectasis usually denotes longstanding and more severe disease.

Bronchial wall thickening is a usual but inconsistent feature of bronchiectasis. Problems with this variable feature have been widely debated and the definition of what constitutes abnormal bronchial wall thickening remains unresolved (Pasteur MC BTS bronchiectasis guidelines 2010).

Figure 7. Signet ring (as indicated by arrows).

Non-specific findings include consolidation or infiltration of a lobe with dilatation of the airways, thickening of bronchial walls, mucus plugs and enlarged lymph nodes (Thomas and Blaquiere 1993). Mucus secretions within bronchiectatic airways are generally easily recognisable as such. The larger plugged bronchi are visible as lobulated or branching opacities. Such airways are usually seen in the presence of non-fluid filled obviously bronchiectatic airways. Mucus plugging of the smaller peripheral and centrilobular airways produces V- and Y-shaped opacities, the so-called 'tree-in-bud' pattern (Gruden and Webb 1995).

In patients with ABPA there is fleeting infiltrates and ultimately patients can develop proximal bronchiectasis. The characteristic distribution with *M. avium* complex infection often involves the middle lobe or lingula (Cartier et al 1999) and typically shows bronchiectasis, nodes and 'tree in bud' appearances. Airway dilatation can be associated with asthma, chronic bronchitis, and pulmonary fibrosis (so-called traction

bronchiectasis), however this thesis deals with clinically significant bronchiectasis, which is radiological diagnosis and clinical features in keeping with bronchiectasis.

In summary, HRCT is the radiological investigation of choice in bronchiectasis, with bronchial wall dilation being most characteristic of bronchiectasis.
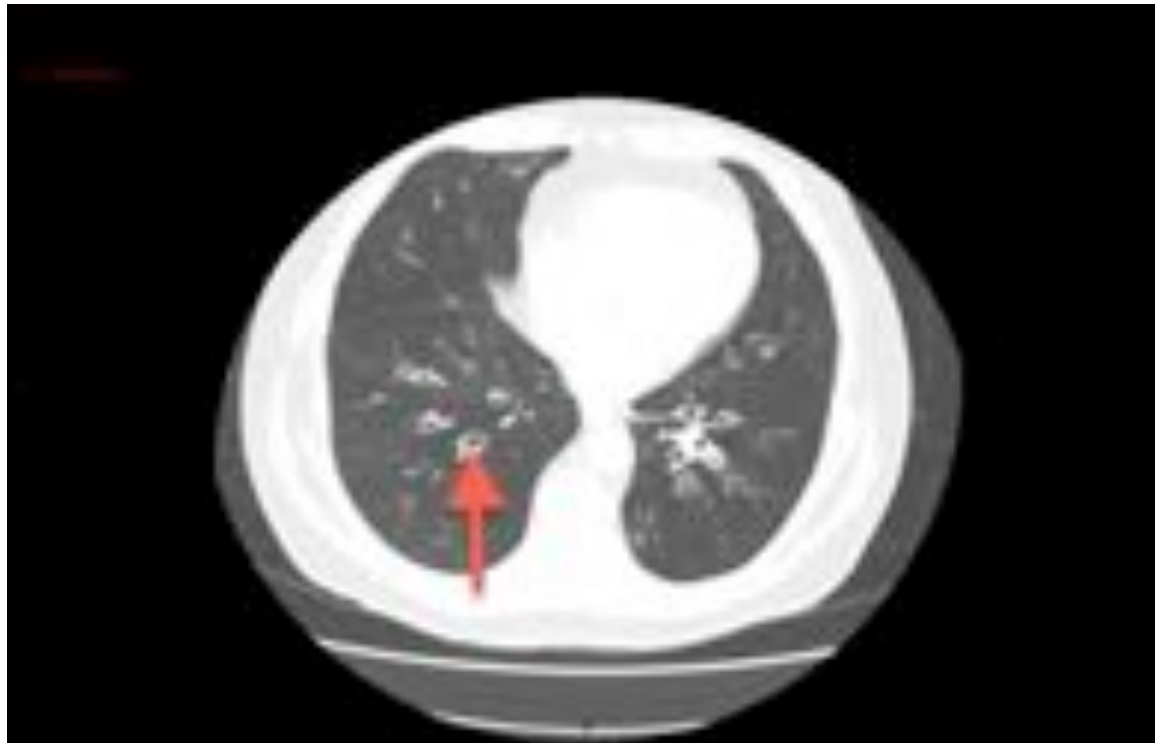
Figure 8. Tubular bronchiectasis (as indicated by arrows).
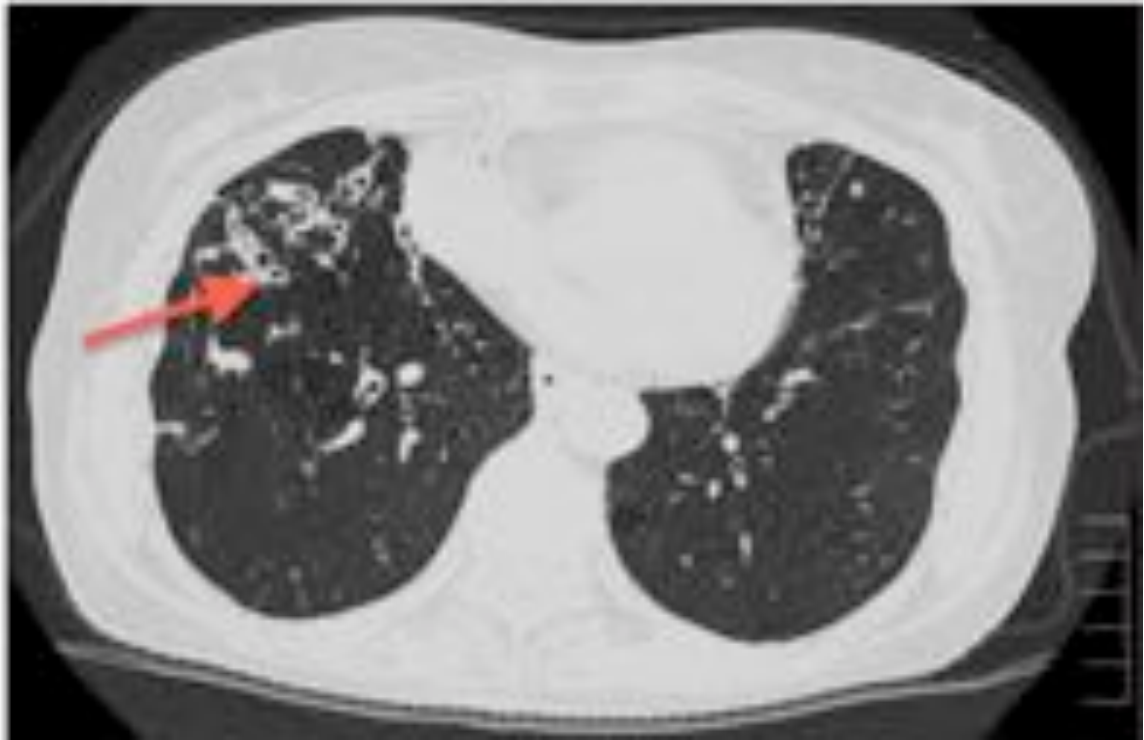
Figure 9. Varicose bronchiectasis (as indicated by arrows).
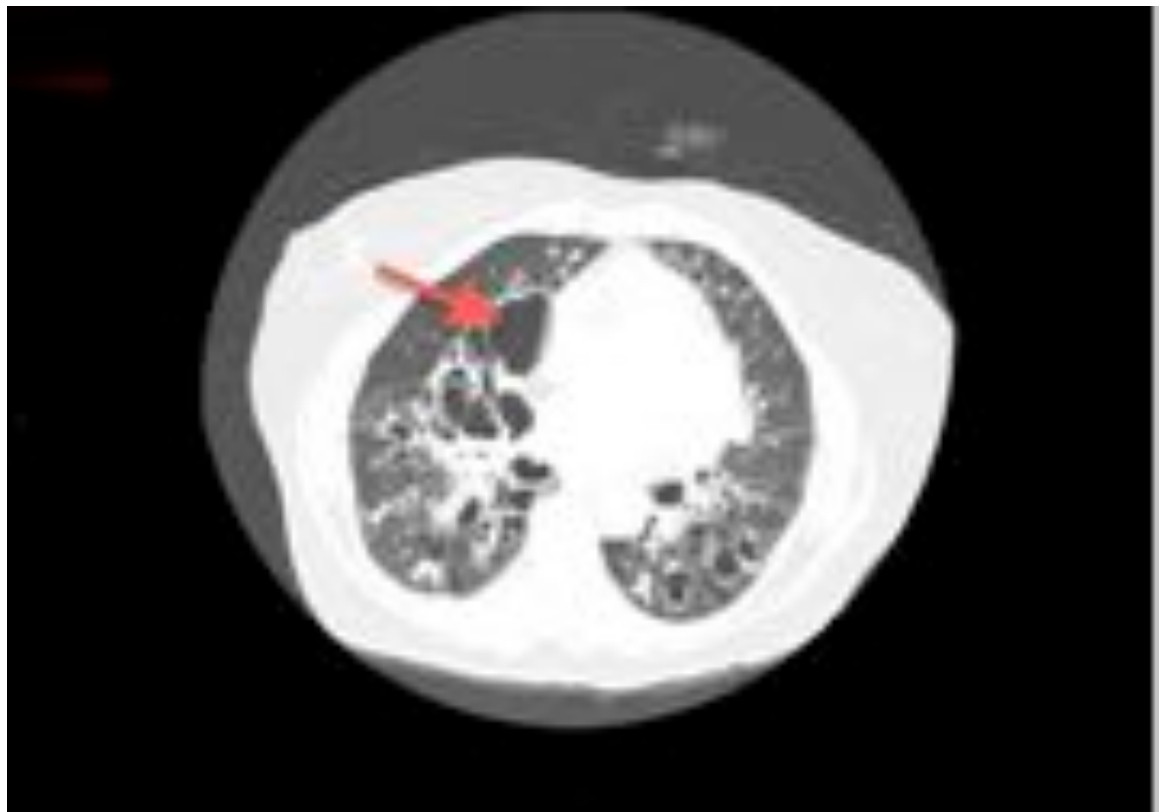
Figure 10. Cystic bronchiectasis (as indicated by arrows).

1.5.2 CLINICAL FEATURES OF BRONCHIECTASIS

1.5.2.1. Cough and sputum

Cough is the commonest symptom of bronchiectasis and occurs in >90% patients (Pasteur MC et al BTS bronchiectasis guidelines 2010). The cough is productive of sputum daily in 75-100%, intermittent in 12-20% and non-productive in 5-8% (Stockley et al 2001). In bronchiectasis, cough occurs due to overproduction of secretions and impaired mucociliary clearance of secretions. Mucus hyper-secretory state in bronchiectasis results from hyper secretion of stored mucin or hypertrophy of goblet cells.

24-hour sputum volume measurement is perhaps a more accurate assessment of volume produced, but is entirely dependent on patient compliance. Sputum purulence is dependent on the release of myeloperoxidase and is classified as mucoid (clear or grey), mucopurulent (pale yellow) and purulent (dark yellow or green) (Stockley et al 2001). Sputum purulence is related to radiological appearances on CT scan and purulent sputum is associated with cystic bronchiectasis on HRCT (O'Brien et al 2000) and correlates well with the presence of potential pathogenic microorganisms (Murray et al 2009).

1.5.2.2. Dyspnoea, hemoptysis, pain and fever

Dyspnoea is present in about 70% of patients and correlates inversely to FEV_1 (Martinez- Garcia et al 2007). Hemoptysis can occur with exacerbations but major hemoptysis can infrequently occur and usually arises from an aberrant blood vessel from the bronchial arteries and can sometimes require interventions such as bronchial embolization or lobar resection. Patients can present with pleuritic chest pain and fever with exacerbations.

1.5.2.3. Infective exacerbations

As previously mentioned, the BTS bronchiectasis guidelines recommended antibiotics for exacerbations that present with an acute deterioration (usually over several days) with worsening local symptoms (cough, increased sputum volume or change of viscosity, increased sputum purulence with or without increasing wheeze, breathlessness, haemoptysis) and/or systemic upset (Pasteur MC et al 2010). Number of exacerbations can vary but from the national BTS bronchiectasis audit, the number

of exacerbations in a secondary care cohort is 2/year (Hill et al 2012). Studies have shown that recurrent exacerbations lead to poor quality of life (Wilson et al 1998).

1.5.2.4. Clinical signs on bronchiectasis

The most characteristic physical finding of bronchiectasis is coarse crackles on auscultation of the lung bases, and is found in about 70% of patients (Pasteur MC et al BTS bronchiectasis guidelines 2010). Some patients may have normal lung function, airflow obstruction or restrictive pattern and/or clubbing.

1.5.2.5. Impact on quality of life

The St George's Respiratory Questionnaire has been validated for use in bronchiectasis (Wilson et al 1997). Higher scores on the St. Georges Respiratory Questionnaire are associated with worse quality of life. In addition, the Leicester Cough questionnaire has also been validated for use in bronchiectasis and a lower score indicates worse cough severity and poorer quality of life (Murray et a 2009).

1.5.2.6. Assessment of disease severity

In the recently published Bronchiectasis Severity Index or BSI (Chalmers et al 2014) and FACED (Martinez- Garcia 2014) scores, bronchiectasis patients can be categorized into disease severity based on a number of clinical and radiological parameters. Parameters included in the BSI score are age; body mass index (BMI), percent predicted FEV_1, previous hospital admission, hospital admissions per year, MRC dyspnea score (Medical Research Council), pseudomonas colonization, colonization with other organisms and radiological severity. A score of 0-4 indicates mild bronchiectasis, 5-8 moderate and >9 severe bronchiectasis. The one-year mortality rates for mild, moderate and severe bronchiectasis are 2.4%, 4.8% and 7.6% respectively. BSI scores can be calculated by the available online tool http://www.bronchiectasisseverity.com. McDonnell and colleagues recently studied the predictive utility of the BSI and FACED across seven European cohorts. They concluded that the BSI accurately predicts mortality, hospital admissions, exacerbations, quality of life, respiratory symptoms, 6 minute walk test, and lung function decline in bronchiectasis, providing a clinically relevant evaluation of disease severity (McDonnell et al 2016). The BSI has been used to assess disease severity for the purpose of this thesis.

1.6. IMMUNE SYSTEM OF THE LUNG AND BRONCHIECTASIS

The innate immune system is the immediate, non-specific and first line of defense against invading organisms. Key cells of the innate immune response include neutrophils, macrophages, eosinophils, basophils, dendritic cells and γδ T cells. Particles >2–3 μm impact on the mucus covering epithelial cells in the upper airways and bronchi and are removed by a combination of normal mucociliary clearance and cough (Loebinger et al 2009). Airway secretions contain several anti-infective proteins and polypeptides, including the bacteriostatic proteins lysozyme and lactoferrin. Secretory leucoprotease inhibitor (SLPI), produced by the mucous glands, macrophages and epithelial cells, has antifungal, antiviral and antibacterial properties (Rogan et al 2006). Secretary IgA is the main antibody isotype in mucosal secretions in the respiratory tract that contributes to the initial defense mechanisms, while IgG predominates in the plasma (Hill et al 2000). If a high bacterial load overwhelms these primary clearance mechanisms, a secondary defense system is activated. Animal experiments demonstrate this relationship between bacterial load and activation of the secondary host response in that a low bacterial load leads to bacterial clearance, but when the bacterial load is greater than 10^6 colony-forming units/ml (cfu/ml) there is a neutrophilic host inflammatory response (Hiemstra 2007). In bronchiectasis, Chalmers et al showed that this response occurs at $\geq 10^5$ cfu/ml (Chalmers et al 2012).

Failure of the local host defenses results in microbial colonization, establishing the 'vicious circle' described by Cole and colleagues. Airway epithelium responds by releasing antimicrobial peptides and inflammatory cytokines into the airways. In human airway epithelial cells the antimicrobial peptides produced are the β-defensins and the cathelicidins (LL-37 in humans). Chemokines and cytokines such as interleukin (CXCL)-8 are released into the sub mucosa (Bals and Hiemstra 2004) and initiate the inflammatory reaction, leading to recruitment of phagocytes (neutrophils, monocytes and macrophages), dendritic cells and lymphocytes (which contribute to the adaptive response) (Weng et al 2010). The airways become inflamed leading to leakage of serum proteins including circulating antibodies and complement, therefore increasing local antibacterial potential.

1.7. KEY INFLAMMATORY CELLS IN BRONCHIECTASIS

1.7.1. NEUTROPHILS

Excessive neutrophilic airways inflammation is the central feature of bronchiectasis. This paradoxically both promotes bacterial colonization and perpetuates damage to the airways creating a vicious cycle of bacterial colonization and inflammation (Cole 1984). The lung is a unique vascular and externally exposed organ that is subject both to PMN-mediated inflammation and PMN-opposed infection.

Granule proteins from activated neutrophils, such as azurocidin and α-defensins, directly alter permeability (Serhan 2007). Moreover, proteases of neutrophilic origin such as neutrophil elastase have been regarded to be important in degradation of surfactant proteins, epithelial cell apoptosis, and coagulation. In addition, neutrophils produce vast quantities of reactive oxygen (ROS) and nitrogen (RNS) species like O_2^- and NO through their oxidant-generating systems such as the phagocyte NADPH oxidase and nitric oxide synthase (NOS), respectively. Besides their important antimicrobial effector function, neutrophil-derived oxidants promote deleterious pro-inflammatory effects thus being a major cause of neutrophil-dependent tissue injury (Morel et al 1991).

1.7.1.1. Key functions of neutrophils relevant to bronchiectasis

1.7.1.1.1. Recruitment and migration

Recruitment is driven by chemo attractants, with interleukin (IL)8 now referred to as CXCL8, leukotriene (LT) B_4, interleukin-1 beta, C5a and tumour necrosis factor (TNF) alpha believed to be of significance in bronchiectasis (Mikami et al 1998). Elevated levels of these pro-inflammatory cytokines have been demonstrated in bronchiectasis airway secretions (Stockley and Bayley 2000)**.** Neutrophil detection of chemotactic stimuli leads to a co-ordinated process of cell signalling, cytoskeletal rearrangement and changes in surface receptor expression to facilitate migration. Key to the process of transendothelial migration is the expression of the integrins CD11/CD18 on neutrophils and the expression of adhesion molecules on endothelial cells, principally intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and the selectins (Cowburn et al 2008).

Neutrophil migration occurs predominantly at the border of endothelial cells where modifications of cell junctions allow this. The cell adhesion molecules, platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31) and junction adhesion molecule (Martin-Padura et al 1998, Muller et al 1993) are involved in neutrophil transmigration. Migration occurs via PECAM-1/PECAM-1 interaction while maintaining the permeability barrier of the endothelial cell monolayer (Muller et al 1993) The endothelial surface density of ICAM-1 is important in regulating this migration (Yang et al 2005).

Neutrophil adhesion to pulmonary endothelial cells and migration into the lung may occur by CD11/CD18 dependent or CD11/CD18 independent mechanisms (Doerschuk et al 2000). Different stimuli within the lung can determine whether CD18 is required for neutrophil migration into the lung. It is felt that stimuli from gram-negative bacteria require CD18, as 75–80% of neutrophil migration is inhibited by CD18 antibodies (Doerschuk et al 2000).

Once neutrophils migrate to the site of infection, neutrophils perform three key actions: phagocytosis of the bacteria, release of products of pre formed granules and release of reactive oxygen species-all three actions are directed towards killing of bacteria.

1.7.1.1.2. Reverse transmigration of neutrophils

In a successful response to an acute injury, it is critical to prevent tissue damage by promoting the local resolution of inflammation through the removal of neutrophils from the site of injury (Soehnlein & Lindbom 2010). This clearance of neutrophils can occur through either apoptosis or necrosis and subsequent phagocytosis by macrophages (Buckley et al 2013). However, there is evidence to suggest that neutrophils at inflamed sites do not necessarily undergo apoptosis. Hughes et al used a rat model of glomerular capillary injury to track the fate of radiolabelled neutrophils and found that ≥70% of neutrophils that entered inflamed glomerular capillaries were able to return to the main circulation and did not undergo apoptosis at the site of inflammation (Hughes et al 1997). Recent studies have shown that neutrophils can leave sites of tissue damage in a process termed neutrophil reverse migration, which describes the interstitial migration of neutrophils away from inflamed sites.

Neutrophils have also been described to re-enter the vasculature in a distinct process referred to as neutrophil reverse transendothelial migration (rTEM).

Neutrophil reverse migration within tissues away from the site of injury was first directly visualized *in vivo* in zebrafish larvae, in which the authors showed that not all recruited neutrophils die at the site of injury, and most leave the site (Mathias et al 2006). Subsequent studies using zebrafish, showed that neutrophils that leave a wound can undergo rTEM and traffic to distal sites post injury (Yoo and Huttenlocher et al 1011; Hall et al 2009). Buckley and colleagues (Buckley et al 2006) described the ability of human neutrophils to reverse transmigrate through an endothelial monolayer *in vitro*, identifying markers that are characteristic of these reverse-transmigrated neutrophils (intercellular adhesion molecule 1 (ICAM1)^{hi}CXCR1^{low}), and found this neutrophil phenotype in the peripheral blood of patients with systemic inflammation. CXC chemokine receptors are membrane proteins that specifically bind and respond to cytokines of the CXC chemokine family, for example CXCL8 binds to both CXCR1 and CXCR2. Neutrophil reverse migration and/or rTEM have now been visualized in multiple models including zebrafish (Ellett et al 2015; Robertson et al 2014; Tauzin et al 2014) mice (Duffy et al 2012; Woodfin et al 2011) and *in vitro* using human neutrophils (Hamza et al 2015; Hamza et al 2014). *In vitro* studies using microfluidics showed that greater than 90% of human neutrophils can reverse their direction away from a chemoattractant and migrate away continually for distances greater than 1,000 μm. Collectively these studies have suggested that reverse migration is a possible mechanism to locally resolve inflammation and is a potential novel target for drug therapy in diseases characterized by excessive neutrophil infiltration. However, a caveat is that neutrophil reverse migration may lead to activated neutrophils being redistributed to other locations in the body, contributing to inflammation elsewhere. It is also important to note that several aspects of neutrophil reverse migration remain controversial including the exact mechanism(s), the fate of reverse-migrated neutrophils and the occurrence of reverse migration in human disease.

1.7.1.1.3. Neutrophil phagocytosis

The internalization of bacteria by neutrophil and formation of a phagosome is facilitated by opsonization. Opsonization is a process where the Fab portion of the IgG antibody binds to the bacteria and the Fc (receptors are FcγRIIa and FcγRIIIB) portion then binds to the phagocyte by specific receptors. Fcγ receptor ligation leads to extension of the pseudopod around the bacteria, which finally engulfs it completely. Both intracellular and extracellular environment is important to regulate neutrophil function (Whitters and Stockley 2012). What is known in bronchiectasis, is that peripheral blood neutrophils function normally in bronchiectasis (Watt et al 2004). Our group demonstrated (unpublished data) that the airway neutrophils do not function normally in bronchiectasis- however I do not know the mechanism for this but hypothesize that the complex airway environment with the overwhelming bacterial load, inflammatory cytokines and chemokines could all in part contribute to the impaired neutrophil function. This will be explored further in this thesis.

1.7.1.1.4. Release of products of degranulation

Granule proteins formed during neutrophil development and released upon activation is one of the key functions of neutrophils. Changes in the cytosolic calcium are required for granule secretion (Lew et al 1986) and for fusion of the granule to the neutrophilic phagosome (Jaconi et al 1990). The granules contain enzymes, host defense proteins with receptors, signaling proteins and adhesion molecules that are expressed on the cell surface upon activation. Azurophilic granules or primary granules release MPO, defensins, neutrophil elastase, proteinase 3 and cathepsin G, among others. Secondary granules release lactoferrin, cathelicidin and tertiary granules release gelatinase acetyltransferase and lysozyme. Excessive degranulation and release of MPO and neutrophil elastase leads to host tissue damage including neutrophils, epithelial cells and alveoli (Mitchell et al 2008).

1.7.1.1.5. Release of superoxide anion

During phagocytosis, neutrophils increase the oxygen consumption by using up NADPH oxidase and releasing superoxide anion (O_2^-). The O_2^- then dismutates to form H_2O_2, releasing MPO from primary neutrophilic granules (Morel et al 1991). This oxidative burst is critical for bacterial killing but simultaneously myeloperoxidase (MPO) causes oxidative damage to the epithelial cells mainly through formation of the cytotoxic oxidant HOCl (Worlitzesh et al 1998). Elevated MPO levels have been found to correlate to disease severity in bronchiectasis (Hill et al 2000). The respiratory burst results in the release of ROS to facilitate bacterial killing. The release of uncontrolled ROS by neutrophils may however also lead to damage to surrounding tissues, amplifying the lung disease process (Tung et al 2009). It had previously been thought that ROS exert a direct toxic effect on ingested pathogens. However Reeves *et al* (Reeves et al 2002) suggested that the respiratory burst sets in motion events, which result in creating a favorable environment for proteases such as elastase to digest the contents within the phagocytic vacuole. By optimizing conditions via ion flux and pH change, the enzymes released from the cytoplasmic granules become more active, facilitating the destruction of ingested pathogens.

As NADPH oxidase is key for respiratory burst, patients with chronic granulomatous disease (defect in one of the subunits of NADPH oxidase genes) are unable to produce ROS and thereby lead to life threatening bacterial infections (Deffert et al 2014). In 2006, King et al demonstrated that in bronchiectasis, neutrophils have normal phagocytic capacity but impaired oxidative burst as compared to controls (King et al 2006). However, in contrast, Pasteur et al failed to detect any difference in oxidative burst between bronchiectatic patients and controls (Pasteur et al 2000). It remains therefore an important abnormality that needs to be explored further in bronchiectasis.

1.7.1.1.6. Apoptosis

It has been well established that there is a key role for apoptosis, or programmed cell death, in the regulation of inflammation and the host immune response. The process of apoptosis, which is distinct from necrosis (toxic cell death), involves a series of coordinated morphologic changes in the affected cell, causing its demise and subsequent recognition and removal by scavenger phagocytes (Wyllie et al 1980). Characteristically, apoptosis initially induces cytoplasmic shrinkage associated with membrane blebbing (zeiosis), followed by chromatin condensation and DNA fragmentation. Although cells usually manifest these changes sequentially when undergoing apoptosis, it is now clear that these events may occur independently under the control of separate and distinct metabolic pathways (Duffin et al 2010).

Among the leukocytes, mature human neutrophils have the shortest life span and die rapidly via apoptosis *in vivo* and *in vitro*, resulting in the demise of the entire population within 72h. As neutrophils proceed through apoptosis, functional activity declines. Apoptotic neutrophils lose CD16 (FcyRIII) expression and demonstrate a reduced ability to degranulate, generate a respiratory burst, or undergo shape changes in response to external stimuli such as the chemotactic bacterial peptide formyl-methyl-leucyl-phenylalanin (fMLF) (Sasmono 2007). Although neutrophils appear to be committed to death via apoptosis, it is now clear that the life span and functional activity of mature neutrophils can be extended significantly by pro inflammatory cytokines, including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, TNF-α and IL-2 (Burdon 2008).

Although elevation of cytosolic-free Ca^{2+} has been associated with apoptosis in a variety of cell types (Whyte et al 1993), transient elevations of cytosolic free Ca^{2+} induced by low doses of calcium ionophores have been reported to inhibit apoptosis in neutrophils *in vitro* (Whyte et al 1993). Other experiments employing inhibitors of tyrosine kinases and phosphatases suggest a possible role for tyrosine phosphorylation events in the signal transduction pathway mediating neutrophil apoptosis (Erttmann et al 2014). Furthermore, it has been shown that intracellular acidification precedes, and may be causally related to, the development of morphologic features characteristic of apoptosis in neutrophils maintained in short term culture (Erttmann et al 2014).

It is also known that the Fas (APO-I; CD95)/ Fas-ligand (FasL) system represents an important cellular pathway responsible for the induction of apoptosis in diverse tissues. Fas is a widely expressed 45-kDa type I membrane protein member of the TNF/nerve growth factor family, which mediates apoptosis following interaction with agonistic anti-Fas IgM Ab or FasL (Wajant et al 2002). FasL is a 40-kDa type II protein member of the TNF family, which includes TNF-α, TNF-β (lymphotoxin), CD40 ligand, and CD30 ligand (Wajant et al 2002). In contrast to Fas, the expression of FasL is relatively restricted in its tissue distribution (Wajant et al 2002) but can be induced in mature T cells following stimulation with phorbol 12-myristate 13-acetate or PMA, ionomycin, anti-CD3, or bacterial antigen (Wajant et al 2005). Furthermore, it has been shown that activated T cells can secrete a functionally active soluble form of FasL that is capable of inducing apoptosis in other cells susceptible to Fas-induced apoptosis (Wajant et al 2005). The relative contributions of the proapoptotic and pro inflammatory functions of Fas/FasL sytem to the pathogenesis of lung disease and the effects of inhibiting this pathway, remains poorly understood.

Binding of Fas to its natural ligand FasL, can trigger apoptosis via activation of caspase 8. FasL exists in a membrane bound form and a soluble form, both of which can activate Fas. In addition to triggering apoptotic pathways, the activation of Fas can also lead to NF-κB translocation and cytokine production (Kreuz et al 2004).

FasL expression is detected on the surface of mature human neutrophils, but not on monocytes or eosinophils suggesting that neutrophils are susceptible to Fas induced cell death.

There is limited data available from two studies in the literature on apoptosis of airway neutrophils in bronchiectasis. Vandivier *et al* (Vandivier et al 2002) concluded that sputum of bronchiectatic patients have more apoptotic neutrophils in comparison to sputum obtained from patients with chronic bronchitis. However, Watt *et al* demonstrated that there were low levels of apoptotic neutrophils in induced sputum as compared to the findings by Vandivier and colleagues (Watt et al 2004). They found no significant differences in the levels of apoptotic neutrophils at the beginning and end of an exacerbation treated with antibiotics. There was however a reduction in the

total number of sputum neutrophils and serum levels of CXCL8, TNF-α, neutrophil elastase (NE) and CRP, at the end of exacerbation. In summary, few studies have investigated neutrophil apoptosis in bronchiectasis and of the two key studies described to date, the opinion remains divided.

1.7.1.1.7. Neutrophil extracellular traps (NETs)

Neutrophil extracellular traps (NETs) are extracellular strands of decondensed DNA with histones and neutrophil granular proteins. NETs were discovered more than a decade ago (Birkmann et al 2004). The authors demonstrated that NETs are generated *in vitro* after stimulation of isolated neutrophils with three different agents: (i) CXCL8, a major neutrophil chemoattractant; (ii) LPS, a component of Gram-negative bacteria; and (iii) PMA, a potent activator of protein kinase C (PKC). Additionally, these *in vitro* generated NETs had antibacterial activity, which was attributed to the associated histones (Hirsch 1958), proteolytic enzymes from granules that could degrade bacterial virulence factors, and enzymatically active myeloperoxidase (MPO). Notably, the antibacterial activity of NETs is abrogated by DNase (Brinkmann et al 2004). Induction of NETs by CXCL8 and LPS indicates that NETs are formed during inflammation and infection. Brinkmann and colleagues were able to demonstrate that neutrophils may undergo an alternative death pathway, termed NETosis, thereby allowing them to serve in innate immune defense even after their death. However, I need to be cautious, as NETosis as a neutrophil death mechanism, may largely be an *in vitro* phenomenon, and NETs seem to be generated *in vivo* by mechanisms different from those described *in vitro*.

1.7.1.1.7.1. NET morphology

The ultrastructure of NETs is unusual. NETs consist of smooth filaments with a diameter of ~17 nm (Brinkmann et al 2004), composed of stacked and modified nucleosomes (Urban et al 2009). This backbone is studded with globular domains that have a diameter of ~50 nm made of granular proteins (Brinkmann et al 2004). This distinct morphology in high-resolution scanning electron microscopy can easily differentiate NETs from other fibrous structures such as fibrin. Interestingly, unfixed, fully hydrated NETs have a cloud-like appearance and occupy a space that is 10–15-fold bigger than the volume of the cells they originate from.

1.7.1.1.7.2. The mechanism of NET formation

NETs are the results of a unique form of cell death that is morphologically characterized by the loss of intracellular membranes before the integrity of the plasma membrane is compromised or lost. Steinberg and Grinstein coined the term "NETosis" for neutrophil cell death that leads to formation of NETs. To release NETs, activated neutrophils undergo exceptional morphological changes (figure 11A). Minutes after activation, neutrophils flatten and firmly attach to the substratum (figure 11B). Over the next hour, the nucleus loses its multi lobulated structure, the chromatin decondenses, and the inner and outer nuclear membranes then progressively detach from each other. Simultaneously, the granules disintegrate. After an hour, the nuclear envelope then disaggregates into vesicles and the nucleoplasm and cytoplasm form a homogenous mass (figure 11C). Lastly, the cells round up and seem to contract until the cell membrane ruptures and the interior of the cell is ejected into the extracellular space, forming NETs (figure 11D; Fuchs et al 2007). NETosis is morphologically different from apoptosis and other forms of cell death (Fuchs et al 2007).

Molecularly, the few events that have been shown to be required, sequentially, are the production of superoxide anion, the migration of the protease neutrophil elastase (NE) and myeloperoxidase (MPO) from granules to the nucleus, the processing of histones, and finally the rupture of the cell (Brinkmann and Zychlinsky 2012).

NETs are removed during the resolution of inflammation. NETs are susceptible to DNase1 (von Köckritz-Blickwede et al 2009; Hakkim et al 2010), an enzyme produced by the pancreas. It is not known what happens to the debris left by DNase1 but perhaps phagocytes, macrophages, and neutrophils newly recruited to the inflammatory site start the process of resolution of inflammation (Bratton and Henson 2011).
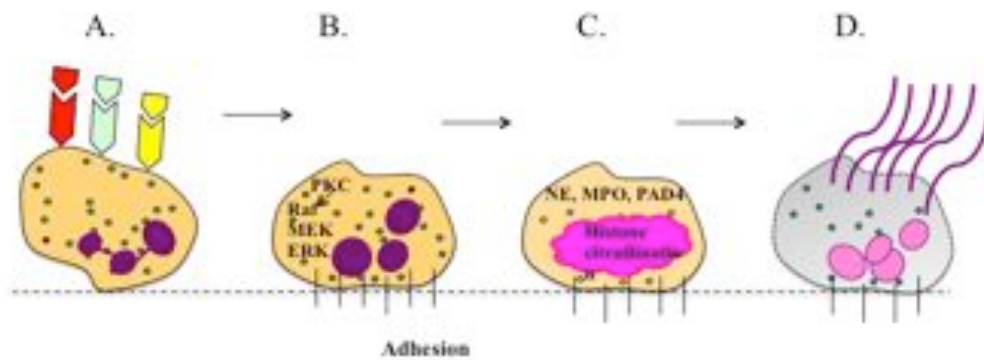
Figure 11. Schematic representation of the NETosis pathway. After stimulation of receptors (A), neutrophils adhere to the substrate (B) and mobilize granule components NE and MPO (C). Granules are depicted as green circles. Histones in the nucleus get processed, and the intracellular membranes degenerate. Finally, the cell membrane ruptures, and the contents of the cytoplasm and nucleoplasm gets expelled to form NETs (D).

Adapted from Brinkmann et al, 2012, J. Cell Biol.

1.7.2. MACROPHAGES IN BRONCHIECTASIS

Little is known about the role of macrophages in bronchiectasis. One of the key functions of macrophages is efferocytosis or "burying" the dead (apoptotic) neutrophils (Serhan 2005). It is well recognized that activated neutrophils must firstly 'switch off' by undergoing apoptosis to limit the inflammatory process and then be cleared by macrophages to prevent secondary necrosis and release of granular products, and thereby promote resolution of inflammation (Koedel et al 2009).

In bronchiectatic patients, Vandivier *et al* demonstrated that neutrophil elastase cleaves phosphatidylserine on the surface of apoptotic cells, preventing efferocytosis by macrophages (Vandivier et al 2002). Further studies are needed to explore this in bronchiectasis.

1.7.2.1. Efferocytosis

A vital event in the resolution of inflammatory responses is the clearance of recruited inflammatory granulocytes, particularly via the co-ordinated induction of programmed cell death (apoptosis) and thereafter the clearance of apoptotic cells by tissue phagocytes (Walker et al 2005). This mechanism has been confirmed in experimental models of inflammation, where accelerating neutrophil apoptosis promotes early resolution and reduction in tissue injury (Rossi et al 2006). Neutrophil apoptosis leads to loss of expression and function of adhesion molecules (Dransfield et al 2005) and greatly reduced responsiveness to external stimuli (Whyte et al 1993) which leads to functional isolation from micro-environmental stimuli. In addition, apoptotic neutrophils are quickly recognised and ingested by neighbouring phagocytes, thereby limiting release of histotoxic intracellular contents (Savill et al 2002). Apoptotic cells once engulfed are contained within a large fluid-filled vesicle called an efferosome that fuses with lysosomes to form the efferolysosome, which then eventually digests the redundant cell. Various cell surface signals help phagocytes distinguish viable cells from apoptotic cells. The primary signal exposed on the surface of apoptotic cells is the membrane phospholipid phospatidylserine (PS) (Fadok et al 1992). In viable cells, PS remains confined to the inner membrane via the transmembrane lipid transporter protein flippase. However, at early-stage apoptosis, PS is translocated from the inner to the outer membrane leaflet via the activity of phospholipid scramblase. PS exposed on the surface of apoptotic cells can be detected

by phagocytes via several recognition mechanisms involving membrane receptors. Although several molecular mechanisms may be involved in the clearance of apoptotic cells by phagocytes (Elliott et al 2010), uptake of apoptotic cells suppresses toll-like receptor-driven production of pro-inflammatory mediators by macrophages and can induce release of IL-10 and transforming growth factor (TGF)-β which have the potential to exert anti-inflammatory effects (Rothlin and Lemke 2010), (Fadok et al 1998).

Phagocytic functions can be enhanced by exposure or treatment with glucocorticoids Glucocorticoids can also stimulate macrophages to switch to an anti-inflammatory phenotype (M2) where they shut down release of pro-inflammatory cytokines and simultaneously release anti-inflammatory cytokines (IL-10, TGFβ, IL-1ra), thereby helping to promote resolution of inflammation and tissue repair (Robb et al 2016). In addition, M2 macrophages have enhanced phagocytic capabilities, with their most important function being the efficient clearance of apoptotic cells (Savill 1997), which contributes significantly to the successful resolution of inflammation. During lung infection and injury, migration and retention of monocyte and macrophage populations are indicated in triggering and sustaining pulmonary inflammation (Kaur et al 2015). Dexamethasone-treated macrophages also display structural reorganisation of the cytoskeleton and an increase in cell motility, both essential for efficient phagocytosis (Giles et al 2001). Furthermore, dexamethasone augmented the expression of active RAC in macrophages, a key signalling protein involved in a variety of cellular functions, including phagocytosis as well as cell motility, mitosis and wound healing (Giles et al 2001).

Professional phagocytes include alveolar macrophages, interstitial lung macrophages and lung 'immature' dendritic cells, whereas non-professional phagocytes include lung epithelial cells - alveolar and bronchial epithelial cells. Defective efferocytosis which thereby results in an increased number of apoptotic cells is implicated in a number of lung diseases including asthma, acute lung injury, cystic fibrosis and COPD (Yun et al 2008; Grabiec and Hussell et al 2016). Efferocytosis has not been studied in bronchiectasis. Furthermore, highly specialised bioactive lipids play key roles during the resolution phase of inflammation.

1.7.3. EPITHELIAL CELLS

1.7.3.1. Airway host defence and damage

The airway mucociliary clearance system is a key component of primary host defence in the lungs, responsible for maintaining airway sterility and health. The system has a mucus gel layer that lines the airway lumen trapping pathogens, inhaled toxins and cellular debris as it is swept by beating cilia from the distal airways to the proximal airways for expectoration and clearance.

In health, the respiratory tract is lined by equal numbers of ciliated columnar epithelial cells and secretory cells. The epithelial cells each have 200 cilia, approximately 7μm length that rest in a thin layer of periciliary fluid. The cilia beat in a 2 stage process, an effective beat where their tips engage with the airway mucus sweeping it through the airways and a recovery beat in the opposite direction where their tips disengage and recover (Rubbo and Lucas 2017). Alveoli produce surfactant to maintain bronchial patency. Bronchioles are lined with ciliated epithelium and secretory cells that produce a thin mucus layer for the airway lumen. The airway mucus is composed predominantly of water (97%) and mucins (3%) and accumulates as it is transported through the larger airways, trachea and pharynx for expectoration.

Dilated, damaged bronchial wall function impairs clearance of mucus and excessive mucus accumulation causes the airways to be persistently exposed to toxic insults and pathogens. In bronchiectasis, the airways are permanently damaged and the normal mucociliary mechanism is believed to be impaired.

1.7.3.2. Abnormal ciliary function

In order to ensure effective mucus transport, the cilia must beat at a normal rate, in a consistent direction and in a coordinated fashion (Eliezer et al 1970). Studies have used ciliated nasal epithelium as a non-invasive means of assessing ciliary function in the respiratory tract. Ciliary beat frequency is significantly slower in bronchiectasis (Rutland and Cole 1981). This finding has been confirmed in a more recent study of 152 patients with idiopathic bronchiectasis, which found ciliary beat frequency to be significantly slower than healthy controls (Tsang et al 2005b). The purulent sputum in bronchiectasis contains excessive quantities of elastase due to the persistent

neutrophilic airways inflammation. Such purulent sputum from patients with bronchiectasis has been shown to slow ciliary beat frequency on normal ciliated nasal epithelium in healthy controls (Smallman et al 1984). The addition to the sputum of an elastase inhibitor (α1- antitrypsin) resulted in no significant change in ciliary beat frequency over the same time period, suggesting that elastase activity had no effect on ciliary function (Smallman et al 1984). The effect of elastase on ciliary function and ciliated epithelium has been shown *in vitro* to increase with a dose dependent effect with progressive disruption of the epithelium and slowing of ciliary beat frequency at higher levels (Amitani et al 1991). The effect of bacterial products on ciliary function has been investigated *in vitro*. Exposure of ciliated nasal epithelium to the supernatants of *Pseudomonas aeruginosa* and *Haemophilus influenzae* over a 4 hour period was associated with significant slowing of ciliary beat frequency and a disorganised beating pattern. A dose related effect was also observed and it may be that the release of a factor or factors by these organisms complements the effect of human neutrophil elastase on ciliary inhibition (Wilson et al 1985).

1.8. INFLAMMATION AND THE ROLE OF LIPID MEDIATORS

Acute inflammation is commonly characterized by the rapid influx of blood granulocytes, typically neutrophils, followed swiftly by monocytes that mature into inflammatory macrophages that subsequently proliferate and thereby affect the functions of resident tissue macrophages. The acute inflammatory response is a protective mechanism that is evolved to eliminate invading organisms and should ideally be self-limiting and lead to complete resolution (Serhan 2007, Mantovani et al 2011, Medzhitov 2010). Chemical mediators biosynthesized locally during acute inflammation give rise to the events characterized by Celsus in the 1st century, namely, rubor (redness), tumor (swelling), calor (heat) and dolor (pain) (Kumar et al 2005). Despite the fact that these cardinal signs of inflammation were evident over 2000 years ago, the cellular and molecular events that regulate the inflammatory response and its timely resolution are only recently beginning to be recognized and understood. Tissue edema is one of the earliest events of the acute inflammatory response and is consequent from increased permeability of microvasculature. Polymorphonuclear neutrophils (PMN) are the first line of defense against microbial invasion, which restrict potentially harmful stimuli via phagocytosis. PMN traverse post-capillary venules at sites of inflammation, degrade pathogens within phagolysosomes, and undergo apoptosis. Next, mononuclear cells infiltrate, differentiate into macrophages, and clear apoptotic PMN by phagocytosis in a non-phlogistic manner termed efferocytosis (Rossi et al 2008). Finally, clearance of microbes and efflux of phagocytes allows for the tissue to return to it normal state (Serhan 2007). Disruption of any of these specific events could potentially give rise to chronic inflammation, which is characterized primarily by excessive leukocyte infiltration and activation, delayed clearance, thereby resulting in tissue damage, and loss of function. **Lipid mediators (LM)** biosynthesized from essential fatty acids play key roles in the defined phases of the inflammatory response (Serhan 2007), with prostaglandin E_2 (PGE_2) and cysteinyl leukotrienes (cysLTs) enhancing early vascular permeability and leukotriene B_4 (LTB_4) stimulating leukocyte chemotaxis (Samuelsson et al 1987). Prostaglandins play additional roles during the acute inflammatory response, which includes the regulation of local changes in blood flow and pain sensitization (Flower 2006). During evolution of an inflammatory exudate, the profile of lipid mediators changes to biosynthesis of counter-regulatory mediators

that would limit further PMN congregation and promote resolution (Levy et al 2001, Serhan et al 2000, Lawrence et al 2010). While the mechanisms that mediate progression from acute to chronic inflammation are not completely understood, it is hypothesized that chronic inflammation is due to excess of pro-inflammatory mediators (Nathan et al 2005). Emerging evidence suggests that it is also plausible that disruptions in endogenous pro-resolving circuits could underlie some of the aberrant mechanisms that lead to chronic inflammation (Nathan et al 2006, Serhan 2007).   Complete resolution of an acute inflammatory response is the ideal outcome after an insult (Kumar et al 2005).

Systematic studies of lipid mediators in the course of acute inflammatory responses have demonstrated that prostaglandins such as prostaglandin E2 (Levy et al 2001) are generated during the initial phase of resolution of inflammation. These pro inflammatory prostaglandins are needed for the control of blood flow and vessel dilation essential for leukocytes to undergo firm adhesion and diapedesis (Williams et al 1977). It is known that essential trafficking of lymphocytes from the post-capillary lumen to the interstitial space is a process that is mediated in part by leukotriene B4 (Pouliot et al 2002). Also 'programmed' in this initial phase is the activation of signaling pathways for the normal self-limiting inflammation (Levy et al 2001,Serhan et al 2000, Serhan et al 2002). Signaling pathways (Gilroy et al 1999) leading to prostaglandins E2 and D2 in turn actively switch on the transcription of enzymes required for the generation of other classes of eicosanoids also generated from arachidonic acid, such as lipoxins (Levy et al 2001), as well as newly identified families of lipid mediators generated from omega-3 polyunsaturated fatty acid called resolvins (resolution-phase interaction products) and protectins (Serhan et al 2000, Serhan et al 2002, Marcheselli et al 2003) which normally can dominate the resolution phase.

1.9. THE CELLULAR PROGRAM IN RESOLUTION

It has been argued that events occurring early in acute inflammation engage an active and coordinated 'resolution program' involving a switch to local production of specialized intercellular messengers, programmed leukocyte death by apoptosis and there after clearance of dying cells by phagocytes that eventually leave the inflamed site through lymphatics (Nathan 2002, Lawrence et al 2002 and Savill 1997).

In order for resolution to follow, further leukocyte recruitment should be halted and accompanied by clearance of leukocytes from inflammatory sites. Resolution of inflammation will occur if granulocytes are eliminated and the tissue mononuclear cell population (macrophages and lymphocytes) returns to normal pre inflammation numbers and phenotypes (Serhan 2007). It is apoptosis of neutrophils, that causes specific recognition and clearance by inflammatory macrophages, an important step in inflammation reduction that is found at several inflammatory sites (Savill 1997, Savill 2001). In addition to clearing dead neutrophils, the process of phagocytosis could serve a dual purpose by initiating a key signal to the inflammatory macrophage (and perhaps the closely related phagocytic dendritic cell) to begin the process of egress from the inflamed local tissue site to the nearest draining lymphatics (Savill 2001), which is the usual fate of inflammatory macrophages (Bellingan et al 1996). This multicellular 'waste elimination program' offers key therapeutic targets. For example, constitutive neutrophil apoptosis, which is normally relatively rapid, can be manipulated to be slowed by a wide range of inflammatory mediators (Lee et al 1993), which in turn may engage activation of NF-κB and other 'prosurvival' transcription factors such as Foxo3a (Johnson et al 2005). These prosurvival and activation factors cannot be left unregulated. Hence, mechanisms must exist that can override such survival signals. One possible mediator of such signals could be the macrophage, whose arrival on the inflammatory scene can cause the release of 'death cytokines' such as Fas ligand, which can then in turn trigger apoptosis in neighboring neutrophils (Brown et al 1999). Alternatively, uptake of apoptotic cells may also initiate macrophages to release mediators that suppress the inflammatory response (Voll et al 1997, Fadok et al 1998). In particular, strong *in vitro* and *in vivo* evidence (Huynh et al 1998, Lucas et al 2003) suggests that macrophage secretion of the anti-inflammatory cytokine transforming growth factor-β1 (TGF-β1) can suppress

proinflammatory signaling from Toll-like receptors.

The reparative properties of TGF-β1 indicate a link between mechanisms governing resolution of inflammation and the 'end game': repair of damaged tissue (Serhan et al 2005). It has been demonstrated that the lipid mediator **Lipoxin A$_4$** increases the appearance of TGF-β1 in resolving exudates (Banneberg et al 2005). Lipoxin A$_4$ is produced during the resolution phase and hence it serves as a mediator of 'pro-resolution' responses (Serhan et al 2005). By its ability to enhance TGF-β, it is also involved in governing resolution and/or its turn to tissue fibrosis. Phagocytosis of apoptotic cells inhibits activated macrophage killing of resident tissue cells (Duffield et al 2001) and also triggers secretion of vascular endothelial growth factor, which is important for repair of endothelial and epithelial injury (Golpon et al 2004). Macrophage clearance of apoptotic leukocytes, can be enhanced by several early inflammatory mediators (Ren et al 2008), which thereafter can be amplified by early interactions with apoptotic cells (Lucas et al 2003). It has been demonstrated that early macrophage exposure to cytokines increases the capacity of macrophages to ingest apoptotic cells (Erwig et al 1998, Erwig et al 2000). It has thereby been proposed that once apoptosis in leukocytes and resident cells is engaged (an early event), subsequent uptake of apoptotic cells causes a switch in macrophage phenotype from activated or injurious to reparative or emigratory (Serhan et al 2005).

The driver for persistent neutrophilic airway inflammation in bronchiectasis is unknown, but infection is considered to play a major role. I hypothesize that **there is failure of resolution in bronchiectasis, which leads to persistent inflammation**. Hence, elucidating the biochemical pathways leading to resolution of the neutrophilic airways inflammation and thereby targeting the host resolution program will help in understanding the resolution process or thereby the lack of it in bronchiectasis, which may lead to development of novel non-antibiotic therapies in this chronic lung disease.

1.10. PRO RESOLVING LIPID MEDIATORS (Figure 12)

Excessive inflammation is widely accepted to be a unifying component in many chronic diseases, including bronchiectasis, vascular diseases, metabolic syndrome and neurological diseases, and thus is a public health concern. Understanding endogenous control points within the inflammatory response could potentially provide us with new perspectives on disease pathogenesis and treatment approaches. Break in the barrier, trauma and microbial invasion encourages the host to neutralize invaders, clear the site, and remodel and regenerate tissue. The acute inflammatory response is protective. Lipid mediators - such as eicosanoids (prostaglandins and leukotrienes) (Flower 2006, Samuelsson 2012) produced from the essential fatty acid arachidonic acid, as well as many cytokines and chemokines (Dinarello et al 2012, Serhan and Savill 2005) have crucial roles in the initial response. Interactions among prostaglandins, leukotrienes and pro-inflammatory cytokines amplify inflammation, the signs and effects of which can be reduced by pharmacological inhibition and receptor antagonists (Flower 2006, Samuelsson 2012, Dinarello et al 2012). However, given that excessive inflammation contributes to several widely occurring diseases, improvements are required in treatment and in our understanding of the mechanisms involved. Phospholipids metabolize to produce omega 6 and omega 3 derivatives. The omega 6 derivative arachidonic acid metabolizes to produce prostaglandins (pro inflammatory), lipoxins (anti inflammatory; pro resolving) and leukotrienes (pro inflammatory). Omega 3 derivatives are eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) (figure 12). The omega-3 fatty acids EPA and DHA, which are found in marine oils, have long been thought to have anti-inflammatory properties and they compete with arachidonic acid, reducing pro-inflammatory eicosanoids (Lands 2009). EPA metabolites are the e-series resolvins and DHA metabolites are d-series resolvins, maresins and protectins. The molecular mechanism by which this occurs is unclear, and the evidence is inconclusive as to whether omega-3 EPA and DHA are beneficial for human health and as treatments for disease.
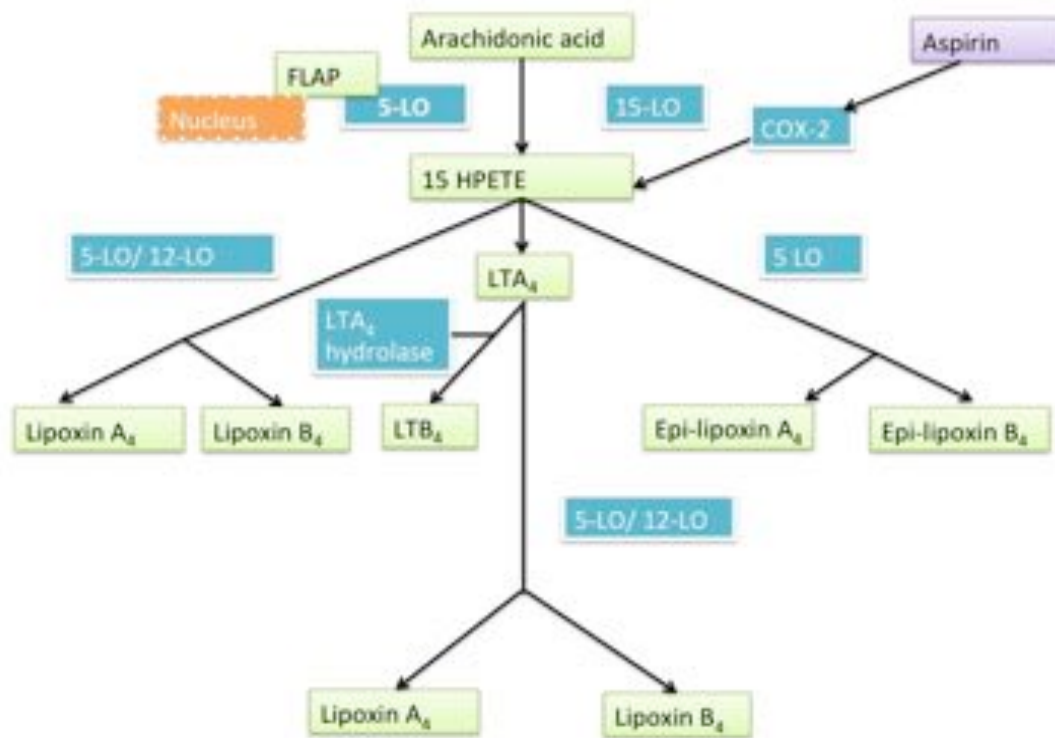
Figure 12. Synthesis of lipoxin. There are three major lipoxygenases (LO): 5-LO, 15-LO, and 12-LO that are involved in lipoxin synthesis from arachidonic acid. In the first pathway of lipoxin synthesis, LTA_4 is acted upon by 12-LO, and is converted to lipoxin A_4 and B_4. The second pathway involves the action of a series of lipoxygenase (5-LO in neutrophils and 15-LO in erythrocytes) activities on arachidonic acid which then gets converted to 15-HEPTE. Lipoxins are formed from 15-HEPTE by the action of either 5-LO or 12-LO. The third pathway is the formation of epi-lipoxin A4 or aspirin-triggered lipoxin (ATL) and epi-lipoxin B4 whose generation is aspirin dependent.

Green filled represent metabolites or end products whereas the blue filled represent the enzymes.

15-HPETE: 15-hydroxyperoxyeicosatetraenoic acid; COX-2: cyclooxygenase-2; FLAP: 5-lipoxygenase-activating protein; LTA_4: leukotriene A_4, LTB_4: leukotriene B_4.

1.11. LIPOXINS

Lipoxins were first isolated from human leukocytes by Serhan et al and were first described as a novel series of compounds with four conjugated double bonds (Sehran et al 1984).

1.11.1. Synthesis (Figure 12)

Lipoxins are a metabolite of the arachidonic acid pathway, which was well studied by Hamberg and Samuelsson (Hamberg et al 1974). Arachidonic acid pathways play an important role in inflammation, producing several anti-inflammatory molecules (Malhotra et al 2012). Lipoxins can be synthesized by two major routes from arachidonic acid. Additionally, lipoxin epimers can be formed under the influence of aspirin treatment as described by Serhan and colleagues (Serhan et al 1984) (figure 13). There are three major lipoxygenases (LO) that are involved in lipoxin synthesis from arachidonic acid namely 5-LO, 15-LO, and 12-LO. The first pathway of lipoxin synthesis occurs in platelets where leukotriene A_4 is metabolized by 12-LO, and is converted to lipoxins (Serhan et al 1990). The second pathway of synthesis involves the action of a series of LO (5-LO in neutrophils and 15-LO in erythrocytes and reticulocytes) activities on arachidonic acid. Arachidonic acid then gets converted to 15-hydroxyperoxyeicosatetraenoic acid, which is subsequently converted to lipoxin A and lipoxin B (Serhan et al 1984). These pathways occur in neutrophils, eosinophils (Serhan et al 1987) and alveolar macrophages (Levy et al 1993), or during cell/cell (tissue) interactions (Serhan et al 1987). This route may perhaps be prevalent in the airways. A reduction in LXA_4 formation and expression of 5-LO and 15-LO isoforms was detected in bronchoalveolar lavage cells and endobronchial biopsies from asthmatic patients (Planaguma et al 2008).

1.11.2. Aspirin triggered lipoxins (ATL)

A series of LX epimers with alcohol at C15 in the *R* configuration was discovered by Claria and Serhan (1995). This is the third pathway for lipoxin generation, which is aspirin dependent, and leads to the generation of 15 epi-lipoxin A_4, also known as aspirin-triggered lipoxin (ATL) and 15 epi-lipoxin B_4 (Claria et al 1995). Aspirin acetylates cyclooxygenase-2 (COX-2) to form 15R-hydroxy eicosatetraenoic acid (15 HETE). 15*R*-HETE is converted thereafter by 5-LO to an epoxide intermediate, enzymatically transformed into 15-epi-LXA_4 and 15-epi-LXB_4 (figure 13). Cellular models of several methods of ATL generation include co-incubations of PMN with aspirin-treated human umbilical endothelial cells (Claria and Serhan 1995), transcellular metabolic exchanges between PMN and A459 lung adenocarcinoma cells (Claria et al 1996) or between hepatocytes and liver cells (Titos et al 1999). In addition, ATL production is induced by aspirin when administered to healthy volunteers (Chiang et al 2004 and Fiorucci et al 2003b).
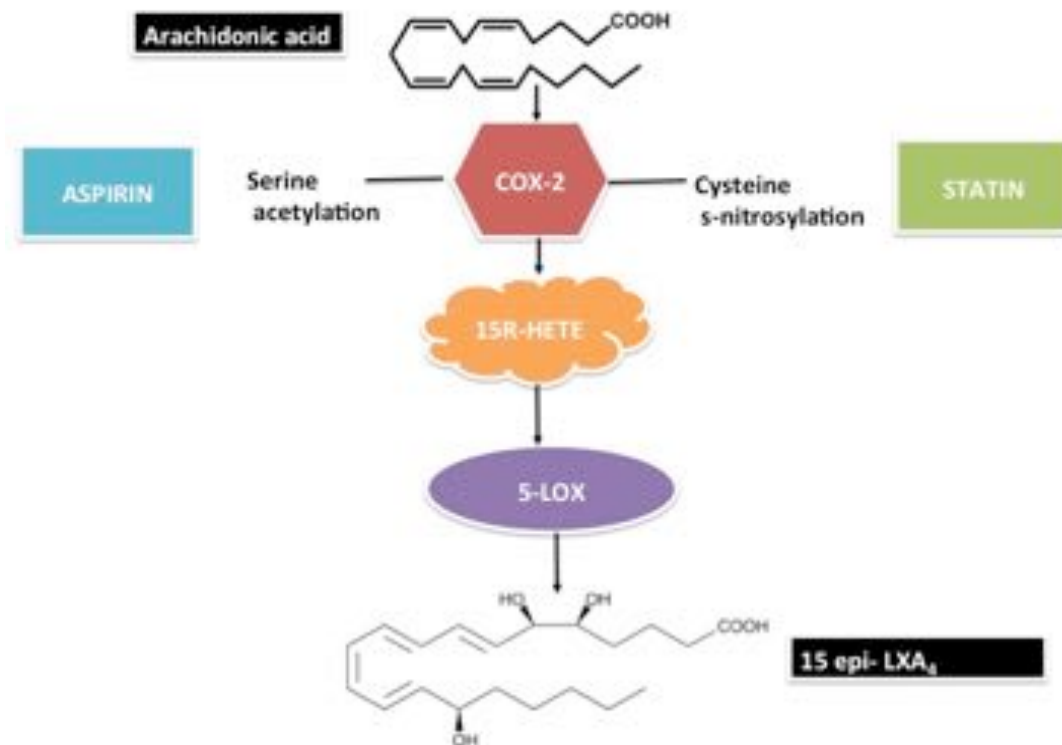
Figure 13. Aspirin and statins promote the formation of 15- epi lipoxin A_4. Aspirin acetylates the active site of COX2 (Asp-COX2) that now is able to metabolize arachidonic acid to 15(R)-hydroxyeicosatetranoic (15R-HETE), which when released from endothelial and epithelial cells is converted by leucocyte 5-LOX to the aspirin-triggered LXs (ATLs), 15-epi-LXA$_4$.
COX-2: cyclooxygenase-2; HETE: hydroxyeicosatetranoic acid; LOX: lipoxygenase; LX: lipoxin.

1.11.3. Biological actions of lipoxins and aspirin triggered lipoxins

There is enough evidence now to suggest that neutrophils change their phenotype to produce different profiles of lipid mediators depending on the cells and substrates present in their local environment (Levy et al 2001, Serhan et al 2000). Neutrophils in resolving inflammatory exudates switch from the production of leukotrienes to that of lipoxins and resolvins, whereas neutrophils in the peripheral blood, on activation, generate and release leukotriene B_4 as one of their main bioactive products (Levy et al 2001). In the same context, local prostaglandin E2 and prostaglandin D2 stimulate the processing of 15-lipoxygenase mRNA in leukocytes to then produce a functional enzyme for lipoxin production (Levy et al 2001). Other cell types can acquire the ability to generate lipoxins when exposed to certain specific cytokines or growth factors (Fiore and Serhan 1990), or in macrophages, for example, when they engulf apoptotic leukocytes (Friere-de-Lima 2006). These findings are of significance to pro-resolution mechanisms because lipoxin A_4 generated by macrophages probably contributes to the stimulation of their phagocytic activity (Cambell et al 2007) without elaborating pro-inflammatory mediators- namely, the non-phlogistic process. Pathogens can also contribute to the provision of the necessary components for lipoxin biosynthesis. It has been demonstrated that *Pseudomonas aeruginosa* encodes the first identified secretory lipoxygenase that converts host arachidonic acid to 15-HETE for local lipoxin production (Vance et al 2004).

Lipoxin A_4 and lipoxin B_4 inhibit neutrophil entry into inflamed sites and counter-regulate some of the main aspects of inflammation (Serhan et al 2008) (figure 14). They act on several cell types including blood cells, neural cells and stromal cells (Takano et al 1998, Chiang et al 2006, Maddox and Serhan 1996). Lipoxin A_4 regulates leukocyte responses *in vitro* and trafficking *in vivo* by activating its specific receptor, known as lipoxin A_4 receptor (ALX/FPR2). ALX/FPR2 is a G protein-coupled receptor (GPCR) that is expressed by leukocytes and has cell-type-specific signaling pathways (Chiang et al 2006). This is explained by the fact that in neutrophils, lipoxin A_4–ALX interactions stop neutrophil migration, whereas in monocytes lipoxin A_4–ALX interactions stimulate monocyte chemotaxis and non-phlogistic responses (Maddox et al 1997). Unlike the classic GPCRs for chemo-attractants that mobilize intracellular Ca^{2+} to evoke chemotaxis, lipoxins instead

induce changes in the phosphorylation of proteins of the cytoskeleton, resulting in cell arrest (Gronert et al 1998 and Patcha et al 2004). In addition to these effects on the resolution of inflammation, lipoxin A_4 reduces organ fibrosis, acts directly on vascular and smooth muscle and has direct action in reducing pain (Svensson et al 2007).

Aspirin impinges on the endogenous lipoxin‑generating system during cell–cell interactions. Inhibition of prostaglandin biosynthesis by aspirin is a well known mechanism in its anti-thrombotic and anti-inflammatory effect (Vane et al 1982). Aspirin triggers the endogenous formation of carbon-15 epimeric lipoxins, namely aspirin-triggered lipoxins (ATLs). Cells that express cyclooxygenase‑2 (COX2), namely vascular endothelial cells, epithelial cells, macrophages and neutrophils, are involved in ATL production. Acetylation of COX2 by aspirin blocks its ability to biosynthesize prostaglandins (Serhan et al 2008).

LX and ATL exert potent anti-inflammatory pro-resolution actions both *in vitro* and *in vivo*. Leukocytes, which express high levels of ALX/FPR2, are elective targets of these eicosanoids. For instance, LXA_4, 15-epi-LXA_4 and or stable analogs inhibit PMN and eosinophil chemotaxis (Bandeira-Melo et al 2000 and Soyombo et al 1994), PMN vascular adhesion, transendothelial and transepithelial migration, superoxide anion generation (Carlo et al 2013 and Serhan et al 1984), azurophilic degranulation (Gewirtz et al 1999), peroxynitrite formation and nuclear factor (NF)-κB activation (Jozsef et al 2002). On the other hand, they stimulate monocyte chemotaxis, macrophage efferocytosis and bacterial phagocytosis, reducing the release of inflammatory cytokines (Godson et al 2000 and Romano et al 1996). LXA_4 also down tones memory B-cell responses, thus modulating adaptive immunity (Ramon et al 2014).
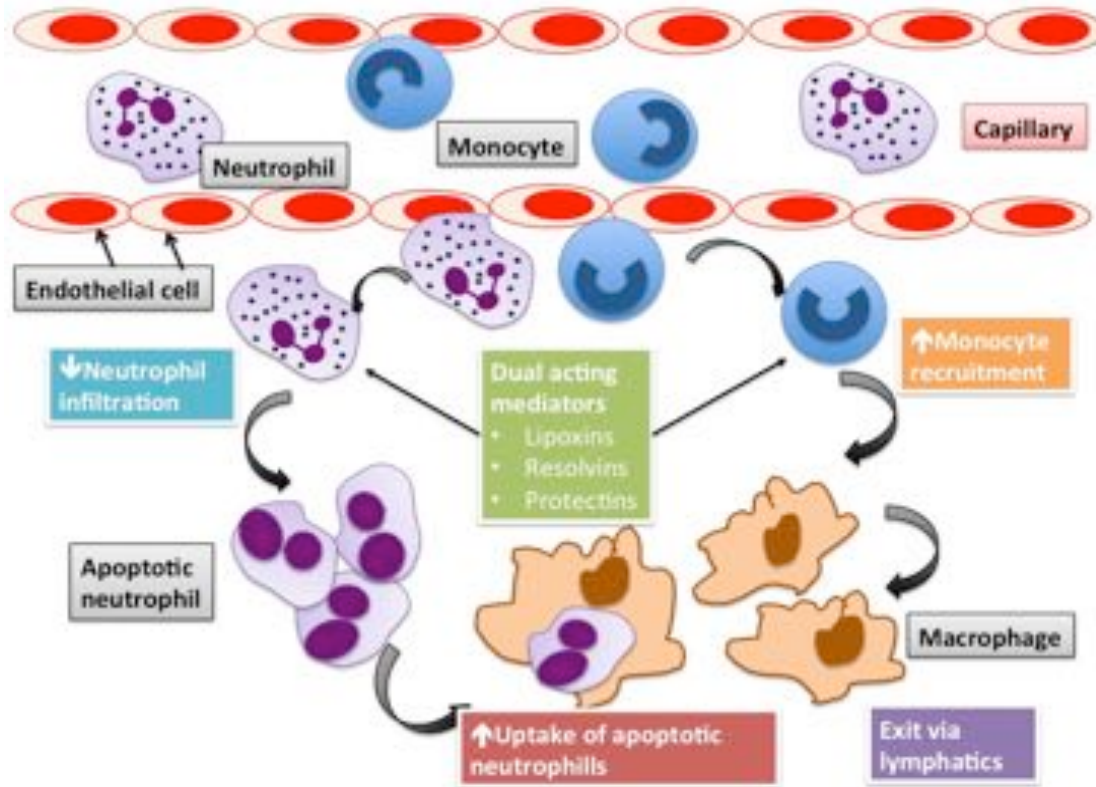
Figure 14: Dual anti inflammatory and pro resolution actions of lipoxins. The key histological feature in the resolution of inflammation is the loss of neutrophils from the local inflamed sites. This is a programmed process that is actively regulated at different levels: by reducing neutrophil infiltration into the exudate, increasing monocyte recruitment to the exudate, stimulating macrophage uptake of apoptotic neutrophils, and enhancing phagocyte exit from the exudate via the lymphatics.

Adapted from Serhan et al; Nature; 2008.

1.12. N- FORMYL- METHYL- LEUCYL- PHENYLALANINE (fMLF) AND

RECEPTORS

1.12.1. fMLF

Neutrophils are first responders in an organism's rapid assault on infectious pathogens. Through genetically conserved receptors, neutrophils recognize chemoattractants, lipid products, and the molecular patterns present on the surface of bacteria, viruses, and fungi (Medzhitov and Janeway 2000). fMLF is recognized by neutrophils and is a potent neutrophil chemoattractant. fMLF, upon binding to its heterotrimeric G protein-coupled receptor, initiates signaling cascades that activate multiple pathways (Haribabu et al 2000). These pathways include the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI-3K) cascades, which are important for the development of the functional responses of neutrophils in inflammation (e.g., the respiratory burst, transmigration, and phagocytosis) (Mocsai et al 2000, Rane et al 1997).

1.12.2. Formyl peptide receptor (FPR) 1

The FPR receptor family was extensively characterised throughout the 1980s as seven membrane spanning, G-protein coupled receptors (GPCR) via pertussis toxin sensitivity; specifically attributed to G-protein coupling (Lavigne et al 2002). The human family was cloned in 1990 (Boulay et al 1990) with three genes encoding FPR1, ALX/FPR2 and FPR3 clustered on chromosome 19q13.3.

FPR1, the first chemotactic receptor to have its primary structure delineated by molecular cloning (Boulay et al 1990), has been extensively studied for its high affinity binding to fMLF. Following agonist ligation, FPR1 undergoes rapid phosphorylation in a concentration and time dependent manner (Ali et al, 1993) leading to conformation changes. Downstream of G-protein interaction there are a number of signalling pathways including calcium (Ca^{2+}) flux, phospholipase (PL) A, C and D, phosphoinositide-3-kinase (PI3K) and MAP kinase pathways (Selvatici et al 2006).

PLC is an essential upstream mediator of both PI3K and mitogen- activated protein kinase pathways via protein kinase C (PKC). PLA is well characterised for mediating PLC activation central to the biosynthesis of eicosanoids. In neutrophils, activation of PI3K following fMLF has been shown to selectively regulate oxidative burst and actin relocalisation, essential for cell polarisation. The MAP kinase pathway, in particular ERK-1/2, selectively regulates chemokinesis as well as signal transducers and activators of transcription proteins and adaptor proteins (Wenzel-Seifert et al 1998).

Signalling via FPR1 is regulated by two processes, receptor desensitisation and agonist-induced internalisation (figure 15). Desensitisation is the result of uncoupling of G-protein from the receptor, and can occur either by direct ligation or following activation of similar GPCRs. It has been shown that receptor desensitisation and internalisation is reliant on PKC following both homologous activation of FPR1 or heterologous activation of other GPCRs (Le et al 2001b). After initial activation of FPR1 with fMLF, the receptor rapidly reduces its responses to secondary stimulation with the same agonist; this is termed homologous desensitisation. FPR1 is also susceptible to heterologous desensitisation via ligation of a GPCR receptor, C5aR (complement receptor) or CXCR2 (interleukin-8 receptor), in a concentration-dependent manner (Ali et al 1999). These processes complement the classical idea of concentration-gradient dependent migration by peripheral cells to a site of inflammation.

Apart from the ability of FPR1 to mediate chemotaxis it was also noted that fMLF induced a rapid Ca^{2+} mobilisation (Andersson et al 1986). Although Ca^{2+} has been a robust functional response to fMLF, its biological relevance is still unclear. Ca^{2+} is required for cytoskeleton reorganisation, but phagocytosis and chemotaxis can occur in Ca^{2+} depleted cells (Fu et al 2006).

Functionally, FPR1 is mostly associated with host defence; fMLF has been associated with eliciting shape change, adhesion, phagocytosis, cytokine production, superoxide production and degranulation in phagocytic cells (Selvatici et al 2006).
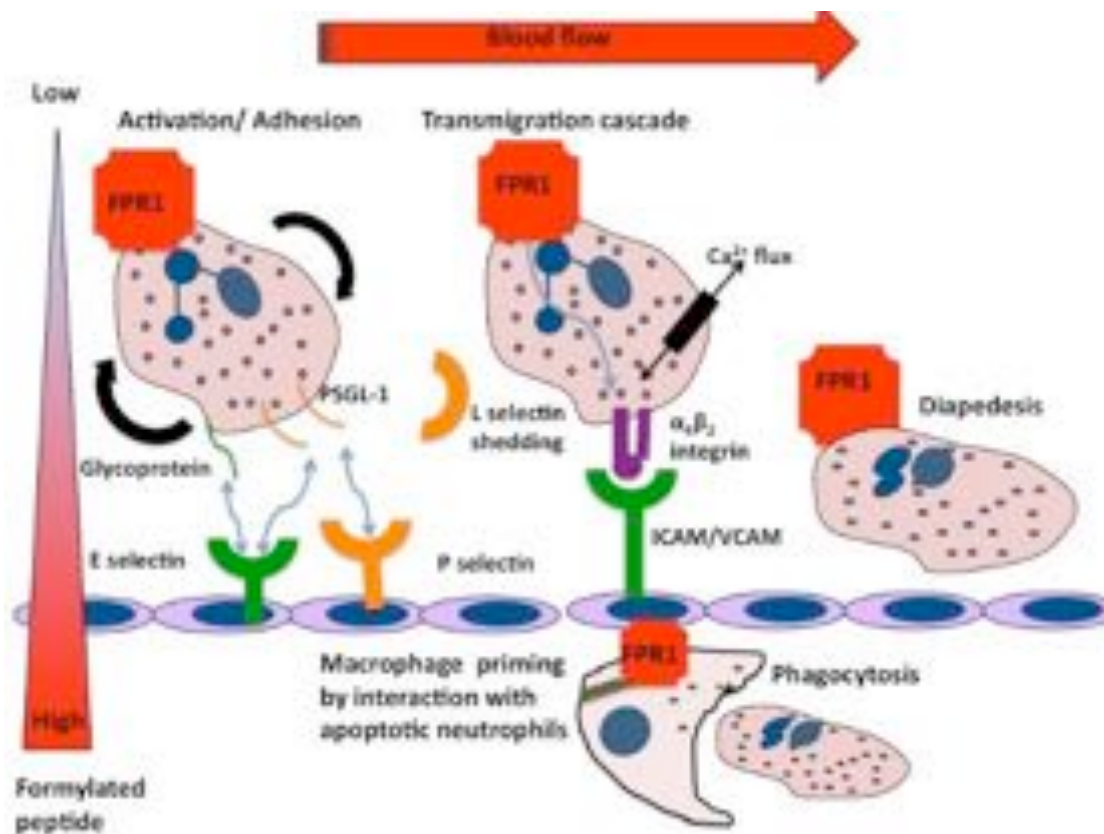
Figure 15. Simplified representation of leukocyte migration cascade via the FPR family (Adapted from Dufton and Peretti 2010). Increasing gradient of formyl peptide leads to a well-characterised sequential response activating circulating and resident leukocytes. FPR1 is activated at low concentration of formyl peptide. FPR activation of leukocytes, results in integrin expression and activation inducing firm adhesion to the endothelial cells. High concentration or repeated stimulation by formyl peptide can lead to receptor desensitisation, which may be a prominent regulatory mechanism. FPRs play a role in macrophage phagocytosis and as yet uncharacterised roles in innate-adaptive cell interactions.

FPR1= Formyl peptide receptor 1; ICAM= intercellular adhesion molecule; PSGL-1= P-selectin glycoprotein 1; VCAM= vascular cellular adhesion molecule.

1.12.3. ALX/FPR2 receptor

Lipoxin A_4 binds to the FPR2 receptor and hence it is known as ALX/FPR2 receptor. Originally characterised as the low-affinity fMLF receptor (efficacy ~1000 fold lower than FPR1; (Gao & Murphy, 1993)), ALX/FPR2 has a short but fairly complicated history. The human receptor was initially reclassified as the receptor for the arachidonic acid-derived Lipoxin A_4, termed ALX (Fiore et al 1994). However it soon became evident that Lipoxin A_4 was not the only competitive agonist capable of binding this receptor, with reports describing HIV peptides and serum amyloid A (SAA; (Su et al 1999)), Annexin A1 (AnxA1; (Perretti et al 2002)), as well as synthetic peptide (W-peptide; (Le et al 2001)) and chemical compounds (C43; (Burli et al 2006)). There are currently ~30 ligands that have been shown to bind the FPR family and there is considerable promiscuity across the FPR receptors, with ligands capable of transducing either pro- or anti-inflammatory actions *in vitro* and, in some cases, *in vivo* (Dufton and Perretti 2010).

The signal transduction of ALX/FPR2 is a difficult topic, however it is fair to assume it shares part of the intracellular machinery of FPR1 as they have close homology in their intracellular domains (Gao & Murphy 1993). A comprehensive study of the FPR1 and ALX/FPR2 signaling, conducted by creating chimaeric receptors with segments of ALX/FPR2 replaced by FPR1, revealed numerous specific binding sites. Of importance is the fact that fMLF recognised the same extracellular loops on both FPR1 and ALX/FPR2 but with different affinity. This analysis also revealed that *N*-glycosylation is required for peptide binding (although not necessarily at the same domain) to ALX/FPR2, but not for the lipid agonist LXA_4 (Le et al 2005). Similar to FPR1, ALX/FPR2 is strongly associated with host defense, but its novel ability to convey both pro- and anti-inflammatory (likewise, activating and inhibitory) signaling makes it an intriguing and unusual GPCR.

Interaction of ALX/FPR2 with fMLF and the acute phase protein serum amyloid protein A (SAA) leads to NF-κB activation and secretion of CXCL8 by human neutrophils (He et al 2003). The reduced affinity for formylated-peptides, such as fMLF, was initially thought to reflect receptor redundancy, where by the desensitisation of one receptor, FPR1, may result in the prevalence of a second lower

affinity counterpart ALX/FPR2 (Hartt et al 1999). Although this hypothesis may be true *in vitro* it is perhaps slightly simplistic when considering the role of heterologous desensitisation *in vivo*.

ALX/FPR2 has been more comprehensively assessed for its anti-inflammatory pharmacology such as ability to provoke deactivation and detachment of leukocytes, leukocyte apoptosis, increment phagocytosis of apoptotic cells by phagocytes and regulation of COX-2 (El Kebir et al 2007 and Lee et al 2006). Indeed, the most striking attribute of ALX/FPR2 pharmacology is related to its putative endogenous ligands, which includes the glucocorticoid-modulated protein AnxA1 and LXA_4 among others. SAA (mentioned above) a peptide-derivative of beta-amyloid-A (termed βA_{42}) and LL-37 are other potent ligands (figure 16).

These dual roles of ALX/FPR2 make association with disease pathogenesis difficult to define.

ALX/FPR2 expression is induced by both pro-inflammatory stimuli, e.g. TNF-α (Cui et al 2002 and Cui et al 2002) and pattern recognition receptors (Chen et al 2009) as well as anti-inflammatory signalling, such as through the glucocorticoid receptor (Sawmynaden and Perretti 2006). The strongest mechanistic links are with autoimmune diseases including airway allergy, arthritis and Alzheimer's.
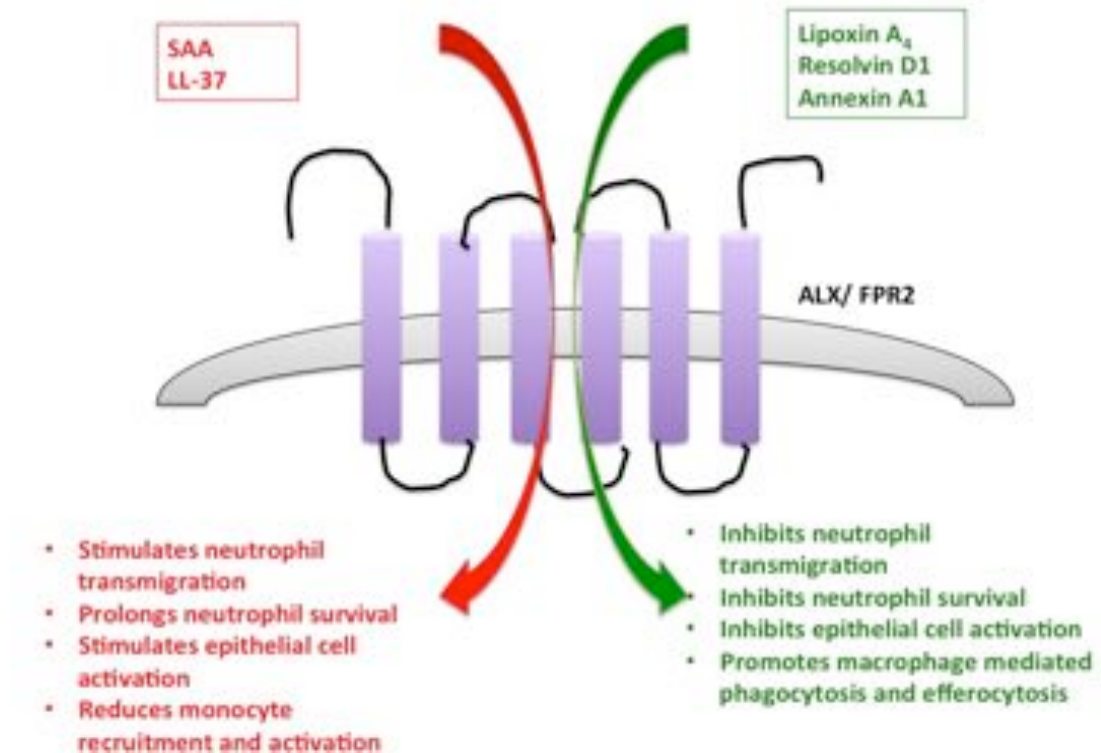
Figure 16. The ALX/FPR2 complex is expressed on leukocytes and the respiratory mucosa. The expression of two known ligands for ALX/FPR2, Serum Amyloid A (SAA) and LL-37 been shown to initiate a pro-inflammatory response. In contrast, alternate ligands Lipoxin A$_4$, Resolvin D1 and Annexin A1 can bind to a different receptor region and promote distinct receptor conformations that translate to an opposing biological action.

1.13. OTHER COMPOUNDS RELEVANT TO THIS THESIS

1.13.1. Roscovitine

Formula: $C_{19}H_{26}N_6O$

Systemic name: 2-(*R*)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine

Molar mass: 354.45 g/mol
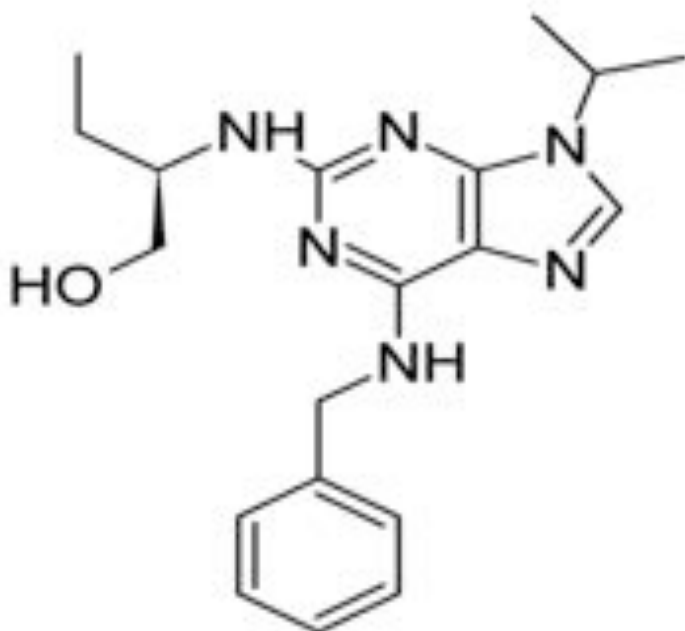
Figure 17. Roscovitine

Neutrophils are key to the immune system and are recruited rapidly to the site of inflammation. Neutrophils then release products of degranulation and activation to defend and destroy invading microorganisms but these byproducts are detrimental to the surrounding tissues (Nathan 2002). Activated neutrophils need to 'switch off' and undergo apoptosis for the resolution of inflammation. Once apoptosis has been engaged, the neutrophil secretory activity is shutdown; the cells remain intact and are phagocytosed by macrophages using recognition mechanisms (efferocytosis) that fail to elicit a pro-inflammatory response (Savill et al 1989, Whyte et al 1993). Impairment of either apoptosis or efferocytosis will lead to persistence of inflammation.

Neutrophil apoptosis is controlled by a complex network of signalling pathways that regulate both the turnover of key molecules, including the anti-apoptotic proteinmyeloid cell leukemia 1 (Mcl-1) and the pro-apoptotic Bcl-2 family member Bax, and activation of the caspase family of proteases. (Riley et al 2006).

Cell division of eukaryotic cells occurs in four phases (G1, S, G2, M) and in some circumstances, where the cell is terminally differentiated, the cell will rest in G0 phase. Neutrophils are terminally differentiated cells. The cyclin dependent kinases (CDK) are key regulators of the cell cycle, whereby different CDKs become activated during cell-cycle progression when complexed with their associated cyclin partners (Vermeulen et al 2003).

Rossi *et al* have been investigating the role of CDK inhibitors in neutrophil apoptosis, both *in vitro* and *in vivo* (Rossi et al 2006). They have shown that human neutrophils express functional CDKs and that different CDK inhibitors directly induce caspase-dependent neutrophil apoptosis and inhibit cell survival induced by several biologically important powerful anti- apoptotic agents. In addition, the CDK inhibitor R-roscovitine down-regulates Mcl-1 expression induced by survival factors in neutrophils. Further studies have demonstrated *in vivo* that roscovitine markedly enhances resolution of inflammation in mouse models of carrageenan-induced acute pleurisy, bleomycin-induced lung inflammation and passively induced arthritis. The roscovitine-enhanced resolution of established pleurisy is driven by a caspase-mediated pro-apoptotic effect (Rossi et al 2006).

Perhaps unsurprisingly, given the terminally differentiated state of these cells, the most significantly expressed CDKs (McGrath et al 2011, Leuenroth et al 2000) have no direct role in the cell cycle but are essential for transcription of a key subset of genes. Mcl-1 (pro survival protein) is down regulated to the level of genes and this is essential for the initiation of apoptosis. It has been further demonstrated that roscovitine manipulates the transcriptional machinery of the neutrophils to thereby promote apoptosis (Leitch et al 2012).

These findings suggest that CDK inhibitors may provide a therapeutic strategy to promote resolution of inflammatory diseases, through specific induction of inflammatory cell apoptosis.

1.13.2. Cytochalasin B

Cytochalasin b is a cell permeable mycotoxin, which can strongly inhibit network formation by actin filaments. It inhibits cytoplasmic division by blocking the formation of contractile microfilaments. In addition, it inhibits cell movement and induces nuclear extrusion. Cytochalasin B shortens and inhibits actin filaments by blocking monomer addition at the fast-growing end of F actin filaments (Theodoropoulos et al 1994). This inhibition can affect all three major steps of actin polymerization. Of interest to us for the purpose of this thesis, is the property of cytochalasin in inhibiting nuclear extrusion. Nuclear extrusion induced by cytochalasin B begins with the movement of the nucleus to the plasma membrane, followed by bulge formation in the membrane. The nucleus then moves to the outside of the membrane, but stays connected to the cell by a thread-like cytoplasmic bridge. If the cells are kept in cytochalasin B containing medium for several hours, the process becomes irreversible. Extrusion is assisted by cytochalasin b induced weakening of the plasma membrane.

1.13.3. Phorbol 12-myristate 13-acetate

Phorbol 12-myristate 13-acetate (PMA), is a diester of phorbol and a potent tumor promoter used in research to activate the signal transduction enzyme protein kinase C (PKC) (Castagna 1982) The effects of PMA on PKC result from its similarity to one of the natural activators of classic PKC isoforms, diacylglycerol. In reactive oxygen species biology, superoxide was identified as the major reactive oxygen species induced by PMA (Swindle 2002). Hence PMA has been routinely used as an inducer for endogenous superoxide production (Huang 2014). PMA is used specifically in cancer diagnostics as a B-cell specific mitogen in cytogenetic testing. PMA is also commonly used together with ionomycin to stimulate T-cell activation, proliferation, and cytokine production.

1.14. MANAGEMENT

In mild bronchiectasis, the mainstay of treatment is regular chest clearance with physiotherapy, annual influenza and prompt treatment of exacerbations. In more advanced bronchiectasis, in addition, there should be consideration of long-term antibiotic therapy and or anti-inflammatory therapy.

1.14.1 Macrolides in bronchiectasis

In bronchiectasis, several studies have been conducted with macrolides, but only three RCTs (randomized control trials) have been conducted to date in adults, assessing the role of macrolide as an anti-inflammatory agent with immunomodulatory properties in bronchiectasis. Key studies are summarized below.

Wong (Wong et al 2012) confirmed these findings in the Effectiveness of Macrolides in patients with Bronchiectasis using Azithromycin to control Exacerbations (EMBRACE) trial. This was a randomised double-blind placebo controlled trial of 141 patients randomised to either taking azithromycin 500mg three times a week or placebo for 6 months. They showed a significant reduction in the rate of event-based exacerbation from 1.57 in the placebo group to 0.59 with the azithromycin group. There was no statistically significant difference in lung function or quality of life.

More recently the Bronchiectasis and long term Azithromycin Treatment (BAT) study in 83 bronchiectasis patients with 3 or more exacerbations in the past year were randomised to either 250mg azithromycin once daily or placebo for 1 year (Altenburg et al 2013). Results showed a significant decrease in exacerbation frequency in the azithromycin group with a longer time to first exacerbation during treatment. FEV_1 and FVC improved with azithromycin as did quality of life as assessed by SGRQ. Sputum microbiology was similar at baseline and at 1 year but significant macrolide resistance was recorded in 88% compared with 26% in the placebo group. *Haemophilus influenzae, Staphylococcus aureus* and *Moraxella catarrhalis* were resistant to macrolides at the end of treatment. Adverse reactions were reported, mainly gastrointestinal symptoms, but not severe enough to discontinue treatment.

The Bronchiectasis and Low dose Erythromycin Study (BLESS) investigated the effect on exacerbations rates and resistance rates post 1-year therapy with low dose erythromycin (Serisier et al 2013). Patients were randomised to low dose erythromycin or placebo for 48 weeks. Results showed a significant reduction in exacerbation frequency, reduced FEV_1 decline but an increase in macrolide resistance. Erythromycin was well tolerated without any evidence of significant adverse effects. However, there was a significant rise in the proportion of erythromycin resistant oropharyngeal *Streptococci*.

The key results of the three studies are summarized in table 4.

Parameters recorded	EMBRACE trial (N= 141)	BAT trial (N=83)	BLESS trial (N=117)
Exacerbation frequency	Reduced	Reduced	Reduced
Macrolide resistance at the end of treatment	Not recorded routinely, but 4% developed resistance in azithromycin group.	88% (35% at baseline) in azithromycin group compared to 26% (28% at baseline) in placebo.	Mean change increased to 27% in the erythromycin group compared to 0.04% in placebo.
Gastrointestinal side effects	13% in placebo compared to 27% in azithromycin group.	21% in placebo compared to 54% in azithromycin group.	26% in placebo compared to 29% in erythromycin group.
QTc	Not recorded.	Not recorded	No change in either group.

Table 4. Comparison of key findings in the macrolide trials.

1.14.1.2 Limitations of long term macrolide use in bronchiectasis

It is indeed very encouraging to see the results from the above three trials using macrolides as an anti inflammatory agent in bronchiectasis in reducing exacerbations. However, the inherent limitation with the use of long-term use of antibiotics (including macrolides), in respiratory diseases is the emergence of new pathogens and increased antimicrobial resistance against the airway microbiota, which limits the enthusiasm for widespread use of antibiotics such as macrolides in bronchiectasis. About 2-10% of patients with bronchiectasis develop non-tuberculous mycobacteria (NTM) disease. The 1ˢᵗ line of treatment for NTM is macrolides and hence there is a concern of macrolide resistance in this group of patients. It seems pragmatic that macrolide maintenance therapy should be considered in patients that have been carefully selected, experience at least 3 exacerbations annually and have had mycobacterial disease excluded.

There remains a drive for novel non-antibiotic therapy in bronchiectasis

1.14.2. Long term antibiotics

Two major RCTs have been doing assessing the role of long term inhaled antibiotics in stable bronchiectasis. Murray et al assessed the role of nebulized gentamicin in stable bronchiectasis patients. Time to next exacerbation was increased with the use of inhaled gentamicin (80mg bd) compared to saline control in a 65 patient RCT with patients who had a potentially pathogenic microbe, who were treated for 1 year with a 3 month follow up (61.5 vs 120 days p=0.02) and the exacerbation number was also reduced in the active group (0 (0-1) vs 1.5 (1-2) p<0.0001) (Murray et al 2011). Quality of life measures improved, sputum was less purulent and exercise capacity increased. Emergence of resistance did not occur, however benefits were lost quickly on stopping treatment during the 3 month follow up. In this study patients were not masked to intervention. Although 7/32 patients in the gentamicin arm reported bronchoconstriction, this only led to withdrawal in 2 patients (the same as the saline arm). Further analysis of this study also documented reductions in sputum inflammatory markers (including MPO activity, neutrophil elastase, CXCL8 and TNF alpha).

Haworth et al studied 144 bronchiectasis patients with chronic pseudomonas infection treated with Promixin (colistin) or 0.45% saline who were followed up until the first

exacerbation or 6 month (Haworth et al 2014). Although the study did not reach significance in its primary endpoint of time to next exacerbation (165 days vs 111days p=0.11), this was significant in a predetermined "compliant" population (those that took the medication for >= 80% of the time (168 vs 103 days p=0.028)). This could be accurately defined because of an electronic chip on the inhaler device. In the whole group analysis they also demonstrated a significant reduction in PA CFU count at 12 weeks and a significant improvement in the SGRQ total score at 26 weeks. There was no difference in FEV_1, sputum weight or adverse events. The incidence of adverse effects leading to discontinuation was low and similar in both groups.

1.14.3. Anti-inflammatory agents in bronchiectasis- statins

I did an RCT in bronchiectasis patients, where 30 patients were randomly allocated to receive either high dose atorvastatin (80 mg) and 30 patients received a placebo, given orally once a day for 6 months, in a double blind RCT. The primary endpoint was reduction in cough from baseline to 6 months, measured by the Leicester Cough Questionnaire (LCQ) score. The change from baseline to 6 months in LCQ score differed between groups, with a mean change of 1.5 units in patients allocated atorvastatin versus –0.7 units in those assigned placebo (mean difference 2.2, 95% CI 0.5–3.9; p=0.01). 12 (40%) of 30 patients in the atorvastatin group improved by 1.3 units or more on the LCQ compared with five (17%) of 30 in the placebo group (difference 23%, 95% CI 1–45; p=0.04). Ten (33%) patients assigned atorvastatin had an adverse event versus three (10%) allocated placebo (difference 23%, 95% CI 3–43; p=0.02). No serious adverse events were recorded. I were able to conclude that 6 months of atorvastatin improved cough on a quality-of-life scale in patients with bronchiectasis. Multicenter studies are now needed to assess whether long-term statin treatment can reduce exacerbations (Mandal et al 2014).

There remains a drive towards novel non-antibiotic therapies in bronchiectasis.

1.15. HYPOTHESIS

Hypothesis 1

Peripheral blood neutrophils are reprogrammed in bronchiectasis and this alters their functional response to infection and inflammation, leading to failure of resolution of inflammation.

Hypothesis 2

There is a dysregulation of lipid mediators in bronchiectasis leading to under expression of the pro resolution and anti-inflammatory mediator Lipoxin A_4 and thereby altered balance with the pro inflammatory mediator leukotriene B_4. This imbalance of lipid mediators contributes to persistent neutrophilic airways inflammation in bronchiectasis

1.16. SPECIFIC AIMS

1. Characterize neutrophils in peripheral blood and airways in bronchiectasis in the stable state and during exacerbations.

2. Cohort study in bronchiectasis to establish if LXA_4 deficiency correlates with disease severity.

3. Characterize lipids in bronchiectasis airways and peripheral blood to establish the correlation of LXA_4 to disease severity.

4. To investigate a potential mechanism for low levels of LXA_4 in bronchiectasis, lipoxin biosynthetic genes expression will be measured.

5. Assess the anti-inflammatory and pro resolution effect of LXA_4 on neutrophils and monocyte-derived macrophages from healthy volunteers.

6. Assess the anti-inflammatory and pro resolution effect of LXA_4 on neutrophils during exacerbations in bronchiectasis and community acquired pneumonia.

CHAPTER 2

METHODS

2.1. STUDY METHODS

2.1.1. NEUTROPHIL ISOLATION

Ethical approval was obtained from the Lothian Research Ethics Committee (Approval #08/S1103/38).

Reagents

- Sodium citrate 3.8 % (3.8 g Sodium citrate tribasic dihydrate (SIGMA 25116) in 100 ml bottled water (BAXTER UKF7114)) (solution is sterile filtered using a 0.22 μm filter unit)

- 6% Dextran [6.0g Dextran 500 (GE Healthcare 17-0320-02*) in 100ml 0.9% NaCl (BAXTER UKF7124)]. Dissolve in 0.9% NaCl Saline warmed to 37°C (solution is sterile filtered using a 0.22 μm filter unit)

- Percoll (GE healthcare 17-0891-02)

- PBS (phosphate buffered saline) – without Ca^{2+}/Mg^{2+} (PAA H15-002)

- PBS (x10) - without Ca^{2+}/Mg^{2+} (Sigma D1408)

- Iscove's modified Dulbecco's modified Eagle's medium (IMDM) (PAA E15-018)

Method

All procedures carried out at room temperature unless otherwise stated.

Freshly drawn blood was collected from healthy volunteers, into sodium citrate; (4ml of citrate per 40 ml of blood in a 50 ml falcon tube) and mixed by gentle inversion of tube, parafilm cap before centrifugation. This was centrifuged at 350g for 20 minutes (Acc1/ Brake 0 Hettich centrifuge, Acc0/Brake 0 Mistral centrifuge).

Platelet-rich plasma (PRP) was aspirated without disturbing the pelleted cells. Autologous recalcified plasma (serum) was prepared by adding 220 μl of 1M CaCl /10 ml plasma in glass tubes at 37°C for 1 hour. "Serum" was separated from platelet plug and transferred to a Falcon tube for later use/storage (4°C for short term or frozen for longer term storage). Leukocytes were separated from erythrocytes by dextran sedimentation: 6 ml of dextran (see above) was added to each tube (adjust

volume of dextran added if necessary – 2.5ml / 10ml cell pellet) and then made to 50ml with saline pre-warmed to 37°C. This was then mixed carefully to ensure cells were fully resuspended then allowed to sediment for between 20 and 30 minutes (not more than 30 minutes) at room temperature.

Percoll gradients were prepared at this point-at room temp. Stock Percoll solution was made isotonic with 10x PBS (WITHOUT Ca^{2+}/Mg^{2+})

81% Percoll made in PBS WITHOUT Ca^{2+}/Mg^{2+} (8.1ml Percoll "stock" +1.9 ml PBS). 70% Percoll made in PBS WITHOUT Ca^{2+}/Mg^{2+} (7ml Percoll "stock" + 3 ml PBS). 55% Percoll made in PBS WITHOUT Ca^{2+}/Mg^{2+} (5.5ml Percoll "stock" + 4.5ml PBS).

Following dextran sedimentation, each leukocyte-rich upper layer was removed and transferred to a fresh 50 ml tube and topped up to 50ml with saline and then centrifuged at 350g for 6 min (Acc5/ Brake 5 for both Hettich and Mistral centrifuges).

The pellets from 2 tubes of cells were resuspended in 3ml of the 55% Percoll (upper layer). 3ml of 81% Percoll (bottom layer) was carefully placed in the bottom of a 15ml Falcon tube. 3ml of 70% Percoll (middle layer) was carefully over layered onto the bottom layer (slowly to avoid mixing of the gradients). 3ml of cells re suspended in the 55% Percoll layer was carefully over-layered onto the middle layer (figure 18).

Gradients were centrifuged at 720g for 20 min, (Acc1/ Brake 0 Hettich centrifuge, Acc0/Brake 0 Mistral centrifuge). Granulocytes were harvested (70/81 interface) and residual erythrocytes pelleted at the bottom of the tube.

Leukocytes were then washed twice in PBS without Ca^{2+}/Mg^{2+} centrifuged at 230g for 6 min, (Acc5/ Brake5 Hettich and Mistral centrifuges). Cells were resuspended at desired concentrations in media and counted using a haemocytometer. 10µl of the granulocyte suspension was placed on the haemocytometer slide and visualised under a light microscope, the number of neutrophils present in 25 squares were counted. The number obtained was equal to the number of neutrophils per 0.1µl of suspension, to calculate the number of neutrophils in 1ml, which was multiplied by 10,000 (figure 19).
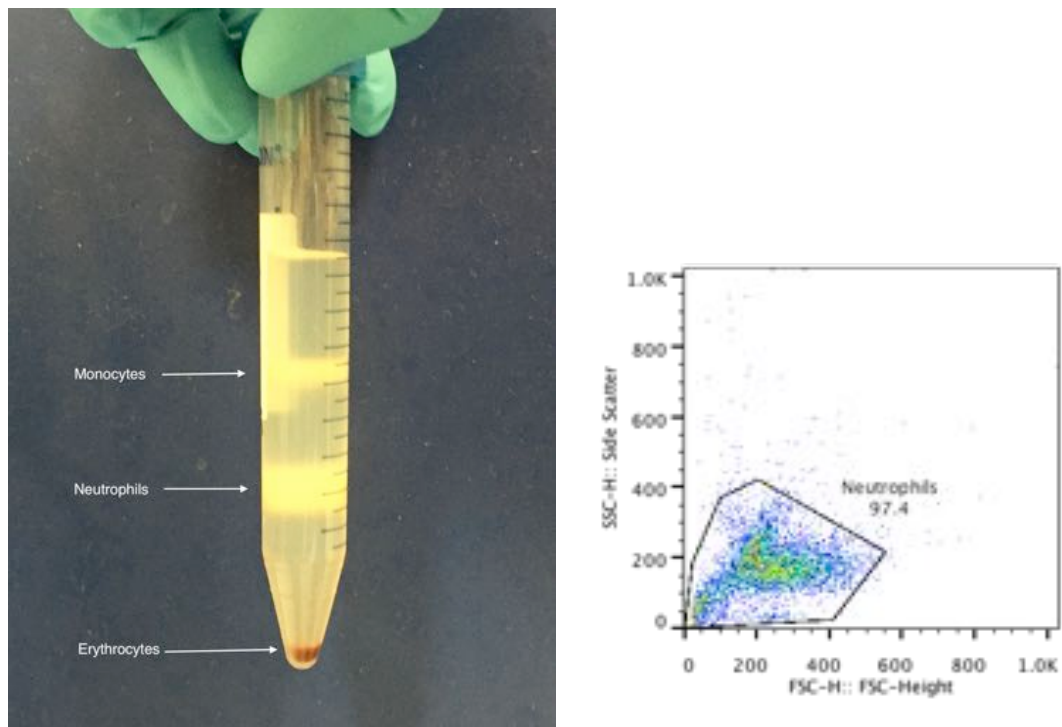
Figure 18. Neutrophil isolation by Percoll gradient (left). Isolated neutrophils on flow plot (right).
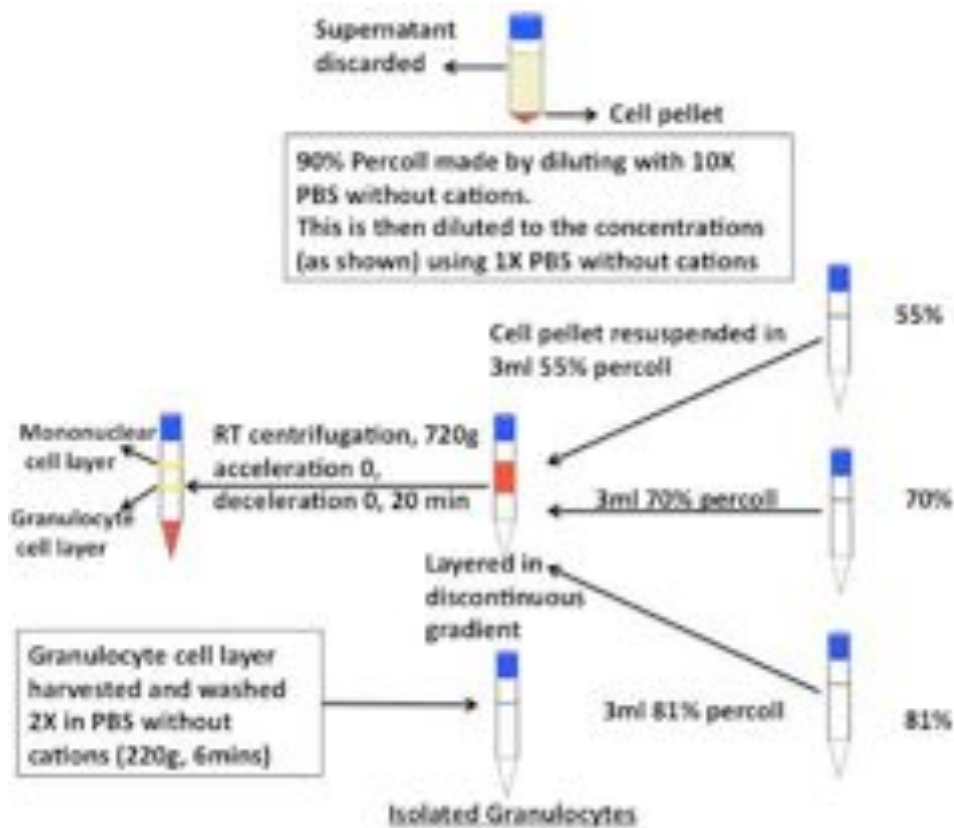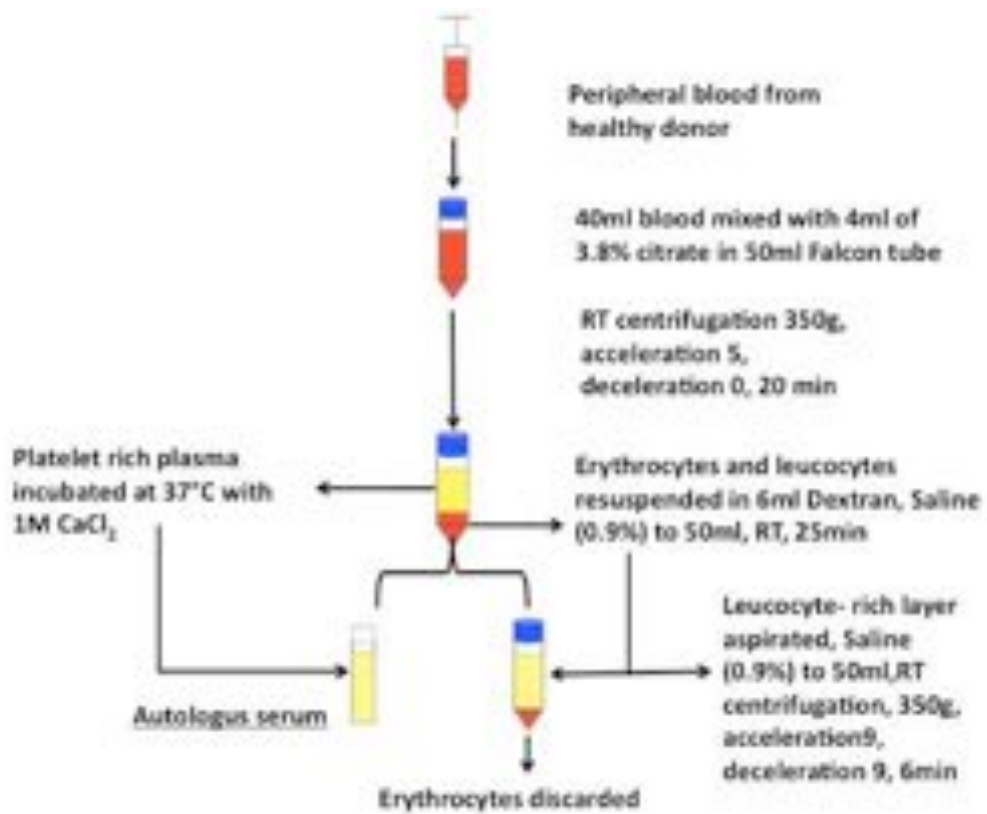
Figure 19. Isolation of neutrophils by Percoll gradient.

2.1.2. APOPTOSIS ASSAY

Reagents

- Lipoxin A_4 (5 (S), 6 (R)- Lipoxin A_4; Cayman Chemicals)
- Roscovitine (Calbiochem)

[Roscovitine is a cyclin dependant kinase inhibitor and has been shown to induce caspase dependent neutrophil apoptosis]

- Annexin V (Roche)
- Propidium iodide (Sigma Aldrich)
- Iscove's Modified Dulbecco's Medium (IMDM; Gibco)
- HBSS (Hanks Balanced Salt Solution; + $CaCl_2$, +$MgCl_2$; Gibco)

Method

Freshly isolated peripheral blood neutrophils (at least 97% purity – performed cytocentrifuge preparation as described above) were suspended at 10×10^6 cells/ml in IMDM supplemented with 10% autologous serum and penicillin/streptomycin (1x). Then 75 μl of neutrophil suspension was added to wells of a 96 well flat-bottom plate. A row of wells was left clear from the periphery of the plate- to allow for addition of reaction media to minimise evaporation. To each well 15μl of treatment (10 times concentration) or 20μM of roscovitine or buffer control was added. Following this, 60μl IMDM with 10% serum was added to each well. If two agents were used in combination only 45μl of IMDM was required. Each treatment was done in duplicate or triplicate. Plates were then covered with a lid, and incubated at $37^{o}C$ in a 5% CO_2 incubator for the desired length of time (8 or 20 hours).

Dose and time response experiments were done. Following incubation for the desired length of time, the plates were taken out of the incubator. Each well was then vigorously pipetted to dislodge adherent cells and transfer 20μl of cells into a flow tube containing 200μl of Annexin V buffer [Annexin buffer= annexin V+ HBBS with Ca and Mg in the ratio of 1:500]. The flow tubes were then incubated for 5 min on ice. Immediately prior to running each sample on a flow cytometer propidium iodide (PI; 1μl of 1mg/ml stock solution) was added. Samples were analysed by flow

cytometry (BD FACSCalibur™) using FL-1/FL-2 channel analysis following appropriate compensation. Cytospins were done for each of the different treatments.

Dextran sedimentation and discontinuous Percoll gradient, as a means to isolate neutrophils from peripheral venous blood, result in cell purity of ≥95%, with between 1% and 5% eosinophils and 1–2% contaminating mononuclear cells. In individuals with atopic conditions, such as hay fever and asthma, eosinophils can represent >5–10% of the granulocyte population.

Treatment conditions and interpretation

Once isolated by Percoll Gradient, human peripheral neutrophils were exposed to the following conditions: Lipoxin A_4 (Sigma Aldrich) at final concentrations of 1nM, 10nM and 100nM, as LXA_4 concentrations <1nM had no effect and LXA_4 concentrations greater than 100nM is not physiological.

For the rest of the thesis, these are the concentrations of lipoxin that will be used.

Flow	Inference
Annexin V negative and PI negative	Live cells
Annexin V positive and PI negative	Apoptotic cells
Annexin V and PI positive	Necrotic cells

Table 5. Interpretation of Annexin V and propidium iodide staining

Apoptosis was assessed by blinded morphological assessment of May–Gründwald–Giemsa stained cytospins, confirmed by quantification of annexin-V–fluorescein isothiocyanate binding and propidium iodide staining.

Cell counts

At 8 (early apoptosis is assessed at 8 hours) and 20hours (late apoptosis is assessed at 20h), cytocentrifuge preparations from treated and control neutrophil suspensions were fixed and stained. Cells were examined under oil immersion light microscopy (x1000 magnification) and apoptotic cells were defined as cells containing darkly

stained pyknotic nuclei.[34] For each time point at least 400 cells were counted from at least 4 different fields.

Crossing serum

- *Bronchiectasis patients*

In addition, apoptosis assays were done using neutrophils from bronchiectasis patients and instead of using autologous serum, serum from a healthy volunteer was used. Apoptosis assays with autologous serum were also done at the same time- as a control.

- *Healthy volunteers*

In addition, apoptosis assays were done using neutrophils from healthy volunteers and instead of using autologous serum; serum from a bronchiectasis patient was used. Apoptosis assays with autologous serum were also done at the same time- as a control.

2.1.3. REACTIVE OXYGEN SPECIES

Reagents

- Lipoxin A_4 (5 (S), 6 (R)- Lipoxin A_4; Cayman Chemicals)
- DHR (Dihydrorhodamine: Sigma Aldrich)

[DHR is an uncharged and non-fluorescent reactive oxygen species (ROS) indicator that can passively diffuse across membranes where it is oxidized to cationic rhodamine, which localizes in the mitochondria and exhibits green fluorescence]

- fMLF (Formyl-Methionyl-Leucyl-Phenylalanine; Sigma Aldrich)
- PMA (Phorbol 12-myristate 13-acetate; Sigma Aldrich)

[PMA activates the signal transduction enzyme protein kinase C]

- DPI (Diphenyleneiodonium chloride; Sigma Aldrich)

[DPI inhibits NADPH oxidase]

- HBSS (Hanks Balanced Salt Solution; + $CaCl_2$, +$MgCl_2$; Gibco)

Method

Freshly isolated peripheral blood neutrophils (at least 97% purity – performed cytocentrifuge preparation as described above) were suspended at $2x10^6$ cells/ml in HBSS with Ca^{2+}/Mg^{2+}. Neutrophils were then loaded with dihydrorhodamine at a final concentration of 10μM for 5minutes. 240μl of neutrophils loaded with DHR were added to a 2ml eppendorf and incubated with 30μl of varying concentrations of Lipoxin A_4 (final concentration: 1nM, 10nM and 100nM) for 30 minutes at 37°C. After 30 minutes, the eppendorfs were put on a shaking heat block (300rpm) for 30minutes. Eppendorfs were then taken off the heat block and 30μl of fMLF (final concentration of 100nM- as this was the dose at which fMLF had maximum response- as demonstrated in the results section) or PMA (final concentration of 10nM- as this was the dose at which PMA had maximum response- as demonstrated in the results section) was added to the eppendorfs and incubated for 15 minutes at 37°C.

Eppendorfs were then taken off and immediately put on ice to stop the reaction. The suspension from each eppendorf was transferred to a flow tube and run on flow cytometry.

DPI (at a final concentration of 1μM- as this is the dose that has been widely used in the literature) was used to as a positive control.

2.1.4. NEUTROPHIL GFP PAO1 PHAGOCYTOSIS ASSAY

Materials

- Lipoxin A_4 (5 (S), 6 (R)- Lipoxin A_4; Cayman Chemicals)
- Fluorescently labeled *Pseudomonas* - GFP PAO1
- Autologous serum
- HBSS (Hanks Balanced Salt Solution; + $CaCl_2$, +$MgCl_2$; Gibco)
- Lysogeny (LB) broth

Method

- Preparation of bacteria

Frozen GFP PAO1 colonies were resuspended in lysogeny broth and put on an incubator (Gallankamp, Panasonic Biomedical Europe) at 200rpm at 37°C overnight, with gentamicin (Sigma Aldrich UK, at a concentration of 50mcg/ml) added to the media to ensure that the bacteria were gentamicin resistant bacteria. Bacteria were then sub cultured the following day and were collected when they were in their log phase (after 1-2hrs of re suspension). The optical density of the bacterial suspension was measured. Bacteria were then opsonized with 20% autologous serum and incubated at 37°C for 1 hour. Cells were then centrifuged at 300rpm (Mistral 3000i, UK) for 5minutes and re suspended in HBSS (with Ca/Mg), to give a 10-fold higher concentration than the phagocytosis assay. Bacteria were re suspended at a final concentration of 10^8 bacteria/ml.

-Preparation of neutrophils

Freshly isolated peripheral blood neutrophils (at least 97% purity – performed cytocentrifuge preparation as described previously) were suspended at 10×10^6 cells/ml in HBSS with Ca^{2+}/Mg^{2+}. 270μl of the cell suspension were put in 2ml eppendorf and incubated with 30μl of varying concentrations of Lipoxin A_4 (final concentration: 1nM, 10nM and 100nM) for 30 minutes at 37°C.

Phagocytosis assay

100μl of bacterial suspension was added to a fresh 2ml eppendorf. 100μl of the neutrophil suspension was then added to the eppendorf and the mixture was gently pipetted up and down to allow the suspensions to mix. They were then incubated for 15 minutes -as this was the maximum time required for phagocytosis to take place (leaving the neutrophils to co incubate with the opsonized bacteria for longer than 15 minutes led to >96% phagocytosis irrespective of the conditions), at 37°C. Spinning the eppendorfs at 300g for 5minutes stopped the reaction. The supernatant was aspirated and the contents of the eppendorfs were transferred to a flow tube, put on ice and then run on flow cytometry (figure 20a,b&c).
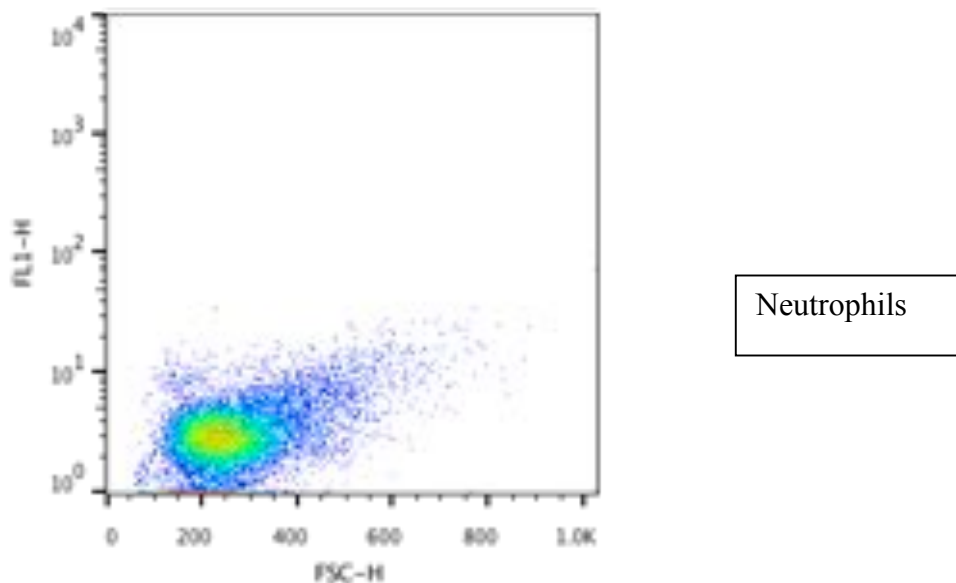
Figure 20a. Flow plot of isolated peripheral blood neutrophils.
X-axis (forward scatter)- represents neutrophils and Y-axis (side scatter) represents Mean Fluorescent Intensity (MFI).
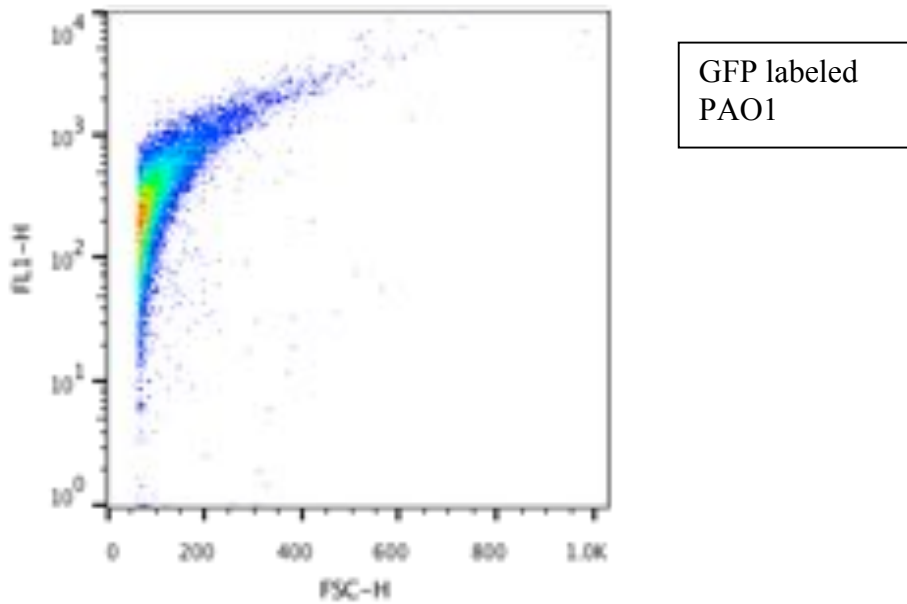
Figure 20b. Flow plot of fluorescent labeled Pseudomonas (GFP PAO1).

X- axis represents forward scatter and Y-axis (side scatter) represents Mean Fluorescent Intensity (MFI) of GFP PAO1.
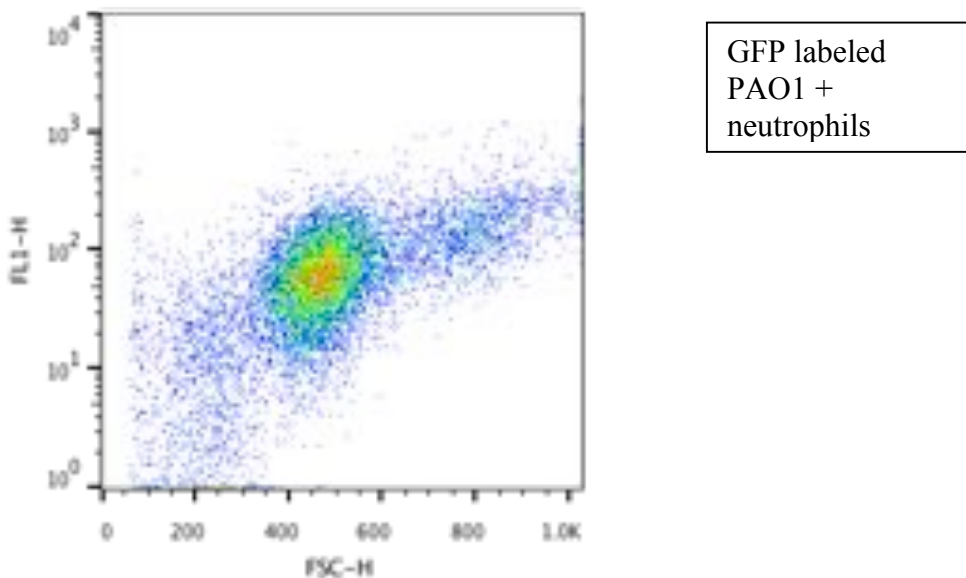
Figure 20c. Flow plot of GFP labeled PAO1 phagocytosed by neutrophils.

X-axis (forward scatter)- represents GFP PAOI phagocytosed by neutrophils and Y-axis (side scatter) represents Mean Fluorescent Intensity (MFI).

2.1.5. NEUTROPHIL GFP PAO1 KILLING ASSAY

Materials

- Lipoxin A_4 (5 (S), 6 (R)- Lipoxin A_4; Cayman Chemicals)
- Fluorescently labeled *Pseudomonas* - GFP PAO1
- Autologous serum
- HBSS (Hanks Balanced Salt Solution; + $CaCl_2$, +$MgCl_2$; Gibco)
- Lysogeny (LB) broth
- Saponin (Sigma Aldrich, UK)

Method

GFP *Pseudomonas* was opsonised with autologous serum and co incubated with neutrophils for 15 minutes [(serum), as >95% phagocytosis obtained after 15 minutes] or 1 hour [(airways neutrophils)- minimum time required for phagocytosis by airway neutrophils] as previously described. Next, spinning the Eppendorfs at 300g for 5minutes stopped the reaction. The supernatant was discarded, the pelleted cells were lysed with saponin and then serially diluted using sterile 0.85% saline to achieve dilutional factors of 10^{-1} to 10^{-4}. *Pseudomonas* isolation agar (Difco) plates were inoculated with 100 µl of dilution. These were incubated at 37°C for 24 hrs. (figure 21). Colonies of the pathogens were then counted to determine the sputum bacterial density, expressed as $\log_{10}$ colony forming units/ml (cfu.ml^{-1}).
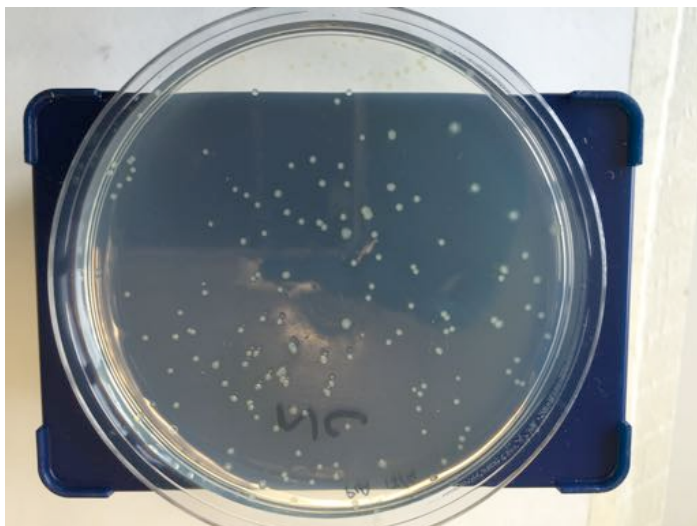
Figure 21. *Pseudomonas* colonies growing after 24 hours.

2.1.6. EFFEROCYTOSIS

Materials

- Lipoxin A_4 (5 (S), 6 (R)- Lipoxin A_4; Cayman Chemicals)
- Cell Tracker green (Invitrogen)- final concentration $20\mu M$
- Anti CD44 antibody (Sigma Aldrich)

[CD44 as a key regulator of macrophage capacity for phagocytosis of apoptotic cells][35]

- Freshly isolated neutrophils
- Monocyte derived macrophages (MDM)
- Iscove's Modified Dulbecco's Medium (IMDM)

Method

Freshly isolated peripheral blood monocytes (at least 97% purity – performed cytocentrifuge preparation as described above) were grown to monocyte derived macrophages in a 24 well plate, over 5-8 days, in IMDM with 20% autologous serum and 1% Penicillin/ Streptomycin. Cells were washed once during this period. Freshly isolated peripheral blood neutrophils were re suspended at $10X10^6$/ml in IMDM. Neutrophils were then stained with cell tracer green (1 in 1000) and left in the incubator at 37°C for 30minutes. The dye was then washed off and neutrophils were re suspended in IMDM. The neutrophils were left in the incubator for 20hours at 37°C, 5% CO_2 to undergo apoptosis. After 20 hours, the neutrophils and MDMs were washed. Neutrophils were re-suspended in IMDM at 4 million/ml. MDMs were treated with of varying concentrations of Lipoxin A_4 (final concentration: 1nM, 10nM, 100nM and 500nM) for 30 minutes at 37°C. Following incubation with Lipoxin A_4, neutrophils were added at a ratio of 3:1 to the MDMs and left in the incubator for 40min – 1hour. Neutrophils that were not phagocytosed were washed off. Trypsin was added to each well and put back in the incubator for 5-10minutes. The plate was immediately put on ice to stop the reaction. The cells were pipetted off the plate and transferred to flow tubes and run on flow cytometry.

2.1.7. RNA extraction

Reagents

- RNeasy® micro kit (Qiagen)
    - Spin column
    - RLT buffer
    - RW1 buffer
    - RDD buffer
    - DNase I stock solution

- β-Mercaptoethanol (Sigma Aldrich)

Homogenizing lung brushings

Lung brushings in 1ml of RNA later were taken from -80 freezer on dry ice. 350µl of RLT plus buffer containing b-mercaptoethanol was added to each of the suspensions. These were then transferred to Precellys® homogenizing kit CK14 (with 1.4 mm ceramic beads) 2 ml tubes and put on Precellys24 for homogenization at a speed of 5000rpm for 30seconds (figure 22). The samples were then returned to ice.

Figure 22. Percellys 24- lung tissue homogenizing machine.

RNA extraction method

Samples were spun at full speed for 3 minutes. 1 volume of 70% ethanol was added to each sample and mixed well by pipetting. 700μl of this suspension was transferred to an RNeasy MinElute spin column and centrifuged for 30s at 10000rpm. Any flow through was discarded. Any remaining sample was added to the spin column and the process was repeated. 350μl of buffer RW1 was added to the spin column. The lid was closed and was centrifuged for 15s at 10000rpm. Any flow through was discarded. Next 10μl of DNase I stock solution was added to 70μl Buffer RDD. Inverting the tube mixed this. The DNase I incubation mix (80μl) was added directly to the RNase MinElute spin column membrane. This was then placed on the bench top for 15minutes. 350μl of buffer RW1 was added to the RNase MinElute spin column. The lid was closed and centrifuged at 10000rpm for 15s. Flow through was discarded. 500μl of 80% ethanol was added to the RNase MinElute spin column. The lid was closed and centrifuged at 10000rpm for 2 minutes. Flow through was discarded. The RNase MinElute spin column was placed in a new 2ml collection tube. The lid of the spin column was left open and centrifuged at full speed for 5minutes to dry the membrane. The flow through and the collection tube were discarded. The RNase MinElute spin column was placed in a new 1.5ml collection tube. 14μl of ultra pure water was added directly to the center of the spin column membrane. The lid was closed gently and centrifuged at full speed for 1minute to elute the RNA.

Nanospec and DNAse treatment

1. Nanospec the samples

   o RNA   3μg
   o 10X buffer   3μL
   o RQ1 DNase   3μL
   o dH2O to make up to   30μL

2. Then Incubate at 37°C for 30 minutes.
3. Add 3μL stop solution and incubate 65°C for 15 minutes.
4. Store clean RNA long term at -80°C.

cDNA generation- RT reaction (for 1X)

10X Reaction buffer	4μL
25mM MgCl2	8.8μL
dNTP	8μL
Random Hexamers	2μL
RNase Inhibitor	0.8μL
Mustiscribe RT enzyme	1μL
'clean' RNA	15.4μL

Total	40μL

1. Use program "SLFRT" on PTC-100 thermal cycler.
2. Conditions:
   - 25°C for 10 mins
   - 48°C for 40 mins
   - 95°C for 5 mins
   - 4°C hold
3. Store cDNA at -20°C

PCR

1. Defrost samples on ice
2. Make up mix for each gene:
   - Master mix (2X) — 12.5μL
   - 18s (20X) — 1.25μL
   - Primer probe for each gene (20X) — 1.25μL
   - cDNA — 2μL
   - dH2O — 8μL

   Total — 25μL

3. Put 25μL sample into each well of a 48 well PCR plate (make sure it is a Fast plate)
4. Run samples in triplicates.
5. Run samples on 48 well PCR machines.

2.1.8. NETs ASSAY

Reagents

- Lipoxin A_4 (5 (S), 6 (R)- Lipoxin A_4; Cayman Chemicals)
- PMA (Phorbol 12-myristate 13-acetate; Sigma Aldrich;

[PMA activates the signal transduction enzyme protein kinase C]

- DPI (Diphenyleneiodonium chloride; Sigma Aldrich)

[DPI inhibits NADPH oxidase]

- HBSS (Hanks Balanced Salt Solution; + $CaCl_2$, +$MgCl_2$; Gibco) +Hepes 20mM
- Sytox® green nucleic acid stain (Thermo fisher 5mM solution in DMSO)
- 24 or 96 well plate (Fostair ®)

Methods

-Fluorescent microscope method

Freshly isolated neutrophils were used in the assay (neutrophils isolated by Percoll gradient as described previously). Neutrophils were resuspended in HBSS+ hepes buffer at a concentration of 125,000 cells/ml. 400μL of this suspension (i.e 50,000cells) were seeded onto a 24 well plate and put in the incubator at 37°C for 30minutes. This is to help the neutrophils to adhere to the bottom of the plate. After 30minutes, the plate is carefully taken out of the incubator so as not to disturb the cells and then treated with 50μL of 1nm, 10nm and 100nm of LXA_4 or 1μM (standard dose used in the literature) of DPI (final concentration). The other wells get 50μL of the vehicle. Then the plate is put back in the incubator at 37°C for 30minutes. Following this, the plate is gently taken out as before and treated with 50μL of 100nm PMA (final concentration). The plate is put back in the incubator for 4hours. After 4 hours, 100μL of 1μM sytox green is added and put back in the incubator for a further 20-30minutes. Following this, the plate is viewed in an EVOS microscope. On the EVOS, representative images of the different treatments are taken in the phase, fluorescent and overlay filters. The images are stored and then counted for NETs at a later date. NETs are visualized as 'diffuse' or 'spread' fluorescent shaped images (figure 23).

-Plate reader method

Serum and airways neutrophils from the above cohort of patients were incubated for 4h in a 96 well plate and then SYTOX green a cell-impermeable nucleic acid stain, with an excitation/emission maxima of 504/523 nm to give a green fluorescent light, was added and NET formation was observed by measuring mean fluorescence in 96 well plates.

b.   Diffuse nets;   Dead cells
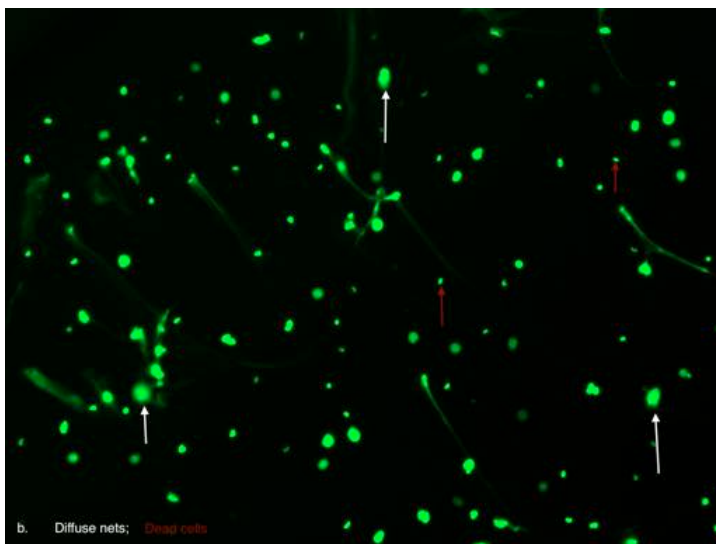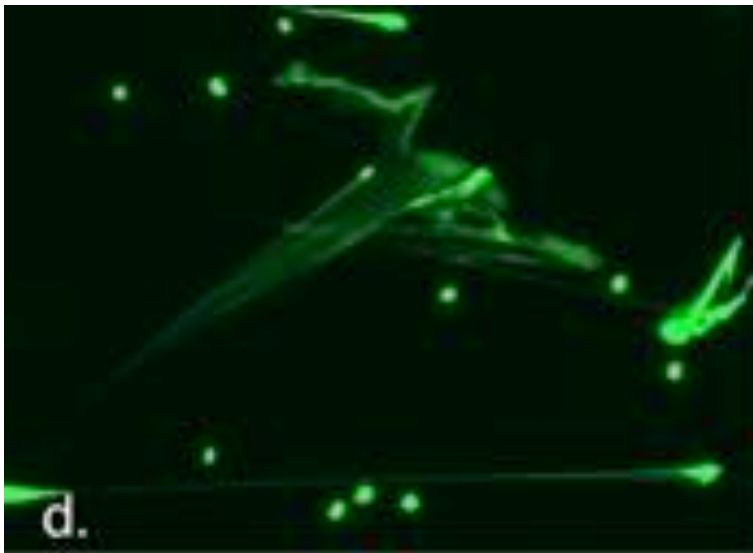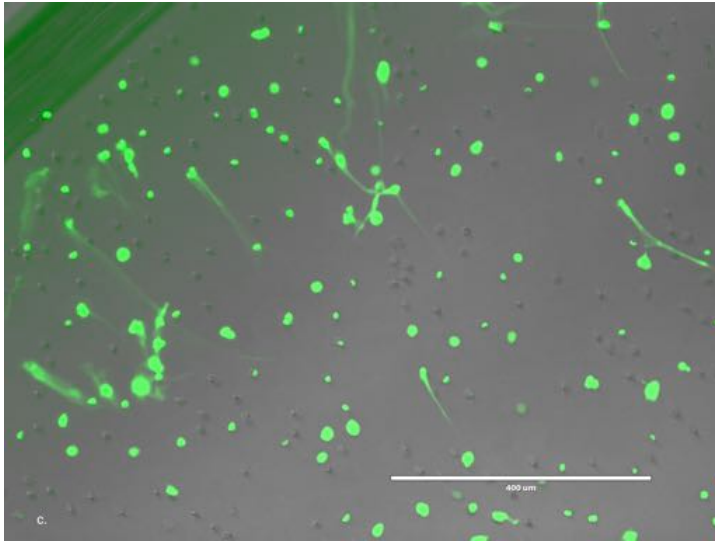
Figure 23a,b&c. X20 magnification images of NETs in EVOS. a. phase image; b. fluorescent labeled neutrophils and c. overlay images of a&b. 6d. X60 magnification showing a typical 'comet' shaped NET. All the above are taken on an inverted EVOS microscope.

2.1.9. CD62L CD11b

Materials

- Lipoxin A_4 (5 (S), 6 (R)- Lipoxin A_4; Cayman Chemicals)
- fMLF (Formyl-Methionyl-Leucyl-Phenylalanine; Sigma Aldrich)
- CD62L antibody (BD Pharmigen ™ PE mouse anti human antibody)
- CD11b antibody (Alexa Fluor$^®$ 488 anti human antibody)
- PBS (Phosphate buffered saline)+ $CaCl_2$, +$MgCl_2$; Gibco
- PBS without $CaCl_2$, or $MgCl_2$; Gibco

Method

Neutrophils were isolated using Percoll gradients as described previously. Freshly isolated neutrophils were re suspended in PBS + $CaCl_2$, +$MgCl_2$, at a concentration of $10X10^6$/ml. 75μL of this suspension was placed in a 2ml eppendorf and treated with 15μL of 1nm, 10nm and 100nm of LXA_4 (final concentration) or vehicle control. This was put in an incubator at 37°C for 30minutes. After 30minutes, the eppendorfs were taken out of the incubator and put on a shaking heat block at 37°C for 30mins at 300rpm. Next, 15μL of fMLF or vehicle control was added and 45μL PBS (with Ca^{2+} and Mg^{2+}) was added on top and further incubated at 37°C for 30 minutes. After 30minutes, 75μL of cells were transferred to a FACS tube containing 75μL PBS (without Ca^{2+} and Mg^{2+}) containing 2μL of CD11b and 2μL CD62L antibodies. These tubes were then placed on ice and kept in the dark for 30minutes. After 30minutes, 1ml of PBS was added to each flow tube and centrifuged at 350g for 5minutes. Supernatants were discarded and the cell pellets were re suspended in 300μL of PBS (without Ca^{2+} and Mg^{2+}) and the samples were analyzed by flow cytometry (BD FACSCANTO™ II).

2.1.10. NEUTROPHIL ACTIVATION ASSAY (MPO MEASUREMENT)

Materials

- Lipoxin A$_4$ (5 (S), 6 (R)- Lipoxin A$_4$; Cayman Chemicals)
- fMLF (Formyl-Methionyl-Leucyl-Phenylalanine,; Sigma Aldrich)
- Cytochalasin B (Sigma Aldrich)
- PBS without CaCl$_2$, or MgCl$_2$; Gibco

Method

Neutrophils were isolated by Percolls gradients as described previously. Once isolated neutrophils were re suspended in PBS without CaCl$_2$, or MgCl$_2$. 75μL of neutrophils were put in a 2 ml eppendorf and treated with 15μL of LXA$_4$ (final concentrations of 1nM, 10nM and 100nM) or vehicle control. 30μL of PBS was added to all conditions and then the eppendorfs were incubated at 37°C for 30 minutes. Following this, 15μL of cytochalasin B (at a final concentration of 1μg/ml- standard concentration) or vehicle control was added to the eppendorfs. The eppendorfs were put back in the incubator at 37°C for 10 minutes. After 10 minutes, 15μL of fMLF (at a final concentration of 10nM- as detected by dose response assay) or vehicle control was added. The eppendorfs were put back in the incubator at 37°C for 30 minutes. The reaction was stopped after 30minutes by centrifuging the samples at 8000g for 5 minutes. The supernatants were collected and stored at -80°C for doing myeloperoxidase assays at a later date.

2.1.11. ALX/FPR2 RECEPTOR EXPRESSION

Materials

- Lipoxin A_4 (5 (S), 6 (R)- Lipoxin A_4; Cayman Chemicals)
- APC conjugated anti FPRL1/ FPR 2 receptor antibody (R&D systems)
- WRW 4 (Torcis®)
- Purified human IgG control antibody (R&D systems)
- Anti IgG antibody (Abcam ab 83786)
- PBS without $CaCl_2$, or $MgCl_2$; Gibco

Methods

Neutrophils were isolated by Percoll gradients as previously described. Neutrophils were re suspended in PBS (without Ca and Mg) with 5% serum at a concentration of 10×10^6/ ml. 180μL of neutrophils were put in 2ml eppendorf. 20μL of IgG was added at a concentration of 1:1000 and put on ice for 15 minutes to prevent non-specific binding. After 15 minutes, the neutrophils were incubated with 30 μL of WRW 4 (at a final concentration of 100nM) or vehicle control, for 30 minutes at 37°C. Following this incubation, samples were treated with 30 μL of Lipoxin A_4 at a final concentration 100nM or vehicle control, for 30 minutes at 37°C. After 30 minutes, fMLF at a final concentration of 100nM was added for 15 minutes at 37°C. After 15 minutes, the APC conjugated anti ALX/FPR2 antibody was added at a final concentration of 1mg/ml (2μL) or IgG (2μL) (as a control antibody). 10 μL of PBS was added to all eppendorfs to bring the final volume of the suspensions to 300 μL. This was then incubated on ice for 1 hour. Samples were then washed and ALX/FPR2 expression was assessed by flow cytometry (BD FACSCalibur™).

2.1.12. LIPID MEDIATOR ANALYSIS METHODS

2.1.12.1. Sample Preparation

BALF samples were received as 50% BALF, 50% methanol. Samples were defrosted on ice and the entire content of a tube was mixed with 500 µL cold methanol containing 1 ng of internal standard mix. Samples were centrifuged at 4°C for 10 minutes at 4000 rpm. The supernatant was diluted with 10 mL HPLC grade water so that the methanol content was below 10%. Samples were acidified with 1N HCl to pH ~3.5.

Serum samples were defrosted on ice and mixed with 2 mL methanol containing 1 ng of internal standard mix. Samples were then stored at -20°C for 1h for protein precipitation and again defrosted on ice. Samples were centrifuged at 4000 rpm for 10 min at 4°C and the supernatant was diluted with 20 mL HPLC grade water. Diluted samples were acidified to pH ~ 3.5 with 1N HCl.

2.1.12.2. Solid Phase Extraction (SPE) Chromatography

ISOLUTE C18 500 mg/6 mL cartridges were conditioned with 3x 6 mL methanol and 2x 6 mL HPLC grade water. Samples were applied and allowed to run through by force of gravity. Afterwards cartridges were first washed with half the sample volume of HPLC grade water and then with 2x 6 mL hexane. Compounds were eluted with 6 mL ethylacetate and dried under vacuum. Samples were re-dissolved in 100 µL methanol/water 50/50 and stored at -80°C until analysis.

2.1.12.3. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

Samples were analysed on a Thermo Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer system coupled to a Thermo Scientific Accela 1250 pump and autosampler. Separation was achieved on a Phenomenex Kinetex XB-C18 LC column of 100 x 2.1 mm with 1.7 µm particle size, maintained at 32°C. The injection volume was 10 µL. A solvent gradient was run with solvent A consisting of 90% water, 10% methanol and 0.1% acetic acid and solvent B consisting of 100% methanol and 0.1% acetic acid. The gradient was the following: linear increase from 45 to 60% solvent B between 0 and 10 minutes, linear increase to 70% solvent B between 10 and 11 minutes, linear increase to 100% solvent B between 11 and 18 minutes, holding at

100% solvent B between 18 and 20 minutes, decrease back to 45% solvent B between 20 and 21 minutes and column equilibration at 45 % solvent B between 21 and 25 minutes.
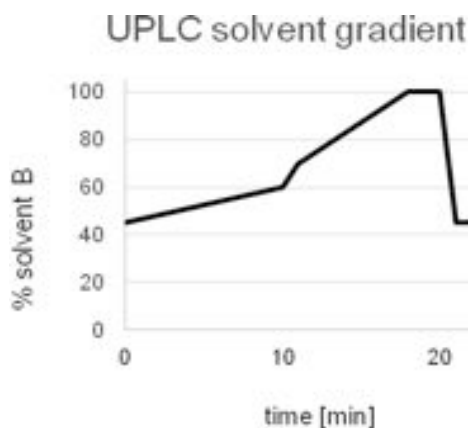
Figure: 24. Graph of a representative sample.

The mass spectrometer was coupled to an electrospray ionisation (ESI) source operated in negative mode and compounds were detected by multiple reaction monitoring (MRM). 56 compounds were monitored, including six deuterium labelled internal standards (see table 6 below, internal standards shown in italics). Compounds were quantified through comparison to original standards. The LC-MS/MS system was controlled and data analysis was performed with Thermo Xcalibur 2.2 software. Results were normalised to the sample volume and recovery of the associated internal standard.

Prof Phil Whitfield and his team (University of Islands and Highlands) did Lipidomics on samples provided by me.

Compound	Precursor ion	Product ion	Compound	Precursor ion	Product ion
6 keto PGF1a	369.2	163.2	9 HETE	319.2	151.3
20 OH LTB$_4$	351.2	195.2	11,12 dHET	337.2	167.2
20 COOH LTB$_4$	365.2	195.1	*14,15 dHET d11*	*348.3*	*207.2*
11 dehydro TxB$_2$	367.2	161.2	15 HEPE	317.2	219.2
8IsoPGF2a	353.2	193.2	8,9 dHET	337.2	127.2
TxB2	369.2	169.1	12 HEPE	317.2	179.2
iPF2a VI	353.2	115.2	5 HEPE	317.2	115.2
11beta PGF2a	353.2	193.1	5,6 dHET	337.2	145.1
PGE2	351.2	271.2	13 HODE	295.3	195.2
dihydro PGE2	351.2	235.0	20 HETE	319.2	245.3
PGE2 d4	*355.2*	*193.2*	7 HDHA	343.2	141.2
PGD2	351.2	233.1	9 HODE	295.3	171.2
dihydro PGD2	351.2	175.2	15 OxoETE	317.2	113.3
PGF2a	353.3	193.2	15 HETE	319.2	219.2
RvD2	375.2	175.1	8 HETE	319.2	155.2
LXA$_4$	351.2	115.3	11 HETE	319.2	167.2
RvD1	375.2	215.2	12 HETE	319.3	179.2
LTD$_4$	495.3	177.0	*15 HETE d8*	*327.3*	*226.1*
5,6 EET	319.2	191.3	17 HDHA	343.3	281.3
Maresin 1	359.2	177.2	14 HDHA	343.3	205.2
LTB$_4$	335.2	195.2	9,10 EpOME	295.2	171.2
LTB$_4$ d4	*339.2*	*197.2*	5 OxoETE	317.2	203.3
10,17 diHDHA	359.2	153.2	5 HETE	319.3	115.2
LTE4	438.2	333.3	*14,15 EET d11*	*330.3*	*175.2*
LTE4 d5	*443.3*	*338.2*	11,12 EET	319.2	167.2
15deoxy D 12,14 PGJ2	315.2	271.2	8,9 EET	319.3	155.2
14,15 dHET	337.2	207.2	14,15 EET	319.3	175.2
9,10 diHOME	313.2	201.2			

Table 6: Compounds monitored by liquid chromatography mass spectrometry.

2.2. COHORT STUDY

169 patients who met inclusion and exclusion criteria were recruited from the Edinburgh Bronchiectasis clinic in the Royal Infirmary of Edinburgh.

Assessments were done at baseline and after one year as shown in table 7. Serum and sputum were collected and stored at -80^0C. ELISAs were performed as per manufacturers instructions in batches. In serum, I measured total white cell count, c-reactive protein and erythrocyte sedimentation rate. Interleukin 8, myeloperoxidase and free neutrophil elastase were measured in sputum.

Baseline Start of Study	End of study At 1 year
Serum Lipoxin A_4	Serum Lipoxin A_4
Sputum LTB_4, MPO, Elastase, CXCL8	Sputum LTB_4, MPO, Elastase, CXCL8
FEV_1 + FVC FEV_1/FVC	FEV_1 + FVC FEV_1/FVC
Exacerbation frequency	Exacerbation frequency

Quality of life questionnaires
  o LCQ
  o SGRQ
	Quality of life questionnaires
	  o LCQ
	  o SGRQ

Table 7. Assessments at each study visit. FEV_1= Forced expiratory volume in one second; FVC= forced vital capacity; LCQ= Leicester cough questionnaire; Leukotriene (LT) B_4, MPO= myeloperoxidase; SGRQ= St. Georges Respiratory Questionnaire.

2.3. BRONCHIECTASIS SEVERITY INDEX

The BSI is a risk stratification tool for mortality and morbidity in bronchiectasis. The minimum score is 0 and the maximum score is 26. A score between 0-4 indicates mild disease; 5-8 indicates moderate disease and a score of >9 indicate severe disease.

The BSI was calculated in all bronchiectasis patients taking part in the study. The BSI was calculated online on the website www.bronchiectasisseverity.com. The breakdown of the BSI is given in table 8. Furthermore table 9 provides the breakdown of the Medical Research Council (MRC) dyspnoea scale, which is a component of the BSI.

Severity marker	Score Points
Age (years)	
<50	0
50-69	2
70-79	4
>80	6
BMI (kg/m^2)	
<18.5	2
18.5-25	0
26-29	0
≥ 30	0
FEV$_1$ % predicted	
>80	0
50-80	1
30-49	2
<30	3
Hospital admission 2years before study	
No	0
Yes	5
Exacerbation 1 year before study	
0	0
1-2	0

≥ 3	2
MRC Dyspnoea score	
1-3	0
4	2
5	3
Pseudomonas **colonization**	
No	0
Yes	3
Colonization with other organisms	
No	0
Yes	1
Radiological severity: ≥ 3 lobes involved or cystic bronchiectasis	
No	0
Yes	1

Table 8. Break down of the bronchiectasis severity index.

MRC Dyspnoea Scale	
Grade 1	Not troubled by breathless except on strenuous exercise
Grade 2	Short of breath when hurrying on a level or when walking up a slight hill
Grade 3	Walks slower than most people on the level, stops after a mile or so, or stops after 15 minutes walking at own pace
Grade 4	Stops for breath after walking 100 yards, or after a few minutes on level ground
Grade 5	Too breathless to leave the house, or breathless when dressing/undressing

Table 9. MRC Dyspnoea scale (Stento et al 2008).

2.4. QUALITY OF LIFE QUESTIONNAIRES

The patients all completed Leicester cough questionnaire and St. George respiratory questionnaire at each visit.

2.4.1. Leicester Cough Questionnaire (LCQ)

Cough is assessed using the Leicester Cough Questionnaire (LCQ). It is a 19 item self-completed quality of life measure of chronic cough. It has 3 domains: physical (8 items), psychological (7 items) and social (4 items). The total severity score ranges from 3-21, a lower score indicating greater impairment of health status due to cough. It assesses the impact of symptoms over the preceding 2 weeks and has been used in COPD and asthma. It has been validated for use in bronchiectasis (Murray et al 2009). The LCQ offers a pertinent, timely and useful clinical tool for bronchiectasis. It focuses purely on the impact of cough severity on HRQoL, unlike other questionnaires that encompass multiple respiratory symptoms. Cough is the dominant symptom of bronchiectasis, present in up to 98% of patients and measuring its impact on HRQoL is critical for both disease assessment and targeted management. Additionally, the LCQ is concise, consisting of only 19 items, offering greater patient acceptance than lengthier questionnaires. The minimum clinically important difference for change (MCID) is 1.3 units (Birring et al 2003).

2.4.2. Health related quality of life- St. George's Respiratory Questionnaire (SGRQ)

Health related quality of life was measured using the St George's Respiratory Questionnaire (Wilson et al 1997). It is a 50-item self-administered health related quality of life questionnaire divided into 3 main domains, symptoms, activities and impacts. The total score ranges from 0-100; a higher score indicates a poorer health related quality of life. The MCID for SGRQ is 4 units (Wilson et al 1997).

2.5. SPUTUM GRAM STAINING

A 10μl sterile loop was used to transfer sputum to a clean, dry, frosted glass slide. 10μl of sterile saline was gently pipetted onto the sputum and the sample smeared uniformly and thinly across the slide. The sample was air-dried and subsequently heat fixed by passing the slide through a low flame several times until all moisture had evaporated. The slide was then flooded with the primary staining reagent methyl violet for one minute followed by gentle rinsing in deionized water. The mordant iodine was then used to flood the slide for one minute followed by further gentle rinsing in deionized water. Following draining, acetone was used as a decolourising agent, flooding the slide until the fluid appeared colourless (approximately ten seconds). The slide was immediately rinsed in deionized water. The counterstain basic fuschin was then used to flood the slide for one minute before further rinsing in deionized water. The slide was gentle blotted using paper towel and allowed to air dry. The results of the staining procedure were observed under oil immersion microscopy. At low (x100) magnification, the number of squamous cells and polymorphonuclear leukocytes per field were counted. At high (x1000) magnification, bacterial cell colour was described as purple (gram positive) or pink (gram negative-figure 25) and morphology was noted (rods or cocci).

All sputum samples were considered to be valid if there were >25 polymorphonuclear leukocytes and <10 squamous cells on gram stain per low-power (x100 magnification) field (Gleckman et al 1988).
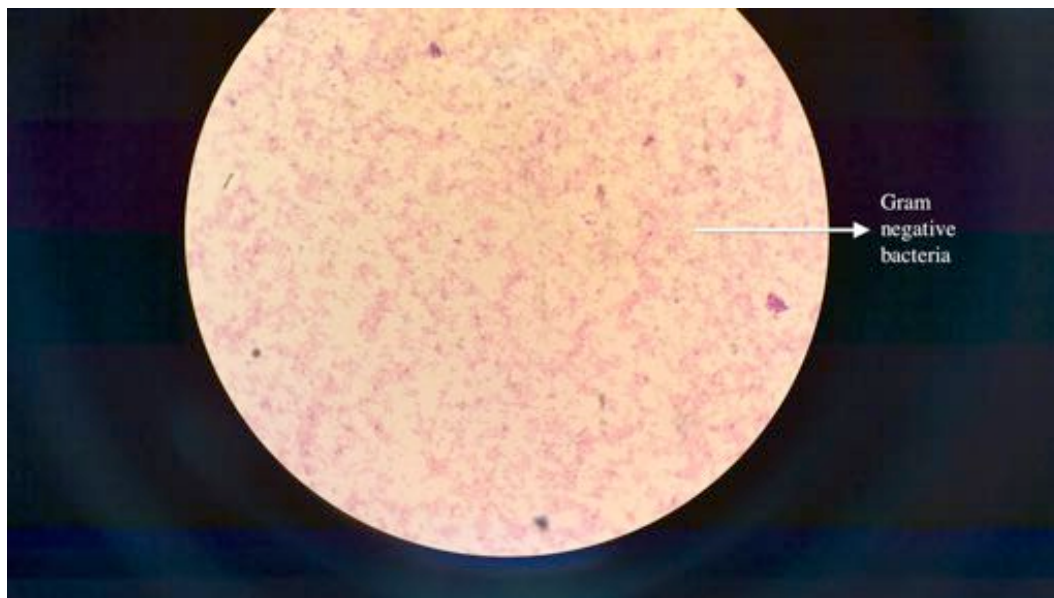

Figure 25. Gram negative stain of sputum

2.6. QUANTITATIVE AND QUALITATIVE SPUTUM MICROBIOLOGY

2.6.1. 24 hour sputum volume: Collected the day before each clinic visit. Patients were advised to store sputum samples at 4°C (in the fridge) during collection.

2.6.2. Induced sputum:

Sputum was induced in all study patients at both study visits. Any upper respiratory secretions were cleared before the procedure was commenced. Patient was positioned in a chair to achieve maximum deposition. 30mls hypertonic saline (3%) was used to induce sputum. Most patients were already practicing active cycle breathing techniques (ACBT) or if patients were physiotherapy naïve, the researcher taught ACBT, before commencing procedure. Once commencing the induced sputum procedure, the aerosol was run for 5 minutes and then stopped. At this point the patient was asked to perform ACBT and try to provide a sample. If this is unsuccessful, the whole procedure was repeated again at 5-minute intervals (up to 20 minutes).

Our senior chest physiotherapist advised us that the procedure was to be discontinued if patient started to spontaneously cough or if the patient became distressed, was coughing excessively or saturations dropped below 90%. Sputum induction was successfully carried out in all patients in the study.

Each sample was confirmed to be a valid sample suitable for processing if there are >25 polymorphonuclear leukocytes and <10 squamous cells present on Gram stain on low power magnification. 1 ml of the sample was used for qualitative and quantitative microbiology.

Sputum colour: Graded as mucoid, mucopurulent or purulent (Murray et al 2009).
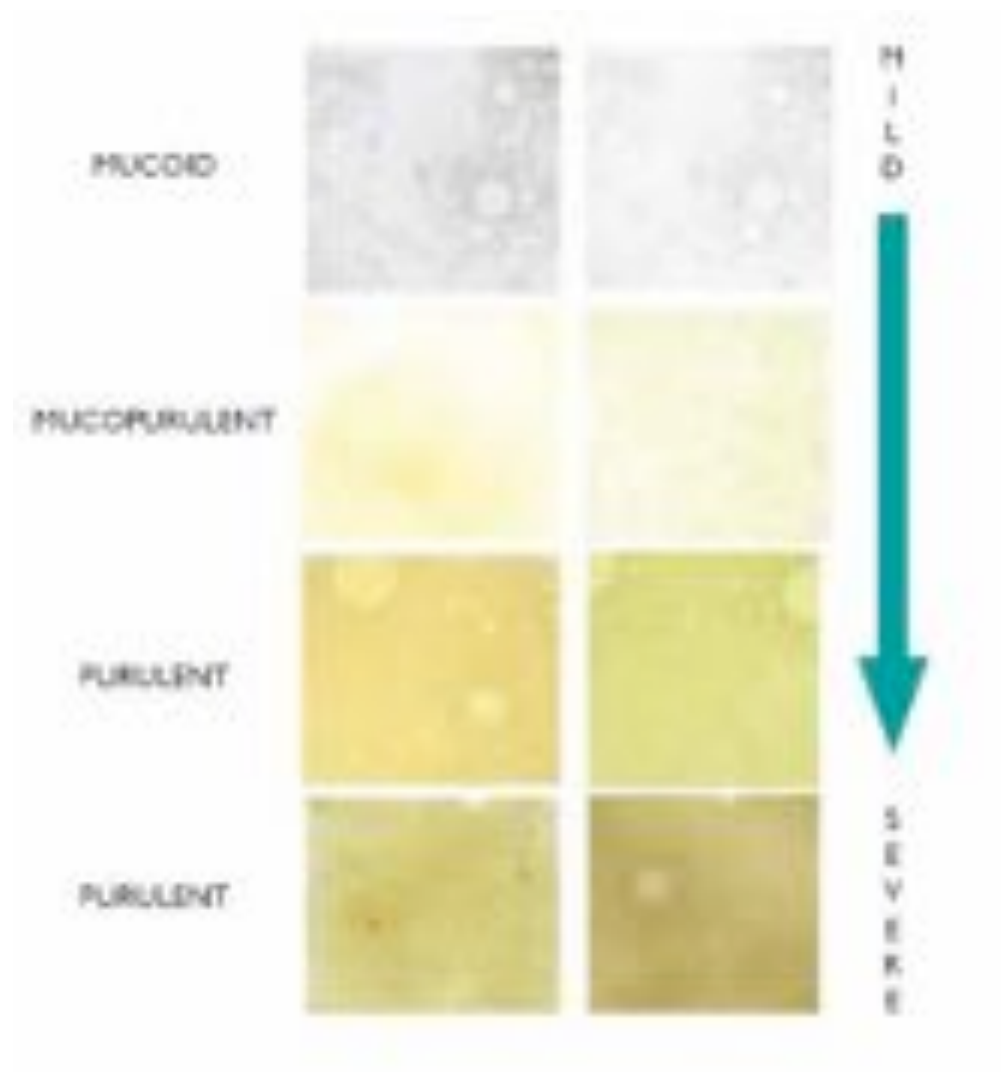
Figure 26. Sputum colour chart (Murray *et al* 2009)

2.6.3. Sputum processing

Sputum was homogenized and liquefied using an equal volume of dithiothreitol and serially diluted using sterile 0.85% saline to achieve dilutional factors of 10^{-1} to 10^{-4}. *Pseudomonas* isolation agar (Difco), chocolate blood agar containing bacitracin (Oxoid) and horse blood agar (Oxoid) plates were inoculated with 100 µl of dilution. These were incubated at 37°C for 48 hrs. Colonies of the pathogens were then counted to determine the sputum bacterial density, expressed as $\log_{10}$ colony forming units/ml (cfu.ml^{-1}). The rest of the sample was divided equally into portions; the first was ultracentrifuged at 30000g for 90 min at 4°C (Hill et al 1999). The sol phase was stored at -70°C until needed for analyzing.

2.6.4. Qualitative sputum bacteriology

Bacterial Count was calculated: Number of bacteria x 2 x 10 x dilution factor to give the number of cfu/ml.

Viable pathogens were identified following incubation based on colonial morphology, Gram stain and further, specific standardised identification tests. The identification methods used for the most common organisms cultured is described.

2.7. *Haemophilus influenzae*

The primary isolation media used for *Haemophilus influenzae* was chocolate with bacitracin agar incubated at 37°C at 5%CO. Colonies are small, round and convex and typically appear after 24hrs incubation. The species appear negative on Gram staining as spherical, oval or rod shaped cells of less than 1μm diameter. Colonies suspected to be *Haemophilus* species from morphology and Gram stain appearance were further identified based on their requirement for X and V factors (figure 27a&b). One or more colonies were selected using a sterile straight wire loop and emulsified in distilled water to produce a light suspension. 100μl of suspension was inoculated onto nutrient agar and spread evenly using a sterile hockey stick spreader. Three filter paper discs incorporating X factor (comprising protoporphyrin IX and haemin), V factor (comprising nicotinamide adenine dinucleotide) and XV factors together respectively, were positioned on the inoculated agar surface in the configuration of an equilateral triangle with a minimum of 3.5cm distance between the discs. Following overnight incubation at 37°C, the agar plate was examined for growth around the discs. *H influenzae* was distinguished from *H parainfluenzae* as detailed in table 10.

Factor	*Haemophilus influenzae*	*Haemophilus parainfluenzae*
X	Negative for growth around disc	Negative for growth around disc
V	Negative for growth around disc	Positive for growth around disc
X and V	Positive for growth around disc	Positive for growth around disc

Table 10. X, V and XV test results for *Haemophilus influenzae* and *Haemophilus parainfluenzae.*
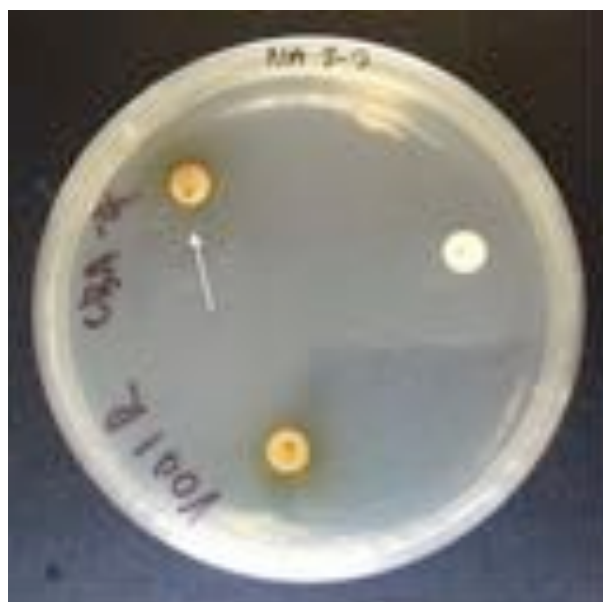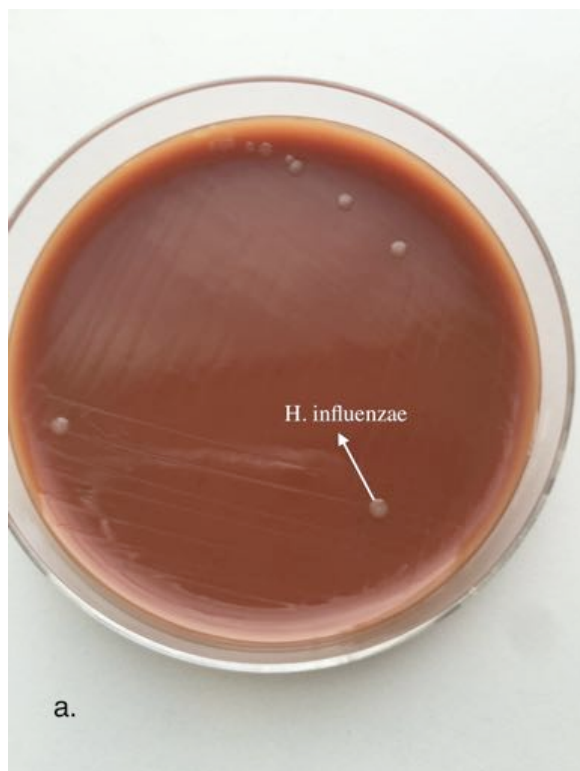
Figure 27. (a) HI influenzae and (b) XV testing: showing growth around the XV disc as indicated by the arrow but no growth around X or V discs. This indicates that the organism isolated is *Haemophilus influenzae*.

2.8. *Streptococcus pneumoniae*

The primary isolation media used for *Streptococcus* species was horse blood agar. Colonies appear white, 1-2mm in diameter and may have a classic draughtsman appearance due to autolysis after incubation. Streptococcus species are positive on Gram staining, appear round and are in pairs, chains or clusters. Colonies suspected from morphology and Gram stain appearance to be *Streptococcus pneumoniae* was further identified by assessing their sensitivity to ethylhydrocupreine hydrochloride (optochin test). Ethylhydrocupreine hydrochloride causes changes in surface tension of the cell membrane causing *Streptococcus pneumoniae* to lyse. Fresh suspicious colonies were selected using a sterile straight wire loop and streaked across a horse blood agar plate. An optochin disc (filter paper disc impregnated with 5μg of ethylhydrocupreine hydrochloride) was placed in the centre of the inoculated agar and incubated at 37°C at 5% CO for 24hours. Following incubation, the inoculated agar was examined for zones of inhibition. A positive result was defined as a radial zone of inhibition measuring 5mm or more from the edge of the disc. A negative result was defined as either no zone of inhibition or a zone of inhibition less than 5mm radius from the edge of the disc (figure 28a&b).
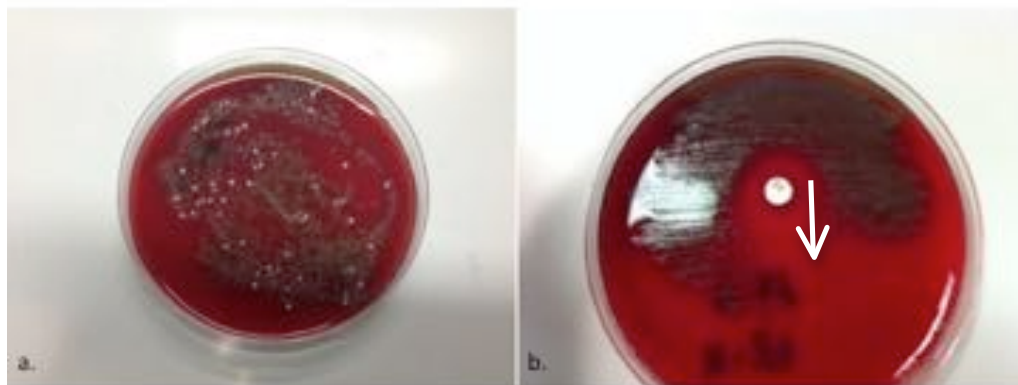
Figure 28a. *S. pneumoniae*. b. Optochin disc test confirming *S pneumoniae*. Solid arrow represents area around optochin disc inhibiting growth of *S. pneumoniae*.

2.9. *Staphylococcus aureus*

The primary isolation media used for *Staphylococcus aureus* was horse blood agar. Colonies are opaque with either a creamy white colour or a yellow-orange colour. Gram staining shows gram-positive cocci occurring either singly, in pairs or in irregular clusters. Colonies suspected to be Staphylococcus aureus were confirmed using the commercial test kit Dryspot Staphytect Plus, a latex slide agglutination test according to the manufacturer's instructions (Oxoid Limited, Basingstoke, Hampshire) (figure 29).
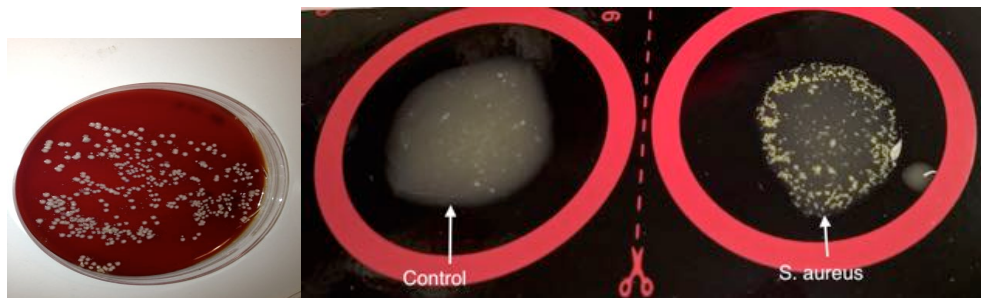
Figure 29. *Staphylococcus aureus* isolated in our laboratory.

2.10. *Pseudomonas aeruginosa*

Cetrimide (Difco) Pseudomonas isolation agar was used as the primary selective media for *Pseudomonas aeruginosa*. Morphological identification of colonies may have included several characteristics. The most common type of colony is a large, low oval convex shape with a rough appearance. There may be a characteristic smell of aminoacetophenone and colonies may have a blue-green appearance due to the production of pyocyanin (blue) and pyoverdin (yellow). The production of this pigment is indicative of *Pseudomonas aeruginosa*, although some strains particularly mucoid strains may not produce pyocyanin. Further identification of colonies was confirmed using the commercially available API20NE kit (bioMerieux UK Limited, Basingstoke, Hampshire) according to the manufacturers' instructions. Briefly, approximately one to four colonies were selected using a sterile straight wire loop and emulsified with 2ml of 0.85% sterile saline to produce a suspension approximating a 0.5McFarland standard. The API20NE test strip consists of twenty microtubes containing dehydrated substances. These microtubes were inoculated with the bacterial suspension and incubated for 24 or 48 hours at 30°C with the addition of reagents and interpretation of reactions done according to the manufacturer's directions. The biochemical reactions were converted accordingly into an octal profile number and decoded using the Analytical Profile Index (API Database Vn6.0, APILAB Software Vn3.3.3, Apilab Plus; bioMerieux) (figure 30 a&b).

Figure 30 a. Mucoid *P. aeruginosa* isolated in our laboratory- green in colour due to the production of pyocyanin; b. API NE test

2.11. *Moraxella catarrhalis*

The primary isolation media used for *Moraxella* species was chocolate brown agar. Colonies are white or buff and convex in shape (figure 31). The species appear as negative cocci on Gram stain, are approximately 0.6-1.0μm diameter, and occur either singly or in pairs. Colonies suspected to be *Moraxella* species from morphology and Gram stain appearance were tested for a positive oxidase reaction. Filter paper was soaked in the test reagent N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride. A sterile wooden stick was used to select at least one suspicious colony and rub it onto the pre- soaked filter paper. A reaction was looked for within ten seconds. A positive result was confirmed with the development of a blue colour, indicating oxidase production. A negative result was indicated by an absence of colour.
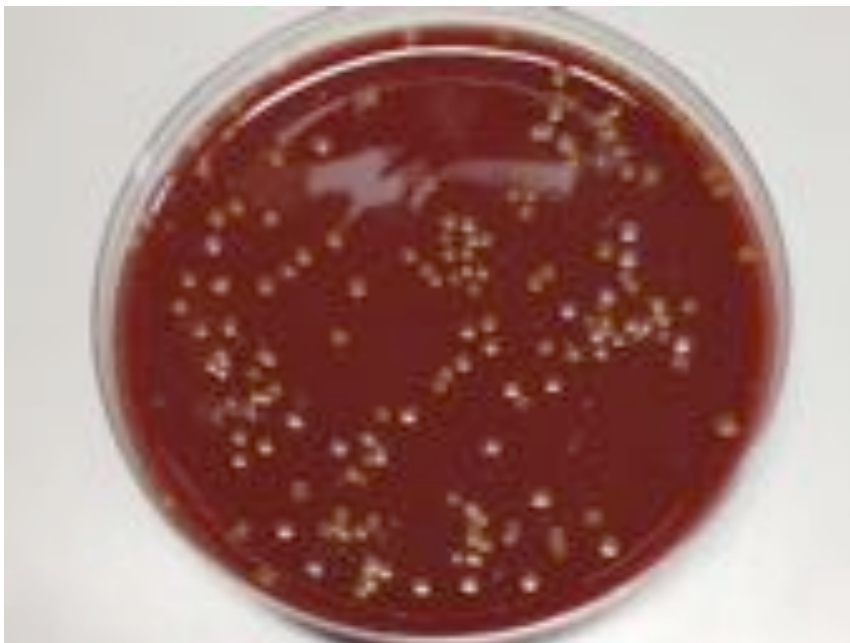
Figure 31. *M. catarrhalis* isolated in our laboratory.

2.12. *Enterobacteriaceae*

Enterobacteriaceae were selected from horse blood agar. The colonies are 2-3mm in diameter, are low, convex, grey and maybe smooth or mucoid (figure 32). The cells appear as Gram negative rods. Further identification of suspicious colonies was initially with a negative oxidase test as described previously and then the commercial kit API20E was used (bioMerieux UK Limited, Basingstoke, Hampshire). The principle and preparation of the API20E strip is similar to the API20NE strip described above, with incubation for only 24 hours at 30°C.
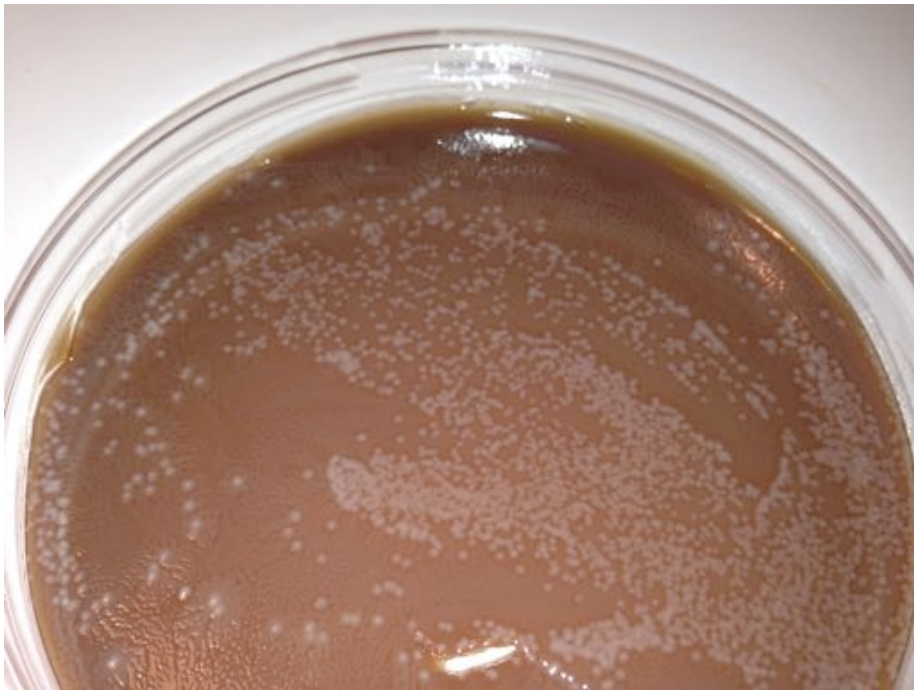
Figure 32. Gram-negative microorganism isolated from sputum of bronchiectasis patient in our laboratory.

2.13. SPUTUM INFLAMMATION MARKERS

For measurement of markers of airway inflammation, sputum, not treated with DTT, was ultracentrifuged (Sorvall™ Ultracentrifuge, UK) at 23200rpm for 90 minutes at 4°C. The sol phase was removed, immediately frozen in aliquots at -80°C. I measured sputum Myeloperoxidase, free neutrophil elastase and CXCL8.

2.13.1. Myeloperoxidase (MPO): MPO activity was measured by a chromogenic substrate assay.

Myeloperoxidase (Calbiochem ®) and samples were diluted as necessary in phosphate buffered solution. All reagents were brought to room temperature first. 25μL of standard or sample was added to the wells of a 96 well microtitre plate (Costar®). 25μL of tetramethylbenzidine (Sigma) was added to each well. The plate was then incubated for 5minutes at 25ºC. Reaction was stopped by adding 50uL of sulphuric acid solution to each well. Absorbance was measured using a dual wavelength of 450 and 560nm and MPO concentration interpolated from the standard curve and expressed as μg/ml. The MPO concentration was determined in duplicate for each sample or standard and the mean determined for each.

2.13.2. Free Elastase activity (NE):

NE activity present in the samples was measured spectrophotometrically using the synthetic substrate methoxysuccinyl-ala-ala-pro-valparanitroanilide
[MeOSAAPVpNa 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid] (Sigma) as described below (Hill et al 1999, Stockley et al 2000).

Standards (NE from Sigma Aldrich) and samples were diluted as necessary in buffer. 40μL of standard or sample were added to each well of a 96 well microtitre plate (Costar®). 40µl of MeoSAAPvn was added to each well and samples read immediately at 37ºC for a minimum of thirty minutes with readings every two minutes.

The rate of change in optical density is converted into elastase activity and expressed in units per milligram. The elastase concentration for each sample is determined in duplicate and the mean determined for each.

2.13.3. Measurement of CXCL8:

CXCL-8 is a key neutrophil chemoatttractant in bronchiectasis (Milkami et al 1998). Untreated sputum sol phase was assayed using commercially available specific enzyme linked immunosorbant assay (ELISA, R+D systems, Abingdon, UK) using kits previously validated for sputum use according to established methodology (Stockley and Bayley 2000).

2.13.4. Validation of sputum ELISA's

All assays for sputum were validated as described (Stockley and Bayley 2000). Standard curves for each assay were obtained using pure mediator provided by the manufacturer following the assay protocol. Three pools of sputum solvent were prepared from 4 patients each with mucoid (grade 1), muco-purulent (grade 2) and purulent sputum (grade 3 or 4) based on a previously published sputum colour chart (Murray et al 2009). Three parameters were assessed in validating assays: Reliability, recovery of spiked mediators and the effect of sample dilution.

Reliability: The quantity of mediator in each sputum pool was determined by interpolation against the standard curve. Individual samples were assayed 5 times on a single plate to obtain the intraassay co-efficient of variation. Each sample was also assayed 5 times on different plates to obtain the inter-assay co-efficient of variation.

Spike and recovery: a known quantity of each mediator was spiked into the 3 pools of sputum. These "spiked" samples were then assayed and compared to the values obtained for the original pool. The obtained value was divided by the predicted value to calculate the % recovery.
Dilution effect: The 3 sputum pools were assayed at dilutions ranging from 1 in 1000, to 1 in 5.

An assay was deemed to be valid if it had a reliable standard curve, an intra-assay and inter- assay coefficient of variation <10%, recovery of spiked samples in the range 80-120% of predicted and a linear dilution effect (Stockley and Bayley 2000). Table 11 shows the validation data for all sputum assays tested.

Mediator	Spike-recovery (median %-IQR)	Intra-assay co-efficient	Inter-assay co-efficient	Linear dilution effect
Myeloperoxidase	102% (94-105)	4.2%	3.7%	Yes
Free neutrophil elastase	102% (83-115)	7.1%	7.7%	Yes
CXCL-8	93% (86-118)	8.5%	8.1%	Yes

Table 11. Validation of assays used in sputum

2.14. SPIROMETRY

Forced expiratory volume in 1 second (FEV_1) and Forced Vital Capacity (FVC) were measured according to standardized guidelines (Miller et al 2005) using a MicroMedical Microloop ML3535 (Viasys Healthcare).

FEV_1 is the maximal volume of air exhaled in the first second of forced expiration following a forced expiration from a position of full inspiration and is expressed in litres. FVC is the maximal volume of air exhaled with maximally forced effort from a maximal inspiration and is expressed in litres. The highest of three technically satisfactory measurements (within 10%) was recorded for each and the ratio was calculated. Actual values were recorded and were also expressed as % predicted for the patients age, sex, and height.

2.15. SYSTEMIC MARKERS OF INFLAMMATION

30 ml of venous blood was collected for full blood count, erythrocyte sedimentation rate and c reactive protein were measured.

5 ml of blood was centrifuged at 750g for 10 min and the supernatant collected and stored at -70C, for measuring pro and anti inflammatory cytokines and chemoattractants by ELISA such as the cathelicidin LL-37 as per manufacturers instructions.

2.16. BRONCHOSCOPY
Requirements

- 1% Xylocaine throat spray

- Topical 2% lidocaine spray to be inserted via the bronchoscope- total 14mls

- Fentanyl 100μg/ml

- Midazolam- 1mg/ml

- Olympus bronchoscope

- Monitor

- Brush; traps

- 0.9% saline

- Lignocaine jelly

- Emergency drugs for reversal of anesthetics administered

  - Flumazenil (for reversal of midazolam)

  - Naloxone (for reversal of fentanyl)

Method

I performed all procedures under supervision of a Consultant Respiratory Physician at the Royal Infirmary of Edinburgh (figure 33).

Ensure patient meets inclusion and exclusion criteria. Patient remains fasted from the midnight before the procedure. On the day of the procedure, patient is put in a monitored bed and his oxygen saturations and heart rate are recorded. The patient is laid flat and just prior to the procedure, 12mls of 1% xylocaine throat spray is sprayed to anaesthetize the vocal cords. Following this, a mouth guard is inserted and midazolam (maximum 5mg) and fentanyl (maximum 100μg) is administered to ensure that the patient is lightly sedated and ready for the procedure. The sterile bronchoscope is checked and then after applying xylocaine jelly around the tip of the bronchoscope, the scope is carefully inserted into the mouth of the patient and the vocal cords are visualized and checked for vocal cord palsy. The scope is then advanced into the trachea and the main bronchus and on reaching the main carina, both the left and right main bronchus are topically anaesthetized with 2% lidocaine spray through the scope. All the while, the anatomy of the bronchi is observed to ensure that there is no abnormality. In bronchiectasis patients, the target sites to be

washed and brushed are identified before starting the procedure- based on the results of an up to date CT scan that the patient had done prior to the procedure. In healthy volunteers, generally the right middle lobe was targeted. In general, I washed and brushed an area of the lung unaffected with bronchiectasis and an area affected by bronchiectasis. Once the target area is reached- 60mls of saline is gently flushed into the segment of the lobe and aspirated back into a trap attached to the outside. After washing out the segments, the same segments are brushed and samples are collected in RNA later. After a quick visualization of the lobes, the scope is withdrawn and the patient is made to sit upright. The research nurses then recover the patient for 2 hours following which the patient is given food and allowed home with a friend/ family member.

Figure 33. a. Bronchoscopic view of vocal cords; b. bronchoalveolar lavage samples;
c. endobronchial brushes.

2.17. ISOLATION OF AIRWAY NEUTROPHILS

Reagents

- Brochoalveolar lavage
- Phosphate buffered saline
- Sputolysin
- Tryptan blue
- 40μm sterile filters

Method

Bronchoalveolar lavage was washed with 8 times volume of phosphate buffered saline (PBS). This was then centrifuged at 2000rpm for 10minutes. The supernatant was discarded and then washed with 4 times volume of PBS at 1500rpm for 10 minutes. Supernatant was discarded and then 4 times volume of sputolysin was added and left on a roller for 10 minutes. Following this, the lavage fluid was filtered through sterile gauze filter (40μm) and viability was ascertained by exclusion of tryptan blue. Viable cells were counted by a hematocytometer. Final centrifuge was done at 2000rpm for 10 minutes at 4˚C. After discarding the supernatant, lavage cells were re suspended in PBS at desired concentration. Cytospins were prepared in a Shandon cytospin 4 (Shandon, Pittsburgh, PA, USA) and stained with Diff-Quick stain (Merz Dade, Switzerland) to check for purity of neutrophils isolated.

2.18. STATISTICAL ANALYSIS

Normality of data was checked using Shapiro-Wilk's test (p>0.05). Overall, parametric data is presented as mean ± standard error of mean (SEM) unless otherwise specified.

-For the neutrophil sub type study and the study assessing the effect of lipoxin on neutrophil function: In experiments that mainly involved three groups, data was analyzed by one-way ANOVA with a Bonferroni's multiple comparison post hoc test. For comparison of two groups, unpaired t tests were done. Flow cytometry analysis was performed using FlowJo v10.0.4 (Tree Star, Ashland, OR, USA).

-For the longitudinal study: Unpaired t tests were done to compare the lipoxin sufficient and deficient groups. Microbiology data is presented as median (inter quartile range).

-For the bronchoscopy study: Continuous data is presented as n (%) and parametric data as mean ± standard error of mean (SEM). For all data with more than 2 groups, one-way ANOVA was done. To analyze data from affected and unaffected segments, paired t tests were done. Unpaired t tests were done to compare two study groups as shown in the results section. Microbiology data is presented as median (inter quartile range).

Significance was accepted with *P* values: *$P < 0.05$.

For statistical tests GraphPad Prism v6; GraphPad Software, La Jolla, CA, USA was used.

CHAPTER 3

NEUTROPHIL AND LIPOXIN STUDIES

3.1. NEUTROPHIL SUBTYPE STUDIES

Study Aim: To characterize neutrophils in the blood and airways in bronchiectasis patients in the stable state and during exacerbations.

Blood neutrophil apoptosis, CD11b and CD62L expression, myeloperoxidase release, and superoxide generation was assessed. Measuring phagocytosis and killing of GFP labeled bacteria (PAO1) assessed neutrophil function. Once neutrophils were characterized, the effects of Lipoxin A_4 on the function of these neutrophils were assessed. The results will be discussed as blood neutrophils and airways neutrophils separately.

For blood neutrophil studies, 8 healthy volunteers, 8 patients with mild (Bronchiectasis Severity Index 0-4) and 8 patients with severe bronchiectasis (Bronchiectasis Severity Index >9) were recruited. 60mls of blood was taken, as described in the methods section. For airways neutrophil studies, only the bronchiectasis patients (8 mild and 8 severe) underwent bronchoscopy. Bronchoalveolar lavage fluid was obtained and airways neutrophils were isolated, as described in materials and methods. No healthy volunteers were bronchoscoped. The experiments described below pertain to the samples from these participants.

3.1.1. Blood neutrophil spontaneous apoptosis rate is altered in bronchiectasis

Blood neutrophils were isolated by percoll gradient as previously described. The assays were done on cells incubated in the presence of 10% autologous serum. Late apoptosis (20 hours) was measured at 20 hours (Figure 34a, b and c). The same effect was observed when analysis of total cell numbers were done as compared to the gold standard microscopic counts, demonstrating that these percentages were not skewed by an artefact of cell loss.

Analysis by one way ANOVA, comparing healthy volunteers, mild and severe bronchiectasis (separate ANOVAs for viable, apoptotic and necrotic cells), showed

that in these **untreated** neutrophils, there was a higher proportion of **viable** cells in the preparations from bronchiectasis patients, with the overall p value being p<0.0001, with the healthy volunteers being the control group. Using Bonferroni's multiple comparison post hoc tests, there were statistically significant differences in the percentage of viable neutrophils between healthy volunteers and mild bronchiectasis patients (p<0.0001), and between healthy volunteers and severe bronchiectasis patients (p=0.0003). There was a corresponding statistically significant, lower percentage of **apoptotic** neutrophils in the preparations from bronchiectasis patients, with the overall p value being p=0.0003, with the healthy volunteers being the control group. Using Bonferroni's multiple comparison, post hoc tests, there were statistically significant differences in the percentage of apoptotic neutrophils between healthy volunteers and mild bronchiectasis patients (p=0.003), and between healthy volunteers and severe bronchiectasis patients (p=0.0002). There was no difference in the necrotic neutrophils, between healthy volunteers, mild and severe bronchiectasis, p=0.7.

Cell counts were also done, for all conditions and this confirmed that there were significantly lower apoptotic cells (p<0.0001) and significantly higher viable cells in bronchiectasis patients compared to healthy volunteers; figure 34d.
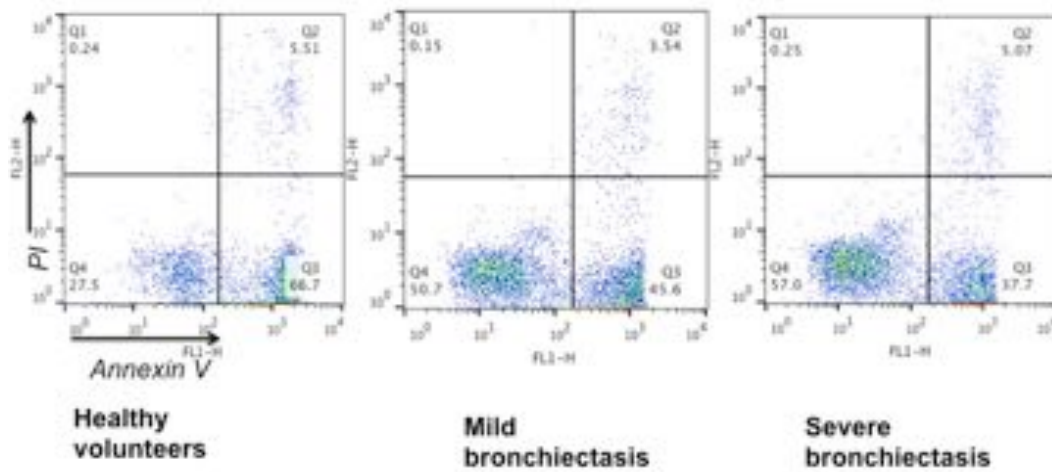
Figure 34a. Blood neutrophils from mild and severe bronchiectasis patients survived longer and underwent later apoptosis as compared to healthy volunteers.

1a. Blood neutrophils from mild and severe bronchiectasis and healthy volunteers were cultured for 20 hours and cell viability (Q4: AnnV-ve/ PI-ve), apoptosis (Q3: AnnV+ve/PI-ve) and necrosis (Q2: AnnV+ve/PI+ve) was assessed by flow cytometry. N=8 in each group; percentage of viable, apoptotic and necrotic neutrophils in each group. Representative flow cytometry plots at 20 hours. PI = propidium iodide.
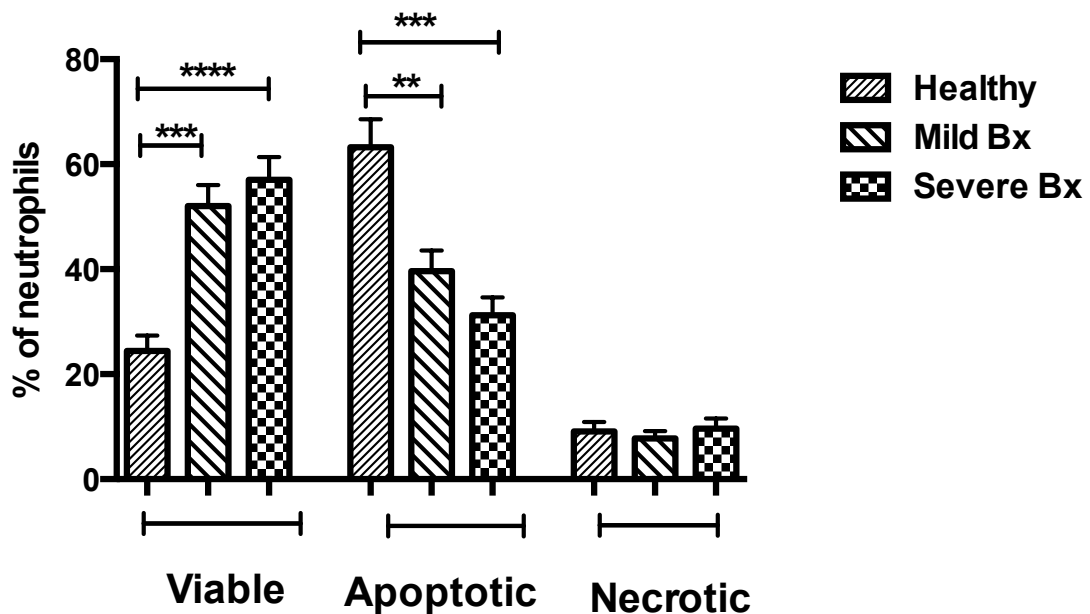
Figure 34b. Cumulative data from N=8 donors in each group. There was no difference in the proportion of necrotic neutrophils between the groups.
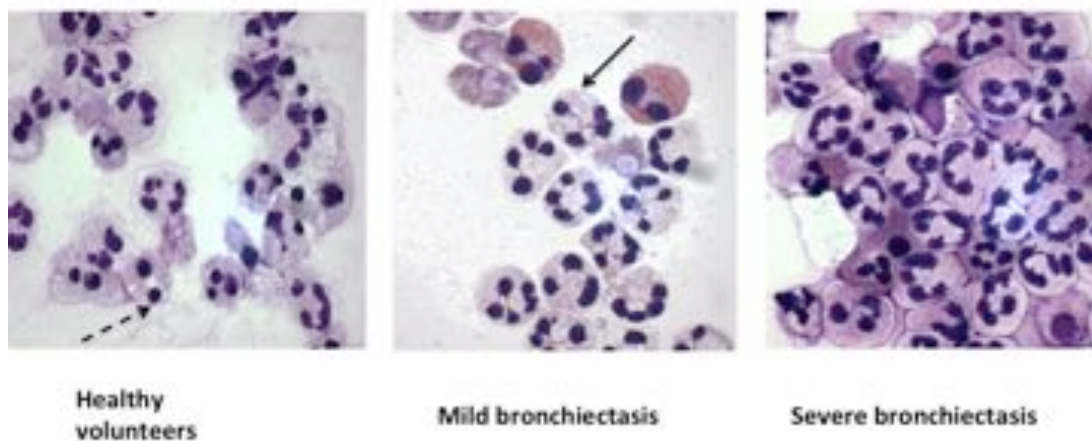
Figure 34c. At 20 hours, cytocentrifuge preparations (gold standard for quantifying apoptosis) from neutrophil suspensions were also fixed and stained. Cells were examined under oil immersion light microscopy and apoptotic cells were defined as cells containing darkly stained pyknotic nuclei. Solid arrow represents healthy neutrophils with multi lobulated nuclei, more abundant in neutrophils from mild and severe bronchiectasis. Dashed arrow represents dark, pyknotic nuclei in apoptotic neutrophils - more abundant in healthy volunteers.
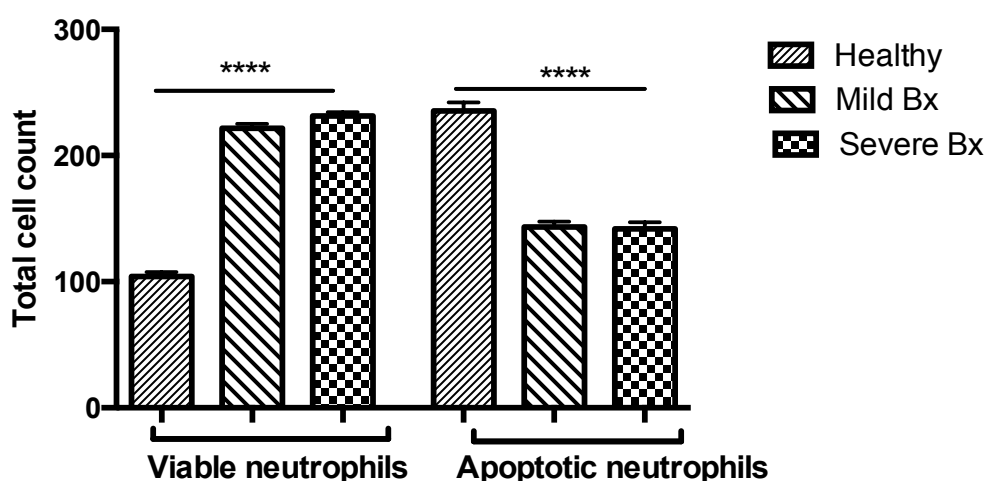
Figure 34d. Significantly higher viable and lower apoptotic neutrophils in bronchiectasis compared to healthy volunteers, at 20 hrs. At 20 hours, at least 400 cells were counted from at least 4 different fields. Pooled data from n=8, presented as mean ± SEM. ****P<0.0001.

3.1.1.1. Bronchiectasis neutrophil apoptosis is increased when incubating cells with serum from healthy volunteers

Having found that the spontaneous apoptosis rate was altered in blood neutrophils from mild bronchiectasis patients, in the presence of autologous serum, the question of whether a patient serum factor was responsible was addressed by assessing apoptosis in the presence of serum from healthy volunteers and patient's autologous serum.

Spontaneous neutrophil apoptosis was assessed as above in 6 mild bronchiectasis patients by comparing rates of apoptosis of their blood neutrophils in the presence of 10% autologous serum, with that in 10% serum from healthy volunteers (figure 35). Using non parametric paired t- tests, after 20 hours, there was a small, but statistically significant (from 51% to 43%, p=0.02) reduction in the percentage of viable neutrophils when incubated with healthy volunteer serum, compared to autologous patient serum, and a corresponding small, significant increase in the percentage of apoptotic neutrophils (from 38% to 43%, p=0.02). There was no change in the percentage of necrotic neutrophils (p=0.3).

This part of the study indicates that there is perhaps a pro survival factor in serum of bronchiectasis patients which leads to prolonged survival and delayed apoptosis.

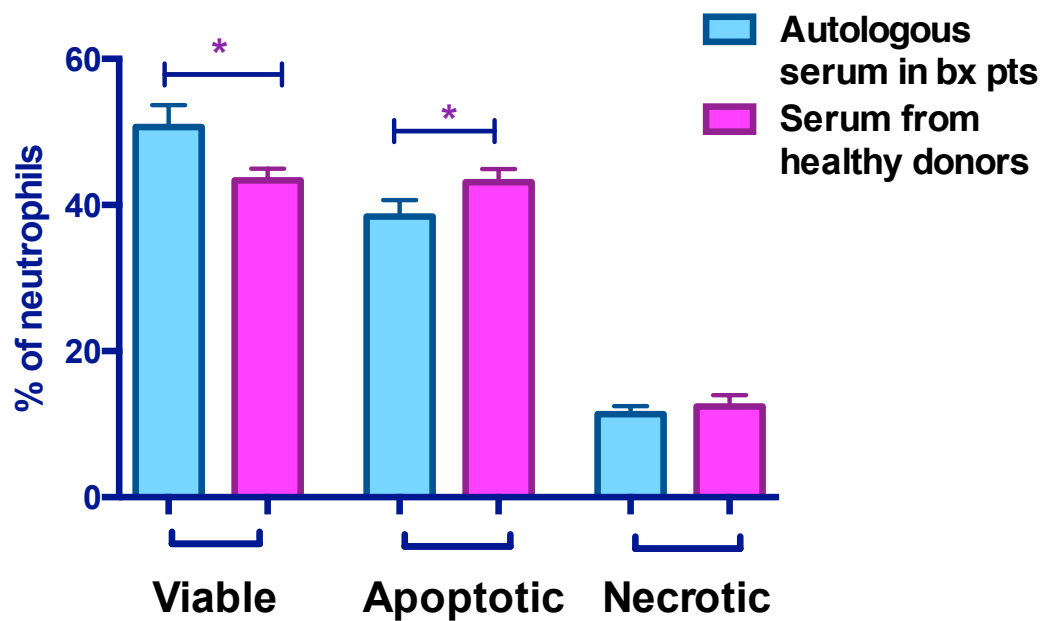Figure 35. A serum or survival factor is missing in mild bronchiectasis serum compared to healthy volunteers. This results in reduction in viability and increased apoptosis when cells are incubated using serum from healthy volunteers. There is no change in necrotic neutrophils. Paired t- tests used for all comparisons. *P<0.05. Pooled data from n=6 donors; data presented as mean (+/- SEM).
Bx = bronchiectasis.

3.1.1.2. Blood neutrophil apoptosis is not altered in healthy volunteers when incubated with bronchiectasis serum

Having found that viability and apoptosis rates were altered when bronchiectatic neutrophils were incubated with serum from healthy volunteers, I wanted to confirm that there was a survival factor in bronchiectasis patient serum that was not present in serum from healthy volunteers.

Spontaneous neutrophil apoptosis was assessed as above in 6 healthy volunteers by comparing rates of apoptosis of their blood neutrophils in the presence of 10% autologous serum, with that in 10% serum from mild bronchiectasis patients.

Using non parametric paired t- test- Wilcoxon test, after 20 hours; there was no statistically significant (from 43% to 42%, p=0.8) change in the percentage of viable neutrophils when incubated with autologous serum, compared to bronchiectasis patient serum. There was no significant change in the percentage of apoptotic neutrophils (from 42% to 39%, p=0.2) when incubated with autologous serum, compared to bronchiectasis patient serum. There was no change in the percentage of necrotic neutrophils (p=0.3) (figure 36).

These data raise that possibility that a factor in healthy serum, deficient in serum from bronchiectasis patients, can promote neutrophil apoptosis. However, the effect of serum was minimal compared to the overall disease-specific differences between the rates of spontaneous apoptosis of neutrophils, suggesting some level of reprogramming or activation of circulating blood neutrophils in bronchiectasis.
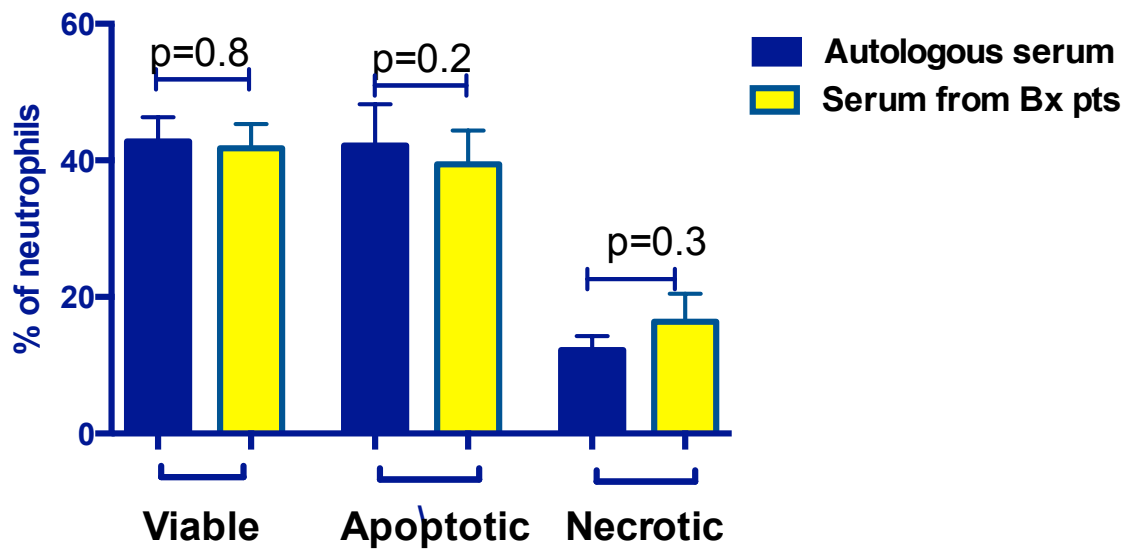
Figure 36. Blood neutrophil apoptosis is not altered in healthy volunteers when incubated with mild bronchiectasis serum. There was no significant change in viable, apoptotic or necrotic neutrophils when healthy volunteers neutrophils were incubated with bronchiectatic serum. Paired t tests used for all comparisons. Pooled data from n=6 donors; data presented as mean (+/- SEM).
Bx= bronchiectasis.

3.1.2. NEUTROPHIL ACTIVATION

Neutrophil activation leads to upregulation of CD11b and shedding of CD62L. 8 healthy volunteers, 8 patients with mild (Bronchiectasis Severity Index 0-4) and 8 patients with severe bronchiectasis (Bronchiectasis Severity Index >9) were recruited and 60mls of blood taken as described in the materials and methods section.

Neutrophils were activated with *N*-Formylmethionine-leucyl-phenylalanine (fMLF) 100nM or vehicle control (PBS with calcium and magnesium) for 30mins. fMLF is a potent neutrophil chemoattractant that upon binding to its heterotrimeric G protein-coupled receptor, initiates signaling cascades that activate multiple pathways. fMLF is found in both gram positive and gram-negative bacteria and is hence used extensively for in vitro experiments in this research (as bronchiectasis patients are infected with both positive and gram negative bacteria). Described in this section, is measurement of CD11b and CD62L in untreated blood neutrophils only. Activation of neutrophils with fMLF and subsequent measurement of CD11b and CD62L is described in section 3.9.2. The reason, CD11b and CD62L measurement has been done in untreated neutrophils is to assess and compare neutrophil activity at baseline in healthy volunteers, mild and severe bronchiectasis.

3.1.2.1. CD11b expression is higher in severe bronchiectasis patients compared to mild bronchiectasis and healthy volunteers

Analysis by one way ANOVA, comparing healthy volunteers, mild and severe bronchiectasis showed that in these **vehicle treated** (PBS with calcium and magnesium) neutrophils, after 30 minutes, there was a higher proportion of **CD11b expression** (via setting a gate against a control) in cells from severe bronchiectasis patients, with the overall p value being p=0.01 using Bonferroni's multiple comparison, post hoc test (p=0.02 for comparison between healthy volunteers and severe bronchiectasis; p=0.02 for comparison between mild and severe bronchiectasis; severe bronchiectasis was the control group against which mild and healthy groups were compared) (figure 37a&b).
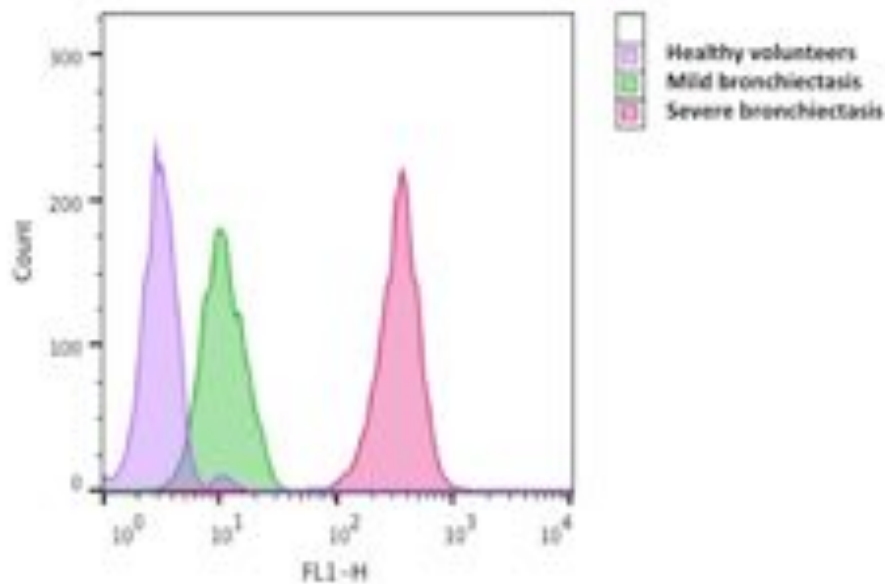
37a.
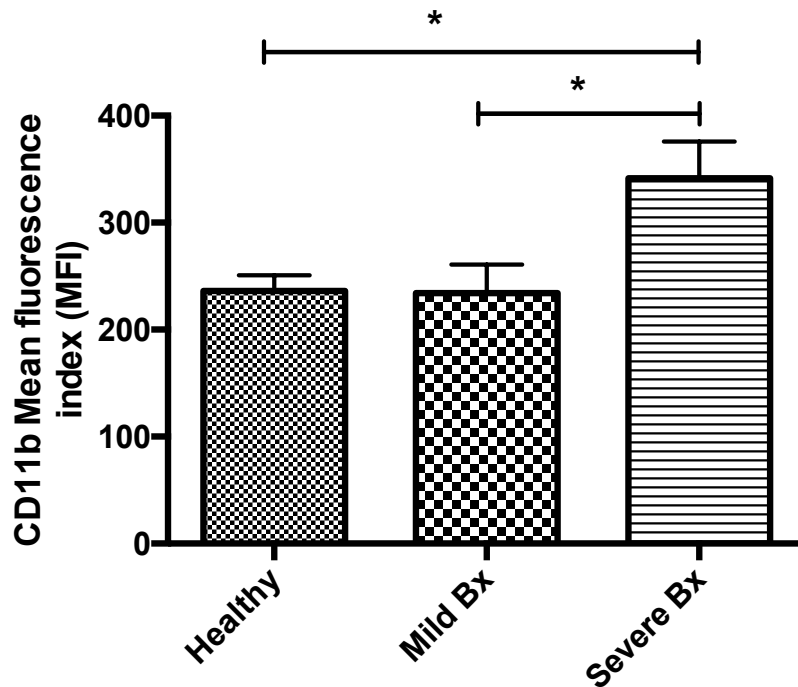
37b.

Figure 37. The baseline level of CD11b was higher in severe bronchiectasis compared to healthy volunteers and mild bronchiectasis.

a. Representative baseline overlay flow plots (from a single experiment) of the three groups. X-axis represents the mean fluorescent index (MFI) and y-axis represents the total cell counts.

b. Cumulative data from N=8 donors in each group; results expressed as mean ± SEM. In untreated blood neutrophils there was significantly higher levels of CD11b in severe bronchiectasis, with the overall p value being p=0.01 using one way ANOVA. Using Bonferonni's post hoc comparison, there was a significant difference in CD11b measured between the healthy volunteers and severe group and between mild and severe bronchiectasis. *P<0.05. Bx= bronchiectasis.

3.1.2.2. CD62L shedding is higher in bronchiectasis patients

Analysis by one way ANOVA, comparing healthy volunteers, mild and severe bronchiectasis showed that in these **vehicle treated** (PBS with calcium and magnesium) neutrophils, after 30 minutes, there was a higher proportion of **CD62L shedding** (via setting a gate against a control) in cells from mild and severe bronchiectasis patients, with the overall p value being p=0.02 using Bonferroni's multiple comparison, post hoc test (p=0.04 for comparison between healthy volunteers and mild bronchiectasis; p=0.03 for comparison between healthy volunteers and severe bronchiectasis; healthy volunteers was the control group against which mild and severe bronchiectasis were compared) (figure 38a&b).
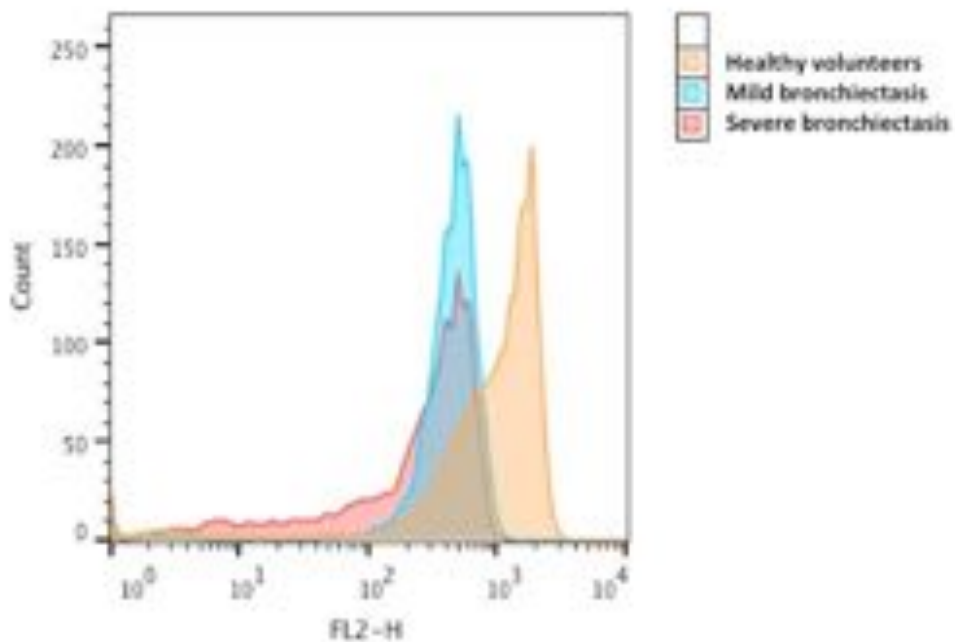
38a.

38b.

Figure 38. At baseline, there was higher proportion of CD62L shedding in mild and severe bronchiectasis compared to healthy volunteers. X-axis represents the mean fluorescent index (MFI) and y-axis represents the total cell counts.

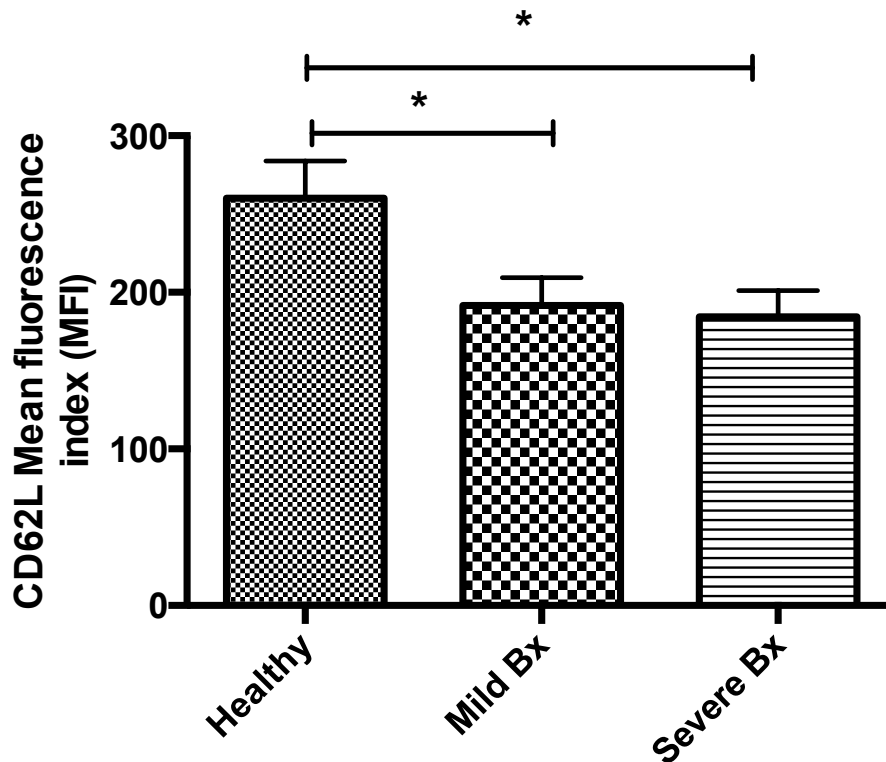a. Representative baseline overlay flow plots (from a single experiment) of the three groups.

b. Cumulative data from N=8 donors in each group; results expressed as mean ± SEM. In untreated blood neutrophils there was significantly higher levels of CD62L shedding in mild and severe bronchiectasis, with the overall p value being p=0.02 using one way ANOVA. Using Bonferonni's post hoc comparison, there was a significant difference in CD62L levels measured between the healthy volunteers and mild group and between healthy volunteer and severe bronchiectasis. *P<0.05. Bx= bronchiectasis.

In summary, this section showed that at baseline, there were higher levels of CD11b expression and higher proportion of CD62L shedding in severe bronchiectasis compared to healthy volunteers and mild bronchiectasis. This indicates that at baseline blood neutrophils are both already primed and activated when obtained (or in response to preparation) OR spontaneously start to activate in severe bronchiectasis. This will be explored in detail with activation of neutrophils using fMLF in section 3.9.2, to distinguish why the neutrophils are more activated in severe bronchiectasis.

3.1.3. NEUTROPHIL DEGRANULATION

MPO release is higher in severe bronchiectasis patients compared to mild bronchiectasis and healthy volunteers

Myeloperoxidase is released from the neutrophil primary granules when these cells are activated. 8 healthy volunteers, 8 patients with mild (Bronchiectasis Severity Index 0-4) and 8 patients with severe bronchiectasis (Bronchiectasis Severity Index >9) were recruited and 60mls of blood taken as described in the materials and methods section. Neutrophils were treated with cytochalasin B (1μg/ml) for 10 minutes and fMLF (10nM) for 30 minutes to activate neutrophils and MPO was measured from supernatants.

As in the studies measuring CD11b and CD62L, described in this section, is measurement of MPO in untreated blood neutrophils only. Activation of neutrophils with fMLF and subsequent measurement of MPO is described in section 3.9.3. The reason, MPO measurement has been done in untreated neutrophils is to assess and compare neutrophil MPO activity to CD11b and CD62L levels, at baseline in healthy volunteers, mild and severe bronchiectasis.

Analysis by one way ANOVA, comparing healthy volunteers, mild and severe bronchiectasis showed that in **untreated** neutrophils, there was a higher proportion of **MPO release** in cells from severe bronchiectasis patients, with the overall p value being p=0.02 using Bonferroni's multiple comparison, post hoc test (p=0.04 for comparison between healthy volunteers and severe bronchiectasis; p=0.02 for comparison between mild and severe bronchiectasis; severe bronchiectasis was the control group against which mild and healthy groups were compared) (figure 39).
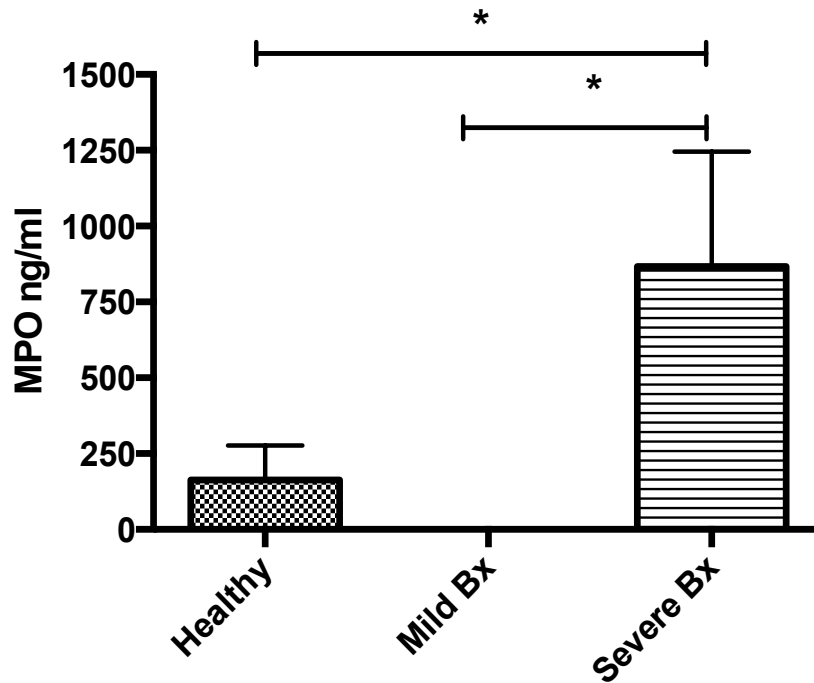
Figure 39. There is more myeloperoxidase release in severe bronchiectasis compared to healthy volunteers and mild bronchiectasis. Cumulative data from N=8 donors in each group; results expressed as mean ± SEM.

In untreated blood neutrophils there was significantly higher levels of MPO release in severe bronchiectasis, with the overall p value being p=0.01 using one way ANOVA. Using Bonferonni's post hoc comparison, there was a significant difference in MPO measured between the healthy volunteer sand severe group and between mild and severe bronchiectasis. *P<0.05. Bx= bronchiectasis.

3.1.4. PHAGOCYTOSIS

Phagocytosis of GFP PAO1 is decreased in bronchiectasis

8 healthy volunteers, 8 patients with mild (Bronchiectasis Severity Index 0-4) and 8 patients with severe bronchiectasis (Bronchiectasis Severity Index ≥9) were recruited and 60mls of blood taken as described in the materials and methods section. Freshly isolated blood neutrophils were co incubated with opsonized GFP labeled *Pseudomonas aeruginosa* PAO1 for 15 minutes (as there was almost 100% phagocytosis at time points greater than 15 minutes across all three groups) and phagocytosis was measured by flow cytometry. Total phagocytosis was calculated on Flow Jo v10.0.4. Once this was done, phagocytosis was divided into high FLI and low FLI (50:50; see figure 40a) to distinguish the neutrophils that had high fluorescence secondary to phagocytosing more GFP PAO1 compared to the neutrophils with low fluorescence. For the purpose of this thesis, all phagocytosis data presented here, represents high FLI. Analysis by one way ANOVA, comparing healthy volunteers, mild and severe bronchiectasis showed that, there was a lower proportion of **phagocytosis of GFP PAO1** in cells from bronchiectasis patients, with the overall p value being p=0.02, with the healthy volunteers being the control group. Using Bonferroni's multiple comparison, post hoc test, there was significantly higher phagocytosis in healthy volunteers compared to mild bronchiectasis, p=0.04; and significantly higher phagocytosis in healthy volunteers compared to severe bronchiectasis, p=0.02 (figure 40b). True phagocytosis as opposed to adherence of PAO1 to the outside of the neutrophils was confirmed by Z stacks done by confocal microscopy (Leica TCS SP5 II, Leica Microsystems; figure 40c).
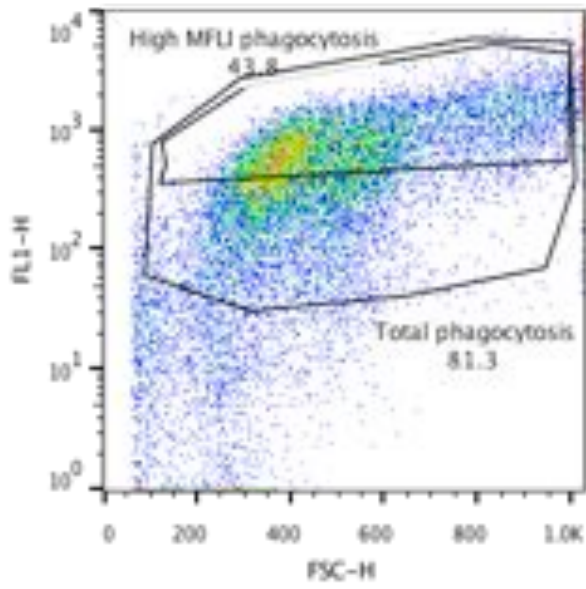
40a.
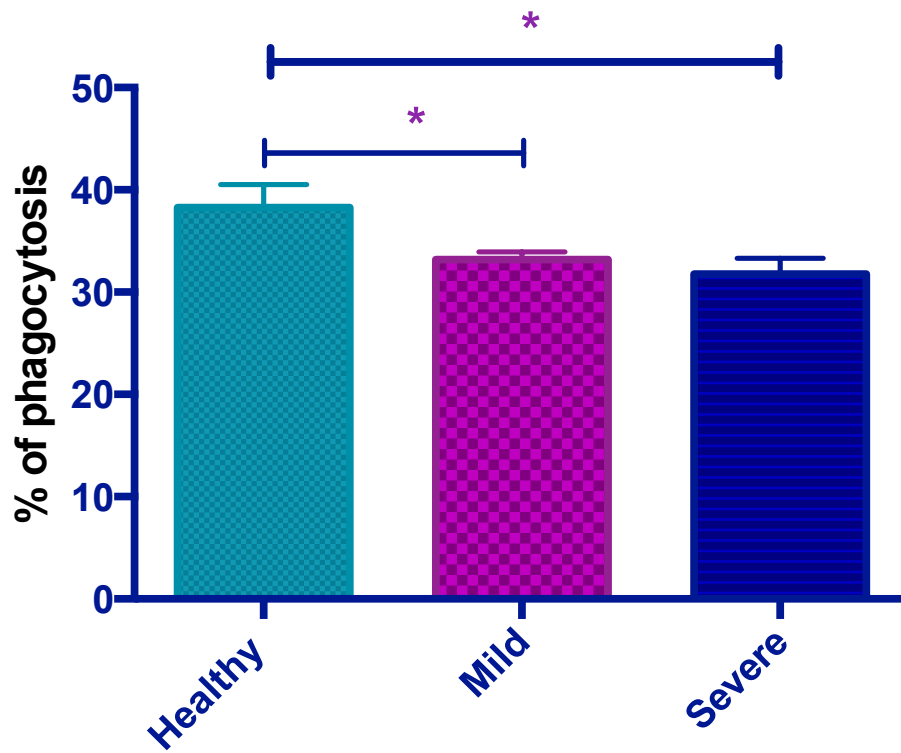
40b.

40c.
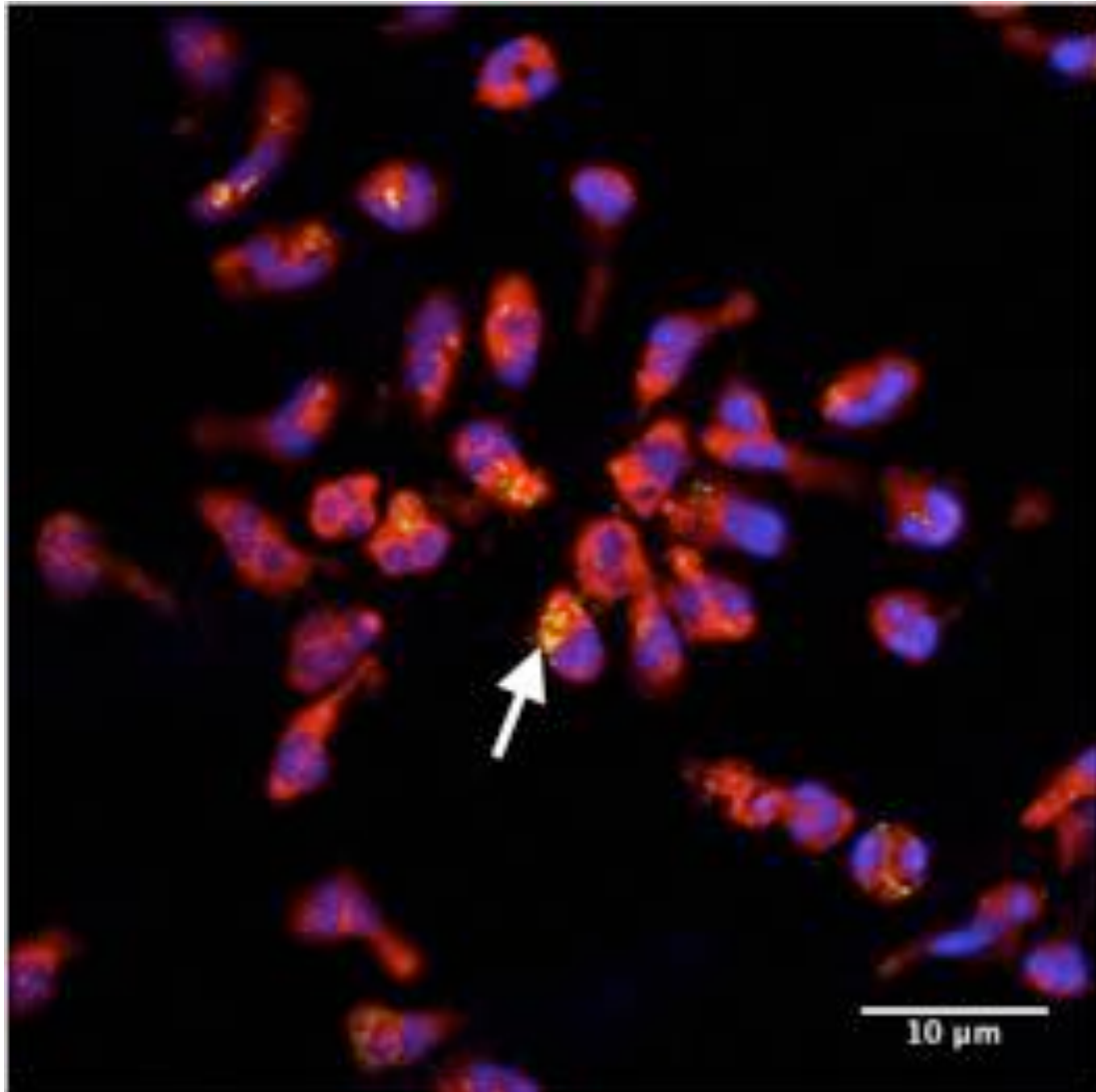
Figure 40. There is a lesser proportion of phagocytosis of GFP PAO1 in bronchiectasis patients compared to healthy volunteers.

a. FACS plot of a representative experiment showing phagocytosis of GFP PAO1 by blood neutrophils. After 15 mins of co incubation of neutrophils with opsonized bacteria, there is at least 75-80% phagocytosis both in healthy volunteers and in bronchiectasis patients. Hence, FACS plots were divided into high and low fluorescence, with the high fluorescence demonstrating neutrophils that have phagocytosed more GFP PAO1, hence have high overall fluorescence.

b. Pooled % neutrophil phagocytosis data, showing means +/- SEM of n=8 per group. One-way ANOVA with Bonferroni's correction for multiple comparisons used; with p values representing the comparison of severe and mild bronchiectasis to healthy volunteers (used as control). *P<0.05.

c. Merged confocal images of neutrophil phagocytosis of GFP labeled bacteria-PAO1.

(Neutrophil cell membrane in red; neutrophil nuclear stain in blue; GFP labeled PAO1 in green). Bx= bronchiectasis.

3.1.5. BACTERIAL KILLING

Blood neutrophil killing of GFP PAO1

Blood neutrophils were isolated by percoll gradients as previously described. Blood neutrophils were co-incubated with GFP *Pseudomonas aeruginosa* PAO1 for 15 mins- as >95% phagocytosis obtained after 15 minutes. Next, spinning the Eppendorfs at 300g for 5minutes stopped the process. The supernatant was discarded to minimize contamination with bacteria that had not been internalized. The pelleted cells were immediately lysed and internalized bacteria were plated out over a dilution series on to *Pseudomonas* isolation agar (Difco) plates to assess bacterial killing, with colony forming units counted after 24 hours of incubation. Hence I assessed the total of surviving PAO1 that interacted with the neutrophils (figure 41).

Analysis by one way ANOVA, comparing healthy volunteers, mild and severe bronchiectasis showed that there was a reduced proportion of **killing of GFP PAO1** in cells from bronchiectasis patients with the overall p value being p=0.0005, with the healthy volunteers being the control group. Using Bonferroni's multiple comparison, post hoc test, p=0.006 for comparison between healthy and mild bronchiectasis; p=0.0003 for comparison between healthy volunteers and severe bronchiectasis.
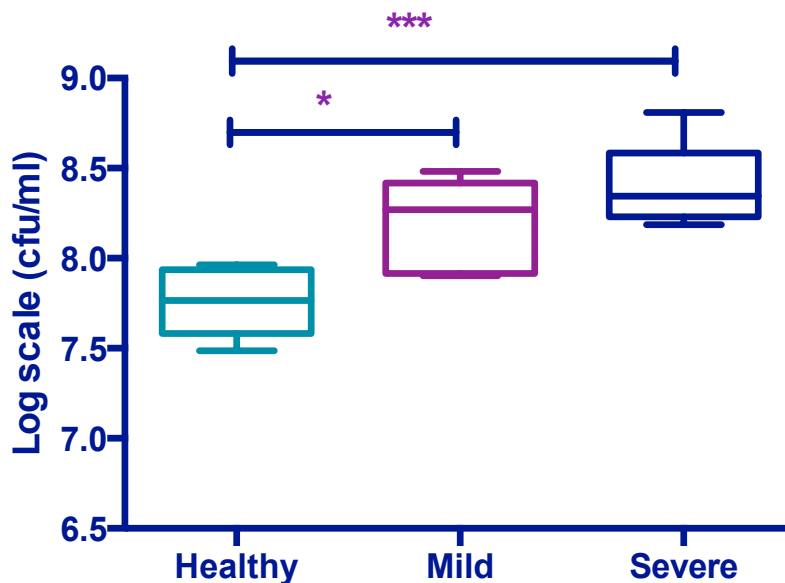
Figure 41. There was significantly lower bacterial killing (PAO1) in severe bronchiectasis compared to healthy volunteers and mild bronchiectasis. Pooled bacterial killing data (in log scale cfu/ml), showing median with IQR of n=6 per group. Significantly higher bacterial killing at the end of exacebation compared to beginning, using paired t tests. Paired t tests used to compare exacebation data and unpaired t tests used to compared stable state to exacerbations. *P<0.05; ***P<0.001.

In summary, this section shows that in bronchiectasis, despite neutrophils being activated and primed when obtained, they phagocytose bacteria less well than neutrophils from healthy volunteers. In addition, these neutrophils have impaired bacterial killing. The ability of neutrophils to kill bacteria is of critical importance in bronchiectasis as recurrent infections is one of the key clinical features in this condition. Can this be regulated or increased extrinsically? This has been explored and discussed in section 3.9.4.

3.1.6. REACTIVE OXYGEN SPECIES (ROS)

- *3.1.6.1. With fMLF as stimulant (Synthetic agonist that reflect aspects of physiological activation).*

Freshly isolated peripheral blood neutrophils from healthy volunteers (n=6) were loaded with dihydrorhodamine for 5minutes. Loaded neutrophils were put on a heat block for 30 minutes and varying doses of fMLF (between 0.1nM and 100nM) were subsequently added for 30 minutes to obtain a concentration response curve. Reactive oxygen species generation was measured by flow cytometry. A maximum response at 100nM fMLF and this was taken as standard for the experiments described below. Described below is measurement of ROS in **untreated neutrophils** to compare ROS generation in neutrophils in healthy volunteers and mild and severe bronchiectasis patients at baseline (as has been done with CD11b, CD62L and MPO generation).
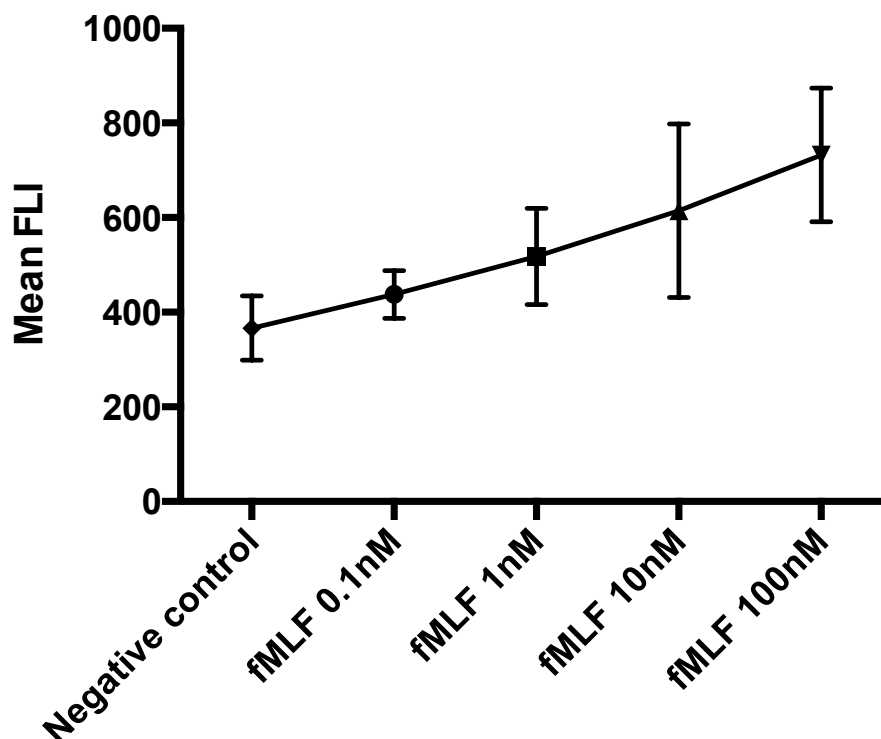
Figure 42. Concentration response curve obtained for fMLF. Neutrophils were obtained from 6 healthy volunteers and stimulated with fMLF (at varying concentrations) for 30 minutes. Reactive oxygen species was measured by flow cytometry. A maximum response at 100nM was standardized and is what is widely been described in the literature.

fMLF= n- formyl- methyl- leucyl- phenylalanine

- ***3.1.6.2. Difference between groups***

Using one way ANOVA for comparison of the three groups for comparison of superoxide release in **untreated** neutrophils, there was no significant difference between healthy volunteers, mild bronchiectasis and severe bronchiectasis; p=0.3, figure 43.
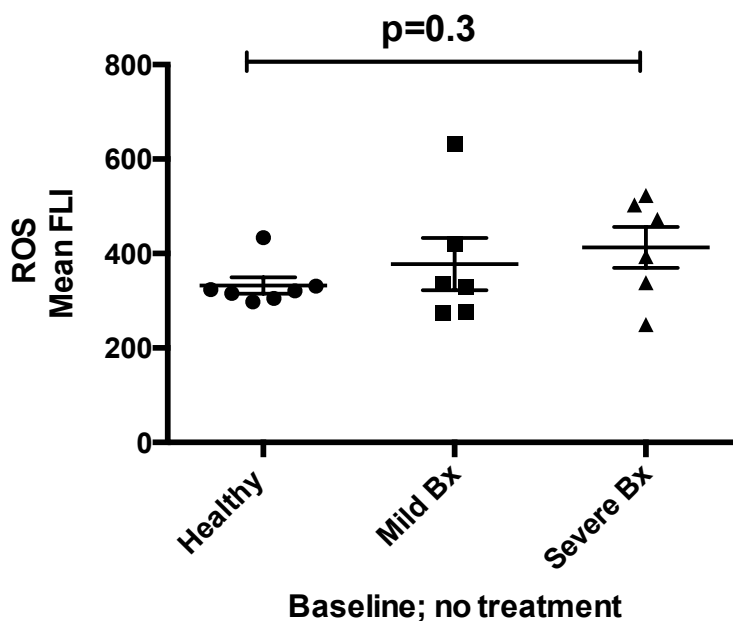
Figure 43. No difference at baseline at reactive oxygen species generation, between healthy volunteers, mild and severe bronchiectasis in untreated neutrophils. Pooled ROS data showing means +/- SEM of n=6 per group. One way ANOVA was done to compare the results obtained.

This section shows that although neutrophils are primed and pre activated in bronchiectasis, there is no difference in the reactive oxygen species generated when compared to healthy volunteers.

3.1.7. COMPARISON OF BLOOD AND AIRWAYS NEUTROPHILS IN BRONCHIECTASIS

6 patients with mild and 6 patients with severe disease participated in this study, all in the stable state. 60mls of blood was taken and all participants underwent bronchoscopy. I assessed phagocytosis and killing of GFP PAO1 by both blood and airways neutrophils, as described before.

- ### *3.1.7.1. Phagocytosis*

Blood neutrophils were isolated by percoll gradients as previously described. Airways neutrophils were obtained from mild and severe bronchiectasis patients from bronchoalveolar lavage, as described previously. Blood and airways neutrophils were co-incubated with GFP labeled bacteria for 15mins (for blood neutrophils) and 60 minutes (for airways neutrophils). Using paired t-test, comparison of the phagocytosis between blood and airways neutrophils showed that there was significantly higher phagocytosis by blood neutrophils compared to airways neutrophils, both in patients with mild and severe bronchiectasis, $p<0.0001$ for mild bronchiectasis and $p=0.0004$ for severe bronchiectasis (figure 44).
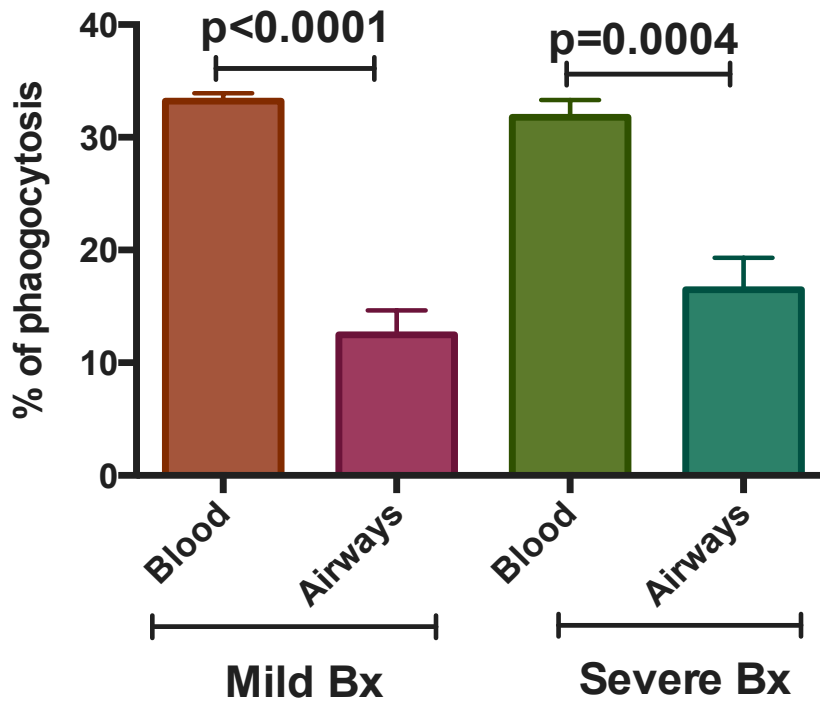
Figure 44. Significantly higher phagocytosis by blood neutrophils compared to airways neutrophils, using paired t tests, p<0.0001 for mild and p=0.0004 for severe bronchiectasis. Phagocytosis was measured at 15minutes for blood neutrophils and at 60 mins for airways neutrophils. Pooled data presented as mean +/- SEM for n=6 per group.

Data is presented here with both mid and severe bronchiectasis on the same graph, to allow better understanding and comparison of the data.

- ### *3.1.7.2. Bacterial killing*

Blood neutrophils were isolated by percolls gradients as previously described. Airways neutrophils were obtained from mild and severe bronchiectasis patients from bronchoalveolar lavage, as described previously. Blood and airways neutrophils were co-incubated with GFP labeled bacteria for 15mins (for blood neutrophils) and 60 minutes (for airways neutrophils). Cells were lysed and internalized bacteria were plated out and bacterial killing was assessed after 24 hours. Using paired t-tests, comparison of the bacterial killing between blood and airways neutrophils showed that there was a significantly higher bacterial killing in blood neutrophils compared to airways neutrophils, both in patients with mild bronchiectasis, p=0.02 and severe bronchiectasis, p=0.02, figure 45.
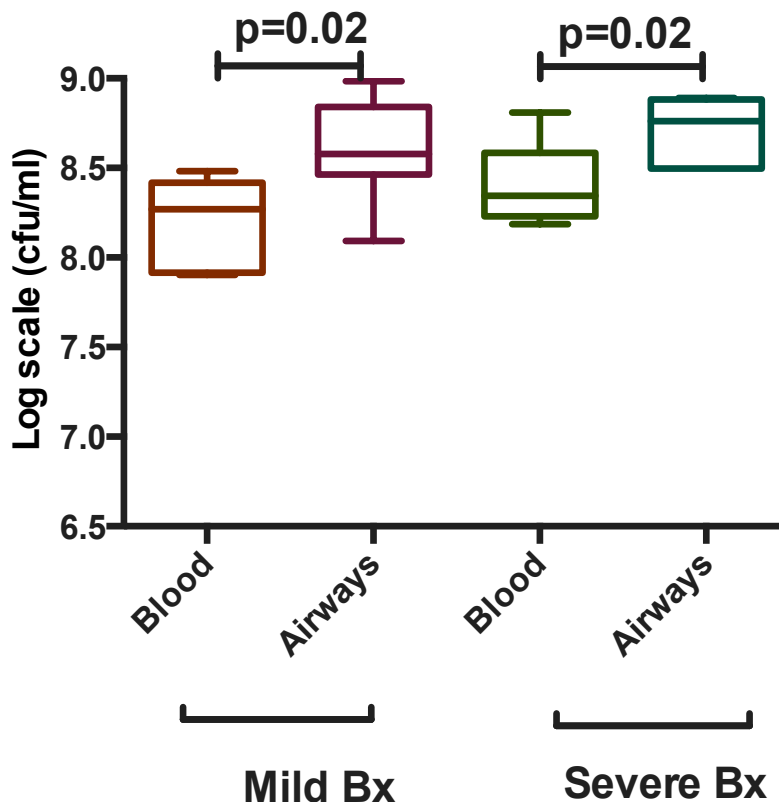
Figure 45. Significantly higher bacterial killing by blood neutrophils compared to airways neutrophils, using paired t tests, p=0.02 for mild and p=0.02 for severe bronchiectasis. Bacterial killing was measured after co incubation with neutrophils-15minutes for blood neutrophils and at 60 mins for airways neutrophils. Plates were read after 24 hours. Pooled data presented as median +/- IQR for n=6 per group.
Data is presented here with both mid and severe bronchiectasis on the same graph, to allow better understanding and comparison of the data.

3.2. EXACERBATIONS

Study aim: To characterize peripheral blood and airways neutrophils in bronchiectasis patients during exacerbations.

- ### 3.2.1. Blood neutrophils

3.2.1.1. Phagocytosis by blood neutrophils during exacerbations and comparison to stable state.

Once I established that blood neutrophils had higher phagocytic and anti bacterial capacity compared to airways neutrophils, I wanted to establish if there was a difference in these functional abilities in neutrophils during exacerbations, and if so, how did it compare to neutrophil function in the stable state. Hence, 6 bronchiectasis patients while in the stable state and 6 bronchiectasis patients with exacerbations were recruited to assess this. *The stable state patients and exacerbation patients were not the same patients.* Patients with exacerbations received intravenous antibiotics for 14 days. Baseline demographics of these exacerbation patients are shown in table 12. These patients were reviewed on Day 1, before the start of antibiotics and on day 14 after completing their last dose of antibiotics. Blood was taken on both occasions and peripheral blood neutrophils were isolated, as previously described. Neutrophils were treated with vehicle control and phagocytosis of opsonized GFP labeled PAO1 was recorded, after co incubating neutrophils with GFP PAO1 for 15 minutes. Using paired t-tests, there was significantly higher phagocytosis at the end of exacerbation compared to beginning of exacerbation, p=0.02. On comparison of stable state to start and end of exacerbation using unpaired t-test Mann Whitney-U test, there was significantly more phagocytosis in the stable state compared to start of exacerbation, p=0.01. There was no difference in phagocytosis between the stable state and the end of exacerbation, p=0.9; figure 46.

Patient characteristics (N=6)	Mean (SEM)
Age	65 (2.7)
Gender (%Female)	50%
CRP (mg/L) • Start of exacerbation • End of exacerbation	123.5 (17.5) 27 (3.2)
WCC (10^9/L) • Start of exacerbation • End of exacerbation	20.6 (0.6) 8.7 (0.3)

Table 12. Baseline demographics. CRP= c- reactive protein; WCC= white cell count.
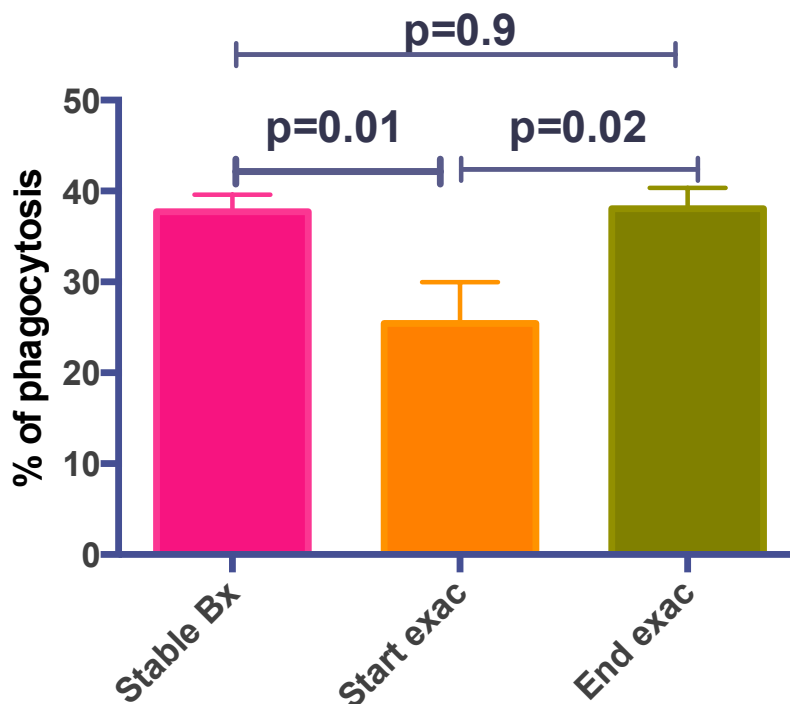
Figure 46. Significantly higher phagocytosis at the end of exacerbation compared to start. There was higher phagocytosis in the stable state compared to start but not at the end of exacerbation by blood neutrophils, using paired t tests. Phagocytosis was measured at 15minutes for blood neutrophils. Pooled data presented as mean +/- SEM for n=6 per group.

3.2.1.2. Comparison of bacterial killing during bronchiectasis exacerbations to stable state bronchiectasis

On comparison of bacterial killing of GFP PAO1 by blood neutrophils (after co incubation for 15 minutes as previously described), at the beginning of exacerbation in bronchiectasis (N=6) to end of exacerbation (N=6) using paired t-tests; there was significantly higher in bacterial killing at the end of exacerbation; p=0.03.

On comparison of bacterial killing at the start of exacerbation in bronchiectasis to stable state bronchiectasis using unpaired t-tests; there was a statistically significant difference in bacterial killing with higher killing in the stable state; p=0.02.

However, there was no significant difference in bacterial killing on comparison of stable state to the end of exacerbation; p=0.3; figure 47.

Figure 47. Significantly higher bacterial killing at the end of exacebation compared to beginning, using paired t tests, in blood neutrophils.
Unpaired t tests demonstrated that there was higher bacterial killing in the stable state compared to start of exacerbation, but not when compared to end of exacerbation.
Pooled bacterial killing data (in log scale cfu/ml), showing median with IQR of n=6 per group.

3.2.2. Airways neutrophils

3.2.2.1. Phagocytosis by airways neutrophils during exacerbations and comparison to stable state.

Sputum from the same 6 bronchiectasis patients while in the stable state and 6 during exacerbations was obtained and airways neutrophils isolated as described in the methods section. Results at the beginning and end of exacerbation were recorded. Phagocytosis of GFP PAO1 was recorded afetr co incubation for 15 minutes as previously described using paired t-tests, there was significantly higher phagocytosis at the end of exacerbation compared to beginning, p=0.008. On comparison of stable state to start and end of exacerbation using unpaired t-test Mann Whitney-U test, there was more phagocytosis in the stable state compared to start of exacerbation although this failed to reach statistical significance, p=0.08. There was no difference in phagocytosis between the stable state and the end of exacerbation, p=0.1; figure 48.
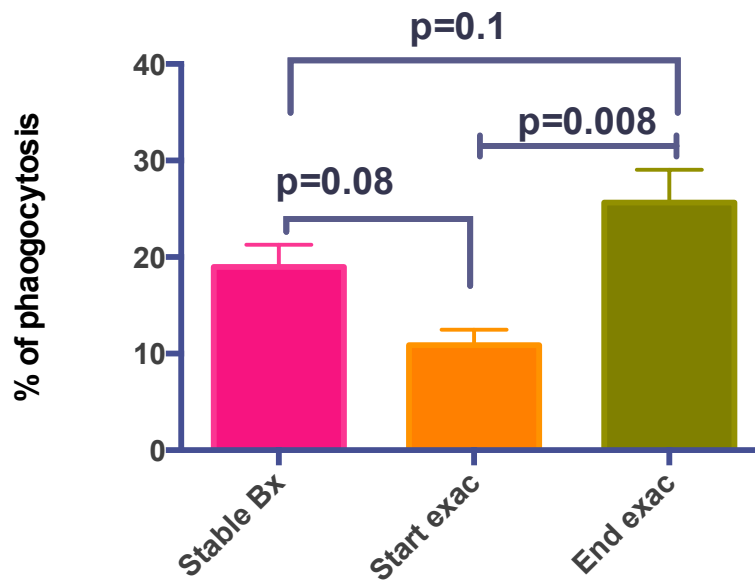
Figure 48. Significantly higher phagocytosis at the end of exacerbation compared to start by airways neurophils (paired t tests). There was higher phagocytosis (although not significant) in the stable state compared to start but not at the end of exacerbation by airways neutrophils, using unpaired t tests. Phagocytosis was measured at 60 minutes for airways neutrophils. Pooled data presented as mean +/- SEM for n=6 per group.

3.2.2.2. Comparison of bacterial killing during bronchiectasis exacerbations to stable state bronchiectasis

On comparison of bacterial killing of GFP PAO1 by airways neutrophils (afetr co incubation for 60 minutes as previously described), at the beginning of exacerbation in bronchiectasis (N=6) to end of exacerbation (N=6); there was a statistically significant higher in bacterial killing at the end of exacerbation; p=0.02, using paired t tests.

Using paired t-tests for comparison of bacterial killing at the start of exacerbation in bronchiectasis to stable state bronchiectasis; there was a statistically significant difference in bacterial killing with higher killing in the stable state; p=0.02.

Using unpaired t-tests, there was trend towards higher killing at the end of exacerbation compared to stable state, but this just failed to reach statitical significance, p=0.06; figure 49.

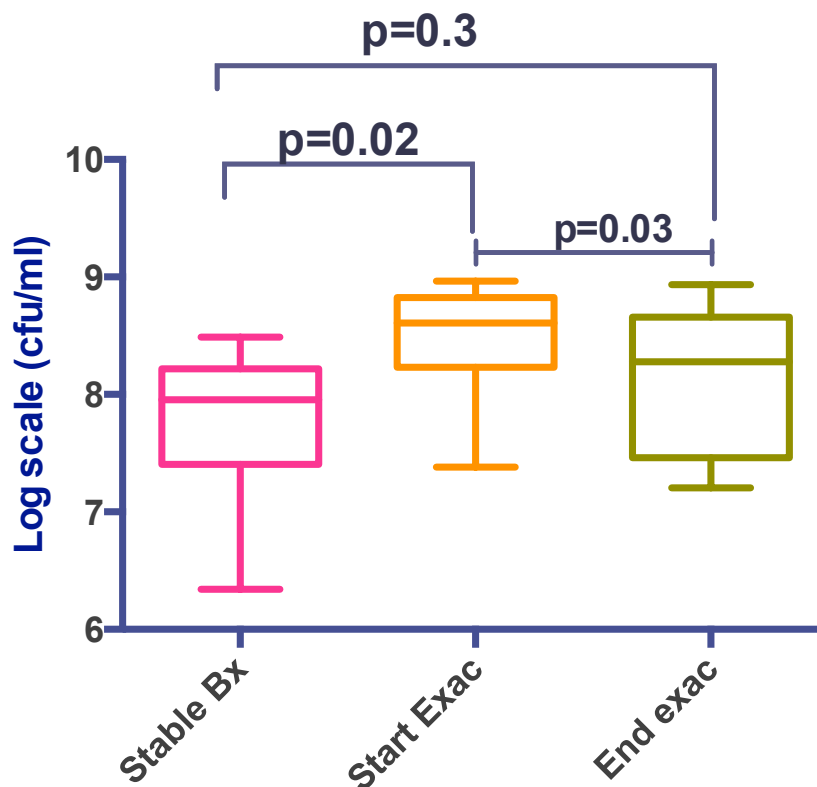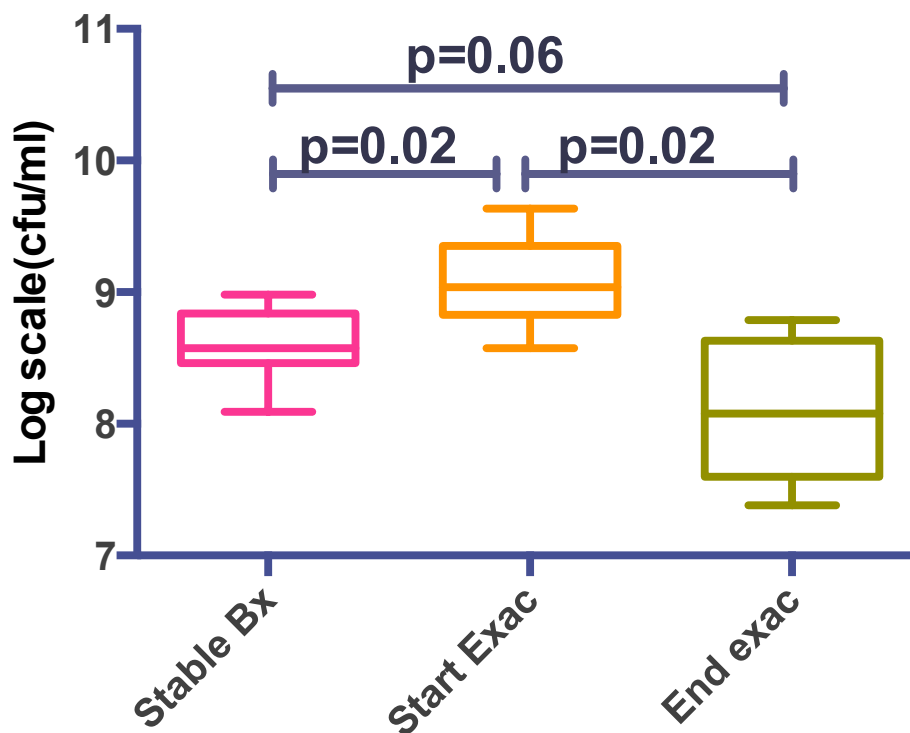Figure 49. Significantly higher bacterial killing at the end of exacebation compared to beginning, using paired t tests, by airway neutrophils.
Unpaired t tests demonstrated that there was higher bacterial killing in the stable state compared to start of exacerbation, but not when compared to end of exacerbation.
Pooled bacterial killing data (in log scale cfu/ml), showing median with IQR of n=6 per group.

In summary, this section shows that in bronchiectasis, blood neutrophils have higher phagocytic and anti bacterial capacity compared to airways neutrophils and this holds in both mild and severe bronchiectasis. Neutrophil function (both blood and airways) was impaired at the start of an exacerbation and this improved significantly with antibiotics. It could be that bronchiectasis neutrophils have impaired phagocytic and killing capacity and to compare this to healthy neutrophils, I assessed the functional ability of neutrophils during pneumonia, in healthy individuals, which is described in the next section.

3.3. COMMUNITY ACQUIRED PNEUMONIA

Study Aim: To assess neutrophil function in pneumonia patients (with no background lung disease) and compare it to neutrophil function during bronchiectasis exacerbations

3.3.1. Phagocytosis

6 community acquired pneumonia patients (with no comorbidities) who were admitted to hospital, were recruited. All patients received intravenous (co-amoxiclav) and oral (clarithromycin) antibiotic therapy. *Streptococcus pneumoniae* was isolated in 4 of the 6 patients and 2 patients did not isolate a bacteria. Results at the beginning (day1) and end of infection (day 5) were recorded. Blood neutrophils were co-incubated with GFP labeled bacteria for 15mins. Phagocytosis was measured by flow cytometry immediately. For bacterial killing, cells were lysed (after 15 minutes co-incubation of neutrophils with GFP PAO1) and internalized bacteria were plated out and bacterial killing was assessed after 24 hours (figure 50a&b).

Baseline demographics of the 6 patients are summarized in table 13.

Patient characteristics (N=6)	Mean (SEM)
Age	54 (4.6)
Gender (% Female)	66.7%
CURB 65	
• 0	0
• 1	1
• 2	2
• 3	3
• 4	0
• 5	0
CRP (mg/L) • Start of exacerbation • End of exacerbation	 190.5 (21.6) 23 (3.3)
WCC (10^9/L) • Start of exacerbation • End of exacerbation	 18.9 (0.9) 9.6 (0.3)

Table 13. Baseline demographics of pneumonia patients recruited in the study.

CRP= c- reactive protein; WCC= white cell count.

Using paired t-tests, there was significantly higher bacterial phagocytosis and killing by blood neutrophils, at the end of treatment with antibiotics compared to beginning, $p=0.03$ for both comparisons. However, phagocytic capacity remains low even after completion of antibiotic therapy compared to phagocytosis at baseline in healthy volunteers (section 3.1.4). Future studies assessing neutrophil phagocytic capacity in these individuals after 4 weeks to determine if phagocytic capacity is improved, would be additive. This has not been explored in this thesis.
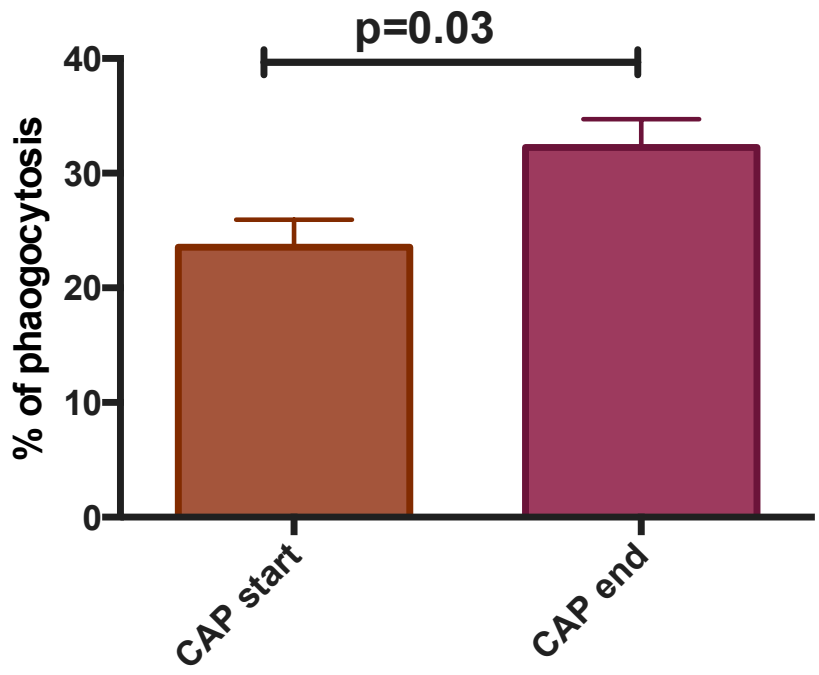
50a.
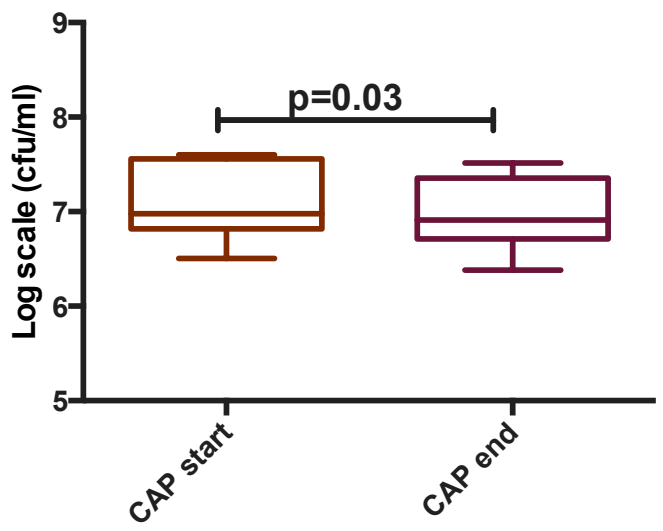
50b.

Figure 17 a&b. There was a significantly higher phagocytosis and killing after antibiotic treatment compared to beginning in pneumonia. Phagocytosis and killing was measured at 15minutes (and plates counted at 24 hours for killing). Pooled data presented as mean +/- SEM (for phagocytosis) and median +/- IQR (for killing); n=6 per group.

3.4. COMPARISON OF BRONCHIETASIS VERSUS PNEUMONIA

Once phagocytosis and bacterial killing was assessed during bronchiectasis exacerbations and pneumonia, I then compared phagocytosis and bacterial killing at the start and end in bronchiectasis to pneumonia start and end. This was done to assess the difference in neutrophil function during and after exacerbations in patients with a chronic lung disease (bronchiectasis) with those of an acute severe lung disease that fully recover (pneumonia patients with no prior history of chronic lung disease).

3.4.1. Phagocytosis

Using unpaired t-test Mann Whitney-U test, for comaprison of phagocytosis of GFP PAO1 by blood neutrophils, during exacerbation in bronchiectasis to pneumonia; there was no statistically significant difference in % phagocytosis at the beginning (p=0.7) or end of infection (p=0.1), between the two groups; figure 51.
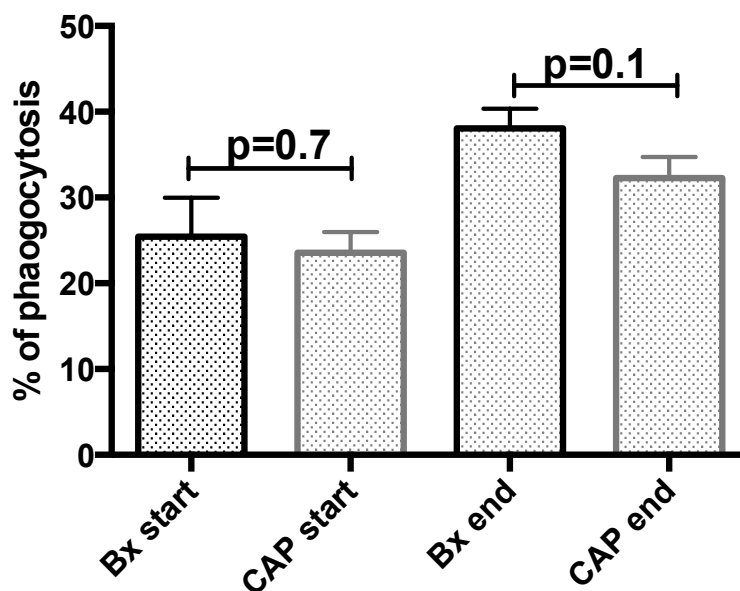
Figure 51. There was no difference in phagocytosis between bronchiectasis and pneumonia, either at the start or at the end of exacerbation. Phagocytosis was measured at 15minutes. Pooled data presented as mean +/- SEM (for phagocytosis); n=6 per group. Unpaired t tests done for both comparisons.
Bx= bronchiectasis; CAP= community acquired pneumonia.

3.4.2. Bacterial killing

Using unpaired t-test Mann Whitney-U test, for comaprison of bacterial killing of GFP PAO1 by blood neutrophils, at the beginning of exacerbation in bronchiectasis to beginning of pneumonia; there was a statistically significant difference; p=0.0007, with lower bacterial killing in bronchiectasis patients.

On comaprison of bacterial killing at the end of exacerbation in bronchiectasis to end of treatment in pneumonia; there was a statistically significant difference; p=0.003, with lower bacterial killing in bronchiectasis patients, figure 52.
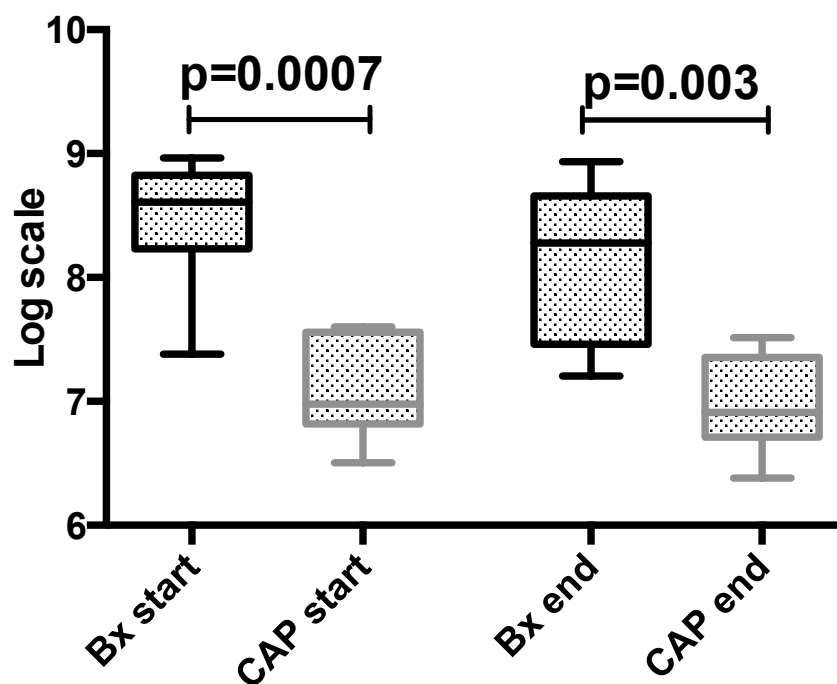
Figure 52. Significantly lesser bacterial killing at the beginning and end of exacerbation in bronchiectasis patients compared to pneumonia patients. Bacterial killing was measured at 15minutes and plates counted at 24 hours. Pooled data presented as median +/- IQR; n=6 per group. Unpaired t tests done for both comparisons.

Bx= bronchiectasis; CAP= community acquired pneumonia. Log scale is in cfu/ml.

3.5. REVERSE TRANSMIGRATING NEUTROPHILS

Once it was established that there was a distinct sub type of neutrophils in bronchiectasis, I wanted to detect if this sub type of neutrophils were reverse transmigrating neutrophils. Neutrophils were isolated from both bronchiectasis patients and healthy volunteers. Reverse migrating neutrophils are ICAM 1 high. However, there was no difference in ICAM 1 staining in bronchiectasis patients as compared to healthy volunteers, as shown in figure 53.
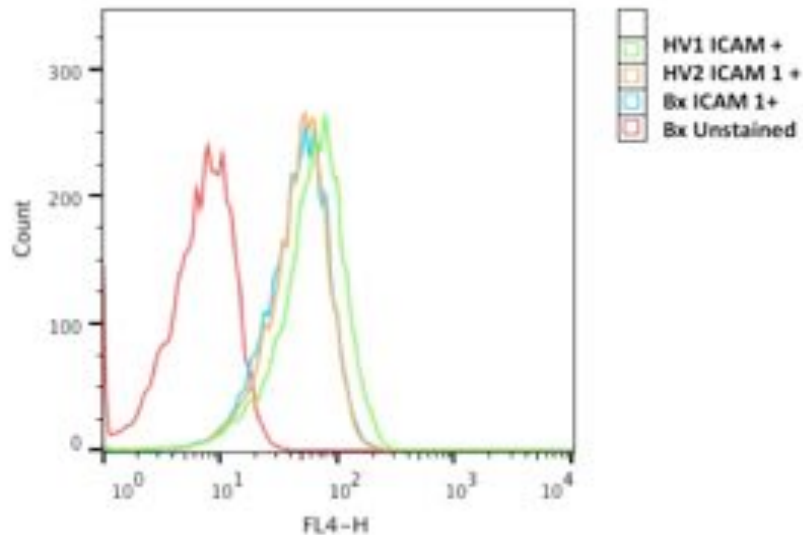
Figure 53. Overlay graphs from a representative experiment showing similar ICAM 1 antibody staining in bronchiectasis patient compared to healthy individuals.
HV= Healthy volunteers; Bx= bronchiectasis.

3.6. NEUTROPHIL EXTRACELLULAR TRAPS (NETs)

3.6.1. NETs by fluorescence

To investigate the fate of neutrophils at the end of exacerbation, I assessed NET formation at the start and end of bronchiectasis exacerbations. Blood samples were obtained from 6 bronchiectasis patients at the beginning and end of exacerbation (day 1 and day 14). Neutrophils were treated with vehicle control or Phorbol 12-myristate 13-acetate (PMA) and blood NET formation was evaluated after 4hours, by fluorescence emitted using a plate reader (figure 54).

3.6.2. NETs production during bronchiectasis exacerbation

Using paired t-tests, for comaprison there was a significant reduction in NETs produced at the end of treatment (with antibiotics) of an exacerbation, both in **untreated** neutrophils and PMA treated neutrophils (figure 54); p<0.0001 for both comparisons.
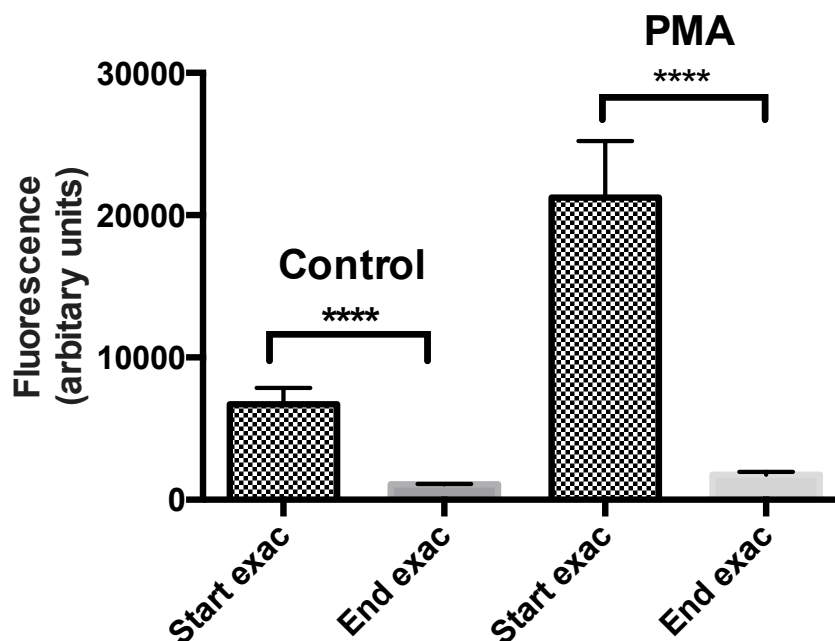
Figure 54. Reduction in NETs formation at the end of exacerbation both in vehicle and PMA treated neutrophils. NET formation was assessed 4 hours after stimulation with PMA or vehicle control, by measuring fluorescence with a plate reader. Pooled data presented as mean +/- SEM; n=6 per group. Paired t tests done for both comparisons, ****P<0.0001.

3.6.3. NETs production during pneumonia

As a control, blood samples were also obtained from 6 pneumonia patients at the beginning and end of pneumonia (day 1 and day 5) and blood NET formation was evaluated after 4 hours by fluorescence emitted by a plate reader.

Using paired t-tests, there was a significant reduction in NETs produced at the end of treatment (with antibiotics) of pneumonia, both in untreated neutrophils (p=0.0003) and PMA treated neutrophils (p=0.004) (figure 55).
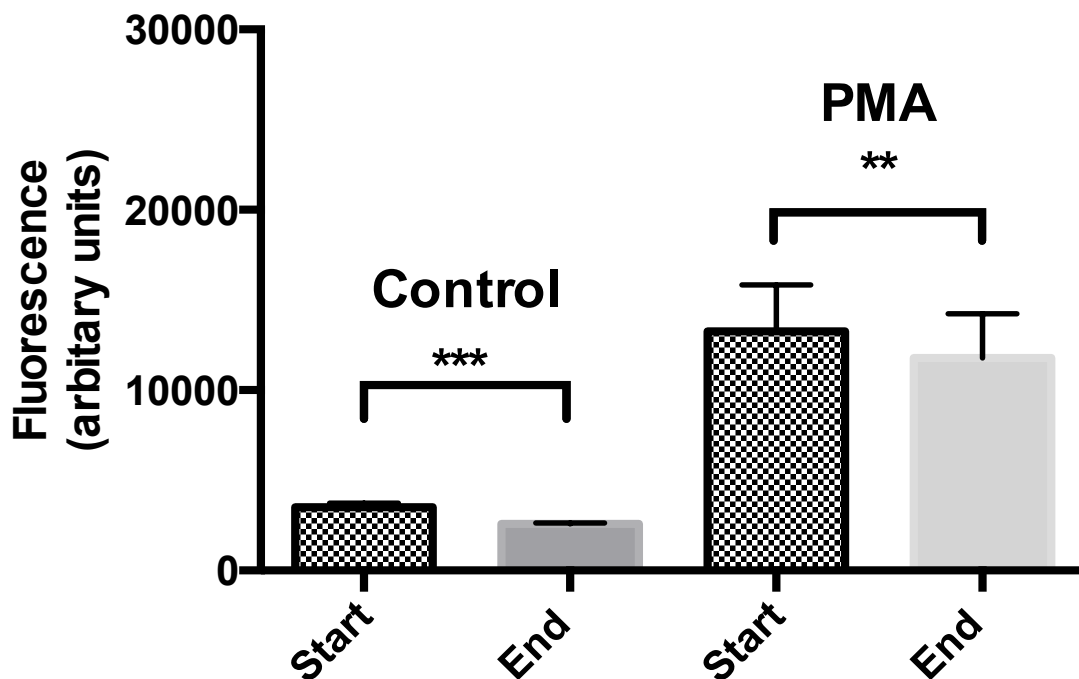
Figure 55. Reduction in NETs formation at the end of pneumonia treatment with antibiotics both in vehicle and PMA treated neutrophils. NET formation was assessed 4 hours after stimulation with PMA or vehicle control, by measuring fluorescence with a plate reader. Pooled data presented as mean +/- SEM; n=6 per group. Paired t tests done for both comparisons, **P<0.01; ***P<0.001.

3.6.4. Comparison of NETs produced in bronchiectasis exacerbations and pneumonia

I now compared the data obtained from bronchiectasis excaerbations and pneumonia, to assess the difference in NETs produced.

Using unpaired t-test Mann Whitney-U test for comparison of the start of exacerbation in bronchiectasis patients compared to pneumonia patients, there was no difference in NETs produced in **PMA treated** neutrophils, p=0.1. Using unpaired t-tests for comparison of the end of exacerbation in bronchiectasis patients to pneumonia patients, there was significantly lower NETs produced in **PMA treated** neutrophils in bronchiectasis patients, p=0.0004 (figure 56a).

Using unpaired t-test Mann Whitney-U test for comparison of the start of exacerbation in bronchiectasis patients compared to pneumonia patients, there was significantly higher NETs produced in **vehicle treated** neutrophils in bronchiectasis patients, p=0.01. Using unpaired t-tests for comparison of the end of exacerbation in bronchiectasis patients compared to pneumonia patients, there was significantly lower NETs produced in **vehicle treated** neutrophils in bronchiectasis patients, p<0.0001 (figure 56b).
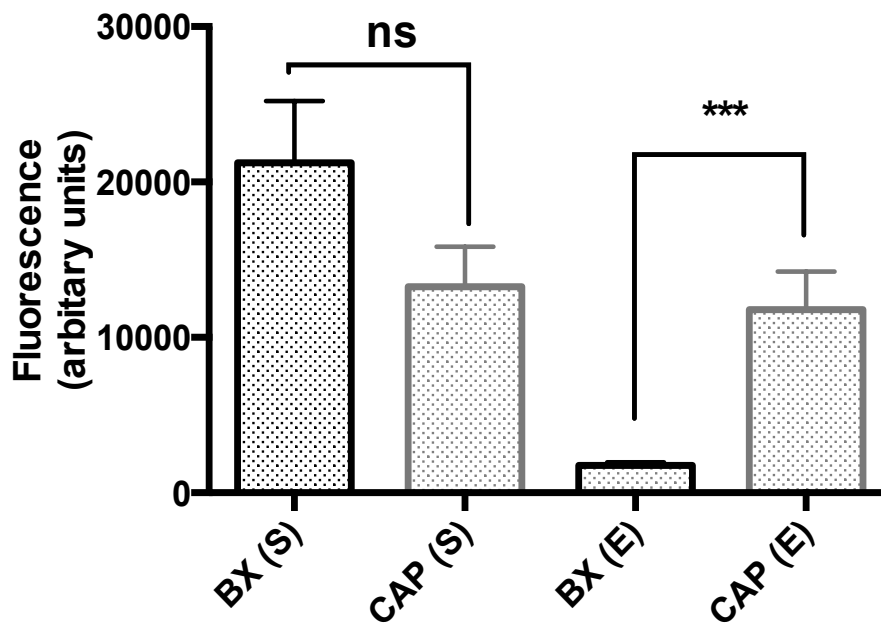
56a.
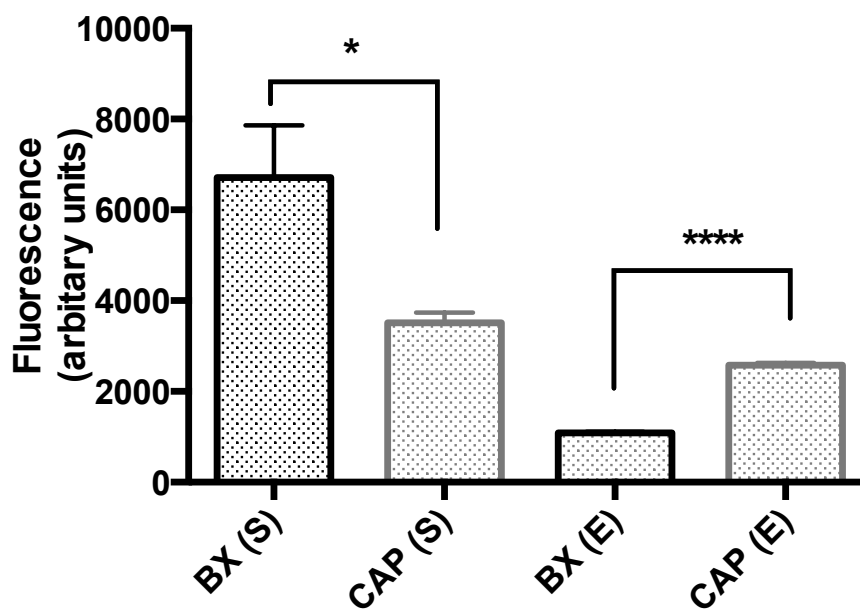
56b.

Figure 56. Higher NETs produced at the beginning (S) and significantly lower NETs produced at the end (E) of exacerbation (in vehicle treated neutrophils), in bronchiectasis compared to pneumonia. NET formation was assessed 4 hours after stimulation with PMA or vehicle control, by measuring fluorescence with a plate reader. Pooled data presented as mean +/- SEM; n=6 per group. Unpaired t tests done for both comparisons, *P<0.05, ***P<0.001, ****P<0.0001

BX= bronchiectasis; CAP= Community acquired pneumonia; E =end; S= start.

3.7. EFFECT OF LXA₄ ON NEUTROPHIL FUNCTION

My main hypothesis for this thesis is that there is failure of resolution of inflammation in bronchiectasis.

Study aim: Assess the anti-inflammatory and pro resolution effect of LXA₄ on blood and airways neutrophils in the stable state and during exacerbations in bronchiectasis.

There is enough evidence in the literature to suggest that Lipoxin A₄ plays a major anti-inflammatory and pro resolution role. However the effects of Lipoxin A₄ have not been studied in bronchiectasis. As bronchiectasis is a neutrophilic airway disease, I have studied the effects of Lipoxin A₄ on neutrophils, as described below.

Blood was obtained from 7 healthy volunteers, 6 patients with mild bronchiectasis and 6 patients with severe bronchiectasis (as calculated by the Bronchiectasis Severity Index). The following studies were done as outlined below.

3.7.1. RESULTS FROM SPONTANEOUS APOPTOSIS ASSAYS

Blood neutrophils were isolated by percoll gradient as previously described and late apoptosis (20hours) assays were done using autologous blood. Roscovitine was used as a positive control and has been described in detail in section 3.9.1.2.

- *3.7.1.1. Effect of Lipoxin A₄*

Spontaneous apoptosis was measured at 20 hours in three groups- healthy volunteers, mild bronchiectasis and severe bronchiectasis patients. Analysis by two way ANOVA, comparing healthy volunteers, mild and severe bronchiectasis showed that, after treatment with vehicle or LXA₄ 1nM, 10nM, and 100nM, LXA₄ was unable to modulate spontaneous apoptosis in any of the three groups with Bonferoni's post hoc analysis of multiple comparisons being $p=0.4$, $p=0.5$ and $p=0.4$ in healthy (figure 57a), mild (figure 57b) and severe (figure 57c) bronchiectasis, respectively.
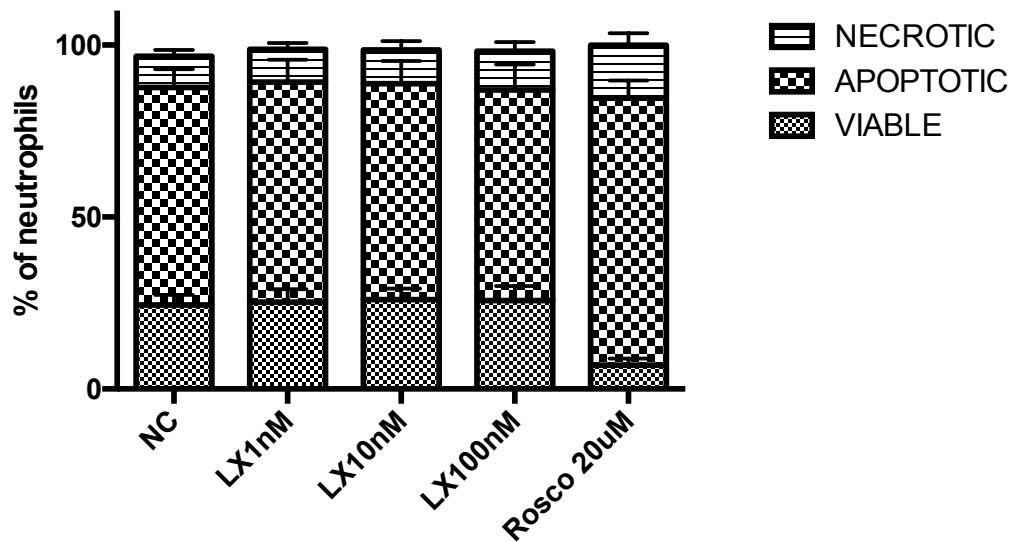
57a. Blood neutrophils from healthy volunteers were cultured for 20 hours and cell viability, apoptosis and necrosis were assessed by flow cytometry. There was no effect of LXA_4 even at a maximum dose of 100nM on spontaneous apoptosis at 20 hours. Pooled data presented as mean +/- SEM; n=6 per group. Analysis done by two way ANOVA.
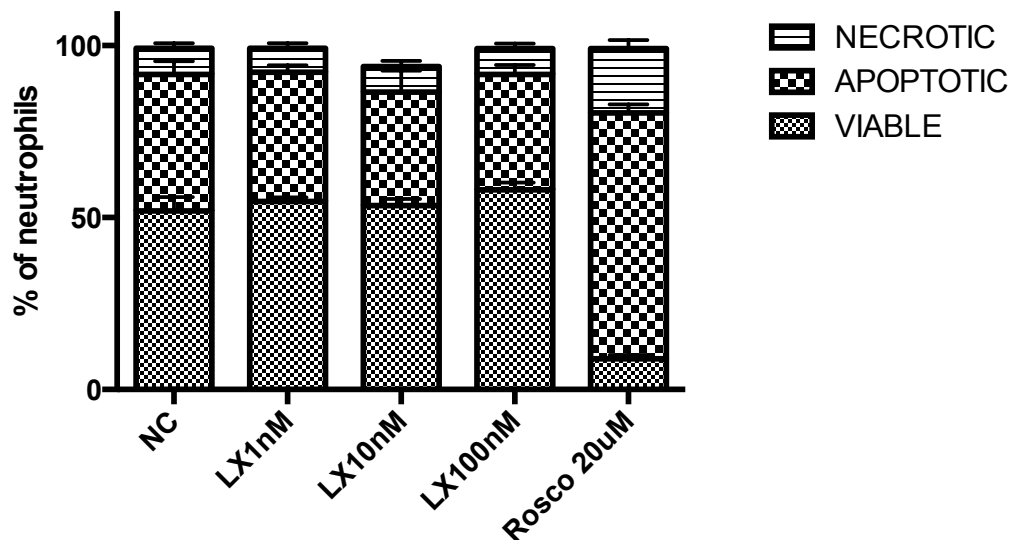
57b. Blood neutrophils from mild bronchiectasis patients were cultured for 20 hours and cell viability, apoptosis and necrosis were assessed by flow cytometry. There was no effect of LXA_4 even at a maximum dose of 100nM on spontaneous apoptosis at 20 hours. Pooled data presented as mean +/- SEM; n=6 per group. Analysis done by two way ANOVA.
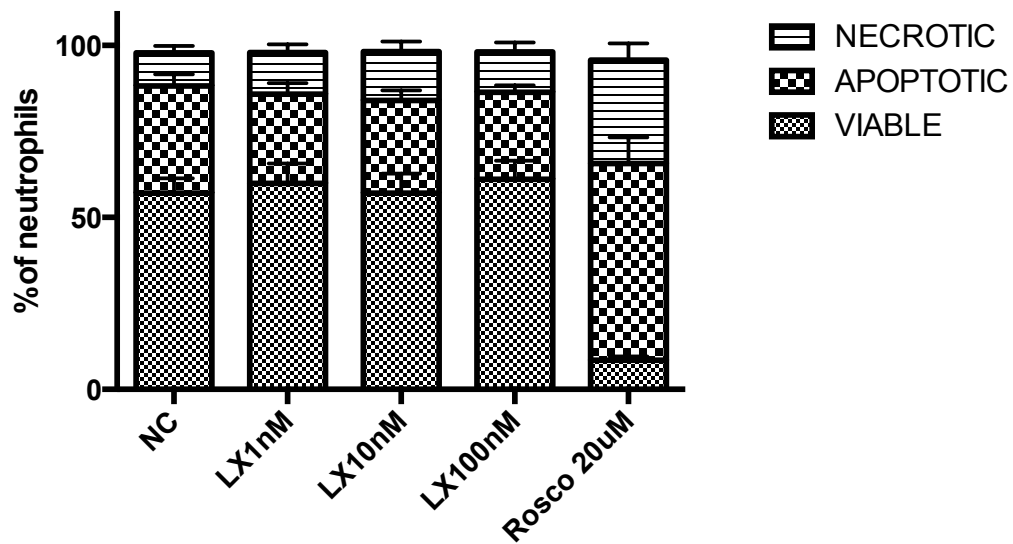
Figure 57c. Blood neutrophils from severe bronchiectasis patients were cultured for 20 hours and cell viability, apoptosis and necrosis were assessed by flow cytometry. There was no effect of LXA_4 even at a maximum dose of 100nM on spontaneous apoptosis at 20 hours. Pooled data presented as mean +/- SEM; n=6 per group. Analysis done by two way ANOVA.

LX= Lipoxin A_4; NC= negative control; rosco= roscovitine.

- *3.7.1.2. Effect of Roscovitine (figure 58)*

Two way ANOVA showed, that in **the roscovitine 20µM** treated neutrophils, there was a statistically significant difference in the percentage of apoptotic neutrophils between healthy volunteers and severe bronchiectasis, p=0.04 but not between healthy and mild bronchiectasis p=0.2. There was a statistically significant difference in in the percentage of necrotic neutrophils between healthy volunteers and severe bronchiectasis, p=0.02 but not between healthy and mild bronchiectasis p=0.4. There was no difference in the viable group, between healthy volunteers and mild bronchiectasis p=0.3 and between healthy and severe bronchiectasis p=0.5.
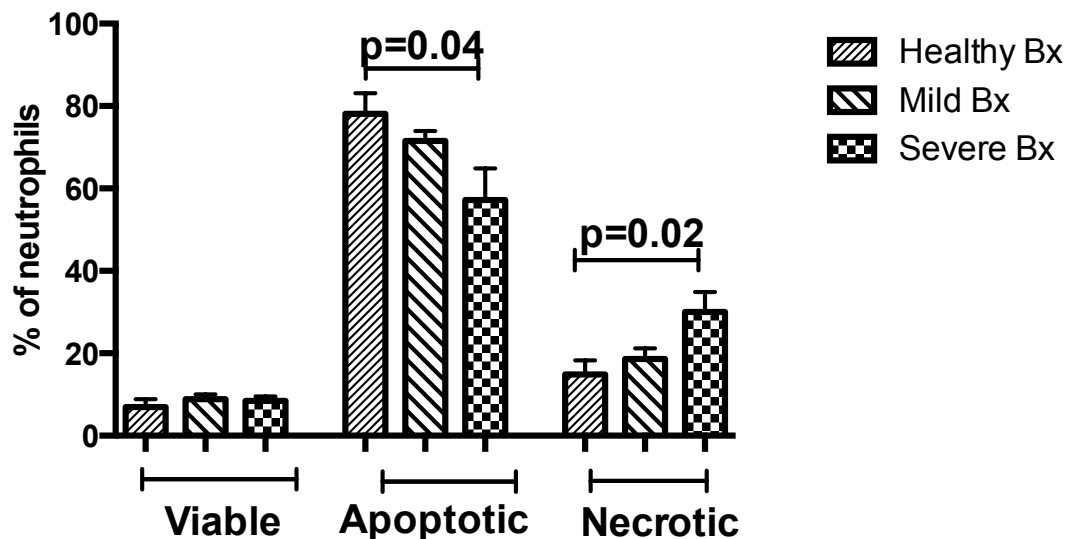
Figure 58. Blood neutrophils from healthy, mild and bronchiectasis patients were cultured for 20 hours and cell viability, apoptosis and necrosis were assessed by flow cytometry. There was a statistically significant difference in the percentage of apoptotic (p=0.04) and necrotic (p=0.02) neutrophils between healthy volunteers and severe bronchiectasis patients, in roscovitine treated neutrophils. Pooled data presented as mean +/- SEM; n=6 per group. Analysis done by one way ANOVA.

3.7.1.3. Cell counts

At 20 hours, cytocentrifuge preparations from treated and control neutrophil suspensions were fixed and stained. Cells were examined under oil immersion light microscopy and apoptotic cells were defined as cells containing darkly stained pyknotic nuclei. For each time point at least 400 cells were counted from at least 4 different fields (figure 59).
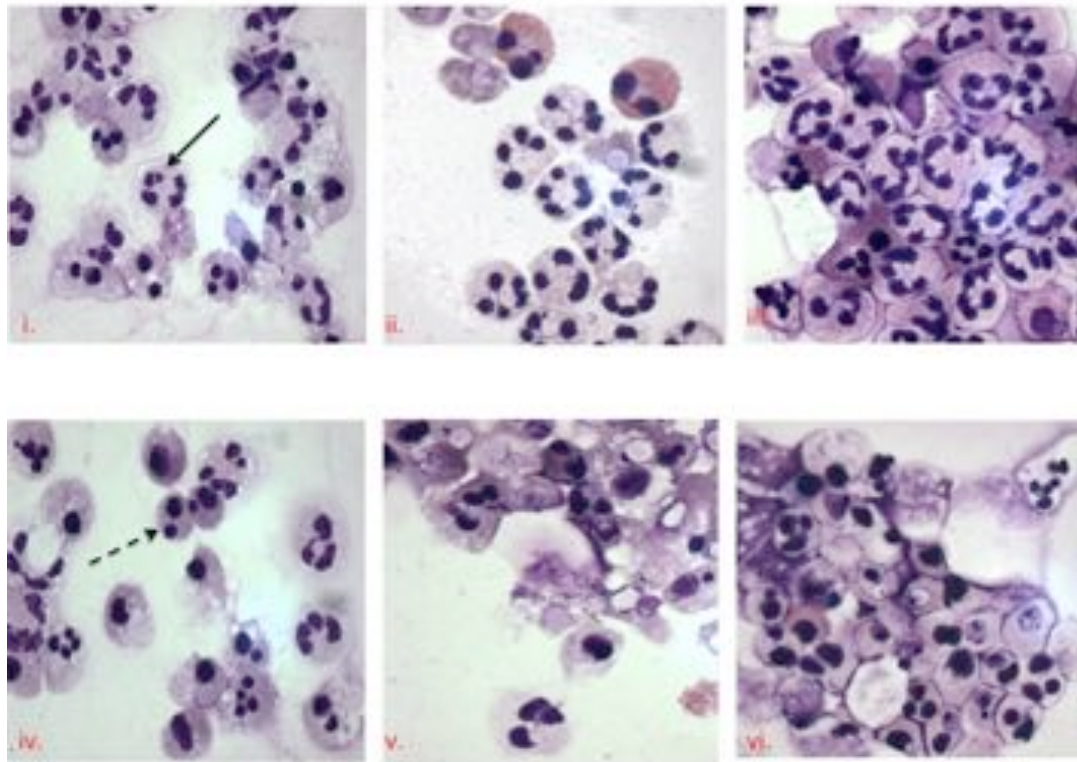
Figure 59. At 20 hours, cytocentrifuge preparations (gold standard for quantifying apoptosis) from neutrophil suspensions were also fixed and stained. Cells were examined under oil immersion light microscopy and apoptotic cells were defined as cells containing darkly stained pyknotic nuclei. For each time point at least 400 cells were counted from at least 4 different fields. Solid arrow represents healthy neutrophils with multi lobulated nuclei, more abundant in neutrophils from mild and severe bronchiectasis. Dashed arrow represents dark, pyknotic nuclei in apoptotic neutrophils - more abundant in healthy volunteers.
(i)& (iv): healthy volunteers
(ii) & (v): mild bronchiectasis
(iii) & (vi): severe bronchiectasis

3.7.2. NEUTROPHIL ACTIVATION

3.7.2.1. CD11b upregulation

Neutrophil activation leads to upregulation of CD11b and shedding of CD62L.

Once neutrophils were isolated (as described previously), they were pre treated with LXA$_4$ 1nM, 10nM and 100nM for 30 minutes and thereafter were activated with fMLF 100nM or vehicle control (PBS) for 30minutes. CD11b expression was measured by flow cytometry.

- ### *3.7.2.1.1. Activation with fMLF*

Analysis by one way ANOVA, comparing healthy volunteers, mild and severe bronchiectasis showed that in **fMLF alone (control) activated and LXA$_4$ (1nM, 10nM and 100nM) pretreated, then activated with fMLF neutrophils**, after 30 minutes, there was a change of fMLF induced CD11b upregulation by LXA$_4$ in a dose dependent manner (via setting a gate against a control)**,** in healthy (p=0.005), mild (p=0.008) and severe (p=0.01) bronchiectasis (figure 60a-c).
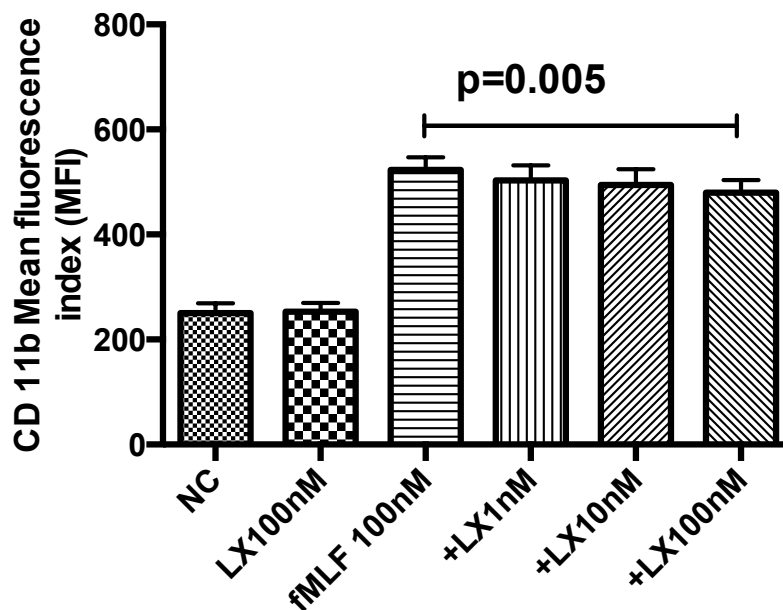
60a. Healthy volunteers: There was a dose dependent reduction of fMLF-induced activation (30 minutes) and CD11b expression by LXA$_4$; p=0.005. Pooled data presented as mean ± SEM; n=6 per group. Analysis done by one way ANOVA.
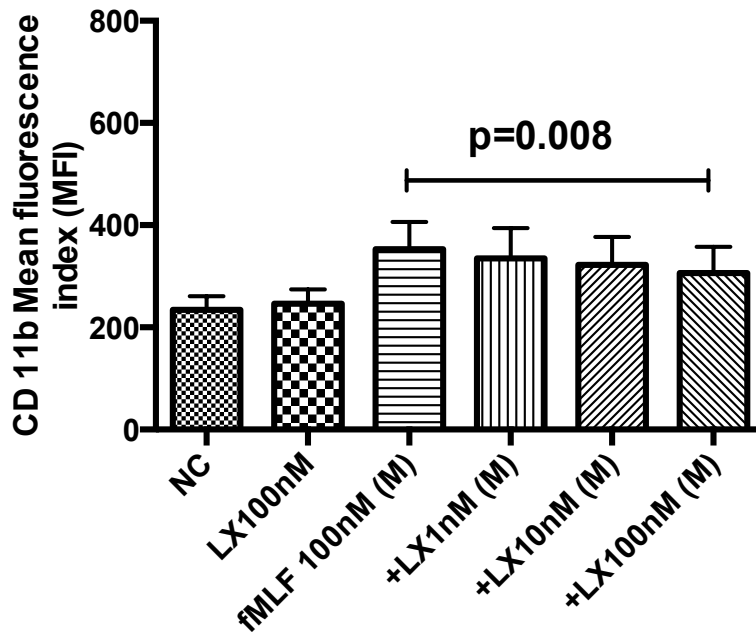
60b. Mild bronchiectasis: There was a dose dependent reduction of fMLF-induced activation (30 minutes) and CD11b expression by LXA_4; p=0.008. Pooled data presented as mean ± SEM; n=6 per group. Analysis done by one way ANOVA.
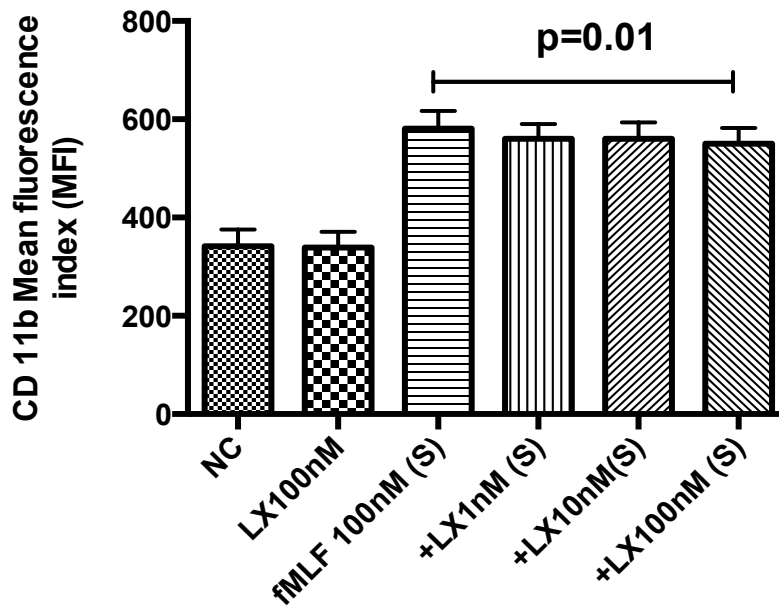
60c. Severe bronchiectasis: There was a dose dependent reduction of fMLF-induced activation (30 minutes) and CD11b expression by LXA_4; p=0.01. Pooled data presented as mean ± SEM; n=6 per group. Analysis done by one-way ANOVA.
NC= negative control; fMLF= n- formyl- methyl- leucyl- phenylalanine; LX= Lipoxin A_4.

3.7.2.2. CD62L

Neutrophil activation leads to shedding of CD62L. Once neutrophils were isolated (as described previously), they were pre treated with LXA$_4$ 1nM, 10nM and 100nM for 30 minutes and thereafter were activated fMLF 100nM or vehicle control (PBS) for 30minutes. CD62L expression was measured by flow cytometry.

- ### 3.7.2.2.2. Activation with fMLF

Analysis by one way ANOVA, comparing healthy volunteers, mild and severe bronchiectasis showed that in **fMLF alone (control) activated and LXA$_4$ (1nM, 10nM and 100nM) pretreated, then activated with fMLF neutrophils**, after 30 minutes, there was a change of fMLF induced CD62L shedding by LXA$_4$ in a dose dependent manner (via setting a gate against a control)**,** in healthy (p=0.01), mild (p=0.03) and severe (p=0.04) bronchiectasis (figure 61a-c).
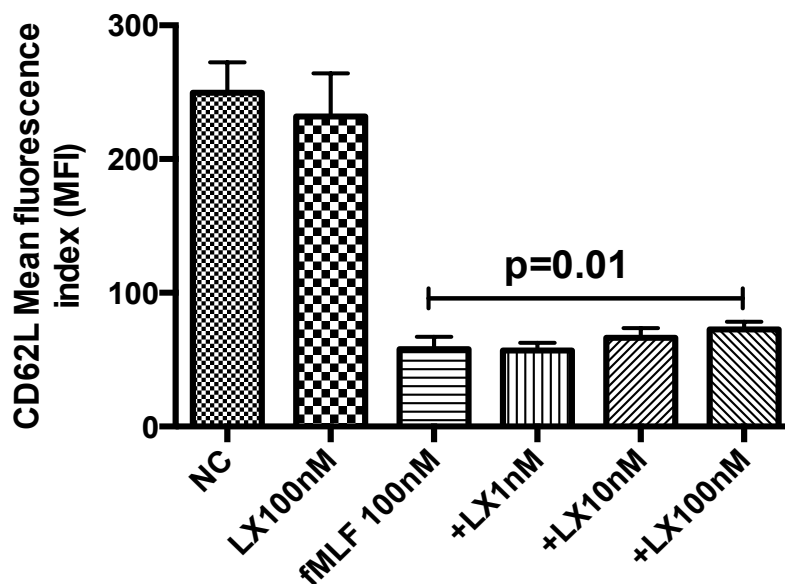
Figure 61a. Healthy volunteers: There was a dose dependent reduction of fMLF-induced activation (30 minutes) and CD62L shedding by LXA$_4$; p=0.01. Pooled data presented as mean ± SEM; n=6 per group. Analysis done by one way ANOVA.
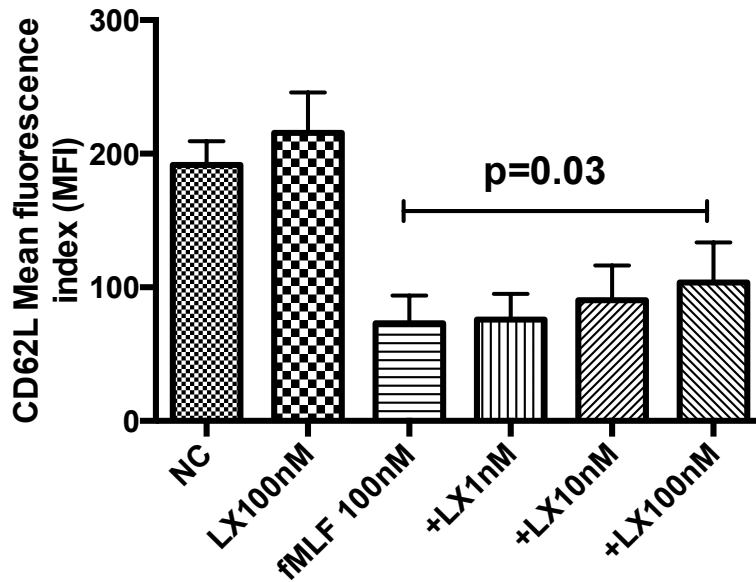
Figure 61b. Mild bronchiectasis: There was a dose dependent reduction of fMLF-induced activation (30 minutes) and CD62L shedding by LXA$_4$; p=0.03. Pooled data presented as mean ± SEM; n=6 per group. Analysis done by one way ANOVA.
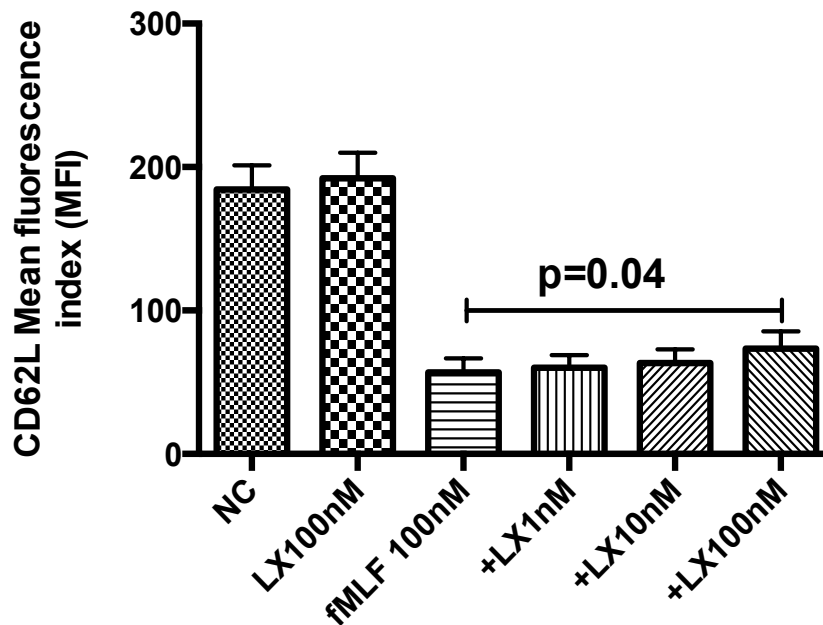
Figure 61c. Severe bronchiectasis: There was a dose dependent reduction of fMLF-induced activation and CD62L shedding by LXA$_4$; p=0.04. Pooled data presented as mean ± SEM; n=6 per group. Analysis done by one way ANOVA.

NC= negative control; fMLF= n- formyl- methyl- leucyl- phenylalanine; LX= Lipoxin A$_4$.

3.7.3. NEUTROPHIL DEGRANULATION

3.7.3.1. MPO measurement

Once isolated, neutrophils were treated with cytochalasin B (1μg/ml) for 10 minutes and fMLF (10nM) for 30 minutes, to activate neutrophils. Myeloperoxidase (MPO) was measured from supernatants by a chromogenic assay to assess neutrophil degranulation.

- *3.7.3.1.1. Activation with cytochalasin B and fMLF*

I have previously demonstrated in section 3.1.3, that there was a significantly higher release of MPO in severe bronchiectasis patients, even at baseline. Here, I was able to demonstrate that there was a significant reduction in cytochalasin B and fMLF-induced activation of neutrophils and release of MPO. A one-way ANOVA showed that there was a reduction of MPO release by Lipoxin A_4 in a dose dependant manner in healthy volunteers (p=0.02), mild bronchiectasis (p=0.02) and even in severe bronchiectasis patients (p=0.02), where significantly higher levels of MPO are released even at baseline; figure 62a-c.
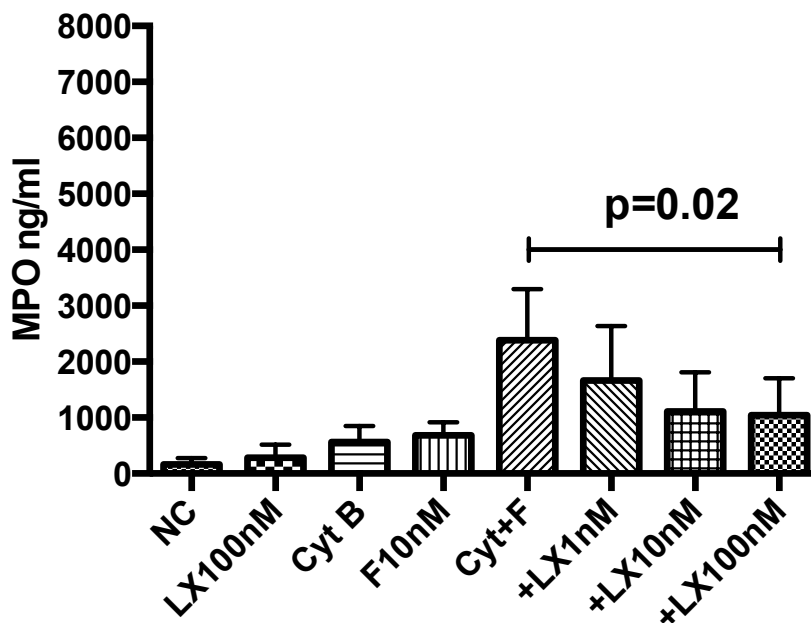
Figure 62a. Healthy volunteers: There was a dose dependent reduction of fMLF (30 minutes) and cytochalasin B (10 minutes) induced activation and release of MPO; p=0.02. Pooled data presented as mean ± SEM; n=6 per group. Analysis done by one way ANOVA.
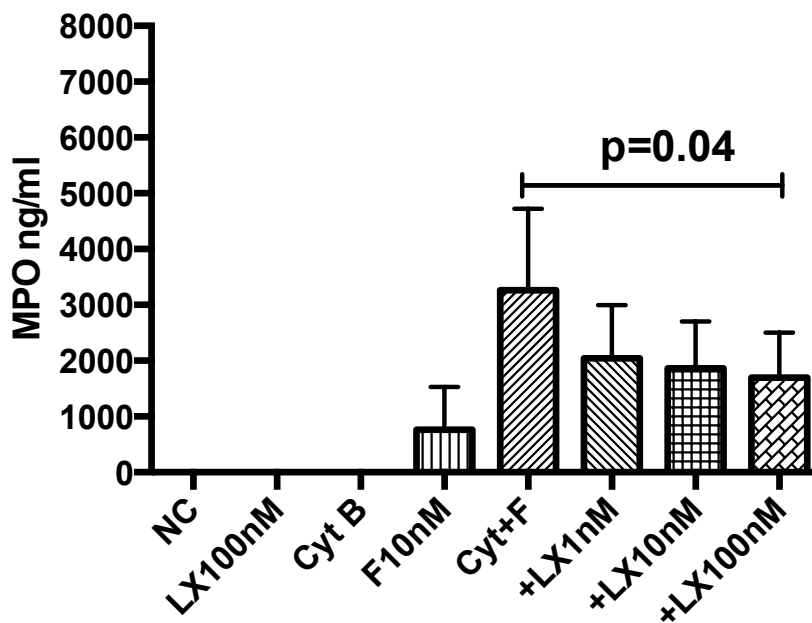
Figure 62b. Mild bronchiectasis: There was a dose dependent reduction of fMLF (30 minutes) and cytochalasin B (10 minutes) induced activation and release of MPO; p=0.04. Pooled data presented as mean ± SEM; n=6 per group. Analysis done by one way ANOVA.
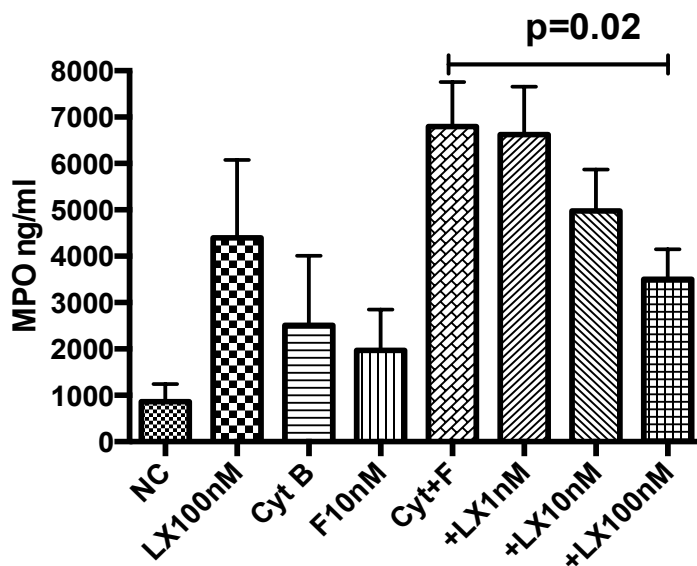
Figure 62c. Severe bronchiectasis: There was a dose dependent reduction of fMLF (30 minutes) and cytochalasin B (10 minutes) activation and release of MPO, p=0.02. Pooled data presented as mean ± SEM; n=6 per group. Analysis done by one way ANOVA.

Cyt= Cytochalasin B; F=fMLF= n- formyl- methyl- leucyl- phenylalanine; NC= negative control; LX= Lipoxin A_4.

*LX100nM on its own seems to increase MPO generation- this is due to the result from one experiment data result which skewed the overall result.

3.7.4. PHAGOCYTOSIS

3.7.4.1. Neutrophil phagocytosis of GFP labeled PAO1

Neutrophils pre treated with LXA_4, 1nM, 10nM and 100nM for 30 minutes were co incubated with opsonized GFP PAO1 for 15 minutes and phagocytosis was assessed by flow cytometry. As before, confocal images and z stacking were done (Leica TCS SP5 II, Leica Microsystems); to ensure that the GFP PAO1 were internalized by the neutrophils and were not simply adhering to the neutrophils.

- *3.7.4.1.1. Effect of Lipoxin A_4 on phagocytosis- figure 63*

There was a statistically significant difference in phagocytosis between healthy controls and mild and severe bronchiectasis, at baseline as previously demonstrated. Pre treatment with LXA_4, led to a dose dependent increase in phagocytosis in healthy volunteers (p=0.0001), mild bronchiectasis (p<0.0001) and severe bronchiectasis (p=0.03); figure 30. There was no significant difference between phagocytosis in healthy volunteers at baseline 38% (±2.2) and severe bronchiectasis treated with LX100nM 42% (±2.3); p=0.2. This demonstrates that LXA_4 is able to restore functional capacity of bronchiectasis neutrophils to that of healthy volunteers.
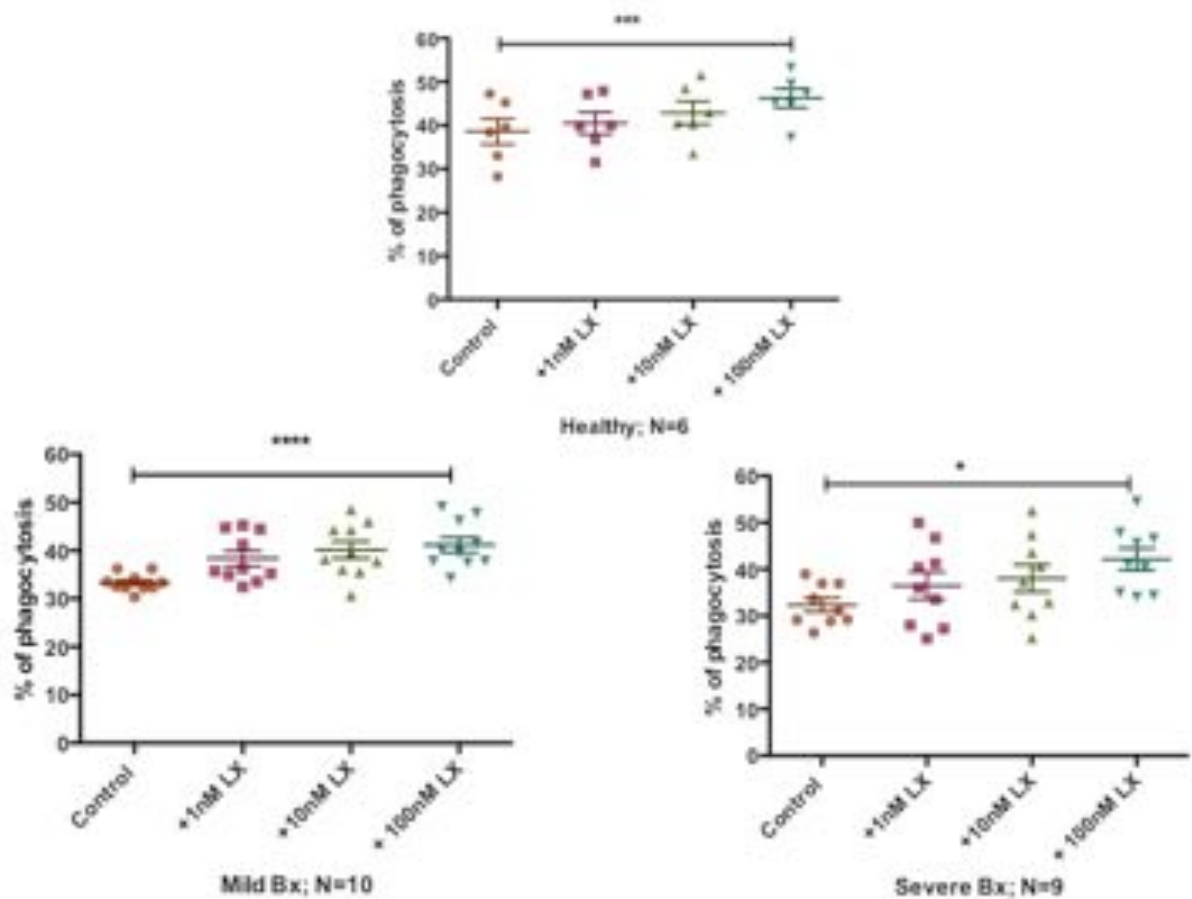
Figure 63. There was a dose dependent increase in phagocytosis with LXA$_4$, in healthy volunteers, mild and severe bronchiectasis. One-way ANOVA with Bonferroni's correction for multiple comparisons used; with p values representing the comparison of control to 1nm, 10nM and 100nM of LXA$_4$. Pooled % neutrophil phagocytosis data, showing means +/- SEM. *P<0.05; ***P<0.001; ****P<0.0001.

3.7.5. BACTERIAL KILLING

3.7.5.1. Neutrophil killing of GFP PAO1

Blood neutrophils were isolated by Percoll gradients as previously described. Blood neutrophils were co-incubated with GFP labeled bacteria for 15mins; cells were lysed and internalized bacteria were plated out and bacterial killing was assessed after 24 hours.

- ### 3.7.5.1.1. Effect of Lipoxin A_4 on bacterial killing- figure 64

There was a statistically significant difference in killing between healthy controls and mild and severe bronchiectasis, at baseline as previously demonstrated. Pre treatment with LXA_4, led to a dose dependent increase in killing in healthy volunteers (p=0.001), mild bronchiectasis (p=0.04) and severe bronchiectasis (p=0.01); figure 31. There was no significant difference between killing in healthy volunteers at baseline 7.7 log units (±0.08) and severe bronchiectasis treated with LX100nM 7.5 log units (±0.1); p=0.1. This demonstrates that LXA_4 is able to restore functional capacity of bronchiectasis neutrophils to that of healthy volunteers.
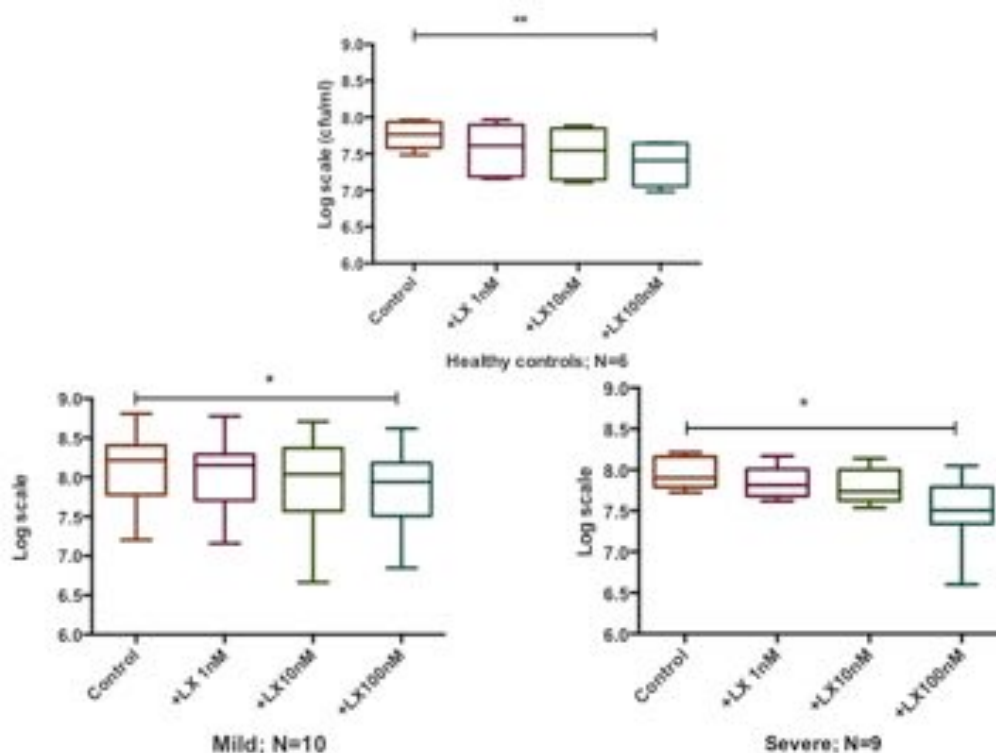
Figure 64. There was dose dependent increase in killing with LXA_4 in healthy volunteers, mild and severe bronchiectasis. One-way ANOVA with Bonferroni's correction for multiple comparisons used; with p-values representing the comparison of control to 1nm, 10nM and 100nM of LXA_4. Pooled % neutrophil killing data, showing median +/- IQR. *P<0.05; **P<0.01.

3.7.6 REACTIVE OXYGEN SPECIES

- *3.7.6.1.With fMLF as stimulant*

As previously demonstrated in section 3.1.6.1, a dose response curve was done with fMLF and a maximum response was attained at fMLF 100nM and this was taken as standard for the superoxide generation experiments performed thereafter.

- *3.7.6.2. Effect of Lipoxin on superoxide anion release*

Freshly isolated peripheral blood neutrophils from healthy volunteers (n=7), mild (n=6) and severe (n=6) were initially pretreated with Lipoxin A_4 1nM, 10nM and 100nM for 30 minutes. Subsequently, they were loaded with dihydrorhodamine for 5minutes. Loaded neutrophils were put on a heat block for 30 minutes, then 100nM fMLF added for 30 minutes and reactive oxygen species generation was measured by flow cytometry (figure 65).

Analysis by one way ANOVA showed that there was a dose dependent reduction in super oxide release by LXA_4 in the healthy volunteers, p=0.004 and in severe bronchiectasis, p=0.03. There was however no effect of superoxide release by LXA_4 in the mild bronchiectasis group; p=0.4 (figure 66).
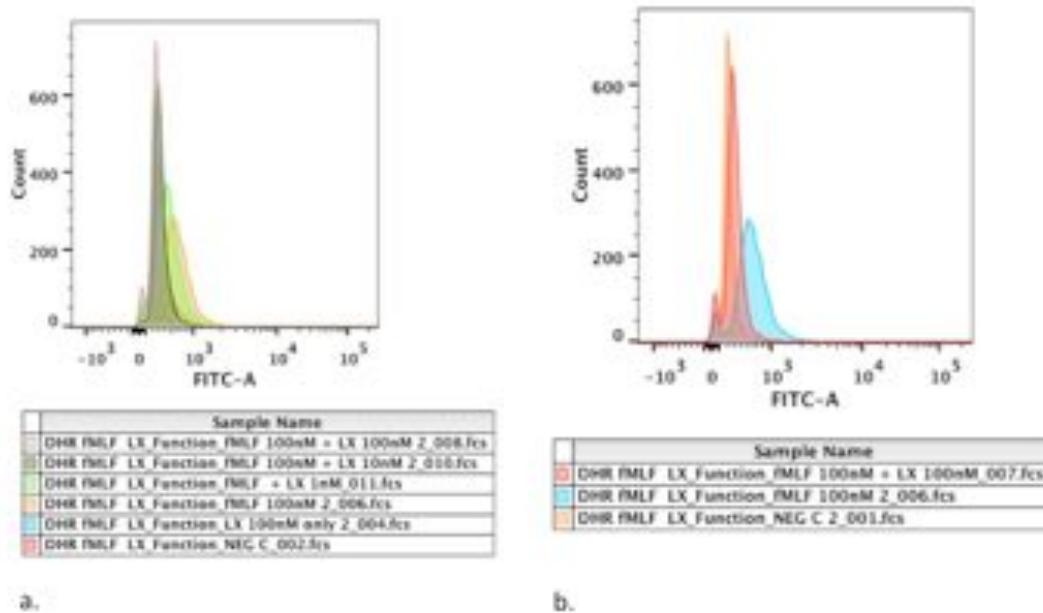
Figure 65a. Representative experiment showing overlay flow plots of LXA$_4$ 1nM, 10nM and 100nM on reduction of fMLF induced superoxide release.

b. Representative experiment showing overlay flow plots of LXA$_4$ 100nM only, on reduction of fMLF induced superoxide release.

DHR= dihydrorhodamine; fMLF= n- formyl- methyl- leucyl- phenylalanine; LX= Lipoxin A$_4$.
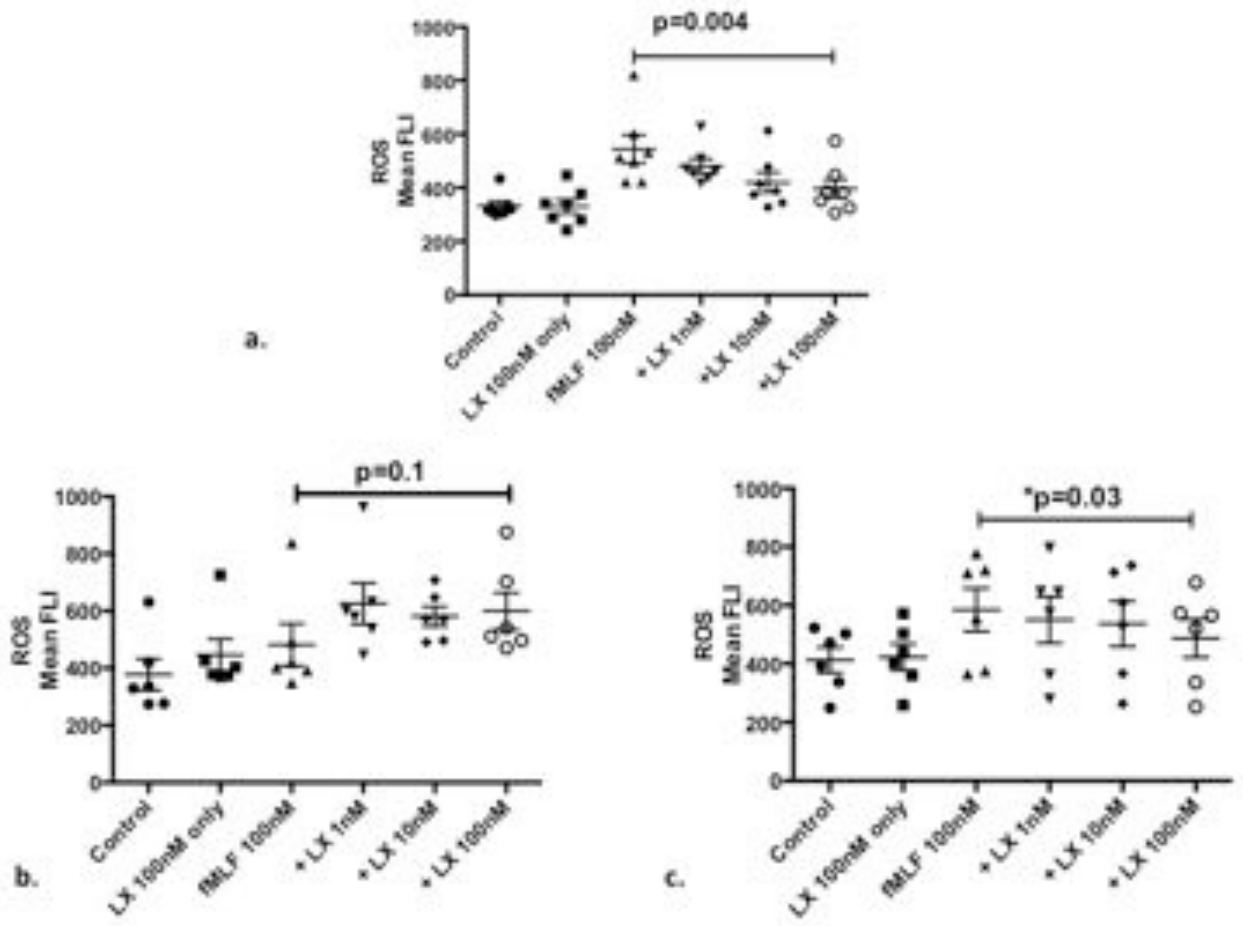
Figure 66. Individual graphs representative of cumulative data from experiments in each group. Pooled ROS data showing means ± SEM of n=7 in healthy, n=6 each in mild and severe bronchiectasis. One way ANOVA showed that there was a dose dependent reduction by LXA_4 of fMLF induced generation of reactive oxygen species in healthy (p=0.004) and severe bronchiectasis ((p=0.03) but not in mild bronchiectasis (p=0.1).

a. healthy volunteers; b. mild bronchiectasis and c. severe bronchiectasis.

fMLF= n- formyl- methyl- leucyl- phenylalanine; LX= Lipoxin A_4.

- *3.7.6.4. With PMA as stimulant (Synthetic agonist that reflect aspects of physiological activation).*

In reactive oxygen species biology, superoxide was identified as the major reactive oxygen species induced by PMA (Swindle 2002). Hence PMA has been routinely used as an inducer for endogenous superoxide production (Huang 2014). Hence after assessing reactive oxygen species generation with fMLF, I next assessed this using PMA as a stimulant, to see if similar results could be obtained.

Freshly isolated peripheral blood neutrophils from healthy volunteers (n=6) were loaded with dihydrorhodamine for 5minutes. Loaded neutrophils were put on a heat block for 30 minutes and varying doses of PMA (between 3nM and 300nM) were subsequently added for 30 minutes to obtain a concentration response curve (figure 67a). Reactive oxygen species generation was measured by flow cytometry. A response at 10nM PMA was taken as standard, as this has been widely used in the literature. In addition, at PMA 10nM, a mean fluorescence of 1000 was attained, and this corresponded to the maximum fluorescence attained by fMLF at 100nM (demonstrated in section 3.1.6). Hence, this value was taken as standard for the superoxide generation experiments performed thereafter with PMA (figure 67b).
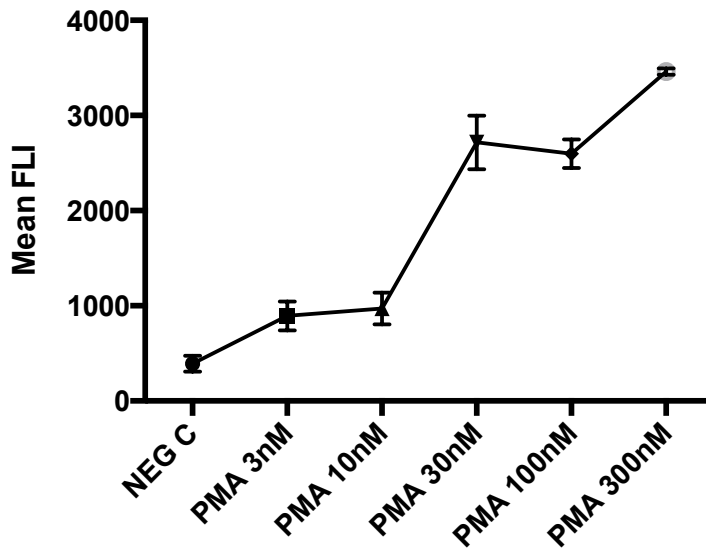
Figure 67a. Concentration response curve obtained for PMA. Neutrophils were obtained from 6 healthy volunteers and stimulated with PMA (at varying concentrations) for 30 minutes. Reactive oxygen species was measured by flow cytometry. Although a maximum response was obtained at PMA 300nM, I used PMA 10nM for the experiments below as this is the concentration at which these experiments have been done in the literature.
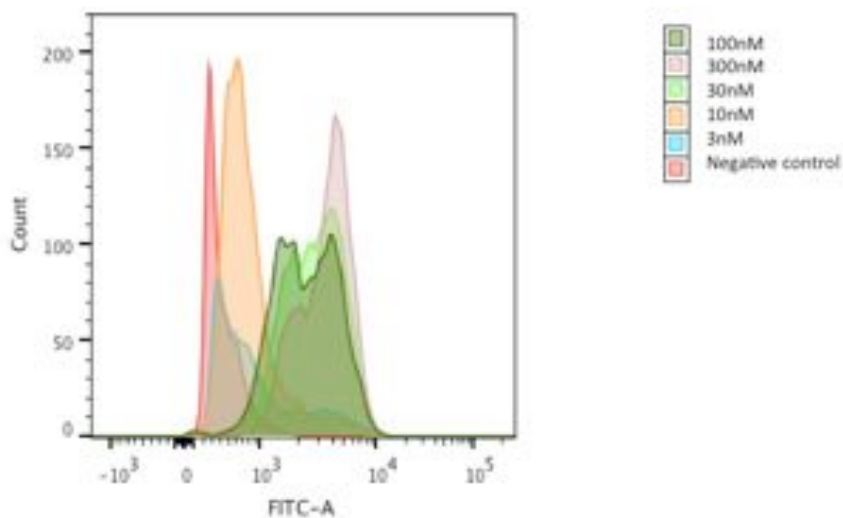
Figure 67b. Representative experiment showing overlay flow plots of PMA 3nM, 10nM, 100nM and 300nM on superoxide release.
PMA= Phorbol myristate acetate.

- ### *3.7.6.5. Effect of Lipoxin on superoxide anion release*

Freshly isolated peripheral blood neutrophils from healthy volunteers (n=6) were initially pretreated with Lipoxin A_4 1nM, 10nM and 100nM for 30 minutes. Subsequently, they were loaded with dihydrorhodamine for 5minutes. Loaded neutrophils were put on a heat block for 30 minutes and then 10nM PMA added for 30 minutes and reactive oxygen species generation was measured by flow cytometry (figure 68).

There was a dose dependent reduction in super oxide release by LXA_4 pre treated neutrophils n the healthy volunteers. Diphenyleneiodonium chloride (DPI) was used as a positive control as it is a known inhibitor of NADPH oxidase.
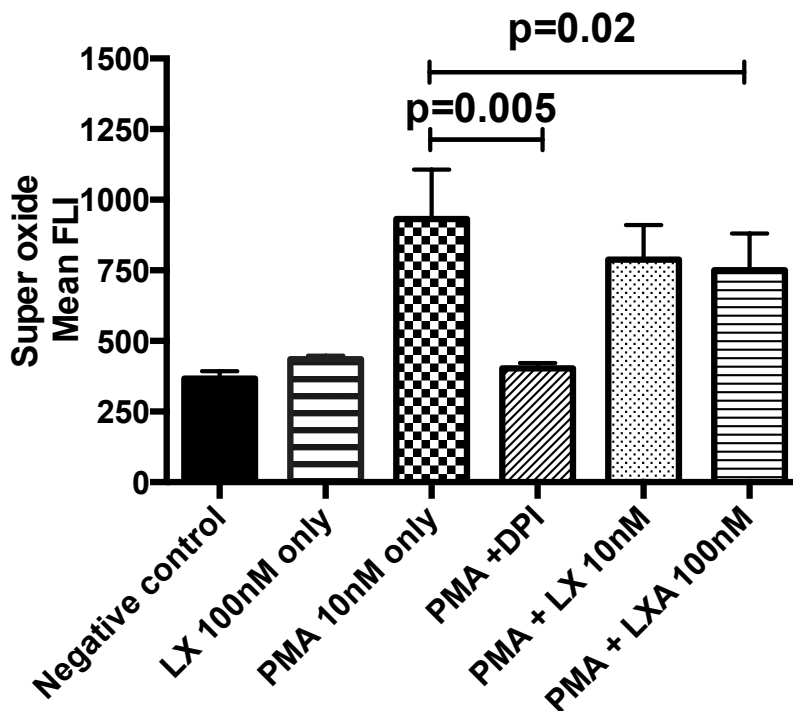
Figure 68. Graph representative of cumulative data from n=6 healthy volunteers. Pooled ROS data showing means ± SEM. One way ANOVA showed that there was a reduction by 100nM LXA_4 of PMA induced generation of reactive oxygen species; p=0.02 and by DPI; p=0.005. DPI was used as a positive control (known NADPH oxidase inhibitor).
DPI= diphenyleneiodonium chloride; LXA_4= Lipoxin A_4; PMA= Phorbol myristate acetate.

3.7.7. ALX/FPR2 EXPRESSION

Neutrophils were isolated from 7 healthy volunteers, 6 mild bronchiectasis and 6 severe bronchiectasis patients. Neutrophils were treated with FPR1 receptor agonist fMLF 100nM (or vehicle), ALX/FPR2 receptor agonist LXA$_4$ 100nM (or vehicle) and ALX/FPR2 antagonist WRW4 100nM [$C_{61}H_{65}N_{15}O_6$] (or vehicle). ALX/FPR2 antibodies were added and ALX/FPR2 expression was assessed by flow cytometry. Key findings are summarized in figure 69. ALX/FPR2 internalization was not measured in these experiments.

One-way ANOVA was done for each of the different conditions (i.e no treatment, treatment with LXA$_4$ 100nM alone, treatment with WRW4 and finally treatment with LXA$_4$ and WRW4 together), separately and has been then put together on a single graph.

At baseline, there was higher detection of ALX/FPR2 receptor in healthy volunteers compared to mild and severe bronchiectasis, although this failed to reach statistical significance; p=0.07. There was statistically significant difference in ALX/FPR2 expression in healthy volunteers compared to mild bronchiectasis, with more expression detected in healthy volunteers; p=0.03, when neutrophils were pre treated with LXA$_4$ 100nM.

Similarly, when pre treated with WRW4 100nM, there was higher detection of ALX/FPR2 receptor in healthy volunteers compared to mild and severe bronchiectasis, although this failed to reach statistical significance; p=0.09.

Finally, pre treatment with WRW4 100nM and LXA$_4$ 100nM, led to higher detection of ALX/FPR2 receptor in healthy volunteers compared to mild and severe bronchiectasis, although this too failed to reach statistical significance in all three conditions; p=0.08 (figure 69).
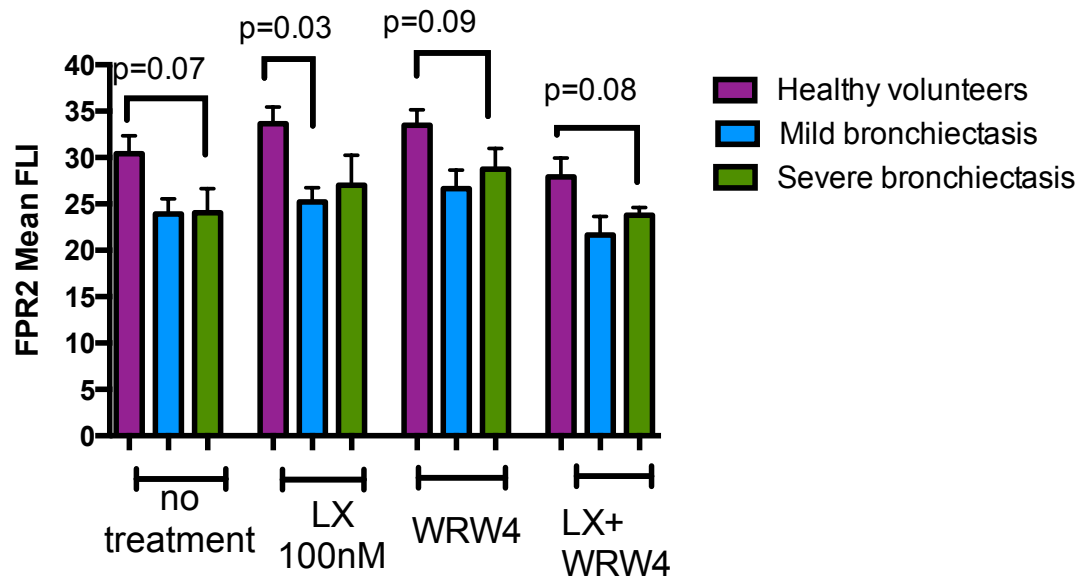
F

igure 69. Pooled ALX/FPR2 expression data showing means ± SEM, from healthy volunteers (n=7), mild bronchiectasis (n=6) and severe bronchiectasis (n=6), subject to 4 different treatment conditions. One way ANOVA showed that there was a trend towards increase ALX/FPR2 expression in healthy volunteers, in untreated neutrophils, neutrophils treated with WRW4 and neutrophils treated with LX and WRW4, compared to mild and severe bronchiectasis; p=0.07, p=0.09 and p=0.08 respectively. There was significantly higher ALX/FPR2 expression in healthy volunteers, in neutrophils pre treated with LX compared to mild bronchiectasis; p=0.03.

WRW4= selective ALX/FPR2 receptor signaling antagonist, LX= Lipoxin A$_4$.

3.7.7.1. FUNCTIONAL ASSESSMENT OF ALX/FPR2 EXPRESSION

Next I wanted to establish the functional assessment of ALX/FPR2 expression. For this, blood neutrophils once isolated were stimulated with cytochalasin B (1µg/ml) for 10 minutes and fMLF 10nM for 30 minutes. This would cause neutrophils to activate and degranulate, releasing MPO. Neutrophils were first treated with WRW4 100nM or vehicle control and then treated with Lipoxin A_4. MPO was measured by a chromogenic assay and results were analyzed.

There was a statically significant reduction of fMLF induced MPO generation by Lipoxin A_4 in a dose dependent manner, as previously demonstrated; p=0.02 (by one way ANOVA; figure 70). Simultaneously performed experiments with the ALX/FPR2 receptor antagonist WRW4 showed that LXA_4 induced attenuation of MPO production was reversed by WRW4 100nM; p=0.07 (by one way ANOVA). This thereby demonstrates that the effect of LXA_4 in MPO release is ALX/FPR2 dependent.
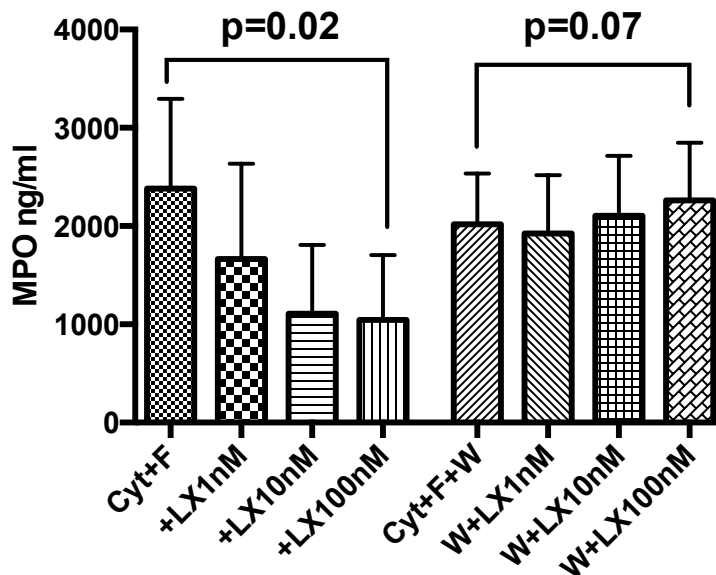
Figure 70. Pooled ALX/FPR2 expression data showing means ± SEM, n=6 in each group. There was a dose dependent reduction of fMLF (30 mins) induced MPO generation by LXA_4, p=0.02; and this was partly reversed by pre treating with WRW4 (for 30 mins); p=0.07. Cyt= Cytochalasin B; F=fMLF= n- formyl- methyl- leucyl-phenylalanine; LX= Lipoxin A_4; WRW4= selective ALX/FPR2 receptor signaling antagonist.

3.8. BRONCHIECTASIS EXACERBATIONS

3.8.1. BLOOD

3.8.1.1. Phagocytosis

6 severe bronchiectasis patients were recruited. All patients received intravenous antibiotics for 14 days. Patients were reviewed on Day 1, before the start of antibiotics and on day 14 after completing their last dose of antibiotics. Blood was taken on both occasions and peripheral blood neutrophils were isolated, as previously described. Neutrophils were pre treated with vehicle control or LX 1nM, LX 10nM or LX100nM. Phagocytosis of opsonized GFP labeled PAO1 was recorded, after co incubating pre treated neutrophils with GFP labeled PAO1 for 15 minutes. The trend was an improvement in phagocytosis in all the patients. One way ANOVA showed that there was a dose dependent increase in phagocytosis with Lipoxin A_4 both at the beginning and at the end of the exacerbation; $p<0.0001$ and $p=0.0002$ respectively, with highest phagocytosis being induced by Lipoxin A_4 100nM (figure 71).
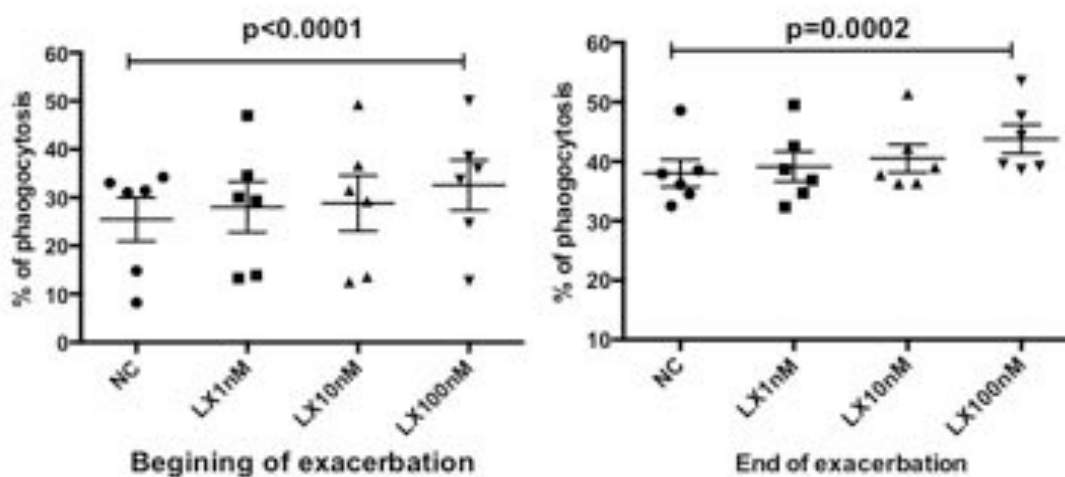
Figure 71. Significantly higher phagocytosis by LXA_4 both at the start and end of exacerbation (by one way ANOVA), $p<0.0001$ and $p=0.0002$ respectively. Neutrophils were pre treated with LXA_4 for 30 minutes and phagocytosis was measured after 15 minutes of co-incubation with GFP PAO1. Pooled data presented as mean ± SEM for n=6 per group. NC= negative control; LX= Lipoxin A_4.

3.8.1.2. Bacterial killing

Blood neutrophils (from n=6 bronchiectasis patients) were co-incubated with GFP labeled bacteria for 15mins. For bacterial killing, cells were lysed (after 15 minutes co-incubation of neutrophils with GFP PAO1) and internalized bacteria were plated out and bacterial killing was assessed after 24 hours. Killing of GFP PAO1 was recorded. One way ANOVA showed that there was a dose dependent increase in blood neutrophil killing of GFP labeled PAO1 with Lipoxin A_4 both at the beginning and at the end of the exacerbation; p=0.01 and p=0.0007 respectively (figure 72).

	Control	LX 1nM	LX10nM	LX 100nM
Start of exacacerbation	6.6 (0.2)	6.5 (0.2)	6.4 (0.3)	6.0 (0.2)
End of exacerbation	6.4 (0.2)	6.3 (0.3)	6.2 (0.2)	5.9 (0.2)

Table 14: Colony counts in log units (standard error of mean), at the start and end of exacerbation.
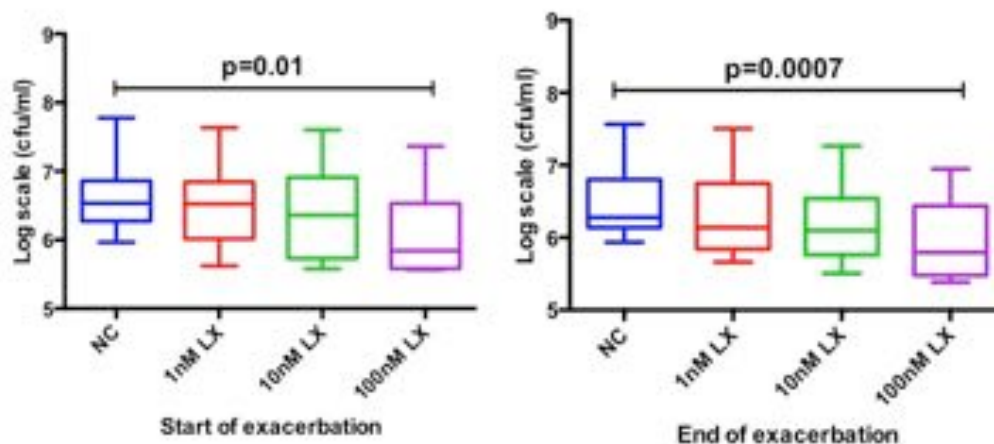
Figure 72. Significantly higher bacterial killing by LXA$_4$ both at the start and end of exacerbation (by one way ANOVA), p=0.01 and p=0.0007 respectively. Neutrophils were pre treated with LXA$_4$ for 30 minutes and killing was measured after 15 minutes of co-incubation with GFP PAO1 and then reading plates after 24 hours. Pooled data presented as median (IQR) for n=6 per group.
NC= negative control; LX= Lipoxin A$_4$.

3.8.1.3. Blood Lipidomics

To evaluate, if there was a change in the interplay of the pro inflammatory and anti inflammatory mediators of arachidonic acid, during exacerbations, I measured certian key metabolites in serum. Pro inflammtory mediators measured were PGE2, 5 HETE, 15 HETE, 9 HODE and LTB_4. Blood was obtained from the 6 patients at the start (Day 1) and end of exacerbation (Day 14) after being treated with intravenous antibiotics (same patients as in the preceding section). Serum samples were prepared with 50% methanol and then subsequently the metabolites were obtained by Liquid Chromatography-Mass Spectometry. . Analysis of the mediators showed that there was no significant reduction of PGE2, 5 HETE, 15 HETE, 9 HODE or LTB_4, after end of treatment with antibiotics (figure 73). Lipoxin was not detected in any of the samples.
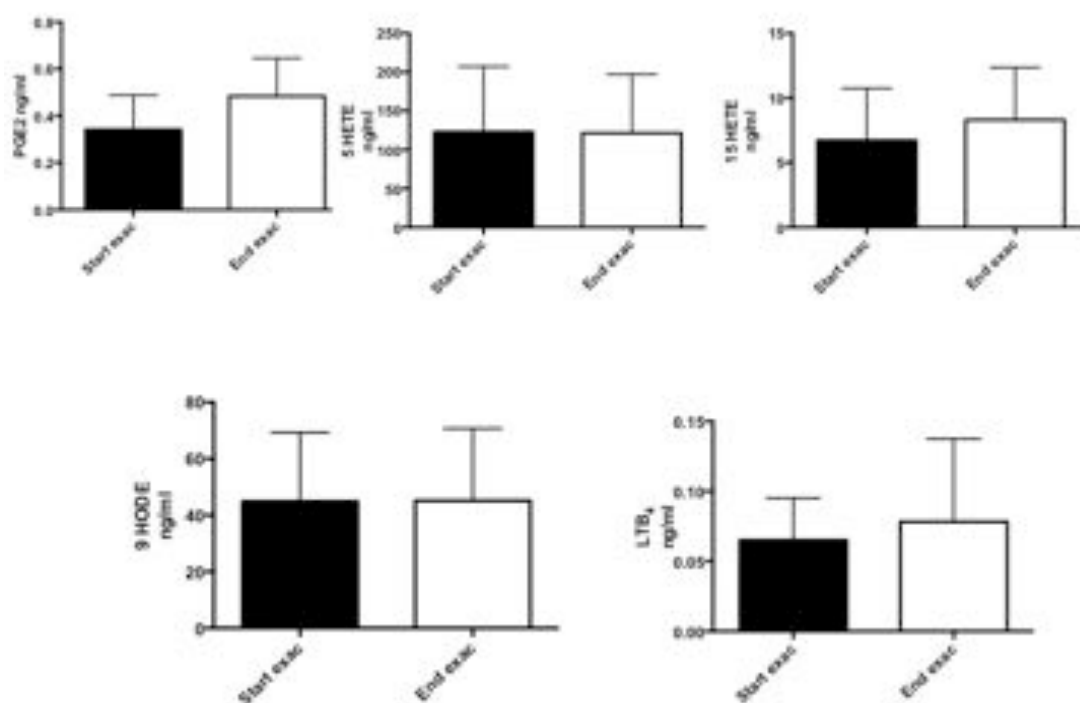
Figure 73. No significant change in blood lipidomics at the start and end of exacerbation, obtained from 6 bronchiectasis patients. Pooled data presented as mean ± SEM. Paired t tests used for all comparisons. p>0.1 for all comparisons.
5 HETE (hydroxyeicosapentanoic acid), 15 HETE hydroxyeicosapentanoic acid), 9-HODE (hydroxyoctadecadienoic acid), LT (leukotriene) B_4 and PG (Prostaglandin) E2.

3.8.2. SPUTUM

3.8.2.1. Phagocytosis

Once I assessed the role of Lipoxin A_4 on blood neutrophils during exacerbations, I wanted to assess its role on sputum neutrophils during exacerbations. Sputum from the same 6 bronchiectasis patients (patients who took part in the exacerbation study in section 3.8.1) was obtained and airways neutrophils isolated as described in the methods chapter. Airways neutrophils were pre treated with vehicle control or LX100nM. Phagocytosis of opsonized GFP labeled PAO1 was recorded, after co incubating pre treated neutrophils with GFP labeled PAO1 for 60 minutes. Results at the beginning and end of exacerbation were recorded. Paired t tests showed that there was no significant difference in phagocytosis with Lipoxin A_4 either at the beginning or at the end of the exacerbation; p=0.8 and p=0.9 respectively (figure 74).
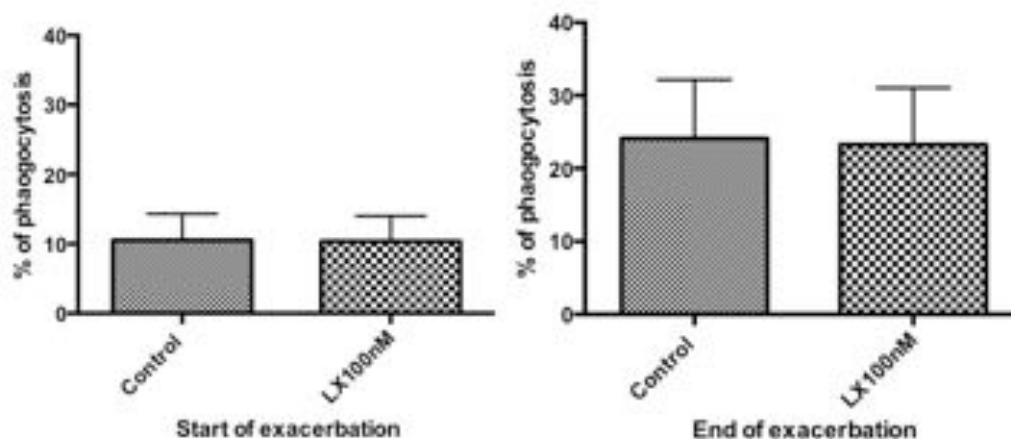
Figure 74. No increase in airways neutrophil phagocytosis of GFP labeled PAO1 by Lipoxin A_4 100nM, at the beginning or end of exacerbation, p=0.8 and p=0.9 respectively (by paired t-tests). Neutrophils were pre treated with LXA$_4$ for 60 minutes and phagocytosis was measured after 60 minutes of co-incubation with GFP PAO1. Pooled data presented as mean ± SEM for n=6 per group.
LX= Lipoxin A_4

3.8.2.2. Bacterial killing

Airways neutrophils (from n=6 bronchiectasis patients) were co-incubated with GFP labeled bacteria for 60mins. For bacterial killing, cells were lysed (after 60 minutes co-incubation of neutrophils with GFP PAO1) and internalized bacteria were plated out and bacterial killing was assessed after 24 hours. Killing of GFP PAO1 was recorded. Paired t-tests showed that there was a significant increase in airways neutrophil killing of GFP labeled PAO1 with Lipoxin A_4 100nM both at the beginning and at the end of the exacerbation; p=0.02 and p=0.01 respectively (figure 75). Colony counts at the start and end of exacerbation in control and in airways neutrophils treated with Lipoxin A_4 100nM are shown in table 15.

	Control	LX 100nM
Start of exacerbation	7.1 (0.2)	6.7 (0.3)
End of exacerbation	6.8 (0.2)	6.6 (0.2)

Table 15: Colony counts in log units (standard error of mean), at the start and end of exacerbation.
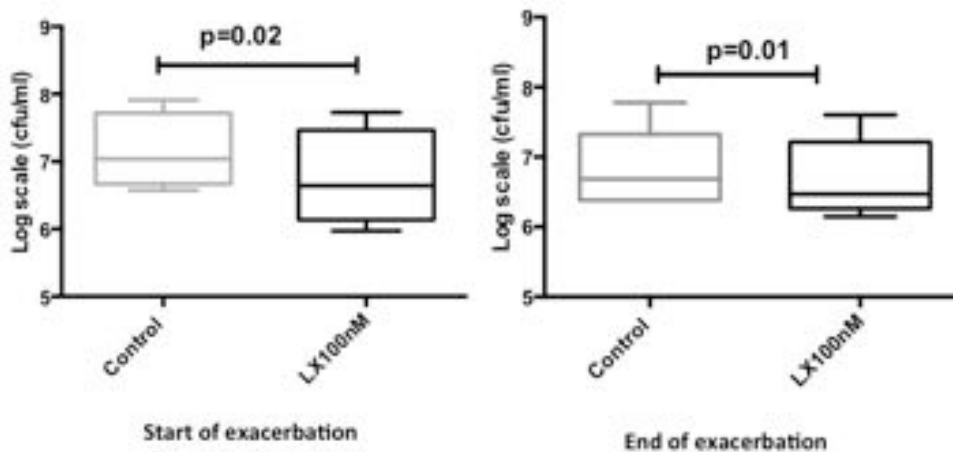
Figure 75. Significantly higher bacterial killing of GFP PAO1 by LXA_4 100nM both at the start and end of exacerbation (by paired t-tests), p=0.02 and p=0.01 respectively. Airways neutrophils were pre treated with LXA_4 for 30 minutes and killing was measured after 60 minutes of co-incubation with GFP PAO1 and then reading plates after 24 hours. Pooled data presented as median (IQR) for n=6 per group.

3.8.2.3. Sputum lipidomics

To evaluate, if there was a change in the interplay of the pro inflammatory and anti inflammatory mediators of arachidonic acid, during exacerbations, I measured certain key metabolites in sputum, similar to what was done in serum. Pro inflammatory mediators measured were PGE2, 5 HETE, 15 HETE, 9 HODE and LTB_4. Sputum was obtained from the 6 patients at the start (Day 1) and end of exacerbation (Day 14) after being treated with intravenous antibiotics (same patients as in the preceding section). Sputum samples were prepared with 50% methanol and then subsequently the metabolites were obtained by LC-MS. Sputum lipidomics was done on patient samples at the beginning and end of an exacerbation, treated with antibitoics. There was a reduction in 15 HETE, p=0.03 and LTB_4 although this just failed to reach statistical significance, p=0.08. Although there was a trend, there was no significant reduction in reduction of PGE2 and 5 HETE p=0.4 and p=0.1 respectively, after end of treatment with antibiotics (figure 76).
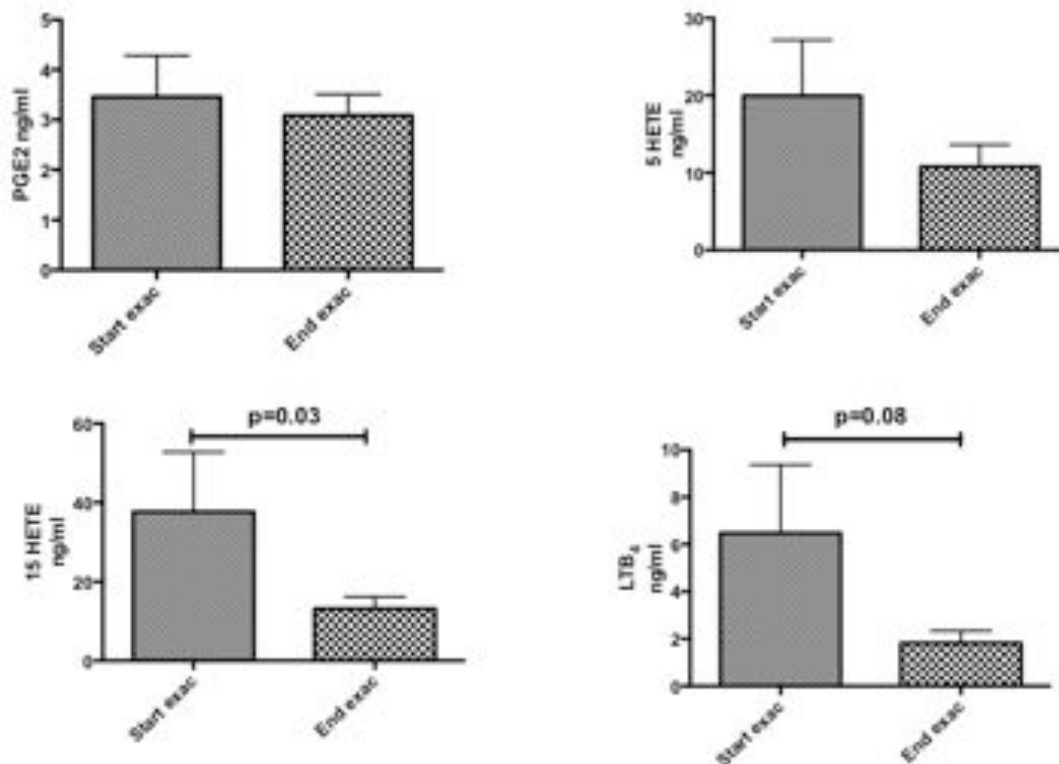
Figure 76. Significant reduction in 15HETE p=0.03 and reduction (not statistically significant) in LTB_4 p=0.08 in sputum, at the end of treatment with antibiotics. No significant change in PDE2 and 5HETE. Pooled data presented as mean ± SEM, n=6 per group. Paired t tests used for all comparisons. 5 HETE (hydroxyeicosapentanoic acid), 15 HETE hydroxyeicosapentanoic acid), 9-HODE (hydroxyoctadecadienoic acid), LT (leukotriene) B_4 and PG (Prostaglandin) E2.

3.9. COMMUNITY ACQUIRED PNEUMONIA

Study aim: Assess the anti-inflammatory and pro resolution effect of LXA_4 on neutrophils at onset and post treatment of community acquired pneumonia.

3.9.1. Phagocytosis

6 community acquired pneumonia patients (with no comorbidities) who were admitted to hospital, were recruited. All patients received intravenous (co-amoxiclav) and oral (clarithromycin) antibiotic therapy. *Streptococcus pneumoniae* was isolated in 4 of the 6 patients and no bacteria could be isolated from 2 patients. Results at the beginning (day1) and end of infection (day 5) were recorded. Blood neutrophils were co-incubated with GFP labeled bacteria for 15mins. Phagocytosis was measured by flow cytometry immediately. For bacterial killing, cells were lysed (after 15 minutes co-incubation of neutrophils with GFP PAO1) and internalized bacteria were plated out and bacterial killing was assessed after 24 hours. These were the same patients who took part in previous section on community-acquired pneumonia (section 3.3)

One way ANOVA showed that there was a dose dependent increase in phagocytosis with Lipoxin A_4 both at the beginning and at the end of infection; p=0.01 and p=0.03 respectively (figure 77).

Baseline demographics of the 6 patients are given in section 3.3.1.
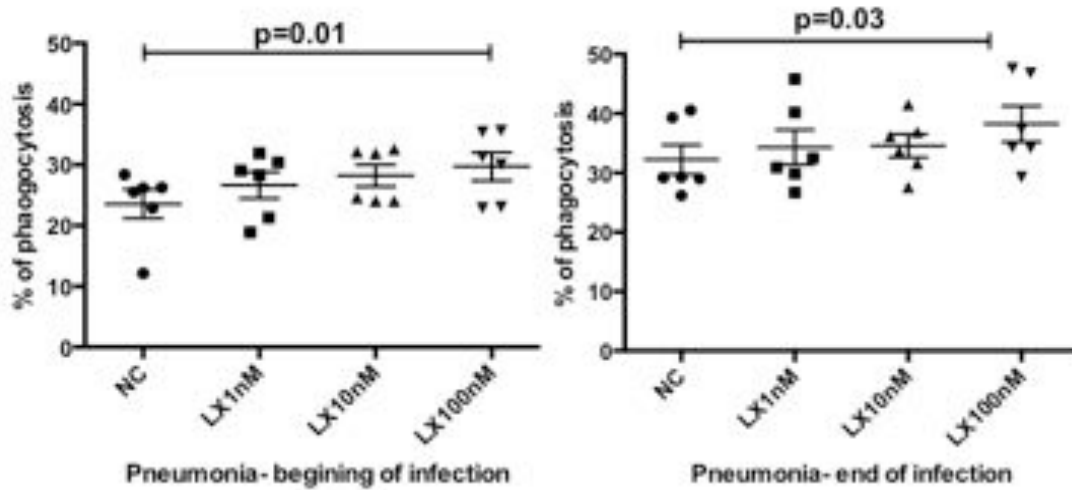
Figure 77. Significantly higher phagocytosis by LXA$_4$ both at the start and end of exacerbation (by one way ANOVA), p=0.01 and p=0.03 respectively. Neutrophils were pre treated with LXA$_4$ for 30 minutes and phagocytosis was measured after 15 minutes of co-incubation with GFP PAO1. Pooled data presented as mean ± SEM for n=6 per group. NC= negative control; LX= Lipoxin A$_4$.

3.9.2. Bacterial killing

Blood neutrophils (from n=6 pneumonia patients) were co-incubated with GFP labeled bacteria for 15mins. For bacterial killing, cells were lysed (after 15 minutes co-incubation of neutrophils with GFP PAO1) and internalized bacteria were plated out and bacterial killing was assessed after 24 hours. Killing of GFP PAO1 was recorded. One way ANOVA showed that there was a dose dependent increase in blood neutrophil killing of GFP labeled PAO1 with Lipoxin A_4 both at the beginning and at the end of the exacerbation; p=0.04 and p=0.01 respectively (figure 78).

	Control	LX 1nM	LX10nM	LX 100nM
Start of exacacerbation	7.1 (0.1)	6.9 (0.1)	6.8 (0.2)	6.6 (0.1)
End of exacerbation	6.9 (0.1)	6.7 (0.2)	6.5 (0.2)	6.2 (0.2)

Table 16: Colony counts in log units (standard error of mean), at the start and end of exacerbation.
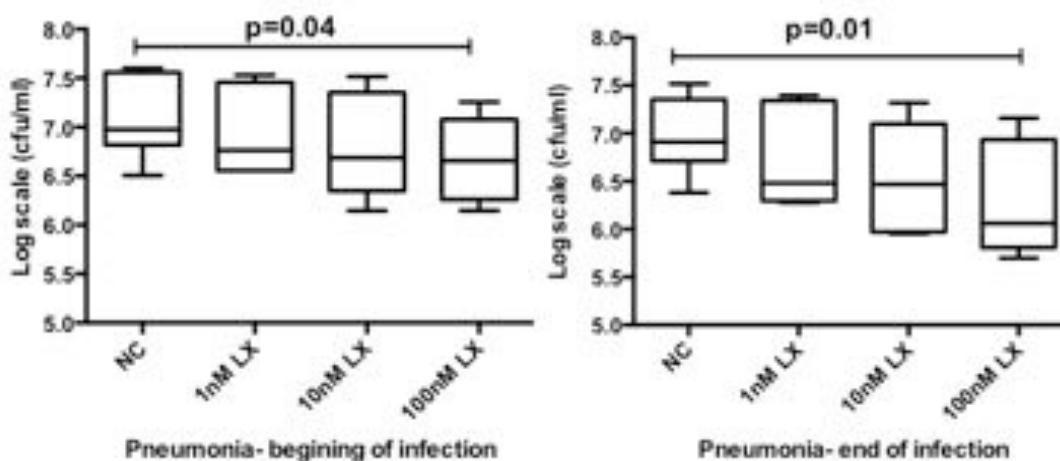
Figure 78. Significantly higher bacterial killing by LXA$_4$ both at the start and end of exacerbation (by one way ANOVA), p=0.04 and p=0.01 respectively. Neutrophils were pre treated with LXA$_4$ for 30 minutes and killing was measured after 15 minutes of co-incubation with GFP PAO1 and then reading plates after 24 hours. Pooled data presented as median (IQR) for n=6 per group. NC= negative control; LX= Lipoxin A$_4$.

3.9.3. Blood lipidomics

Next I wanted to assess if there was a change in blood lipidomics at the start and end of treatment for pneumonia, as assessed in the previous sections with bronchiectasis patients. I measured PGE2, 5HETE, 15HETE, 9HODE and LTB$_4$, as before. Paired t-tests showed that although there was a trend towards reduction in blood PGE2, 15 HETE and 9 HODE, these were not statistically significant, with p values being 0.1, 0.2 and 0.1 respectively (figure 79). LXA$_4$, LTB$_4$ and 5 HETE levels were not detected in these samples.
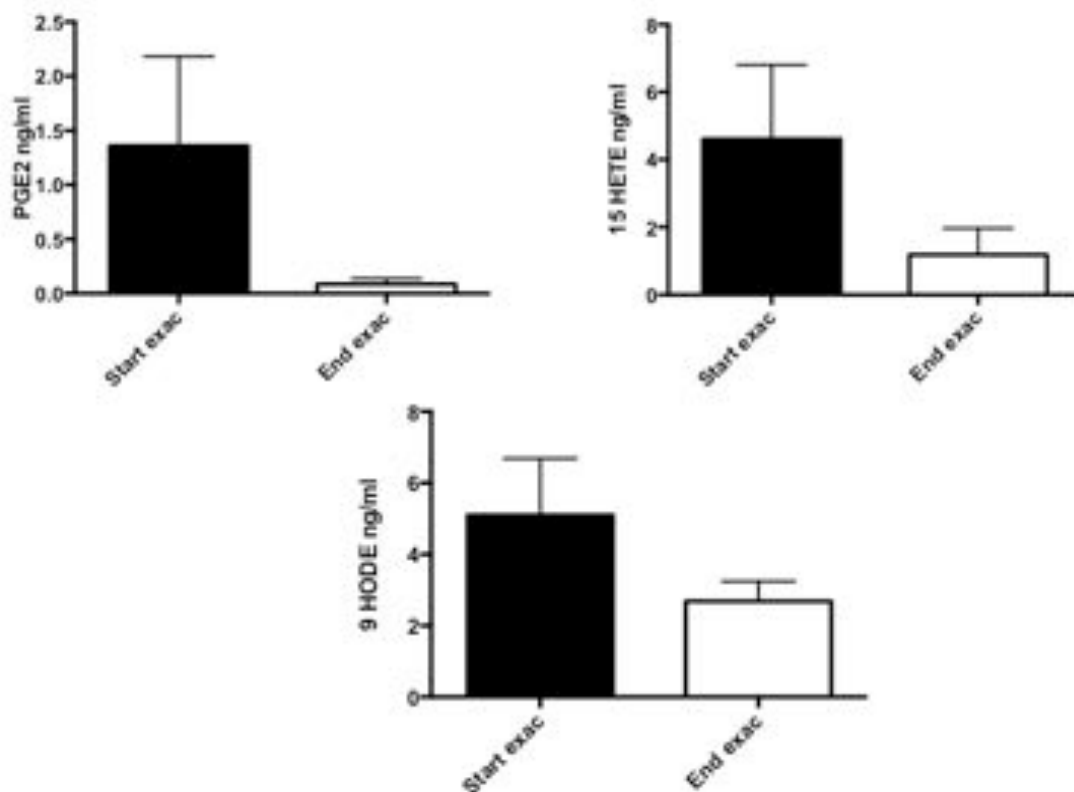
Figure 79. No significant change in blood lipodimics on pneumonia blood at the start and end of infection, in pneumonia. Pooled data presented as mean ± SEM, n=6 per group. Paired t tests used for all comparisons.
5 HETE hydroxyeicosapentanoic acid), 9-HODE (hydroxyoctadecadienoic acid) and PG (Prostaglandin) E2.

3.10. NEUTROPHIL EXTRACELLULAR TRAPS (NETs)

Study aim: To assess alternative ways of cell death during bronchiectasis exacerbations and pneumonia.

3.10.1. NETs by fluorescent microscopy

Peripheral blood neutrophils were isolated from 6 healthy volunteers, as previously described and were pretreated with Lipoxin A_4 doses of 1nM, 10nM and 100nM or vehicle control. Neutrophils were then stimulated with PMA 10nM and NETs were counted after 4 hours. Cells were visualised by fluorescent microscopy carried out on an Evos fl inverted microscope (AMG, Bothwell, WA).

After 4 hours incubation, there was an 11% (from 27% in PMA treated and 16% in LXA_4 100nM treated) statistically significant reduction in the percentage of NETs formed by PMA, by Lipoxin A_4 100nM; p=0.01 (figure 80). DPI is a known NADPH oxidase inhibitor and was used as a positive control.
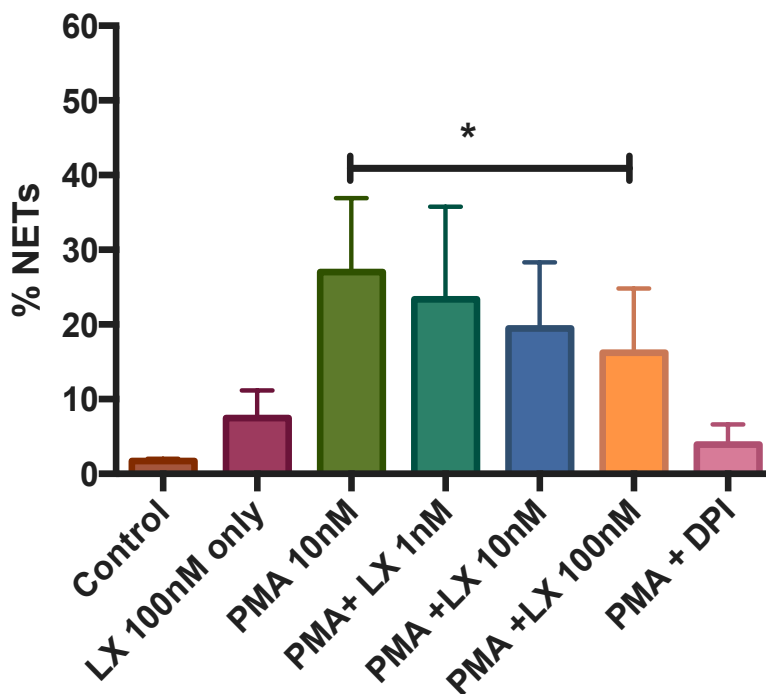
Figure 80. Reduction of PMA 10nM (after 4 hours) induced NETs by Lipoxin A_4 in a dose dependent manner. Pooled data presented as mean ± SEM, n=5. One way ANOVA used for comparison. *P<0.05.
DPI= diphosphoinositide; LX= Lipoxin A_4; PMA= phorbol myristate acetate.

3.10.2. NETs by fluorescence

Blood samples were obtained from 6 bronchiectasis patients (same patients as in section 3.2) at the beginning and end of exacerbation (day 1 and day 14) and blood NET formation was evaluated by fluorescence emitted using a plate reader.

As a control, blood samples were also obtained from 6 pneumonia patients (same patients as in section 3.3) at the beginning and end of pneumonia (day 1 and day 5) and blood NET formation was evaluated by fluorescence emitted by a plate reader.

Briefly blood neutrophils were incubated for 4h in a 96 well plate and then SYTOX green a cell-impermeable nucleic acid stain, with an excitation/emission maxima of 504/523 nm to give a green fluorescent light, was added and NET formation was observed by measuring mean fluorescence in 96 well plates.

3.10.3. NETs, LXA$_4$ and bronchiectasis exacerbations

3.10.3.1. Start of exacerbation- figure 81a

Paired t-tests showed that there was no reduction on PMA stimulated NETs production, even by the highest concentration of LXA$_4$ 100nM. On the contrary, there was an increase in NETs when LXA$_4$ 100nM was added to PMA stimulated NET formation, p=0.001. However, LXA$_4$ only treated neutrophils were able to reduce NETs produced by untreated neutrophils (NC- neutrophils treated with vehicle control only); p=0.0005.

3.10.3.2. End of exacerbation- figure 81b

Similar to findings at the start of exacerbation, there was no reduction by LXA$_4$ on PMA stimulated NETs production at the end of exacerbation, but on the contrary there was an increase in NETs when LXA$_4$ 100nM was added to PMA stimulated NET formation, p<0.0001. However, LXA$_4$ only treated neutrophils were able to reduce NETs produced by untreated neutrophils (NC- neutrophils treated with vehicle control only); p=0.0003.
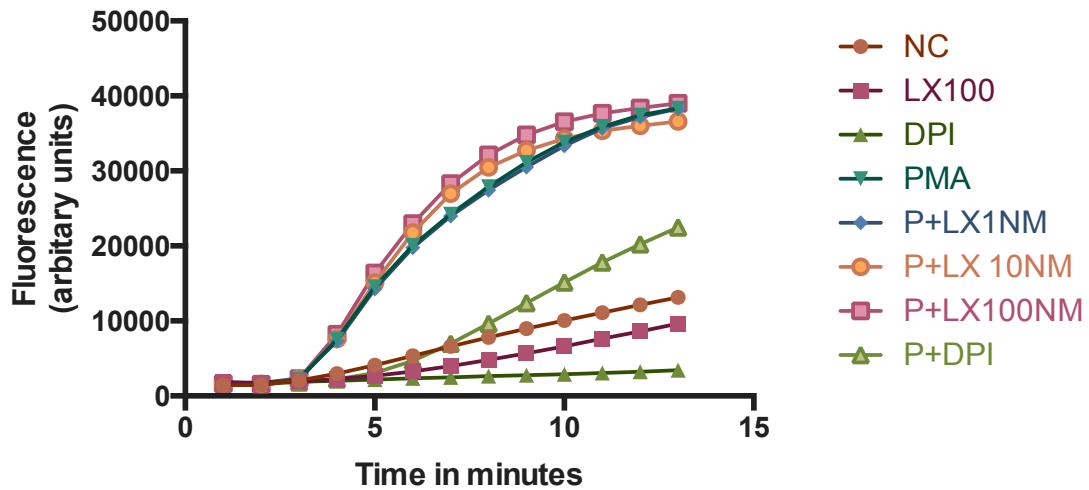
Figure 81a. Representative graph from blood neutrophils of bronchiectasis patient producing NETs as measured by fluorescence, at the start of an exacerbation. LX only treated neutrophils were able to significantly reduce NETs produced at baseline (NC).
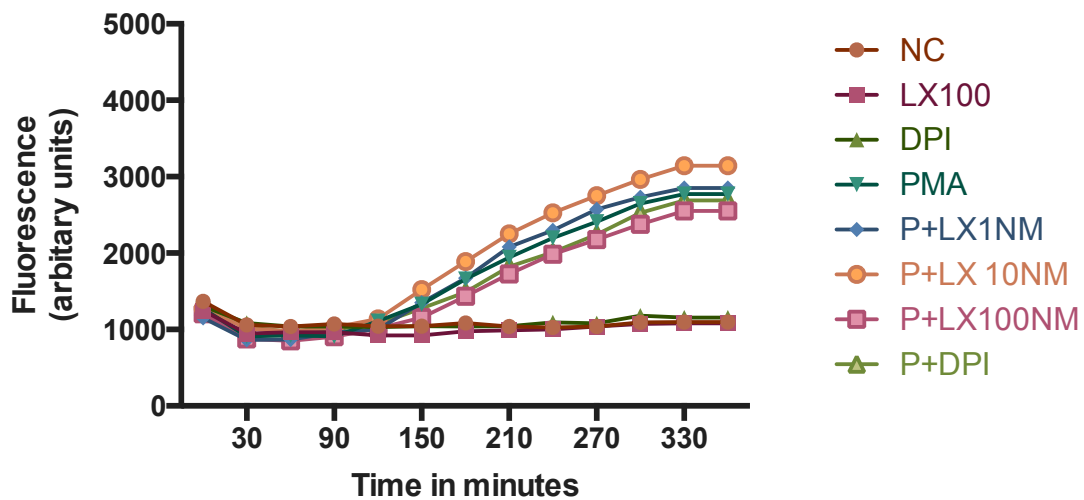
Figure 81b. Representative graph from blood neutrophils of bronchiectasis patient producing NETs as measured by fluorescence, at the end of an exacerbation.

LX only treated neutrophils were able to significantly reduce NETs produced at baseline (NC).

DPI= diphosphoinositide; LX= Lipoxin A$_4$; PMA= phorbol myristate acetate.

Although, lipoxin A_4 was not able to reduce PMA induced NET formation, paired t-tests showed that there was a significant reduction in NETs produced at the beginning (p=0.0005) and at the end of treatment with antibiotics (p=0.0003) in bronchiectasis patients (figure 81c).
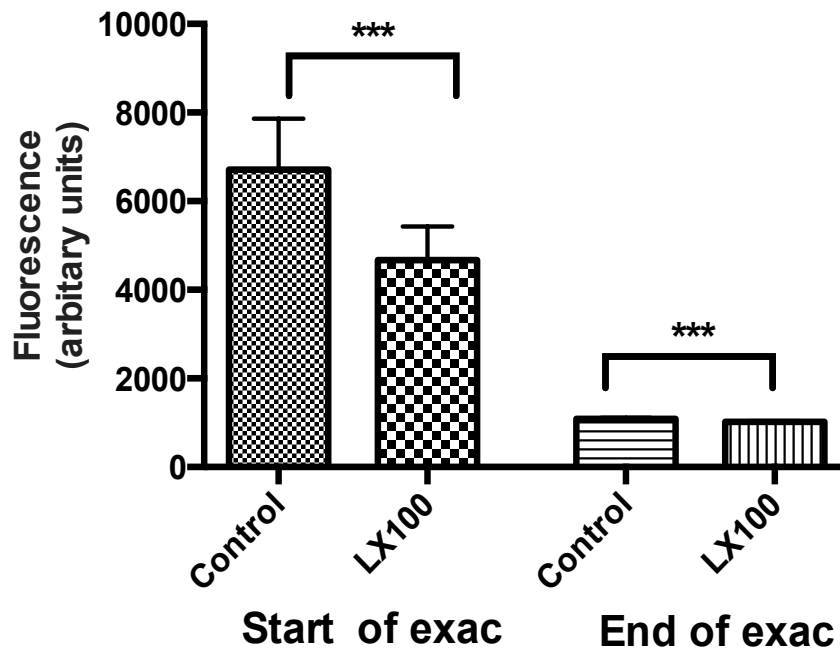
Figure 81c. Reduction in NET formation by LXA_4 in untreated neutrophils at the start and at the end of exacerbation. Pooled data presented as mean ± SEM, n=6. Paired t-test used for comparison. ***P<0.001.

3.10.4. NETs, LXA$_4$ and pneumonia

3.10.4.1. Start and end of infection- figure 82a&b

Paired t-tests showed that there was no reduction on PMA stimulated NETs production in blood neutrophils, even by the highest concentration of LXA_4 100nM. On the contrary, there was an increase in NETs when LXA_4 100nM was added to PMA stimulated NET formation, p=0.0003. Additionally, LXA_4 only treated neutrophils were not able to reduce NETs produced by untreated neutrophils (in contrast to bronchiectasis patients), and on the contrary increased NETs production; p=0.006.

Similar to findings at the start of infection, there was no reduction on PMA stimulated NETs production, at the end of pneumonia treatment. Additionally LXA4 only treated neutrophils were not able to reduce NETs produced by untreated neutrophils (NC-neutrophils treated with vehicle control only).
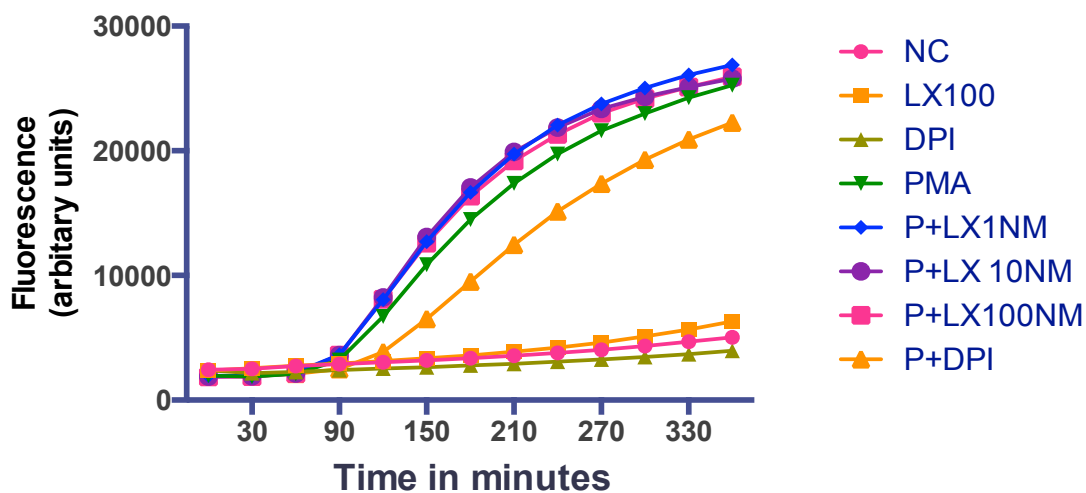
Figure. 82a. Representative graph from blood neutrophils of pneumonia patient producing NETs as measured by fluorescence, at the beginning of an infection. LX only treated neutrophils were **not** able to significantly reduce NETs produced at baseline (NC).
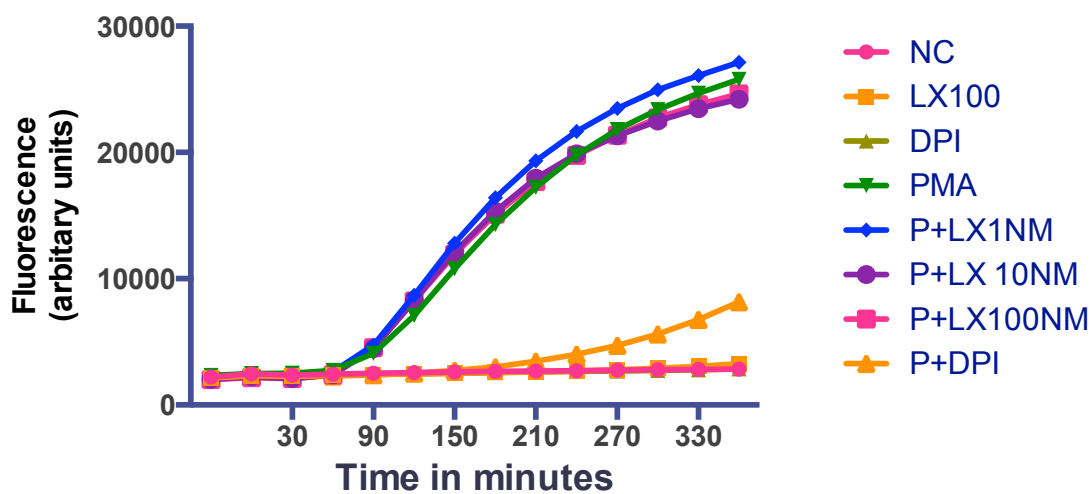
Figure 82b. Representative graph from blood neutrophils of pneumonia patient producing NETs as measured by fluorescence, at the end of treatment with antibiotics. LX only treated neutrophils were **not** able to significantly reduce NETs produced at baseline (NC).
DPI= diphosphoinositide; LX= Lipoxin A4; PMA= phorbol myristate acetate.

Although, Lipoxin A_4 was not able to reduce PMA induced NET formation, when comparing NETs produced at the start and end of pneumonia, there was a significant reduction in NETs produced at the end of treatment (with antibiotics) of pneumonia, both in untreated neutrophils (p=0.0003) and PMA treated neutrophils (p=0.004) (figure 82c).
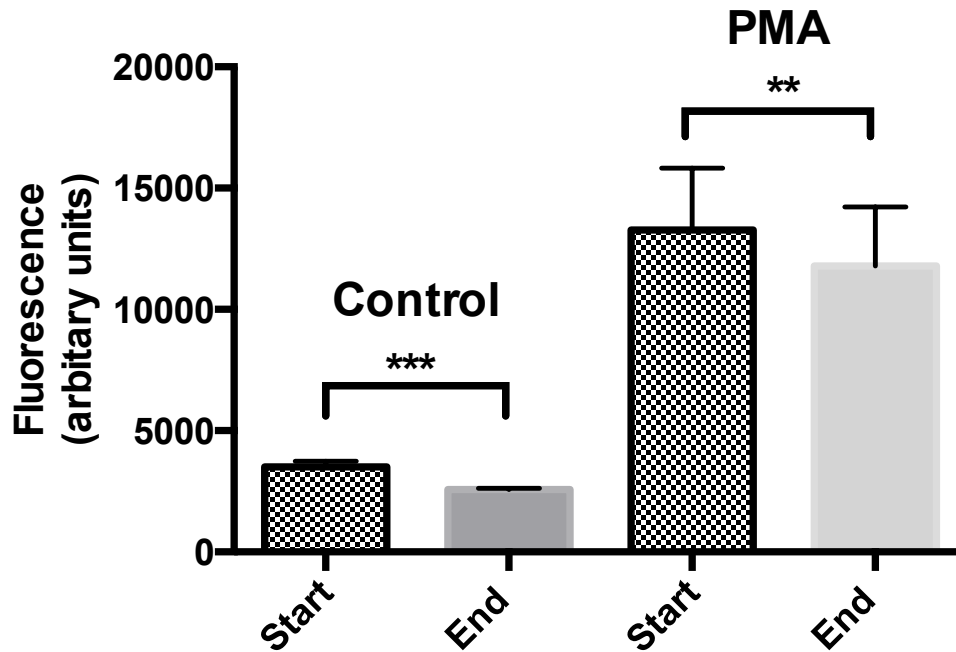
Figure 82c. Significant reduction in NETs formation at the end of pneumonia treatment with antibiotics in untreated and PMA treated neutrophils. Pooled data presented as mean ± SEM, n=6. Paired t-test used for comparison. **P<0.01; ***P<0.001.

3.10.5. Comparison of NETs in bronchiectasis to pneumonia

In the previous section, I concluded that for both bronchiectasis and pneumonia there was no effect of Lipoxin A_4 on PMA treated neutrophils. However, LXA_4 was able to reduce NETs produced in unstimulated neutrophils both at the start and end of exacerbations in bronchiectasis only. I now wanted to assess if there was a difference in NETs produced in in unstimulated neutrophils during exacerbations. On comparison, I found that there was significantly higher NETs produced at the start of exacerbations in bronchiectasis (p=0.01) and significantly lower at the end of exacerbations (p=0.0003) compared to pneumonia patients (figure 83). This shows that NETs are perhaps one of the methods of cell death in bronchiectasis but fewer NETs at the end of exacerbations compared to pneumonia suggests that perhaps cells are directed towards another pathway for clearance that contributes to ongoing inflammation. My hypothesis is that this could be by secondary necrosis.
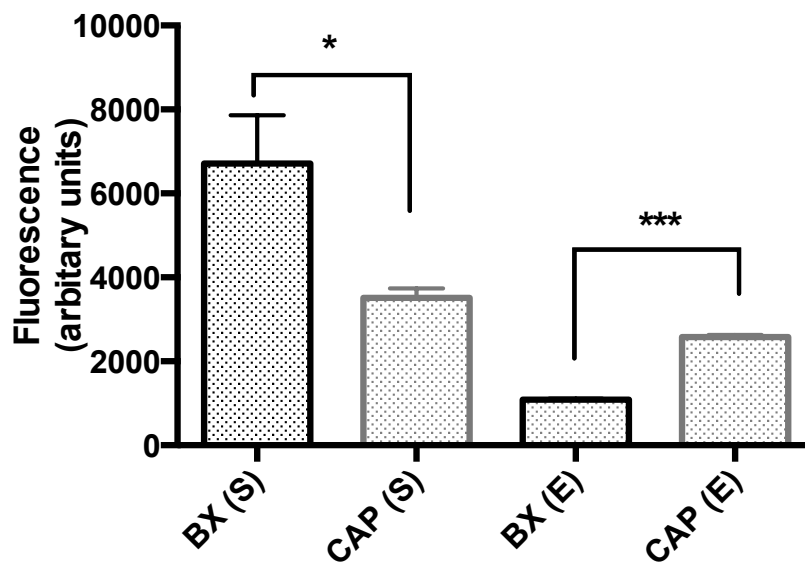
Figure 83. Significant difference in NETs formation at the start and end of bronchiectasis exacerbations compared to pneumonia, in untreated neutrophils. Pooled data presented as mean ± SEM, n=6. Paired t-test used for comparison. **P<0.01;   ***P<0.001.

3.11. EFFEROCYTOSIS

<u>Study aim:</u> Assess the anti-inflammatory and pro resolution effect of LXA$_4$ on monocyte-derived macrophages from healthy volunteers.

Monocytes were isolated from 6 healthy volunteers and were grown to monocyte-derived macrophages in 5-7 days. Upon, co incubating MDMs with apoptotic neutrophils for 40-60 minutes, there was a dose dependent increase in the phagocytosis of apoptotic neutrophils (also known as efferocytosis) by MDMs that were pre-treated with Lipoxin A$_4$, however this failed to reach statistical significance; p=0.07 (one way ANOVA).

CD44 was added as a positive control. The cell surface receptor CD44 is a known to be a key regulator of macrophage capacity for phagocytosis of apoptotic cells. Paired t tests of vehicle treated (MDM and neutrophils only) and CD44 treated cells showed a statistically significant improvement in efferocytosis; p=0.04 (figure 84).
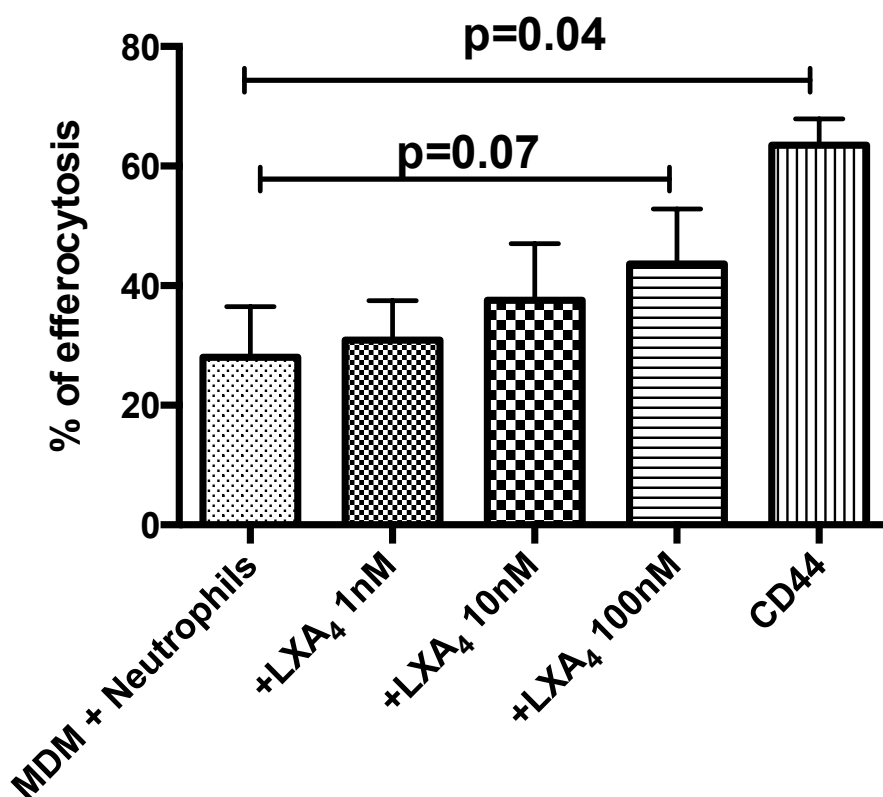
Figure 84. Significant increase in MDM phagocytosis of apoptotic neutrophils when MDM were pre-treated with CD44. No significant increase in phagocytosis when neutrophils were pre treated with LXA$_4$. Pooled data presented as mean ± SEM, n=5. One way ANOVA used for comparisons.
LXA$_4$= Lipoxin A$_4$; MDM= monocyte derived macrophages.

3.12. REPROGRAMMING OF BLOOD NEUTROPHILS IN BRONCHIECTASIS

3.12.1. Characteristics and functions of blood neutrophils in bronchiectasis

I studied the behavior of peripheral blood neutrophils in mild (6) and severe bronchiectasis (6) and compared it to the activity of peripheral blood neutrophils isolated from healthy volunteers (7). Untreated neutrophils from bronchiectasis patients (both in mild and severe groups) had increased survival and decreased apoptosis when compared to healthy volunteers. Further studies done demonstrated that when neutrophils from bronchiectasis patients underwent apoptosis using serum from healthy volunteers (and not autologous serum), there was a reduction in viability and increased apoptosis in bronchiectasis neutrophils. However, the reverse was not true when treating healthy volunteers patients neutrophils with bronchiectasis serum. This suggests that there is perhaps a re programming of neutrophils in bronchiectasis and the findings are not secondary to blood factors.

Neutrophil activation was measured by assessing expression of CD11b and CD62L. Upon activation, neutrophils express more CD11b and shed CD62L. At baseline (untreated neutrophils), there was an increase in expression of CD11b in severe bronchiectasis compared to healthy volunteer sand mild bronchiectasis patients. There was more shedding of CD62L in mild and severe bronchiectasis patients compared to healthy volunteers indicating that, even when unstimulated, neutrophils in bronchiectasis remain more activated. Having more baseline activation would thereby explain why these neutrophils have a longer life span. This could be speculated to be secondary to more activating survival factors in a more proinflammatory environment in bronchiectasis patients blood and airways.

Measuring myeloperoxidase release assessed neutrophil degranulation. In untreated blood neutrophils, there was significantly more myeloperoxidase release in bronchiectasis patients compared to healthy volunteers. Reactive oxygen species generation by neutrophils upon stimulation with fMLF was measured. There was no difference in superoxide generation by unstimulated neutrophils in the groups.

Assessment of phagocytosis and killing of GFP labeled *Pseudomonas aeruginosa* by

blood neutrophils was done in all three groups. There was a significantly higher phagocytosis and killing in healthy volunteers compared to mild and severe bronchiectasis.

Table 17 summarizes the overall key blood neutrophil functions that were assessed in mild and severe bronchiectasis patients, in comparison to blood neutrophil function from *healthy volunteers*.

Function	Mild bronchiectasis N=8	Severe bronchiectasis N=8
Apoptosis	↓	↓
Viable neutrophils	↑	↑
CD11b upregulation	↔	↑↑
CD62L shedding	↓	↓
Myeloperoxidase release	↔	↑
Phagocytosis	↓	↓
Bacterial killing	↓	↓↓
Superoxide release	↔	↔

Table 17. Summary of key neutrophil functions evaluated at baseline in mild bronchiectasis and severe bronchiectasis; n=8 per group.

3.12.2. Comparison of blood neutrophils to airways neutrophils in bronchiectasis

Mild and severe bronchiectasis patients were recruited in this study. Blood neutrophils were isolated from blood and airways neutrophils were isolated from bronchoalveolar lavage fluid obtained during bronchoscopy. Phagocytosis and killing of GFP labeled bacteria by both blood and airways neutrophils were assessed. There was significantly higher bacterial phagocytosis and killing by blood neutrophils in both mild and severe patients compared to airways neutrophils.

3.12.3. Neutrophil function during exacerbations in bronchiectasis

6 patients during stable state and 6 patients with exacerbations were recruited in this study. There was a significantly higher bacterial phagocytosis and killing by blood neutrophils at the end of exacerbations compared to beginning. This suggests that neutrophil function is improved by treatment with antibiotics. In addition, comparison of neutrophil function during exacerbations was made to stable state. There was significantly higher phagocytosis and bacterial killing in the stable state compared to the beginning of exacerbation. However, there was no difference in phagocytosis or bacterial killing in the stable state compared to end of exacerbation. This thereby suggests that antibiotic treatment is able to restore phagocytic and killing ability of neutrophil to the stable state. However, what still remains unexplained is the ongoing inflammation in bronchiectasis. Can addition of an anti-inflammatory or pro resolution mediator, such as Lipoxin A_4 be additive and negate the persisting inflammation?

On assessing airways neutrophil function, obtained from sputum, there was a significantly higher bacterial phagocytosis and killing by airways neutrophils at the end of exacerbations compared to beginning. In addition, comparison of neutrophil function during exacerbations was made to stable state. There was higher phagocytosis (although not significant), and significantly higher bacterial killing in the stable state compared to the beginning of exacerbation. However, there was no difference in phagocytosis or bacterial killing in the stable state compared to end of exacerbation, in airways neutrophils.

3.12.4. Blood neutrophil function in community acquired pneumonia

In this study, 6 patients with community-acquired pneumonia were recruited and neutrophils were isolated from blood obtained at the start and end of infection. There was significantly higher bacterial phagocytosis and killing at the end of infection compared to beginning, in community acquired pneumonia. On comparison of neutrophil function in bronchiectasis exacerbation to community-acquired pneumonia, there was no difference in bacterial phagocytosis between the two groups, either at the beginning or at the end of exacerbation. However, on comparison of bacterial killing, there was significantly higher bacterial killing by blood neutrophils in community acquired pneumonia compared to bronchiectasis, both at the beginning and at the end of infection. This suggests that there is perhaps a defect in the killing

and clearance of bacteria in bronchiectasis that antibiotics are unable to do. Antibiotics do perhaps not attenuate the survival signals for bacteria and hence the roles of anti-inflammatory agents need to be explored. This is discussed in the next section.

Neutrophil extracellular trap (NETs) formation was evaluated in both bronchiectasis exacerbations and pneumonia. There was a reduction in neutrophil extracellular trap formation at the end of treatment of exacerbation both in bronchiectasis and in pneumonia, compared to beginning of exacerbation. On comparison of NETs in bronchiectasis to pneumonia, I found that there was significantly higher NET formation in bronchiectasis than pneumonia at the start of infection. At the end of treatment of infection, there was significantly lesser NETs produced in bronchiectasis, suggesting perhaps another alternate pathway for cell death in bronchiectasis.

3.12.5. Bronchiectasis and Lipoxin A_4

Once I had established neutrophil function in bronchiectasis, I wanted to assess the role of Lipoxin A_4 on these neutrophil functions. Would LXA_4 be able to restore the neutrophil function in bronchiectasis patients to healthy volunteers? Lipoxin A_4 improved CD11b upregulation, CD62L shedding, myeloperoxidase and superoxide release (except in mild bronchiectasis) release, neutrophil phagocytosis and killing of GFP PAO1, in a dose dependent manner. Neutrophil activation was measured by assessing expression of CD11b and CD62L. There was no effect of LXA_4 on apoptosis. This part of the study established that LXA_4 stabilizes neutrophils, which improves phagocytosis and killing of GFP PAO1, which is key in bronchiectasis where a recurrent bacterial infection is the most common clinical presentation in these patients.

3.12.6. NETs and Lipoxin

I assessed NETs formation in bronchiectasis and in pneumonia patients at the start and end of treatment with antibiotics. Although, *in vitro*, in the healthy volunteers (with no infection) lipoxin was able to reduce PMA induced NET formation in a dose dependent manner, there was no reduction in PMA induced NETs production in bronchiectasis or pneumonia patients, either at the start or at the end of infection. However, in unstimulated neutrophils, LXA_4 100nM was able to significantly reduce NETs formation compared to untreated neutrophils. Additionally, as expected there were reduced PMA induced NETs at the end of infection in both bronchiectasis and pneumonia. Significantly higher NETs were detected at the start of infection in bronchiectasis and lesser NETs were detected at the end of bronchiectasis exacerbation compared to end of pneumonia treatment.

3.12.7. Lipoxin and function of blood neutrophils

I studied the behavior of peripheral blood neutrophils in mild and severe bronchiectasis and compared it to the behavior of peripheral blood neutrophils from healthy volunteers, as discussed earlier. In addition, I assessed the role of lipoxin A_4 in modulating the response of these neutrophils under various stimuli. Three groups were of participants were recruited for this study- 7 healthy volunteers, 6 patients with mild bronchiectasis and 6 patients with severe bronchiectasis. Lipoxin A_4 was unable to increase or decrease spontaneous apoptosis in blood neutrophils from healthy volunteers, mild bronchiectasis and severe bronchiectasis patients. When treated with roscovitine, a known inducer of apoptosis, there was less apoptosis and more necrosis of neutrophils from bronchiectasis patients compared to healthy volunteers. Neutrophil activation was measured by assessing expression of CD11b and CD62L. Upon activation, neutrophils express more CD11b and shed CD62L. Lipoxin was able to reduce fMLF-induced activation and further increase in CD11b expression and CD62L shedding, in a dose dependent manner, in all three groups. Next I assessed neutrophil degranulation by measuring myeloperoxidase. Upon stimulation with fMLF and cytochalasin B, lipoxin was able to reduce myeloperoxidase release in all groups in a dose dependent manner. Reactive oxygen species generation by neutrophils upon stimulation with fMLF was measured. When stimulated with fMLF, LXA_4 was able to reduce superoxide release in a dose dependent manner in healthy

volunteers and severe bronchiectasis patients. There was no effect of lipoxin on superoxide generation in mild patients.

Effect of Lipoxin on neutrophil function was assessed next. Lipoxin was able to enhance phagocytosis and killing of GFP labeled *Pseudomonas aeruginosa* by blood neutrophils in a dose dependent manner across all three groups.

Lipoxin A_4 binds to the ALX/FPR2 receptor. Although this just failed to reach statistical significance, there was an increased expression of the ALX/FPR2 receptor in healthy individuals compared to bronchiectasis patients, at baseline. Upon treatment with LXA_4, there was significant increase in ALX/FPR2 expression in neutrophils from healthy volunteers compared to bronchiectasis patients.

In summary, pretreatment of blood and airways neutrophils with Lipoxin A_4, stabilizes and improves neutrophil function.

3.12.8. Efferocytosis

Clearance of apoptotic neutrophils is one of the key functions of macrophages to initiate the process of resolution of inflammation. Although, other studies (Mitchell et al 2002) have demonstrated that lipoxin does increase efferocytosis (albeit the methods of assessing by Mitchell and colleagues were by electron microscopy and I assessed efferocytosis by flow cytometry), in this study lipoxin was not able to enhance clearance of apoptotic neutrophils. However, addition of the cell surface receptor CD44 was significantly able to increase efferocytosis. Assessing efferocytosis with higher doses of lipoxin is needed but has not been investigated further in this thesis.

CHAPTER 4

IN HUMAN STUDIES

Study aim: Assess the anti-inflammatory and pro resolution effect of LXA$_4$ on neutrophils during stable state in bronchiectasis.

4.1. BRONCHOSCOPY STUDY

55 patients meeting the study criteria were approached to take part in the study of which 34 bronchiectasis patients consented and completed the study. 6 healthy volunteers took part and completed the study. Patients were all given a bronchiectasis severity score by calculating their respective bronchiectasis severity index (BSI). Of the 34 patients, 10 were mild, 15 moderate and the remaining 9 severe bronchiectasis.
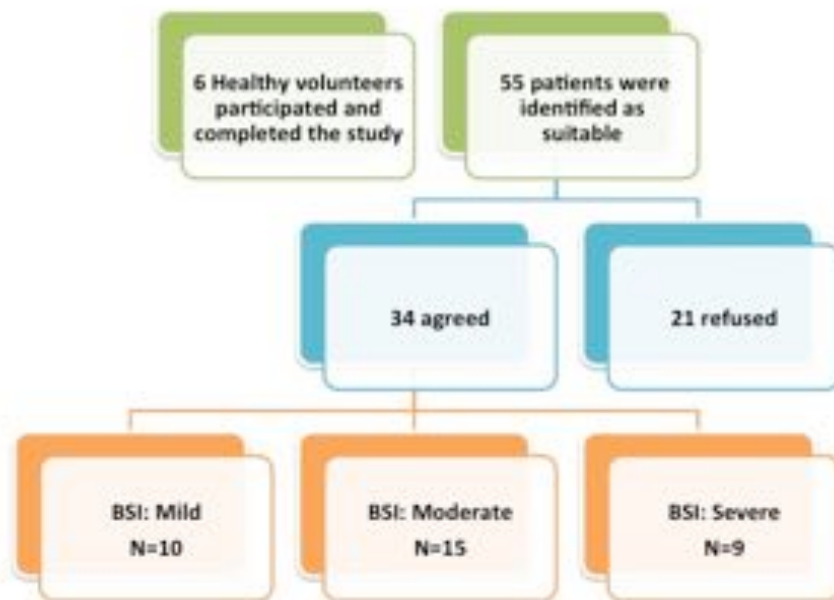
Figure 85. Flow chart depicting the recruitment of healthy volunteers and bronchiectasis patients and classification of patients as per the bronchiectasis severity index (BSI) (Chalmers et al 2014).

Baseline demographics of study participants are shown in table 18. Section 4.1.1.1 to 4.1.1.5 describes the baseline demographics in further detail.

Parameters	Bronchiectasis patients N=34			Healthy volunteers N=6
	Mild n=10	Moderate n=15	Severe n=9	
Age	55 (4.1)	65 (2.2)	64 (2.2)	52 (6.8)
Gender (% female)	40%	60%	22%	80%
Total WCC	6 (0.5)	6.3 (0.4)	9.3 (1.1)	5.9 (0.5)
Neutrophils	3.3 (0.3)	4.1 (0.3)	6.6 (1.1)	3.5 (0.3)
Eosinophils	0.2 (0.04)	0.3 (0.07)	0.2 (0.06)	0.2 (0.06)
Monocytes	0.5 (0.03)	0.6 (0.05)	0.7 (1)	0.5 (0.05)
ESR	6.7 (1.8)	13.2 (2.9)	19.6 (6.8)	4.8 (1)
CRP	2.8 (0.5)	4 (1)	16 (7.4)	3.2 (1.1)
FEV$_1$% predicted	95 (5.5)	82 (4)	55 (6.5)	-
FVC % predicted	111 (6)	97 (4)	84 (6)	-
TLCO % predicted	94% (4.9)	82% (4.2.)	74% (7.8)	-
KCO % predicted	106% (4.5)	97% (3.7)	100% (7.2)	-
Chronic colonisation	8 (89%)	12 (86%)	5 (50%)	-
Exacerbations in the last year	0.4 (0.3)	2.4 (0.5)	4.2 (0.9)	-
Hospital admissions in the last year	0	0.05 (0.05)	0.7 (0.2)	-

Table 18. Baseline demographics of the study population. Data presented as mean (± standard deviation).

CRP= c reactive protein; ESR= erythrocyte sedimentation rate; FEV$_1$= forced expiratory volume in 1sec; FVC= forced vital capacity; KCO= transfer coefficient; TLCO= transfer factor for the lung for carbon monoxide; WCC= white cell count.

4.1.1. STUDY PARTICIPANTS

4.1.1.1. Age

There was no statistically significant difference in the age across the groups in the healthy volunteers and bronchiectasis patients, p=0.09; figure 86.
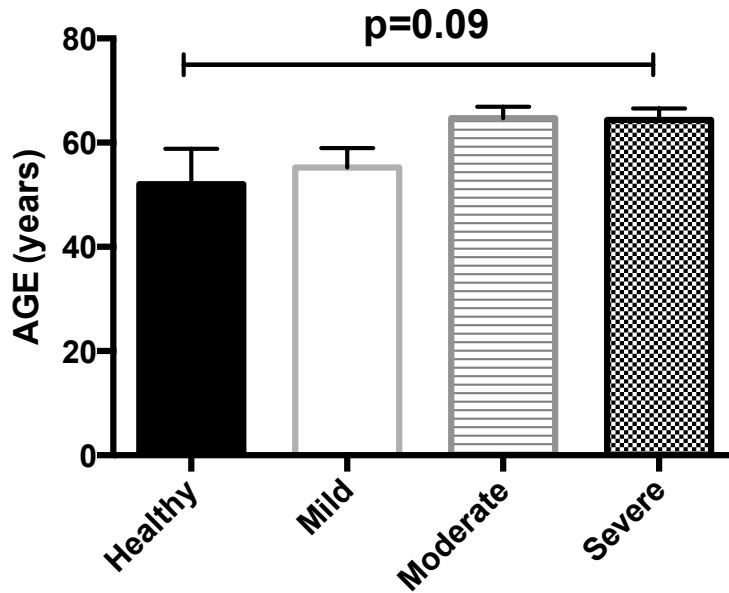
Figure 86. There was no significant difference in age across the group. Pooled data presented as mean ± SEM. One way ANOVA was done of the age in the four groups.

4.1.1.2. Serum inflammatory markers

- *4.1.1.2.1. White cell counts, CRP and ESR*

Using one-way ANOVA with Bonferroni's multiple comparison post hoc test, there was a statistically significant difference in the total white cell count (p=0.005), neutrophils (p=0.001) and c reactive protein (p=0.04). Although there was a trend, there was no significant difference in erythrocyte sedimentation rate; p=0.1. Severe patients had higher white cell counts, neutrophil counts, c- reactive protein and a trend towards higher ESR compared to the other three groups. There was no statistical difference in the monocyte count (p=0.4) or eosinophil count (p=0.4) between the groups; figure 87.
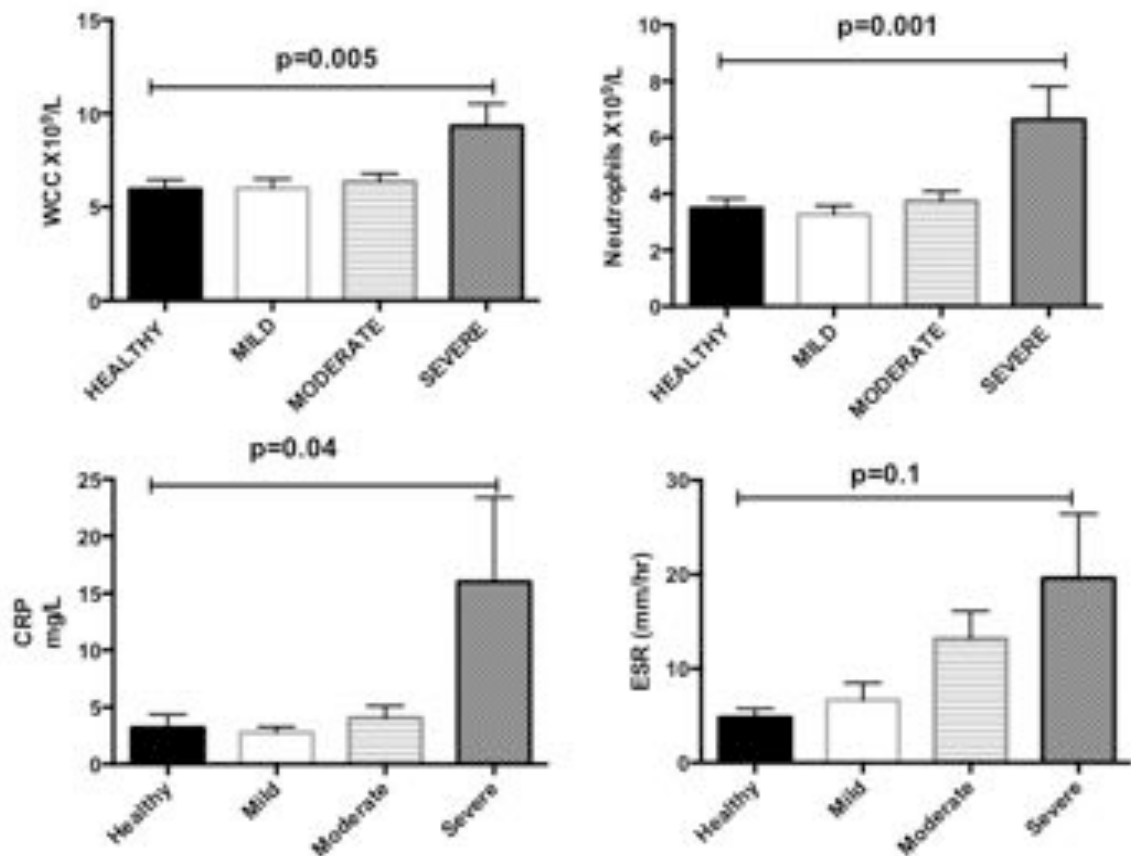
Figure 87. Significantly higher white cell counts, neutrophils, c- reactive protein (CRP) across the four groups. No difference in erythrocyte sedimentation rate. Pooled data presented as mean ± SEM. One way ANOVA was done for all comparisons.

4.1.1.3. Lung physiology

Using one-way ANOVA with Bonferroni's multiple comparison post hoc test, there was a statistically significant difference in percent predicted FEV_1 and FVC across the groups; p=0.0001 and p=0.008 respectively. Severe patients has lower % predicted FEV_1, % predicted FVC compared to the other two groups. Unpaired t tests showed a p value of p=0.052, when comparing % predicted TCO between mild and severe patients. There was no statistically significant difference in percent predicted KCO; p=0.4, by one way ANOVA; figure 88.
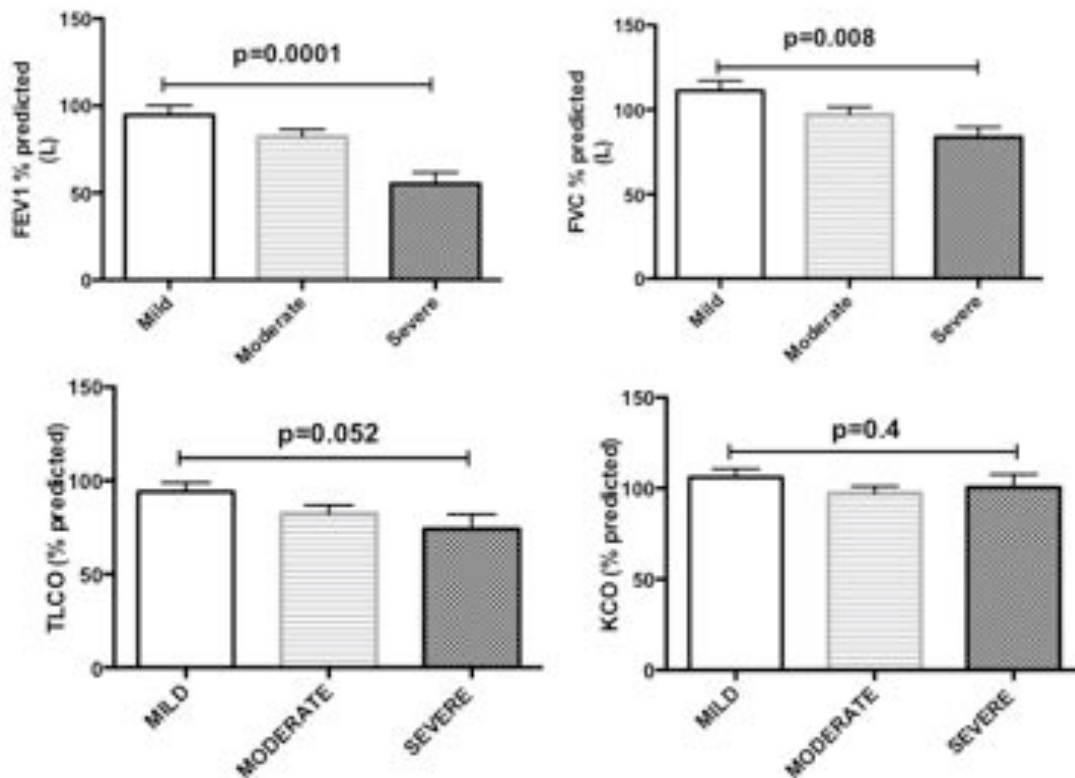
Figure 88. Significantly higher % predicted forced expiratory volume in 1 second, % predicted forced vital capacity % predicted transfer factor in mild compared to severe bronchiectasis. Pooled data presented as mean ± SEM. One way ANOVA was done for all comparisons.

4.1.1.4. Exacerbations and hospital admissions

The number of exacerbations (as defined by the British thoracic society guidelines) (British Thoracic Society bronchiectasis guidelines, 2010, Thorax) and hospital admissions secondary to bronchiectasis, in the year preceding participation in the study were recorded. Using one-way ANOVA with Bonferroni's multiple comparison post hoc test, there was a statistically significant difference across the groups, in exacerbations requiring antibiotics and hospital admissions; $p=0.0002$ and $p=0.0008$ respectively (figure 89). Severe patients had more exacerbations requiring antibiotics and hospital admissions, compared to mild and moderate patients.
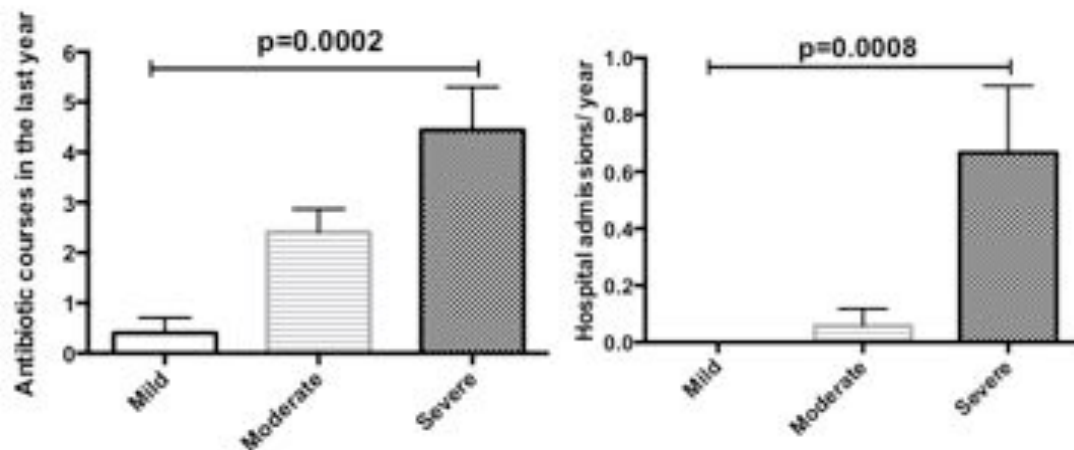
Figure 89. Significantly more exacerbations requiring antibiotic therapy and hospital admissions secondary to bronchiectasis exacerbations, in the preceding year, in severe bronchiectasis compared to mild and moderate bronchiectasis.

4.1.1.5. Microbiology from bronchoalveolar samples in study participants

All 34 patients and 6 healthy volunteers underwent bronchoscopy. In healthy volunteers 33% isolated an organism from the bronchoalveolar lavage fluid. In the mild group 40%, moderate 43% and severe 56%, isolated an organism from the bronchoalveolar lavage fluid.

Chi square analyses showed that there was a statistically significant increase in percentage of bacteria isolated in the severe group compared to healthy and mild groups; p=0.001 and p=0.03 respectively. There was no significant difference between the moderate and severe groups, p=0.08. The breakdowns of the organisms isolated are listed in table 19.

	Mild N=10	Moderate N=14	Severe N=9	Healthy volunteers N=6
Pseudomonas aeruginosa	0	1 (7%)	1 (11%)	0
Gram negative organisms	1 (10%)	2 (14%)	3 (34%)	0
Haemophilus influenzae	2 (20%)	2 (14%)	1 (11%)	1 (17%)
Streptococcus pneumoniae	0	1 (7%)	0	0
Moraxella catarrhalis	1 (10%)	0	0	1 (17%)
Mixed normal flora/ no growth	6 (60%)	8 (57%)	4 (44%)	4 (66%)

Table 19. Qualitative microbiology across the groups.

4.1.2. BLOOD NEUTROPHIL AND LIPOXIN

4.1.2.1. Phagocytosis

Peripheral blood neutrophils were isolated and phagocytosis assays were performed by co incubating the pre treated neutrophils (30 minutes with LXA_4) with GFP PAO1 for 15 minutes. Using one-way ANOVA with Bonferroni's multiple comparison post hoc test, there was a statistically significant improvement in phagocytosis by LXA_4 in a dose dependent manner in the healthy volunteers group ($p=0.0001$), mild ($p<0.0001$), moderate ($p<0.0001$) and severe bronchiectasis $p=0.03$; figure 90. These findings are summarized in table 20.

	Healthy volunteers N=6 %phagocytosis	Mild N=10 %phagocytosis	Moderate N=14 %phagocytosis	Severe N=9 %phagocytosis
Control (Neutrophils + GFP PAO1)	38.6 (2.9)	33.3 (0.5)	34.4 (1.3)	32.3 (1.4)
+ LXA$_4$ 1nM	40.5 (2.5)	38.3 (1.5)	37.2 (1.2)	36.4 (2.9)
+ LXA$_4$ 10nM	42.9 (2.6)	40.1 (1.7)	37.3 (1.7)	38.1 (2.9)
+ LXA$_4$ 100nM	46. 3 (2.2)	41.2 (1.7)	39.4 (1.2)	42.1 (2.3)

Table 20. Percentage phagocytosis across the bronchiectasis groups including the different treatments the neutrophils were exposed to- vehicle treated, 1nM, 10nM and 10nM Lipoxin A$_4$.
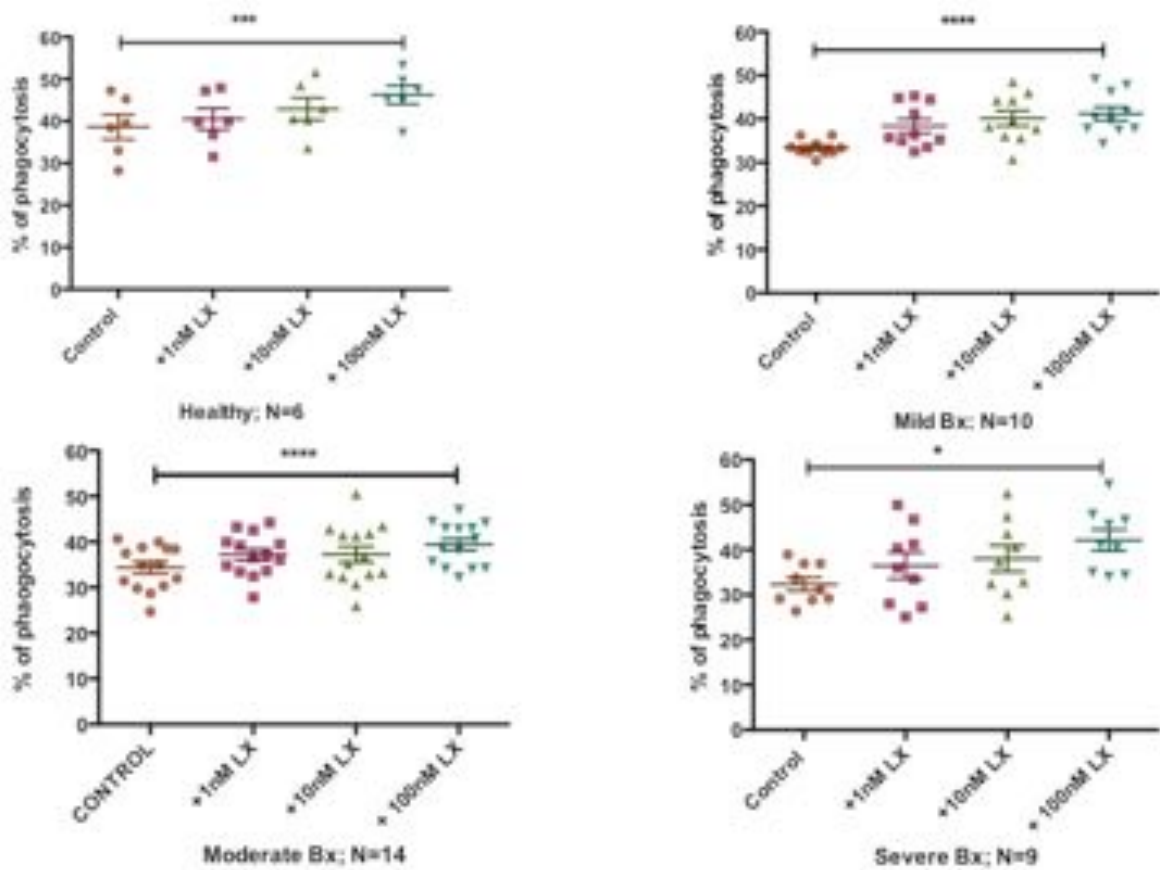
Figure 90. There was a dose dependent increase in phagocytosis with LXA_4. One-way ANOVA with Bonferroni's correction for multiple comparisons used; with p values representing the comparison of control to 1nm, 10nM and 100nM of LXA_4. Pooled % neutrophil phagocytosis data, showing means +/- SEM. *P<0.05; ***P<0.001; ****P<0.0001.
Bx= bronchiectasis; LX= Lipoxin A_4.

4.1.2.2. Bacterial killing

Following co incubation of the peripheral blood neutrophils with the GFP PAO1 for 15 minutes, the cells were lysed and the suspensions were plated out on pseudomonas isolation agar and bacterial counts were read after 24 hours. Data is presented as median (inter quartile range).

Using one-way ANOVA with Bonferroni's multiple comparison post hoc test, there was a statistically significant improvement in phagocytosis by LXA_4 in a dose dependent manner in the healthy volunteers group (p=0.001), mild (p=0.04), moderate (p=0.03) and severe bronchiectasis (p=0.01); figure 91.
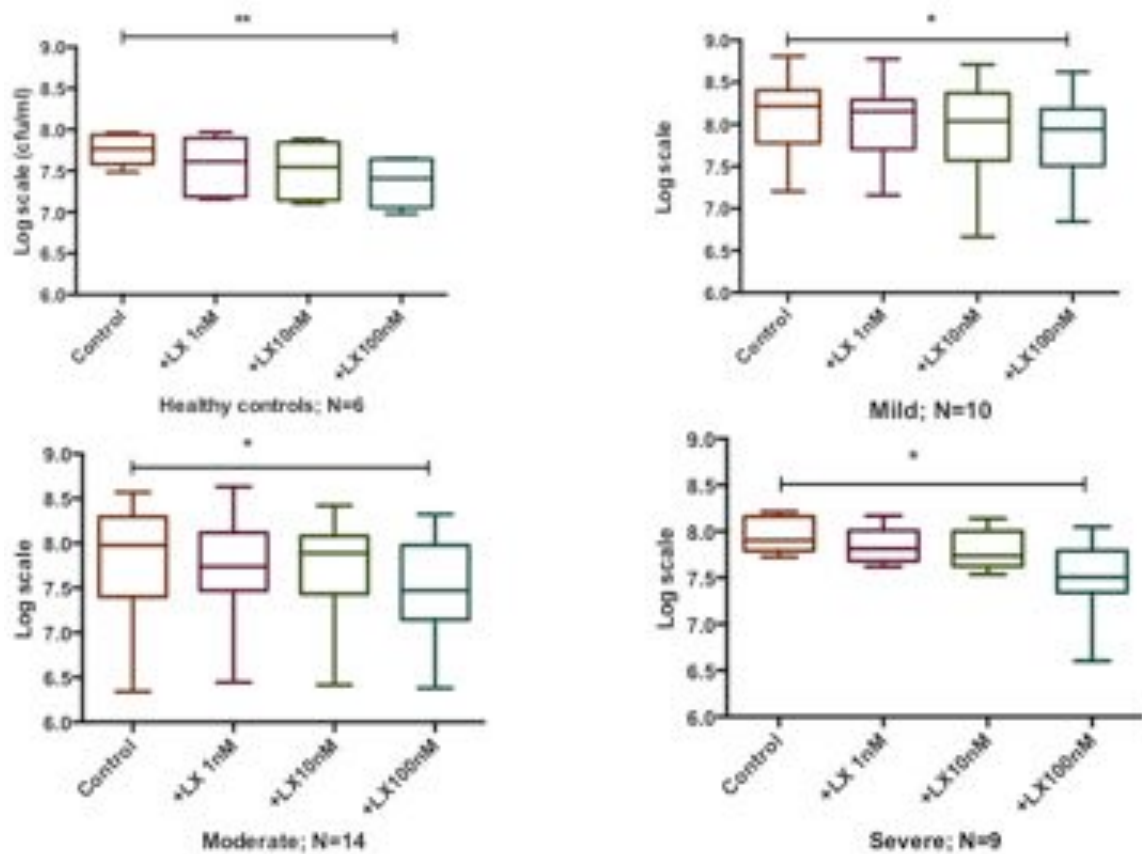
Figure 91. Lipoxin A_4 significantly improved bacterial killing in a dose dependent manner in all 4 groups. Box plot of killing assay of GFP labelled PAO1 by peripheral blood neutrophils, across the groups. Scale is log bacterial load in cfu/ml. One-way ANOVA with Bonferroni's correction for multiple comparisons used; with p values representing the comparison of control to 1nm, 10nM and 100nM of LXA_4. Pooled % neutrophil killing data, showing means +/- SEM. *$P<0.05$; **$P<0.01$.
LX= Lipoxin A_4.

4.1.3. AIRWAYS NEUTROPHILS AND LIPOXIN

Bronchoalveolar lavage was done in an area affected by bronchiectasis and in an area unaffected by bronchiectasis. Neutrophils were isolated and counted from the lavage fluid; table 21. In all three groups there was a similar return of neutrophils/ml in areas affected by bronchiectasis compared to unaffected areas, with no significant statistical difference (using unpaired t-tests); figure 92. This suggests that in bronchiectasis, there is an altered phenotype of neutrophils and that this is perhaps effected at the bone marrow, hence disease severity does not affect it.

	Affected Neutrophils/ ml	Unaffected Neutrophils/ ml	P value
Mild	$7.9X10^5$ $(3X10^5)$	$6.9X10^5$ $(2.4X10^5)$	0.7
Moderate	$1.4X10^6$ $(6.7X10^5)$	$7.5X10^5$ $(3.3X10^5)$	0.07
Severe	$9.9X10^5$ $(4.8X10^5)$	$7.5X10^5$ $(2.7X10^5)$	0.4

Table 21. Total neutrophils isolated from bronchoalveolar lavage fluid.
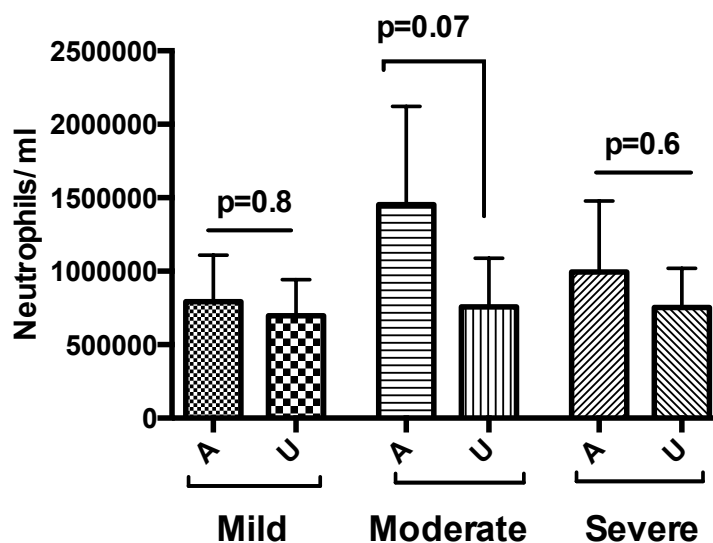
Figure 92. There was no significant difference in total cell counts between affected and unaffected segments in mild, moderate and severe bronchiectasis. Paired t tests used for comparisons. Pooled data presented as mean ± SEM.
A= affected segment; U= unaffected segment.

4.1.3.1. Phagocytosis

Airways neutrophils were isolated from bronchoalveolar lavage fluid from bronchiectasis patients and phagocytosis assays were performed with GFP PAO1.
Using paired t-tests, in the unaffected segments, there was no significant improvement in phagocytosis with LXA_4 100nM in any of the groups. In the affected segments, there was a statistically significant improvement in phagocytosis by LXA_4 100nM in the mild, moderate and severe bronchiectasis groups by LXA_4 100nM, p=0.01, p=0.02 and p=0.04 respectively, figure 93. The percentage phagocytosis is summarized in table 22.

	Unaffected segment %phagocytosis			Affected segment %phagocytosis		
	Control	+LX 100nM	P value	Control	+LX 100nM	P value
Mild Bx; N=10	11.3 (1.9)	11.2 (2.1)	0.9	12.5 (2.1)	13.9 (2.3)	**0.01**
Moderate Bx; N=14	15 (2.1)	15.8 (2.5)	0.3	19 (2.3)	22.2 (2.2)	**0.02**
Severe Bx; N=9	16.8 (2.9)	16.3 (2.2)	0.7	16.5 (2.8)	18.5 (2.7)	**0.04**

Table. 22. Percentage phagocytosis in all 3 groups presented as mean (SEM).
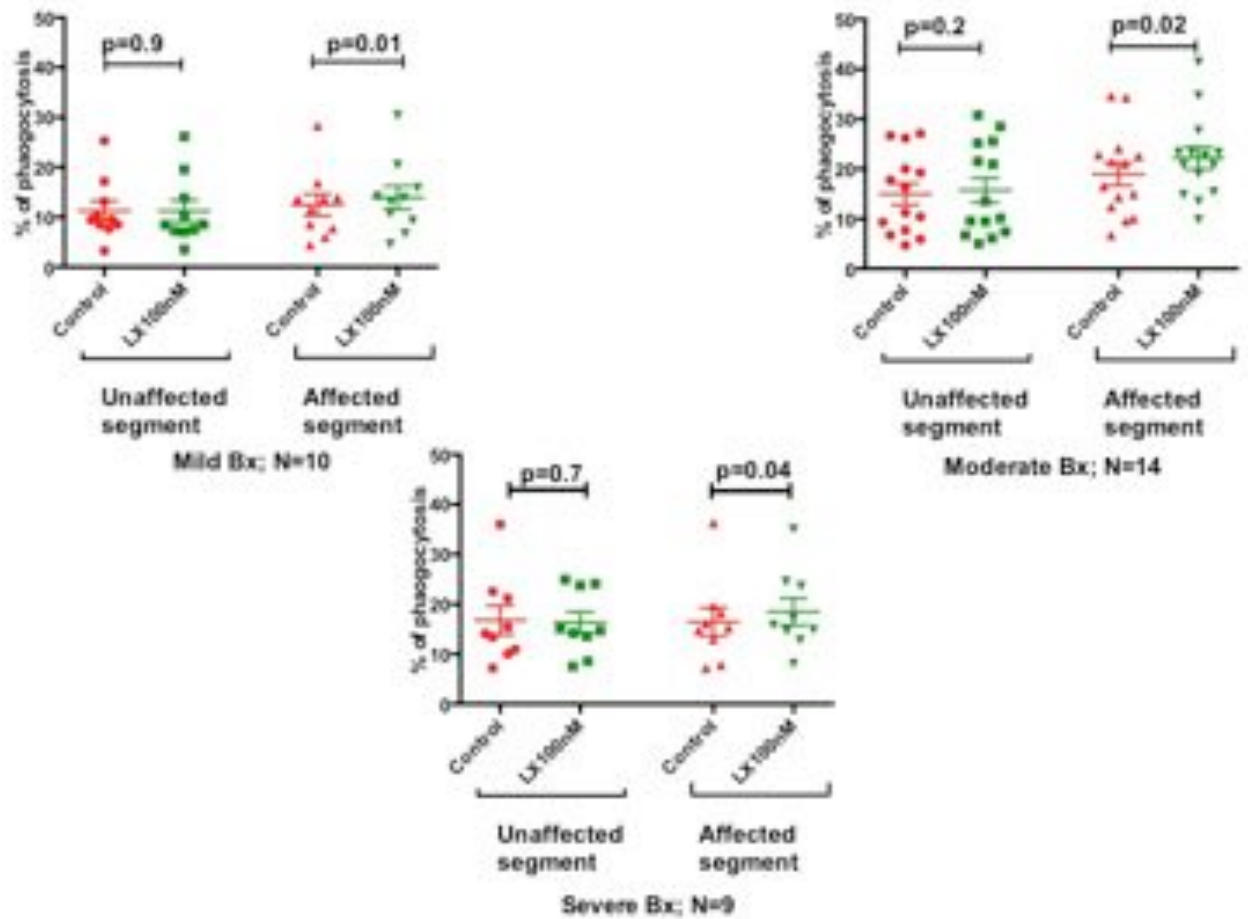
Figure 93. Lipoxin A$_4$ was able to significantly increase phagocytosis by airways neutrophils in the segments affected by bronchiectasis in mild, moderate and severe disease. There was no effect of LXA$_4$ on the unaffected segments. Paired t tests used for comparisons. Pooled data presented as mean ± SEM. Paired t tests used for all comparisons.

Bx= bronchiectasis; LX= Lipoxin A$_4$.

4.1.3.2. Bacterial killing

Following co incubation of the airway neutrophils (isolated from bronchoalveolar lavage fluid) with the GFP PAO1, the cells were lysed and the suspensions were plated out on pseudomonas isolation agar and bacterial counts were read after 24 hours. Data is presented as median (interquartile range).

Using paired t-tests, in the unaffected segments, there was a statistically significant improvement in bacterial killing with LXA$_4$ 100nM in the severe group of patients only; p=0.02. In the affected segments, there was a statistically significant improvement in killing by LXA$_4$ in the mild, moderate and severe bronchiectasis groups by LXA$_4$ 100nM, p=0.02, p=0.0005 and p=0.04 respectively, figure 94 and table 23.

	Unaffected segment cfu/ml			Affected segment cfu/ml		
	Control	**+LX 100nM**	**P value**	**Control**	**+LX 100nM**	**P value**
Mild N=10	$7.7X10^7$ $(5.4X10^7-$ $1X10^8)$	$6.8X10^7$ $(3.6X10^7-$ $9X10^7)$	0.07	$6.9X10^7$ $(4.1X10^7-$ $1.1X10^8)$	$4.6X10^7$ $(2.2X10^7-$ $8.6X10^7)$	**0.02**
Moderate N=14	$1.8X10^8$ $(6.2X10^7-$ $2.7X10^8)$	$1.9X10^8$ $(9.6X10^7-$ $2.5X10^8)$	0.1	$2.1X10^8$ $(8.3X10^7-$ $3.9X10^8)$	$1.8X10^8$ $(6.2X10^7-$ $2.8X10^8)$	**0.0005**
Severe N=9	$9.9X10^7$ $(3.6X10^7-$ $3.1X10^8)$	$7.9X10^7$ $(2.4X10^7-$ $1.9X10^8)$	**0.02**	$1X10^8$ $(2.7X10^7-$ $2.5X10^8)$	$4.6X10^7$ $(1.7X10^7-$ $1.6X10^8)$	**0.04**

Table 23. Bacterial counts after 24 hours; cfu= colony forming units.
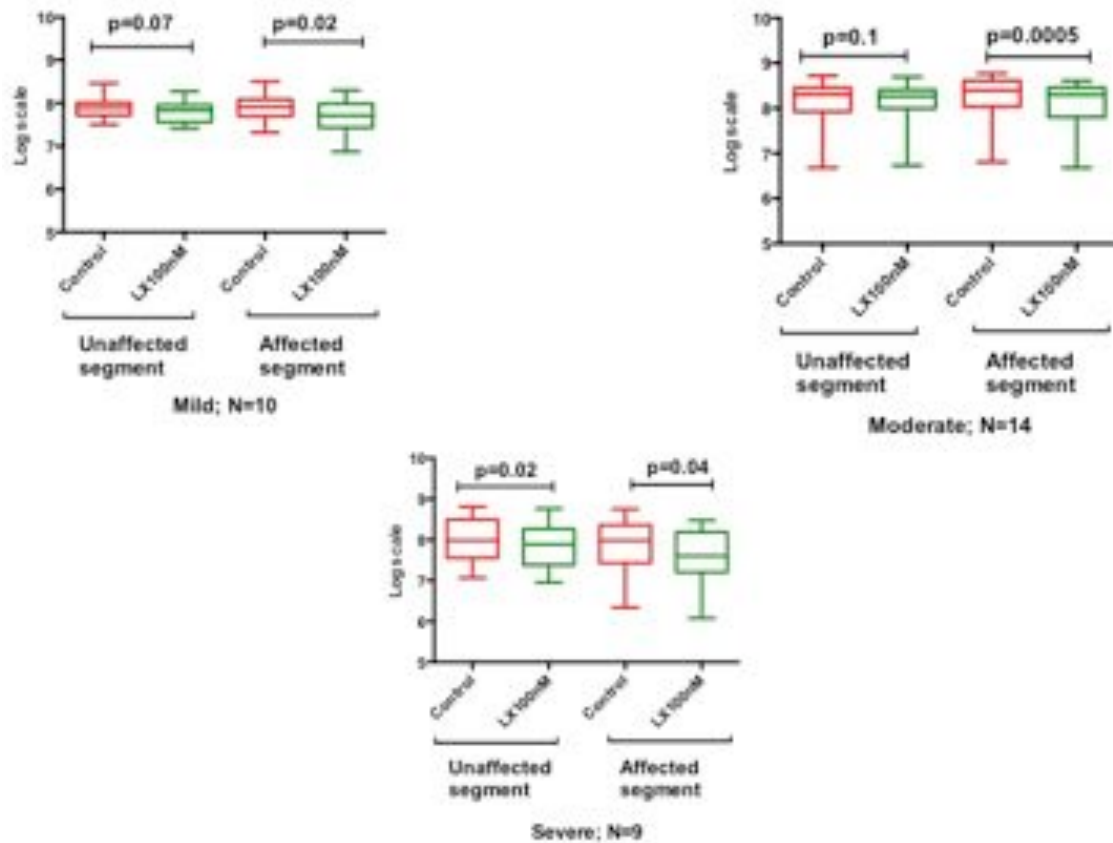
Figure 94. Lipoxin A_4 was able to significantly increase killing by airways neutrophils in the segments affected by bronchiectasis in mild, moderate and severe disease. However, in the unaffected segments, LXA_4 was able to increase killing in severe disease only. Paired t tests used for comparisons. Pooled data presented as median $\pm$ IQR. Unpaired t tests used for all comparisons. Scale is log bacterial load cfu/ml. Bx= bronchiectasis; LX= Lipoxin A_4.

The results from the phagocytosis and killing assays and the effect of Lipoxin A_4 suggests that Lipoxin A_4 predominantly has an effect on neutrophils from segments of the lung affected with bronchiectasis. In the unaffected segment- there is no further role for LXA_4 to improve neutrophil function, in comparison to affected segments where LXA_4 is able to significantly improve neutrophil phagocytosis and killing. Assessing neutrophil maturity and determining the ratio in which low-density and high-density neutrophils would be present in the unaffected and affected segments would be additive.

4.1.3.3. BALF inflammatory markers from affected airways

- *4.1.3.3.1. Myeloperoxidase*

   Myeloperoxidase was measured in all 4 groups from BALF. Using one-way ANOVA with Bonferroni's multiple comparison post hoc test showed a statistically significant increase in myeloperoxidase across the groups; p=0.002, with highest levels of MPO in the severe group. Comparison between groups (as measured by ANOVA) showed a significant difference between healthy and moderate group (p=0.01), between healthy and severe group (p=0.001). There was no statistically significant difference between healthy and mild group (p=0.3); figure 95.
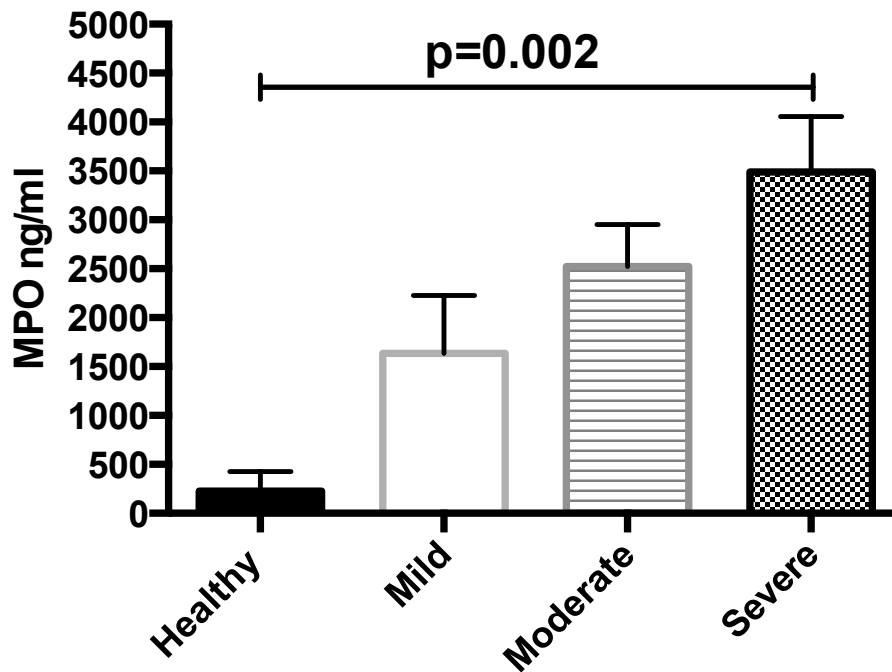
Figure 95. Myeloperoxidase measured was significantly higher in moderate and severe bronchiectasis compared to healthy volunteers. Pooled data presented as mean ± SEM. One way ANOVA with post test used for comparisons.
Healthy=6 volunteers; mild= 10 patients; moderate=14 patients and severe =9 patients.

- *4.1.3.3.2. Neutrophil Elastase*

Neutrophil elastase was measured in all 4 groups from bronchoalveolar lavage fluid. Using one-way ANOVA with Bonferroni's multiple comparison post hoc test showed a statistically significant increase in neutrophil elastase across the groups; p=0.002; with highest levels of neutrophil elastase in the severe group. Comparison between groups (as measured by ANOVA) showed a significant difference between healthy and severe group, p=0.01. There was no significant difference between healthy and mild (p=0.2) and healthy and moderate bronchiectasis (p=0.1) (figure 96).
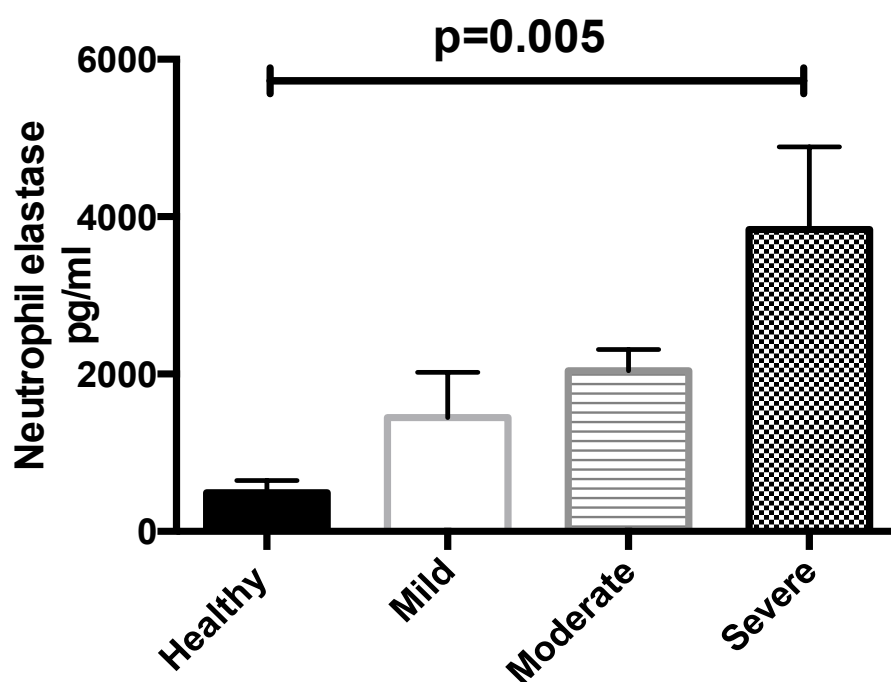
Figure 96. Neutrophil elastase was significantly higher in severe bronchiectasis compared to healthy volunteers. Pooled data presented as mean ± SEM. One way ANOVA used for comparisons.
Healthy=6 volunteers; mild= 10 patients; moderate=14 patients and severe =9 patients.

4.1.4. SERUM LL37

As one of the relevant anti microbial cathelicidins, I wanted to measure serum LL37 in all 4 groups. Although there was a trend towards increase in serum LL37 with disease severity, one way ANOVA with Bonferroni's post hoc analysis for multiple comparisons, showed no difference in the groups, p=0.1; figure 97.
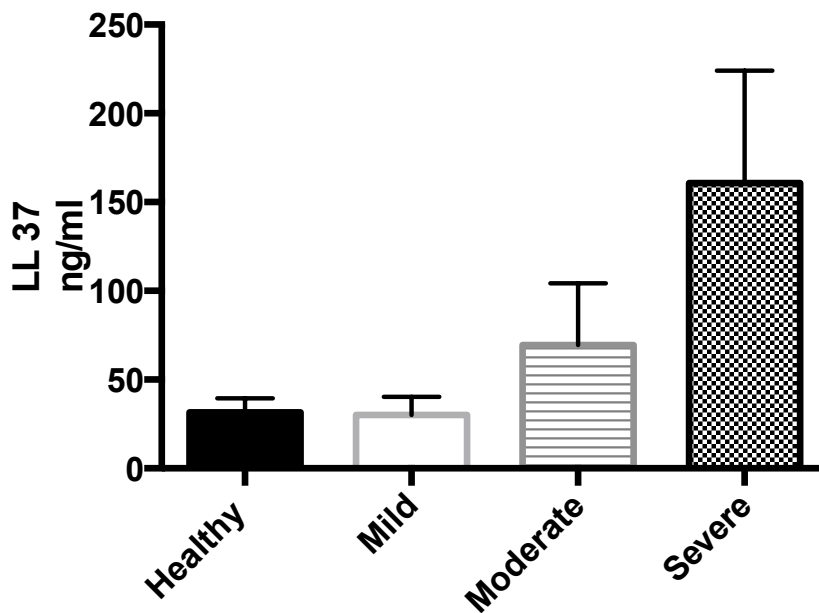
Figure 97. There was no difference in LL-37 measured across the groups, by one way ANOVA. Pooled data presented as mean ± SEM
Healthy=6 volunteers; mild= 10 patients; moderate=14 patients and severe =9 patients.

4.1.5. SERUM LIPOXIN A$_4$

Serum Lipoxin A$_4$ was measured in all study participants. One way ANOVA showed that LXA$_4$ was reduced in severe bronchiectasis compared to healthy volunteers; p=0.04. There was no significant difference in LXA$_4$ levels between healthy volunteers and mild bronchiectasis (p=0.2) or between healthy volunteers and moderate bronchiectasis (p=0.4); figure 98.
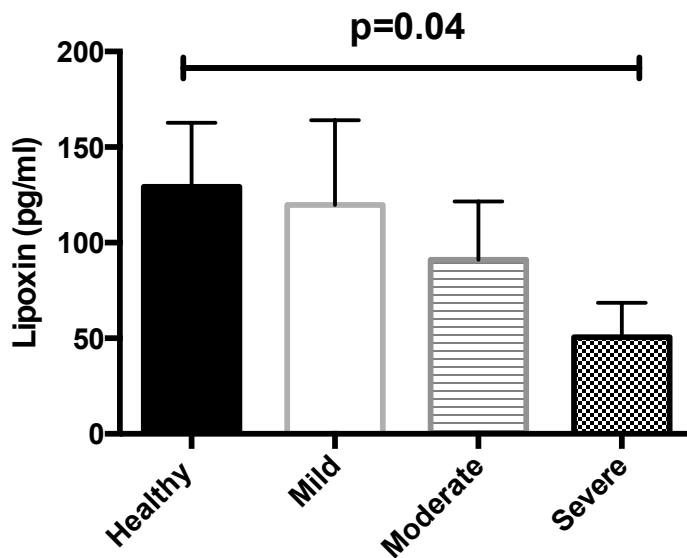
Figure 98. Serum Lipoxin A$_4$ was significantly reduced in severe bronchiectasis, on comparison to healthy volunteers, mild and moderate bronchiectasis. Pooled data presented as mean ± SEM. One way ANOVA used for comparisons.

4.1.6. SERUM LIPIDOMICS

Liquid chromatography and mass spectrometry was done on all serum samples obtained, where available. Samples were divided into mild (9) and moderate-severe (15) groups, based on their score calculated by the bronchiectasis severity index.

The main lipids assessed were: Lipoxin A_4, Resolvins, Maresins, Prostaglandin (PG) E2, 5 hydroxyeicosatetranoic acid (HETE), 15 hydroxyeicosatetranoic acid (15 HETE), LTB_4 and 9-hydroxyoctadecadienoic acid (9-HODE). Lipoxin A_4, Resolvins and Maresins were not detected in any of the samples. 15 HETE is a precursor of Lipoxin A_4 and is a pro inflammatory mediator. LTB_4 is a pro inflammatory cytokine and 9HODE is a pro inflammatory metabolite produced from arachidonic acid.

Using unpaired t-tests, there were significantly higher levels of PGE2, 15 HETE and LTB_4 in patients with moderate-severe disease compared to healthy controls, p=0.03, p=0.03 and p=0.02 respectively. Although there was a trend towards higher 5HETE and 9 HODE levels in moderate-severe disease compared to healthy volunteers, this failed to reach statistical significance; p=0.3 and 0.2 respectively (figure 16a&b). There was no difference in the PGE2, 15 HETE, LTB_4 , HETE and 9 HODE levels between the healthy volunteers and mild bronchiectasis (figure 99a&b).
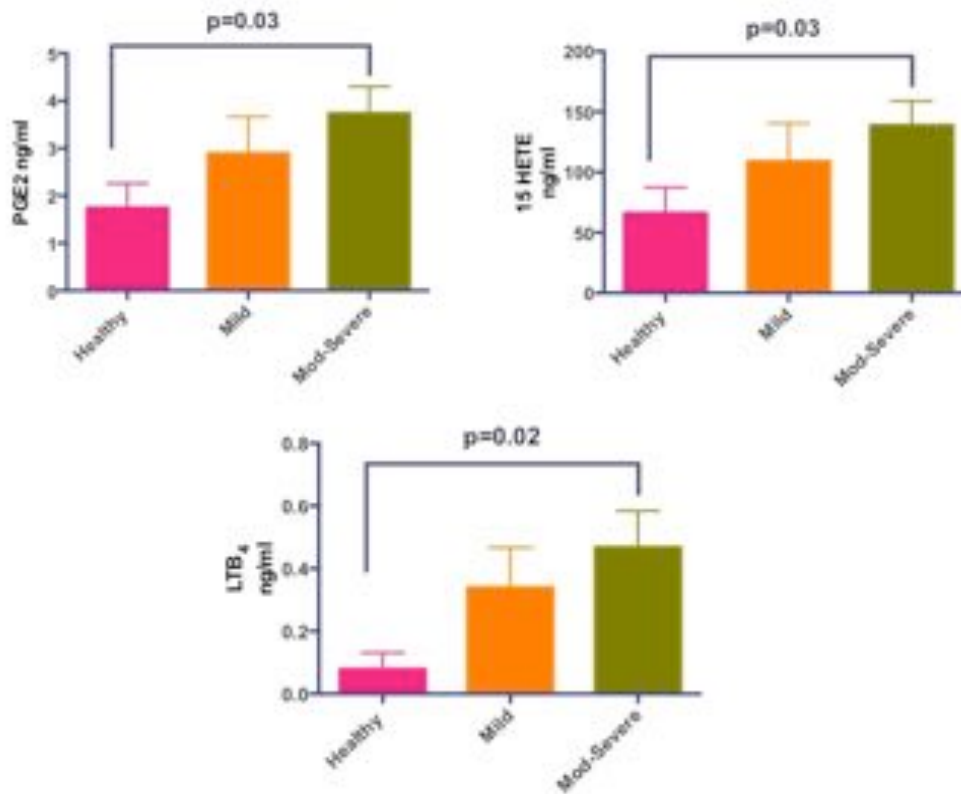
Figure 99a: Significantly higher levels of PGE2, 15 HETE and LTB$_4$ detected in patients with moderate- severe disease compared to mild healthy volunteers, p=0.03, p=0.03 and p=0.02, respectively. Lipidomics were obtained by mass spectrometry and liquid chromatography. Pooled data presented as mean ± SEM. One way ANOVA used for comparisons.

Healthy=6 volunteers; mild= 9 patients; moderate- severe =15 patients.
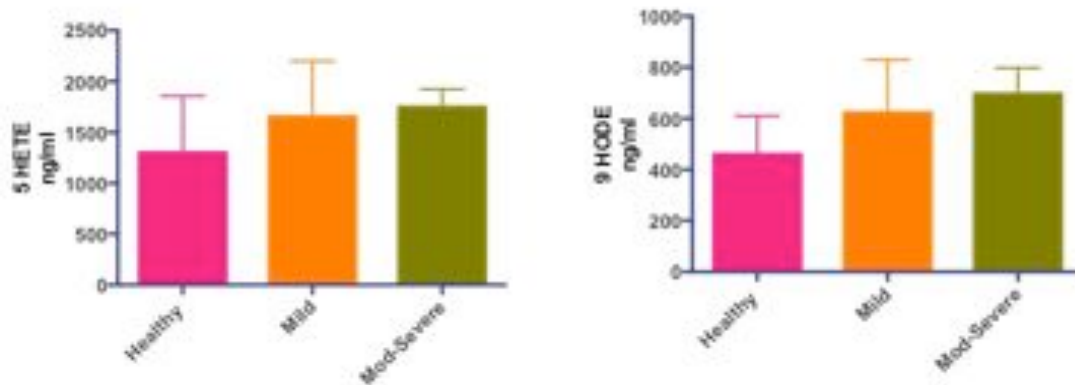
Figure 99b: Higher levels of 5 HETE and 9 HODE detected in patients with more moderate-severe disease compared to mild disease, but not statistically significant. Lipidomics were obtained by mass spectrometry and liquid chromatography. Pooled data presented as mean ± SEM. One way ANOVA used for comparisons.

5 HETE (hydroxyeicosatetranoic acid), 15 HETE hydroxyeicosatetranoic acid), 9-HODE (hydroxyoctadecadienoic acid), LT (leukotriene) B$_4$ and PG (Prostaglandin) E2.

Healthy=6 volunteers; mild= 9 patients; moderate- severe =15 patients.

4.1.7. BALF LIPIDOMICS

Liquid chromatography and mass spectrometry was done on all samples obtained at bronchoalveolar lavage, where available. In addition, samples were obtained from an area affected by bronchiectasis and an area unaffected by bronchiectasis; hence patients could act as their own internal control. As before, samples were divided into mild (9) and moderate-severe groups (15), based on their score calculated by the bronchiectasis severity index.

PGE2 was not detectable in BALF from healthy individuals. Using unpaired t-tests, there were significantly higher levels of PGE2, 5HETE and 15 HETE in patients with moderate-severe disease compared to healthy volunteers, $p<0.0001$, $p=0.004$ and $p=0.005$ respectively. There were significantly higher levels of 9HODE and LTB_4 in moderate-severe patients compared to healthy volunteers, $p=0.04$ and $p<0.0001$ respectively (figure 100 a&b). There was no difference in the PGE2, 15 HETE, LTB_4, HETE and 9 HODE levels between the healthy volunteers and mild bronchiectasis.
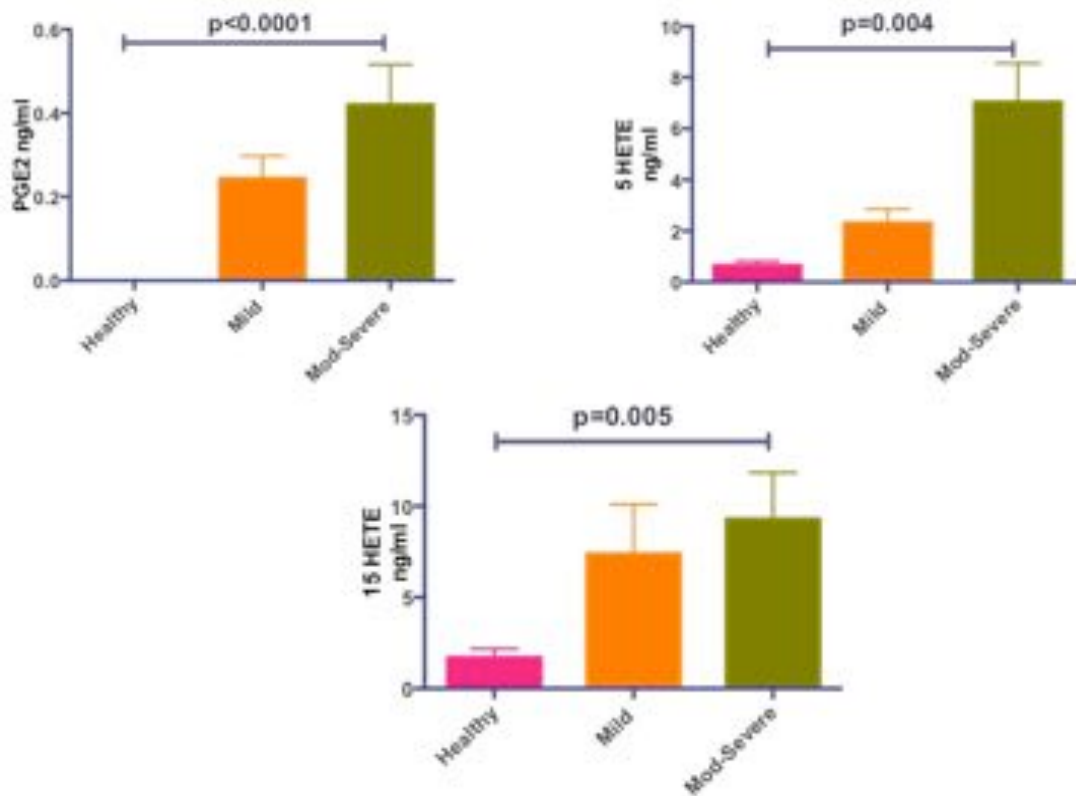
Figure 100a: Significantly higher levels of PGE2, 5 HETE and 15 HETE detected in patients with moderate- severe disease compared to mild disease. Lipidomics obtained by mass spectrometry and liquid chromatography. Pooled data presented as mean ± SEM. One way ANOVA used for comparisons.

Healthy=6 volunteers; mild= 9 patients; moderate- severe =15 patients.
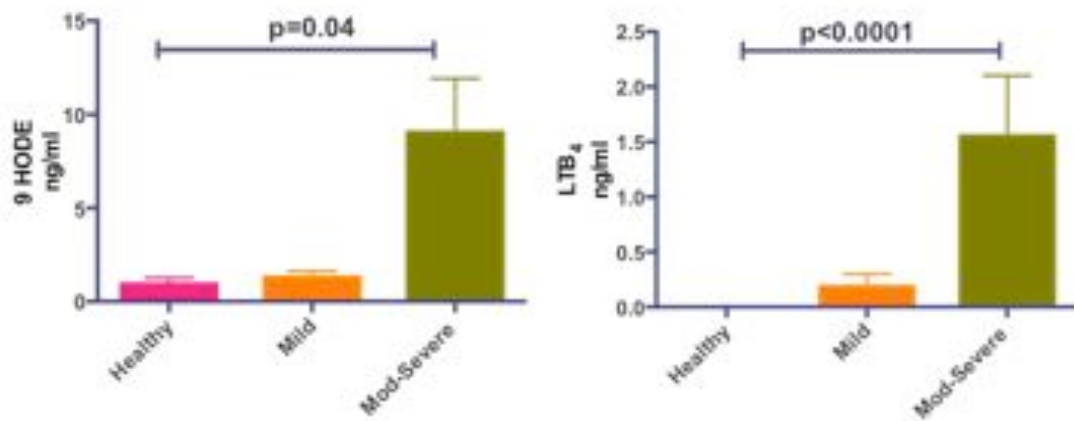
Figure 100b: Significantly, higher levels of 9 HODE and LTB$_4$ detected in patients with moderate-severe disease compared to mild disease and healthy volunteers. Lipidomics obtained by mass spectrometry and liquid chromatography. Pooled data presented as mean ± SEM. One way ANOVA used for comparisons.

5 HETE (hydroxyeicosapentanoic acid), 15 HETE hydroxyeicosapentanoic acid), 9-HODE (hydroxyoctadecadienoic acid), LT (leukotriene) B$_4$ and PG (Prostaglandin) E2.

Healthy=6 volunteers; mild= 9 patients; moderate- severe =15 patients.

4.1.8. Comparison of lipidomics in samples obtained from affected and unaffected areas

Data was then analysed to compare the mediators obtained from the affected and unaffected area of the same patient and to assess if this there was a significant difference in samples obtained from an individual patient.

Using paired t-tests, there were no significant differences between affected and unaffected segments in PGE2 (p=0.6 in mild and p=0.8 in mod-severe), 5HETE (p=0.7 in mild and p=0.9 in mod-severe), 15 HETE (p=0.5 in mild and p=0.1 in mod-severe), and 9 HODE (p=0.5 in mild and p=0.3 in mod-severe; figure 101). It was not possible to sub-analyse the results for LTB$_4$ as there were not enough samples in which LTB$_4$ was detected.
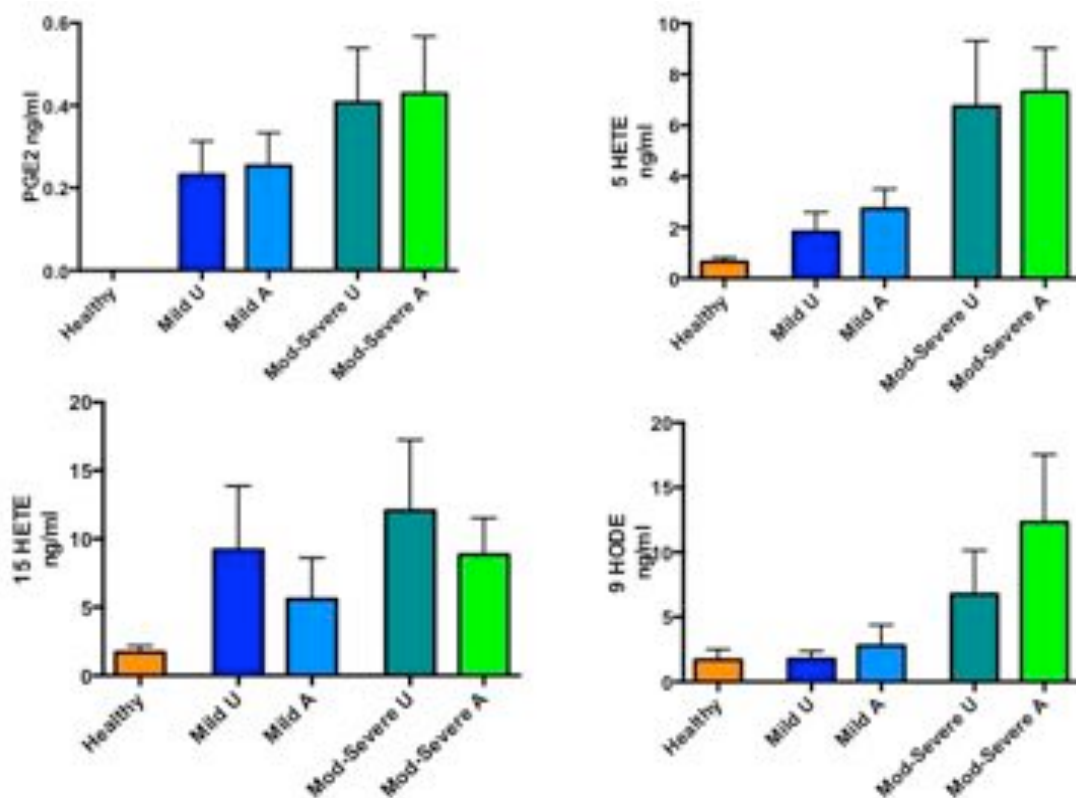
Figure 101. No significant difference in PGE2, 5 HETE, 15 HETE and 9HODE obtained by lipidomics between unaffected and affected segments in the mild and moderate-severe groups. Pooled data presented as mean ± SEM. Unpaired t tests used for comparisons.
5 HETE (hydroxyeicosapentanoic acid), 15 HETE hydroxyeicosapentanoic acid), 9-HODE (hydroxyoctadecadienoic acid) and PG (Prostaglandin) E2.
Healthy=6 volunteers; mild= 9 patients; moderate- severe =15 patients.

4.1.9. RT PCR from bronchial brushings

Study aim: To investigate a potential mechanism for low levels of LXA$_4$ in bronchiectasis, lipoxin biosynthetic genes expression was measured.

Bronchial brushings were obtained from all 34 patients and 6 healthy volunteers. RNA was extracted from the brushings where adequate samples were obtained. There was insufficient samples for RNA extraction from samples that were obtained from healthy volunteers. RT PCR was done for the following genes: 5 Lipoxygenase (5LOX), Leukotriene A$_4$ hydrolase (LTA$_4$H), 15 Lipoxygenase A (15 LO-A) and 15 Lipoxygenase B (15 LO-B). The lipoxin generation pathway and the important biosynthetic enzymes are shown in figure 12 section 1.10.

By quantitative PCR, the delta Ct values for these four pivotal LX biosynthetic genes were determined. LTA$_4$ hydrolase was the most abundant RNA (lowest DCt) in bronchial brushings. In contrast, the least abundant gene (highest delta Ct) was 15 LO-B. Using unpaired t-tests, the delta Ct values for 5-LOX and 15-LO-B were both significantly increased (p=0.01 and p=0.01 respectively) in subjects with moderate-severe bronchiectasis compared with subjects with mild bronchiectasis, indicating decreased expression of all these regulatory genes in moderate-severe bronchiectasis. Unpaired t- tests showed that, delta Ct value of LTA$_4$ hydrolase was significantly decreased in subjects with moderate-severe bronchiectasis compared with subjects with mild bronchiectasis (p=0.007), indicating increased expression of in moderate-severe bronchiectasis. There was no significant difference in 15 LO-A gene expressions between mild bronchiectasis and moderate-severe bronchiectasis (p=0.2); figure 102.
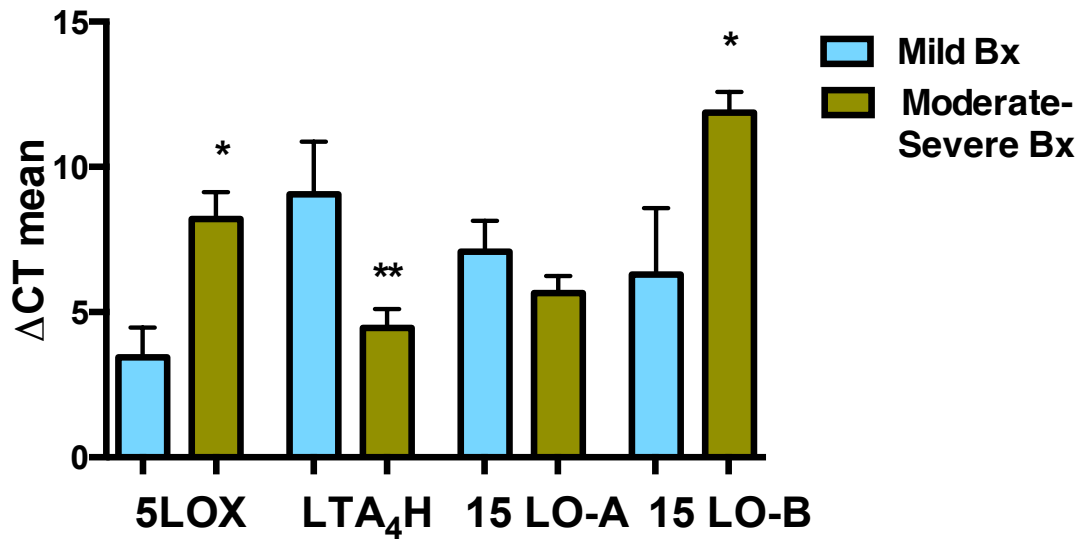
Figure 102: 5LOX and 15 Lipoxygenase B genes were significantly lower and LTA_4 hydrolase was significantly higher in moderate –severe bronchiectasis. No difference in 15 LO-A between the groups. Pooled data presented as mean ± SEM. Unpaired t tests used for comparisons.

5 Lipoxygenase (5LOX), Leukotriene A_4 hydrolase (LTA_4H); 15 Lipoxygenase A (15 LO-A) and 15 Lipoxygenase B (15 LO-B).

Mild= 9 patients; moderate- severe =15 patients.

4.2. LIPOXIN FUNCTION IN HEALTHY VOLUNTEERS AND IN BRONCHIECTASIS PATIENTS

4.2.1. Clinical parameters

This study was done to assess the role of Lipoxin A_4 on serum and airways neutrophil function in bronchiectasis patients, and compare it to its role on serum neutrophil function from healthy volunteers. A total of 40 study participants were recruited in the study- 34 bronchiectasis patients and 6 healthy volunteers. Of the 34 patients, 10 had mild, 15 had moderate and 9 had severe disease, as calculated by the bronchiectasis severity index. Several parameters were assessed to measure if diseases severity on the bronchiectasis severity index correlated to clinical and laboratory markers. There was no significant difference in age between the groups. There was a statistically significant higher values of total white cell count, neutrophil count and c- reactive protein, in the severe group compared to the other three groups. There was no difference in erythrocyte sedimentation rate across the groups, although there was a trend towards increasing ESR in the severe group. Lung function was measured in the bronchiectasis groups. There was a statistically significant lower values in the percent predicted forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and transfer factor for the lung carbon monoxide (TLCO), with worsening lung function in the severe group. There were more antibiotic courses and hospital admissions required in the severe group compared to mild and moderate group. Analysis of the percentage of bacteria isolated in each group showed that there was a statistically significant difference between healthy and severe groups, mild and severe groups, but failed to reach statistical significance between moderate and severe groups. There was however no difference in the colony counts across the groups.

4.2.2. Lipoxin and serum neutrophil function

Peripheral blood neutrophils were isolated from all four groups. Phagocytosis and killing of GFP labeled *Pseudomonas aeruginosa* by peripheral neutrophils and the effect of Lipoxin A_4 was measured. In the healthy group, there was a statistically significant improvement in phagocytosis by LXA_4 in a dose dependent manner. There was a statistically significant improvement in phagocytosis by LXA_4 in mild, moderate and severe bronchiectasis groups. In the healthy group, there was a statistically significant improvement in bacterial killing by LXA_4 in a dose dependent

manner;. There was a statistically significant improvement in bacterial killing by LXA$_4$ in mild, moderate and severe bronchiectasis groups.

4.2.3. Lipoxin and airways neutrophil function

Bronchoscopy was done and bronchoalveolar lavage fluid was obtained from two separate segments in the same patient- a segment affected by bronchiectasis and a segment unaffected by bronchiectasis. By this, patients were able to act as their own internal controls.

Airway neutrophils were isolated from bronchoalveolar lavage fluid. There was no statistically significant difference in neutrophil counts in the bronchoalveolar lavage fluids obtained from the unaffected segments compared to the affected segments.

In the unaffected segments, there was no statistically significant improvement in phagocytosis with LXA$_4$ 100nM in any of the groups. In the affected segments, there was a statistically significant improvement in phagocytosis by LXA$_4$ 100nM in the mild, moderate and severe bronchiectasis groups.

In the unaffected segments, there was a statistically significant improvement in bacterial killing with LXA$_4$ 100nM in the severe group of patients only; p=0.02. In the affected segments, there was a statistically significant improvement in bacterial killing by LXA$_4$ in the mild, moderate and severe bronchiectasis groups by LXA$_4$ 100nM, p=0.02, p=0.0005 and p=0.04 respectively.

4.2.4. Lipoxins, cytokines and cathelicidins in bronchiectasis

Serum Lipoxin A_4 levels were measured and statistically significant lower levels were detected in severe patients compared to healthy volunteers. There was no significant difference in lipoxin levels between mild and moderate disease and healthy volunteers.

Inflammatory markers were measured in the bronchoalveolar lavage fluid. There was a statistically significant difference in myeloperoxidase and free neutrophil elastase across the groups, with higher levels detected in the severe group. There was a trend towards increase in the anti microbial cathelicidin LL-37 in severe patients, but this was not statistically significant.

4.2.5. Lipidomics

Lipidomics was done on serum and bronchoalveolar samples, as available. For analyzing the lipidomics- three groups were used- healthy, mild and moderate-severe bronchiectasis. Lipoxin A_4, resolvins and maresins were not detected in any of the samples- in serum or lavage. In serum, significantly higher levels of PGE2, 15 HETE and LTB_4 were detected in moderate- severe disease compared to healthy individuals. 5 HETE land 9 HODE levels were higher in moderate to severe disease but not statistically significant.

There were significantly higher levels of PGE2, 5-hydroxyeicosapentanoic acid (HETE) and 15 HETE in patients with severe disease compared to patients with mild disease and healthy volunteers. There were significantly higher levels of 9-hydroxyoctadecadienoic acid (HODE) and LTB_4 in severe patients compared to mild patients and healthy volunteers.

Further sub analysis of lipidomics was done to assess if there was a difference in lipidomics between the affected and unaffected segments in the groups. Although, there was a trend towards increased levels of prostaglandin E2, 5HETE and 9HODE, and decreased levels of 15 HETE in the affected segments compared to the unaffected segments, none of these were statistically significant. LTB_4 was detected in fewer samples and hence enough data sets were not available for a sub analysis.

What was very different in the levels of 5 HETE and 15 HETE detected was that there was an at least 100 fold higher level of these metabolites detected in serum compared to bronchoalveolar lavage fluid. This indicates that these lipids are expressed in different quantities in different compartment of the body. Would gene expression be different in serum compared to bronchial cells? This needs to be explored further.

4.2.6. Gene expression in bronchial brushings

Gene expression of key lipoxin biosynthetic enzymes was measured in samples obtained from bronchial brushings. Statistically significantly higher ΔCt levels of 5LOX, p=0.01 and 15LO-B, p=0.01 were detected in moderate-severe bronchiectasis patients compared to mild bronchiectasis, indicating decreased expression of these genes in bronchial brushings from moderate-severe patients. In addition, lower ΔCt levels of LTA$_4$ hydrolase, p=0.007 were detected in severe bronchiectasis, compared to mild disease indicating higher gene expression of LTA$_4$ hydrolase in moderate-severe disease. There were lower levels of 15 LO-A in moderate-severe bronchiectasis compared to mild bronchiectasis although this was not statistically significant. The lowest ΔCt level was that of LTA$_4$ hydrolase indicating it was the most abundant gene isolated in bronchial brushings from moderate-severe patients. The highest ΔCt level was of 15 LO-B indicating it was the least abundant gene isolated in bronchial brushings from moderate-severe patients.

CHAPTER 5

LONGITUDINAL STUDY

5.1. PATIENT CHARACTERISTICS

169 patients were included in the study. All patients were seen at baseline and one year later, whilst clinically stable. Patients were categorized in to mild, moderate and severe bronchiectasis severity, based on the BSI index. There were 61 patients with mild disease, 69 with moderate and 39 with severe bronchiectasis.

Baseline demographics of the patients are listed in table 24.

Parameters	Mild N=61	Moderate N=69	Severe N=39
Age	61 (1.4)	69 (1.1)	70 (1.4)
Gender (% female)	46%	54%	51%
Smoking			
Ex	19 (31%)	28 (40%)	20 (51%)
Never	38 (62%)	33 (48%)	18 (46%)
Current	4 (7%)	8 (12%)	1 (3%)
Cause of bronchiectasis			
Idiopathic	42 (69%)	48 (70%)	27 (69%)
Post infectious	8 (13%)	8 (11%)	8 (21%)
ABPA (inactive)	8 (13%)	6 (9%)	2 (5%)
Rheumatoid arthritis	1 (1%)	2 (3%)	1 (2%)
Ulcerative colitis	2 (3%)	5 (7%)	1 (2%)
Asthma	23 (38%)	29 (42%)	17 (43%)
COPD	4 (7%)	11 (16%)	9 (23%)
IHD	13 (21%)	10 (14%)	9 (23%)
Previous malignancy	5 (8%)	9 (13%)	3 (8%)

Table 24. ABPA= allergic bronchopulmonary aspergillosis; COPD= chronic obstructive pulmonary disease; IHD= ischaemic heart disease.

5.2. SERUM LIPOXIN A$_4$ AND BRONCHIECTASIS

5.2.1. At baseline

Serum Lipoxin A$_4$ from healthy volunteers was measured. The mean was 269.3 pg/ml, with the lower 95% CI of 135 pg/ml. Based on these results, bronchiectasis patients in the stable state, with a serum level of LXA$_4$ <135 pg/ml were taken as deficient in Lipoxin A$_4$ and a value of ≥ 135 pg/ml was taken to be sufficient in LXA$_4$ (figure 103). Using unpaired t-tests (for all comparisons), at baseline, bronchiectasis patients deficient in serum LXA$_4$ had more severe disease p=0.01 (as calculated by the Bronchiectasis severity index), had significantly higher serum levels of serum c-reactive protein and erythrocyte sedimentation rate; p=0.04 and p=0.02 respectively. LXA$_4$ deficient patients had higher levels of sputum myeloperoxidase and LTB$_4$ levels; p=0.01 and p=0.03 respectively, figure 104 a&b. In addition, LXA$_4$ deficient patients had a worse quality of life, difference of 4units compared to LXA$_4$ sufficient patients, as measured by the St. Georges Respiratory Questionnaire. Other parameters measured are shown in table 25 below.
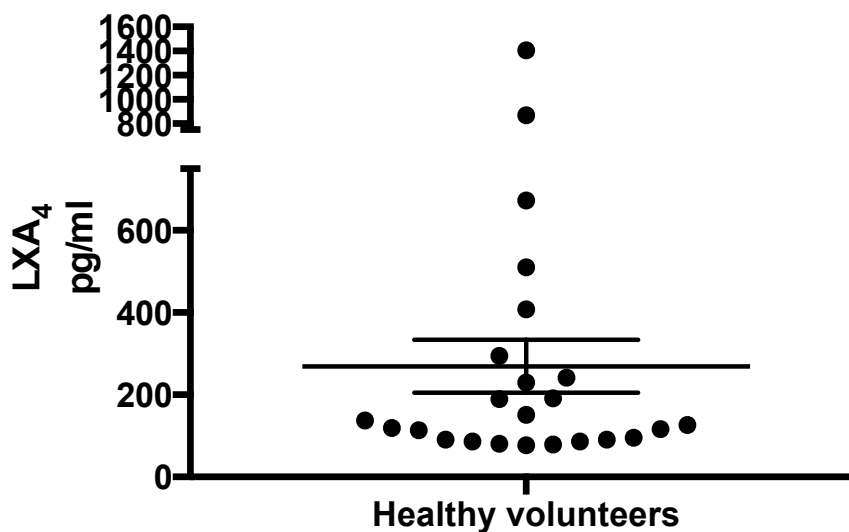
Figure 103. LXA$_4$ as measured in healthy volunteers. Pooled data presented as mean ±SEM.

	Deficient patients (N=95)	Sufficient patients (N=74)	p value
LXA_4 (pg/ml)	62.3 (8.8)	234 (28)	**<0.0001**
Bronchiectasis severity index	8.3 (0.6)	5.8 (0.7)	**0.01**
WCC (10^9/L)	8.2 (0.6)	6.9 (0.4)	0.09
CRP (mg/L)	12.1 (3.4)	4.6 (0.9)	**0.04**
ESR (mm/hr)	21.7 (2.8)	13.4 (1.6)	**0.02**
LCQ (Units)	17.1 (0.5)	16.8 (0.5)	0.7
SGRQ (Units)	34.1 (2.6)	25.3 (2.9)	**0.03**
FEV_1 % predicted (L)	77.3 (3.2)	77.5 (4.4)	0.9
FVC % predicted (L)	95.7 (3.8)	99.7 (4)	0.5
Antibiotic courses	1.9 (0.2)	1.8 (0.3)	0.8
Hospital admissions per year	0.4 (0.1)	0.4 (0.1)	0.8
Sputum neutrophil elastase (ng/ml)	9 (2.9)	12.5 (7.7)	0.6
Sputum myeloperoxidase (ng/ml)	21400 (4400)	1900 (700)	**0.01**
Sputum CXCL 8 (ng/ml)	23.3 (3.3)	29.6 (6.8)	0.3
Sputum leukotriene B_4 (pg/ml)	196.4 (30.7)	99.4 (29.5)	**0.03**

Table 25. Baseline inflammatory markers, quality of life and spirometry in lipoxin deficient and sufficient patients. Data presented as mean (±SEM).
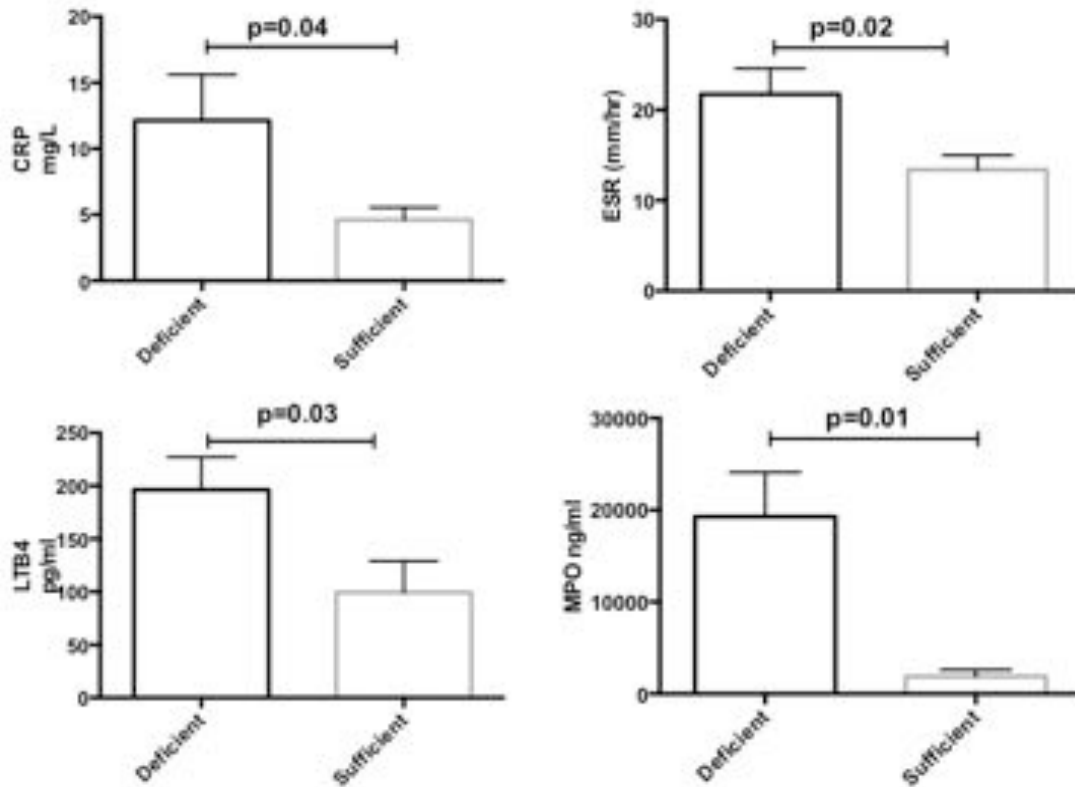
Figure 104a.
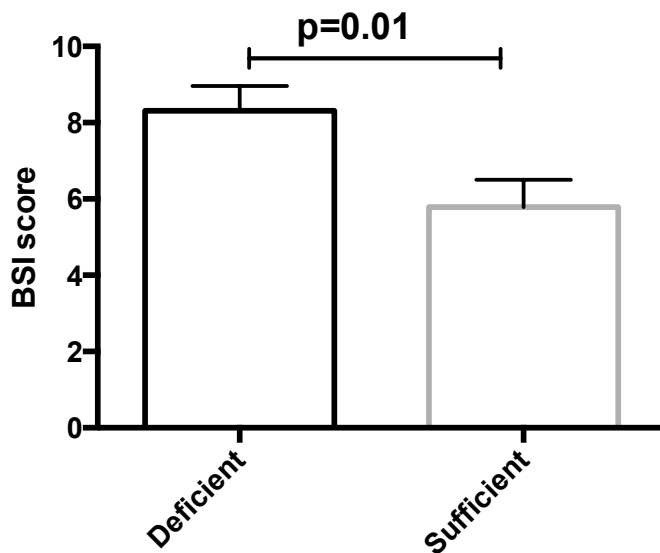
Figure 104b.
Figure 2. (a) CRP, ESR, LTB$_4$ and MPO were significantly different at baseline between the deficient (N=95) and sufficient (N=74) patients.
(b) BSI was significantly higher in the deficient group.
Pooled data presented as mean ±SEM. Unpaired t –tests used for comparison.
BSI= Bronchiectasis Severity Index; CRP= c reactive protein; ESR = erythrocyte sedimentation rate; LTB$_4$ = Leukotriene B$_4$; MPO= myeloperoxidase.

5.2.2. Follow-up after 1 year

All 169 patients were followed up after 1 year and the same parameters were measured again to assess disease progression. Using unpaired t-tests, patients who were lipoxin sufficient, had lesser exacerbations requiring antibiotics (p=0.02) and significantly lesser amounts of LTB_4 (p<0.0001) and myeloperoxidase (p<0.0001) in sputum, compared to lipoxin deficient patients; figure 105.
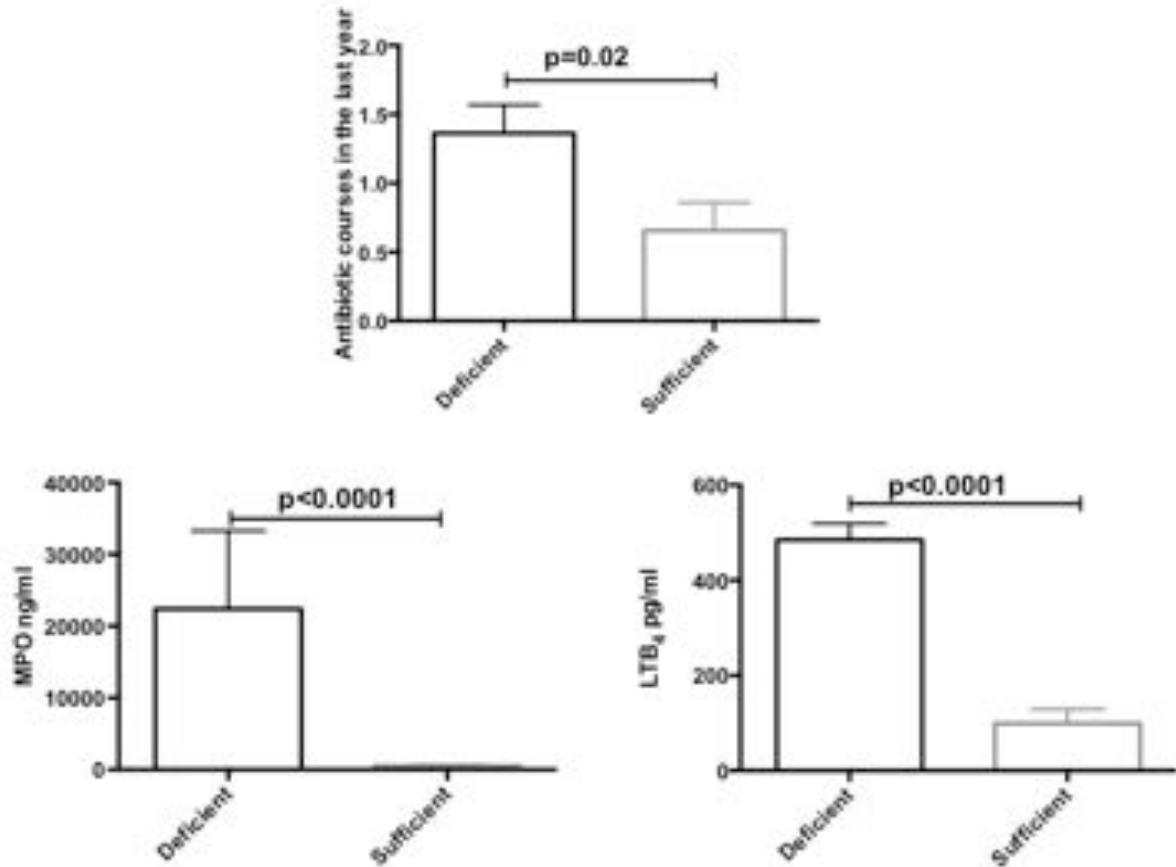
Figure 105. Significantly lesser exacerbations, MPO and LTB_4 were detected in the Lipoxin A_4 sufficient patients after 1 year. Pooled data presented as mean ±SEM. Unpaired t –tests used for comparison.
LTB_4= Leukotriene B_4; MPO= myeloperoxidase.
Deficient patients N=95, sufficient patients N=74.

5.3. SUMMARY OF LONGITUDINAL STUDY

This study was done to assess the role of lipoxin and its deficiency in bronchiectasis in a large cohort of bronchiectasis patients, over a year. 169 bronchiectasis patients were recruited into the study. All patients were reviewed at baseline and then after a year. Several parameters were checked at both time points including serum lipoxin A_4, white cell count, c reactive protein, erythrocyte sedimentation rate, sputum inflammatory markers (myeloperoxidase, neutrophil elastase and interleukin 8), quality of life questionnaires and spirometry. Bronchiectasis severity index was calculated on all patients. Hospital admissions and antibiotic courses for bronchiectasis exacerbations only were also recorded. Serum lipoxin was measured in healthy volunteers and followed up longitudinally. Based on these results, bronchiectasis patients in the stable state, with a serum LXA_4 level of $LXA_4 <135$ pg/ml were taken as deficient in Lipoxin A_4 and a value of ≥ 135 pg/ml was taken to be sufficient in LXA_4. Patients were categorized into lipoxin sufficient and deficient groups. At baseline, the ***LXA_4 deficient*** group had more severe disease as calculated by the bronchiectasis severity index; significantly raised c reactive protein; erythrocyte sedimentation rate and sputum myeloperoxidase and LTB_4 levels, compared to lipoxin sufficient patients. Patients with LXA_4 deficiency had worse quality of life as measured by St. Georges Respiratory Questionnaire. There was no difference in forced expiratory volume in one second or forced vital capacity between the groups. There was no difference in hospital admissions or antibiotic courses required for exacerbations at baseline, between the groups.

All patients were followed up after one year. On comparison of the two groups after 1 year, the ***lipoxin sufficient*** group had lesser and fewer exacerbations needing antibiotic therapy. In addition, the ***lipoxin sufficient*** group had significantly lesser amounts of the pro inflammatory cytokine interleukin 8 and leukotriene B_4 in sputum, indicating that lesser inflammation in the airways perhaps leads to fewer exacerbations. No changes in other parameters were noted.

CHAPTER 6

DISCUSSION

6.1. REPROGRAMMING OF BLOOD NEUTROPHILS IN BRONCHIECTASIS

6.1.1. Are blood neutrophils reprogrammed in bronchiectasis?

Neutrophils from bronchiectasis patients live longer and undergo less apoptosis compared to healthy volunteers. There was no difference in apoptosis between mild and severe bronchiectasis, suggesting that in bronchiectasis, peripheral blood neutrophils persist longer irrespective of disease severity. Given the number of neutrophils in bronchiectasis airways, late neutrophil apoptosis could have devastating consequences. Slowing neutrophil apoptosis would mean delayed removal by macrophages and thereby perpetuating the ongoing inflammation. To understand, if delayed apoptosis was a constitutive or adaptive defect, I investigated apoptosis by using serum from healthy volunteers. Delayed apoptosis was reversed by incubating neutrophils with serum from healthy volunteers- suggesting a constitutive defect rather than an acquired mechanism.

In bronchiectasis, I have established that peripheral neutrophils have increased expression of CD11b, more shedding of CD62L, and increased release of myeloperoxidase in the stable state compared to healthy volunteers. Results from these neutrophil activation experiments demonstrate that in bronchiectasis, peripheral peripheral blood neutrophils are in a primed and pre activated state at all times, perhaps secondary to chronic and undetectable infection. All patients in this study had no infective exacerbation of bronchiectasis for at least 4 weeks prior to giving blood for this study. Hence, acute infection can be safely ruled out as a driver for inflammation in this study. Thereby, this study helps distinguish between 'adaptive' and 'constitutive' inflammation in bronchiectasis. I have established earlier in this thesis, that there is increased myeloperoxidase, interleukin 8, leukotriene B_4 and neutrophil elastase in the airways in these patients. Do these pro inflammatory mediators lead to inflammatory reprogramming of neutrophils in bronchiectasis? It is known that inflammatory reprogramming of neutrophils lead to increased viability

(Chakravarti et al 2009). To my best knowledge, peripheral blood and airways neutrophils have not been studied in detail in the literature so far.

Once I established that peripheral neutrophils are primed and pre activated in bronchiectasis, I wanted to assess what functional correlates this would have. These neutrophils showed a very characteristic profile. Super oxide generation was increased in both mild and severe patients compared to healthy volunteers at baseline, although this was not statistically significant. The ability of neutrophils to produce toxic oxygen radicals constitutes a major weapon against microbial intruders. This will be discussed in detail later in the next section. I found that neutrophils from healthy volunteers were able to phagocytose and kill bacteria, significantly higher when compared to neutrophils from bronchiectasis patients. Thus so far these 'reprogrammed' neutrophils have demonstrated that despite being pre activated their ability to phagocytose and kill bacteria was lesser than that compared to healthy volunteers. This would thereby lead to persistent infection and inflammation in bronchiectasis. Is there a failure in the resolution of inflammation in bronchiectasis leading to chronic inflammation? The neutrophils must "switch off" and be "buried" for the inflammation to resolve. I need to understand the biochemical pathways that can trigger the process of resolution of inflammation in bronchiectasis. Hence, there is a need for anti inflammatory and pro resolving mediators in bronchiectasis.

6.2. NEED FOR ANTI INFLAMMATORY AND PRORESOLUTION MEDIATOR IN BRONCHIECTASIS

So far, I have established that in bronchiectasis, blood neutrophils are primed and are in a pre-activated state even in the stable state. These 'reprogrammed' neutrophils undergo delayed apoptosis, which contributes to the ongoing persistent inflammation. These neutrophils are more activated at baseline, with significantly higher CD62L shedding and CD11b upregulation. Additionally, these reprogrammed neutrophils degranulate to release significantly high levels of myeloperoxidase. This affects the functional ability of these neutrophils- lesser phagocytosis and killing of bacteria. I found that, airways neutrophils have lesser phagocytic and killing capacity than blood neutrophils. This would in part explain why there is persistent airways neutrophilic inflammation in bronchiectasis. Are airways neutrophils also reprogrammed in bronchiectasis? This is beyond the scope of this thesis and needs to be explored further.

On comparing neutrophil function during exacerbations to stable state, I found that both in blood and airways neutrophils, there was higher bacterial phagocytosis and killing in the stable state than during exacerbations. Antibiotic therapy improves neutrophil function during exacerbations and restores it to its functional ability in the stable state.

In community-acquired pneumonia, similarly, antibiotic therapy improved both bacterial phagocytosis and killing. However, there was significantly higher bacterial killing by neutrophils from healthy volunteers in community-acquired pneumonia than bronchiectasis, both at the start and end of infection.

Reprogrammed neutrophils in bronchiectasis have lesser phagocytic and killing ability compared to healthy volunteers during exacerbations. This is perhaps secondary to their pre-activated state even in the stable state. I hypothesize that pro resolving and anti-inflammatory agents such as lipid mediators, administered in the stable state may improve neutrophil function during exacerbation. This would thereby provide a role of non-antibiotic therapy in improving neutrophil function.

6.2.1. Role of lipids in the inflammatory process: Oxidative lipid products including, notably, the products of unsaturated lipids are increasingly being recognized as important contributors to chronic inflammatory diseases (Bonnans and Levy 2007, Serhans et al 2007). The resolution of inflammation is a highly coordinated and active process that is controlled by endogenous 'pro-resolving' mediators (Gilroy et al 2004). Lipid mediators derived from arachidonic acid (**lipoxins**) (Serhan and Savill 2005, Serhan 2005, Samuelsson et al 2007) are known to regulate the inflammatory process and generate pro-inflammatory, anti-inflammatory and pro-resolving mediators. Lipoxins display selective actions on leukocytes that include inhibition of polymorphonuclear neutrophils (PMN) chemotaxis (Levy et al 2001), inhibition of PMN adhesion to and transmigration through endothelial cells (Papayanni et al 1996), as well as inhibition of PMN-mediated increases in vascular permeability. LXs are potent stimuli for peripheral blood monocytes, stimulating monocyte chemotaxis and adherence (Maddox and Serhan 1996), without causing degranulation or release of reactive oxygen species (Joszef et al 2002). More recently LX has been shown to attenuate release of cytokine [Interleukin (IL)-1β, IL-13, Interferon (IFN) gamma] and CXCL8 (Hachicha et al 1999, Gronert et al 1998), eosinophil trafficking and stimulation of phagocytosis of apoptotic PMN *in vitro* (Godson et al 2000).

6.3. LIPOXIN AND THE INFLAMMATORY PROCESS IN BRONCHIECTASIS

6.3.1. Lipoxin and apoptosis

Bronchiectasis is a neutrophilic condition where recurrent cough, excessive sputum production and recurrent chest infections are the key symptoms. Precise control of the neutrophil death program provides a balance between their defense functions and safe clearance, whereas impaired regulation of neutrophil death is thought to contribute to a wide range of inflammatory pathologies. Apoptosis is essential for neutrophil functional shutdown, removal of the emigrated neutrophils, and timely resolution of inflammation (Rossi et al 2006). Neutrophils receive survival and pro-apoptosis cues from the inflammatory microenvironment and integrate these signals through surface receptors and several downstream mechanisms. A complex network of intracellular death and survival pathways regulates neutrophil apoptosis and the balance of these pathways would ultimately determine the fate of neutrophils. Since neutrophils undergo apoptosis even in the absence of any extracellular stimuli, this type of death is called spontaneous or constitutive programed cell death (Savill et al 2002; Rossi et al 2006). It has been demonstrated that during inflammation, extending the lifespan of neutrophils during transendothelial migration and at the sites of infection is critical for efficient destruction of pathogens (Watson et al 1997; Savill et al 2002; Nathan 2006). Once this is achieved, neutrophils may undergo necrosis, apoptosis, NETosis (neutrophil extracellular trap cell death) (Brinkmann et al 2004; Fuchs et al 2007), or autophagy (Remijsen et al 2011) with the type of death profoundly affecting the outcome of the inflammatory response.

Apoptotic neutrophil death *in situ* has multiple pro-resolution actions. In addition to be coming unresponsive to agonists and stopping the production of inflammatory mediators, apoptotic neutrophils can thereafter sequester cytokines (Ariel et al 2006; Ren et al 2008) and their phagocytosis by macrophages induces macrophage polarization from a pro-inflammatory (M1) to a pro-resolution (M2) phenotype (Fadok et al 1998). M2 macrophages secrete mediators, such as IL-10 and TGFb, which mediate resolution and tissue repair (Ariel and Serhan, 2012; Sica and Mantovani, 2012).

In non-resolving inflammation, such as in bronchiectasis, it is speculated that neutrophils persist at the inflamed site and are liable to cause tissue destruction (Nathan and Ding, 2010; Soehnlein, 2010). Neutrophil recruitment may occur normally or may become excessive, but neutrophils persist as a result of delayed apoptosis or decreased clearance by macrophages (Haslett 1999; Savill et al 2002). The abnormal host response creates a persistent inflammatory microenvironment with ongoing release of inflammatory mediators and with it the damage-associated molecular patterns (Nathan and Ding 2010; Serhan 2011).

6.3.2. Myeloperoxidase prolongs neutrophil life span and delays resolution of inflammation

Acute elevation of plasma myeloperoxidase levels are known to prolong the life span of rat neutrophils by suppression of apoptosis as assayed ex vivo by Kebir et al. MPO also suppresses neutrophil apoptosis in a mouse model of carrageenan-induced lung injury and delays spontaneous self-resolution of pulmonary inflammation (El Kebir et al 2008). Thus, combined administration of carrageenan and MPO evokes persisting lung injury/inflammation with few airway neutrophil-exhibiting signs of apoptosis even 5 days post-injection, compared to complete resolution of pulmonary inflammation in the lungs of carrageenan-injected mice. The effects of MPO closely resemble those of zVAD-fmk, a pan-caspase inhibitor, which aggravates and prolongs carrageenan-elicited acute pleurisy (Rossi et al., 2006) and lung inflammation (El Kebir et al, 2008).

Lipoxins exert multipronged actions to counter neutrophil responses to MPO. Down-regulation of Mac-1 expression on neutrophils adhesion and transendothelial migration is one of the key components of the anti-inflammatory activities of LXA_4 and 15-epi-LXA_4 (Serhan et al, 2008). 15- epi-LXA_4 also prevents MPO-induced up-regulation of Mac-1 expression and MPO release, thereby interrupting MPO-mediated autocrine/paracrine loop for perpetuation of the inflammatory response (El Kebir et al, 2009). Inhibition of neutrophil adhesion and transendothelial migration are important components of the anti-inflammatory activities of LXA_4 and 15-epi-LXA_4.

Lipoxins themselves do not appear to interfere with the apoptotic machinery in neutrophils, whereas they can override the potent outside-in Mac-1-mediated survival

signal and redirect neutrophils to apoptosis *in vitro* (El Kebir et al, 2009). 15-epi-LXA$_4$ attenuates MPO-evoked ERK and Akt-mediated phosphorylation of the pro-apoptotic protein Bad and decreases Mcl-1 expression, critical events in enhancing neutrophil apoptosis. Non-phosphorylated Bad associates with Mcl-1 and prevents its anti-apoptotic actions (Reed, 2006). These would aggravate mitochondrial dysfunction, ultimately leading to caspase-3 mediated cell death (El Kebir et al, 2009; Wardle et al, 2011). Treatment of mice with 15-epi-LXA$_4$ at the peak of inflammation enhances resolution of carrageenan plus MPO-induced and *E. coli* septicemia-associated acute lung injury and improves the survival rate (El Kebir et al, 2009). 15-epi-LXA$_4$ reduces pulmonary neutrophil accumulation with concomitant increases in the percentage of apoptotic neutrophils in the airways, facilitates recruitment of monocytes/macrophages and phagocytosis of apoptotic neutrophils and other cells (El Kebir et al, 2009), all of which is consistent with tissue repair (Godson et al, 2000; Mitchell et al, 2002). Furthermore, LXA$_4$ released at sites of inflammation protects macrophages from apoptosis (Prieto et al, 2010). The beneficial actions of 15-epi-LXA$_4$ can be prevented in the presence of a pan-caspase inhibitor, thereby indicating the importance of neutrophil apoptosis in inflammatory resolution.

In one of the sections of my study I have demonstrated the neutrophils have delayed apoptosis and live longer in bronchiectasis. I have also demonstrated that lipoxin was unable to modulate spontaneous or constitutive neutrophil apoptosis and this is in keeping with the current available literature. In addition, I have also demonstrated that there are significantly higher levels of myeloperoxidase detected in bronchiectasis patients. The raised myeloperoxidase levels could explain in part why there is prolonged survival of neutrophils in bronchiectasis and why lipoxin is unable to modulate spontaneous apoptosis. Is lipoxin able to modulate apoptosis in neutrophils isolated from bronchiectasis patients during infection, when there are several pro survival factors in the environment? This warrants further investigation.

6.4. LIPOXIN AND ITS FUNCTIONAL EFFECT ON BRONCHIECTASIS NEUTROPHILS

In health, acute inflammatory responses in the lung are almost-daily occurrences that resolve swiftly. Most respiratory pathogens and noxious stimuli elicit an acute inflammatory response that again swiftly resolves. This immune response is critical for host defense, but it is equally important for the lung's inflammatory response to resolve in a timely manner. If unrestrained, acute respiratory distress syndrome can develop (Serhan and Savill 2005). If acute inflammation becomes chronic, then asthma, COPD or bronchiectasis can develop. Healthy resolution of inflammation is an active process that programs specific signals and cellular mechanisms to control the intensity and duration of acute inflammation. The past decade's discovery of resolution mediators and mechanisms is shining new light on the pathobiology of important chronic inflammatory diseases including bronchiectasis. At present, much more information is available on lipoxins than on other specialized pro resolving mediators (SPMs) such as protectins and maresins. SPM are rapidly formed and rapidly inactivated to serve as local autacoids to influence cellular responses (Serhan et al 2007).

In my study, I demonstrated several key functions of lipoxins. Lipoxin A_4 was not able to augment or inhibit spontaneous apoptosis in neutrophils from healthy volunteers or bronchiectasis patients. LXA_4 was able to reduce fMLF induced activation of neutrophils in a dose dependent manner in all groups- measured by flow cytometry assessment of CD62L shedding and CD11b upregulation. In addition, LXA_4 was able to reduce fMLF and cytochalasin B induced degranulation and release of myeloperoxidase, in a dose dependent manner. These anti-inflammatory functions of LXA_4 have previously been demonstrated in the literature. However, this is the first time that the effects of lipoxins have been demonstrated on a subset of reprogrammed neutrophils. A head to head assessment with neutrophils from healthy volunteers has demonstrated that lipoxins are able to override the pre activated state in these neutrophils and are able to exert anti inflammatory actions. This needs to be explored further in randomized controlled trials with Lipoxin A_4 in bronchiectasis.

Lipoxins have dual anti inflammatory and pro resolving functions. Key pro resolving actions include super oxide generation and microbial clearance. The enzyme system responsible for this respiratory burst, the NADPH-oxidase, functions as a transporter of electrons from NADPH on the cytosolic side of the membrane to oxygen on the other side. However, there are two pools of NADPH oxidase- one extracellular on the plasma membrane and the other on granules. Different agonists activate the two pools of NADPH oxidase differently, thereby suggesting that the signaling and molecular mechanisms for regulation differ depending on the localization of the oxidase (Dahlgren et al 1987, Watson 1991, Karlsson 1998). fMLF is a G protein coupled receptor and activation and activation of these receptors leads to extracellular release of superoxide anion. Lipoxin was able to reduce fMLF induced superoxide release in a dose dependent manner. In addition, neutrophils pre treated with lipoxin was able to increase phagocytosis of and killing bacteria. Generation of superoxide anions and bacterial clearance are key functions in the host defense as well as are important key steps in resolution of an inflammation. There is evidence from our studies that lipoxin is able to initiate both anti inflammatory and pro resolution activities in peripheral blood neutrophils from bronchiectasis patients.

It might be argued that if lipoxin is able to reduce the respiratory burst and limit superoxide release, will this affect bacterial killing? Although ROS production is critical for the killing and degradation of internalized bacteria and particles, it can also contribute to inflammatory damage of host tissue. It is perhaps important to control the 'collateral' damage that superoxide generation does. I have assessed the effect of lipoxin on super oxide generation by fMLF and PMA. fMLF mimics oligopeptides produced by bacteria and PMA is a well known endogenous ROS producer. Activation of G-protein-coupled seven-transmembrane spanning cell surface receptors, such as the formyl peptide receptors, generates signals that lead to assembly of the NADPH-oxidase in the plasma membrane, releasing oxidants to the extracellular environment. That lipoxin is able to attenuate fMLF and PMA induced ROS production indicates that lipoxin perhaps attenuates only extracellular superoxide release. Further studies using chemiluminescense (isoluminol and luminol) will help distinguish this.

6.5. LIPOXINS AND OTHER EICOSANOIDS IN BRONCHIECTASIS

This is the first study where bronchoscopy has been done to study the effects of lipid mediators in bronchiectasis. Lipoxin A_4 has previously been studied in other airway diseases such as asthma and cystic fibrosis. Lipoxin A_4 levels are decreased in severe asthma and cystic fibrosis. Bronchiectasis is characterized by recurrent chest infections and exacerbations. In this study, I have demonstrated that lipoxin A_4 can modulate neutrophil phagocytosis of bacteria in a dose dependent manner, both in the airways and peripheral blood. In addition, lipoxin was able to enhance bacterial killing of GFP labeled bacteria in a dose dependent manner. In blood, lipoxin was able to restore phagocytosis and killing of GFP PAO1, back to levels comparable to healthy volunteers. This study thereby demonstrates that lipoxin A_4 can potentially enhance bacterial clearance in bronchiectasis, when neutrophils are pre treated with Lipoxin A_4. Perhaps, this translates into treating bronchiectasis patients with lipoxin in the stable state- prior to an infection to enable quicker clearance and resolution of infection? Lipoxin can perhaps be considered as an additive therapy to antibiotics to augment the process of resolution, during an infection. It is known that lipoxins influence host control of *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Trypanosoma cruzi* and *Plasmodium berghei* cerebral malaria in mice (Russell and Schwarze 2014). The role of lipoxin in bacterial infections needs to be explored further.

Lipoxin A_4, protectins or maresins were not detected in the bronchoalveolar lavage samples in bronchiectasis patients. This could be due to the volumes of samples that were analysed and perhaps larger volumes (lesser than 500μL that was used in this study) are needed for future studies. However, there were several intermediates in the lipoxin generation pathway that were detected in these bronchoalveolar lavage samples. There were statistically significant higher levels of PGE2, 15 HETE and LTB_4 in serum and PGE2, 5HETE, 15 HETE 9-HODE and LTB_4 levels bronchoalveolar lavage fluid, in the moderate-severe bronchiectasis group compared to the mild group. As all these metabolites are pro inflammatory mediators, and the fact that they remain high even in the stable state, these findings explain in part the why more moderate-severe bronchiectasis patients have a higher mortality rate and more hospital admissions compared to patients with mild disease. In severe disease, as per the bronchiectasis severity index, the 1-year outcomes are 7.6 % - 10.5 % for

mortality rate and between 16.7 - 52.6 % for hospitalisation rates. The 4-year outcomes are between 9.9 - 29.2 % for mortality and between 41.2 - 80.4 % hospitalisation rates. This is almost 5-6 times higher than the mortality and hospital admission rates in patients with mild disease: 1 year outcomes: 0 - 2.8 % mortality rate, 0 - 3.4 % hospitalisation rate; 4 year outcomes: 0 - 5.3 % mortality rate, 0 - 9.2 % hospitalisation rate (Chalmers et al 2014). LTB_4 has been studied in bronchiectasis and is known to be one of the major chemotactic factors in the bronchial airways in bronchiectasis (Mikami et al 1998). Additionally, there is evidence to suggest that LTB_4 is raised during an exacerbation and reduces with antibiotic therapy (Chalmers et al 2012). This is the first study demonstrating however, the role of other lipid mediators (including LTB_4) in bronchiectasis. This part of the study suggests that lipid mediators play a key role in the ongoing inflammation both in the serum and in the airways in bronchiectasis patients and thereby necessitates further exploration in to the biochemical pathway of lipids and the mechanism by which they trigger the process of resolution.

6.6. NETs AND LIPOXIN

Increased levels of neutrophils lead to chronic neutrophilic inflammation, observed in bronchiectasis patients, mostly caused by chronic bacterial and viral infections enhanced by conditions favoring microbial growth. NET production, additionally promoted by bacterial infection in cystic fibrosis airways, is often ineffective in bacterial killing as was presented for *Pseudomonas aeruginosa* (Marcos et al 2010), and may facilitate bacterial airway colonisation and biofilm formation. In bronchiectasis, the liberated elastase (which I have demonstrated is significantly higher in bronchiectasis) as well as other proteolytic NET components can damage lung tissue and enhance the immune response by modulating the inflammatory factors. Neutrophil elastase cleaves endothelial actin cytoskeleton, E-cadherin and VE-cadherin, thereby increasing the permeability of the alveolar-capillary barrier. Moreover, neutrophil elastase induces apoptosis of epithelial cells and the release of proinflammatory cytokines (Saffarzadeh et al 2012). Other proteinases (PR3, cathepsin G) are able to regulate the inflammatory process by activating pro inflammatory and degrading anti-inflammatory proteins (Grommes & Soehnlein 2011). The antimicrobial peptide LL-37, detected in NET structures (Urban et al 2009), has cytotoxic and pro apoptotic properties directed towards endothelial and epithelial cells (Aarbiou et al 2006). In addition, ROS produced by MPO cause epithelial cell injury, which leads to apoptosis or necrosis (Grommes & Soehnlein, 2011), besides ROS-promoted netosis (Nishinaka et al 2011).

NETosis is a specific form of cell death different from apoptosis and necrosis. NET production by neutrophils plays an essential role in immune response to infection. The chromatin scaffold binds pathogens preventing their dissemination and limiting the inflammation area while the components of NETs very efficiently kill the trapped pathogens by oxidative and non-oxidative mechanisms (Yipp and Kubes 2013). Regardless of antimicrobial function, NETs participate also in many non-infectious diseases, autoimmune and inflammatory disorders, including chronic lung disease, sepsis, and vascular disorders. The increased auto reactivity towards NET constituents is a result of excessive netosis or diminished NET clearance (Ma and Kubes 2008). Although a range of biological events activating NET release is currently under excessive exploration, the mechanisms of its regulation are still unknown. It is

perhaps somewhat clear that there is excessive NETs formation in bronchiectasis and this in part contributes to the ongoing inflammation in this neutrophilic disease. While the mechanism by which lipoxin reduces NETs needs further investigation, it is encouraging that lipoxin is able to attenuate NETs formation in neutrophils isolated from individual during an infection, that have not be stimulated *ex vivo*.

6.7. REGULATION OF LIPIDS BY GENE EXPRESSION

LTA_4 hydrolase is a key enzyme in the lipoxin generation pathway. Sequential biosynthesis of eicosanoid classes in inflammatory exudates (termed "class switching") directs the temporal progression of acute inflammation – right from initiation to propagation and finally to active resolution (Levy et al 2001). Prostaglandins initiate the inflammatory response. Leukotrienes follow and are typified by LTB_4 which amplifies and propagates inflammation (Levy et al 2001). LTA_4 hydrolase is a key enzyme in the class switching that metabolizes LTA_4 to LTB_4. In the absence of LTA_4 hydrolase, LTA_4 is metabolized to produce LXA_4. Increased gene expression of LTA_4 hydrolase in moderate-severe bronchiectasis patients indicates that there is higher levels of LTB_4 in moderate-severe patients compared to LXA_4. This is in keeping with our study findings where higher levels of LTB_4 were found in serum and in bronchoalveolar lavage fluids in moderate-severe bronchiectasis patients.

There were significantly lower levels of 15 LO-B and 5LOX genes expressed in moderate-severe bronchiectasis patients compared to mild patients. LXA_4 is biosynthesised in the respiratory tract by transcellular cooperation of different cells-neutrophils (Chavis et al 1996), eosinophils (Serhan et al 1987), alveolar macrophages (Levy et al 1993) or airway epithelial cells (Claria et al 1996), each expressing different lipoxygenase (LO) enzymes (Haeggström JZ and Funk CD 2011). The neutrophil donates the LTA_4 intermediate formed by the activity of 5LOX on arachidonic acid to the airways epithelial cells or alveolar macrophages whereby 15 LO-A/B catalyses LXA_4 formation. Additionally, airway epithelial cell or alveolar macrophage 15 LO-A/B activity catalyses the conversion of AA to 15(S)HETE which is donated to the acceptor neutrophil and converted to LXA_4 by 5LOX (Levy et al 2001, Claria et al 1996, Fiore and Serhan 1990). Hence, it is evident that 15-Lipoxygenase plays a key role in the "eicosanoid mediator class switching." Statistically significant lower levels of 15LO-B and 5 LOX genes detected in moderate-severe airways in bronchiectasis would mean that there are lower levels of 15 and 5 lipoxygenase in moderate-severe airways. This would thereby lead to lesser biosynthesis of 15 HETE and LXA_4 (as 15 HETE is metabolized by 5LOX to form LXA_4).

Lower 15LO-B would necessarily lead to more LTA_4 availability for LTA_4 hydrolase to metabolize into LTB_4 and thereby lesser production of LXA_4. However, this is a complex system whereby a host of different enzymes produced by different cells act in a complex airways condition. Further studies in larger patient groups are needed to elucidate these findings more clearly.

6.8. LONG TERM EFFECTS OF LIPOXINS

This study elucidates that patients who are deficient in lipoxin A_4 have more severe disease with higher levels of serum and sputum inflammatory markers compared to patients who are sufficient in lipoxin A_4. Recent studies have established that disease severity in bronchiectasis, predicts mortality, hospital admissions, exacerbations, quality of life, respiratory symptoms, exercise capacity and lung function decline in bronchiectasis (McDonnell et al 2016). Lipoxin deficient patients had raised sputum LTB_4 at the end of one year, remaining parameters remained stable. Interestingly, there were lesser antibiotic courses required in the lipoxin sufficient group in the one year follow up period and this was demonstrated in their sputum which detected lower levels of myeloperoxidase and interleukin 8. This is a large study where several clinical parameters used to assess disease progression and severities were recorded. Lipoxins are the lead family in specialized pro resolving mediators, and have both anti inflammatory and pro resolution properties to stop acute inflammation. In bronchiectasis, there is chronic, unresolved and unremitting inflammation. LXA_4, LXB_4, and their 15-epimers are active in doses ranging from picogram to nanogram. They are generated at sites of vascular inflammation and are known to down regulate neutrophil transmigration, vascular leakage, proinflammatory cytokine release and function, and inflammatory pain signals. When exogenous specialized pro resolving mediators are administered during experimental inflammation, they exert their protective actions in low-nanogram amounts (Serhan 2007). The capacity of these molecules to jump-start several aspects of resolution suggests intriguing pharmacological roles for them as potential biotemplates for the design of new therapeutics (Serhan 2007), some of which are the subject to ongoing clinical research. That lipoxin sufficient patients had more stable, less severe disease and lesser exacerbations requiring antibiotic courses can be explained in part due to the above anti inflammatory and pro resolving functions of lipoxin.

6.9. ANTI INFLAMMATORY AND PRO RESOLVING

"Anti-inflammatory" and "pro resolving" do not have the same meaning, however. The definitions of these terms have important differences. Anti-inflammatory actions decrease granulocyte recruitment and activation, resulting in a predisposition to infection. Mediators with anti-inflammatory properties lead to decreased leukocyte: endothelial cell interactions, platelet aggregation, vascular permeability, and generation of reactive oxygen species from leucocytes. In sharp contrast, pro resolving actions activate tissue-resident cells to decrease vascular and tissue inflammation to restore organ function-a process termed catabasis to represent a return from the battle of inflammation. Mediators with pro resolving properties stimulate endothelial nitric oxide and prostacyclin release, mucosal epithelial expression of antimicrobial peptides, and macrophage phagocytosis, including efferocytosis, microbial clearance, removal of cellular debris and noxious stimuli such as antigens, and their cellular efflux to lymph nodes (Levy and Serhan 2014). These pro resolving actions increase tissue host defense either directly or indirectly via the restoration of tissue homeostasis. Thus, anti-inflammation is not synonymous with resolution.

6.10. FUTURE DIRECTIONS

In this thesis, I have addressed two important areas in bronchiectasis. I have demonstrated that blood neutrophils are reprogrammed in bronchiectasis and that lipid mediators are key regulators in the on going inflammatory process in bronchiectasis. Findings in this thesis suggest that lipoxin A_4 can target one of the key arms of the vicious circle in bronchiectasis as shown in figure 106.

The reprogrammed neutrophils remain pre activated in the stable state and are undergo lesser spontaneous apoptosis. These neutrophils tend to live longer. Through further studies, I have demonstrated that this is a constitutive defect. Why do neutrophils live longer in bronchiectasis? Do they inhibit caspase 3 and cause persistence of the neutrophils? Additionally these reprogrammed neutrophils release more myeloperoxidase and superoxide anions thereby contributing significantly to the ongoing inflammation in bronchiectasis. Persistence of these pre activated reprogrammed neutrophils can have deleterious effects in bronchiectasis. Despite being primed and activated, blood neutrophils from bronchiectasis patients had lesser killing ability than neutrophils from healthy volunteers. This is not desirable and explains in part why exacerbation in bronchiectasis need longer antibiotic therapy duration, compared to other infections. I also demonstrated that airways neutrophils have lesser phagocytic and bacterial killing ability than blood neutrophils, in bronchiectasis. Are airways neutrophils also reprogrammed in bronchiectasis? This needs further investigation.
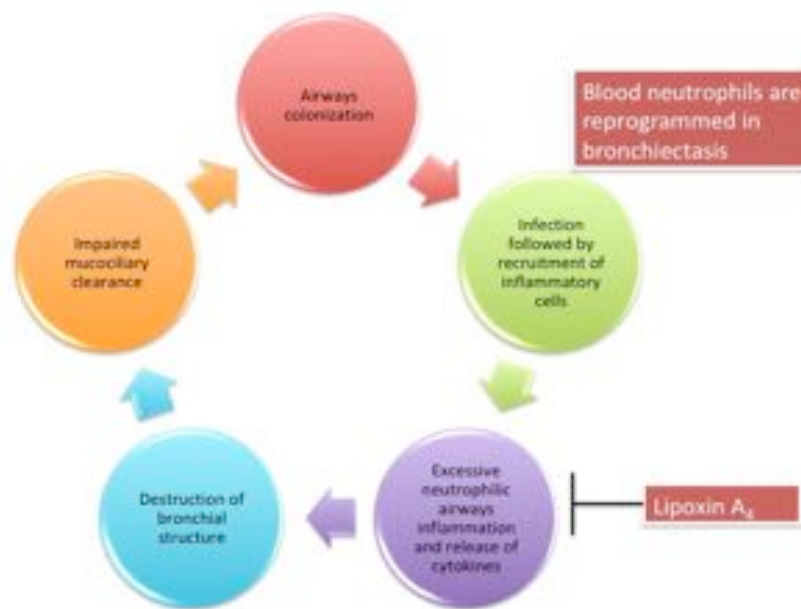
Figure 106. Peripheral blood neutrophils are reprogrammed in bronchiectasis and Lipoxin A4 has key anti inflammatory and pro resolution effects in bronchiectasis.

There is a dysregulation of the eicosanoids in bronchiectasis serum and airways. The key products studied were the omega 6 derivatives produced by the arachidonic acid pathway. Several mechanistic and functional studies have been done to demonstrate the efficacy of lipoxins in bronchiectasis. Lipoxin did not affect spontaneous or constitutive apoptosis. Could lipoxin affect apoptosis during an infection? Studies assessing the role of lipoxin on blood neutrophil apoptosis during an exacerbation would be interesting. Additionally, lipoxin was able to reduce superoxide generation from neutrophils. However, further studies are needed to assess if lipoxin affects intra cellular or extracellular superoxide generation. There is excessive myeloperoxidase in bronchiectasis and it is known that for super oxide generation, myeloperoxidase present in the azurophilic granules must fuse with the free radical in the same compartment. Does lipoxin inhibit the fusion by slowing down neutrophil degranulation? Further mechanistic studies are needed.

From a clinical point of view, I was able to demonstrate that lipoxin sufficient patients did better and had lesser exacerbations when followed up after one year, compared to lipoxin deficient patients. Functionally, lipoxin was able to increase phagocytosis and bacterial killing across all groups of patients. This is desirable in bronchiectasis. Across the studies I have demonstrated the various anti inflammatory and pro resolving activity of lipoxin in bronchiectasis neutrophils, both in blood and in the airways. Should lipoxin be used as an adjunct to antibiotics during exacerbations or should they be administered in the stable state too? There is evidence in this thesis to warrant randomised control trials with lipoxins.

6.11. CONCLUSION

In this thesis, I have demonstrated through different methods and techniques that there is failure of resolution of inflammation in bronchiectasis. There is on going persistent neutrophilic inflammation. Blood neutrophils are reprogrammed and persist longer in bronchiectasis. These reprogrammed neutrophils are the key contributors to the ongoing persistence of inflammation in bronchiectasis. This prolonged survival, made neutrophils primed and pre activated. These reprogrammed neutrophils were functionally different and produced higher levels of myeloperoxidase but had less phagocytic and bacterial killing ability. However, lipoxin was able to override the pro survival signals in these neutrophils. Although, lipoxin could not modulate spontaneous apoptosis, lipoxin was able to decrease neutrophil activation and degranulation and enhance their functional ability by enhancing neutrophil phagocytosis and bacterial killing.

I have demonstrated that there is a dysregulation of lipid mediators in the blood as well as in the airways of bronchiectasis patients. There is more production of the pro inflammatory mediators and decreased production of the anti inflammatory and pro resolution mediators in bronchiectasis. This impairs the resolution process thereby perpetuating the vicious circle of infection-triggered inflammation. Decreased gene expression of 15 Lipoxygenase B and increased expression of LTA_4 hydrolase in bronchial brushings demonstrated that in the airways, the direction of the arachidonic acid metabolism pathway is more steered towards production of leukotriene B_4 and lesser production of lipoxin A_4. Lipoxin overrides these proinflammatory signals and commences the resolution process by enhancing bacterial phagocytosis and killing, both in blood and in the airways. I demonstrated that patients that were deficient in lipoxin at baseline had higher levels of LTB_4. LTB_4 is one of the main chemoattractants in bronchiectasis, and key pro inflammatory signal.

There is now evidence from this thesis, that blood neutrophils are reprogrammed in bronchiectasis and that there is an imbalance of lipids in bronchiectasis, which contributes to the persistence of inflammation. Further studies assessing if airways neutrophils are reprogrammed in neutrophils will be needed. Additionally, I need to investigate further, the mechanisms by which lipid mediators regulate the inflammatory process in bronchiectasis.

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Title Page

Blood Neutrophils are Reprogrammed in Bronchiectasis.

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AT A GLANCE COMMENTARY:

Scientific knowledge on the subject
Bronchiectasis is a predominantly neutrophilic airways disease and despite this, there is persistent airways infection leading to chronic inflammation. The are no studies that have assessed neutrophil phenotype and how this alters neutrophil function in bronchiectasis.

What the study adds to the field
This is the first study that demonstrates that there is a distinct subset of neutrophils that are reprogrammed in bronchiectasis. Bronchiectasis neutrophils have prolonged survival and delayed apoptosis. In stable bronchiectasis patients, blood neutrophils are pre-activated and reprogrammed leading to an inability to kill PAO1 as effectively as healthy controls or patients that have recovered from community acquired pneumonia, leading to persistent infection and inflammation. Blood neutrophil phagocytosis and killing of PAO1 are further impaired at start of exacerbations needing intravenous antibiotic therapy that improved following treatment.

<u>**Contribution of authors:**</u>
PB performed the experiments, collected and interpreted the data and wrote the manuscript.
DJD contributed to experimental design, interpretation of data and writing of the manuscript.
BJMCH contributed to experimental design and writing of the manuscript.
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ATH contributed to experimental design, interpretation of data and writing of the manuscript.

ABSTRACT

Introduction
Excessive neutrophilic airways inflammation is the central feature of bronchiectasis, but little is known about neutrophils in bronchiectasis. The aim of this study was to assess blood neutrophil phenotype in bronchiectasis patients both whilst stable and during exacerbations.

Methods
In the clinically-stable arm of this study were 8 healthy volunteers, 8 mild and 8 severe bronchiectasis. In addition, 6 severe bronchiectasis patients were compared with 6 community-acquired pneumonia patients at the start and end of an exacerbation. We assessed neutrophils for spontaneous apoptosis rate, cell surface marker expression, degranulation, reactive oxygen species generation, phagocytosis and killing of *Pseudomonas aeruginosa* (PAO1). In addition, blood neutrophil function was compared to airway neutrophil function in bronchiectasis.

Results
In stable bronchiectasis, compared to healthy volunteers, blood neutrophils had: significantly prolonged viability; delayed apoptosis; increased CD62L shedding; upregulated CD11b expression; increased myeloperoxidase release; impaired neutrophil phagocytosis and killing of PAO1.

Bronchiectatic airway neutrophils had significantly lower bacterial phagocytosis and killing, than their matched autologous blood neutrophils. Both blood and airway neutrophil phagocytosis and killing was impaired at the start of an exacerbation and improved following antibiotic treatment.

In pneumonia patients, there was a significant improvement in phagocytosis and killing after treatment with antibiotics. On comparing phagocytosis and killing during exacerbations in bronchiectasis to pneumonia patients, there was no difference in phagocytosis, but there was significantly increased bacterial killing at the start and end of infection in pneumonia patients compared to bronchiectasis patients.

Conclusion
In the stable state, in bronchiectasis, peripheral blood neutrophils are reprogrammed and have prolonged survival.This impairs their functional ability of bacterial phagocytosis and killing, thereby perpetuating the vicious circle in bronchiectasis.

TEXT

Introduction

Excessive neutrophilic airways inflammation is the central feature of bronchiectasis. This paradoxically both promotes bacterial colonization and perpetuates damage to the airways creating a vicious circle of bacterial colonization and inflammation [1]. The acute inflammatory response is a protective mechanism that is evolved to eliminate invading organisms and should ideally be self-limiting and lead to complete resolution [2-4]. However, there is failure of resolution of inflammation in bronchiectasis, leading to irreversible damage and dilatation of the bronchial airways with loss of mucociliary function. The driver for persistent neutrophilic airways inflammation in bronchiectasis is unknown.

The traditional definition of the neutrophil, is that it is an effector of acute inflammation, and is short lived, with lifespan being measured in hours, and having a predefined set of functions [5]. However, this concept has been developing primarily by studying blood neutrophils (before extravasation to the tissues). It is subject to challenge as evidence emerges that neutrophil lifespan is considerably prolonged in response to stimuli such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), interleukins (ILs), interferons and bacterial products [6-10] with consequent functional significance. During inflammation, leukocytes leave the circulation, enter new environments, and are exposed to multiple factors, such as cytokines, endogenous growth factors and bacterial products. Indeed, the activity of infiltrating neutrophils has been closely linked to disease evolution in a variety of clinical conditions [11-12]. Thereby these cells contribute not only to acute inflammatory reactions, but also to the evolution of a variety of chronic inflammatory diseases.

Despite being a predominantly neutrophilic disease, bronchiectasis results in patients having recurrent chest infections. In more severe bronchiectasis there are high levels of sputum myeloperoxidase, free elastase activity and chemo attractants such as interleukin 8, leukotriene B$_4$ and C5a [7,8], contributing to the persistent neutrophilic airways inflammation. Watt and colleagues have shown there is prolonged airway neutrophil survival in bronchiectasis [10] with less airway neutrophil apoptosis. Is this consequent to the altered inflammatory milieu in bronchiectasis? How does this impact on neutrophil function? There have been no studies to date assessing and phenotyping blood neutrophil function in bronchiectasis.

The aim of this study was to assess the phenotype of blood neutrophils in bronchiectasis patients whilst stable and during exacerbations.

Methods

Three study groups were included. Study 1: all patients were in a stable state (no infective exacerbation of bronchiectasis for at least 4 weeks prior to giving blood for this study) and included 8 patients with mild bronchiectasis, 8 with severe bronchiectasis and 8 healthy volunteers. Bronchiectasis patients that took part in the study were not on any inhaled corticosteroids, long term antibiotics or immunosuppressive therapy. All participants had 60 mls of blood taken and underwent a bronchoscopy with a broncho-alveolar lavage targeted to the most affected segment with bronchiectasis.

Study 2: Six severe bronchiectasis patients were recruited during an exacerbation requiring intravenous antibiotics and patients were reviewed at day 1 (start of antibiotic therapy) and day 14 (end of antibiotic therapy). All patients had 60 mls of blood taken and sputum induced on day 1 and day 14.

Patients in study 2 were not the same patients as in study 1.

Study 3: Six community acquired pneumonia patients were recruited and reviewed at day 1 (hospital admission) and day 5 (when stable following antibiotic therapy). All patients had blood taken at day 1 and day 5. Patients required oral or intravenous antibiotics. We were not able to induce sputum in these patients.

Bronchiectasis severity
The severity of bronchiectasis was based on the Bronchiectasis Severity Index (BSI) [13]. The BSI is a risk stratification tool for morbidity and mortality in bronchiectasis. The minimum score is 0 and the maximum score is 26. A score between 0-4 indicates mild disease; 5-8 indicates moderate disease and a score of ≥9 indicate severe disease. The BSI was calculated in all bronchiectasis patients taking part in the study.

Consent
Lothian Research Ethics Committee gave consent for the study, 10/S1402/33.

Isolation of blood and airways neutrophils
Freshly drawn blood was collected into 3.8% sodium citrate and granulocytes were subsequently isolated by dextran sedimentation and discontinuous Percoll gradient, as described [14].

Sputum and bronchoalveolar lavage was washed, treated with sputolysin and airways neutrophils isolated (described in detail in online supplement). Anti CD16 antibodies (Abcam) were used to identify neutrophils by flow cytometry.

Blood neutrophil apoptosis at 20 hours
Freshly isolated blood neutrophils (at least 97% purity) was suspended at 10×10^6 cells/ml in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10% autologous serum and penicillin/streptomycin (1x). The assay was done on a 96 well flat-bottom plate. Following 20-hour incubation, apoptosis was examined by flow cytometry (Annexin-V (Roche) and propidium iodide (Sigma); BD FACS Calibur™) and confirmed by cytocentrifuge and Diff-Quick staining (Gamidor) [15].

Neutrophil activation
Freshly isolated neutrophils were activated with N-Formyl-methionyl-leucyl-phenylalanine, (fMLF) or vehicle control. Anti CD62L (BD Pharmigen ™) and CD11b (Alexa Fluor®) antibodies were added and the samples analyzed by flow cytometry (BD FACS Calibur™) [15].

Neutrophil degranulation and MPO measurement
Neutrophils were activated with cytochalsin B (Sigma Aldrich) and fMLF (Sigma Aldrich). Supernatants were collected and stored at -80°C. We measured myeloperoxidase activity (Sigma Aldrich) [16] with a chromogenic substrate assay.

Reactive oxygen species generation
Freshly isolated blood neutrophils were loaded with dihydrorhodhamine (Sigma Aldrich). fMLF was added and superoxide release was measured by flow cytometry (BD FACS Calibur™) [15].

Neutrophil phagocytosis of Green fluorescent protein (GFP) labeled *Pseudomonas* and killing
GFP bacteria in the log phage were resuspended in PBS at a final concentration of 10^8 bacteria/ml. Following this, bacteria were opsonised with autologous serum for 1 hour at 37°C. The neutrophils (10×10^6 per condition) were then co-cultured with opsonised GFP PAO1 (1×10^8 bacteria per condition) and phagocytosis was measured by flow cytometry (BD FACS Calibur™) [17]. Cells were lysed with saponin (Sigma Aldrich, UK) before serial dilutions were plated out on *Pseudomonas* isolation agar. Colony counts were performed after 24 hours incubation of plates at 37°C in 5% CO_2. (Full method in online supplement).

Bronchoscopy
All patients in study 1 underwent a bronchoscopy. Patients were sedated with midazolam +/- fentanyl. Bronchoalveolar lavage was done and samples obtained for neutrophil studies.

Inducing sputum
Sputum was induced as per standard protocol using 3% saline [18].

Statistical analysis
Flow cytometry analysis was performed using FlowJo v10.0.4 (Tree Star, Ashland, OR, USA). Results are presented as mean ± standard error of mean (SEM). Paired and unpaired t tests were used to compare two groups, where applicable. Data was analyzed by one-way ANOVA with a Bonferroni's multiple comparison post hoc test (GraphPad Prism v6; GraphPad Software, La Jolla, CA, USA), when three groups were involved; significance was accepted with *P* values: *$P < 0.05$.

Results

Study design
The groups examined, and the timepoints for collection of biological samples, in the three elements of this study are shown in Figure 1.
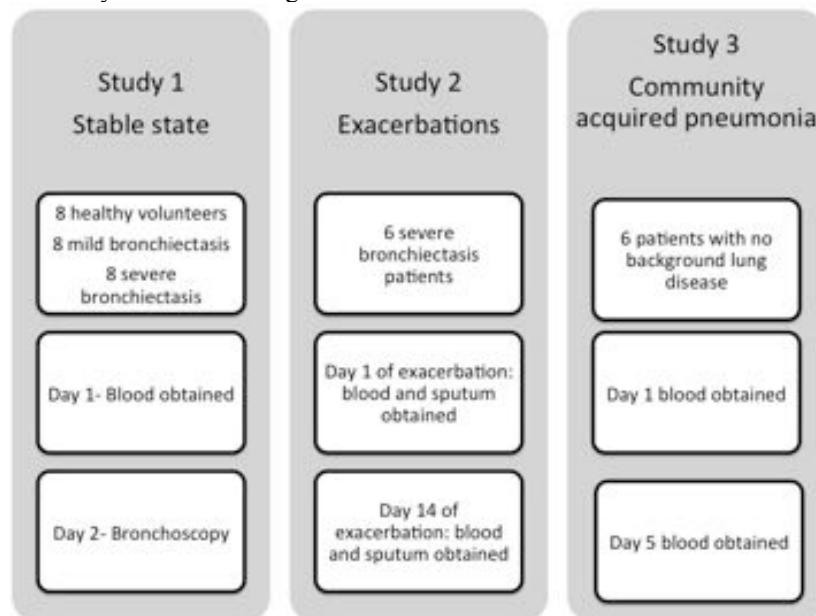

Figure 1. The three groups recruited for this study.
Study 1: All study participants – healthy volunteers, mild and severe bronchiectasis (no exacerbations for at least 4 weeks prior to taking part in the study) had blood obtained and underwent bronchoscopy.
Study 2: Six severe bronchiectasis patients had blood and sputum obtained at the start (day 1) and end of antibiotic therapy (day 14).
Study 3: Six pneumonia patients had blood obtained at the start (day 1) and end of antibiotic therapy (day 5).

Baseline demographics of the participants for Study 1 are shown in Table 1 (see online supplement Table E3 and E4, for baseline demographics of study 2&3 participants). In comparison to the patients with mild bronchiectasis, the severe group had significantly higher white cell counts, neutrophil counts and C reactive protein in blood; lower FEV_1 % predicted, FVC % predicted and TLCO % predicted; higher rates of bacterial lung colonization; and a greater number of exacerbations and hospital admissions in the preceding year.

Parameters	Healthy volunteers N=8	Mild N=8	Severe N=8	P value
Age	52 (6.8)	55 (3.8)	64 (2.2)	p=0.09
Gender (% female)	80%	40%	22%	**p<0.0001**
Cause of bronchiectasis -Idiopathic -Post infective	-	8 (100%) 0	8 (100%) 0	-
Total WCC ($x10^9$/L)	5.9 (0.5)	6 (0.5)	9.3 (1.1)	**p=0.005**
Neutrophils ($x10^9$/L)	3.5 (0.3)	3.3 (0.3)	6.6 (1.1)	**p=0.001**
Eosinophils ($x10^9$/L)	0.2 (0.06)	0.2 (0.04)	0.2 (0.06)	p=0.7
Monocytes ($x10^9$/L)	0.5 (0.05)	0.5 (0.03)	0.7 (1)	p=0.7
ESR (mm/hr)	4.8 (1)	6.7 (1.8)	19.6 (6.8)	p=0.1
CRP (mg/L)	3.2 (1.1)	2.8 (0.5)	16 (7.4)	**p=0.04**
FEV_1% predicted	-	95 (5.5)	55 (6.5)	**p=0.0001**
FVC % predicted	-	111 (6)	84 (6)	**p=0.008**
TLCO % predicted	-	94% (4.9)`	74% (7.8)	**p=0.002**
KCO % predicted	-	106% (4.5)	100% (7.2)	p=0.4
Chronic bacterial lung colonization- bacteria	-	3 (37.5%) *H.influenzae* (3)	5 (62.5%) *P.aeruginosa* (2) *S.pneumoniae* (2) *H.influenzae* (1)	**p<0.0001**
Exacerbations in the last year	-	0.4 (0.3)	4.2 (0.9)	**p=0.002**

Hospital admissions in the last year	-	0	0.7 (0.2)	**p=0.008**

Table 1. Baseline demographics of study participants. Results expressed as mean (SEM). Chronic colonization defined as at least two isolates of an organism whilst clinically stable, at least 3 months apart over 1 year [13,19]. CRP= c reactive protein; ESR= erythrocyte sedimentation rate; FEV_1= forced expiratory volume in 1 sec; FVC= forced vital capacity; KCO= transfer coefficient; TLCO= transfer factor for the lung for carbon monoxide; WCC= white cell count.

Blood neutrophil - spontaneous apoptosis

Healthy blood neutrophils undergo well-described spontaneous apoptosis in culture; a process which can be altered by exposure to survival factors and by activation [19]. Blood neutrophils from both mild and severe bronchiectasis patients, in stable state, had greater viability, after 20 hours in culture, than healthy controls (p=0.002, p=0.005 respectively; figure 2). A correspondingly lower number of apoptotic neutrophils was observed (p=0.0003 for mild and p<0.0001 for severe), with no difference in the percentage of necrotic cells. The proportion of apoptotic neutrophils from severe bronchiectasis patients was reduced to ~50% of that of controls. There was no difference in the proportions of viable (p=0.4) or apoptotic cells (p=0.2) when comparing cells from mild and severe bronchiectasis patients. The effect was confirmed by light microscopic counting and assessment of total cell numbers demonstrated that these percentages were not skewed by an artefact of cell loss (data not shown).
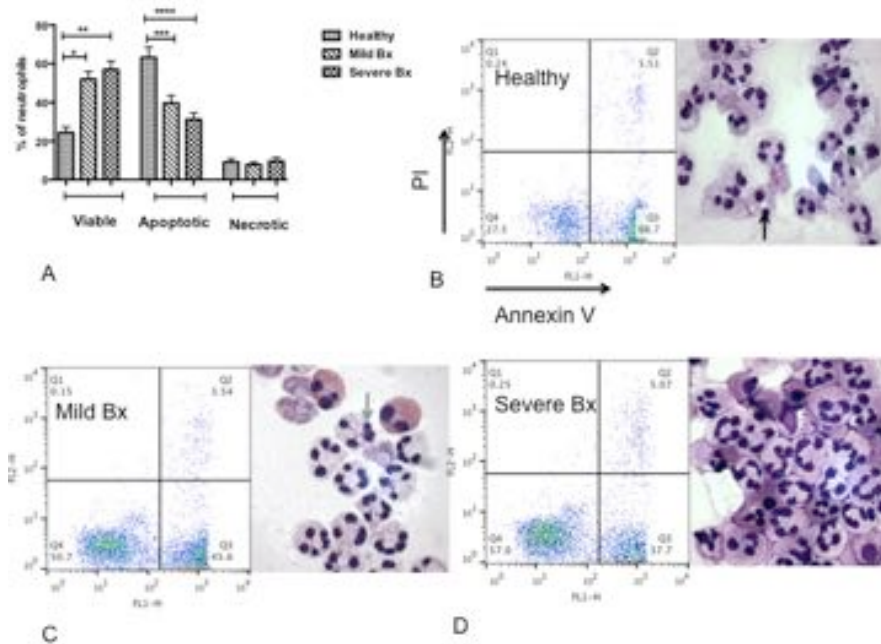

Figure 2. Blood neutrophils from mild and severe bronchiectasis patients survived longer and underwent less apoptosis when compared to healthy volunteers.
Blood neutrophils from mild and severe bronchiectasis patients in a stable state and healthy volunteers were cultured for 20 hours and cell viability (AnnV-ve/ PI-ve), apoptosis (AnnV +ve/PI-ve) and necrosis (AnnV+ve PI+ve) were assessed by flow cytometry. (A) N=8 in each group; percentage of viable, apoptotic and necrotic neutrophils in each group; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. One-way ANOVA with Bonferroni's correction for multiple comparisons used for all 3 groups compared; comparing mild and severe bronchiectasis to healthy controls in viable, apoptotic and necrotic neutrophils (B)-(D) Representative flow cytometry plots and cytocentrifuge preparations at 20 hours (x1000 magnification). PI= propidium iodide. Black arrow: Dark pyknotic apoptotic nucleus. Grey arrow: multi lobulated viable nucleus.

In order to determine whether disease-related factors in the autologous serum used in these cultures differentially impacted the rate of neutrophil apoptosis, serum swap studies were conducted (Supplementary Figures E1 and E2). Firstly, spontaneous apoptosis was assessed in neutrophils from bronchiectasis patients in the presence of serum from healthy volunteers. After 20 hours, there was a

significant reduction in the percentage of viable neutrophils when cultured in healthy control serum, as opposed to autologous serum (from 43.4% (±1.5) versus 50.7% (±3) respectively, p=0.02) and a significant increase in the percentage of apoptotic neutrophils (43% (±1.8) versus 38% (±2) respectively, p=0.02). There was no change in the percentage of necrotic neutrophils. In contrast, the nature of the serum used had no effect on the apoptosis of neutrophils from healthy volunteers

These data raise that possibility that a factor in healthy serum, deficient in serum from bronchiectasis patients, can promote neutrophil apoptosis. However, the effect of serum was minimal compared to the overall disease-specific differences between the rates of spontaneous apoptosis of neutrophils, suggesting some level of reprogramming or activation of circulating blood neutrophils in bronchiectasis.

Blood neutrophil - cell surface expression of CD62L and CD11b

To evaluate the baseline activation status of unstimulated blood neutrophils from stable bronchiectasis patients, surface expression of CD62L and CD11b were contrasted to heathy controls (figure 3). Surface expression of CD62L on neutrophils from mild and severe bronchiectasis patients was significantly lower than on healthy control neutrophils (p=0.04 and p=0.02 respectively). There was no difference between mild and severe bronchiectasis. Compatible with this, CD11b levels were significantly higher on neutrophils from severe bronchiectasis patients compared to healthy controls (p=0.01) and mild bronchiectasis (p=0.01), but in this case neutrophils from patients with mild bronchiectasis were unchanged compared to controls.

Blood neutrophil - degranulation and reactive oxygen species generation

To further evaluate the baseline activation status of blood neutrophils from stable bronchiectasis patients, myeloperoxidase (MPO) release from granules and superoxide generation were evaluated in unstimulated cells. A significantly higher level of MPO was detected in the supernatant of neutrophils from severe bronchiectasis patients, after 90 minutes in culture, compared to neutrophils from mild bronchiectasis patients (p=0.03) and healthy controls (p=0.04), with the latter two groups comparable. However, on comparison of superoxide release at baseline, there was no significant difference between neutrophils from healthy volunteers, mild and severe bronchiectasis patients; p=0.3 (figure 3).
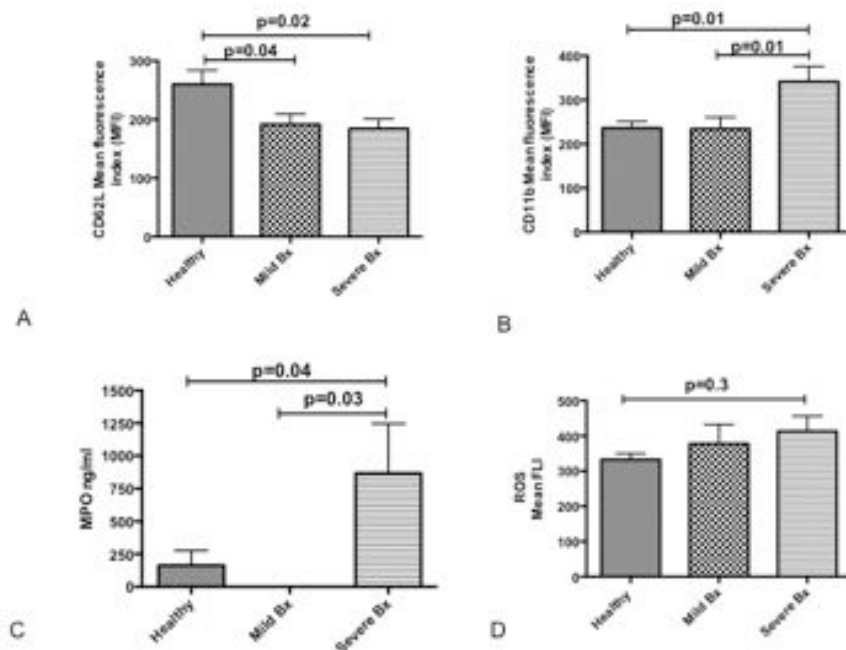

Figure 3. Blood neutrophils from stable bronchiectasis patients are more activated in the unstimulated state than neutrophils from healthy volunteers.

(A)-(B) Blood neutrophils were isolated and cell surface markers CD62L and CD11b were measured by flow cytometry, when unstimulated. N=8 in each group.

(C) Blood neutrophils were isolated and MPO measured by chromogenic assay, when unstimulated. N=8 in each group.

(D) Blood neutrophils were isolated, loaded with dihydrorhodamine and spontaneous reactive oxygen species generation was measured by flow cytometry. N=8 in each group.

One-way ANOVA with Bonferroni's correction for multiple comparisons used for all 4 experiments; A, C&D: comparison of severe and mild bronchiectasis to healthy volunteers (used as control); B: comparison of healthy volunteers and mild bronchiectasis to severe bronchiectasis (used as control). Pooled data expressed as mean ± SEM.

Blood neutrophil - phagocytosis and killing of *Pseudomonas aeruginosa*
Having determined baseline differences in parameters that suggest differential activation of blood neutrophils from stable bronchiectasis patients, their functional efficacy in uptake and killing of pathogens was evaluated using a GFP *P. aeruginosa* strain PAO1 (Figure 4). Cells were spun at 300g for 5 minutes, supernatants discarded to remove free bacteria, and then cells lysed before plating. Phagocytosis was confirmed with z-stacking using confocal microscopy. In separate experiments phagocytosis was blocked by doing the experiments at 4°C, which demonstrated that at 4°C bacteria were still bound to cells but not internalized (data not shown).

Data was analysed by gating the overall phagocytosis first (figure 4a). There was no difference in the overall phagocytosis between the groups. Next, we gated the neutrophils that had taken up much higher number of bacteria, as indicated by the mean fluorescence (MFLI). This was done at 50% of the total phagocytosis and we called this high MFLI phagocytosis. There was significantly higher phagocytosis (comparing only the high MFLI and not total phagocytosis) by blood neutrophils from healthy volunteers compared to those from bronchiectasis patients, both the mild and severe groups (p=0.04; p=0.02 respectively). There was no difference between mild and severe groups, p=0.4. Comparison of the subsequent neutrophil-mediated bacterial killing showed significantly lower numbers of bacteria following exposure to healthy control neutrophils when compared to neutrophils from patients with mild or severe bronchiectasis (p=0.006; p=0.0003 respectively). There was no difference between mild and severe groups, p=0.1. These data demonstrate that despite increased activation, neutrophils from the blood of bronchiectasis patients have impaired phagocytosis and killing capacity (figure 4).
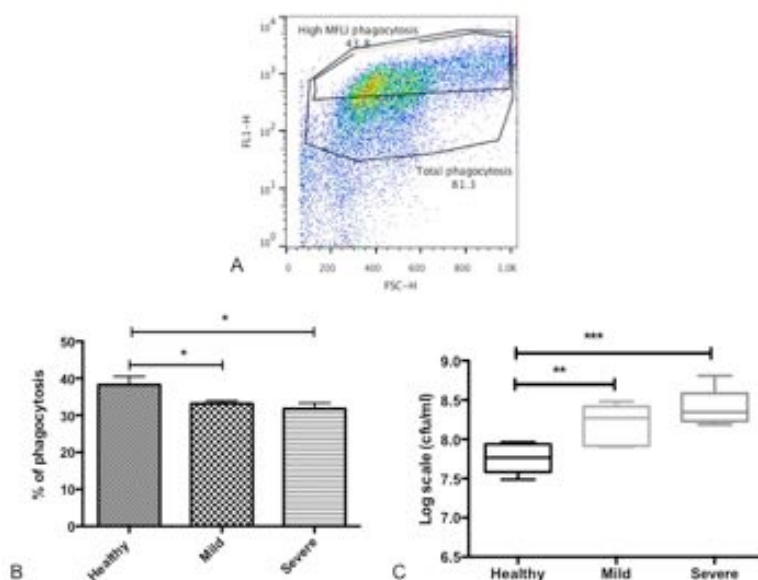

Figure 4. Impaired bacterial phagocytosis and killing by blood neutrophils from bronchiectasis patients compared to in healthy controls.
Blood neutrophils were isolated and co cultured with autologous serum-opsonized GFP-labelled *P. aeruginosa* PAO1 (at a concentration of 10^8/ml) for 15 minutes. Bacterial phagocytosis was measured by flow cytometry and serial dilutions of lysed cells were plated on *Pseudomonas* isolation agar, with colony forming units (cfu) counted 24 hours after plating to assess killing.
(A) Representative flow cytometry plot of phagocytosis, with high MFLI. Gates distinguish total cells having phagocytosed GFP-labelled bacteria, with "High MFL1 Phagocytosis", indicating cells that had phagocytosed the most bacteria. (B) Pooled % neutrophil phagocytosis data, showing means +/- SEM of n=8 per group for Hi MFL1 gating. (C) Pooled bacterial killing in log scale units cfu/ml, data, showing median with IQR of n=8 per group. (B)-(C) One-way ANOVA with Bonferroni's correction for multiple comparisons used for both experiments; with p values representing the comparison of severe and mild bronchiectasis to healthy volunteers (used as control). *P<0.05; **P<0.01, ***P<0.001.

Comparison of antibacterial function of blood and airways neutrophils from patients with bronchiectasis

Having established differential function of blood neutrophils isolated from bronchiectasis patients as compared to healthy controls, the antibacterial function of bronchoalveolar lavage-derived airway neutrophils from these patients was then evaluated (figure 5). Significantly lower levels of bacterial phagocytosis were observed for bronchoalveolar lavage-isolated neutrophils, when compared to donor-matched blood neutrophils, both from patients with mild and severe bronchiectasis (p<0.0001; p=0.0004 respectively). Comparison of the bacterial killing capacity showed a compatible significantly lower antibacterial function of bronchoalveolar lavage derived neutrophils compared to autologous blood neutrophils from the same patient, both in patients with mild and severe bronchiectasis (p=0.003; p=0.001 respectively). No differences between the antimicrobial functions of neutrophils from patients with mild and severe bronchiectasis was observed. This demonstrates that bronchoalveolar lavage neutrophils have impaired function compared to blood neutrophils in bronchiectasis.
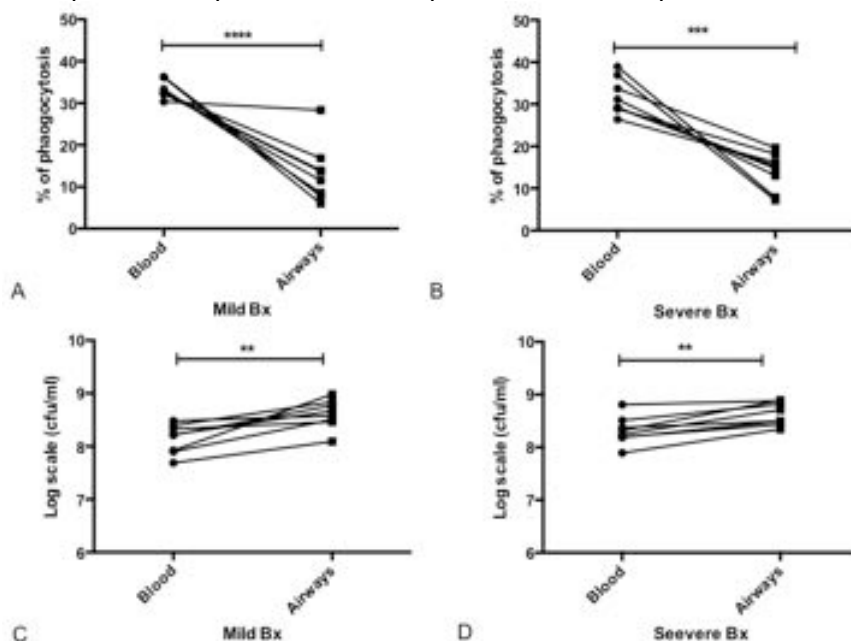

Figure 5. Impaired antibacterial function of bronchoalveolar lavage (BAL) derived neutrophils compared to matched blood neutrophils from patients with mild and severe bronchiectasis.
Neutrophils were isolated from BAL and blood from patients with mild and severe bronchiectasis, and co-cultured with serum-opsonized GFP PAO1 for 15 minutes (blood neutrophils) or 60 min (BAL neutrophils). Bacterial phagocytosis was measured by flow cytometry, and serial dilutions of lysed cells were plated on *Pseudomonas* isolation agar, with colony forming units (cfu) counted to assess killing. (A) Mild and (B) Severe bronchiectasis: Matched neutrophil phagocytosis data in blood and BAL derived neutrophils in the same patient; n=8 per group. (C) Mild and (D) Severe bronchiectasis: Matched bacterial killing in log scale units cfu/ml, in blood and BAL derived neutrophils in the same patient; n=8 per group. 2 way ANOVA showed no significant difference in the antibacterial activity between mild and severe bronchiectasis; p=0.2. **P<0.01; ***P<0.001; ****P<0.0001.

Comparison of antibacterial function of *blood* neutrophils from patients with bronchiectasis in stable and exacerbation states

The observation that blood neutrophils from patients with bronchiectasis had a higher baseline level of activation, with impaired antibacterial function, raised the question of the extent to which this might be further modulated by disease exacerbations. Matched samples collected from the same individuals at the beginning and end of an exacerbation were analysed, and compared to unmatched stable state controls. There was significantly higher bacterial phagocytosis by neutrophils collected at the end of an exacerbation compared those from the beginning of exacerbation (p=0.02). There was significantly more phagocytosis in the stable state compared to start of exacerbation, p=0.01. There was no difference in phagocytosis between the stable state and the end of exacerbation, p=0.9 (figure 6).

There was significantly higher killing at the end of exacerbation compared to beginning of exacerbation, p=0.03. There was significantly more killing in the stable state compared to start of exacerbation, p=0.02. There was no difference in bacterial killing between the stable state and the end of exacerbation, p=0.3. This demonstrates that blood neutrophil function is restored to levels comparable to the stable state in bronchiectasis, at the end of treatment of exacerbations.
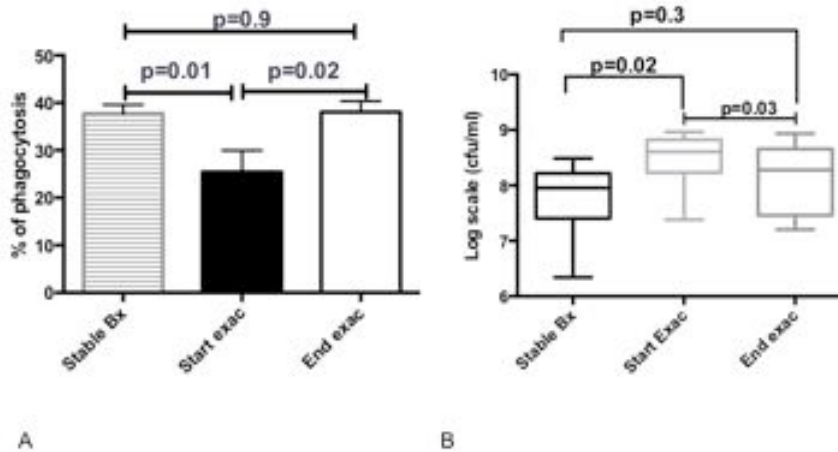

Figure 6. Phagocytosis and killing of bacteria by blood neutrophils significantly improves after antibiotic therapy in bronchiectasis.
(A)-(B) N=8 in stable state and N=6 at the start and end of exacerbation. Blood neutrophils isolated and phagocytosis and killing of GFP PAO1 assessed as previously described. Unpaired t-tests for comparison of stable state to start and end of exacerbation and paired t-tests for comparison of start to end of exacerbation. Pooled data presented as mean ± SEM for phagocytosis data and median (IQR) for bacterial killing data.

Phagocytosis and killing by *sputum derived* neutrophils during exacerbations and comparison to stable state

Having established that blood neutrophil function improved after treatment with antibiotics, the extent to which sputum-derived neutrophils had a similar response was assessed. There was significantly higher phagocytosis at the end of exacerbation compared to cells from the same donor taken at the beginning, p=0.008. There was more phagocytosis in the stable state compared to start of exacerbation although this observation, between unmatched donors, failed to reach statistical significance, p=0.08. There was no difference in phagocytosis between the stable state and the end of exacerbation, p=0.1.
There was significantly higher bacterial killing at the end of exacerbation compared to the beginning, p=0.02. There was more killing in the stable state compared to start of exacerbation p=0.02. There was no significant difference in killing between stable state and end of exacerbation, p=0.06, demonstrating that sputum neutrophil function is restored to stable state after treatment with antibiotics in exacerbations (figure 7).
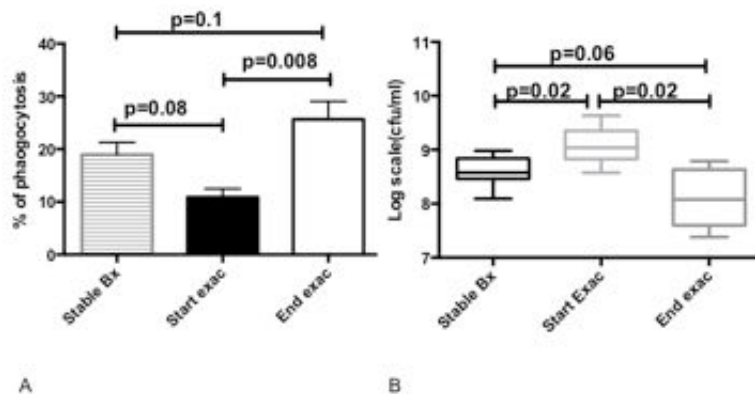
Figure 7. Phagocytosis and killing of bacteria by airways neutrophils significantly improves after antibiotic therapy in bronchiectasis.
(A)-(B) N=8 in stable state and N=6 at the start and end of exacerbation. Airway neutrophils isolated and phagocytosis and killing of GFP PAO1 assessed as previously described. Unpaired t-tests for comparison of stable state to start and end of exacerbation and paired t-tests for comparison of start to end of exacerbation. Pooled data presented as mean ± SEM for phagocytosis data and median (IQR) for bacterial killing data.

COMMUNITY ACQUIRED PNEUMONIA

Comparison of bronchietasis versus pneumonia
In order to determine the disease specificity of the neutrophil phenotypes determined for individuals with bronchiectasis, 6 pneumonia patients (with no comorbidities) who were admitted to hospital, were also recruited and studied. Baseline demographics of this group are available in the online supplement (Table E3).

Phagocytosis and killing of GFP PAO1 by blood neutrophils
The phagocytic and antibacterial properties of matched blood neutrophils collected at the beginning and day 5 of treatment of pneumonia, from the same patients, were compared. There was significant improvement in phagocytosis (p=0.03) and killing (p=0.03) at the end of treatment in pneumonia patients.

Comparison of phagocytosis by blood neutrophils collected in CAP and bronchiectasis exacerbation showed no statistically significant difference at the beginning (p=0.7) or end of infection (p=0.1), between the two conditions. This suggested that the phagocytosis phenotype may not be disease specific, but a more general consequence of severe infection. In contrast, there was significantly lower bacterial killing in patients with bronchiectasis both at the start (p=0.0007) and end of exacerbation (p=0.003). These data demonstrate a bronchiectasis-specific neutrophil bacterial killing defect, that may be exacerbated by impaired phagocytosis. Furthermore, at the end of bronchiectasis exacerbations treated with antibiotics, this remains highly impaired in comparison to neutrophils from healthy volunteers with a recent pneumonia
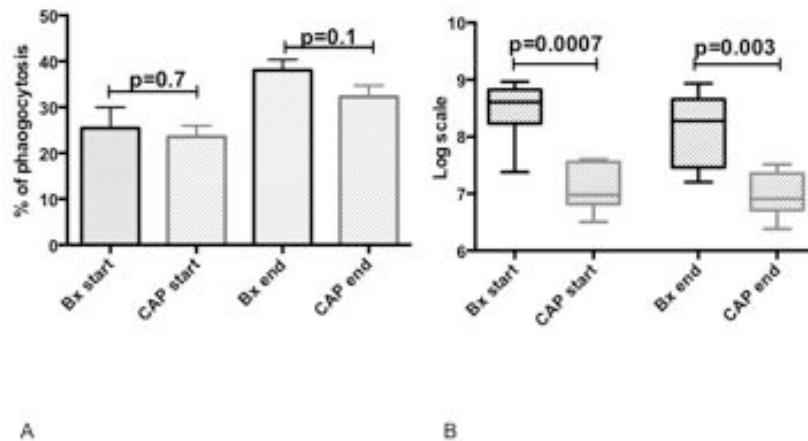
Figure 8. There is significantly higher bacterial killing at the start and end of pneumonia compared to bronchiectasis.
(A)-(B) N=6 in each group. Blood neutrophils isolated and phagocytosis and killing of GFP PAO1 assessed as previously described. Unpaired t-tests for all comparisons. Pooled data presented as mean ± SEM for phagocytosis data and median (IQR) for bacterial killing data. Bx= bronchiectasis; CAP = community- acquired pneumonia.

Discussion

We studied the functional phenotype of blood neutrophils in mild and severe bronchiectasis and compared it to the activity of blood neutrophils isolated from healthy volunteers. In bronchiectasis patients, we also assessed the function of airways neutrophils and compared it to blood neutrophil function from the same patient. In addition, we studied neutrophil function during bronchiectasis exacerbations and compared it to neutrophil function in healthy patients admitted with community-acquired pneumonia. The authors chose experiments with *Pseudomonas aeruginosa*, as this organism is associated with a worse outcome in bronchiectasis [13]. Future work investigating other pathogens in bronchiectasis is warranted.

Blood neutrophils from bronchiectasis patients (both in mild and severe groups) had prolonged survival and delayed apoptosis when compared to healthy volunteers. Further studies demonstrated that when blood neutrophils from bronchiectasis patients were treated with serum from healthy volunteers, there was a reduction in viability and increased apoptosis in bronchiectasis neutrophils. However, the reverse was not true when treating healthy volunteers neutrophils with bronchiectasis serum. The overall effect of delayed apoptosis and prolonged survival of bronchiectasis neutrophils was more pronounced than the effect of serum swap. The reason for this remains to be determined, but the effect was minimal compared to the overall disease-specific differences between the rates of spontaneous apoptosis of neutrophils, suggesting some level of reprogramming or activation of circulating blood neutrophils in bronchiectasis. There was more CD62L shedding in mild and severe bronchiectasis compared to healthy volunteers. There was higher CD11b expression and myeloperoxidase release in severe bronchiectasis compared to healthy volunteers and mild bronchiectasis. There was no difference in reactive oxygen species generation in the three groups. There was significantly higher phagocytosis and bacterial killing of PAO1 in healthy volunteers compared to mild and severe bronchiectasis. Although the significantly higher proportion of females in the healthy volunteers group, compared to bronchiectasis patients, could be considered a minor limitation of this study, we have no reason to believe that this had any impact on the results of the study [20]. Studies have shown that older adults have impaired neutrophil migration that contributes to poorer outcomes during infection [21,22]. In this study, both in the stable state (healthy volunteers and bronchiectasis patients) and during exacerbations (pneumonia patients and severe bronchiectasis patients), there was no significant difference in the age of the study groups.

Having established that blood neutrophil phagocytosis and killing is impaired irrespective of disease severity, we wanted to explore whether airway neutrophil function was impaired. We demonstrated that phagocytosis and killing of PAO1 is impaired in airways neutrophils compared to blood neutrophils in both mild and severe bronchiectasis. Although the etiology of bronchiectasis is heterogeneous, to address this as a confounding factor, for the purpose of this study we included patients with idiopathic bronchiectasis only. Despite the neutrophils being preactivated, the phagocytic and bactericidal ability of blood and airways neutrophils is impaired (more marked in the airways) which suggests that the inflammatory milieu with myeloperoxidase and proteinase excess in patients with bronchiectasis is perhaps a contributory factor for this [8] leading to impaired function. It is known that inflammatory reprogramming of neutrophils leads to increased viability [23]. To the authors best knowledge airways neutrophil function in other disease such as COPD and cystic fibrosis conditions remain limited. Studies on broncho-alveolar lavage fluid neutrophils from other chronic lung diseases is warranted in the future. We hypothesize that airway neutrophils are also reprogrammed in bronchiectasis.

In acute infective exacerbations of bronchiectasis, phagocytic and bactericidal activity of blood and airways neutrophil function improved with antibiotic therapy and was restored to levels comparable to the stable state. On comparison of bronchiectasis exacerbations and patients admitted with community-acquired pneumonia, there was no difference in blood neutrophil phagocytosis between the two groups; there was however significantly higher bacterial killing of PAO1 at the start and end of infection in community-acquired pneumonia patients compared to bronchiectasis exacerbations. Indeed, even at the end of treatment with antibiotics during bronchiectasis exacerbations, although phagocytosis and killing ability of neutrophils are restored to stable state, it was still lesser than in patients recovered from community acquired pneumonia suggesting that even at the end of exacerbation, there is failure of resolution of inflammation in bronchiectasis. This is important, as reduced functional activity of neutrophils would mean slower and lesser clearance of bacteria in bronchiectasis. Reduced phagocytosis and killing was not secondary to anti-pseudomonal antibodies as only 25% were positive for *P aeruginosa* in the severe group and in sub-group analysis excluding *P aeruginosa,* killing was still impaired (data not shown). We hypothesize that this failure of resolution could be secondary to persistence of reprogrammed neutrophils at the sites of inflammation or impaired clearance of these neutrophils.

Bronchiectasis is a predominantly neutrophilic condition, where despite excess neutrophils, patients suffer daily cough, chronic sputum production and recurrent chest infections. The lung is continuously exposed to inhaled pathogen and it is the primary and secondary defense of the lung that maintains the microbiome of the lung. The excessive neutrophilic airways inflammation leads to damage of the bronchial wall and paradoxically promotes more airways inflammation and bacterial infection creating a vicious cycle [24, 1]. During natural resolution, polymorphonuclear neutrophils are required for antimicrobial defense [3], but these cells must then apoptose and are removed from the inflammatory site by macrophages [25]. Is this natural resolution impaired in bronchiectasis? There are no studies exploring the resolution mechanism in bronchiectasis or perhaps the lack of it, in the literature to date.

Apoptotic neutrophil death *in situ* has multiple pro-resolution actions [26-28]. Bronchiectasis neutrophils live longer and undergo delayed apoptosis compared to healthy volunteers. Given the number of neutrophils in bronchiectasis airways, late neutrophil apoptosis could have devastating consequences. Slowing neutrophil apoptosis would mean delayed removal by macrophages and delaying the process of resolution, thereby perpetuating the ongoing inflammation. Vandivier and colleagues had previously demonstrated that neutrophil elastase cleaves phosphatidylyserine receptor on neutrophils in cystic fibrosis and bronchiectasis, thereby impairing its clearance by macrophages [29]. This along with the data from our study suggest that bronchiectasis neutrophils undergo an alternative method of cell death. Could this be NETosis or secondary necrosis? It is known that inefficient clearance of apoptotic cells results in secondary necrosis of cells and exacerbation of the inflammatory response [30]. Recent studies have demonstrated that in cystic fibrosis, there is increased NETosis as these neutrophils engage less in apoptosis [31]. This needs to be studied further in bronchiectasis.

Our studies demonstrate that bronchiectasis neutrophils are reprogrammed. There is emerging evidence of subpopulations of mature and immature neutrophils coexisting in the circulation performing

immunosuppressive or proinflammatory functions [32]. These have been identified as low-density neutrophils (LDNs) and are known to have phenotypic and functional heterogeneity [32]. LDNs have not been investigated in this study. Is there a release of chronic immature neutrophils in bronchiectasis or is there an imbalance between mature and immature neutrophils released? Antibiotics improve neutrophil function during exacerbations. However, neutrophils are preactivated even in the stable state. Do anti inflammatory or pro resolution mediators have a role as a long-term treatment in bronchiectasis? This needs to be investigated further.

Overall, there was higher c-reactive protein, CD11b and myeloperoxidase generation in severe disease compared to mild disease. For the rest of the other parameters measured- there was no difference in viability, apoptosis, CD62L, reactive oxygen species generation, phagocytosis and killing between mild and severe groups. These suggest that the effect of the disease on peripheral blood neutrophil activity and function is more pronounced than disease severity. However, we accept that a limitation of the study is the size of the study and therefore further differences may have been found with a larger sample size. We accept that exacerbation data from the same patients in study 1 would be ideal but this is a technical difficulty. It was difficult to predict exacerbations in the stable patients and hence we recruited consecutive patients who had an exacerbation and presented to hospital. The data remains a valuable addition despite this. Another limitation is we assessed patients following 14 days of antibiotic therapy in bronchiectasis patients. A different time point was chosen for patients with pneumonia at day 5. All patients with pneumonia, were however clinically improved at day 5. These time points were chosen to reflect end of antibiotic therapy.

To the authors' best knowledge, this is the first study demonstrating that peripheral blood neutrophils are reprogrammed in bronchiectasis. This is important preliminary work and would lead to further research in the area. Key future work would be to assess the airway neutrophil function. In this study, we have shown that in the vicious circle of infection and inflammation, the dynamics of cell death and clearance are not altered leading to overwhelming neutrophilic inflammation and thereby cytokine release. Neutrophil apoptosis and clearance and how this can be modified needs to be investigated further.

Conclusion
In the stable state, peripheral blood neutrophils are reprogrammed and persist longer in bronchiectasis. This impairs their functional ability of bacterial phagocytosis and killing, thereby perpetuating the vicious circle in bronchiectasis.

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